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Organ specific optical imaging of mitochondrial redox state in a rodent model of hereditary hemorrhagic tel-angiectasia-1

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Abstract

Hereditary Hemorrhagic Telangiectasia-1 (HHT-1) is a vascular disease caused by mutations in the endoglin (Eng)/CD105 gene. The objective of this study was to quantify the oxidative state of a rodent model of HHT-1 using an optical imaging technique. We used a cryofluorescence imaging instrument to quantitatively assess tissue metabolism in this model. Mitochondrial redox ratio (FAD/NADH), FAD RR, was used as a quantitative marker of the metabolic status and was examined in the kidneys, and eyes of wild-type and Eng +/- mice. Kidneys and eyes from wild-type P21, 6W, and 10M old mice showed, respectively, a 9% (± 2), 24% (± 0.4), 15% (± 1), and 23% (± 4), 33% (± 0.6), and 30% (± 2) change in the mean FAD RR compared to Eng +/- mice at the same age. Thus, endoglin haploinsufficiency is associated with less oxidative stress in various organs and mitigation of angiogenesis.

Images of FAD for one representative eye (10 months, Eng +/-). Left: volume rendering of half of the eye (with connected optic nerve) including the lens. Right: volume rendering of the eye excluding the lens.

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Keywords

Optical imaging; NADH; FAD; redox ratio; Endoglin; Hereditary Hemorrhagic Telangiectasia-1; oxygen-induced ischemic retinopathy; oxidative stress

1. Introduction

Endoglin (Eng)/CD105 is a type I membrane glycoprotein, which is highly expressed in vascular endothelial cells (EC). Loss-of-function mutations in the human endoglin gene cause a dominant vascular dysplasia known as HHT-1. HHT disease-causing genes encode proteins that modulate transforming growth factor (TGF)- β superfamily signalling in vascular endothelial cells. It has been shown that heterozygous mutations in Endoglin (Eng) and Activin receptor-like kinase 1 (Alk1) cause HHT-1 and HHT-2, respectively [1–7]. Both of these genes are expressed predominantly in endothelial cells [8, 9]. Also mutations in the common down stream mediator of TGF-family signaling [10], Smad4, causes HHT in association with juvenile polyposis which is called JPHT. The majority of HHT patients (>80%) will have mutations in either *Eng* or *ACVRL*, *Eng* mutations being more common (61%) than *ACVRL1* mutations (37%) or *Smad4* (2%) [11]. HHT-1 is associated with frequent nose bleeds, telangiectases, mucosa, and arteriovenous malformations in lung, liver, and brain [1, 12]. Ocular involvement has been reported in patients with hereditary hemorrhagic telangiectasia. Intraocular vascular lesions, including retinal telangiectasia, arteriovenous malformation in the retina, and abnormal choroidal vascular changes were reported [13–17]. Sudden visual loss has been described because of ocular involvement [17], bleeding from kidney and urinary vascular malformations are other complications reported in HHT patients [18, 19]. *Here, the Eng-related hereditary haemorrhagic telangiectasia, HHT-1, is investigated.*

Endoglin functions as a transmembrane accessory receptor for TGF- β [20–22]. Endoglin expression is markedly up-regulated in proliferating EC, playing a crucial role in angiogenesis during development and in adult animals [23–25]. Complete deletion of endoglin is embryonically lethal. Indeed, endoglin knockout mice (Eng $^{-/-}$) die *in utero* from cardiovascular defects due to inappropriate remodeling of their mature vascular network [26–29]. Additionally, some of the human vascular defects with endoglin haploinsufficiency are seen in endoglin heterozygote (Eng $^{+/-}$) mice [30]. Endoglin is also over-expressed in EC of healing wounds, embryos, and psoriatic skin [28, 29, 31, 32], suggesting that it is an endothelial proliferation marker. Various angiogenic processes including regulation of EC proliferation and migration, induction of pericyte differentiation, and production of the basement membrane depend on TGF- β activity. TGF- β regulates angiogenesis through balancing the pro-proliferative and pro-differentiation signaling pathways of EC [33–35]. The impaired ability of these cells under certain diseases conditions [36] contribute to retinal degeneration [37, 38] and vascular disease during diabetes [39, 40]. *However, a better understanding of how EC function in vascular disease is required in order to develop therapies that can be used to influence or control them.*

Oxygen-induced ischemic retinopathy (OIR) in a mouse is a highly reproducible model of angiogenesis *in vivo* and recapitulates the human retinopathy of prematurity condition [41].

