The Vasoneuronal Effects of AT1 Receptor Blockade in a Rat Model of Retinopathy of Prematurity

Kate M. Hatzopoulos,1 Kirstan A. Vessey,1 Jennifer L. Wilkinson-Berka,2 and Erica L. Fletcher1

1Department of Anatomy and Neuroscience, University of Melbourne, Victoria, Australia
2Department of Immunology, Monash University, Melbourne, Victoria, Australia

Retinopathy of prematurity (ROP) is an iatrogenic condition of premature infants caused by the use of high oxygen therapy to aid survival.1 It is a major cause of visual impairment in these children. Although the incidence of ROP varies from country to country,2 in developed nations such as Sweden, as many as 73% of infants with a gestational age less than 27 weeks develop some form of ROP, with severe ROP affecting 35%.3 Despite major improvements in clinical care of neonates, the incidence of ROP has not declined.4–6 Retinopathy of prematurity is caused by the effects that high levels of oxygen, required for infant survival, have on blood vessel development; blood vessel growth is attenuated in response to high oxygen, rendering the peripheral retina avascular, and reducing the extent of blood vessel growth into the deep layers of the retina.3 On returning to normal air, the retina is rendered hypoxic because of the lack of normal vessel development, and consequently pathological neovascularization develops that can ultimately lead to retinal detachment and blindness.5 Rodent models of ROP are referred to as oxygen-induced retinopathy (OIR) owing to the development of retinal pathology during postnatal development.4–6

In addition to the extensive vascular pathology, OIR in animals and ROP in humans is characterized by significant changes in the integrity of neurons.4,7 Scotopic thresholds are reduced in infants with ROP8 and photoreceptor dysfunction has been reported even after mild ROP has resolved.9,10 In addition, OIR in rodents is also associated with the loss of amacrine cells, especially the AII amacrine cell,11 a key interneuron in the rod pathway. Alterations in horizontal cells and neurochemical changes across a variety of inner retinal interneuron in the rod pathway. Alterations in horizontal cells and neurochemical changes across a variety of inner retinal

Keywords: retinopathy of prematurity, oxygen-induced retinopathy, electroretinogram, valsartan, neovascularization, hypoxia
Valsartan and Oxygen-Induced Retinopathy

be more complex than hitherto considered. Our previous work showed that blockade of AT$_1$ receptors partially prevents the loss of amacrine cells and neurochemical changes across a variety of retinal neuronal subtypes in the rat model of OIR. What is not clear from these previous studies is whether treatment with an AT$_1$ receptor antagonist can restore retinal function.

The aim of this study was to examine whether treatment with the AT$_1$ receptor antagonist, valsartan, prevents retinal dysfunction in a rat model of OIR. Valsartan was used to treat animals in this study because it is known to reduce retinal neovascularization$^{16,20}$ and partially ameliorate neuronal loss in a rat model of OIR$^{12}$ and has been shown previously to prevent retinal dysfunction in a rat model of diabetes.$^{22}$ We evaluated the effect of a range of doses of valsartan on blood vessel growth, microglial activation, and retinal function. Oxygen-induced retinopathy was characterized by aberrant blood vessel growth, microgliosis, and significant attenuation of retinal function, both photoreceptor function and also inner blood vessel growth, microgliosis, and significant attenuation of retinal function.

### Methods

#### Animals

Animal experimental procedures were performed in accordance with the animal ethics committees of both The University of Melbourne and Monash University and also complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were housed in a controlled 12-hour light/dark cycle environment and given rat chow and water ad libitum.

#### OIR and Valsartan Treatment

Sprague-Dawley (SD) rats were obtained from the Animal Resources Centre (Perth, Western Australia) and housed in the Alfred Medical Research and Education Precinct (AMREP) animal house in Melbourne, Australia. At birth, pups and their mothers were randomly assigned to control and OIR groups. Oxygen-induced retinopathy was induced as previously described.$^{17}$ Newborn SD pups were exposed to 80% ± 2% O$_2$ (<2% CO$_2$) for 22 h/d and to room air for 2 h/d from P0 to P11. Gas composition was monitored every 4 to 8 hours (ML 205; AD Instruments Pty. Ltd., Australia). Flow rate was maintained at ~2.5 L/min to minimize any variations in CO$_2$ and O$_2$ tensions caused by metabolic activity. At postnatal day (P)12, pups were transferred to a normoxic environment until P18. Age-matched controls were raised simultaneously in room air from P0 to P18.

### Electroretinography

Retinal function was assessed in P18 animals using the flash ERG as previously described.$^{10,22,24}$ The ERG is a gross retinal potential that provides information about the function of cohorts of different classes of retinal neurons, including photoreceptors (called the a-wave) and postreceptorial neurons (called the b-wave and oscillatory potentials). In addition, the function of neurons forming the rod pathways was segregated from those of the cone pathways by presenting two rapid light stimuli.$^{25,26}$

Following overnight dark adaptation (>12 hours), the rats were anesthetized under red light illumination ($\lambda = 650$ nm) with an intramuscular injection of ketamine hydrochloride (Ketamil; Troy Laboratories, NSW, Australia: 60 mg/kg) and xylazine (Ilium xylazine; Troy laboratories, NSW, Australia: 5 mg/kg). The anterior eye was anesthetized with topical proparacaine hydrochloride (Alcaine 0.5% Alcon, Frenchs Forest, New South Wales, Australia) and pupils dilated (tropicamide, 0.5% Alcon Frenchs Forest, New South Wales, Australia). Each rat was placed on a heat pad, an Ag/AgCl electrode was placed on the cornea to record the ERG waveforms and a reference electrode was placed in the mouth. Responses were amplified (gain × 5000: ~3 dB at 1 Hz and 1 kHz, ADInstruments, Castle Hill, New South Wales, Australia) and digitized at 10 kHz over a 200-ms epoch.

