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Oxygen Tension and Gradient Measurements in the Retinal Microvasculature of Rats

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Abstract

Background—Oxygen delivery from the retinal vasculature plays a crucial role in maintaining normal retinal metabolic function. Therefore, measurements of retinal vascular oxygen tension (PO_2) and PO_2 longitudinal gradients (gPO_2) along retinal blood vessels may help gain fundamental knowledge of retinal physiology and pathological processes.

Methods—Three-dimensional retinal vascular PO_2 maps were generated in rats by optical section phosphorescence lifetime imaging. A major retinal artery and vein pair, and a smaller blood vessel (microvessel) between them were segmented and PO_2 along each blood vessel was measured. In each blood vessel, an average PO_2 (mPO_2) was calculated and gPO_2 was determined by linear regression analysis. Reproducibility of measurements was assessed by calculating intraclass correlation coefficient (ICC) of repeated measurements. The correlation of mPO_2 and gPO_2 measurements with systemic arterial oxygen tension (P_aO_2) and carbon dioxide tension (P_aCO_2) was determined.

Results—Measurements of mPO_2 and gPO_2 in retinal arteries, microvessels and veins were reproducible (ICC > 0.86; $p < 0.01$; $N = 8$), except for retinal arterial gPO_2 . Retinal arterial, microvessel and venous mPO_2 were 41 ± 8 , 32 ± 8 and 25 ± 7 mmHg, respectively (mean $\pm$ SD; $N = 27$). Retinal arterial mPO_2 was correlated with P_aO_2 and P_aCO_2 ($R > 0.44$; $p < 0.03$), while retinal microvessel and venous mPO_2 were only correlated with P_aCO_2 ($R > 0.68$; $p < 0.01$). Retinal microvessel gPO_2 (-3.8 ± 1.5 mmHg/100 μ m) was significantly steeper (more negative) than venous gPO_2 (0.02 ± 0.43 mmHg/100 μ m) ($p < 0.01$; $N = 27$), and neither were significantly correlated with P_aO_2 or P_aCO_2 .

Conclusions—Quantitative measurement of mPO_2 and gPO_2 in the retinal microvasculature was demonstrated. A significant decrease in PO_2 was observed along most retinal microvessels, indicative of substantial oxygen extraction by the retinal tissue. This method has the potential to help elucidate retinal microvascular oxygen transport in health and disease.

Keywords

Phosphorescence lifetime imaging; Oxygen tension; Oxygen tension gradients; Retina

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Introduction

Retinal metabolic function depends on an adequate delivery of oxygen by the chorioretinal vasculature and consumption of oxygen by the retinal tissue. Abnormalities of oxygenation of the retinal tissue have been implicated in the development of many retinal diseases, such as retinal vascular occlusion, diabetic retinopathy, retinopathy of prematurity and glaucoma [1–5]. Additionally, retinal hypoxia is known to be a factor in the development of tissue damage and neovascularization [6]. Since oxygen tension (PO_2) changes in the retinal microvasculature are thought to reflect local tissue oxygen consumption, measurements of PO_2 in and along retinal vessels may serve as useful parameters for evaluation of retinal metabolic function and understanding of local physiologic and pathologic oxygen dynamics.

Retinal tissue PO_2 across the retinal depth [1, 3, 7, 8] and vascular PO_2 and oxygen saturation (SO_2) in major retinal blood vessels have been quantified previously [9–12]. However, limited data have been published assessing PO_2 or SO_2 longitudinal gradients along major retinal vessels [12–14], and none in smaller order retinal vessels. In other tissues, while PO_2 in blood vessels of different size and radial PO_2 profiles outward from the vessel have been measured [15–17], PO_2 or SO_2 longitudinal gradients along blood vessels were reported only in arterioles of the hamster's dorsal skinfold [18], and capillaries of the rat's brain [19] and hamster's retractor muscle [20, 21]. In this study, a method for measurement of vascular PO_2 and PO_2 longitudinal gradients (gPO_2) in the retinal microvasculature of rats was demonstrated.

