Choroidal Involution Is a Key Component of Oxygen-induced Retinopathy

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PURPOSE. Retinopathy of prematurity (ROP) is a major cause of visual handicap in the pediatric population. To date, this disorder is thought to stem from deficient retinal vascularization. Intriguingly, functional electrophysiological studies in patients with mild or moderate ROP and in the oxygen-induced retinopathy (OIR) model in rats reveal central photoreceptor disruption that overlies modest retinal vessel loss; a paucity of retinal vasculature occurs predominantly at the periphery. Given that choroidal circulation is the major source of oxygen and nutrients to the photoreceptors, the authors set out to investigate whether the choroidal vasculature system may be affected in OIR.

METHODS. Rat models of OIR treating newborn animals with 80% or 50/10% alternated oxygen level for the first two postnatal weeks were used to mimic ROP in humans. Immunohistology staining and vascular corrosion casts were used to investigate the vessel layout of the eye. To investigate the effect of 15-deoxy-D12,14-PGJ2 (15d-PGJ2); a nonenzymatic product of prostaglandin D2) on endothelial cells, in vitro cell culture and ex vivo choroid explants were employed and intravitreal injections were performed in animals.

RESULTS. The authors herein demonstrate that deficient vascularity occurs not only in the retinal plexus but also in the choroid. This sustained, marked choroidal degeneration is specifically confined to central regions of the retina that present persistent photoreceptor loss and corresponding functional deficits. Moreover, the authors show that 15d-PGJ2 is a prominent contributor to this choroidal decay.

CONCLUSIONS. The authors demonstrate for the first time pronounced, sustained choroidal vascular involution during the development of ROP. Findings also suggest that effective therapeutic strategies to counter ROP should consider choroidal preservation.

R etinopathy of prematurity (ROP) is the leading cause of severe visual impairment and blindness in neonates and children in North America and Europe.1 Our current understanding of the pathogenesis of ROP does not fully explain the associated functional deficits. ROP is thought to originate as a result of an initial phase of defective vascularization foremost at the periphery of the retina.2 The oxygen-induced retinopathy (OIR) models in rodents has contributed significantly to elucidating the pathogenesis of ROP.3,4 The relatively high oxygen tension to which preterm infants are exposed compared with that in utero5 and a general loss of maternally derived factors6,7 are important components in the genesis of ROP. As the retina develops, rising oxygen demand in avascular regions (i.e., the periphery) drives abnormal and exaggerated angiogenesis aberrantly into the vitreous, predisposing to retinal detachment.7 Using multifocal electroretinogram (mERG) (which allows analysis of distinct regions of the retina) evaluation, one detects that even in mild forms of ROP (wherein the risk of retinal detachment is diminished), the most pronounced functional deficits are found in central regions, whereas peripheral zones are less affected.8 Of particular relevance one notes abnormal cone function,9 which contributes to disorders of color discrimination,10 defective central rod function and associated dark adaptation,11,12 and increasingly recognized foveal dysplasia.13-15 Hence, photoreceptor deficits seem to predominate in the central retina, where the extent of retinal vessel dropout in ROP is relatively small compared with that observed in the periphery. Similar observations are detected in OIR in rats, wherein the retinal vasculature is substantially more compromised in the periphery than in the center,16,17 whereas the reverse is seen for photoreceptor density and function.18-20

A possible explanation for the discrepant topography in photoreceptor integrity and retinal vascular deficits may lie in the choroidal vascular system. The choroid is the prominent supplier of oxygen and nutrients to the outer retina (i.e., the photoreceptor layer).21 The choroid begins to develop before...
the retinal vasculature, albeit its maturation continues to progress after birth.\textsuperscript{22} Importantly, choroidal circulation in newborn animals is significantly controlled by high local levels of prostaglandin D\textsubscript{2} (PGD\textsubscript{2}), which, in turn, curtails the autoregulation of choroidal blood flow in response to hyperoxic exposure,\textsuperscript{23} resulting in increased oxygenation of the outer retina.\textsuperscript{24} Notably, a high density of PGD\textsubscript{2} receptors is found in the choroid.\textsuperscript{25,26} The ensuing oxidative environment, as that seen in the immature subject,\textsuperscript{27,28} facilitates the nonenzymatic conversion of PGD\textsubscript{2} into the electrophile 15-deoxy-\Delta\textsubscript{12,14}-PGJ\textsubscript{2} (15d-PGJ\textsubscript{2}),\textsuperscript{29,30} which participates in redox cell signaling\textsuperscript{31-35} and is cytotoxic to endothelial cells under high concentrations.\textsuperscript{36,37}

To date, the involvement of the choroid in ROP has not been addressed. We hereby demonstrate an unprecedented pronounced, sustained degeneration of the choroid in models of ROP, largely through the actions of 15d-PGJ\textsubscript{2}.

