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Retinal blood flow abnormalities following six months of hyperglycemia in the Ins2(Akita) mouse

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Abstract

The aim of this study was to characterize the microvascular flow abnormalities and oxygenation changes that are present following six months of hyperglycemia in the diabetic Ins2(Akita) mouse. Previous studies have shown decreased retinal blood flow in the first several weeks of hyperglycemia in rodents, similar to the decreases seen in the early stages of human diabetes. However, whether this alteration in the mouse retina continues beyond the initial weeks of diabetes has yet to be determined, as are the potential consequences of the decreased flow on retinal oxygenation. In this study, male Ins2(Akita) and age-matched C57BL/6 (non-diabetic) mice were maintained for a period of six months, at which time intravital microscopy was used to measure retinal blood vessel diameters, blood cell velocity, vascular wall shear rates, blood flow rates, and transient capillary occlusions. In addition, the presence of hypoxia was assessed using the oxygen-sensitive probe pimonidazole. The diabetic retinal microvasculature displayed decreases in red blood cell velocity (30%, $p < 0.001$), shear rate (25%, $p < 0.01$), and flow rate (40%, $p < 0.001$). Moreover, transient capillary stoppages in flow were observed in the diabetic mice, but rarely in the non-diabetic mice. However, no alterations were observed in retinal hypoxia as determined by a pimonidazole assay, suggesting the possibility that the decreases seen in retinal blood flow may be dictated by a decrease in retinal oxygen utilization.

Keywords

Diabetes; blood flow; retina; hypoxia; mouse

1. Introduction

The leading cause of blindness in western countries is diabetic retinopathy (DR) (Rossing 2005); yet, the mechanisms for DR are still poorly understood. Diabetic retinopathy, characterized by microaneurysms and intraretinal hemorrhages, progresses to proliferative DR characterized by retinal neovascularization (Fong et al., 2003; Yam and Kwok 2007). It

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is believed that retinal hypoxia plays a role in the development of DR by upregulating vascular endothelial growth factor (VEGF) as a key molecule in the angiogenic sequence (Pe'er et al., 1996; Ayalasomayajula and Kompella 2003).

The hypoxia that develops in the diabetic retina is thought to result from decreased retinal perfusion, which could result from decreased flow rates and/or capillary dropout (Curtis et al., 2009). Retinal blood flow has been reported in humans to decrease by approximately 30-35% prior to the development of clinical manifestations as described previously (Clermont et al., 1997). Following this initial decrease in retinal blood flow is a return to normal values that then may even increase to supranormal levels as the severity of DR continues to worsen (Clermont et al., 1997). Similar results have been observed in a separate study (Konno et al., 1996); and furthermore, autoregulation of blood flow is dysregulated in both initial and subsequent phases of the disease (Berkowitz and Roberts 2008).

Similar to early human DR, we observe significant decreases in retinal blood flow rates in the initial weeks of hyperglycemia in diabetic rats and mice (Lee and Harris 2008; Lee et al., 2008; Wright and Harris 2008; Wright et al., 2009; Wang et al., 2010; Wang et al., 2011; Yadav and Harris 2011). The magnitude of the decrease within the first several weeks of developing hyperglycemia in rodents is ~25-40%, very similar to the change seen in early human DR. While it could be speculated that retinal hypoxia would accompany this decrease in blood flow rate, we see little if any evidence of this within the first 3-12 weeks (Wright et al., 2010). However, it is certainly possible that the retina could become hypoxic at subsequent time points, especially if blood flow remains low.

Accordingly, the purpose of the current study is to measure retinal blood flow and hypoxia following six months of hyperglycemia. To our knowledge, this is the first study to investigate retinal blood flow and hypoxia measurements at the same time point of experimental diabetes in rodents. This characterization of diabetic retinal pathology uses the Ins2(Akita) mouse, which is a model of type 1 diabetes that has been shown to exhibit abnormalities similar to early clinical findings in humans, with vascular lesions and leukostasis having been shown to occur in this model following 31 weeks of hyperglycemia (Barber et al., 2005).

