Resistance of Diabetic Rat Electroretinogram to Hypoxemia

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Purpose. To investigate the mechanisms of the known electroretinographic abnormalities of diabetic rats and to explore effects of hypoxemia.

Methods. Subretinal and vitreal microelectrodes were used to isolate the retinal and retinal pigment epithelial components of the electroretinogram. Normoxic and hypoxicemic recordings were taken from nine normal and six streptozotocin-diabetic, anesthetized, paralyzed, and ventilated pigmented rats.

Results. When inspired O$_2$ was reduced the retinal pigment epithelial c-wave component of most of the normal rats diminished, whereas those of the diabetic rats, though initially smaller, were more resistant to the episode of hypoxemia ($P = 0.0061$). A similar trend was seen in other components.

Conclusion. It is proposed that the reduced sensitivity of the diabetic electroretinogram to hypoxemia results from a reduced dependency of the diabetic retina on oxygen. This reduced dependence may follow from a shift in adenosine triphosphate production whereby oxidative phosphorylation is reduced by the high level of retinal intracellular glucose (Crabtree effect). A reduced oxygen demand would cause a transient increase in retinal PO$_2$, leading to a reduction in retinal blood flow. The resulting chronic hypoperfusion of the retinal circulation may deprive the retina of vital, non-energy-related substances.

The absence of (endogenous) insulin, estimated by low levels of C-peptide, has recently been shown to be unrelated to the frequency or severity of retinopathy within specified groups based on age of onset and use of insulin. This leaves chronic hyperglycemia as the most likely fundamental cause of retinopathy, though other factors are involved. One abnormality that has been demonstrated in hyperglycemic (streptozotocin-diabetic) rats is a reduction in amplitude or even an inversion of polarity of the c-wave of the electroretinogram (ERG). The c-wave is the sum of a component from the neural retina, slow P III, and a component from the retinal pigment epithelium (RPE), which can be recorded separately by placing a microelectrode in the subretinal space. The c-wave was divided into its two components because they are in opposite polarity; therefore, any perturbation affecting both of them could well be masked in the resulting, conventionally recorded c-wave. The recordings were taken from anesthetized normal and streptozotocin-diabetic pigmented rats. This report is confined to the effects of a transient episode of hypoxemia on the b- and c-waves of the ERG.

METHODS

Experiments were performed on 11 normal and 7 diabetic Long-Evans pigmented rats and the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research was strictly adhered to in all respects. More details of the methods employed are available elsewhere. Housing of the rats was standard with a light regimen of 14 hours on (60 to 100 lux at the cornea) and 10 hours off.

Diabetes Induction

After overnight fasting, streptozotocin (Sigma, St. Louis, MO) 70 mg/kg in saline at pH 4.5, was injected intraperitoneally. The diabetic state (taken as blood glucose > 300 mg/dl or 16.7 mM) was confirmed in all
animals by blood glucose measurements (Glucometer II, Ames, Elkhart, IN) 2 days after induction and during experiments. Experiments were carried out on the diabetic rats at 7, 9, 11, 13, 15, 26, and 28 weeks after diabetes induction. ERGs were recorded from each animal in one session. The animals were then killed while still anesthetized.

**Preparation**

After fasting and dark adapting overnight, anesthesia was induced and maintained with 2% isoflurane/35% oxygen during surgery. Cannulas of polyethylene tubing (outer diameter 0.96 mm) were inserted into the femoral artery and vein. The animal’s temperature was maintained at 37°C by a water blanket that was thermostatically controlled (Model 73A, Yellow Springs Instruments, Yellow Springs, OH). Electrocardiographic leads were attached to the forelimbs and the signal was fed to an oscilloscope and an audio amplifier. The heart rate at this stage was slow (200 to 280 beats/minute) and the mean blood pressure low (60 mm Hg) because of the anesthetic agent. A urethane loading dose (800 mg/kg intravenously for an 1 hour in 4 ml) was delivered with a pump.