**METHODS**

**Animal Models of Oxygen-Induced Ischemic Retinopathy**

We used rat models of OIR because they reproduce the predominant peripheral deficit vasculization and central outer retinal neurostructural and functional deficits observed in human ROP. In contrast, the mouse OIR model presents a central vaso-oblitration.\textsuperscript{40} Newborn Sprague-Dawley rats (Charles River, St. Constant, Quebec) were used in accordance with the Hôpital Sainte-Justine Animal Care Committee-approved protocol and in adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. According to Penn et al.,\textsuperscript{16} newborn rats were placed in alternated room air (21% O\textsubscript{2}). Details of OIR/ROP models have been described.\textsuperscript{16,41} Given the centro-temporal predominance of cones in rodents,\textsuperscript{83} the centers of mfERG stimuli were aligned accordingly. mfERG responses were evoked using a multifocal system (Veris 5.1b; Electro-Diagnostic Imaging, Inc., Redwood City, CA) connected to a video camera display positioned at the center of the eye as viewed on the monitor. The stimulus matrix consisted of 37 hexagons presented in a pseudorandom (m-sequence) fashion at a frame rate of 75 Hz for a total recording time of 4 minutes.

**Retina Flatmount**

Detailed methods on flatmount preparation were described previously.\textsuperscript{42} TRITC-conjugated lectin (Sigma-Aldrich, St. Louis, MO) was used to reveal the vasculature layout.

**Ocular Tissue Preparation**

Animals were perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA). For histology sections, eyes were immediately collected, dehydrated by alcohol, and embedded in paraffin. Sagittal sections measuring 7 μm were cut by microtome (RM 2145; Leica, Wetzlar, Germany). Eyes for cryosectioning were further fixed in 4% PFA overnight, rinsed with water, and air dried. To exclude corrosion casts with incomplete perfusion, only casts with completely filled iris vessels were used in the analysis. Retinal vasculature was removed with forceps. The casts were then fixed on a metal stub and coated with gold/platinum for scanning electron microscopy (JSM 35; JEOL Ltd., Tokyo, Japan).

**Corrosion Casts and Scanning Electron Microscopy**

The protocol for resin preparation and perfusion was previously described by Krucker et al.\textsuperscript{45} 65% resin was perfused intracardially. Eyes were then collected and dissolved with 40% KOH at 40°C overnight, rinsed with water, and air dried. To exclude corrosion casts with incomplete perfusion, only casts with completely filled iris vessels were used in the analysis. Retinal vasculature was removed with forceps. The casts were then fixed on a metal stub and coated with gold/platinum for scanning electron microscopy (JSM 35; JEOL Ltd., Tokyo, Japan).

**Western Blot Analysis**

Protein extracts (40 μg) were loaded, separated by voltage potential, and transferred to polyscreen polyvinylidene difluoride transfer membrane (PerkinElmer, Waltham, MA). Respective proteins were revealed by rabbit anti-cleaved caspase 3 (Cell Signaling, Beverly, MA); rabbit anti-proliferating cell nuclear antigen (PCNA; Abcam, Cambridge, MA); rabbit anti-cleaved caspase-9 (Cell Signaling, Beverly, MA); and mouse anti-β-actin (Novus Biological, Littleton, CO) antibodies.

**15d-PGJ\textsubscript{2} Enzyme Immunoassay**

The extraction and detection of 15d-PGJ\textsubscript{2} from tissues were conducted according to the protocols described in the manufacturer’s instruction of for the 15d-PGJ\textsubscript{2} enzyme-linked immunosorbent assay kit (Assay Designs Inc., Ann Arbor, MI). Four eyes from two animals were pooled together to obtain a sufficient amount of 15d-PGJ\textsubscript{2} for detection.

