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Retina-specific activation of a sustained hypoxia-like response leads to severe retinal degeneration and loss of vision

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ABSTRACT

Loss of vision and blindness in human patients is often caused by the degeneration of neuronal cells in the retina. In mouse models, photoreceptors can be protected from death by hypoxic preconditioning. Preconditioning in low oxygen stabilizes and activates hypoxia inducible transcription factors (HIFs), which play a major role in the hypoxic response of tissues including the retina. We show that a tissue-specific knockdown of von Hippel-Lindau protein (VHL) activated HIF transcription factors in normoxic conditions in the retina. Sustained activation of HIF1 and HIF2 was accompanied by persisting embryonic vasculatures in the posterior eye and the iris. Embryonic vessels persisted into adulthood and led to a severely abnormal mature vessel system with vessels penetrating the photoreceptor layer in adult mice. The sustained hypoxia-like response also activated the leukemia inhibitory factor (LIF)-controlled endogenous molecular cell survival pathway. However, this was not sufficient to protect the retina against massive cell death in all retinal layers of adult mice. Caspases 1, 3 and 8 were upregulated during the degeneration as were several VHL target genes connected to the extracellular matrix. Misregulation of these genes may influence retinal structure and may therefore facilitate growth of vessels into the photoreceptor layer. Thus, an early and sustained activation of a hypoxia-like response in retinal cells leads to abnormal vasculature and severe retinal degeneration in the adult mouse retina.

Introduction

Retinitis pigmentosa (RP) is a major cause of severe visual impairment or blindness in humans. It is characterized by an initial loss of photoreceptors in the peripheral retina causing tunnel vision. As photoreceptor degeneration progresses, affected patients loose vision also in the center leading to complete blindness. Although mechanisms of cell death in RP and other degenerative diseases of the retina have been a subject of intense investigations, the exact molecular events leading to loss of photoreceptors have not been defined. As a consequence, only little is known about possible ways to inhibit cell death by neuroprotective approaches. In experimental model systems, preconditioning by hypoxia or light – the pre-exposure of mice to low oxygen concentrations or to non-damaging levels of light, respectively – protects photoreceptors from light induced degeneration (Grimm et al., 2002; Grimm et al., 2006; Chollangi et al., 2009). These systems may thus be valuable tools to study and develop neuroprotective measures to delay photoreceptor apoptosis.

Conditions of low oxygen availability (hypoxia) activate hypoxia inducible transcription factors (HIFs), which may regulate potentially protective genes during hypoxic preconditioning. HIFs are heterodimeric factors consisting of an α- and a β-subunit. Three isoforms of the α-subunit, HIF1A, HIF2A and HIF3A, and one form of the β-subunit, called aryl-hydrocarbon receptor nuclear translocator (ARNT), are known. ARNT is constitutively expressed and located in the nucleus. In conditions of normal oxygen availability (normoxia) HIF-α subunits are constantly degraded through the proteasomal pathway. Prolyl hydroxylases (PHDs or EGLNs) hydroxylate HIF-α subunits turning them into a binding substrate for the von Hippel-Lindau (VHL) protein complex that includes VHL, Cullin-2, Rbx1, the Elongins B and C and an E3-ubiquitinase (Kibel et al., 1995; Iwai et al., 1999; Kamura et al., 1999). Subsequent ubiquitination targets the HIF-α subunits to proteasomal degradation (Huang et al., 1998; Ivan et al., 2001). The function of PHDs depends on oxygen as a substrate. Hence, during hypoxia, hydroxylation does not occur and the VHL complex cannot bind to HIF-α. HIF-α is stabilized, enters the nucleus, binds ARNT and p300 as a transcriptional co-activator and participates in the regulation of gene expression.

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It has been shown that hypoxic exposure of mice stabilizes and activates HIF1A and HIF2A in the retina (Grimm et al., 2002; Thiersch et al., 2009). However, it is unknown whether activation of HIF transcription factors is sufficient to protect photoreceptors from light induced degeneration as observed after hypoxic preconditioning. Inactivation of VHL prevents degradation of HIF-α subunits and leads to constitutively active HIFs even in normoxia (Haase et al., 2001). Long-term stabilization of HIF transcription factors during normoxia in the retina might provide information about the contribution of HIF to neuroprotection by hypoxic preconditioning. However, overactivation of HIFs may lead to excessive blood vessel growth, a phenomenon particularly important in tumor biology (Senger et al., 1983). In addition, a recent report shows that untimely activation of HIFs during retinal development causes a severely disturbed retinal vasculature (Kurihara et al., 2010).

To activate HIFs in normoxia, we generated a *Vhl* knockdown mouse using the Cre-lox system with Cre-recombinase being expressed under the control of the α-element of the Pax6-promotor. This promoter is active as early as E10.5 and leads to the deletion of floxed sequences in the distal retina and in the iris, but not in the lens or the retinal pigment epithelium (Marquardt et al., 2001). In accordance with a recent report, we show that development of the retinal vasculature is severely disturbed early during development in the *vhl* knockdown mouse (Kurihara et al., 2010). In addition we show that embryonic vasculature persists into adulthood and that the *vhl* knockdown also results in severe cell death in the ageing retina with an increase of *Casp-8* and *Casp-1* expression and induction of HIF-independent stress response pathways.