A specially designed barrel was sutured to the sclera 1 mm from the limbus. Through this a needle entered the globe bearing a voltage microelectrode, which would be advanced to the subretinal space. A second voltage microelectrode was pushed through the sclera into the vitreous humor, a reference electrode was placed in the conjunctival fornix, and a ground electrode was placed under the skin of the scalp. Atropine drops were instilled. The light stimulus was delivered from a tungsten iodide 50W bulb through a fiber optic bundle, producing 100 lux at the cornea at zero attenuation. The stimuli were controlled by an electronic shutter (Uniblitz, Vincent Associates, Rochester, NY) and timer (Model S-100, Winston Electronics, San Francisco, CA).

On finishing the eye surgery the animal was paralyzed with pancuronium bromide (0.5 mg/kg), anticoagulated (heparin 120 u/kg), and ventilated mechanically (Harvard rodent respirator). A maintenance intravenous infusion was then commenced of urethane 75 mg/kg/hr, pancuronium bromide 0.2 mg/kg/hr, and heparin 12 U/kg/hr combined in 3 ml saline/kg/hr. The isoflurane was stopped, which allowed the blood pressure to rise to 90 to 110 mm Hg and the heart rate to rise to about 320 beats/minute. Differential residual effects of the isoflurane on the ERGs of the two groups of rats were considered unlikely because the cardiovascular systems of the rats recovered from it in seconds and ERGs were recorded more than 1 hour after it had been stopped. Although any anesthetic may affect the ERG, urethane is known to affect it less than others. Blood pH was maintained at 7.35 to 7.45 and PaO₂ at 90 to 100 mm Hg. Blood gases were measured with a Corning 158 pH/blood gas analyzer (Corning, Medfield, MA).

**Recording**

Both the vitreal and microelectrode signals were recorded with respect to the orbital electrode in the light-tight Faraday cage. The vitreal signal was recorded with a unity gain amplifier (Model FD-223, WP Instruments, New Haven, CT) the output of which was amplified by a 5A18N module of a storage oscilloscope (Model 5111A, Tektronix Instruments, Beaverton, OR). The intraretinal voltage from the microelectrode was recorded with a model M4A amplifier (W-P Instruments), passed through a 60-Hz notch filter and amplified by the 5A22N module of the oscilloscope. ERGs were recorded on FM tape (Model Store 4DS, Racal Recorders Inc., Irvine, CA) and a chart recorder (Model 440, Gould Inc., Cleveland, OH) and were also digitized online and sent to a computer. Signals were digitized at 100 Hz during the b-wave and 20 Hz during the c-wave (data acquisition board: DT2811-PGH, Data Translation Inc, Marlboro, MA; software: Labtech Notebook, Labtech, Wilmington, MA).

The tip of the microelectrode was advanced hydrodynamically to the subretinal space using the appearance of the ERG as a guide. The light shutter was programmed to expose the retina to a 6-second stimulus every 60 seconds. The intensity of the stimulus was 1 lux at the cornea, which had been shown to just saturate the b-wave. The low intensity stimulus needed for repeated and reproducible b- and c-waves precluded study of the a-wave. After several minutes the concentration of inspired oxygen was reduced. After 5 to 10 minutes, when the recordings had stabilized, the PaO₂ was measured before the inspired oxygen concentration was reduced further. Recordings were made at one or two steps of hypoxemia before the inspired oxygen concentration was returned to the prehypoxemia level. Recordings were only included in the analysis if the wave amplitudes returned to the prehypoxic level.

**Data Analysis**

The b-wave amplitudes were taken as the maximum values in the first 500 ms after stimulation minus the baseline voltage. The c-wave amplitudes were taken as the average deflection between 3.8 and 4.3 seconds after stimulus onset minus the baseline voltage. This time interval was chosen because the recordings did not have a distinct peak and because some vitreal recordings had a trough in this time. Wave amplitudes were plotted against PaO₂ and the average slope of the normal rats was compared with that of the diabetic group. This was done using a normal errors linear model that included terms for diabetic status and for
rat within status. In the resulting analysis of variance the F value for the intersection between PaO₂ and status was used to test for the effect of diabetic status on the slope. The analysis was performed using the General Linear Models Procedure (SAS Institute Inc, Cary, NC).