Electroretinogram responses were elicited by a photographic flash unit (Mecalbitz 60CT4; Metz, Zirndorf, Germany) presented using a Ganzfeld sphere. The light source was attenuated with calibrated neutral-density filters (Wratten; Eastman Kodak, Rochester, NY, USA) from 1.5 to 2.1 log cd/m$^2$ and a twin flash paradigm was used to isolate rod and cone photoreceptor contributions of the ERG waveforms.$^{24,27,28}$ Two flashes were presented in succession with a short interstimulus interval (ISI) of 0.8 seconds and the rod contribution was isolated by digital subtraction of the cone response from the initial mixed response (rod + cone).

### Rod Photoreceptor Function

The initial downward deflection of the ERG is referred to as the a-wave and is thought to reflect photoreceptor function.$^{25}$ When it is modelled, it is referred to as the PIII.$^{25}$ In this study, the rod ERG was isolated and the leading edge of the a-wave was modelled by a modified computational description of the phototransduction cascade as described by the equation: PIII $(t,i) = \left(1 - \exp\left[-IS(t-td)/Rmax\right]\right)$ $\times$ S$^3$. The component PIII gives the summed photocurrent as a function of luminous exposure, $i$ (cd/m$^2$) and time, $t$ (seconds). The component $Rmax$ (μV) is the saturated amplitude of the PIII, $S$ is its sensitivity (m$^2$·cd$^{-1}$·s$^{-1}$), which represents the gain of the phototransduction process, and $td$ (seconds) is a brief delay that accounts for recording latencies between the stimulus and response start.
Rod Postphotoreceptor Function

The PII (b-wave) and the oscillatory potential (OP) response are thought to reflect inner retinal function, especially depolarizing bipolar function (b-wave/PII) and amacrine cells (OPs). The postphotoreceptor PII component was derived by digitally subtracting the modelled photoreceptor PII from the raw waveform. The PII waveform was modelled using an inverted gamma function so that its maximum amplitude (μV) and implicit time (time to peak in milliseconds) could be described for the response to the highest stimulus intensity 2.1 log cd/m². Oscillatory potentials were isolated by subtracting the modelled PII from the raw data waveform. Three major OP peaks (OP2, OP3, and OP4) could consistently be isolated at the highest luminosity exposure and the amplitude (μV) and the implicit time (time until the maximum response was reached, ms) from these three OPs were assessed and expressed as the summed amplitude and also summed implicit time for comparison between treatment groups.

Cone Postphotoreceptor Function

In view of the small number of cones present in the rodent retina, the cone a-wave was too small for reliable extraction. The cone PII (b-wave) was analyzed by measuring the maximum amplitude (μV) from the prestimulus baseline to the peak of the waveform, and time to peak (implicit time; ms) of the cone b-wave, termed the cone PII.

Immunohistochemistry

Following ERG recording, animals were culled with an overdose of sodium pentobarbital (Nembutal; Merial Australia, NSW, Australia: 72 mg/kg). Eyes were enucleated and the anterior segment and vitreous were removed. The posterior eyecups were then fixed for 30 minutes in chilled 4% paraformaldehyde in 0.1M phosphate buffer (PB). Eyes were then washed three times in PB and cryoprotected in graded sucrose solutions (10%, 20%, and 30% in PB). The eyecups were embedded in a commercial solution (Tissue-Tek O.C.T. Compound; Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands) frozen and sectioned vertically at 12 μm with a cryostat maintained at a temperature of −20°C. Sections were collected on glass slides coated with polylysine and stored at −20°C until required. The remaining eyecups were used as retinal flatmounts.

In order to assess the effect of valsartan on angiogenesis and microglia, retinal flatmounts and vertical sections were processed for fluorescent labeling. Blood vessels were labelled using the lectin Bandeiraea simplicifolia BS-I Isolecitin B4 (IB4) FITC conjugate (diluted 1:75, catalogue number L2895; Sigma-Aldrich, St. Louis, MO, USA). Microglia were labeled (IB4) FITC conjugate (diluted 1:75, catalogue number L2895; Sigma-Aldrich, St. Louis, MO, USA). Microglia were labeled with BS-I Isolecitin B4 (IB4) FITC conjugate (diluted 1:75, catalogue number L2895; Sigma-Aldrich, St. Louis, MO, USA). Microglia were labeled (IB4) FITC conjugate (diluted 1:75, catalogue number L2895; Sigma-Aldrich, St. Louis, MO, USA). Microglia were labeled with BS-I Isolecitin B4 (IB4) FITC conjugate (diluted 1:75, catalogue number L2895; Sigma-Aldrich, St. Louis, MO, USA).

DAPI (diluted 1:3000; Invitrogen) for 1 hour. Flatmounts and vertical sections were then cover-slipped in antifade mounting medium prepared using Mowiol (Polysciences, Inc., PA, USA). Confocal micrographs were taken using a confocal microscope (Zeiss LSM 5; Zeiss Microscopy, Stuttgart, Germany) equipped with an argon and a helium-neon laser. Using ×20 magnification air and ×40 magnification oil immersion objectives, images were captured at a resolution of 1024 × 1024 pixels using image browser software (Zeiss LSM; Zeiss Microscopy). Fluorescence filters were used (Alexa TM 594/ CY3: excitation at −568 nm, emission filter 605/32; Alexa TM 488/FITC: excitation at −488 nm, emission filter 522/32) in order to differentially visualize the fluorophores. Red and green fluorescence channels were scanned as separate images and appropriate adjustments for black levels, brightness and contrast applied using a commercial graphics editing program (Adobe Photoshop CS2 version 9.0; Adobe Systems, Inc., San Jose, CA, USA).