Materials and methods

Animals

Twenty eight male Long Evans pigmented rats (450 – 650 g) were used in this study. The animals were treated in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twelve rats were ventilated with 21% ($N=11$) or 30% ($N=1$) fractions of oxygen to maintain normal systemic arterial PO_2 (P_aO_2) and carbon dioxide tension (P_aCO_2) levels. For ventilation, an endotracheal tube was connected to an animal ventilator (Harvard Apparatus Inc., South Natick, MA, USA). Sixteen rats breathed 30% oxygen spontaneously with a high flow face mask system, which maintained the P_aO_2 near normoxic levels, while P_aCO_2 was elevated. The range of systemic P_aO_2 and P_aCO_2 in the rats allowed investigation of retinal vascular PO_2 and gPO_2 as functions of systemic P_aO_2 and P_aCO_2 . Blood gas analysis was performed with a blood gas analyzer (Radiometer, Westlake, OH, USA) on arterial blood drawn from the femoral artery in 27 of 28 rats. Systemic arterial blood gas analysis was not performed in one rat due to hardware malfunction.

The rats were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg) intraperitoneally. Anesthesia was maintained by supplemental injections of ketamine (20 mg/kg) and xylazine (1 mg/kg) as needed. Pd-porphine (Frontier Scientific, Logan, UT), an oxygen sensitive molecular probe, was dissolved (12 mg/mL) in bovine serum albumin solution (60 mg/mL) and injected intravenously (20mg/kg). Body temperature was maintained at 37°C using an animal holder with a copper tubing water heater. The pupils were dilated with 2.5% phenylephrine and 1% tropicamide. One percent hydroxypropyl methylcellulose and a glass cover slip were applied to the cornea to eliminate the cornea's refractive power and to prevent corneal dehydration. The animal's physiological condition (blood pressure and heart rate) was continuously monitored with a data acquisition system (Biopac Systems, Goleta, CA, USA) linked to a pressure transducer connected to a catheter positioned in the femoral artery.

Instrumentation

The optical section phosphorescence imaging system has been described previously [11]. In short, a laser beam was focused to a vertical line, and projected at an oblique angle on the retina after intravenous injection of the Pd-porphine solution. An optical section phosphorescence image of the retina was acquired with an intensified charge-coupled device camera attached to a slit lamp biomicroscope. Since the incident laser and imaging axis were not coaxial, structures at various retinal depths were laterally displaced in the section image according to depth. The laser line was scanned horizontally across the retina in small steps. For each step, a set of 10 phase delayed phosphorescence optical section images was acquired by incrementally delaying the modulated intensifier of the imaging camera with respect to the modulated intensity of the laser beam. Imaging was performed at temporal or nasal areas within 3 disc diameters (600 μm) from the edge of the disc. During imaging, the laser power incident on the retina was approximately 40 μW , which is safe for 1 hour of continuous viewing according to the American National Standard Institute for Safety Standards [22].

Image Processing and Analysis

Dedicated software algorithms were developed with Matlab (The Mathworks Inc, Natick, MA, USA) to reconstruct three-dimensional retinal vascular PO_2 maps from the acquired phosphorescence section images. First, phosphorescence lifetimes and PO_2 values were obtained from the 10 phased delayed phosphorescence section images [9, 11, 23]. Second, PO_2 section images were stacked contiguously to form a three-dimensional vascular PO_2 map with a voxel size of 8 by 8 by 10 μm^3 with no gaps or overlaps. Third, the PO_2 map was flattened with respect to the curvature of the retina. A three-dimensional phosphorescence map was also generated with the same reconstruction process from the zero-phase delayed phosphorescence section images.