**Microvascular Sprouting from Choroidal Explants**

Central choroids from juvenile rats were cut into approximately 2 mm × 1 mm pieces, placed in growth factor-reduced basement membrane matrix (Matrigel; BD Biosciences, Franklin Lakes, NJ), and incubated in 37°C and 5% CO\textsubscript{2} for 48 hours before drug administration. Photographs of individual explants were taken before and 36 hours after drug administration. The sprouting covered areas were quantified with ImageJ 1.42q software (Wayne Rasband).

**Cell Viability Assay**

Cell viability of ocular endothelial cells (kindly provided by Pierre Hardy; Sainte-Justine Hospital), porcine brain microvascular endothelial cell cultures (BMECs; Cell Systems, Kirkland, WA; catalog no. CSC 2PB3), human umbilical vein endothelial cells (HUVECs; American Type Culture Collection, Manassas, VA; catalog no. PCS-100-010), astrocytes (Lonza, Walkersville, MD; catalog no. CC-2565), and rat retinal ganglion cells (RGC-5; kindly provided by Adriana DiPolo, University of
Montreal) were determined by 3-(4,5-dimethyl trazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) assay. Twenty-four hours after treatments, cells were starved for 4 hours before incubation with MTT for 3 hours at 37°C. After lysis of cells by acidified isopropanol, the optical densities (ODs) of the supernatants were measured by spectrophotometry at 560 nm and corrected at 690 nm. Cell viability was expressed as OD relative to control.

Flow Cytometry

Annexin V binding was determined on ocular endothelial cells incubated with 15d-PGJ2 (5 μM) for 24 hours. Cells were trypsinized, washed with PBS, and stained with fluorescein conjugated Annexin-V (excitation 488 nm, emission 527 nm) according to the manufacturer’s instructions. Fluorescence intensity was monitored with a flow cytometer (BD Biosciences, Lincoln Park, NJ), and data were analyzed using the accompanying software (Cell Quest; BD Biosciences).

DNA Fragmentation Analysis

Ocular endothelial cells were treated with either 15d-PGJ2 (5 μM) or vehicle control in reduced PBS media (2% DMEM) for 24 hours. Both adherent and floating cells were harvested and concentrated by centrifugation. The protocol for DNA extraction and RNA degradation refers to Kotamraju et al.46 DNA fragmentation was revealed by resolving 10 μg DNA for each sample.

DCFH-DA Assay

The formation of intracellular reactive oxygen species (ROS) was assessed by 2′,7′-dichloro fluorescein diacetate (DCFH-DA).47 Ocular endothelial cells were incubated with 7 μM DCFH-DA for 15 minutes before treatment with either 5 μM 15d-PGJ2 or vehicle for 5 minutes. The fluorescence signal was measured at 525 nm by a microplate fluorescence reader with background fluorescence corrected by the inclusion of parallel blanks.

Intravitreal and Intrapitoneal Injection

15d-PGJ2 (final estimated intraocular concentration based on eye volume of 10−4–5 μM) or vehicle control was injected intravitreally at P0, P3, and P7 to mimic the 15d-PGJ2 elevations in OIR choroids. The choroidal thicknesses of these room air–raised animals were examined at P14. To block 15d-PGJ2 synthesis in the hyperoxic choroid, ibuprofen was administered intraperitoneally (30 mg/kg) twice daily throughout the first week of OIR treatment, and choroidal thickness was examined at P7. Furthermore, 15d-PGJ2 or vehicle control was readministered intravitreally into the ibuprofen–injected OIR animals, as described.

Statistical Analysis

Data are presented as mean ± SEM for all the histograms unless otherwise indicated. Comparisons between groups were made by either independent t-test or analysis of variance (ANOVA) followed by post hoc Bonferroni’s correction for comparison among means. P < 0.05 was considered statistically significant.

RESULTS

Photoreceptor Density and Retinal Function Are Predominantly Altered Centrally in OIR/ROP Models, whereas Retinal Vasculature Degenerates Primarily in the Periphery

Previous studies in OIR models of ROP revealed a robust association between loss of retinal vasculature and inner retinal structure and corresponding functional deficits.19,41,49 However, a central photoreceptor deficit has also been observed in patients who previously had mild or moderate ROP and in OIR rodent models.20 On the other hand, defective vascularization is detected primarily at the periphery of the retina.

