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Inhibition of 20-HETE attenuates diabetes-induced decreases in retinal hemodynamics

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Abstract

The mechanisms of early diabetes-induced decreases in retinal blood flow have yet to be fully determined. The aim of this study was to explore the hypothesis that 20-hydroxyeicosatetraenoic acid (20-HETE) plays a role in the early decrease of retinal hemodynamics in diabetic mice. 20-HETE has been implicated previously in the diabetes-enhanced vasoconstriction of mesenteric and renal vessels; however, its role in the diabetic retinal microcirculation has not been investigated. Diabetes was induced by multiple low-dose injections of streptozotocin (STZ; 50 mg/kg for 5 consecutive days), then ~2 weeks later the mice were administered daily intraperitoneal injections with or without the 20-HETE inhibitor HET0016 (2.5 mg/kg/day) for the following 2 weeks. Non-diabetic age-matched mice were included as controls. Intravital microscopy was used to obtain measurements of retinal vascular diameters and red blood cell (RBC) velocities for the feed arterioles and draining venules extending out of and into the optic disk. From these values, wall shear rates and blood flow rates were calculated. Diabetes induced approximate 30–40% decreases in RBC velocity, wall shear rate, and blood flow rate. These decreases were attenuated to 5–10% in the mice given HET0016. In summary, the 20-HETE inhibitor HET0016 is able to attenuate the retinal hemodynamic changes induced by diabetes.

Keywords

diabetic retinopathy; blood flow; 20-HETE

INTRODUCTION

Diabetic retinopathy is classified as a microvascular disease, and is characterized by microaneurysms, hemorrhages, pericyte loss, increased microvascular permeability, exudates, capillary basement membrane thickening, capillary occlusion, shunts, venous beading, edema, and neovascularization (Blair et al., 2009; Kador et al., 2009). Diabetes also induces changes in vascular reactivity (induction of altered vasodilation or vasoconstriction) in various tissues throughout the body, including the retina (Justesen et al., Lorenzi et al.,

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Pemp et al., 2009). Animal experiments, including those from our own lab (Lee and Harris 2008; Wright and Harris 2008; Wright et al., 2009; Wang et al., 2010), have demonstrated that diabetes induces significant decreases in retinal blood flow; however, the complete mechanisms of these decreases have yet to be determined.

The cytochrome P450-dependent metabolism of arachidonic acid can result in the production of 20-HETE, which has been found to be a potent vasoconstrictor. The mechanisms of 20-HETE-induced vasoconstriction have been attributed to increased Ca^{++} sensitivity, increased Ca^{++} entry via the depolarization of K_{Ca} channels, and activation of L-type Ca^{++} channels via protein kinase C (Roman et al., 2006). Additionally, the vasoactive actions of 20-HETE have been determined to be associated with a number of other constrictor pathways, including those of angiotensin II, vasopressin, endothelin, serotonin, and thromboxane (Miyata and Roman 2005; Roman et al., 2006). 20-HETE has been implicated previously in the diabetes-enhanced mesenteric and renal responses to vasoconstrictors (Yousif et al., 2009); however, to date, its role in the diabetic retinal microcirculation has not been investigated.

HET0016 (N-hydroxy-N'-(4-n-butyl-2-methylphenyl)Formamidine) is a potent and selective inhibitor of 20-HETE formation, and demonstrates three orders of magnitude higher inhibition of 20-HETE than of epoxyeicosatrienoic acids (EETs) (Miyata and Roman 2005). In the current study, we investigate the possibility that daily administration of HET0016 can attenuate the retinal hemodynamic changes induced in mice injected with streptozotocin. Measurements included arteriolar and venular diameters, red blood cell (RBC) velocities, wall shear rates, and blood flow rates.