To ensure the reliability of PO_2 measurements, voxels that did not fit well ($R^2 < 0.9$) to the theoretical relationship between phosphorescence intensity and phase delay were excluded. Exclusion of data based on fitting error is a standard method used with the phosphorescence lifetime imaging technique [11, 24]. The remaining noise was reduced by filtering the data based on physiological validity of the calculated PO_2 values. In each animal, PO_2 measurements between 0 and the systemic P_aO_2 were included, and the mean upper tenth percentile of the feeding arterial PO_2 was used as an upper limit for PO_2 in the smaller blood vessels. Overall, 96 and 85% of the measurements were retained from large and small retinal blood vessels, respectively. As expected, a higher percentage of data were excluded from the smaller blood vessels because their size led to a lower phosphorescence signal as compared to retinal arteries and veins.

The phosphorescence map was used to segment the retinal vessels. For each vessel, a three-dimensional volume of interest was identified by thresholding, which assigned voxels within the vessel to 1 and outside the vessel to 0 based on their intensities. Along each blood vessel, upstream and downstream locations were manually selected based on the extent of the imaged retinal area, which was contingent upon the curvature of the eye and dilation of the pupil. To obtain the most reliable measurement along vessels and minimize errors due to variability, the upstream and downstream locations were chosen by selecting the longest possible vessel length to be analyzed. A centerline along the vessel was identified using distance transformation [25, 26] between vessel upstream and downstream locations. The centerline was then smoothed with cubic smoothing spline functions [27], resulting in an average distance of 7 μm between centerline points. For visualizing three dimensional maps, images were rendered with Paraview 3.8.0 software (Kitware Inc, Clifton Park, NY, USA).

From the PO₂ maps generated in each rat, 3 types of blood vessels were selected consisting of a major retinal artery and vein pair, and a smaller blood vessel (microvessel) between them. The selected microvessels were side branches of major retinal arteries (N = 5), connected to both arteries and veins (N = 3), or side branches of major retinal veins (N = 19). They had diameters less than half of the major blood vessels, and had lengths of 100 μm or more.

Data Analysis and Statistics

A mean PO₂ (mPO₂) was calculated in each blood vessel by averaging PO₂ measurements over the segmented vessel. PO₂ at each centerline point along the vessel was calculated by averaging PO₂ over a cross-sectional area perpendicular to the centerline. Linear regression analysis was performed on PO₂ measurements along the vessel centerline and the slope of the best fit line was defined as PO₂ longitudinal gradient (gPO₂).

Reproducibility of measurements was assessed by determining intraclass correlation coefficient (ICC) and absolute difference between 2 repeated measurements in 8 sets of vessels. Linear regression analysis was performed to relate mPO₂ and gPO₂ measurements with systemic P_aO₂ and P_aCO₂ for each vessel type. Analysis of variance and post hoc multiple comparisons (Bonferroni) were performed to compare mPO₂ and gPO₂ measurements among vessel types. Statistical significance was accepted at $p < 0.05$.

Results

Systemic Physiologic Status

Systemic arterial P_aO₂, P_aCO₂ and blood pH measurements were 97 ± 18 mmHg, 52 ± 15 mmHg and 7.33 ± 0.08 , respectively (mean $\pm$ SD; N = 27). Systemic P_aO₂ and P_aCO₂ were not linearly correlated ($R = 0.1$; $p = 0.64$). Systemic P_aCO₂ and pH were inversely correlated ($R = -0.93$; $p < 0.01$). Blood pressure and heart rate measurements were 106 ± 20 mmHg and 218 ± 50 beats per minute, respectively.

Reproducibility

Mean PO₂ measurements in arteries, microvessels and veins were reproducible (ICC > 0.86; $p < 0.01$; N = 8). Mean absolute differences of mPO₂ in arteries, microvessels and veins were 1.5, 3.1 and 1.2 mmHg (N = 8), respectively. PO₂ gradient measurements in retinal microvessel and veins were reproducible (ICC > 0.88; $p < 0.01$; N = 8). Mean absolute differences of gPO₂ in microvessels and veins were 0.56 and 0.29 mmHg/100μm, respectively. The ICC of gPO₂ measurements in retinal arteries was relatively low (ICC = 0.58; $p = 0.04$; N = 8), therefore retinal arterial gPO₂ measurements were excluded from further analysis.

