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Abnormal Retinal Vascular Oxygen Tension Response to Light Flicker in Diabetic Rats

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Abstract

Purpose—To test the hypothesis that the intravascular oxygen response to light flicker is abnormal in diabetes.

Methods—Ten eyes of normal rats and 10 eyes of rats made diabetic with streptozotocin were examined. Oxygen tension (P_{O_2}) was measured noninvasively in the retinal arteries and veins on optical section retinal images. P_{O_2} was estimated based on the quenching by oxygen of the phosphorescence of an intravenously injected palladium porphyrin molecular probe. Measurements were conducted with and without light flicker at 10 Hz. Oxygen saturation (S_{O_2}) was calculated with adjustment for the arterial pH.

Results—In the normal rats flicker induced an increase in arterial P_{O_2} and in the difference in arterial and venous (A-V difference) P_{O_2} from 51 ± 5 (mean and SD) to 55 ± 7 mm Hg and from 22 ± 3 to 26 ± 5 mm Hg, respectively ($P < 0.002$ and 0.015 , respectively). Flicker induced an increase of arterial S_{O_2} and A-V S_{O_2} difference from $64\% \pm 8\%$ to $68\% \pm 7\%$ and from $34\% \pm 4\%$ to $38\% \pm 6\%$, respectively ($P < 0.002$ and 0.035 , respectively). No changes in P_{O_2} or S_{O_2} were observed with flicker in the veins. In the diabetic rats, no significant flicker-induced changes were seen in P_{O_2} or S_{O_2} in the retinal arteries, veins, or A-V differences.

Conclusions—The diabetic rats lacked the flicker induced increase in arterial P_{O_2} and S_{O_2} and also the A-V difference in P_{O_2} and S_{O_2} observed in the normal rats. The best explanation appeared to be that diabetes impairs the increase in oxygen consumption normally provoked by light flicker.

Diabetic retinopathy is a major cause of visual loss in our society, especially in the most productive years between 20 and 65.¹⁻⁴ The ophthalmoscopically visible lesions, such as microaneurysms, hemorrhages, exudates, intraretinal microvascular abnormalities, venous beading, edema, and neovascularization, indicate involvement of the retinal vasculature. In the healthy retina, retinal diameter, and blood flow are regulated by factors affecting systemic blood parameters (e.g., hypoxia,⁵⁻⁷ hyperoxia,⁵⁻¹⁴ hypercapnia,^{7,12,13,15} and hyperglycemia^{7,10,11,14,16}) and ocular perfusion (e.g., changes in intraocular pressure,^{17,18} changes in systemic blood pressure^{5-8,14}). However, diabetes has been shown to impair regulatory vessel diameter and blood flow responses.^{5-11,14,16,18}

Some evidence indicates involvement of the retinal neural tissue, even early in the course of the disease.¹⁹⁻³⁰ Light flicker offers a way to probe vascular function by manipulating neural activity. It causes an increase in retinal metabolism, especially in the inner retina.³¹⁻³⁸ This is

associated with an increase in blood vessel diameter and flow in the retina and optic nerve.^{32,35-37,39-51} Reduced sensitivity in flicker perimetry,²³ impaired flicker-induced vascular diameter responses,⁵² and abnormalities in electroretinography due to light flicker^{20,29} have been observed in patients and animals with diabetes.

We have developed a method of measuring the oxygen tension in the retinal vessels that avoids choroidal influence.⁵³⁻⁵⁵ Previously, we showed that light flicker leads to an increase in the difference between the arterial and venous oxygen tension (P_{O_2}) in normal rats.³⁸ Since no information is available on the effect of flicker on oxygen in the diabetic retinal vasculature, we tested the hypothesis that the intravascular oxygen response to light flicker is abnormal in diabetes.

Methods

Animals

Male Long Evans pigmented rats (450–650 g) were used for the study. The animals were treated in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. We studied 10 eyes of 10 normal and 10 eyes of 8 diabetic animals. Diabetes was induced by injecting 55 mg/kg of streptozotocin intravenously. Diabetic animals had blood glucose levels of 385 ± 119 mg/dL, as measured 3 to 6 days after injection; the lowest being 192 mg/dL. The studies were performed after the animals had had diabetes for 4 weeks. Both the diabetic and normal animals were studied at two or more months of age.

