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Elimination of signaling by the luteinizing hormone receptor reduces ocular VEGF and retinal vascularization during mouse eye development

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Abstract

Purpose/Aim: VEGF dysregulation is implicated in the pathogenesis of retinopathy of prematurity (ROP). Identifying the factors that contribute to VEGF regulation during normal retinal vascularization is the key to ROP prevention. Currently, physiologic hypoxia is thought to be responsible for retinal VEGF regulation in utero. However, a potential hormonal contribution to VEGF regulation during eye development has not been fully investigated. The placental hormone, human chorionic gonadotropin and the pituitary hormone, luteinizing hormone (LH) induce VEGF expression in several tissue types. Both of these gonadotropins activate the same LH receptor (LHR) in the human body; LHRs are expressed in the retina. In this study, we aimed to show that LHR signaling participates in VEGF regulation in the developing eye.

Methods: When offspring from breeding pairs of LHR knockout mice (lhrkos) reached 21 days old, eyes and serum were extracted from homozygote lhrkos and wildtype (WT) siblings. VEGF levels were measured using Mouse VEGF Quantikine immunoassay kit. Retinas were incubated with isolectin for endothelial cell staining, flat mounted and imaged by confocal microscopy. Retinal vascular density was quantified using Imaris software. Some eyes were sectioned and stained for histopathologic review.

Results: Ocular VEGF and retinal vascular volumes were significantly reduced by ~15% in lhrko eyes. Serum VEGF was not changed. The lhrko retinas did not display any anomalies.

Conclusions: We provides evidence that LHR signaling plays a role in VEGF regulation and vascularization in the developing eye. Given that human preterm infants may have altered LHR-activity, the effect of gonadotropins on eye development should be further studied to identify novel strategies for ROP prevention.

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Disclosure of Potential Conflicts of Interest: Zietchick Research Institute (ZRI) is a private, (for-profit) research institute and Dr. Tammy Movsas (Founder and Director of ZRI) has a pending patent application for the use of gonadotropins for promoting normal infant development. Other than being a fulltime employee (ocular biochemist) at ZRI, Dr. A Muthusamy has no other financial conflicts to report. Dr. Robert Sigler is the Director of Unit Laboratory of Animal Medicine at University of Michigan and has no financial or other potential conflicts to report.

Keywords

VEGF; developing eye; eye development; ROP; retinopathy of prematurity; luteinizing hormone

Introduction:

Retinopathy of prematurity (ROP), a leading cause of pediatric visual disability, afflicts preterm infants of all races, ethnicities and socioeconomic groups; infants of very low birthweight (<1250 g) are at highest risk.(1) Vascular endothelial growth factor (VEGF) dysregulation is an important factor in the pathogenesis of ROP. (2–4) Low levels of systemic (and presumably intraocular) VEGF in the first weeks of life are associated with early stage ROP. (3, 5, 6) Inadequate retinal vascularization (from early stage ROP) is theorized to be responsible for retinal ischemia which in turn, promotes VEGF over-expression. (2–4) The high levels of retinal VEGF stimulates the sprouting of pathologic vessels, the hallmark of late stage (proliferative) ROP. (2–4) Thus, VEGF in the developing eye needs to be carefully choreographed such that its levels do not fall too low nor rise too high.

What factors drive VEGF regulation during eye development? The answer to this holds the key to ROP prevention. The conventional theory is that “physiologic hypoxia” (term coined by Chan-Ling & Stone) is responsible for retinal VEGF induction in utero. (7) Indisputably, oxygen levels influence intraocular VEGF production.(8, 9) However, the potential additional contribution of hormones to VEGF regulation during physiologic retinal vascularization has not been fully studied.

The placental hormone, human chorionic gonadotropin (hCG) and the pituitary hormone, luteinizing hormone (LH) are both known to induce VEGF expression and promote blood vessel formation in several gonadal tissue types.(10–13) Both of these gonadotropins activate the same LH receptor (LHR) in the human body.(11) Surely, these hormones must also play non-gonadal roles since LHRs have been identified in several non-gonadal organs including the eyes.(14–18) In fact, we have recently shown a strong correlation between LH and VEGF in mammalian eyes.(19) In addition, we have also shown that LH is reliably present in adult human vitreous fluid.(20) In this study, we utilize neonatal LHR knock outs (lhrkos) (15) to demonstrate that the elimination of LHR signaling reduces retinal VEGF and vessel density in the developing mouse eye.