Vascular PO₂

An example of a three-dimensional phosphorescence map obtained in one rat is shown in Fig 1a. Vessel segmentations and centerlines of a microvessel and vein are displayed in Fig 1b. The segmented vascular PO₂ map is shown in Fig 1c. Measurements of mPO₂ in retinal arteries, and veins were on average 41 ± 8 and 25 ± 7 mmHg, respectively (N = 27). Measurements of mPO₂ in retinal microvessels side branching from major retinal arteries, extending from arteries to veins, and side branching from major retinal veins were progressively lower, but the differences were not significant statistically ($p = 0.42$). The average mPO₂ in all retinal microvessels was 32 ± 8 mmHg (N = 27). Measurements of mPO₂ were significantly different among arteries, microvessels and veins ($p < 0.01$). Retinal arterial mPO₂ was significantly higher than retinal venous mPO₂ ($p < 0.01$). Retinal

microvessel mPO_2 were significantly lower than retinal arterial and higher than venous mPO_2 measurements ($p < 0.01$).

The relationships between mPO_2 in retinal blood vessels and systemic P_aO_2 and P_aCO_2 are shown in Fig 2. Retinal arterial mPO_2 was correlated with both systemic P_aO_2 ($R = 0.45$; $p = 0.02$) and P_aCO_2 ($R = 0.51$; $p = 0.01$). Retinal microvessel mPO_2 was highly correlated with P_aCO_2 ($R = 0.69$; $p < 0.01$), but not correlated with systemic P_aO_2 ($R = 0.21$; $p = 0.3$). Similarly, retinal venous mPO_2 was highly correlated with P_aCO_2 ($R = 0.84$; $p < 0.01$), but not correlated with systemic P_aO_2 ($R = 0.21$; $p = 0.3$).

PO_2 Longitudinal Gradients

An example of PO_2 measurements as a function of distance along a retinal microvessel is shown in Fig. 3. The slope of the best fit line to the PO_2 measurements (gPO_2) was -3.65 mmHg/100 μ m. Measurements of gPO_2 among retinal microvessels side branching from arteries, veins or both were not significantly different ($p = 0.4$). In all retinal microvessels, gPO_2 measurements were negative (decrease from upstream to downstream) and were statistically significant in 22 of 27 vessels. Retinal venous gPO_2 measurements were close to zero, and statistically significant in only 14 of 27 vessels. Mean microvessel gPO_2 was -3.8 ± 1.5 mmHg/100 μ m ($N = 27$), significantly steeper (more negative) than venous gPO_2 (0.02 ± 0.43 mmHg/100 μ m) ($p < 0.01$).

The relationships between gPO_2 in retinal blood vessels and systemic P_aO_2 and P_aCO_2 are shown in Fig 4. Retinal microvessel gPO_2 was not correlated with systemic P_aO_2 ($R = 0.35$; $p = 0.07$) or P_aCO_2 ($R = 0.07$; $p = 0.7$). Retinal venous gPO_2 was not correlated with systemic P_aO_2 ($R = 0.21$; $p = 0.3$) or P_aCO_2 ($R = -0.18$; $p = 0.4$).

Discussion

Longitudinal changes in PO_2 along retinal blood vessels are indicative of retinal tissue oxygen extraction and are expected to be related to the retinal metabolic function. In the current study, a method for measuring mPO_2 and gPO_2 in retinal arteries, microvessels and veins of rats was established. We report for the first time to our knowledge, quantitative and reproducible measurements of PO_2 in retinal blood vessels that are approximately 20 μ m in diameter. In previous studies, these microvessels have been termed precapillary arterioles and postcapillary venules [28, 29]. Supporting the validity of our measurements, retinal microvessel mPO_2 was between mPO_2 of the feeding arteries and draining veins.