In this model, postnatal day 7 (P7) mice are exposed to 75% oxygen for 5 days. During this time, the developing retinal vasculature is highly sensitive to high levels of oxygen. The high oxygen impedes further growth of blood vessels and causes obliteration of the existing vessels. At postnatal day 12, the mice are returned to room air (20% oxygen) for 5 days. During this stage, the lack of sufficient blood vessels causes the retina to become ischemic [41], up-regulating VEGF (Vascular Endothelial Growth Factor) expression and inducing angiogenesis. Unfortunately, these vessels are fragile and leaky, sprout into the vitreous, and hemorrhage, causing further damage to the retina. We have recently shown that endoglin haploinsufficiency results in attenuation of retinal neovascularization during OIR [42].

Reactive oxygen species (ROS) play an important role during angiogenesis, and their aberrant production is linked to retinopathy of prematurity and diabetic retinopathy [43, 44]. In fact, antioxidants inhibit microvascular degeneration in models of diabetes and OIR [44–46]. The cellular mechanisms, which modulate intracellular oxidative state, however, are not fully characterized [47]. To gain detailed insight into the cellular oxidative state, we prepared EC from retina, kidney, and lung of Eng^{+/+} and Eng^{+/-} mice. We recently showed that Eng^{+/-} retinal EC are more resistant to oxidative challenge compared to Eng^{+/+} cells [42]. *How these changes in vascular EC oxidative state translate to the organ specific alterations and the underlying mechanisms, however, need further investigation.*

Fluorescence imaging have been used to probe tissue redox state and energy homeostasis in organs such as the heart [48], brain [49], kidney [50–52], liver [53], skeletal muscle [54], cervix [55], and colon [56], and in breast cancer tumor localization and oxygenation [57, 58]. Tissue metabolic state, which is an indicator of cellular oxygen consumption, can be extracted from fluorescence images of intrinsic fluorophores [59]. Mitochondrial metabolic coenzymes nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂) are the primary electron carriers in oxidative phosphorylation. NADH and FAD (the oxidized form of FADH₂) are autofluorescent and can be monitored without exogenous labels. Our studies have demonstrated that the ratio of these fluorophores, (FAD/NADH), called the FAD redox ratio (FAD RR), is a quantitative marker of the metabolic state of tissue *ex vivo* [60] and *in vivo* [61]. *Here, we investigated the effect of endoglin haploinsufficiency on the FAD RR in different organs of mice spatiotemporally at different conditions (normal and OIR).*

2. Materials and Methods

We evaluated the mitochondrial redox state quantitatively in the *in situ* kidney and eyes using a 3-D fluorescent optical imaging instrument called the cryoimager to detect the oxidative state of the kidney and eyes in Eng ^{+/-} and Eng ^{+/-} OIR mice and their proper controls, Eng^{+/+} and Eng^{+/+} OIR mice. We also examined the oxidative state of retinal and lung EC *in vitro* from Eng ^{+/-} and Eng ^{+/+} mice using quantitative assessment.

2.1 Animal preparation

All experiments were carried out in accordance with 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 Care Committee of the University of Wisconsin School of Medicine and Public Health.

Endoglin heterozygous (Eng +/-) mice were provided by Dr. Dean Lee (University of Utah, St Lake City, UT) and the mice were screened as previously described [28]. These mice were crossed to Immorto mice expressing a temperature-sensitive SV40 large T antigen (Charles River Laboratories, Wilmington, MA) to generate Eng +/- mice which carry the Immorto transgene. The Immorto/Eng +/- mice were identified by PCR analysis of DNA isolated from tail biopsies. The PCR primer sequences were as follow: Immorto-forward: 5'-CCTCTGAGCTATT CCAGAAGTAGTG-3', Immorto-reverse: 5'-TTAGAGCTTTAAATCTCTGTAGGTAG-3'; Neo-forward: 5'-TGCTCTCCATCTGCACGAGAC TAG-3', Neo-reverse: 5'-GAGTTGCTTGTGGTGAA CGCTCAG-3'; Endoglin-forward: 5'-CACAGCTGTA ATCT CAGCACTTG-3', and Endoglin-reverse: 5'-GATTGGATCCATTGTGGTAGCTG-3'. These mice were used for isolation of EC.