Materials and methods

Mice and genotyping

Mice were treated in accordance with the regulations of the Veterinary Authority of Zurich and with the statement of 'The Association for Research in Vision and Ophthalmology' for the use of animals in research. 129S-Vhlh^{tm1jae}/J-mice (from now on referred to as *vhl*$^{flox/flox}$ mice), which have *lox*P sites flanking exon 1 and part of the promoter of the *Vhl* gene (Haase et al., 2001) were purchased from Jackson Laboratory (Bar Harbor, USA). To generate retina-specific *Vhl* knockdown mice, *vhl*$^{flox/flox}$ mice were crossed with mice expressing Cre-recombinase under the control of the α-element of the Pax6-promotor (α-Cre), which leads to the deletion of floxed sequences in the distal retina and in the iris (Marquardt et al., 2001). Breeding pairs were established to generate *vhl*$^{flox/flox}$;*α-cre* and *vhl*$^{flox/flox}$ control littermates. The following primers were used to detect wild-type (wt) and *Vhl*-flox alleles: forw (5′-TGAGTATGGGATAACGGGTTGAAC-3') and rev (5'-AGAACTGACTGACTTC CACTGATGC-3′). The wt allele was identified as a 125-bp and the *Vhl*-flox allele as a 317-bp long fragment on a 1.5% agarose gel. Presence of the α-Cre transgene was tested by PCR using the following primer pair: forw (5′-AGGTGTAGAGAAGG CACTTAGC-3′) and rev (5′-CTAATCGCCATCTTCCAGCAGG-3′). Amplification resulted in a 411-bp fragment.

To detect excision of floxed sequences in the *Vhl* gene, genomic DNA from retina was isolated and tested by PCR using the following primers: forw_un-excised (5′-CTGGTACCCACGAAACTGTC-3′), forw_excised (5′-CTAGGCACCGAGCTTAG AGGTTTGCG-3′) and rev_both (5′-CTGACTTCCACTGATGCTTGTCACAG-3′). The excised allele was identified as a 260-bp and the un-excised allele as a 460-bp fragment.

RNA, DNA and protein preparation

Retinas were removed through a slit in the cornea and immediately frozen in liquid nitrogen. Total RNA was prepared using the RNeasy RNA isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's directions including a DNase treatment to digest residual genomic DNA. Equal amounts of RNA were used for reverse transcription using oligo(dT) primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA).

For retinal genomic DNA isolation, 10 µl of the homogenate prepared for RNA isolation (RNeasy RNA isolation; see above) was processed with the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's directions but without proteinase K treatment and heating.

For protein isolation retinas were homogenized by sonication in 100 mM Tris/HCl, pH 8.0, and analyzed for protein content using Bradford reagent. The 4× Laemmli buffer was added and samples were heated for 10 min at 75 °C before loading onto an SDS–PAGE gel for Western blotting (see below).

Semi-quantitative real-time polymerase chain reaction (PCR)

Relative quantification of cDNA was done by semi-quantitative real-time PCR using the LightCycler 480 SybrGreen I Master kit, a LightCycler 480 instrument (Roche Diagnostics, Basel, Switzerland) and specific primer pairs (Table 1). Three animals per time point were analyzed in duplicates and normalized to *β-actin* using the Light Cycler 480 software (Roche Diagnostics, Basel, Switzerland). Values of experimental retinas were expressed relative to the first time point tested, which was set to 1. Significance of differences between expression levels at specific time points in wild-type and knockdown mice was tested using an unpaired Student's *t*-test.

Western blotting

Standard SDS–PAGE (10%) and Western blotting of 40 µg of total retinal extracts were performed. For immunodetection, the following antibodies were used: anti-HIF1A (#Nb100-479 Novus Biologicals, Cambridge, United Kingdom, 1:1000), anti-HIF2A (#Nb100-122, Novus Biologicals, 1:1000), anti-pSTAT3 (#9131 Cell Signaling Technology, 1:500), anti-STAT3 (#9132 Cell Signaling Technology, 1: 1000), anti-GFAP (MAB302 Chemicon/Millipore, Billerica, USA, 1:500), CASP1, CASP3, CASP8 (all kindly provided by Peter Vandenabeele, Ghent University, Belgium, all 1:10000), CASP9 (#9504, Cell Signaling Technology, 1:500) and anti-ACTB (#A5441 Sigma, St. Louis, MO, USA, 1:5000). Blots were incubated overnight at 4 °C with primary antibodies followed by a 1-hour incubation at room temperature (22 °C) with HRP-conjugated secondary antibodies. Immunoreactivity was visualized using the Western Lightning Chemiluminescence reagent (Perkin-Elmer, Boston, MA, USA).

Confocal scanning laser ophthalmoscopy (cSLO)

cSLOs were obtained according to previously reported procedures (Seeliger et al., 2005). Briefly, mice were anaesthetized by a subcutaneous injection of ketamine (66.7 mg/kg) and xylazine (11.7 mg/kg). Pupils were dilated with tropicamide eye drops (Mydriaticum Stulln, Pharma Stulln, Stulln, Germany) in anesthesia. cSLO imaging was performed with a Heidelberg Retina Angiograph (HRA I) equipped with an argon laser featuring two wavelengths (488 nm and 514 nm) in the short wavelength range and two infrared diode lasers (795 nm and 830 nm) in the long wavelength range. The 488-nm and the 795-nm lasers were used for fluorescein (FL) and indocyanine green (ICG) angiography, respectively. FL angiography was performed using a subcutaneous injection of 75 mg/kg body weight fluorescein-Na (University pharmacy, University of Tuebingen, Germany), and ICG angiograhy following an s.c. injection of 50 mg/kg body weight ICG (ICG-Pulsion, Pulsion Medical Systems AG, Munich, Germany).