MATERIALS AND METHODS

Animals

Nine-week old C57BL/6 male mice (Jackson Laboratories) were randomly assigned to intraperitoneal (i.p.) injection of STZ (Sigma, St. Louis, MO; 50 mg/kg dissolved in pH 4.5 sodium citrate buffer), or sodium citrate buffer alone, for 5 consecutive days, with the STZ injection performed within 15 min of mixing into solution. Changes in blood glucose levels were detected with a tail vein puncture after the 5th day with an AlphaTRAK glucometer (Abbott; Abbott Park, IL); diabetic mice were included in the study if glucose values exceeded 300 mg/dl. Fourteen days after the STZ injection, subsets of the mice were injected i.p. (in DMSO) with 0 or 2.5 mg/kg/day HET0016 (BIOMOL, Plymouth Meeting, PA) with the injections continuing for the final 14 days of the protocol. Pharmacokinetic data and efficiency of HET0016 as a 20-HETE inhibitor have been provided previously (Poloyac et al., 2006; Mu et al., 2008). The four groups of mice were (1) untreated non-diabetic controls injected with vehicle, (2) non-diabetic controls administered HET0016, (3) diabetics injected with vehicle, and (4) diabetics administered HET0016. Mice were housed one per cage, received standard chow, and were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Experimental preparation

For each mouse in the study, anesthesia was induced with pentobarbital (50 mg/kg, i.p.) followed 4–5 min later with an injection of ketamine (50 mg/kg, i.p.) The right femoral vein and left femoral artery were cannulated, with the femoral artery connected to a Pressure Monitor BP-1 (World Precision Instruments, Sarasota, Florida, USA). Body temperature was maintained during the procedures using a heating pad under the mouse.

The eye under examination was dilated with a drop of 1% tropicamide ophthalmic solution (Akron Inc., Buffalo Grove, IL) followed by a drop of 2.5% GonakTM hypromellose

ophthalmic demulcent solution (Akron Inc.) covered by a 5 mm glass coverslip. Using a Nikon Eclipse E600FN microscope with an attached CoolSNAP ES digital camera (Photometrics, Tucson, AZ), the optic disk was brought into focus for intravital microscopy.

Microvascular diameters

A 40–50 μ l bolus of 4.5 mg/kg fluorescein isothiocyanate (FITC)-dextran (MW 2×10^6) was injected at a rate of 20 μ l/min with a syringe pump (Harvard Apparatus, South Natick, MA), and the vessels filling first with FITC-dextran were identified as the arterioles.

Approximately 2–4 min following the injection of FITC-dextran, baseline retinal diameters were recorded (10 $\times$ objective; fluorescein filter) by sequentially focusing on each of the primary arterioles and venules (Wright and Harris 2008). The video was analyzed using NIS Elements Basic Research software (version 2.1–2.3; Nikon Instruments Inc, Melville, NY). Diameter measurements were obtained at three different locations along each vessel within 450 μ m of the optic disk, with an average of the three measurements reported.

Red blood cell velocity and calculated parameters

Red blood cells (RBCs) obtained from C57BL/6 donor mice were fluorescently labeled with 1,1'-dioctadecyl-3, 3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI; Invitrogen Molecular Probes, Eugene, OR) as described previously (Lee and Harris 2008; Wright and Harris 2008). After the optic disc was visualized via intravital microscopy and diameters recorded, labeled RBCs (20–25% by volume) were infused for 2 min via syringe pump into the right femoral vein, at a rate of 20 μ l/min. Images obtained through a rhodamine filter and 10 $\times$ objective were video-recorded using a camera exposure time of 10 ms. In the video-recording, DiI-labeled RBCs appeared as fluorescent streaks in the vessels (Lee et al., 2008), with the length of the streak proportional to RBC velocity. Figure 1 provides example images, showing a 4 $\times$ view of the dextran-filled superficial arterioles and venules (Fig 1A), a 10 $\times$ view used for measurements of diameter (Fig 1B), and a 10 $\times$ view of the fluorescent RBC streaks (Fig 1C), from which velocities were calculated. Ten consecutive RBC streaks were averaged per vessel, with the mean velocity V_{mean} reported in mm/s. Assuming cylindrical geometry, microvessel wall shear rate (SR) was estimated using the Newtonian definition $SR = 8(V_{\text{mean}}/D)$, with D the mean diameter. Vessel blood flow rate was estimated as $\pi V_{\text{mean}} D^2/4$.

