Normalization of Retinal Blood Flow in Diabetic Rats With Primary Intervention Using Insulin Pumps

Allen C. Clermont, Mariel Brittis, Teruo Shiba, Terence McGovem, George L. King, and Sven-Erik Bursell

Purpose. Prior results have demonstrated a significant reduction in retinal blood flow in streptozotocin (STZ)-induced diabetic rats. These studies were extended to investigate whether retinal blood flow changes, in the diabetic rat model, could be prevented with strict glycemic control using insulin pumps. Retinal blood flow changes also were measured during hyperoxia and after intravitreal histamine infusion to validate the methodology.

Methods. Retinal blood flow changes were measured using video-based fluorescein angiography and computer-assisted image analysis. A total of 48 male Sprague-Dawley and 9 Brown Norway rats were used in these experiments. Retinal blood flow after primary insulin intervention was evaluated in diabetic rats implanted with miniosmotic insulin pumps within 24 hours of STZ-induced diabetes. Diabetic rats, not treated with insulin, were used for comparison.

Results. Hyperoxia caused a significant (P = 0.001) reduction (54%) in retinal blood flow, whereas intravitreal infusion of 10^-3 M histamine caused a significant (P = 0.009) increase (108%) in retinal blood flow. Retinal blood flow in the primary insulin intervention group, after 1 week of diabetes, was not statistically different from retinal blood flow of nondiabetic controls as measured at baseline from the animals used in the hyperoxia and histamine infusion experiments. In contrast there was a significant (P = 0.0001) retinal blood flow reduction in the untreated diabetic group.

Conclusions. The results showed that the local effect of histamine and hyperoxia on the retina produced the expected responses in retinal blood flow, further confirming the validity of the methodology. Primary insulin intervention demonstrated that strict glycemic control initiated immediately after induction of diabetes was sufficient to maintain normal retinal blood flow in STZ-induced diabetic rats. Invest Ophthalmol Vis Sci. 1994;35:981-990.

Vascular complications in diabetes mellitus are the result of metabolic and hormonal abnormalities caused by insulin deficiency. The identification of specific factors causing vascular cell dysfunction and the mechanisms by which they exert their influence is very important in the treatment of patients with diabetes. Hemodynamic changes have been implicated in the process of vascular complications. Blood flow changes in the retina have been shown to be associated with diabetic retinopathy, and various methodologies have been used to investigate this phenomenon.

We have used a video-based fluorescein angiography (VFA) system coupled with computer-assisted image analysis to determine retinal blood flow both in the rat model and in human subjects. In a prior study, we have reported that there was a significant reduction in retinal blood flow in streptozotocin (STZ)-induced diabetic rats as early as 1 week after diabetes induction. In that study, the results showed that retinal mean circulation times (MCT) were significantly prolonged, and that there was a 37% reduction in retinal blood flow in the rats with 1 week's duration of diabetes compared to the matched control group. In addition, there were no significant changes in retinal vessel diameters between the diabetic and control groups, indicating that the reduction in flow was being mediated at the microcirculatory level, and reflected primarily a prolongation in the retinal MCT. Measurements made from human subjects using our VFA system also show significantly reduced retinal blood flow...
in patients with type 1 diabetes with less than 5 years' duration of disease and no clinically evident diabetic retinopathy. Other investigators have observed reduced blood flow velocities in the perimacular capillaries of diabetic subjects with no or mild diabetic retinopathy, and reduced retinal blood flow in children with diabetes with poor glycemic control.

The current studies were performed to characterize the effect of histamine and hyperoxia on retinal circulatory parameters in the rat. Furthermore, we have extended our previous studies to investigate whether primary insulin intervention can adequately prevent the abnormality in retinal blood flow found in the diabetic rats.

MATERIALS AND METHODS

Instrumentation

The VFA system has been previously described. This system has since been modified to include a Recognition Concepts, Inc. (Carson City, NV) Trapix Plus and DataStore, which enable direct digitization and storage of the analogue video signal. Video angiograms were digitized at the video rate of 30 frames/second and stored as 512 X 512 X 8 monochrome images. Direct digitization permits the maintenance of full video camera resolution (700 video lines), in contrast to prior video tape storage medium, which provided approximately 400 lines. The system memory permits the storage of up to 20,000 digitized images, and thus can be used to record all the angiograms from an experiment. The subsequent analysis of the angiograms was performed on a frame-by-frame basis to determine the retinal circulatory parameters of interest.