Blood Vessel Profile and Microglial Number Analysis

Entire transverse retinal sections located near the optic nerve labelled with IB4 or rabbit anti-IbA1 were used to analyze the number of blood vessel profiles or microglia respectively. An overview of each section was captured by tile scanning using a ×20 magnification air objective. Blood vessels and microglia from control, OIR, sham-valsartan (10 mg/kg/d), OIR-valsartan (10 mg/kg/d), sham-valsartan (40 mg/kg/d), and OIR-valsartan (40 mg/kg/d) rats (n = 6 per group) were analyzed. Beginning at the periphery, each retinal section was divided into four equal parts such that, based on eccentricity, parts 1 and 4 were considered “peripheral” and parts 2 through 3 were considered “central.” Blood vessel lumen profiles were counted from entire transverse retinal sections according to their location in the retina. Vessels considered part of the superficial plexus were located in the GCL, the intermediate plexus at the inner (proximal) edge of the inner nuclear layer (INL), and the deep plexus at the outer edge (distal) of the INL. In OIR rats, there were also the extraretinal vessels located in the vitreous. All vessel profiles and microglia counts were normalized per length of retinal section (mm) and also analyzed based on eccentricity.

Microglial Morphological Analysis

To assess for morphological changes, microglia were imaged using a ×40 magnification oil objective with 12-μm optical thickness sections collected as a Z-stack and the image collapsed to a maximum intensity projection for analysis. A total of 134 microglia were analyzed from six control rats, 235 microglia from six OIR rats, 142 microglia from six sham valsartan (40 mg/kg/d) rats and 179 microglia from six OIR valsartan (40 mg/kg/d) rats. Microglial activation was assessed based on the area of the soma and area of their dendritic spread. As a means to assess microglial activation, the ratio of somal area to dendritic area was determined.

Statistical Analysis

The compilation and manipulation of all data was completed using spreadsheet software (Excel; Microsoft Corp., Redmond, WA, USA) and all data was graphed and statistically analyzed using commercial software (GraphPad Prism 4; GraphPad Software, San Diego, CA, USA). All data are presented as mean ± SEM. Data was analyzed using two-way ANOVAs to compare the effects of oxygen history (OIR versus control) and treatment (different doses of Valsartan). Post hoc analysis was
performed where appropriate using Tukey’s post hoc test. Differences between control and OIR and/or treatment effects were considered statistically significant at \( P < 0.05 \).

**RESULTS**

**AT\(_1\) Receptor Blockade Reduces Retinal Angiogenesis in OIR**

The aim of this study was to examine whether treatment with the AT\(_1\) receptor antagonist valsartan prevents vascular and neuronal dysfunction in a rat model of OIR. We first evaluated the effect of a range of doses of valsartan on blood vessel growth in control and OIR animals (Fig. 1). Figures 1A, 1D, and 1G shows low magnification micrographs of the retinal vasculature (superficial plexus) at P18 in retinae from control (Fig. 1A), OIR (Fig. 1D), and valsartan-treated OIR animals (40 mg/kg; Fig. 1G). The extent of the retina covered by the vasculature is markedly reduced in OIR compared with control. In addition, in the OIR retinae there are numerous vascular tufts, which appear to be reduced in number by treatment with valsartan. This finding is in accordance with our previous, quantitative studies which show that valsartan reduces neovascular tuft formation and improves vascular extent of the superficial plexus in OIR.\(^{18,20}\) Previous studies have also shown that OIR can have differential effects on the deep compared with the superficial vascular plexuses.\(^{18}\) In the control retina at P18, both the deep (red) and superficial plexus (green) had developed across the entire retina from central (Fig. 1B) to peripheral eccentricities (Fig. 1C). In OIR retinae, the deep and superficial plexus in the central retina appeared well formed and similar to control (Fig. 1E). In contrast, in the peripheral OIR retina, the extent of the retina covered by the deep and superficial plexus was reduced (Fig. 1F). In addition, there were also a number of vascular tufts extending from the superficial plexus into the vitreous in OIR (Fig. 1F; arrows). Valsartan treatment (40 mg/kg) had little effect on the appearance of the deep and superficial plexus in the central retina (Fig. 1H), but appeared to qualitatively reduce the number of vascular tufts in the peripheral retina (Fig. 1H), consistent with our previous findings.\(^{18,20}\)