RESULTS

Results from 9 of the 11 normal and 6 of the 7 diabetic rats were eligible for inclusion in the analysis. The intraretinal amplitudes of the remainder did not return to their prehypoxic levels, probably because of movement of the electrodes. Recordings were stable and not progressively diminished by the light stimuli. Sequential RPE c-waves were measured in a normal rat. Five prehypoxic and four posthypoxic amplitudes together showed a coefficient of variation of 3.0%. Amplitudes had fully recovered 3 to 4 minutes after the end of hypoxemia.

Representative traces from a normal rat are shown in Figure 1. The vitreal ERG, in the middle, consists of a b-wave followed by the slower c-wave. Both of these components are seen in the trans-RPE recordings at the top and the transretinal recordings at the bottom of the figure. Based on the recordings and analysis of the ERG in cat retina, where the ERG has been analyzed more fully, it appears that the b-wave recorded across the RPE is the result of a passive voltage drop across the high resistance of the RPE and sclera, and that the transretinal recording reflects the origin of the b-wave. The c-wave, however, is known to have components generated by both the RPE (RPE c-wave) and by the neural retina (retinal c-wave or slow PIII), and these sum to give the smaller vitreal ERG c-wave. The RPE component is usually larger, so the c-wave in the vitreous is usually positive. The signal from the vitreous humor is the same as would be recorded more commonly at the cornea, although it is slightly larger because it avoids the voltage drop across the corneal resistance.

Figure 1 (left) shows individual responses (i.e., not averaged) to a 6-second light stimulus, which is just bright enough to saturate the b-wave. A recording at a PaO₂ of 30 mm Hg is superimposed over one at a PaO₂ of 92 mm Hg. There was a 30% reduction of the vitreal b-wave, a 58% reduction of the RPE c-wave, and a 68% reduction of the retinal c-wave during hypoxemia. However, because these two components of the c-wave summed to produce the vitreal recording the latter showed virtually no change. Corresponding tracings from a diabetic rat are also shown in Figure 1. The vitreal c-wave was smaller than normal, in agreement with other studies and the two components were smaller than normal. An analysis of these normoxic components will be the subject of a future report. The important finding here is that both components appeared to be less susceptible to the episode of hypoxemia. Raw tracings from another normal and another diabetic rat are shown in Figure 2. The normoxic c-wave amplitudes of this diabetic rat were by far the largest of the diabetic group but provided a good comparison with the normal rat because of the steps of hypoxia. Again, the responses of the diabetic rat show much less effect of hypoxia.

Figure 3 shows a plot of the amplitude of individual RPE c-wave responses versus the PaO₂ for each rat in the study. The average slope of the data points for each normal rat (not drawn) was steeper than that for the diabetic rats (P = 0.0061) indicating that the diabetic retinas were less affected by the hypoxic episode. The amplitudes from one diabetic rat were much larger than the rest of the diabetic rats (Fig. 2), which is puzzling, but nevertheless the resistance to hypoxia was consistent. A corresponding plot of the transretinally recorded c-wave versus PaO₂ (not illustrated) showed a similar trend of resistance of the diabetic amplitudes to hypoxia that was marginally significant (P = 0.063). A plot of vitreal b-wave amplitudes versus PaO₂ is shown in Figure 4. There was again a tendency of less resistance to hypoxemia in the normal group.
Diabetic ERG and Hypoxemia

FIGURE 2. Individual ERGs recorded from a second pair of normal and diabetic pigmented rats. The component amplitudes of this diabetic rat were similar to those of the normal rats but were still resistant to hypoxemia.

FIGURE 3. RPE c-wave amplitude vs arterial oxygen tension for nine normal and six diabetic pigmented rats. Each data point represents the single recording taken at approximately the time of the measurements of PaO₂, i.e., during normoxemia and one or two steps of hypoxemia and shows the maximal effect at each level. There was a trend of reduced sensitivity to hypoxemia of the diabetic rats but this was not significant (P = 0.25).