Animal Protocols

Forty-eight male albino Sprague-Dawley (Taconic, MA) and 9 Brown Norway pigmented rats were used for the experiments described below. All the animals were handled and cared for according to the ARVO Resolution on the Use of Animals in Research. For all the animals used in these studies, the following procedures were performed: on the day before VFA measurements, all animals had a surgically implanted Silastic catheter placed into the right jugular vein. The catheter was subcutaneously externalized to the back of the neck. Each catheter was flushed with approximately 0.1 ml solution of 1000 U heparin/30 ml 0.9% NaCl before and after catheter placement. The rats were anesthetized for this procedure with 0.1mg/kg sodium amobarbital injected intraperitoneally.

Immediately before VFA measurements, each rat was again anesthetized as above, and the left eye was dilated using 1% tropicamide. VFA measurements were made only from the left eye to avoid possible changes in blood flow delivery related to the right-sided jugular vein catheterization. A 40-μl Hamilton syringe filled with 10% sodium fluorescein dye was connected to the jugular vein catheter. The rat was positioned on a platform attached to the chin rest of the retinal fundus camera, and a focused image of the retina with the optic disk centered in the field of view was obtained. Figure 1 illustrates an image obtained from a VFA recording of a Sprague-Dawley rat.

With the barrier filter in place, the VFA recording sequence was initiated and a 5-μl bolus of fluorescein dye was injected rapidly (approximately 0.1 seconds) into the jugular vein catheter. The time of injection was marked on the video recording. The dye first appeared in the retinal arteries 1 to 2 seconds after the time of injection, and cleared from the retinal veins approximately 5 seconds later.

Hyperoxic Effect

In this experiment, nine Sprague-Dawley rats, weighing approximately 200 g, were used. For each rat, two to three angiograms were recorded with the rat breathing room air. These recordings provided the baseline measurements. A latex breathing mask was then attached over the nose and mouth of the rat. The rat was exposed to a 100% oxygen breathing gas for a period of 2 minutes. With the mask still in place, and the rat still breathing 100% oxygen, a further series of four to six angiograms was recorded.

FIGURE 1. Image taken from a fluorescein angiogram recording of a Sprague-Dawley albino rat.
Histamine Effect

For these experiments, 11 male Sprague-Dawley rats and 9 male Brown Norway rats were used. The 11 Sprague-Dawley animals were separated into 2 groups. Eight animals underwent intravitreal infusion of a 10-μl volume of 10^{-3} M histamine dissolved in phosphate-buffered saline (PBS), and three animals underwent intravitreal infusion of 10 μl of PBS alone to provide the controls for this experiment. Before intravitreal infusions, a series of two to three VFA was recorded to provide baseline retinal circulatory parameters. Intravitreal infusion was performed by first gently manipulating the eye so that it propulsed from the orbit, allowing visualization of the limbus. The 30-gauge needle attached to a 10-μl Hamilton syringe was inserted carefully into the vitreous cavity at a site 2 mm posterior to the limbus. The insertion of the needle was performed under direct visualization, taking care to avoid the spherical lens. The tip of the needle was positioned directly above the optic disk region. The agent was then slowly infused into the vitreous cavity, with observation of the central retinal artery to ensure that no acute elevation of intraocular pressure occurred during infusion or as a result of the injected agent.

Five minutes after the intravitreal infusions of either histamine or vehicle, a series of four to six postinfusion VFA recordings was taken. This 5-minute period between infusion and VFA measurement was the time of maximal histamine effect, as assessed from prior experiments that measured the retinal response to histamine at different times after intravitreal infusion. In addition, the investigators were masked as to whether histamine or vehicle alone was infused until after the analyses had been completed.

The retinal circulatory dose–response characteristics to different concentrations of infused histamine were investigated using the nine pigmented Brown Norway rats. After recording baseline angiograms from each animal, histamine was infused intravitreally, at a concentration of 10^{-7} M (one animal), 10^{-6} M (two animals), 10^{-5} M (two animals), 10^{-4} M (two animals), and 10^{-3} M (two animals). Postinfusion VFA recordings were again made at 5 minutes. The value of the infused dose of histamine was masked from investigators until after the analyses had been completed.