We sought to determine whether photoreceptor cell density exhibited an uneven distribution in the rat model of OIR; notably, despite a lack of a distinct macula, eccentricity of cone distribution with central predominance is also observed in rodents.53 In rats exposed alternately to 50% and 10% O2 (OIR/ROP) or exclusively to 80% O2 (OIR) from P0 to P14, the photoreceptor cell count was decreased in the central retina compared with normal–raised rats at P60 (and a similar tendency was detected by P21) (Fig. 1A). The photoreceptor cell count in the periphery of the retina did not differ among the groups of animals; from here on we used the cycled O2 (OIR/ROP) model (Fig. 1B). Correspondingly, mfERG analysis of P60 rats subjected to OIR/ROP revealed pronounced functional changes, primarily in central regions (Supplementary Fig. S1A, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6742/-/DCSupplemental). P1 wave amplitude was significantly attenuated in the fERG (most central responses) in OIR/ROP rats compared with controls, whereas no difference between groups of animals was observed for ring 4 (most peripheral responses) (Supplementary Fig. S1B, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6742/-/DCSupplemental).

Figure 1. Retinal structure is altered in OIR/ROP. (A) Hematoxylin and eosin–stained central and peripheral retinal cross-sections of eyes of P60 rats, raised in normal (21% O2; n = 6), 80% O2 (OIR; n = 4), and 50%/10% alternating O2 (OIR/ROP; n = 4). Scale bar, 25 μm. (B) Corresponding quantification of photoreceptor cell density showing reduced photoreceptor cell counts in the central retina of OIR/ROP animals with no difference in the periphery. Values are mean ± SEM of four to six experiments per group. **P < 0.01 compared with normal. (C) Representative retinal flat mounts from P14 animals (near peak [peripheral] retinal vascular deficiency) raised in room air or subjected to OIR/ROP. Scale bar, 1 mm.
DCSupplemental). On the other hand, even at the peak loss of vessels in the OIR rat model, the central vasculature was largely conserved whereas the amply documented deficiency in the peripheral vasculature was clearly observed (Fig. 1C). Together our results in rat models of OIR revealed predominant central geographic anomalies consistent with those recently documented in this species and in humans with ROP.

**OIR/ROP Is Associated with Central Choroidal Vascular Involution**

Given that photoreceptor disruption overlies a relatively less damaged retinal vasculature zone (i.e., the central retina) in rats subjected to OIR/ROP, we speculated that the choroidal vascular bed, which provides the primary supply of oxygen and nutrients to the outer retina, could be injured. Immuno-histochemistry was performed on sections of the choroid, divided into equal lengths across the ocular globe (central, intermediate [middle], and peripheral) (Fig. 2A). Choroidal vascular thickness of animals at P14 exposed to cycled and constant O2 in the most central region was half that in normoxic controls, was modestly affected in the intermediate zone, and remained unaffected in most peripheral zone (Fig. 2B; Supplementary Figs. S2A–C, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6742/-/DCSupplemental). Indeed, though the choroidal thickness of normoxia-raised rats increased gradually from P0 to P14, the choroidal thickness of O2-exposed animals decreased to reach a plateau as early as P7 consistent with cell death. In contrast, choroidal thickness at the periphery of the ocular globe was unaffected by O2 exposure (Figs. 2C, 2D).

To obtain a more thorough 3-dimensional assessment of the choriocapillary vascular bed, we performed corrosion casts of normal (21% O2) rats and of animals exposed to hyperoxia at P14 (immediately after O2 exposure; Figs. 2E, 2F) and at P60 (Figs. 2G, 2H). Central (but not peripheral) choriocapillary involution was observed at P14 and remained sustained at P60. Lower magnitude scanning electron microscopy images covering the whole ocular vascular casts were used to select completely perfused samples with filled iris vessels (Supplementary Fig. S2D, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6742/-/DCSupplemental). In addition, we witnessed a systematic presence of resin balls in casts of OIR/ROP but not of control animals (Fig. 2I), suggesting microvesSEL leakage. Increased capillary leakage at P14 was indeed confirmed by increased Evans blue leakage in the choroid (Fig. 2I), in line with endothelial disruption.

**Choroidal Involution in OIR/ROP Is Associated with Choroidal Cell Apoptosis and Elevated Levels of 15d-PGJ2**

Central choroidal involution secondary to high oxygen exposure was found to be associated with an early marked increase in cleaved caspase 3 expression indicative of cell death (Figs. 3A, 3B) but not in changes in cell division, as evidenced by the unaffected expression of proliferating cell nuclear antigen (PCNA) (Figs. 3C, 3D); of note, PCNA decreased slightly with age, in line with tissue maturation.