2. Materials and methods

2.1 Animals

Male C57BL/6^{Ins2Akita/J} diabetic mice (Jackson Laboratories) and age-matched male C57BL/6J control mice (Jackson Laboratories) were received at an age of 5-6 weeks. The mice were given standard chow and water and were housed one per cage for 25 weeks with no insulin administered. By this endpoint, the Ins2(Akita) mice should have been diabetic for ~26 weeks (6 months), given the onset of hyperglycemia at 4 weeks of age (Barber et al., 2005). All mice were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all procedures were approved by the Louisiana State University Health Sciences Center – Shreveport institutional animal care committee.

2.2 Measurement of retinal blood flow and complete blood count

On the day of the experiment, body weight and non-fasting blood glucose levels were checked via a tail vein puncture using an AlphaTRAK blood glucose monitoring system (Abbott Laboratories, IL). The mice were anaesthetized using pentobarbital (50 mg/kg i.m.) and ketamine (50 mg/kg i.m.) 5 min apart. The right carotid artery was cannulated and mean arterial pressure (MAP) was measured using a Pressure Monitor BP-1 (World Precision Instruments, Sarasota, FL). Intravital microscopy was used to measure retinal vessel

diameters and red blood cell (RBC) velocities in the left eye, using methods described in several of our recent studies (Wang et al., 2010; Wang et al., 2011; Yadav and Harris 2011). Briefly, measurements of diameters were enhanced by vascular filling with a systemic infusion of high molecular weight (2×10^6) fluorescein isothiocyanate (FITC)-dextran, and RBCs from a C57BL/6 non-diabetic donor mouse were visualized by fluorescent labeling with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI), with velocity calculated from the length traveled per image exposure time (10 ms). Following the intravital microscopy measurements on five arterioles and five venules per retina, an arteriolar blood sample was taken from the cannulated carotid artery and a complete blood count analysis was performed (Beckman Coulter, LH750; Brea, CA). Mice with MAP < 50 mmHg were excluded from the intravital microscopy studies; however, blood samples were still collected from these mice for complete blood count analyses.

The intravital microscopy measurements were used to calculate vessel wall shear rates and volumetric flow rates, using an assumption of uniform circular cylindrical diameters. Flow rates were calculated as $\pi V D^2 / 4$, with V the mean RBC velocity and D the vessel diameter. Vessel shear rate was calculated as $8 \times V / D$. Additional intravital microscopy videos were analyzed for the number of times per minute per $10 \times$ field of view that RBCs came to a complete stop (stationary for 1 video frame of 70 ms) in the underlying capillary bed.

2.3 Measurement of retinal hypoxia

The Hypoxyprobe™-1 Omni Kit (Natural Pharmacia International Inc., Burlington, MA) was used to measure retinal hypoxia, with the kit including pimonidazole hydrochloride (HCl) and rabbit anti-pimonidazole antisera. Pimonidazole forms irreversible adducts with thiol groups in low oxygen environments (Raleigh and Koch 1990), with the sensitivity to oxygen beginning with levels below 10 mmHg, and with adduct formation increasing throughout the range from 10 to 0 mmHg (Koch 2008). Pimonidazole has been used in a number of studies of retinal oxygenation (de Gooyer et al., 2006; Moon et al., 2010; Mowat et al., 2010), including from our own lab (Wright et al., 2010; Wright et al., 2011), with its lower sensitivity to factors other than oxygen discussed previously (Kleiter et al., 2006; Wright et al., 2011).

Mice were injected i.p. with pimonidazole at a dose of 60 mg/kg as we have performed previously (Wright et al., 2010), and were anesthetized three hours later with pentobarbital (50 mg/kg i.m.) and ketamine (50 mg/kg i.m.) 5 min apart. Eyes were enucleated and placed in phosphate buffered 4% paraformaldehyde (FD Neurotechnologies, Inc., Baltimore, MD) for 1 hour. The whole eye was then placed in chilled Optimal Cutting Temperature (OCT) compound and immediately submerged in liquid nitrogen for 20 s, and placed into a -80°C freezer until cut. Retinal cross-sections were cut at a $10 \mu\text{m}$ thickness and stored at -80°C until labeled. Retinal cross-sections were fluorescently labeled using the anti-pimonidazole antisera, allowing for localized hypoxia analysis of the individual layers of retinal tissue.