FIGURE 4. Vitreal b-wave amplitude vs arterial oxygen tension for nine normal and six diabetic pigmented rats. Each data point represents a single recording taken at approximately the time of the measurements of PaO₂, i.e., during normoxemia and one or two steps of hypoxemia and shows the maximal effect at each level. There was a trend of reduced sensitivity to hypoxemia of the diabetic rats but this was not significant (P = 0.25). Only two of the six diabetic rats showed a marked reduction in b-wave amplitude at a PaO₂ of 20 to 30 mm Hg, while seven of the nine normal animals showed an effect at these levels of PaO₂ or higher. The transretinally recorded b-wave and the vitreal c-wave versus PaO₂ (not illustrated) also showed this trend but not to any significant degree. No correlation was found between any parameter measured and duration of diabetes, but the number of animals was not large enough to exclude the possibility.

Blood glucose levels, measured after approximately 3 hours of isoflurane and 2 hours of urethane anesthesia, were 7.2 ± 2.7 mM (mean ± SD) for the normal group (range 5.4 to 10.5 mM) and 31.3 ± 17 mM for the diabetic group (range 17.6 to 57.5 mM). Measured 2 to 3 days after induction of diabetes the blood glucose levels of the diabetic rats were generally about threefold higher than the normal values (approximately 24 vs 8 mM). At the time of the experiments the diabetic rats weighed less than the normals (324 ± 53 g vs 448 ± 82 g).

DISCUSSION

Effect of Hypoxemia on the ERG of the Normal Rat

The b-wave was reduced by hypoxia as in the cat. The most likely explanation is a reduction of the signal at
some stage in b-wave generation, whether in the Müller cell, bipolar cell, or photoreceptor. The b-wave could possibly be reduced by an increase in RPE resistance, but this is most unlikely because, assuming ocular resistances are the same as in the cat, a 50% increase would be needed to reduce the b-wave by only 15%. In cat the c-wave increases at a milder degree of hypoxia than that which diminishes the b-wave. This increase in the cat is due to subtle effect, a depolarization and reduction of resistance of the RPE basal membrane during hypoxemia, which is explained fully elsewhere.10 In cat there is little effect of hypoxemia on the light-evoked K+ change underlying the RPE c-wave, and therefore no effect on the RPE apical membrane generator or on the retinal component of the c-wave. By contrast, in the normal rat both c-wave components decrease, suggesting that the generators themselves, rather than the RPE basal membrane, are probably the main site of action of hypoxemia. It may be that the light evoked K+ change is reduced in the rat during hypoxemia, which would most likely implicate the photoreceptors, but more complex possibilities cannot be ruled out at this time.

The b-wave is probably generated by the Müller cells, which are largely dependent on the retinal circulation that can autoregulate. The c-wave components, however, are passive manifestations of potassium ions being pumped out of the subretinal space while their leakage back into this space has been indirectly blocked by light.11 The photoreceptors and RPE (and hence the Na+/K+ pumps) are nourished by the choroidal circulation, which is not believed to have the capacity to autoregulate. Thus, it might be expected that the c-wave would be more vulnerable than the b-wave to hypoxemia, though both the b- and c-waves are dependent on photoreceptor function. This subject has been reviewed in detail elsewhere. In the human Brown et al reported sensitivity of the b-wave to hypoxemia, especially using red light.12

Relative Resistance of Diabetic Rat ERG to Hypoxia

Under normoxic conditions the RPE c-wave component was clearly reduced in the diabetic rats, 2.22 ± 1.09 vs 3.92 ± 0.50 mV (mean ± SD, P = 0.003, unpaired t test), as seen in Figure 3. This result has been discussed elsewhere. The mechanism was believed to be a reduction of the c-wave generators in the presence of reduced RPE resistance and will form the basis of a future report. Although initially of lower amplitude, the diabetic RPE c-wave was barely affected even by quite severe episodes of hypoxemia. Three general possibilities could explain this.

