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Modulation of cellular signaling pathways in P23H rhodopsin photoreceptors☆

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ABSTRACT

We previously reported activation of the unfolded protein response (UPR) in P23H rhodopsin (RHO) retinas with autosomal dominant retinitis pigmentosa (ADRP). Knowing that the UPR can trigger Ca^{2+} release from the endoplasmic reticulum and regulate cellular signaling we examined the level of Ca^{2+}-regulated proteins. We also looked for changes in the expression of Bcl2 family proteins, autophagy proteins and the mTOR/AKT pathways, as well as for the induction of mitochondria-associated apoptosis in the P23H RHO retina. Our data demonstrated that the elevation of calpain and caspase-12 activity was concomitantly observed with a decrease in the BCL2-XL/BAX ratio and an increase in mTor levels in the P23H-3 RHO retina suggesting a vulnerability of P23H RHO photoreceptors to apoptosis. The translocation of BAX to the mitochondria, as well as the release of cytochrome *C* and AIF into the cytosol supports this conclusion and indicates the involvement of mitochondria-induced apoptosis in the progression of ADRP. The level of autophagy proteins in general was found to be decreased in the P21–P30 P23H RHO retina. Injections of rapamycin, however, protected the P23H RHO rod photoreceptors from experiencing physiological decline. Despite this fact, the downregulation of mTOR did not alter the level of autophagy proteins. Our results imply that in addition to activation of the UPR during ADRP progression, photoreceptors also experience alterations in major proapoptotic pathways.

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1. Introduction

Retinitis pigmentosa (RP) is a heterogeneous group of eye disorders that result in irreversible blindness. Visual symptoms include night blindness, followed by photopia and decreasing visual fields, leading to tunnel vision and eventually legal blindness [1,2]. Most frequently autosomal dominant RP (ADRP) is associated with mutations in rhodopsin (RHO), with such mutations accounting for approximately 25% of all ADRP cases [1]. Based on their biochemical and cellular properties, ADRP rhodopsin mutations have been classified into six groups, however most fall into the class I or class II categories [3]. Class I mutations can fold normally, but are not correctly transported to the outer segment. The S334ter RHO protein expressed in transgenic rats represents a Class I mutation. The substitution of proline for histidine at position 23 within the rhodopsin (P23H RHO) gene yields a Class II mutation. These RHO mutants are defective for proper folding. They are retained in the endoplasmic reticulum (ER) and are unable to form a functional chromophore with 11-cis-retinal. They are then transported to the cytoplasm for degradation by the proteasome [4].

In 1990 the P23H RHO mutant was proposed to cause ADRP [5]. Since then, the expression of P23H mutant rhodopsin (RHO) in photoreceptors has been studied in transgenic mice [6–8], rats [9–11] and frogs [12–14]. Loss of photoreceptors and the course of visual decline have been previously described for rats expressing both the S334ter and P23H *RHO* transgenes (http://www.ucsfeye.net/mlavailRDratmodels.shtml) [15,16]. However, molecular mechanism involved in the loss of vision in Class I and II mutations has not been studied in detail. Previously, we have demonstrated that activation of the unfolded protein response (UPR) occurs during retinal degeneration in both the S334ter and P23H RHO rat models [11,17,18]. In the S334ter transgenic retina we have also determined that activation of the UPR is associated with increased expression of the JNK, Bik, Bim, Bid, Noxa, and Puma genes and cleavage of caspase-12 that, together with activated calpains, presumably compromise the integrity of the mitochondrial MPTP in ADRP photoreceptors [18]. In P23H-3 *RHO* rats there have been no cellular signaling pathways identified as being involved in the mechanism underlying photoreceptor degeneration with the exception of the UPR [11,17,19]. Therefore, there is a critical need to describe any pathways that are modified concomitantly with the activated UPR in P23H-3 *RHO* photoreceptors in order to validate new therapeutic targets.

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The UPR is initiated by the activation of three signaling pathways (PERK, ATF6 and IRE1) and is required for controlling ER protein folding capacity and reestablishing homeostasis in the cell. Upon acute or unresolved ER stress, the UPR also triggers apoptosis, which eliminates any cell that is incapable of restoring proper protein folding and orchestrating a coordinated adaptive downstream response. Previously, we demonstrated that P23H-3 *RHO* photoreceptors have an activated ER stress-induced caspase-7 [17] and a consistent increase in Bip and CHOP gene expression [11]. These results suggested that ER stress persists in P23H-3 *RHO* photoreceptors, which might stimulate apoptotic signaling in these animals. Subsequently the persistence of the UPR has been confirmed in our other study [11]. However, apart from the UPR, other signaling pathways have been found to originate from the ER and those pathways have not been properly investigated. For example, the Ca^{2+}-mediated signaling pathway could be triggered by the ER stress response leading to activation of calpains and caspase-12 [20,21]. The Bcl2 family proteins are known to be upregulated during UPR activation *via* the transcriptional activity of ATF4 and CHOP (Puma, Noxa and Bim). They can also be upregulated by activated JNK, which phosphorylates the BCL-2/BCL-XL proteins and additionally, promotes autophagosome formation by releasing the active beclin-1/PI3K complex from the ER. This complex is known to regulate ATG12–ATG5 formation and to promote the LC3-II conversion during the formation of autophagosomes [22]. Therefore, autophagy, the major degradation pathway after UPR activation in neuronal cells, could also be induced by ER stress. Both the PERK/eIF2α and IRE1 arms of the UPR have been implicated in the regulation of autophagy [23].

mTOR/AKT signaling is another example of a pathway that is tightly regulated by the UPR. We have previously reported that a T17M *RHO* retina that experiences UPR activation also demonstrates an elevation of mTor protein and a decrease in phosphorylated AKT [21]. It has been demonstrated that the UPR can activate mTOR *via* ATF6a signaling [24]. The ATF6 UPR pathway was found to be upregulated in both P23H and T17M RHO retinas [17,21], thus implying that similar to T17M RHO, P23H RHO photoreceptors could also have modified mTOR/AKT signaling.