To prepare the oxygen-induced ischemic retinopathy organs (eyes and kidneys), the 7-day-old (P7) pups and mothers were placed in an airtight incubator and exposed to an atmosphere of $75 \pm 0.5\%$ oxygen for 5 days. Incubator temperature was maintained at $23 \pm 2^\circ\text{C}$, and oxygen was continuously monitored with a PROOX model 110 oxygen controller (Reming Bioinstruments Co., Redfield, NY). Mice were then brought to room air for 5 days, and then were sacrificed [62].

2.2 Isolation and culture of endothelial cells

EC were isolated from retina and lung of 4-week-old Eng+/+ and Eng +/- immorto mice as previously described [63, 64]. The EC were grown in gelatin-coated culture dishes and maintained as previously described [63, 64]. The intracellular ROS level was determined by dihydroethidium (DHE; Invitrogen/Molecular Probes, Eugene, OR) staining. For DHE staining, EC were plated on fibronectin-coated (5 $\mu\text{g}/\text{ml}$) 4-well chamber slide (4×10^4 cells/well) and incubated for 1 day. Cells were then incubated with 5 μM DHE at 33°C for 20 min. Following incubation, cells were fed with normal growth medium without DHE for 1 h, and rinsed with phosphate buffered saline (PBS; Sigma). Fluorescence images were captured using a Zeiss microscope (Carl Zeiss) in digital format (x200). For the quantitative assessment, fluorescence intensities measured in at least 80 cells/cell type using Image J software (National Institutes of Health, Bethesda, MD). Mean cellular fluorescence intensity of WT control cells was regarded as 100% and relative mean intensity of Eng +/- cells was reported as percentage of control (Mean $\pm$ SEM).

2.3 Freezing and embedding

For the cryoimaging studies, kidneys and eyes were extracted from a total of 4 groups of mice in different ages, Eng +/- and Eng+/+ mice in 21 postnatal day (P21), 6 week (6W), and 10 month (10M) and Eng +/- OIR and Eng+/+ OIR mice in 17 postnatal day (P17). Before fluorescence imaging, the extracted tissue were frozen and embedded as previously described [65].

2.4 Cryoimager

The cryoimager is an automated image acquisition and analysis system consisting of software and hardware designed to acquire fluorescence images of tissue sections. Details of this instrument has been previously reported [65]. For this study, we used a resolution of 50 μm for kidney and 10 μm for eyes in the z direction, which resulted in 300 and 250 z-slices per kidney and eye, respectively.

2.5 Imaging calibration

To compensate for day-to-day variation of light intensity and non-uniformity of the illumination pattern, the calibration method described previously [65] was carried out.

2.6 Image processing

FAD and NADH autofluorescence images (containing 300 slices per kidney and 250 slices per eye) from each group of kidney and eyes were processed using MATLAB. Composite images were created using all of the image slices for each tissue, for both FAD and NADH signals. The ratio of FAD and NADH was calculated voxel by voxel using MATLAB, according to equation (1).

$$\text{FAD Redox Ratio} = \text{FAD RR} = \text{FAD}/\text{NADH} \quad (1)$$

The 3D representation of each tissue was then calculated using z-stacks of slices of the FAD and NADH 3D volume. In this method, a 3-D volume of each FAD and NADH image was rendered. The retina is the target layer to investigate the effect of retinopathy on tissue metabolism and therefore we segmented out the lens, choroidal and sclera layers [66, 67]. Then, the 3-D volume FAD RR of retina was calculated and the corresponding histograms were plotted. The bottom row in figure 1 shows the NADH, FAD and FAD RR rendered volume images of the retinal shell with connected optic nerve. The histogram of the FAD RR values in each group, diseased and normal was created for quantitative comparison between normal and diseased groups. The mean of this histogram was calculated according to Eq. (2).

$$\text{Mean} = \frac{1}{N_x \times N_y \times N_z} \sum_{i=1}^{N_x} \sum_{j=1}^{N_y} \sum_{k=1}^{N_z} \text{tissue_slice}(i, j, k) \quad (2)$$

Where N_x , N_y and N_z are the number of pixels in the x, y and z directions. The voxel size in the z direction is different for eyes (10 μm) and kidney (50 μm) but in the x and y directions, it is the same for both tissues and equal to 10 μm .