We have previously described that PGD2 is abundant in the choroid and plays a major role in interfering with choroidal blood flow autoregulation in newborn animals, resulting in increased peroxidation. The latter can, in turn, facilitate the nonenzymatic formation of 15d-PGJ2 from PGD2, and 15d-PGJ2 has been shown to be antiangiogenic. We therefore investigated the contribution of 15d-PGJ2 in the choroidopathy of OIR. A twofold increase in 15d-PGJ2 was observed in the central choroid but not in the retina during the first week of 50%/10% alternating O2 exposure (Figs. 4A, 4B), corresponding to approximately 1 μM concentrations in vivo in the choroid; in the peripheral choroid, concentrations of 15d-PGJ2 were hardly detectable (<10 nM).

**15d-PGJ2 Provokes Apoptotic Death of Endothelial Cells by Induction of Oxidative Stress**

We determined endothelial cytotoxic properties for 15d-PGJ2. 15d-PGJ2 caused the dose-dependent apoptotic death of various endothelial cell types with LD50 of 1.4 μM for human umbilical vein endothelial cells, 1.6 μM for human umbilical vein endothelial cells (HUVECs), and 2.3 μM for brain microvascular endothelial cells (BMECs) (Figs. 4C–E). In contrast, astrocytes and neuron-type retinal ganglion cells (RGC-5) were minimally toxic on exposure up to 10 μM 15d-PGJ2. The effects of 15d-PGJ2 on endothelial cells were independent of its potential actions through the PPAR-γ or the PGD2 receptor DP1 because the PPAR-γ agonist ciglitazone, PGD2, and the DP1 agonist BW245c did not decrease cell viability, whereas the PPAR-γ antagonist GW9662 did not modulate 15d-PGJ2-induced cell death (Supplementary Figs. S3A, S3B, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6742/-/DCSupplemental). Analogous effects were observed on endothelial sprouting of choroidal explants placed in basement membrane matrix (Matrigel; BD Biosciences) (Supplementary Fig. S3C, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6742/-/DCSupplemental). 15d-PGJ2 caused a concentration-dependent inhibition of choroidal explant endothelial sprouting (Fig. 4F) such that concentrations >5 μM induced the degeneration of existing (baseline) endothelial sprouting (Fig. 4G). Under high magnification, the sprouting endothelial cells from vehicle-treated choroidal explants showed characteristic tube formation within the basement membrane matrix, whereas 15d-PGJ2 (5 μM) caused cell shrinkage and nuclear condensation consistent with apoptosis (Fig. 4H); this was confirmed by immunoreactivity to activated caspase 3 (Fig. 4I). Similarly, ocular endothelial cells treated with 15d-PGJ2 (5 μM) exhibited a robust sevenfold increase in Annexin V immunoreactivity determined by flow cytometry (Figs. 4J, 4K; P < 0.001).

Because of the important role of oxidant stress in the pathogenesis of ROP and OIR, the link between the formation of ROS and apoptotic processes, and the induction of ROS by higher doses of 15d-PGJ2, we evaluated whether 15d-PGJ2 caused an increase in endothelial cell ROS. 15d-PGJ2 (5 μM) caused a significant increase in intracellular ROS generation, assessed by DCFH-DA oxidation (Fig. 4I; P < 0.0001). Moreover, the precursor to the anti-oxidant glutathione N-acetylcysteine (NAC) prevented 15d-PGJ2-induced cytotoxicity (Supplementary Fig. S3, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6742/-/DCSupplemental). Collectively, the data reveal that 15d-PGJ2 levels are elevated in the central choroid of animals subjected to OIR/ROP and that 15d-PGJ2 induces the apoptosis of ocular endothelial cells through the induction of oxidant stress.