Primary Insulin Intervention

Twenty-eight male albino Sprague-Dawley rats, weighing approximately 200 g, were used in these experiments. All 28 animals were made diabetic with intraperitoneal injection of 100 mg/kg of STZ (100 mg STZ/1 ml 10 mM citric acid at pH 4.5). Animals were fasted for 12 hours before STZ injection. Initial blood glucose levels were obtained by tail vein samples 24 hours after STZ injections to determine diabetic status. All rats with blood glucose levels greater than 250 mg/dl were considered diabetic and were included in the study. Blood glucose levels were then obtained every other day for the duration of the study.

Seventeen of these diabetic rats, selected at random, underwent placement of a 7-day intraperitoneal miniosmotic insulin pump (Alzet [Palo Alto, CA] model 2001) immediately after initial blood glucose determination. Eleven of the rats remained as insulin-deprived, untreated animals. The rats were anesthetized for insulin pump emplacement using 0.1 mg/kg sodium amobarbital injected intraperitoneally. Before placement, each pump was filled with U-100 regular insulin (Humulin, Lilly, Indianapolis, IN) buffered with 7 mg glutamic acid/1 ml insulin. The duration of diabetes for all animals was 1 week. Table 1 describes the characteristics of the animals in this experiment. All animals were housed two to a cage and supplied with two water bottles. The condition of the animals, whether they were on insulin pumps or were insulin deprived, was masked from the investigators until the conclusion of the data analysis.

Data Analysis

VFAs were recorded and stored on the Trapix Plus system. Each angiogram was analyzed densitometrically for changes in fluorescence intensity over time. Sample sites were chosen from the primary retinal vessels at a fixed radial distance from the optic disk center. Densitometric measurements were made typically from six artery–vein pairs and from sites adjacent to the vessels for the determination of the local background fluorescence.

<table>
<thead>
<tr>
<th>TABLE 1. Characteristics for Primary Insulin Intervention Groups</th>
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</thead>
<tbody>
<tr>
<td><strong>Diabetic, insulin intervention</strong></td>
</tr>
<tr>
<td>(n = 17)</td>
</tr>
<tr>
<td>Body weight (g)</td>
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<tr>
<td>Blood glucose (mg/dl)</td>
</tr>
<tr>
<td><strong>Diabetic, untreated (n = 11)</strong></td>
</tr>
<tr>
<td>Body weight (g)</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
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</table>
Vessel diameters were determined from images recorded before fluorescein dye injection and barrier filter insertion, at the chosen vessel sample sites. The width of the vessel defined the size of the window used for measuring vessel fluorescence intensities. The diameter of each vessel was calculated in pixels (video picture elements) by locating the edge of the vessel wall with a boundary-crossing algorithm from four distinct sites. The difference between half-heights for two opposite sites yielded the vessel width at that site. Each vessel width could be measured to within ±0.5 pixels. Vessel widths were calculated from the average of the four distinct sites. The resulting vessel measurement window formed a rectangular area that was the width of the vessel and ten pixels in length along the vessel. The average vessel diameters calculated for each eye represent the averages of the individual artery and vein diameters for that eye, quoted as average ± 1 standard deviation (SD).

The average retinal artery and vein fluorescence intensities within these vessel sample site windows were determined on a frame-by-frame basis. In addition, background fluorescence intensity levels were obtained from sample windows set adjacent to the respective vessels. These temporal fluorescence intensity levels yielded background-corrected dye dilution curves for each retinal vessel.

The dye dilution curves for each vessel were fit to a log-normal distribution function. The fitted parameters of the log-normal function were used to determine the average retinal artery and vein circulation times.

The resulting dye dilution curves provided two parameters used to characterize the circulation. The first parameter, the arterial appearance time (AT), was defined as the time between dye injection and the first detectable appearance (vessel fluorescence intensity greater than the background intensity level by twice the SD of the average background intensity) of the dye in the central retinal artery at the optic disk. The AT represents a relatively macroscopic view of the systemic delivery of dye to the eye. The second parameter, the retinal MCT, was calculated as the time difference between the average arterial circulation time and the average venous circulation time for corresponding retinal arteries and veins. The MCT was calculated for each of the six artery–vein pairs. By averaging the six MCT values, an average retinal MCT for each animal was obtained. Retinal blood flow has been shown to be proportional to the sum of the squares of the arterial and venous diameters divided by the retinal MCT. This measure of retinal blood flow is based on the assumption that the vessel diameter measurements reflect the perfused retinal blood volume. This assumption will not be valid if areas of nonperfusion or capillary shunting develop. In nondiabetic animals and in the early stages of diabetes, however, capillary nonperfusion is unlikely to occur. The calculated retinal blood flow values are in units of pixels²/second, and all results are reported as average ± SD.