First, the diabetic RPE c-wave could appear resistant to hypoxia if the current generator was diminished and one of the RPE membrane resistances (basal or apical) increased simultaneously, because of Ohm’s Law, V = I X R. An increase in RPE resistance is unlikely because it would increase the b-wave current through the retina itself (therefore increasing the transretinal b-wave), but would decrease the b-wave current through the parallel ERG circuit (therefore reducing the b-wave recorded in the vitreous humor). Neither of these two events occurred in relatively mild hypoxia. Alternatively, no change would have been seen in the c-wave if the generator was increased at the same time as RPE resistance was reduced. In this combination, however, the former is improbable.

Second, the diabetic ERG c-wave may have appeared resistant to hypoxia because it had been so diminished by diabetes before the episode of hypoxemia, for reasons given earlier, that no further deterioration was possible. This remains a possibility, but the normal component amplitudes of the diabetic rat illustrated in Figure 2, which were nevertheless resistant to hypoxia, would argue against this to some extent. It can be safely assumed that this animal was diabetic because two blood glucose measurements during the experiment were high and the vitreal c-wave was virtually extinguished.

Third, the retinas of the diabetic rats may have been more resistant to hypoxia because they had become less dependent on oxygen, and this seems the most likely explanation. This would be reasonable in the diabetic eye, in which increased entry of glucose into retinal cells is independent of insulin,13 and elevated glucose would be expected to promote a Crabtree effect. The Crabtree effect, whereby oxidative phosphorylation is reduced in the presence of raised glucose levels, possibly by competition for inorganic phosphate, has been demonstrated in bovine retinal tissue in phosphate buffer.15 The retina is capable of high levels of glycolysis and so in the hyperglycemic situation with the proposed reduction of oxidative metabolism, a compensatory increase in glycolysis would be expected, and indeed was reported recently.18

Crabtree Effect as a Hypothesis for Diabetic Retinopathy

In peripheral nerves in experimental diabetes Greene and Winegrad found oxygen consumption to be reduced by 32% and glycolysis to be increased by 94%.19 These findings were consistent with the resistance to tourniquet-induced hypoxia of the excitability of peripheral sensory nerves in diabetic patients, the effect correlating with the presence of neuropathy. In the human brain Grill et al demonstrated a reduction in the ratio of oxygen to glucose consumption in persons with well-controlled diabetes.21 Rubinstein et al found reduced oxygen consumption of the corneal epi-
The diabetem of diabetic patients compared to control subjects,\textsuperscript{22} though whether insulin is required for glucose entry into these cells is open to question. Thus there is evidence for a shift in metabolism in other parts of the nervous system, as we proposed here for the retina.

Any diminution of oxygen consumption would reduce retinal blood flow by vascular autoregulation,\textsuperscript{23} assuming that autoregulation also occurs in the rat retina. Diabetes probably reduces cerebral blood flow\textsuperscript{24} but its effect on retinal blood flow is not clear.\textsuperscript{25-31} Could there be a shift in adenosine triphosphate (ATP) generation in the human diabetic retina, from respiration to glycolysis? Enzymatic adaptation to increased glycolysis could explain the puzzling observation that suddenly imposing tight glucose control in a poorly controlled patient can cause inner retinal "ischemia."\textsuperscript{32,33} When the primary substrate was suddenly reduced to, say, a third of its previous level the cells may have become starved of ATP; "hypoATPic" or relatively hypoglycemic rather than hypoxic (Fig. 5).

If control of retinal blood flow is dominated by oxygen tension, rather than ATP availability, the proposed reduction in oxygen consumption, and hence blood flow, could deprive the retina of adequate ATP generation (perhaps causing preretinopathic functional disturbances\textsuperscript{34-35}) and vital, non–energy-related substances (e.g., antioxidants, growth factor inhibitors) with harmful consequences.

**Key Words**

diabetic retinopathy, etiology, ERG, hypoxia, Crabtree effect.

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**References**