The effect of OIR on intraretinal and intravitreal vessel growth was quantified by counting the number of blood vessel profiles in vertical sections of control, OIR, and valsartan treated OIR retinae (Fig. 2). Figures 2A through 2C shows representative micrographs of vertical sections of peripheral retinae of control (Fig. 2A), OIR (Fig. 2B), and valsartan-treated OIR retinae (Fig. 2B). The total number of blood vessel profiles in all vascular plexuses (i.e., deep, intermediate, superficial plexuses, and if apparent, intravitreal) in both the central (Fig. 2D) and peripheral retina (Fig. 2E) of control (clear bars) and OIR-rats (black bars) treated with different doses of valsartan is shown. There was no effect of either OIR or valsartan on the total number of blood vessel profiles in the central retina (Fig. 2D). In the peripheral retina, in OIR (sham treated, 0 mg/kg valsartan), there was a significant increase in the number of blood vessel profiles (Fig. 2E; two-way ANOVA for oxygen history \( P < 0.05 \) and valsartan treatment \( P < 0.05 \); control versus OIR for 0 mg/kg valsartan \( P < 0.05 \)) consistent with the pathological intravitreal neovascularization described in previous studies.\(^{17,18,20,55}\) Valsartan treatment was found to reduce the number of blood vessel profiles across the peripheral retina of OIR animals in a dose-dependent fashion. At 10 mg/kg, valsartan reduced and restored the number of blood vessel profiles in OIR to that observed in control rats. At 40 mg/kg, valsartan had a potent anti-angiogenic effect such that there were fewer vessels in the peripheral OIR-rat retina than in control (Fig. 2E; two-way ANOVA for oxygen history \( P < 0.05 \) and valsartan treatment \( P < 0.05 \); control versus OIR for 40 mg/kg valsartan \( P < 0.05 \)). These antiangiogenic effects in the peripheral retina were apparent on both the development of the deep/intermediate plexus (Fig. 3A; two-way ANOVA, control versus OIR for 10 and 40 mg/kg valsartan, \( P < 0.05 \) and the development of neovascular tufts into the vitreous (Fig. 3C; two-way ANOVA, control versus OIR for 40 mg/kg valsartan, \( P < 0.05 \)). The number of vessels in the superficial plexus was unaffected by OIR or valsartan (Fig. 3B; two-way ANOVA for oxygen history \( P > 0.05 \) and valsartan treatment \( P > 0.05 \)). These results suggest that AT\(_1\) receptor blockade inhibits both physiological (deep plexus) angiogenesis and pathological (intravitreal) neovascularization in OIR rats, particularly affecting vessels that form from the main superficial plexus.

**Valsartan Restores the Number and Morphology of Microglia in OIR**

Microglia are cells of immune origin that are known to constantly survey the retina and respond in situations of disease or injury.\(^{56}\) Microglia have also been implicated in vascular development and in the maintenance of neuronal integrity in the central nervous system.\(^{56,57}\) Moreover, release of proinflammatory cytokines from microglia is thought to contribute to the development of vascular pathology in OIR.\(^{58}\) We examined whether microglia were altered in OIR or by valsartan treatment (Fig. 4). Oxygen-induced retinopathy induced a significant increase in the number of microglia in the retina (Fig. 4B) compared with controls (Fig. 4A). When this was quantified, the increase in microglia number in OIR was found to be significant regardless of eccentricity, affecting both the central and peripheral retina (Figs. 4A, 4D, and 4E, respectively; two-way ANOVA for oxygen history \( P < 0.05 \); control versus OIR for 0 mg/kg valsartan \( P < 0.05 \)). Treatment with valsartan, both 10 and 40 mg/kg, reduced the number of microglia in OIR-treated retinae to control levels (representative micrograph, Fig. 4C; quantification, central Fig. 4D and peripheral Fig. 4E; In both cases, two-way ANOVA, control versus OIR for 40 mg/kg valsartan \( P > 0.05 \). This data suggests that there is a greater number of microglia present in the retina in OIR and that this increase in number occurs regardless of eccentricity-dependent changes in vascular pathology and that valsartan inhibits this response.

Microglial activation is observed when microglia change morphology from a stellate, highly ramified cell to one that has an amoeboid shape (process retraction and enlarged cell body).\(^{10,33,54}\) We quantified changes in the morphology of microglia in control and OIR retinae. As shown in Figure 5, although soma size did not change in OIR (Fig. 5A), there was a reduction in process area (Fig. 5B; two-way ANOVA for oxygen history \( P < 0.05 \), control versus OIR for 0 mg/kg valsartan \( P < 0.05 \)). The microglial activation index was then assessed as a ratio of somal:dendritic area, where an increase in this ratio signifies an increase in microglial activation or microgliosis. The activation index was increased in OIR compared with control rats (Fig. 5C; two-way ANOVA for oxygen history \( P < 0.05 \), control versus OIR for 0 mg/kg valsartan \( P < 0.05 \)). When the effects of valsartan on microglial morphology were investigated, valsartan was found to have an unexpected effect. In animals treated with 40 mg/kg valsartan, there was a significant reduction in the soma area of microglia in both control and OIR animals (Fig. 5A; two-way ANOVA for valsartan \( P < 0.05 \), control, 0 vs. 40 mg/kg, \( P < 0.05 \) and OIR, 0 vs. 40 mg/kg, \( P < 0.05 \)). This suggests...
that valsartan may have a direct effect on the morphology of retinal microglia during development in control, normoxic-reared animals, as well as in OIR. Process area was also differentially affected by valsartan treatment such that this parameter was restored to control levels by AT1 receptor blockade in OIR (Fig. 5B; two-way ANOVA for valsartan, \( P < 0.05 \); OIR, 0 vs. 40 mg/kg, \( P < 0.05 \)). When the activation index of microglia was considered (Fig. 5C), valsartan reduced microglial activation in OIR rats compared with sham-treated OIR (OIR: valsartan 0 versus 40 mg/kg, \( P < 0.05 \)).
and restored this morphological index to that seen in untreated controls (Fig. 5C; control, 0 mg valsartan versus OIR, 40 mg valsartan, \( P > 0.05 \)). Overall, these results indicate that OIR is associated with microgliosis across the entire retina and that treatment with valsartan prevents this activation response.