The results from the hyperoxia and histamine infusion experiments were evaluated using a paired Student’s t test comparing baseline values to values determined during hyperoxia or 5 minutes after intravitreal histamine infusion. Population differences were tested for normality using the Wilks-Shapiro test. In cases where the differences were non-normally distributed, the Wilcoxon rank sum test was used for the paired data. Group comparisons were evaluated using the two-tailed Student’s t test. The populations were tested for normality using the Wilks-Shapiro test, and in this case, non-normal populations were evaluated using the Mann-Whitney rank sum test. In addition, if the variances of the compared groups were not equal, an unequal variance t test was used. Values of P < 0.05 were considered to be statistically significant.

**RESULTS**

**Hyperoxic Response**

The results obtained from the nine nondiabetic Sprague-Dawley rats (average weight = 205.6 ± 29.0 g) breathing room air followed by breathing 100% oxygen are summarized in Table 2. The changes in retinal circulatory parameters were evaluated in a paired fashion, comparing values measured while breathing room air to values measured while breathing 100% oxygen.

The paired t test evaluations showed a significant arterial (0.72 ± 0.65 pixels) and venous (0.80 ± 0.49 pixels) diameter changes. The results are summarized in Table 2.

<table>
<thead>
<tr>
<th>Breathing Mixture</th>
<th>Room Air</th>
<th>100% Oxygen</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT (sec)</td>
<td>1.39 ± 0.18</td>
<td>1.71 ± 0.22</td>
<td>0.0001</td>
</tr>
<tr>
<td>MCT (sec)</td>
<td>0.87 ± 0.27</td>
<td>1.64 ± 0.49</td>
<td>0.001</td>
</tr>
<tr>
<td>Artery diameter (pixel)</td>
<td>7.0 ± 0.3</td>
<td>6.3 ± 0.5</td>
<td>0.01</td>
</tr>
<tr>
<td>Vein diameter (pixel)</td>
<td>8.3 ± 0.5</td>
<td>7.5 ± 0.5</td>
<td>0.002</td>
</tr>
<tr>
<td>Blood flow (pixel²/sec)</td>
<td>144.0 ± 29.9</td>
<td>65.8 ± 30.5</td>
<td>0.001</td>
</tr>
</tbody>
</table>

**TABLE 2. Retinal Circulatory Response to Hyperoxia (n = 9)**
pixels) constriction during hyperoxia. This vasoconstriction, together with the significant prolongation of the retinal MCT (0.77 ± 0.45 second), resulted in a significant reduction in average retinal blood flow (78.4 ± 36.7 pixels²/second) while breathing 100% oxygen compared to breathing room air. In addition, the AT during hyperoxia was significantly (P = 0.0001) prolonged compared to baseline (0.35 ± 0.09 second), reflecting the hyperoxia-induced reduction in cardiac output and an overall decreased extraocular flow.

**Histamine Response**

The results obtained from the eight nondiabetic Sprague-Dawley rats (average weight = 338.8 ± 17.5 g) that had been infused intravitreally with 10⁻³ M histamine and the three animals (average weight = 220.0 ± 10.0 g) infused with vehicle alone are summarized in Table 3. The changes in the circulatory parameters were evaluated in a paired fashion, comparing preinfusion baseline to postinfusion values.

Five minutes after intravitreal histamine infusion, there was significant dilation of the retinal arteries (0.67 ± 0.41 pixel) and veins (0.97 ± 0.37 pixel) compared to preinfusion values. This vasodilation, together with the significant reduction in MCT (0.34 ± 0.29 second), resulted in a significant increase in retinal blood flow (165 ± 131 pixels²/second) after 10⁻³ M intravitreal histamine infusion. In contrast, there was no significant changes in any of the above retinal circulatory parameters 5 minutes after intravitreal infusion of vehicle alone. In addition, there were no significant changes in retinal AT both after histamine infusion and after vehicle infusion. Of note, AT was increased in the group of histamine-infused animals compared to the vehicle-infused animals. A prior study¹⁵ had demonstrated a significant association between increasing AT and increasing body weight in the rat model. The increased AT noted in the heavier animals used for the histamine infusion was a reflection of this phenomenon, and indicates that the AT parameter can provide a useful assessment of extraocular systemic circulation.