Thus, several signaling pathways have been identified that are mediated by the activation of the UPR, which is found in P23H-3 *RHO* photoreceptors [11,17,19]. They could be modified either by ER stress or independently altered to contribute to the mechanism of ADRP. In both scenarios, these signaling pathways could provide alternative therapeutic strategies in the P23H RHO retina in addition to gene therapy against mutant rhodopsin. Therefore, the major focus of this study is to identify whether UPR activation in P23H RHO photoreceptors is accompanied by changes in Ca^{2+}-induced signaling, autophagy, and mTOR/AKT pathways during ADRP progression and whether alterations in mTOR cellular signaling could be responsible for slowing the rate of retinal degeneration.

2. Materials and methods

2.1. Animal use

All animal procedures conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Institutional Animal Research Committee of the University of Alabama at Birmingham. The P23H-3 *RHO* (line 3) rats were kindly provided by Dr. LaVail (UCSF). Rats were housed in specific pathogen-free (SPF) conditions with a 12-hour light and 12-hour dark cycle. Animals were sacrificed by thoracotomy, and retinas were rapidly excised (removal of the lens), placed on an ice-cold plate, and stored at −80 °C.

2.2. RNA preparation and quantitative real-time PCR

Total RNA was isolated from individual SD and P23H-3 RHO (N = 4) retinas using an RNeasy Mini kit (Qiagen, Valencia, CA). We then used a high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, California, USA). Two cDNA reactions were prepared from each RNA sample and 10 ng cDNA was used for qRT-PCR using Applied Biosystems TaqMan assays that were validated for each selected gene on a One Step Plus instrument (Applied Biosystems, Foster City, CA). To analyze the samples, we compared the number of cycles (Ct) needed to reach the midpoint of the linear phase and normalized all observations to the GAPDH housekeeping gene. The replicated RQ (Relative Quantity) values for each biological sample were averaged.

2.3. Retinal protein extract for western blot analysis

We obtained retinal protein extracts from dissected retinas that were sonicated in a buffer containing 25 mM sucrose, 100 mM Tris–HCl, pH = 7.8, and a mixture of protease inhibitors (PMSF, TLCK, aprotinin, leupeptin, and pepstatin). The total protein concentration from individual retinas was measured using a Bio-Rad protein assay, and 40 μg of total protein was used to detect individual proteins. The detection of proteins was performed using an infrared secondary antibody and an Odyssey infrared imager (Li-Cor, Inc.). Antibodies against PUMA (7467S), ATG5 (8540P), ATG7 (78558P), LC3 (#2775), mTOR (2983P), caspase-3 (9662P), caspase-9 (9507S) and caspase7 (9492P) were purchased from Cell Signaling (1:1000). Antibodies against AIF (sc5586), Cytochrome *C* (sc13156), NOXA (sc11718), and active BAX, which detects only the active form of the BAX protein (sc23959) were purchased from Santa-Cruz Biotechnology (1:1000). Anti-caspase-12 (ab62484) and anti-pAKT (ab66138), which detects phosphorylated AKT, were purchased from Abcam (1:1000). We used an anti-BAX antibody (B8429) and an anti-Lamp2 (L0668) antibody from Sigma-Aldrich (1:1000). β-Actin was used as an internal control and was detected using an anti-β-actin antibody (Sigma-Aldrich).

2.4. Intraperitoneal injection of rapamycin and ERG analysis

A 10 mg/kg dose of rapamycin was used for injections. A water solution of 5% ethanol, 5% tween-20 and 5% polyethylene glycol 400 was used to dissolve rapamycin and was also used in the vehicle-treated control injections. Starting at P15, daily intraperitoneal injections (IPs) were performed in rats over the course of 10 days, during which no signs of weight loss were recorded. The *E*lectro*R*etino*G*rams (ERG) of treated and untreated animals were recorded as previously described [25] at P30 and P45, which correlate with 2 and 4 weeks post-treatment, respectively. Briefly, rats were dark-adapted overnight, then anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). Their pupils were dilated in dim red light with 2.5% phenylephrine hydrochloride ophthalmic solution (Akorn, Inc.). The KC UTAS-3000 Diagnostic System (Gaithersburg, MD) was used to perform scotopic ERGs. The measurements were conducted using a wire contacting the corneal surface with 2.5% hypromellose ophthalmic demulcent solution (Akorn, Inc.). The ERG was performed at different intensities including 0 dB (2.5 cd*s/m2), 5 dB (7.91 cd*s/m2), 10 dB (25 cd*s/m2), and 15 dB (79.1 cd*s/m2).

2.5. Calpain activity assay

The detection of calpain activity was performed using the Calpain Activity Assay kit from BioVision in accordance with the manufacturer's recommendations. The detection of the cleaved substrate Ac-LLY-AFC was performed using a fluorometer that was equipped with a 400-nm excitation filter and 505-nm emission filter.

2.6. Isolation of mitochondria from rat retinas

We separated the cytosolic fraction from the mitochondrial fractions from five individual P23H RHO and SD rats using the Mitochondria

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