2.7 Statistical evaluation of data

Statistical analysis was also carried out for each group of retinas, kidneys, and lungs using a one-tailed Student's t-test with $P < 0.05$ as the criterion for statistical significance.

3. Results

3.1 In vitro results

We examined the levels of reactive oxygen species (ROS) in retinal and lung EC prepared from Eng^{+/+} and Eng^{+/-} mice by DHE staining. Figure 2 shows a 34% decrease in ROS levels detected in Eng ^{+/-} compared to Eng ^{+/+} cells. Eng ^{+/-} lung EC also showed a 25% decrease in cellular ROS levels compared to Eng ^{+/+} lung EC. These results demonstrate that Eng^{+/-} EC exhibit reduced levels of oxidative stress.

3.2 Cryo-fluorescence redox imaging (in situ) results

In the present study, we used the cryo-fluorescence redox imaging method to quantitate the kidney and eye mitochondrial redox state over time. To determine the effect of age and OIR on the FAD RR, mitochondrial redox state was determined on kidneys and eyes from Eng ^{+/-} mice and control littermates at postnatal day 21, 6 weeks, and 10 months of age, as well as kidneys and eyes from Eng ^{+/-} OIR mice and control littermates at postnatal day 17. This method detects changes in the oxidation state of the mitochondrial respiratory chain. Thus, FAD RR can be used as a quantitative marker to evaluate the oxidative stress in affected and normal kidney and eye.

FAD, NADH, and FAD RR 3-D rendered images show changes in the oxidation state of the mitochondria in Eng ^{+/-} and Eng ^{+/+} kidney and eye. Figures 3 and 4 display the 3-D rendering of FAD and NADH fluorescence signals, and their ratio (FAD RR = FAD/NADH) as well as the FAD RR histogram from a representative kidney and eye of each of the three groups (P21, 6W, 10M) of Eng^{+/-} mice in comparison with corresponding controls. The images show a slight decrease in the mean FAD RR in kidneys from P21 Eng ^{+/-} compared to Eng ^{+/+}. This decrease became more significant in the mean FAD RR of kidney from 6W and 10M old Eng ^{+/-} mice compared to their Eng ^{+/+} controls. However, as Figure 4 shows eyes from Eng ^{+/-} mice exhibit a significant decrease in the mean FAD RR in all three age groups compared to Eng ^{+/+} mice.

To better compare the intensity of the FAD RR 3-D rendered images in Eng ^{+/+} and Eng ^{+/-} groups, the histograms of FAD RR for each set of kidneys and eyes were plotted. These histograms display the result in which, in the Eng ^{+/-} group, the FAD RR indicates a more oxidized biochemical state compared to Eng ^{+/+}. The results presented in the histogram indicate that the mean FAD RR of kidney and eye from 6 week-old Eng ^{+/-} mice showed a more significant decrease compared with control mice (Eng ^{+/+}). There is a blue shift in histograms from Eng ^{+/-} versus the Eng ^{+/+} kidney and retina. The histogram shows 9%, 24%, and 15% change in the mean FAD RR in P21, 6W, and 10M kidneys and 23%, 33%, and 30% change in P21, 6W, and 10M retinas, respectively.

We also compared the FAD RR in the kidney and eye during OIR in Eng^{+/-} and Eng^{+/+} mice. Figures 5 and 6 display the 3-D rendering of FAD and NADH fluorescence signals, and FAD RR as well as the FAD RR histogram from a representative kidney and eye of each of the Eng^{+/-} and Eng^{+/+} mice during OIR. The images show a remarkable decrease in FAD signal and therefore a substantial decrease in the FAD RR of both organ, kidney and eye from P17 Eng ^{+/-} OIR compared to Eng ^{+/+} OIR. The presented histograms

demonstrate 29%, and 41% change in the mean FAD RR and also 58% and 37% change in the deviation of FAD RR in P17 kidneys and eye of Eng+/- mice compared to Eng+/+ mice during OIR.