Nine pigmented Brown Norway rats were used to investigate the possible dose–response characteristics of the retina to different concentrations of infused histamine. The results are illustrated in Figure 2, which shows the average percent decrease (± SD) in MCT from baseline for infused histamine concentrations ranging from 10⁻⁷ M to 10⁻³ M. Two animals were measured at each concentration except 10⁻⁷ M. The results demonstrate decreasing MCT changes with decreasing concentrations of infused histamine, showing an EC₅₀ of approximately 5 x 10⁻⁶ M injected concentration of histamine.

**Primary Insulin Intervention**

VFA measurements were made from two groups of Sprague-Dawley rats. The primary insulin intervention group consisted of 17 diabetic rats that had had an Alzet miniosmotic insulin pump implanted immediately after confirmation of diabetes (blood glucose > 250 mg/dl). The comparison group consisted of 11 untreated diabetic rats that were used as a matched control group to the insulin-treated group. Unpaired t test evaluations were used to compare the group characteristics summarized in Table 1 and the retinal circulatory parameters summarized in Table 4.

The diabetic group implanted with insulin pumps showed a significant weight gain compared to the untreated diabetic animals. The blood glucose levels dropped to euglycemic levels the day after the pump was implanted, and these were maintained for the 1-week duration of the experiment (82.4 ± 35.0 mg/dl compared to 398.6 ± 50.3 mg/dl). The weight gain in the insulin-treated animals was consistent with that observed for nondiabetic animals, approximately 50 g/week. The untreated diabetic rats showed no significant change in weight, although the glucose levels did show a significant elevation during this 1-week period. The untreated group of diabetic animals showed a

**TABLE 3. Retinal Response to Intravitreal Histamine Infusion**

<table>
<thead>
<tr>
<th></th>
<th>Preinfusion</th>
<th>Postinfusion</th>
<th>P Value</th>
</tr>
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<tbody>
<tr>
<td><strong>10⁻³ M histamine (n = 8)</strong></td>
<td></td>
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</tr>
<tr>
<td>AT (sec)</td>
<td>1.68 ± 0.3</td>
<td>1.77 ± 0.22</td>
<td>0.12</td>
</tr>
<tr>
<td>MCT (sec)</td>
<td>0.87 ± 0.23</td>
<td>0.53 ± 0.16</td>
<td>0.015</td>
</tr>
<tr>
<td>Artery diameter (pixel)</td>
<td>6.8 ± 0.3</td>
<td>7.5 ± 0.4</td>
<td>0.002</td>
</tr>
<tr>
<td>Vein diameter (pixel)</td>
<td>8.9 ± 0.3</td>
<td>9.9 ± 0.2</td>
<td>0.0005</td>
</tr>
<tr>
<td>Blood flow (pixel²/sec)</td>
<td>152.5 ± 40.7</td>
<td>317.6 ± 126.8</td>
<td>0.009</td>
</tr>
<tr>
<td><strong>Vehicle (PBS) (n = 5)</strong></td>
<td></td>
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<tr>
<td>AT (sec)</td>
<td>1.47 ± 0.11</td>
<td>1.48 ± 0.07</td>
<td>0.7</td>
</tr>
<tr>
<td>MCT (sec)</td>
<td>0.88 ± 0.06</td>
<td>0.90 ± 0.06</td>
<td>0.6</td>
</tr>
<tr>
<td>Artery diameter (pixel)</td>
<td>7.3 ± 0.3</td>
<td>7.2 ± 0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Vein diameter (pixel)</td>
<td>9.0 ± 0.8</td>
<td>8.9 ± 0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>Blood flow (pixel²/sec)</td>
<td>152.3 ± 18.3</td>
<td>146.1 ± 25.1</td>
<td>0.3</td>
</tr>
</tbody>
</table>
lower average weight than the insulin-treated diabetic animals; however, this difference in weight was not statistically significant.