The rats were anesthetized with 85 mg/kg ketamine and 3.5 mg/kg xylazine administered intraperitoneally. Anesthesia was maintained by an intraperitoneal infusion of ketamine and xylazine at the rates of 0.5 mg/kg/min and 0.02 mg/kg/min, respectively. The animals breathed room air spontaneously. A femoral artery was cannulated for drawing arterial blood and monitoring the blood pressure and heart rate. These were monitored via a pressure transducer linked to a data-acquisition system (Biopac Systems Inc., Goleta, CA). Femoral arterial blood (200 μ L) was drawn without air exposure, and P_{O_2} and pH were measured with a blood gas analyzer (Radiometer, Westlake, OH). The animal holder incorporated copper tubing with flowing warm water to maintain body temperature, which was monitored via a rectal thermometer.

The pupils were dilated with 2.5% phenylephrine and 1% tropicamide. An oxygen-sensitive molecular probe, Pd-porphine (Frontier Scientific, Logan, UT), was dissolved (12 mg/mL) in physiological saline containing bovine serum albumin (60 mg/mL) buffered to a pH of 7.4, and this was injected intravenously (20 mg/kg). Hydroxypropyl methylcellulose 1% was applied to the cornea and a glass coverslip was placed on it to eliminate its refractive power and to prevent corneal dehydration. The rat was placed in front of the imaging instrument. The laser power was adjusted to 100 μ W, which is safe for viewing according to the American National Standard Institute for Safety Standards.⁵⁶

Phosphorescence Imaging

The principle of the imaging technique has been described in detail.^{53-55,57} Briefly, a diode laser beam at 532 nm was expanded, focused to a narrow line, and projected at an oblique angle on the retina after injection of the probe. A two-dimensional (2D) phosphorescence optical section retinal image perpendicular to the retinal plane was acquired by placing a near-infrared filter in front of the CCD imaging camera. The filter's transmission overlapped the phosphorescence emission of the oxygen probe. Since the incident laser beam was not coaxial with the viewing path, structures at various depths appeared laterally displaced on the phosphorescence optical section image according to their depth location. Imaging was

performed at areas within 2 disc diameters (600 μm) from the edge of the optic nerve head. The intensity of the excitation laser beam and the sensitivity of the image intensifier interfaced to the imaging camera were modulated at 1600 Hz. At each location, a set of phase-delayed phosphorescence optical section images was acquired by incrementally delaying the modulated excitation laser beam with respect to the modulated imaging intensifier sensitivity.

Calculation of Oxygen Tension by Image Analysis

The methodology for quantitative measurement of P_{O_2} based on 2D phase-delayed phosphorescence intensity images has been published.⁵⁴ The phase-delayed images were analyzed by using a frequency-domain approach for measurement of phosphorescence lifetime. The lifetime was used to determine the P_{O_2} (mm Hg) according to the Stern-Volmer expression: $\text{P}_{\text{O}_2} = [(\tau_0/\tau) - 1]/(\kappa Q)(\tau_0)$, where τ is the phosphorescence lifetime (μs) and κQ and τ_0 are the probe's quenching constant and lifetime in a 0 oxygen environment, respectively. P_{O_2} was calculated in the major arteries and veins on the images with the use of previously published κQ and τ_0 values of 381 mm Hg $^{-1}$ s $^{-1}$ and 637 μs , respectively.⁵⁸ Arterial and venous P_{O_2} were derived from the average of P_{O_2} measurements in three images.

Stimulation of Metabolism by Light Flicker

For visual stimulation, the illumination light from a slit lamp biomicroscope was used as the source for light flicker.³⁸ A filter that transmitted light at a wavelength of 568 ± 5 nm (Edmund Industrial Optics, Barrington, NJ) was placed in the slit lamp illumination housing to eliminate overlap with the phosphorescence excitation or emission and thus to permit imaging during light flicker. The light power was 110 μW at the pupil. A shutter was attached to a solenoid and placed in front of the illumination housing of the slit lamp biomicroscope to alter the flicker frequency. The solenoid was externally driven by a square wave generator (Temma, Springboro, OH) to produce a flicker frequency of 10 Hz, which has been found to maximally stimulate vascular P_{O_2} changes.³⁸

Calculation of Oxygen Saturation of Hemoglobin

The oxygen saturation (S_{O_2}) of hemoglobin was calculated using the following equation⁵⁹: $\text{S}_{\text{O}_2} = \text{P}_{\text{O}_2}^n / (\text{P}_{\text{O}_2}^n + \text{P}_{50}^n)$, where S_{O_2} is the percentage of saturation of hemoglobin, P_{50} is the P_{O_2} at which hemoglobin is half saturated, and n is the Hill coefficient.⁵⁹ P_{50} and n were taken to be 36.0 mm Hg and 2.6, respectively, according to Cartheuser.⁵⁹ The hemoglobin dissociation curve is altered by pH. Accordingly, S_{O_2} was calculated using the Bohr coefficients published by Cartheuser.⁵⁹ In one rat in each group, pH data were not available. In those cases we used the mean pH value of the other rats in their groups. This seemed reasonable because the coefficients of variation of pH were 0.4% and 0.6% in the normal and diabetic rats, respectively.