To evaluate the statistical significance of the results, four kidneys and eyes in the P21 groups (Eng +/+ and Eng +/-), ten kidneys and eyes in the 6W groups and two kidneys and eyes in the 10M groups were imaged. Also three kidneys and six eyes from Eng +/+ and Eng +/- mice were studied during OIR. The mean values of the histograms of 3-D rendered images were compared, the results of which are represented in Figures 7 and 8. These figures display a bar graph comparing the mean values of the histograms of 3-D rendered images of kidneys and retinas from Eng +/- mice versus their appropriate control Eng +/+ mice in three different ages as well as Eng +/- versus Eng +/+ mice during OIR at the age of P17.

4. Discussion and conclusion

The studies performed here suggest that mitochondrial oxidative stress can be detected in various organs in the absence of sufficient endoglin expression. Oxidative stress and mitochondrial dysfunction can lead to apoptotic cell death early in the pathology of many kidney, lung, and eye diseases [68–75]. Thus, the measurement of mitochondrial metabolic activity could potentially be an early indicator of organ dysfunction during the disease. Our present method measures fluorescence from both NADH and FAD, allowing calculations of the redox ratio, which has proven to be a powerful diagnostic marker, independent of the number of mitochondria and hemodynamics [50, 65, 76]. Furthermore, this study demonstrates the ability of cryoimaging to measure FAD RR of various organs.

The cryoimaging results for the kidney and eye are consistent. Both affected tissues demonstrated a decrease in tissue FAD signal and, as a consequence, a decrease in tissue FAD RR. This decrease is due to a deficiency in the endoglin protein (Eng +/-), which makes the tissue more resistant to oxidative challenges, yielding a lower FAD RR (24% in kidney and 33% in eye) compared to their control (Eng +/+). However, diminished oxidative response may be responsible for mitigation of angiogenesis in these organs, as we have shown in the eye during OIR [42].

The results from the kidney and eye of Eng +/- show same direction shift in FAD RR compared to Eng+/+ at all three ages. The changes in FAD RR were the lowest at P21 mice, then reached its maximum at 6 weeks, but decreased in the 10 month mice.

To gain further insight into the physiological role endoglin plays during vascular development and neovascularization, we compared the FAD RR as a marker of oxidative stress in the kidneys and eyes of Eng +/- and Eng+/+ mice during OIR. Eng +/- tissue exhibited much lower FAD RR compared to the Eng +/+ (41% in eye and 29% in kidney), which means it is less oxidized. The noticeable change of FAD RR in the eye during OIR is consistent with significant up-regulation of endoglin expression in retinal vasculature, and enhanced retinal neovascularization in the Eng +/+ mice. These mice showed higher FAD RR than Eng +/- mice which exhibits little or no neovascularization during OIR [42].

The changes of FAD RR in both eyes and kidneys from Eng+/- in comparison with Eng+/+ presented greater value during OIR compared to normal condition. Due to high oxygen-mediated vessel obliterations, the percentage of FAD RR changes in P17 mice during OIR is even bigger than 6W old mice during normal condition. The histogram of FAD RR in the eyes and kidneys from Eng +/+ OIR is broader than that of Eng+/- OIR (Figures 5 and 6). The standard deviation of FAD RR in the eyes of Eng+/+ was 37% higher than that of Eng+/- and the histogram of FAD RR in kidneys showed even greater increase, 58%. These results correspond to influence of enhanced ocular and renal neovascularization in the Eng +/+ mice during OIR.

The results show a significant difference in the mean redox ratio of kidneys and eyes with and without endoglin haploinsufficiency in both room air and OIR conditions. As expected, the decrease in the FAD redox ratio of the Eng +/- kidneys and eyes indicated an overall decrease in oxidative stress.

The *in vitro* result of retinal and lung ECs demonstrated a noticeably higher level of oxidative stress in the Eng +/+ EC compared to Eng +/- EC. This is consistent with the result of cryo imaging studies. The cell experiments confirmed that EC from retina and lung from the Eng+/- mice exhibit 34% and 25% decrease in the level of oxidative stress compared to the EC from the retina and lung of Eng +/+ mice, respectively. These experiments demonstrated that tissue and EC in the different organ of Eng +/- mice have indeed significantly less oxidative stress. In addition, compared to kidney, eyes showed the greatest change in the oxidative stress.