The arterial and venous diameters were not significantly different between the two groups. The MCT, however, was significantly shorter for the animals on insulin pumps compared to the prolonged MCTs measured in the untreated diabetic group. The large significant decrease in MCT for the animals on insulin pumps yielded a significantly higher value for retinal blood flow than those measured in the untreated diabetic animals. The higher retinal blood flow in the insulin intervention group is attributable to the change in MCT because there were no significant differences in the primary vessel diameters. The systemic circulation time (AT) also was significantly shorter in the group implanted with insulin pumps, compared to the untreated diabetic group. The AT parameter measured from the animals implanted with insulin pumps was not statistically different from that measured in the nondiabetic animals (Table 2). In addition, there was no significant correlation between MCT and AT (slope = 0.12, r² = 0.05, P = 0.2) for the rats used here. These results indicated that the change in AT did not account for the total variation in the measured retinal MCT.

Following the suggestion of one of the reviewers, we have made measurements from two nondiabetic rats that had been chronically infused with 50% glucose for 48 hours, and one control animal infused with normal saline for 48 hours. This hyperglycemic model, using chronic in vivo glucose infusion in nondiabetic rats, has been previously described. Briefly, indwelling jugular vein catheters were surgically implanted and exteriorized between the shoulders. The infusion device allowed the rats to have unrestrained access to food and water. The glucose infusions were started 24 hours after catheter emplacement, and VFA recordings were obtained 48 hours after the initiation of glucose infusions.

The saline-infused rat had a 48-hour blood glucose level of 154 mg/dl, and demonstrated retinal hemodynamic parameters comparable to those of nondiabetic animals (MCT = 1.0 second, artery diameter = 6.4 pixels, vein diameter = 8.9 pixels, blood flow = 118.1 pixel²/second), whereas the two 50% glucose-infused animals had an average blood glucose level at 48 hours of 340 mg/dl, and demonstrated average retinal hemodynamic parameters comparable to our STZ-induced diabetic rats (MCT = 1.8 ± 0.9, artery diameter = 6.1 ± 0.07 pixels, vein diameter = 8.9 ± 0.8 pixels, and blood flow = 78.5 ± 46.5 pixels²/second).

**DISCUSSION**

A number of different noninvasive techniques have been used to measure retinal blood flow; the most widely used technique is laser Doppler velocimetry. The disadvantage with most of these methodologies is that retinal blood flow measurements must be made separately in each individual artery or vein. We have chosen to use a methodology based on VFA recordings. These recordings can be made easily from both humans and animal models, which facilitates translation of basic research studies to clinical applications. This methodology also allows the characterization of retinal blood flow from all the retinal vessels in the retinal field of view using a single angiogram recording. The small amount of dye required for these measurements ensures a sharp dye front in the retinal cir-

<table>
<thead>
<tr>
<th>TABLE 4. Retinal Circulatory Parameters and Primary Insulin Intervention</th>
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<tr>
<td></td>
</tr>
<tr>
<td>Number</td>
</tr>
<tr>
<td>AT (sec)</td>
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<tr>
<td>MCT (sec)</td>
</tr>
<tr>
<td>Artery diameter (pixel)</td>
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<tr>
<td>Vein diameter (pixel)</td>
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<tr>
<td>Blood flow (pixel²/sec)</td>
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culation, enhancing the accuracy of retinal circulation time determinations. In addition, because such small bolus injections of dye are used, multiple angiograms can be performed in the same animal.

The effects of known vasoactive stimuli on the retinal circulation were investigated to demonstrate the validity of this methodology in determining retinal circulatory parameters. We chose to measure the retinal response to hyperoxia by having the rats breathe 100% oxygen, and the local retinal response to histamine by infusing histamine directly into the vitreous.