**Choroidal Microvascular Involution Associated with OIR/ROP Is Prevented by Cyclooxygenase Inhibition and Reproduced by 15d-PGJ2**

We next determined whether 15d-PGJ2 could induce central choroidal involution in vivo. Intravitreal injections of 15d-PGJ2 (intravitreal concentration, ~5 μM) caused significant involution of the central choroid from a thickness of approximately 27 μm to 15 μm at P14, without affecting the peripheral choroid (Figs. 5A, 5B) (note that the choroid at P0 measured ~20 μm and thickened with early age [Fig. 2C]). This pattern of choroidal involution by 15d-PGJ2 is similar to that seen in...
FIGURE 2.  Choroidal vasculature degenerates in OIR/ROP. (A) Lectin (red) and DAPI (blue) staining of retinal cross-sections of normal (21% O₂) and OIR/ROP (50%/10% alternating O₂) rats at P14. White bars: central, intermediate (middle), and peripheral regions of the eye. Scale bar, 0.5 mm. (B) Representative confocal microscopy images used to assess choroidal thickness. Arrows: choriocapillary layer. Vertical bars: average choroidal thickness. Scale bar, 20 µm. (C, D) Quantification of choroidal thickness during the first 2 weeks of postnatal development. Values are mean ± SEM of four to six experiments per group; *P < 0.05, **P < 0.01, ***P < 0.001 (two-way ANOVA with Bonferroni correction) compared with corresponding values in normal (21% O₂) rats. (E, G) Representative images of scanning electron microscopy of choriocapillary corrosion casts and quantification at P14 (F) and P60 (H) of OIR/ROP suggest a pronounced and persistent loss of central choriocapillary vessels into adulthood. Values are mean ± SEM of six experiments per group. **P < 0.01, ***P < 0.001 compared with corresponding values for normal O₂-raised rats (two-way ANOVA with Bonferroni correction). Scale bar, 20 µm. (I) Images of central choroidal casts of normal and OIR/ROP animals at P14 show ball-shaped resin leaks (arrow) between the retinal and choroidal vascular beds. Scale bar, 100 µm. (J) Vessel leakage was quantified by intracardiac perfusion with Evans blue and revealed a higher rate of vessel leakage within choroidal beds. Values are mean ± SEM of seven experiments per group. *P < 0.05 compared with room air-raised controls.
OIR/ROP animals (Figs. 2B, 2C). More relevantly, we made similar choroidal measurements in OIR/ROP (P0–P7) in rats treated or not treated with the prostaglandin synthase inhibitor ibuprofen. Ibuprofen preserved central choroidal thickness, whereas the addition of 15d-PGJ2 reproduced central (but not peripheral) choroidal involution (Figs. 5C, 5D); selective PGD2 synthase inhibitors are not available. Analysis of retinal function at P30 of animals treated with ibuprofen without or with 15d-PGJ2 was consistent with choroidal changes, such that the a-wave derived from photoreceptors (predominantly supplied by choroidal circulation21) was (largely) preserved by ibuprofen and depressed by 15d-PGJ2 (Fig. 5E). As reported, OIR/ROP also depressed the b-wave,18–20 which was protected by ibuprofen (Supplementary Fig. S4, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10–6742/-/DCSupplemental).

The central vulnerability of the choroid to OIR/ROP and 15d-PGJ2 suggests the regional presence of susceptibility factors. Because 15d-PGJ2 is cytotoxic by inducing oxidant stress (Fig. 4L; Supplementary Fig. S3C, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6742/-/DCSupplemental) and because glutathione, a key component of the redox state, modulates the cytotoxic effects of 15d-PGJ2 in a redox potential-dependent manner,55 we measured at P3 the baseline (in normoxia-raised rats) ratio of oxidized to reduced forms of glutathione (GSSG/GSH) in the central and peripheral choroid. As anticipated, the ratio of GSSG/GSH was greater in the center than in the periphery of the choroid (31.1% ± 1.1% in the central choroid of P3 pups compared with 26.2% ± 1.7% in the peripheral choroid; \( P < 0.05, n = 6 \)), suggesting a more oxidized environment in the central choroid, making it more vulnerable to oxidative damage.

**DISCUSSION**

Deficient central photoreceptor density and function are features of ROP and OIR animal models.8,10–12,18–20,56,57 The outer neuroretina is highly metabolically active21 and presents the greatest oxygen consumption, supplied by the high blood flow choroid, per unit weight of any tissue58. The current view that ROP uniquely affects the retinal vascular bed fails to explain photoreceptor deficiency since this vasculature exclusively perfuses the inner regions of the retina (from the ganglion cell layer to the outer plexiform layer).59 In addition, the geographic discrepancy between the extent of photoreceptor function loss (central) and that of retinal vascular degeneration (peripheral) (Fig. 1) prompted us to explore the integrity of the choroidal vascular bed as a possible explanation to account for this inconsistency. The present study is the first to identify marked choroidal involution (confined to the central portions of the eye) in OIR models that remains detectable in the young adult animal; this degeneration would explain the central functional visual deficits associated with this disorder. We also uncovered 15d-PGJ2, an electrophile product of PGD2 (the most abundant prostaglandin produced in the choroid23), as a key mediator of this choroidal vaso-obliteration through the induction of oxidant stress.