As a positive control for the Hypoxyprobe assay, an aged-matched control mouse was injected i.p. with 60 mg/kg pimonidazole that was allowed to circulate for 20 minutes before the right carotid artery was tied off under anesthesia. After 5 minutes of ischemia to the right eye, both the right and left eyes were enucleated and processed as described in the previous paragraph. It should be mentioned that due to the short time frame of the positive control procedure, the pimonidazole was administered with the mouse under anesthesia, rather than 3 hours prior to anesthesia as described for the other experiments.

2.4 Immunofluorescence labeling

Slides were labeled using methods previously described (Wright et al., 2011). A rabbit polyclonal anti-pimonidazole antibody (NPi Inc, Burlington, MA) was used as the primary antibody at a 1:1000 dilution and a rabbit polyclonal IgG antibody (Abcam, Cambridge, MA) was used for an isotype control. An FITC goat anti-rabbit IgG-Fc fragment antibody (Jackson Immuno Research Labs, West Grove, PA) was used as a secondary antibody against anti-pimonidazole at a 1:200 dilution.

2.5 Immunofluorescence image analysis

A Nikon Eclipse E600FN microscope with a CoolSNAP ES camera (Photometrics, Tucson, AZ) attached to an X-Cite® 120 Fluorescence illumination system was used for image acquisition, using a 20× objective and an exposure time of 500 ms. A radiometer photometer (model ILT 1400-A; International Light Technologies; Peabody, MA) was used to obtain a light intensity measurement before and after images were obtained from each slide, and then averaged. The software NIS Elements Basic Research version 3.0 (Nikon Instruments Inc., Melville, NY) was used for capturing and analyzing the images.

Images were obtained from both the central and peripheral retina. The individual layers (ganglion cell layer, GCL; inner plexiform layer, IPL; inner nuclear layer, INL; outer plexiform layer, OPL; outer nuclear layer, ONL; and photoreceptor layer – inner segment, PR-IS) were analyzed, with the GCL and IPL combined. A region of interest was drawn around the layer of interest in a DAPI counterstained image, with the same region of interest analyzed in the matching FITC-conjugated stained image. Low-level background intensities were measured from the FITC image and subtracted from both the sample and isotype control. The values presented represent the sample minus isotype fluorescence, normalized to the light intensity of the microscope.

2.6 Statistics

Data are expressed as means $\pm$ standard error of the mean. Statistical t-tests were performed with GraphPad Instat version 3.05 software (San Diego, CA), using a criterion of $p < 0.05$ as statistically significant.

3. Results

Control and diabetic mouse body weight, blood glucose, and mean arterial pressure (MAP) measurements are presented in Table 1. Included in the study were two different sets of control and diabetic animals: one group was used for retinal blood flow measurements and complete blood count analyses, while the other group was used for hypoxia measurements. For the latter group, body weight and blood glucose measurements were measured when the mice were 6 weeks of age, and at this time point, the Ins2(Akita) mice had already become diabetic as indicated by the lower body weight and higher plasma glucose compared to the non-diabetic controls. Table 1 also provides the measurements (with the two experimental protocols combined) on the day of the experiments, when the mice were $\pm$ a few days being 31 weeks old. As shown in the table, the diabetic mice gained 2-3 grams of weight over the course of the study, while controls gained 8-9 grams. Values of MAP (obtained from a subset of mice) were similar between the control and diabetic mice.

Retinal hemodynamic measurements were obtained via intravital microscopy in both non-diabetic controls and diabetic Ins2(Akita) mice. No statistically significant differences were found in vessel diameters between the diabetics and controls (Figure 1). However, significant changes were observed in RBC velocities between control and diabetic mice: Figure 2 shows that blood cell velocities are ~30% slower in Ins2(Akita) retinal arterioles

and venules than in non-diabetic C57BL/6 mice. Similar differences of ~25% were seen in both arteriolar and venular shear rates, with the lower values seen in the diabetic mice (Figure 3). The largest differences between the C57BL/6 and Ins2(Akita) mice were found in the vascular blood flow rates: ~40% lower in both diabetic arterioles and venules, as shown in Figure 4.

During the same intravital experiments, the microscope objective was focused down into the capillary beds to monitor the frequency with which RBCs came to a complete stop (for 1 video frame lasting 70 ms). This was a rare occurrence in C57BL/6 controls, but occurred almost 4 times per minute per 10× field of view in the Ins2(Akita) mice (Figure 5).