MATERIALS AND METHODS

Animal Model

Breeding pairs of heterozygous luteinizing hormone receptor knockout (lhrko) mice [B6;129X1-*Lhcgr*^{tm1Zmlei/J}] (15) were procured from Jackson Laboratory (Maine, USA), and bred at the University Laboratory of Animal Medicine (ULAM) at University of Michigan (UM). All procedures were approved by the UM Committee on the Use and Care of Animals and all animal treatment was consistent with ARVO Statement for the Use of Animals in Ophthalmic and Visual Research.

When offspring from the breeding pairs reached 21 days of age (post-natal age of ocular maturation in the mouse)(21), eyes and serum were extracted from homozygote lhrkos and wildtype (WT) siblings and processed for VEGF and retinal vascular density quantitation. No heterozygous lhrko eyes were included. Four additional eyes were extracted from young adult (10 week old) lhrkos and WT siblings and fixed in 10% buffered neutral formalin, sectioned at 4 micron thickness and stained with hematoxylin and eosin (H&E) for histopathologic review by a board-certified animal pathologist.

Quantitation of mouse VEGF-A

Dissecting vitreous from small, neonatal mouse eyes may introduce a source of error (if the vitreous is not 100% separated from the lens or retina). To ensure more uniformity of our samples, we utilized homogenized, whole mouse eyes. Whole mouse eyes (10 lhrkos, 10 WT; mixed gender in each group) were extracted with ice cold Stuart's buffer, and the whole eye supernatant was used for analyte quantification. VEGF-A levels in the whole eye supernatant and serum were measured using Mouse VEGF Quantikine immunoassay kit (MMV00, R&D Systems, Minneapolis, MN) following the manufacturer's instructions. Total protein was measured by using Micro BSA protein assay kit (Thermo Fisher, Catalogue # 23235). VEGF levels were normalized to total protein levels.

Retinal whole mount immunofluorescence

8 WT and 9 lhrko eyes (WT and lhrko eyes of mixed gender) were removed and fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, trimmed of extraneous tissue followed by removal of the anterior segment and lens. The neural retina was then detached and immunolocalization of retinal vessels was performed as previously described (22) In brief, retinas were incubated with isolectin GS-IB4 (for endothelial cell staining) from Griffonia simplicifolia conjugated with *Alexa Fluor*TM 488 (Thermo Fisher) for 3 days at 4°C followed by extensive washes with TBST for 24 h. The retinas were flat mounted on glass slides with Prolong Gold anti-fade mounting media (Invitrogen), and imaged by a Nikon A1-Spectral confocal microscope A confocal Z-stack of 10 images from the center of each lobe were collected over a depth of 5 μ m and projected as one image. Volume of the isolectin GS-IB4 signal (representing vascular density) in the retinas was quantified using Imaris software (Bitplane, South Windsor, CT).

Statistical Analysis

Mann Whitney U test was utilized to assess the statistical significance between conditions. using Prism 4.0 (GraphPad Software, San Diego, CA); $p < 0.05$ was considered as significant.

Results:

Whole eye VEGF was significantly reduced by 15.3% [U(10,10)=13, $p < 0.05$] and retinal vascular density was significantly reduced by 15.9% [U(8,9) =13, $p < 0.05$] in 21-day-old lhrko pup eyes compared to WT. (Fig 1) However, serum VEGF was not significantly different between groups. [U(15,10)= 79]. (Fig 1) Review of H&E stained slides of adult lhrko and WT retinas demonstrated apparent normal development in all retinal layers; there

was no evidence of hypoplasia, thinning, or disorganization of nuclear or photoreceptor layers in either the *lhrko* or WT eyes. (Fig 2)

Whole eye VEGF measurements were also performed on 14 week old WT and *lhrko* eyes (collected for a different study) were not significantly different between male and female groups [U(8,18)=72]. Mean ocular VEGF (pg/total protein): males 12.9 (SD 2.0); females 13.5 (SD 3.0). [Of note, in our breeding colony, twice as many female *lhrkos* are born than male *lhrkos*. We compensated for the small number of available male *lhrko* eyes by performing the VEGF assay on an equal ratio of *lhrko*:WT eyes in each gender group (male group= 4 *lhrkos* + 4 WTs; female group = 9 *lhrkos* +9 WTs)