Statistical Analysis

The systemic data were analyzed by Student's two-tailed unpaired t -test. The P_{O_2} and S_{O_2} data were analyzed by Student's one-tailed paired t -test, since our previous work showed that flicker will not reduce P_{O_2} .³⁸ The results were considered significant at $P \leq 0.05$.

Results

Systemic Cardiovascular Status

The blood pressures in the normal and diabetic rats were 100 ± 15 (mean $\pm$ SD) and 91 ± 10 mm Hg, respectively ($P = 0.07$). The heart rates in the normal and diabetic animals were 222 ± 80 and 232 ± 19 beats per minute, respectively ($P = 0.37$). The arterial P_{O_2} was 77 ± 11 mm Hg in the normal rats and 64 ± 12 mm Hg in the diabetic rats ($P = 0.015$). The arterial P_{CO_2} was

51 ± 3.0 mm Hg in the normal rats and 53 ± 3.8 mm Hg in the diabetic rats ($P = 0.22$). The arterial pH in the normal and diabetic rats was 7.31 ± 0.03 and 7.31 ± 0.05 , respectively ($P = 0.71$).

Intravascular P_{O_2} in Normal and Diabetic Rats without and with Light Flicker

The intravascular P_{O_2} in the normal and diabetic rats is presented in Table 1. In the normal rats, the arterial P_{O_2} without and with flicker was 51 ± 5 and 55 ± 7 mm Hg, respectively ($P = 0.002$). The venous P_{O_2} without and with flicker was 30 ± 3 and 30 ± 4 mm Hg, respectively ($P = 0.42$). The differences in arterial and venous (A-V difference) P_{O_2} without and with flicker were 22 ± 3 and 26 ± 5 mm Hg, respectively ($P = 0.015$).

In the diabetic rats, the arterial P_{O_2} without and with flicker was 47 ± 7 and 46 ± 7 mm Hg, respectively ($P = 0.25$). The venous P_{O_2} without and with flicker was 30 ± 4 and 30 ± 3 mm Hg, respectively ($P = 0.32$). The A-V P_{O_2} differences without and with flicker were 17 ± 7 and 17 ± 8 mm Hg, respectively ($P = 0.40$).

Intravascular S_{O_2} in Normal and Diabetic Rats without and with Light Flicker

The intravascular S_{O_2} in the normal and diabetic rats is presented in Table 2. In the normal rats, the arterial S_{O_2} without and with flicker was $64\% \pm 8\%$ and $68\% \pm 7\%$, respectively ($P = 0.002$). The venous S_{O_2} without and with flicker was $30\% \pm 6\%$ and $30\% \pm 7\%$, respectively ($P = 0.42$). The A-V S_{O_2} differences without and with flicker were $34\% \pm 4\%$ and $38\% \pm 6\%$, respectively ($P = 0.035$).

In the diabetic rats, the arterial S_{O_2} without and with flicker was $58\% \pm 9\%$ and $57\% \pm 10\%$, respectively ($P = 0.24$). The venous S_{O_2} without and with flicker was $31\% \pm 8\%$ and $31\% \pm 7\%$, respectively ($P = 0.33$). The A-V S_{O_2} differences without and with flicker were $27\% \pm 9\%$ and $26\% \pm 11\%$, respectively ($P = 0.42$).

Discussion

In this study, we found no detectable light flicker-induced changes in arterial or venous P_{O_2} , arterial or venous S_{O_2} , or A-V difference in P_{O_2} or S_{O_2} in diabetic rats. However, in the normal rats, light flicker induced an increase in arterial P_{O_2} and S_{O_2} and an increase in A-V P_{O_2} and S_{O_2} difference, as has been reported elsewhere.³⁸ This finding confirmed our hypothesis that intravascular oxygen response to light flicker is abnormal in diabetic rats, consistent with previous reports of impaired retinal vessel autoregulation in diabetes.^{5-11,14,16,18} The S_{O_2} measurements in the present study were low compared with the S_{O_2} measured with spectrometry in humans.⁶⁰ We attribute this largely to the hypoxic state of our rats during measurement caused by the respiratory depressant effect of the anesthetics.⁶¹