Complete blood count analyses indicated a similar hematocrit between the control and diabetic mice, as shown in Table 2, with an essentially equal number of RBCs per volume blood in both groups. The tendency for an approximate 3% increase in hematocrit in the Ins2(Akita) mice is due to the increased volume of the individual RBCs in the diabetic mice ($p < 0.05$ for mean corpuscular volume, MCV). Hemoglobin amounts per RBC were identical between the controls and diabetics (15.5 pg), but the mean corpuscular hemoglobin concentration (MCHC) was slightly smaller in the diabetics due to the increased RBC volume per cell. No differences were observed in the number of circulating platelets or in platelet volume per cell. Finally, although no statistical differences were found in white blood cells counts due to some variability, there was a tendency ($p = 0.05$) for a diabetes-induced decrease in the number of circulating lymphocytes.

Retinal hypoxia was assessed using the hypoxia probe pimonidazole (Hypoxyprobe). As shown in Figure 6, staining intensities were very similar among retinal layers between control and diabetic mice, both in the central retina as well as in the peripheral retina. Sample cross-sectional staining images are provided in Figure 7. As a positive control, bright Hypoxyprobe staining is observed in the retina when a carotid artery is occluded (Fig 7D).

4. Discussion

Current reviews of diabetic retinopathy emphasize the importance of investigating the consequences of microvascular dysfunction (Barber et al., 2011; Durham and Herman 2011; Hammes et al., 2011). A general premise is that a decrease in perfusion of the diabetic retina can contribute to hypoxia, with the hypoxia in turn helping stimulate the production of VEGF that induces pathological retinal angiogenesis. (However, it should be mentioned that factors other than decreased oxygenation can upregulate VEGF and angiogenesis.) A role for hypoxia in retinal neovascularization is supported experimentally (Pournaras 1995; Zhang et al., 2003). However, to date, such angiogenesis has not been consistently demonstrated in rodent models of diabetic retinopathy, possibly because of the short lifespan of rodents relative to the time course of retinopathy/angiogenesis seen in human diabetic patients. However, it should also be considered that the absent retinal angiogenesis in rodent diabetes might be due to a lack of substantial hypoxia.

Our current study of looking at both retinal perfusion and hypoxia at the 6-month time point of diabetes is a continuation of our previous studies looking at earlier time points. At 3-4 weeks of diabetes, we find no increase in retinal HIF-1 α or Hypoxyprobe staining in either mice or rats, and in fact, we have found tendencies for *less* Hypoxyprobe and HIF-1 α staining in diabetic rats at the 3-4 week time point (Wright et al., 2010; Wright et al., 2011). We did, however, previously observe a small increase in HIF-2 α staining at the 3-week time point in rats, but this one change in itself is insufficient to conclude the existence of significant hypoxia. These results were in contrast to our expectations that hypoxia might

accompany the 20-40% decreases in retinal blood flow that we observe in rats and mice at the 3-4 week time point of hyperglycemia (Lee and Harris 2008; Lee et al., 2008; Wright and Harris 2008; Wright et al., 2009; Wang et al., 2010; Wang et al., 2011; Yadav and Harris 2011).

At the subsequent 12-week period of diabetes in streptozotocin-injected rats, we previously found that the lack of hypoxia continues, with evidence even of *hyperoxygenation* based on statistically significant 20-50% decreases in Hypoxyprobe staining at various sites in the retina (Wright et al., 2010). These findings were complimented by the oxygen microelectrode measurements by another group (Lau and Linsenmeier 2010), who used the same diabetic model (12 weeks streptozotocin-induced hyperglycemia in rats) and obtained the same results as we did, that is, increased diabetic retinal oxygenation.

In the current study, we doubled this period of hyperglycemia, going out to 26 weeks of diabetes, to further extend and characterize the retinal blood flow and oxygenation time course in a diabetic mouse model. As presented in this study, we found no statistical changes in Hypoxyprobe staining in the diabetic mouse retina despite substantial 40% decreases in retinal blood flow rates. Our findings may, or may not, be in agreement with the findings reported by another group (de Gooyer et al., 2006), who detected an approximate 8% increase in retinal Hypoxyprobe staining (ELISA assay) in mice diabetic for 5 months following injection with streptozotocin. Although they found this increase to be statistically significant, we did not find the same to be true for our results of approximate $\pm 10\%$ changes in Hypoxyprobe immunostaining. In any case, it should be considered that the small changes in Hypoxyprobe staining reported by their group (de Gooyer et al., 2006) and in our current study (where we found little if any change) might not be sufficient to initiate hypoxia-dependent responses.