A significant linear decrease in PO_2 along most retinal microvessels (negative gPO_2) was found. Although retinal capillaries are commonly thought to be the primary site for oxygen delivery, the substantial oxygen loss along most retinal microvessels indicates oxygen extraction by the retinal tissue occurs not only at the capillary level. PO_2 measurements in retinal arteries were more variable because of the lower sensitivity of the Pd-porphine dye at high PO_2 [9]. This variability coupled with the presumed small change in PO_2 along retinal arteries [14, 30], limited measurements of retinal arterial gPO_2 . The magnitude of retinal venous gPO_2 was relatively small and its sign was variable. This finding concurs with reported oxygen flux measurements in the vitreous of cats, showing the direction of oxygen flux to be either going inward or outward from the veins [14]. Additionally, diffusional shunting directly from retinal arteries to veins may also contribute to variations in venous gPO_2 [31].

Retinal arterial mPO_2 was correlated with both systemic P_aO_2 and P_aCO_2 , and was on average 42% of the P_aO_2 , similar to previous reports of 47% and 39% in rats and pigs [32, 33]. Retinal microvessel and venous mPO_2 were correlated with systemic P_aCO_2 , but not

with P_aO_2 . Previous studies have shown that elevated systemic P_aCO_2 triggers nitric oxide mediated vasodilation [34, 35] which in turn, increases retinal blood flow [35–37] and tissue PO_2 [38]. Therefore, retinal vascular mPO_2 should increase with systemic P_aCO_2 , although this effect may have been dampened by an opposing vasoconstriction factor if hyperoxia was present.

Retinal microvessel gPO_2 did not correlate with systemic P_aO_2 or P_aCO_2 . Since an increase in systemic P_aCO_2 has been shown to increase retinal blood flow [36, 37], gPO_2 would be expected to decrease (less negative), as long as oxygen consumption remains unchanged, according to Fick's principle [39]. The finding that retinal microvessel gPO_2 did not change with P_aCO_2 may imply an increase in retinal oxygen consumption as suggested in previous studies [38, 40]. Future studies combining retinal vascular PO_2 and blood flow measurements are needed to determine any effect of systemic P_aCO_2 on inner retinal oxygen consumption.

In summary, a method for quantitative measurements of mPO_2 and gPO_2 in retinal microvasculature of rats was established. The steepness of the retinal microvessel gPO_2 indicates substantial oxygen loss along their lengths, similar to the expected oxygen loss from retinal capillaries. Since the retinal capillary network is anastomotic, its branching and ensuing short vessel segments hinders reliable quantification of gPO_2 by any existing technique. Therefore, retinal microvessels may serve as a valuable surrogate for studying retinal oxygen transport. In future studies, PO_2 mapping of multiple retinal areas to increase the microvessel sample size will allow a comprehensive assessment of retinal oxygen dynamics in response to physiological challenges or pathological conditions.

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Fig. 1.

(a) An example of a three dimensional phosphorescence map of the retinal microvasculature in one rat. (b) Segmented artery (A), microvessel (M) and vein (V) and their corresponding centerlines (red solid lines). (c) Segmented vascular PO₂ map; color bar shows PO₂ measurements in mmHg.

Fig. 2.

Mean oxygen tension (mPO₂) in retinal arteries, microvessels and veins plotted as functions of (a) systemic arterial oxygen tension (P_aO₂), and (b) carbon dioxide tension (P_aCO₂). Each data point represents a measurement obtained in one vessel of one rat (N = 27). Regression lines are displayed when the correlations were statistically significant.

Fig. 3. PO₂ measurements plotted as a function of distance along a retinal microvessel in one rat. The best fit regression line and corresponding linear equation are displayed. Error bars represent standard errors of the means of 6 to 12 measurements.

Fig. 4. PO_2 longitudinal gradients (gPO_2) in retinal microvessels and veins plotted as functions of (a) systemic arterial oxygen tension (P_aO_2) and (b) carbon dioxide tension (P_aCO_2). Each data point represents a measurement obtained in one vessel of one rat ($N = 27$). Note the lack of correlation, clustering of venous gPO_2 around zero, and uniformly negative microvessel gPO_2 .