It is known that CO_2 generated from respiration reduces venous pH and hence the binding of oxygen to hemoglobin. One limitation of the present study was that we had to use systemic arterial pH for all S_{O_2} calculations. Therefore, differences in pH between systemic arteries and the retinal vasculature could not be accounted for in our calculations of S_{O_2} . Systemic arterial pH was essentially the same in both normal and diabetic animals, and should be very similar to retinal arterial pH near the optic disc. This factor should, therefore, not have largely affected retinal arterial S_{O_2} . However, since both hyperglycemia and diabetes have been shown to reduce retinal tissue pH,^{62,63} venous pH in diabetic animals was probably lower than that in normal animals. Therefore, measured retinal venous P_{O_2} would be associated with an overestimation of S_{O_2} and an underestimation of A-V S_{O_2} differences, and this phenomenon would be more prominent in the diabetic group. Nonetheless, without flicker stimulation, large A-V S_{O_2} differences existed in both groups, even though the diabetic retinas may well have been more

acidic. The A-V S_{O_2} difference enlarged further during flicker in the normal group, indicating extension of the same process. Thus, it seems unlikely that pH-mediated oxygen delivery would dominate with flicker in the diabetic state if it did not dominate in the absence of flicker. It would be especially unlikely to completely eliminate enlargement of the A-V difference, as we observed. We note that any enhanced lactate production with flicker would not have had a major effect on our calculated venous S_{O_2} , because lactate-induced pH changes have a much smaller, and possibly negligible, effect on hemoglobin saturation than those induced by CO_2 .⁵⁹

One interpretation of the results is that the diabetic retina did not increase its oxygen consumption with flicker stimulation. According to the Fick principle, oxygen consumption equals the blood flow rate times the difference in concentration of oxygen between the artery and the vein.⁷ The ability of the diabetic retinal vasculature to regulate its blood flow in response to several stimuli has been shown to be impaired in multiple studies,^{5-11,14,16,18} and there is some evidence that light flicker does not increase the blood flow rate in the diabetic state as it does in normal eyes.⁵² Furthermore, it is known that in cats with diabetes for more than 2 years, the retina is hypoxic,⁶⁴ suggestive of inadequate vascular regulation. As previously discussed, in the diabetic retina the intraretinal pH is low, which can also serve as a stimulus for compensatory regulation.⁶³ These observations suggest that the retinal vascular compensatory mechanisms may have been stimulated to maximal capacity in the diabetic state, even before light flicker challenge. In that case, light flicker stimulation could induce no further increase in blood flow and oxygen delivery, thereby limiting oxygen consumption and enlargement of the A-V S_{O_2} difference. On the other hand, retinal venous P_{O_2} was 30 mm Hg, indicating that at least some oxygen still may have been available for extraction, particularly since retinal cells can consume substantial oxygen even in an environment of 15 mm Hg or less.^{65,66} This raises an alternative possible interpretation that the diabetic state rendered the retina unable to respond to light flicker with increased oxygen consumption even though oxygen was available. This would be consistent with other evidence of abnormal neural function in diabetes.¹⁹⁻³⁰

Further complementary studies are needed to clarify the roles of retinal acidity, impaired vascular regulatory responses, and intrinsic neural retinal dysfunction in response to light flicker in diabetic retinopathy. We expect that the results will aid in comprehending the pathophysiology of diabetic retinopathy and eventually lead to better treatment options.

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Table 1Retinal Intravascular P_{O2} in Normal and Diabetic Rats with and without Light Flicker

	P _{O2} without Flicker	P _{O2} with Flicker	<i>P</i>
Normal			
Artery	51 ± 5	55 ± 7	0.002
Vein	30 ± 3	30 ± 4	0.42
Artery-Vein	22 ± 3	26 ± 5	0.015
Diabetes			
Artery	47 ± 7	46 ± 7	0.25
Vein	30 ± 4	30 ± 3	0.32
Artery-Vein	17 ± 7	17 ± 8	0.40

Data are mean mm Hg ± SD.

Table 2Retinal Intravascular SO_2 in Normal and Diabetic Rats with and without Light Flicker

	SO_2 without Flicker	SO_2 with Flicker	<i>P</i>
Normal			
Artery	64 ± 8	68 ± 7	0.002
Vein	30 ± 6	30 ± 7	0.42
Artery-Vein	34 ± 4	38 ± 6	0.035
Diabetes			
Artery	58 ± 9	57 ± 10	0.24
Vein	31 ± 8	31 ± 7	0.33
Artery-Vein	27 ± 9	26 ± 11	0.42

Data are mean percentage $\pm$ SD.