A relevant question related to these studies is why the retina does not become more overtly hypoxic given the substantial decreases in retinal blood flow. One possibility is that the diabetic retina no longer has the same oxygen requirements, with some cells becoming apoptotic and dying, given reports of a decrease in the number of oxygen-consuming photoreceptors (Martin et al., 2004; Park et al., 2006; Zhang et al., 2008; Zhang et al., 2009). Alternatively, other physiological changes may lead to a decrease in energy demand (Ottlecz and Bensaoula 1996; Kowluru et al., 1998). In fact, the decrease in blood flow could be considered to be a response to a decreased metabolic state, a scenario that has been postulated previously (Small et al., 1987; Rimmer and Linsenmeier 1993). Possibly due to one or more of these factors, reports indicate that oxygen consumption decreases in diabetic animals (Illing and Gray 1951; Sutherland et al., 1990; Linsenmeier et al., 1998).

Despite the results from the initial months of experimental diabetes, more prolonged periods of hyperglycemia appear to substantially increase hypoxia. In a study of diabetes in three cats, a period of 6-8 years resulted in an approximate 50% decrease in retinal oxygen concentrations (Linsenmeier et al., 1998), with the data obtained by oxygen microelectrodes. Similarly, in humans, vitreous fluid obtained from patients with diabetic retinopathy contains only two-thirds as much oxygen as non-diabetic patients (Holekamp et al., 2006). Although the number of years of diabetes was not provided for this latter study, most of the diabetic patients had progressed to the advanced stage of proliferative diabetic retinopathy.

As mentioned, we find very substantial 40% decreases in retinal blood flow rates at 6 months of hyperglycemia in Ins2(Akita) mice. However, we can only speculate regarding the mechanisms of this decrease. The two main mechanisms by which flow could be reduced are 1) increased resistance of the retinal microcirculation, and 2) decreased perfusion pressure. With respect to the former, increased resistance could result from

smaller microvessel diameters and/or decreased numbers of perfused capillaries. Microvascular flow resistance is very sensitive to changes in the diameters of the resistance vessels (to the 4th power), with 5-10% decreases in diameter potentially resulting in 19-34% decreases in flow. Although the 5-10% decreases in retinal vascular diameters in our study did not reach statistical significance, we cannot exclude this possible influence. Moreover, our diameter measurements were limited to the primary arterioles/venules extending into and out of the optic disk, and we cannot state whether more substantial changes in microvascular resistance could occur closer to or within the capillary bed.

We did not note any obvious alterations in capillary density, which could influence microvascular resistance, but we did find that the circulating red blood cells would pause, indicative of temporary capillary occlusion, much more frequently in diabetic mice than in controls (Figure 5). We can speculate that the RBC 'pausing' was a result of transient leukocyte entrapment in the capillaries, which would be expected given previous reports of increased leukocyte-endothelial cell interactions in the diabetic retina. In-vivo measurements of altered retinal capillary leukostasis in rodents, diabetic for up to one month, include increases of 2-fold (Leal et al., 2007), 3-4 fold (Miyamoto et al., 1999; Barouch et al., 2000) and ~20-fold (Chen et al., 2006), with the latter approaching the relative increase in transient capillary occlusions observed in our 6-month study. It should be considered that capillary tortuosity (not monitored in this study) could contribute to the difficulty in the microvascular passage of blood cells. Although not as likely, it is also possible that the increased size of the RBCs in the diabetic mice (3% increase; Table 2) could interfere with capillary transit. Although these capillary occlusions may contribute to the decrease in retinal perfusion by increasing the resistance to flow, it could also be considered that the cause-and-effect may involve the opposite, that is, the decreased blood flow rate and decreased microvascular shear rates may contribute to the increased frequency of microvascular occlusions.