We noted in an OIR/ROP model a striking involution of the central choroid (while the periphery remained intact) that directly overlaid the reduced central photoreceptor density. In normal newborn animals, a predominance of both larger caliber central capillaries and a denser microvascular network favors increased oxygenation. In addition, the inability of the choroid of newborn animals to autoregulate its blood flow...
FIGURE 4. 15d-PGJ2 accumulates in the choroid of OIR/ROP and induces endothelial cell apoptosis through the induction of oxidative stress. (A, B) Immunoreactivity of 15d-PGJ2 production in the central choroid (A) and central retina (B) during the first 2 weeks of postnatal development. Values are mean ± SEM of four experiments per group. *P < 0.05 compared with age-matched normoxic controls. (C) MTT assay demonstrating that 15d-PGJ2 dose dependently reduces endothelial cell viability on three cell lines: human ocular microvascular endothelial cells, HUVECs, and BMECs. In contrast, astrocytes and neuron-type retinal ganglion cells (RGC-5) were minimally affected on exposure up to 10 μM 15d-PGJ2; n = 6 per cell type. Treatment of ocular endothelial cells with 5 μM 15d-PGJ2 induced chromatin condensation, as determined by nuclear staining with Hoechst 33342 (D; scale bar, 100 μm) and DNA laddering visualized by agarose gel electrophoresis (E). Representative micrographs (F) and quantification (G) of choroid sprouting after 36 hours of exposure to different concentrations of 15d-PGJ2. Scale bar, 2 mm. High-magnification micrographs of endothelial cell tube formation in basement membrane matrix (H) and immunohistochemical staining of activated caspase 3 (green) and DAPI (blue) (I) in vehicle versus 5 μM 15d-PGJ2 treatment. Arrows: normal chromatin staining in vehicle-treated controls or activated caspase 3 associated with chromatin condensation after exposure to 5 μM of 15d-PGJ2. Scale bar, 100 μm. (J) Flow cytometric analysis of 15d-PGJ2–induced cell death (5 μM for 24 hours) shows an increase in Annexin V–positive cells with no propidium iodide (PI) indicative of apoptosis (K). Values are mean ± SEM of three experiments. **P < 0.001 compared with corresponding vehicle-treated controls. (L) Ocular endothelial cells preloaded with the fluorescent ROS probe DCFH-DA produce more ROS after treatment with 5 μM 15d-PGJ2, as monitored by dichlorofluorescein (DCF) fluorescence intensity. Values are mean ± SEM, expressed as a percentage of control from three separate experiments, each performed in quadruplicate. ***P < 0.0001 compared with vehicle-treated control.
results in further excessive O2 delivery and peroxidation to the choroid and outer retina24 at a time in ontogenesis when anti-oxidants are not fully developed.27 Accordingly, the more vigorous oxidative environment of the central choroid may explain the greater susceptibility of this region to vaso-obliteration. In line with neuroretinal19,41 and cerebral endothelial cells,63 choroidal endothelium was also found to be particularly vulnerable to hyperoxic exposure from P0 to P14 (Figs. 2C, 2E, 2F), which induced choroidal involution as of the first postnatal week. In contrast, glia and neurons, including photoreceptors, are less sensitive to hyperoxia, and ensued neurotoxicity is delayed to the second postnatal week.19,41,63 This relatively increased susceptibility of endothelial cells is consistent with the greater sensitivity of the endothelium to oxidant stress.62 The impact of the choroidal microvascular degeneration observed on local hemodynamics also seems profound: the approximately 50% decrease in cross-sectional choroidal vessel diameter in OIR models of ROP would result in a severe 16-fold decrease in choroidal blood flow62 and corresponding O2 and nutrient delivery to the outer retina.54 Hence, the choroidal vaso-obliteration observed is consistent with the relative hypoxia of the central retina of rats subjected to OIR despite greater preservation of the overlying retinal vasculature.17 It is thus tempting to postulate that central photoreceptor disruption is likely secondary, at least in part, to the pronounced choriocapillary decay. However, one cannot exclude a contribution of direct effects of oxidant stress on the neural retina independently of the vascular degeneration17; ibuprofen fully prevented choroidal vascular degeneration in OIR/ROP (Figs. 5C, 5D), as reported for the retinal vasculature,65 but incompletely prevented retinal functional deterioration (Fig. 5E; Supplementary Fig. S4, http://www.iovs.org/lookup/ suppl/doi:10.1167/iovs.10-6742/-/DCSupplemental).