OIR Reduced Function of Rod and Cone-Mediated Pathways and These Responses Were Not Salvaged by Valsartan

Having established that treatment with valsartan prevents angiogenesis in OIR and also restores the number of microglia...
and their activation state, we next examined the effect of valsartan treatment on neuronal function in control and OIR rats. Here, we used the twin flash ERG to assess retinal function of the rod- and cone-mediated pathways. The ERG is a gross retinal potential that enables the function of groups of specific types of neurons in the inner or outer retina to be assessed. Figure 6 shows representative rod ERG waveforms in response to a 2.1 log cd/m² flash, derived from control (grey) and OIR rats (black; Fig. 6A), as well as control and OIR rats treated with valsartan (Fig. 6B). Oxygen-induced retinopathy induced a significant reduction in amplitude of the rod photoreceptor response (Fig. 6C; rod PIII amplitude; two-way ANOVA, oxygen history, $P < 0.05$, and valsartan, $P < 0.05$; control versus OIR for 0 mg/kg valsartan $P < 0.05$) without altering sensitivity (data not shown). In addition, OIR was also associated with a loss of function of inner retinal neurons as indicated by the reduction in amplitude of the rod PII (b-wave/PII amplitude; Fig. 6D; two-way ANOVA, oxygen history, $P < 0.05$ and valsartan, $P < 0.05$; control versus OIR for 0 mg/kg valsartan, $P < 0.05$) and oscillatory potentials (summed OP

**Figure 4.** Changes in microglial number in OIR retinae in response to valsartan. Vertical sections of retinae from control- (A), OIR- (B), and OIR-treated rats with valsartan (C), labeled for the microglial cell marker, Iba1 (red) and cell nuclei, DAPI (blue). Microglial number were quantified per mm of retina in central (D) and peripheral (E) retinae. There was an increase in the number of microglia in both central and peripheral OIR retinae compared with controls. In animals treated with valsartan, the number of microglia appeared similar to controls. Statistical analysis was completed using a two-way ANOVA for oxygen history and dose of valsartan and Tukey’s post hoc test where appropriate for $n = 6$ in all groups. In central and peripheral retinae, there was a significant effect of oxygen history ($P < 0.05$) and valsartan ($P < 0.05$) and post hoc significance between control and OIR retinae is indicated by $* P < 0.05$. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer. Scale bar: 20 μm.

**Figure 5.** Changes in microglial morphology in OIR and treatment with valsartan. Graphs showing the somal area (A), process area (B) and activation index (C) of microglia from control (clear bars), OIR retinae (black bars), and those treated with 40 mg/kg valsartan. The microglial activation index was assessed as a ratio of somal:dendritic area, where an increase in this ratio signifies an increase in microglial activation or microgliosis. Oxygen-induced retinopathy induced a significant reduction in amplitude of the rod photoreceptor response (Fig. 6C; rod PIII amplitude; two-way ANOVA, oxygen history, $P < 0.05$, and valsartan, $P < 0.05$; control versus OIR for 0 mg/kg valsartan $P < 0.05$) without altering sensitivity (data not shown). In addition, OIR was also associated with a loss of function of inner retinal neurons as indicated by the reduction in amplitude of the rod PII (b-wave/PII amplitude; Fig. 6D; two-way ANOVA, oxygen history, $P < 0.05$ and valsartan, $P < 0.05$; control versus OIR for 0 mg/kg valsartan, $P < 0.05$) and oscillatory potentials (summed OP

**Figure 4.** Changes in microglial number in OIR retinae in response to valsartan. Vertical sections of retinae from control- (A), OIR- (B), and OIR-treated rats with valsartan (C), labeled for the microglial cell marker, Iba1 (red) and cell nuclei, DAPI (blue). Microglial number were quantified per mm of retina in central (D) and peripheral (E) retinae. There was an increase in the number of microglia in both central and peripheral OIR retinae compared with controls. In animals treated with valsartan, the number of microglia appeared similar to controls. Statistical analysis was completed using a two-way ANOVA for oxygen history and dose of valsartan and Tukey’s post hoc test where appropriate for $n = 6$ in all groups. In central and peripheral retinae, there was a significant effect of oxygen history ($P < 0.05$) and valsartan ($P < 0.05$) and post hoc significance between control and OIR retinae is indicated by $* P < 0.05$. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer. Scale bar: 20 μm.

**Figure 5.** Changes in microglial morphology in OIR and treatment with valsartan. Graphs showing the somal area (A), process area (B) and activation index (C) of microglia from control (clear bars), OIR retinae (black bars), and those treated with 40 mg/kg valsartan. The microglial activation index was assessed as a ratio of somal:dendritic area, where an increase in this ratio signifies an increase in microglial activation or microgliosis. Oxygen-induced retinopathy induced a significant reduction in amplitude of the rod photoreceptor response (Fig. 6C; rod PIII amplitude; two-way ANOVA, oxygen history, $P < 0.05$, and valsartan, $P < 0.05$; control versus OIR for 0 mg/kg valsartan $P < 0.05$) without altering sensitivity (data not shown). In addition, OIR was also associated with a loss of function of inner retinal neurons as indicated by the reduction in amplitude of the rod PII (b-wave/PII amplitude; Fig. 6D; two-way ANOVA, oxygen history, $P < 0.05$ and valsartan, $P < 0.05$; control versus OIR for 0 mg/kg valsartan, $P < 0.05$) and oscillatory potentials (summed OP
amplitude; Fig. 6E; two-way ANOVA, oxygen history, \( P < 0.05 \) and valsartan, \( P < 0.05 \); control versus OIR for 0 mg/kg valsartan, \( P < 0.05 \). Treatment with valsartan did not prevent retinal dysfunction at any concentration tested. In contrast, valsartan was found to reduce both photoreceptor and inner retinal function in control and OIR at a dose of 40 mg/kg compared with untreated control or OIR-retinae, respectively (two-way ANOVA, \( P < 0.05 \) significant difference of control 0 versus control 40 mg/kg, and OIR 0 versus OIR 40 mg/kg, indicated by \#).