Discussion:

To the best of our knowledge, we are the first to demonstrate that LH receptor (LHR) signaling participates in retinal development. We show a ~15% reduction in ocular VEGF levels with a corresponding reduction (of same magnitude) in retinal vascular density in neonatal, genetically-modified mice with no LH receptors (homozygous *lhrkos*). We examined mouse eyes at post-natal day 21 (P21) since this is the age of mouse eye maturity. (21)

Of important note, this study was conducted at normoxia; thus, the observed decrease in retinal vascular volume is not caused by any abnormal fluctuation in O₂ level. In future work, we plan to examine the interaction between reduced LHR signaling and oxygen-induced retinopathy, the standard model of proliferative ROP. The lack of any difference in serum VEGF between *lhrkos* and WTs suggests that the relationship between LHR signaling and VEGF production is organ-specific.

In humans, the same LH receptor is activated by two related hormones (LH and hCG); therefore, it is possible that both LH (from the fetal pituitary) and hCG (from the placenta) contributes to LHR signaling in the human fetus. Premature human birth leads to the early separation of infants from their exposure to placental hCG. Thus, altered levels of gonadotropins may potentially exist in preterm infants and possibly account for problems with retinal vascularization. Because mice produce only LH (and do not produce an hCG equivalent)(11), the *lhrko* mouse model does not fully represent the potential role of LHR signaling in human organogenesis.

Histopathologically, the *lhrko* retinas do not display any apparent anatomic abnormalities. This suggests that LHR signaling is not a requirement for the development of the various components of the mouse retina. However, this does not rule out subtle retinal defects that are not visible on H&E staining. In fact, the electroretinograms of adult *lhrkos* show a moderate impairment of cone-mediated responses. (20) In agreement with this finding, LHRs in the retina are most densely expressed in cone photoreceptor cells.(14, 18) The visual impairment in adult *lhrkos* may represent residual impairment from anomalous development during eye maturation and/or perhaps, LHR signaling plays an ongoing role in visual processing beyond development. This latter possibility is supported by a strong correlation of VEGF and LH in healthy, adult mammalian eyes.(19)

Because gonadal organs in *lhrko* mice are severely underdeveloped, it is challenging to accurately determine the sex of *lhrkos* in the first weeks of life. Therefore, our groups contained unsexed, mixed gender mouse pups. However, the VEGF-linked role of LH in the eye, appears not to be related to gender. In bovine, porcine and non-diabetic human eyes, LH and/or VEGF levels are not significantly impacted by gender. (19, 20) In this current study, we also did not find any gender-related VEGF difference in (WT/*lhrko*) adult mouse eyes. Thus, a potential difference in gender ratios between groups is unlikely to have mediated our reported results.

Despite dramatically elevated serum LH levels, the male and female gonads of homozygous *lhrkos* are severely hypoplastic (15); this suggests that the LH hormone does not exert any effect when the LH receptor is not present. Because only *lhrko* homozygotes (with no LH receptors) were included in this study, ocular LH would not have impacted the findings and thus, was not measured.

All in all, we have provided evidence that LHR signaling likely plays a physiologic role in VEGF regulation and retinal vascularization in the developing eye. Given that human preterm infants may have altered LHR-activity, the participation of gonadotropins in eye development should be further studied to identify novel strategies for ROP prevention.

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Figure 1. Knockdown of LH-R expression results in the reduction of retinal vascular density and ocular VEGF levels in LHR-KO mice relative to WT mice.

A). Representative images under 10X (left panel) and 40X (right panel) of IB4 stained retinal vasculature of WT and LHR-KO mice; 40X image on the right panel is a magnified portion of the inset in 10X image. **B).** Vessel volume of WT and LHR-KO mice; **C).** Mean ocular VEGF protein levels of WT and LHR-KO mice; **D).** Mean serum VEGF protein levels of WT and LHR-KO mice.

Figure 2. Representative images of retinal cross section of wild type (WT) and LH receptor knockout (LHRKO) mice stained with H&E. All layers of both WT and LHRKO retinas appear normal.

GCL: ganglion cell layer; *IPL*: inner plexiform layer, *INL*: inner nuclear layer; *OPL*: outer plexiform layer; *ONL*: outer nuclear layer; *OIS*: outer and inner segments.