Our data is more consistent with the notion that some level of reactive oxygen species is essential during angiogenesis, and perhaps other cellular activity. A significant decrease in nitric oxide (NO) level, consistent with decreased expression of endothelial nitric oxide synthase (eNOS) and inducible NOS (iNOS) in Eng +/- EC, was observed in our previous study [42]. Our study here shows reduced oxidative stress in Eng+/- tissues and vascular EC. This is also consistent with reduced sensitivity Eng+/- EC compared to Eng+/+ when challenged under oxidative stress [42]. Therefore, uncoupling of eNOS is an unlikely possibility since Eng+/- EC express less eNOS and exhibit lower oxidative stress. How endoglin deficiency contributes to reduced level of oxidative stress and eNOS expression/function is beyond the scope of the present study and subject of future investigation.

These presented results suggest that endoglin functions maintain the oxidative state in several organs. These results will inspire future studies that will allow to establish a diagnostic tool for early detection of different diseases and a correlation between predicted target organ damage and changes in the FAD RR levels, as a marker of oxidative stress caused by disease progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Images of NADH, FAD and FAD RR for one representative eye (10 months, Eng+/-). The top panel is the result of volume rendering of half of the eye (back of the eye which includes retinal layer) with the lens part and the bottom panel is the volume rendering of the retina shell of the eye.

Figure 2. Reduced levels of ROS in EC from Eng^{+/-} mice. A: A representative fluorescence image of DHE staining of retinal and lung EC from Eng^{+/+} and Eng^{+/-} mice (x200). B: The quantitative assessment of DHE staining. Please note that DHE fluorescence was significantly decreased in Eng^{+/-} EC compared to Eng^{+/+} EC (****P*<0.001). Scale bars represent 20 μm.

Figure 3. Representative 3-D reconstructions of a kidney from each of the three groups (P21, 6W, 10M) and their related histograms. From left to right, images shown are FAD, NADH, and FAD redox ratio (FAD RR). The histogram shows 9%, 24%, and 15% change in the mean FAD RR in P21, 6W, and 10M kidneys.

Figure 4.

Representative 3-D reconstructions of a eye from each of the three groups (P21, 6W, 10M) and their related histograms. From left to right, images shown are FAD, NADH, and FAD redox ratio (FAD RR). The histogram shows 23%, 33%, and 30% change in the mean FAD RR in P21, 6W, and 10M eyes.

Figure 5.

Representative 3-D reconstructions of kidneys from P17 *Eng*^{+/+} mice (first panel) and P17 *Eng*^{+/-} mice (second panel) during OIR and related histogram (bottom panel). From left to right, images shown are FAD, NADH, and FAD redox ratio (FAD RR). The histogram shows 29% and 58% change in the mean and deviation of FAD RR.

Figure 6.

Representative 3-D reconstructions of eyes from P17 Eng^{+/+} mice (first panel) and P17 Eng^{+/-} mice (second panel) during OIR and related histogram (bottom panel). From left to right, images shown are FAD, NADH, and FAD redox ratio (FAD RR). The histogram shows 41% and 37% change in the mean and deviation of FAD RR.

Figure 7.

Bar graph plot comparing the mean values of the histograms of 3D rendered images from Eng +/- kidneys and their respective controls in three different ages and also from Eng +/- and Eng +/+ kidneys at the age of P17 during OIR.. The results show a significant difference between kidneys at P17, P21, 6 week, and 10M old Eng +/- and Eng +/+ mice (**p < 0.01, ****p < 0.0001) during both normal condition and OIR. P values were obtained from a one-tailed student's t-test.

Figure 8.

Bar graph plot comparing the mean values of the histograms of 3D rendered images from Eng +/- eyes and their respective controls in three different ages and also from Eng +/- and Eng +/- eyes at the age of P17 during OIR. The results show a significant difference between eyes from 17 days, 21 days, 6 week and 10M old Eng +/- and Eng +/- mice (* $p < 0.05$). The most significant change was demonstrated by 6 week old Eng +/- compared to Eng +/- mice and by 17 day old Eng +/- compared to Eng +/- mice during OIR (**** $p < 0.0001$). P values were obtained from a one-tailed student's t-test.