Hyperoxia is known to produce a reduction in cardiac output, vessel constriction, and reduced organ blood flow. In the retina, breathing 100% oxygen has been shown to reduce retinal blood flow by between 40% and 60%. In this study, nondiabetic Sprague-Dawley rats, after 2 minutes of breathing 100% oxygen, showed a significant constriction of retinal arteries (10.5%) and veins (10.2%), a significant prolongation of the retinal MCT (90.7%), and a significant reduction in retinal blood flow (54.3%). Compared to retinal circulatory parameters measured while the rats breathed room air. In these experiments there was also a significant prolongation of AT (23.9%). This probably reflects the expected reduction in cardiac output during 100% oxygen breathing. The prolongation in AT, a measure of the systemic delivery of dye to the eye, is a relatively macroscopic parameter reflecting prolonged circulation times in the extraocular tissue beds. The change in AT, however, does not account for the total increase in measured retinal MCT, indicating that although the prolonged AT may contribute to the prolonged retinal MCTs, the primary effect appears to be the local prolongation of retinal circulation times, reflecting the retinal autoregulatory response to hyperoxia. These results agree with other investigations on retinal blood flow changes in human subjects breathing pure oxygen. The results from the hyperoxia experiments demonstrated that the VFA methodology could be used to quantitate expected retinal circulatory responses to hyperoxia.

Histamine is an intrinsic regulator of the microcirculation, causing vasodilation and increased blood flow in a number of microcirculatory vascular beds. Histamine and its related enzymes also have been detected in rat and bovine retina, suggesting that histamine may play a role as a modulator of the retinal circulation. Studies on isolated bovine retinal arteries demonstrated that histamine-induced vascular dilation involved primarily the activation of H1-receptors. Retinal histamine synthesis also has been shown to be increased in STZ-induced diabetic Sprague-Dawley rats.

In this study, using nondiabetic Sprague-Dawley rats, and comparing values measured 5 minutes after histamine infusion to preinfusion baseline values, intravitreal infusion of 10^{-3} M histamine induced significant vasodilation in the retinal arteries (9.7%) and retinal veins (10.9%), a significant reduction in MCT (39.1%), and a twofold increase in retinal blood flow. In contrast, the infusion of PBS, the vehicle in which histamine was dissolved, produced no significant changes in any of the retinal circulatory parameters. In addition, there was no significant change in AT (a measure of systemic delivery of the dye to the eye) after intravitreal infusions of histamine or PBS alone, indicating that the histamine effect was localized to the retina and did not induce any measurable systemic flow changes. Histamine also demonstrated a retinal dose–response characteristic, with an EC50 of approximately 5 × 10^{-6} M. The histamine dose–response experiment was performed using pigmented Brown Norway rats, and the measured histamine responses indicated that this retinal response was not restricted to a single rat species. Although it is difficult to assess the actual concentration of histamine reaching the retinal vessels, an estimate can be made assuming that the volume of the rat vitreous is approximately 250 μl. Thus, one would expect, at the most, a dilution factor of approximately 25 to the initial infused concentration of histamine, and an EC50 for the retinal vessels of approximately 2 × 10^{-7} M. This half-maximal response is lower than that found in isolated bovine retinal artery segments. This difference may be associated with the fact that the latter results were obtained from an in vitro situation, whereas the results reported here were from in vivo experiments. Nevertheless, the intravitreal histamine infusion results show that the VFA methodology could be used to quantitate retinal responses of vasodilation and increased retinal blood flow, as well as hyperoxia-induced vasoconstriction and reduced retinal blood flow.

The results from the insulin intervention studies showed that, in the group of diabetic animals implanted with insulin pumps to maintain euglycemia, retinal blood flow was not significantly different from that measured in the nondiabetic animals used in these studies (Tables 2, 3). In addition, the retinal blood flow after 1 week of diabetes in the untreated diabetic rats was significantly (P = 0.0001) lower than that in insulin-treated diabetic rats and nondiabetic rats. The reduction in retinal blood flow appears to be primarily associated with a significant prolongation of the retinal MCT in the untreated diabetic animals, because there were no significant changes in arterial or venous diameters between untreated and insulin pump-implanted diabetic animals and nondiabetic animals. These results confirm our previous findings, and indicate that at this stage of early diabetes, the reduction in blood flow in the untreated diabetic animals was most probably associated with increased vascular resistance to flow at the microcirculatory level, rather than
with vascular diameter changes in the larger retinal vessels.

The prolonged AT time in the untreated diabetic rats was most likely a reflection of diabetes-related changes in hemorheology, such as increased viscosity, reduced flow in extraocular tissues, or both. The AT time in the insulin-treated rats was not significantly different from that in nondiabetic animals of comparable weight, and there was no significant association between MCT and AT. The change in AT does not account for the total variation in retinal MCT in the diabetic animals. Thus, although extraocular changes in AT may contribute to the prolonged retinal MCT in the diabetic animals, the data indicate that the measured retinal hemodynamic changes are primarily characteristic of diabetes-associated changes in the retinal circulation.