Besides an increase in microvascular resistance, the other major mechanism that can potentially decrease flow is a decrease in perfusion pressure. We found only a non-statistically significant 6% decrease in mean arterial pressure, which if real, could be a minor contributing factor towards the decrease in retinal perfusion. However, others have found that despite altered diastolic (but not systolic) heart function (Basu et al., 2009), there is no change in either mean arterial blood pressure (Faulhaber-Walter et al., 2008) or heart rate (Basu et al., 2009) near this age of Ins2(Akita) mice. We did not measure intraocular pressure, so we do not know its potential influence. Overall, it is certainly possible that several factors, including smaller microvascular diameters, capillary occlusions, increased tortuosity, and decreased perfusion pressure, may combine to decrease flow.

It should be mentioned that our measurements were performed with the mice under anesthesia (ketamine and pentobarbital), and that pentobarbital can cause respiratory depression. The extent to which the alterations in respiration may have affected retinal blood flow are not known; however, the decrease in respiratory rate has been reported to be very similar between diabetic and non-diabetic animals (specifically, rats) anesthetized with pentobarbital (~30% in both groups) (Yamazaki et al., 2002). We do not know whether a different choice of anesthetic would alter any of the results and/or interpretations.

In summary, this study determined that diabetic Ins2(Akita) mice, at a time point of 6-months hyperglycemia, showed significant alterations in retinal microvascular perfusion, as we have seen previously in the initial weeks (from 3 weeks up to 8 weeks) in rodent models of type 1 diabetes (Lee and Harris 2008; Lee et al., 2008; Wright and Harris 2008). Although it is possible that the decrease in retinal perfusion persists throughout the first 6 months in the Ins2(Akita) mice, we can only speculate that this may be the case. Most previous measurements of retinal blood flow in experimental type 1 diabetes have been

performed using chemically induced hyperglycemia in contrast to the current genetic model that is noncatabolic. Additionally, to our knowledge, this is the first study to report retinal blood flow and hypoxia measurements at the same time period of experimental diabetes in rodents. Decreases were observed in red blood cell velocity, vascular wall shear rates, and volumetric blood flow rates, with increased incidence of transient capillary occlusions. However, despite these hindrances to oxygen delivery, no evidence of substantially enhanced hypoxia was observed when using the oxygen-sensitive probe pimonidazole.

The lack of enhanced hypoxia, seen in this study and in our previous work using both rats and mice (Wright et al., 2010; Wright et al., 2011), in the presence of attenuated blood flow (Lee and Harris 2008; Lee et al., 2008; Wright and Harris 2008) could be consistent with a decrease in the retinal utilization of oxygen in the early months of hyperglycemia. Therefore, it should be considered that mice and rats in the first months of diabetes do not have a sufficient driving force to mimic the angiogenic stage of retinopathy seen after many years of the disease in human diabetic patients. Despite this limitation, studying the retinal microvascular changes in Ins2(Akita) mice and streptozotocin-injected rodents may be useful to model the initial pathological mechanisms operating in the human diabetic retina, in which the same magnitude of early attenuations of perfusion is found. Attenuations in retinal perfusion could contribute to transient capillary leukostasis (as observed in this study) and eventual capillary dropout.

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- Six months of diabetes in Ins2-Akita mice causes deficient retinal perfusion.
- Retinal blood cell velocity, wall shear rate, and blood flow decreased by 25-40%.
- Transient retinal capillary stoppages were observed in the diabetic mice.
- No evidence of retinal hypoxia (using the probe pimonidazole) was found.

Figure 1. Retinal arteriolar and venular diameters in control (N=9) and diabetic (N=8) mice obtained via intravital microscopy. No statistical differences were observed. Data are provided as means $\pm$ SEM.

Figure 2. Retinal arteriolar and venular RBC velocities in control (N=9) and diabetic (N=8) mice, with lower values found in the diabetic mice. Data are provided as means $\pm$ SEM. *** $p < 0.001$ vs control.

Figure 3. Retinal arteriolar and venular shear rates in control (N=9) and diabetic (N=8) mice, with lower values found in the diabetic mice. Data are provided as means $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$ vs control.

Figure 4. Retinal arteriolar and venular blood flow rates in control (N=9) and diabetic (N=8) mice, with lower values found in the diabetic mice. Data are provided as means $\pm$ SEM. *** $p < 0.001$ vs control.

Figure 5.