Choriocapillary involution associated with OIR has not previously been described. Although the choriocapillary network was not found to be compromised on wet flatmounts of newborn dogs exposed to 4 days of hyperoxia,66 the duration of O2 exposure was probably insufficient to cause decay consistent with our observations wherein no difference in choroidal thickness was detected after 4 days of hyperoxia in rats (Fig. 2C). Evidence for choroidal changes in humans has been limited by available anatomic specimens, partly because of the survival rate of preterm infants; on the other hand, rare isolated reports have documented choroidal involution in ROP.67 Choriocapillary degeneration has also been observed in humans and in animal models of a distinct form of ischemic retinopathy, notably secondary to diabetes.68,69

A salient finding in this study is that the mediators of the choroidal decay in OIR originate from the tissue’s vasoactive modulators. Of these, PGD2 has previously been established as the principal prostaglandin in the choroid, and its abundance in newborn compared with juvenile animals has been shown to impede choroidal blood flow autoregulation,25,27 resulting in increased peroxidation.24 Under these conditions the high levels of PGD2 provide the substrate for the generation of 15d-PGJ2,54,55,70 which exerts proapoptotic effects in endothel-
GSSG/GSH ratio), the difference may only partially explain the markedly greater vulnerability of the central choroid to 15d-PGJ2 relative to the peripheral choroid (Figs. 5A, 5B); the precise mechanisms of action of 15d-PGJ2 on cytotoxicity have yet to be fully determined.

Interestingly, the choriocapillary involution associated with OIR remained sustained into young adulthood, although there was some resolution because the choriocapillary network was augmented from birth to adulthood (Figs. 2G, 2H). In contrast, retinal revascularization is rapid because it resumes by the middle of the second postnatal week.41 Mechanisms for these differences are not clear but may involve vascular endothelial growth factor (VEGF), which participates in retinal revascularization.75 In line with this inference, VEGF expression was found to be decreased in the chorioids of 1-month-old rats subjected to OIR/ROP (Supplementary Fig. S5, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6742/-/DCSupplemental). Correspondingly, the benefits of antioxidants on the visual acuity of human infants have been reported.72 However, although the central choroid contains a more oxidized state relative to the peripheral choroid (based on GSSG/GSH ratio), the difference may only partially explain the markedly greater vulnerability of the central choroid to 15d-PGJ2 relative to the peripheral choroid (Figs. 5A, 5B); the precise mechanisms of action of 15d-PGJ2 on cytotoxicity have yet to be fully determined.

The present study focused on damage to the choroid, which supplies the outer retinal structure, notably the photoreceptors. This does not take away from the importance of the inner retinal disorders in OIR and ROP, which is, for the most part, beyond the scope of this article. Nonetheless, inner retinal function and structure are considerably compromised in OIR and ROP, as we and others have reported.14,17,75 and are even more compromised in central regions.8,20 Indeed, exposure to hyperoxia for the first 14 postnatal days leads to marked thinning of the inner nuclear layer (predominantly bipolar cells) and its adjoining synaptic outer and inner plexiform layers and a decreased amplitude of the corresponding b-wave.19,41 The profound loss of the penetrating retinal vascular network,76 along with the marked involution of the main source of O2 supply to the developing, incompletely vascularized retina, namely the choroid (Fig. 2), are likely to contribute to inner neuroretinal structural decay in OIR.

In summary, this study demonstrated an unprecedented sustained, pronounced central choroidal involution in OIR, which, given the importance of this ocular vascular bed, would explain associated outer retinal structural and functional deficits. It is also tempting to speculate that in patients with regressed ROP, the thinning of the central choroid may perturb and elongate the focal distance and contribute to myopia,77 the most common complication of ROP. Finally, we expose 15d-PGJ2 as the prominent mediator of central choroidal involution in ROP/OIR, thus introducing a target pathway for pharmacologic intervention.

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References