Cone pathway function was also investigated. Representative cone ERG waveforms from control (grey) and OIR rats (black, Fig. 7A), as well as from control and OIR rats treated with valsartan (Fig. 7B) are shown. Significant loss of cone pathway-mediated function was observed in OIR rats compared with controls rats (Fig. 7C; cone PII amplitude; two-way ANOVA, oxygen history, \( P < 0.05 \) and valsartan, \( P < 0.05 \); control versus OIR for 0 mg/kg valsartan, \( P < 0.05 \)). Valsartan did not prevent the loss of cone pathway–mediated function observed in OIR. Instead, as was seen with the rod pathway, valsartan treatment reduced the cone PII amplitude (b-wave) in control rats and exacerbated the loss of the cone PII at doses of 10 mg/kg and 40 mg/kg in OIR (Fig. 7C; two-way ANOVA, \( P < 0.05 \) significant difference of control 0 mg/kg versus control 10 and 40 mg/kg, and OIR 0 mg/kg versus OIR 10 and 40 mg/kg, indicated by \#).

The ERG generates a serial waveform, where a loss in the photoreceptor-mediated a-wave (PIII) is carried through as a loss in inner retinal function (e.g., PII or OP loss). Thus, in order to determine whether OIR preferentially affected outer or inner retinal function of the rod pathway, we calculated the amplitude of the rod PIII, PII, oscillatory potentials, and cone b-wave in OIR as a percentage of the average control responses (Fig. 7D). Although there was a slightly greater loss of the rod PIII amplitude in OIR rats compared with the loss of the rod PIII amplitude, this was not statistically significant (OIR PIII \( \sim 30\% \) reduction versus PII \( \sim 38\% \) reduction, one-way ANOVA, \( P > 0.05 \)). This indicates that the loss of the PII in OIR rats was not greater than what would be expected due to the reduction in photoreceptor input. However, unlike the PII amplitude, the loss of the summed OP amplitude in OIR rats was significantly greater than the loss of the rod PIII amplitude (OIR PIII \( \sim 30\% \) loss versus summed OP \( \sim 52\% \) loss, one-way ANOVA, \( P < 0.001 \)). This suggests that in addition to the photoreceptor disturbance in rats with OIR, there was also a specific inner retinal neuronal dysfunction. A comparison of the rod-mediated with cone-mediated function also revealed differences; the loss in the cone PII was greater than the rod PII suggesting that cone pathway function may be more severely affected in the rat model of OIR than rod pathway function (one-way ANOVA, \( P < 0.001 \)). Next, we evaluated the effect that valsartan treatment had on the function of the inner and outer retina. We reasoned that valsartan might influence inner retinal function in particular, because AT\(_1\) receptors are known to be expressed by neurons of the inner retina, glia, and blood vessels. The amplitude of each waveform from sham valsartan–treated rats was expressed as a percentage of the amplitude recorded from untreated control rats for the rod PIII, PII and OPs (Figs. 8A, 8C, 8E, respectively). While, neither 4 nor 10 mg/kg valsartan significantly affected the rod photoreceptor PIII amplitude, there was a significantly greater loss of the PIII amplitude in sham rats treated with 40 mg/kg/d valsartan compared with the PII of untreated controls (Fig. 8A; PIII \( \sim 27\% \) reduction, one-way ANOVA, \( P < 0.05 \)). The apparent increase in photoreceptor function in animals treated with 4 mg/kg valsartan was not statistically different from untreated animals. This suggests that, at high doses, valsartan has a toxic effect on...
rod photoreceptor function in control animals. Valsartan did not significantly affect the rod PII (Fig. 8C), however the OPs were significantly reduced in control retinae at all doses evaluated (Fig. 8E). This indicates that valsartan alters the normal processing of inner retinal neurons in rat retinae at doses 10 times lower than those that induce photoreceptor dysfunction, suggesting a specific and differential modulation of inner retinal neurons.

The effect of valsartan treatment on retinal function in OIR retinae was also examined. For this analysis, the response amplitudes of rod ERG components determined from valsartan-treated OIR rats was expressed as a percentage of untreated OIR rats at all doses evaluated (Fig. 8B). This indicates that valsartan alters the normal processing of inner retinal neurons in rat retinae at doses 10 times lower than those that induce photoreceptor dysfunction, suggesting a specific and differential modulation of inner retinal neurons.

The effect of valsartan treatment on retinal function in OIR retinae was also examined. For this analysis, the response amplitudes of rod ERG components determined from valsartan-treated OIR rats was expressed as a percentage of untreated OIR rats. This revealed that the rod PIII in OIR rats was significantly reduced following valsartan treatment at the highest dose (Fig. 8B; one-way ANOVA, 40 mg/kg, \( P < 0.05 \)). Thus, valsartan did not prevent photoreceptor dysfunction in OIR rats, but instead potentiated the functional loss observed in OIR alone. Similarly, the rod PII response was reduced in OIR following 40 mg/kg valsartan treatment (Fig. 8D; one-way ANOVA, 40 mg/kg, \( P < 0.05 \)). At all doses of valsartan tested, the rod OPs were attenuated in OIR-treated rats compared with untreated OIR rats (Fig. 8F; one-way ANOVA, 4, 10, and 40 mg/kg, \( P < 0.05 \)). In combination, this suggests that valsartan attenuated rod photoreceptor function and inner retinal function over and above that induced by OIR and that inner retinal function is particularly susceptible to valsartan treatment.