Frequency of RBC pausing within the retinal capillary bed, quantified as the number of RBCs per minute coming to a complete stop for at least 70 ms (video exposure time) in a 10× field of view. Data are provided for control (N=9) and diabetic (N=8) mice, with higher values found in the diabetic mice. Data are provided as means $\pm$ SEM. ** $p < 0.01$ vs control.

Figure 6.

Fluorescent staining intensities for an anti-pimonidazole antibody (a measure of hypoxia) in the central and peripheral retina of control (N=6) and diabetic (N=6) mice. Layers are abbreviated as GCL (ganglion cell layer), IPL (inner plexiform layer), INL (inner nuclear layer), OPL (outer plexiform layer), ONL (outer nuclear layer), and PR-IS (photoreceptor layer – inner segment). No statistical differences were observed. Data are provided as means $\pm$ SEM.

Figure 7.

Representative cross-sectional images of anti-pimonidazole (Hypoxyprobe) staining in the retina from a non-diabetic control mouse (A) and a diabetic Ins2(Akita) mouse (B). Panels (C) and (D) show the left eye and right eye, respectively, of a control mouse in which the right carotid artery was occluded. An increase in spatial distribution and magnitude of staining is seen in panel D in each layer of the retina, with the greatest increases seen in the inner nuclear and ganglion cell layers. Retinal layers are labeled to the right of panel D as GCL (ganglion cell layer), IPL (inner plexiform layer), INL (inner nuclear layer), OPL (outer plexiform layer), ONL (outer nuclear layer), PR-IS (photoreceptor inner segment), and PR-OS (photoreceptor outer segment). In panels A-C, the inner retina is toward the left and the outer retina is toward the right.

Table 1

Body weight, blood glucose, and mean arterial blood pressure (MAP) values in control mice (C57BL/6J) and diabetic mice (Ins2(Akita)). Glucose levels are reported as median values, without statistical tests performed, due to several values obtained over the 750 mg/dl meter limit.

	6 weeks of age		31 weeks of age	
	Control	Diabetic	Control	Diabetic
Weight (g)	22.9 ± 0.5 (N=6)	18.8 ± 0.3 ^{***} (N=6)	31.8 ± 0.8 (N=17)	21.5 ± 0.6 ^{***} (N=17)
Glucose (mg/dl)	198 (N=6)	729 (N=6)	210 (N=14)	701 (N=17)
MAP (mmHg)			67.8 ± 3.8 (N=9)	63.5 ± 3.0 (N=8)

^{***}
p<0.001 vs corresponding control mice.

Table 2

Complete blood count analysis measurements from control mice (C57BL/6; N=11) and diabetic mice (Ins2(Akita); N=9).

Parameter	Units	Control (C57BL/6)	Diabetic (Ins2(Akita))	p value
RBC	M/ μ l	9.10 $\pm$ 0.15	9.09 $\pm$ 0.16	0.96
Hct	%	41.8 $\pm$ 0.6	43.2 $\pm$ 1.0	0.24
MCV	fL	45.9 $\pm$ 0.2	47.5 $\pm$ 0.6	0.02 *
Hb	g/dL	14.2 $\pm$ 0.3	14.1 $\pm$ 0.3	0.94
MCH	pg	15.5 $\pm$ 0.1	15.5 $\pm$ 0.2	0.94
MCHC	g/dL	33.8 $\pm$ 0.3	32.7 $\pm$ 0.3	0.02 *
Platelets	K/ μ l	1124 $\pm$ 27	1234 $\pm$ 62	0.10
Mean Platelet Volume	fL	5.11 $\pm$ 0.13	5.37 $\pm$ 0.12	0.18
WBC	K/ μ l	4.78 $\pm$ 0.61	3.50 $\pm$ 0.33	0.10
Neutrophils	K/ μ l	1.98 $\pm$ 0.30	1.82 $\pm$ 0.24	0.70
Lymphocytes	K/ μ l	2.61 $\pm$ 0.40	1.59 $\pm$ 0.24	0.05
Monocytes	K/ μ l	0.14 $\pm$ 0.07	0.08 $\pm$ 0.03	0.43

Abbreviations: red blood cell (RBC); hematocrit (Hct); mean corpuscular volume (MCV); hemoglobin (Hb); mean corpuscular hemoglobin (MCH); mean corpuscular hemoglobin concentration (MCHC); white blood cell (WBC).

*
p<0.05 vs Controls.