The preliminary results obtained from the chronic hyperglycemia experiments showed, in nondiabetic rats after 48 hours of chronic glucose infusion, that the measured retinal blood flows were similar to those observed in the STZ-induced diabetic rats. The levels of blood glucose measured after 48 hours of glucose infusion also were comparable to the blood glucose levels measured in the group of STZ-induced diabetic rats. These results indicate that the measured reduction in retinal blood flow is a response to the chronic hyperglycemic insult in diabetes.

The response to chronic hyperglycemia described above is different from that observed after acute hyperglycemia, where investigators have reported a transient increase in retinal blood flow after a bolus infusion of glucose in diabetic and nondiabetic dogs, nondiabetic cats, and nondiabetic humans. We have reported a similar effect in patients with diabetes using the VFA methodology. In this study, patients with diabetes underwent a stepped glucose clamp, and the retinal blood flow measured at a blood glucose level of 300 mg/dl was found to be significantly greater than that measured at 100 mg/dl.

The results from the primary insulin intervention study demonstrate that the retinal blood flow can be maintained in a normal range in diabetic animals if strict glycemic control is initiated within 24 hours of diabetes induction. Other investigations have demonstrated that the maintenance of euglycemia in diabetic dogs, by insulin, at the initiation of diabetes, will completely prevent the development of retinal vascular lesions. This suggests that there may be an association between early diabetes-related retinal blood flow changes and the subsequent development of retinal vascular complications.

The phenomenon of decreased retinal blood flow also has been observed in patients with diabetes of less than 5 years' duration, and no clinical evidence of diabetic retinopathy, using the same VFA methodology. Other studies have demonstrated reduced retinal flow in patients with diabetes with no diabetic retinopathy, and in diabetic dogs before any observable retinopathy development. In contrast, increased retinal perfusion has been measured in diabetic rats of 4 to 6 weeks' duration using the microsphere impaction technique, and using a modified hydrogen clearance technique in animals with 5 weeks' duration of diabetes. In these studies, the methodologies used were more invasive, the levels of blood glucose attained were much higher (400 to 600 mg/dl), and the duration of disease was considerably longer than in the current study. We chose to investigate much earlier diabetes-associated changes in the retina related to lower overall levels of hyperglycemia. Thus, the differences in results may reflect only the differences in level and duration of diabetes obtained in the different animal groups studied.

Retinal blood flow also has been shown to be increased in patients with diabetes with background diabetic retinopathy. These patients, however, already had clinically observable retinopathy, and characteristic were at a later stage in the disease process. Nevertheless, using the VFA methodology, we have demonstrated the same retinal blood flow changes in the very earliest stages of diabetes in both humans and in the rat model. In addition, the expected retinal circulatory responses to local histamine infusion and hyperoxia were measured here, giving further credence to the retinal blood flow changes measured in diabetes.

It has been postulated that the reduction in retinal blood flow observed in the diabetic animals may be related to elevated levels of protein kinase C (PKC) and diacylglycerol (DAG). PKC and DAG have been shown to be activated in response to a hyperglycemic stimulus, and elevated levels have been measured from retinal endothelial cells obtained from diabetic animals. Investigations also have shown that elevated levels of PKC and DAG in the rat retina are correlated to retinal blood flow reduction. PKC has been shown to modulate smooth muscle cell contraction, which may play a role in this reduction of retinal blood flow in diabetes. Results also have demonstrated that the elevated levels of PKC and DAG measured in diabetic animals can be reversed with the institution of insulin therapy. These biochemical measurements parallel the normalization of retinal blood flow in insulin-treated diabetic animals, and indirectly support the hypothesis that retinal blood flow can be modulated by levels of PKC and DAG in diabetes. These results also indicate that insulin therapy applied immediately after diabetes induction to maintain normal blood glucose levels is sufficient to maintain normal retinal blood flow for the duration of diabetes studied here.
Retinal Blood Flow in Insulin-Treated Diabetic Rats

Key Words
retina, hemodynamics, diabetes, insulin, rats

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References
29. Ernest JT, Goldstick TK, Engerman RL. Hyperglycemia impairs retinal oxygen autoregulation in normal