**DISCUSSION**

The main findings of this study were that treatment with valsartan prevented pathological angiogenesis and microglial activation in a rat model of OIR, but did not prevent retinal neural dysfunction. These findings highlight that treatments of OIR and ROP that ameliorate retinal vascular pathology may not necessarily prevent neuronal dysfunction and that more work is required to understand the mechanisms underlying neural loss in OIR.

**Valsartan Reduces Pathological Neovascularization and the Microglial Response in OIR**

There are a wealth of studies examining the effects that antagonists of the RAS have in preventing or reducing retinal vascular pathology. In particular, treatment with ACE inhibitors, \( \text{AT}_1 \) receptor antagonists, or inhibitors of other components of the RAS, such as the prorenin receptor and renin, all reduce angiogenesis in the retina. The results of the current study are in line with this previous work. In OIR specifically, some studies have found that blockade of \( \text{AT}_1 \) receptors can inhibit pathological blood
vessel growth into the vitreous whilst having no effect on physiological intraretinal angiogenesis. In accordance with these previous studies, in the current study valsartan was found to suppress pathological neovascularization in OIR in a dose-dependent manner (Figs. 1–5). Furthermore, valsartan did not affect normal retinal angiogenesis in the sham-treated animals; a likely finding given that at P12, the retinal vasculature is largely established and is less vulnerable to antiangiogenic agents. However, unlike these previous OIR studies, valsartan treatment inhibited physiological blood vessel growth within the intermediate and deep plexus when compared with untreated OIR animals (Fig. 3). The current results imply that the extension of vessels from the main superficial plexus, regardless of the direction of growth, is blocked by valsartan, but that the superficial plexus is unaffected by blockade of the RAS. The mode of action for the antiangiogenic effects of RAS inhibitors is not fully understood. Notably, blockade of the RAS reduces vascular endothelial growth factor (VEGF) expression, especially VEGF164, the isoform of VEGF associated with pathological angiogenesis.

**Figure 8.** The effect of valsartan on different components of the electroretinogram in control and OIR rats. In order to assess the effect of valsartan on the normal retina, (A) the rod PI1, (C) rod PI2, and (E) summed OP amplitude measured in animals treated with 4 mg/kg, 10 mg/kg, and 40 mg/kg valsartan was expressed as a percentage of the average from untreated controls. Notably, valsartan treatment reduced the amplitude of the rod photoreceptor PI1 at 40 mg/kg and OP amplitudes at all doses examined. In order to assess the effect of valsartan on the OIR retina, the percentage loss of the (B) rod PI1, (D) rod PI2, and (F) OPs was calculated by comparing the amplitude measured in OIR animals treated with 4 mg/kg, 10 mg/kg, and 40 mg/kg valsartan compared with untreated OIR rats. Again, valsartan treatment reduced the amplitude of the rod photoreceptor PI1 at 40 mg/kg and the summed OP amplitude at all doses examined. The rod PI1 was also affected at a dose of 40 mg/kg. Statistical analysis between doses of valsartan was completed using a one-way ANOVA and Tukey’s post hoc test and significance between 0 mg/kg and valsartan doses indicated by \( *P < 0.05 \).
Angiogenesis. More work is required to determine the molecular pathways underlying AT\(_1\) receptor regulation of angiogenesis; however, the current study indicates that blockade of the receptor suppresses both pathological and physiological retinal angiogenesis in rats with OIR.

Microglia, the resident immune cells of the retina and CNS, are known to be recruited to sites of damage and release proinflammatory cytokines (e.g., interleukin 1 beta, tumor necrosis factor alpha, interleukin), which potentially contributes to retinal vascular pathology. Previous studies have shown an increase in microglial numbers to be associated with neovascularization in OIR. and this has been found to correlate with increased expression of proangiogenic and proinflammatory factors such as basic fibroblast growth factor, metalloproteinases, and cytokines such as TNF\(\alpha\). In this study, microglial numbers were increased in the retinae of OIR rats and their morphology was altered (Figs. 4, 5), indicative of an activated phenotype. This change in number and morphology of microglia in OIR was found across the entire retina, not specifically at the site of vascular damage in the peripheral retina. Changes in microglial activation and number were observed in central regions, where the retinal vasculature was normal. This suggests that neovascularization may not be the only factor instigating increased microglial activity. It has been suggested that neuronal signaling can modulate microglial activation during ischemic-induced conditions through transmitters such as purines and glutamate, and via the actions of chemokines.

Therefore, microglia may not only be recruited and activated in response to vascular abnormalities but also in response to neuronal damage outside the areas of overt vascular pathology. In OIR, valsartan treatment was found to restore microglial number to baseline (Fig. 4). Previous studies have shown that AT\(_1\) receptor activation recruits microglia in vitro and in several injury models. In addition, Ang II, the principal effector molecule of the RAS, is a key mediator of inflammation and has been shown to evoke cytokine release from microglial cell cultures via activation of the AT\(_1\) receptor. In vivo also, AT\(_1\) receptor blockade in OIR and other ocular diseases has been linked to a reduction in leukostasis and inflammatory mediators, such as monocyte chemoattractant protein-1 and angiogenic factors such as VEGF and angiopeptins. Therefore, use of valsartan to block AT\(_1\) receptors may inhibit Ang II-induced microglial responses in OIR, suggesting a possible positive effect on the overall inflammatory state of the retina. These findings are consistent with the emerging role of the RAS in the activation status of microglia in both the brain and retina.

**Rats With OIR Have Deficits in Both Rod and Cone Pathway Function and Valsartan Treatment Does Not Improve This Loss**

Retinal dysfunction, especially of the rod pathway, has been reported in patients with ROP and in the rat model of OIR. The results of this study show that OIR is associated with significant neuronal dysfunction in both the rod and cone pathways (Figs. 6, 7). Notably, rod photoreceptor and inner retinal function were reduced and there was a significant reduction in cone-mediated function. The reduction in photoreceptor function (P3/a-wave amplitude) could be explained by a reduction in photoreceptor numbers, outer segment length, or the number of cGMP gated channels expressed within photoreceptor outer segments. Previous studies have reported a reduction in outer segment length in the rat and mouse models of OIR, with no change in the number of photoreceptor nuclei. Until recently, it was not clear why photoreceptors were affected in OIR, given that the disease was generally believed to specifically affect the intraretinal blood vessels, while the choroidal vasculature, which supplies the photoreceptors, was largely believed to be intact. However, recent work indicates that the choroid is also negatively impacted in ROP and OIR and that this may underlie the alterations in rod photoreceptor function.

Analysis of the rod ERG revealed that there was also a specific dysfunction of the inner retinal neurons that underlie the oscillatory potentials. This finding is consistent with previous studies, which have shown that there is a loss in postphotoreceptor, inner retinal function in rats with OIR. The oscillatory potentials are thought to reflect the function of amacrine cells. Specific amacrine cell subpopulations are highly susceptible to retinal ischemia.

In the OIR rat, glycinergic amacrine cells are significantly reduced in number, particularly parvalbumin-immunoreactive All amacrine cells, which may contribute to the rod pathway ERG deficits observed in this model. As the All amacrine cells are the major interneurons of the rod pathway, a loss of these neurons may contribute to deficits in scotopic vision reported during ROP.

Having shown that valsartan treatment reduced retinal vascular pathology and microglial activation and number, we anticipated that treatment with valsartan might ameliorate any neuronal effects induced by OIR. Indeed, valsartan has been found to be protective of glial and neuronal histology in OIR, in particular rescuing inner retinal neurons such as the All amacrine cell, and so we anticipated retinal function would be restored. Our current results showed that treatment with valsartan did not prevent retinal dysfunction in OIR. It is possible that neural dysfunction had occurred prior to the onset of treatment at P11, because of exposure to high levels of oxygen during early postnatal development. However, valsartan induced photoreceptor and inner retinal dysfunction in control and OIR animals over and above that attributable to OIR alone. The 40 mg/kg/d dose of valsartan significantly reduced the rod photoreceptor ERG response in control and OIR animals, while all concentrations of valsartan reduced the inner retinal derived, summed OP response. This suggests inner retinal responses are selectively vulnerable to AT\(_1\) receptor blockade. The mechanisms underlying this response are unclear. As valsartan did not affect the growth of the deep or intermediate vascular plexus in sham-treated controls, the ERG changes are likely to be independent of the effect of valsartan on the development of retinal vasculature. Instead, it is likely the RAS is involved in modulating neuronal function via an alternative pathway. The AT\(_1\) receptors are found on blood vessels, macroglia, retinal ganglion cells, and bipolar cells in the rat retina. The blockade for the AT\(_1\) receptor on inner retinal neurons may contribute to the specific reduction in the inner retinal ERG responses. Furthermore, maturation of the retinal circuitry may be interrupted by inhibiting the activation of AT\(_1\) receptors that are expressed on inner retinal neurons at the time of valsartan treatment. Conversely, the effects of valsartan may be on vascular tone rather than neuronal function. Although valsartan administration had no effect on the development of the retinal vasculature, it may still modulate choroidal and retinal blood flow as systemic administration of AT\(_1\) receptor inhibitors causes hypotension and would also cause vasodilatation of the ocular vessels directly, which could negatively impact neuronal function at the time of ERG assessment. Given that valsartan treatment has been found to prevent loss of amacrine cells in OIR, it is possible that the amacrine cells are present but remain dysfunctional during the period of treatment with valsartan. If function were assessed at a later time point after valsartan treatment ceased, the ERG response might be improved.
Further research is required to ascertain the specific mechanism of action of valsartan on retinal function and also to determine if this effect is permanent or reversible.

In conclusion, the results of this study show that valsartan treatment of OIR in rats reduces angiogenesis and microglial activation, but has no effect on salvaging retinal dysfunction at any dose examined. These results question the potential use of this class of drugs for the treatment of ROP, and highlight that treatments that prevent pathological angiogenesis may not necessarily prevent retinal dysfunction.

Acknowledgments

Supported by grants from the National Health and Medical Research Council of Australia, +560815 and +1038220 to (ELF) and a Senior Research Fellowship of the National Health and Medical Research Council of Australia (JW-B).

Disclosure: K.M. Hatzopoulos, None; K.A. Vessey, None; J.L. Wilkinson-Berka, None; E.L. Fletcher, None.

References

34. Wilkinson-Berka JL, Heine R, Tan G, et al. RILKMKPSV influences the vasculature, neurons and glia, and (pro)renin