Statistics

Statistical differences between groups were determined via analysis of variance using Student-Newman-Keuls post-hoc corrections (InStat software v 3.05; GraphPad, San Diego, California, USA). Linear regression analyses were used to evaluate the associations between diameters and blood velocity or microvessel wall shear rate. Error bars are presented as $\pm$ standard error (SE), and statistical significance was set at $p < 0.05$.

RESULTS

The study included four groups of animals, i.e., control and diabetic mice with or without daily treatment with HET0016. However, the dose of 2.5 mg/kg/day HET0016 was not tolerated by the non-diabetic controls: of the eight mice initially in this group, one died prior to the day of the experiment, and another five died following anesthesia, prior to the collection of retinal hemodynamic data. However, the same dose of HET0016 had no such lethal effects on the diabetic mice.

Table 1 provides the body weight, plasma glucose concentrations, and mean arterial blood pressures for the three remaining experimental groups. As indicated, STZ induced an approximate 2-fold increase in plasma glucose, with the increase slightly greater in the

untreated diabetics compared with the HET0016-treated diabetics (although this difference was not statistically significant). No differences in mean arterial blood pressure were found between the three groups. Although both STZ groups gained less weight than the controls, this difference was more significant in the group injected with HET0016.

Figure 2 shows the arteriolar diameters and RBC velocities of the three groups, presented as regression analyses. In the non-diabetic control mice, RBC velocities varied from ~20–35 mm/s, with no correlation to arteriolar diameter (Fig 2A). However, RBC velocity decreased in the untreated STZ mice, and to a greater extent in the smaller diameter arterioles, as indicated by the statistically significant slope shown in Figure 2B. Treatment with HET0016 was able to attenuate the decrease in RBC velocities (Fig 2C). The arteriolar velocities from Figure 2 were averaged and are shown in Figure 3 along with the venular averages for all three experimental groups, showing the ~33% decrease induced by diabetes and the partial (and statistically significant) attenuation of this decrease with HET0016 treatment. As shown in Figure 4, vessel diameters tended to be smaller in the untreated STZ mice than in either the controls or HET0016-treated STZ, with this tendency close to statistical significance ($p=0.08$) for the arterioles.

Microvessel wall shear rate varies inversely with diameter as shown in Figure 5A; however, this relationship was significantly altered by STZ, with the slope disappearing ($p=0.97$; Fig 5B). In the diabetic mice, HET0016 preserved the relationship between shear rate and diameter as found in controls (Fig 5C). The shear rates from Figure 5 were averaged and are presented in Figure 6, which shows that for both arterioles and venules, there is a significant decrease (~26–30%) in the untreated STZ mice, and a significant attenuation of this decrease in the HET0016-treated diabetics.

For each mouse, the sum of blood flow rates in the primary arterioles were calculated, and averaged with the sum of blood flow rates in the primary venules. Figure 7 presents the mean blood flow rates for the three experimental groups. STZ induced a 37% decrease in retinal blood flow rate; this decrease was significantly attenuated (5% below controls; $p<0.01$ vs untreated STZ) in the HET0016-treated STZ mice.

DISCUSSION

To our knowledge, this is the first study to investigate the influence of 20-HETE in the diabetic retinal vasculature, with a unique feature of the study being the direct in vivo microscopic measurement of retinal blood flow and hemodynamics. The results indicate that 20-HETE inhibition attenuates the significant decrease in retinal RBC velocity, shear rate, and blood flow rate induced by diabetes. This finding could have clinical relevance, since decreases in retinal blood flow and shear force could be responsible, at least in part, for diabetes-induced events such as retinal capillary occlusion by leukocytes, ischemia, and endothelial dysfunction.

Although this may be the first study of 20-HETE in the diabetic retinal vasculature, the role of the vasoconstrictor also has been investigated in a model of hyperoxia-induced reductions in retinal blood flow in newborn pigs (Zhu et al., 1998). In this previous study, a different 20-HETE inhibitor, dibromododecenyl methyl sulfonimide, was able to significantly attenuate the 42% decrease in retinal blood flow (similar decrease as seen in our current study) induced by breathing 100% oxygen. The similar magnitude decrease in retinal blood flow between our diabetic mouse model and their retinal hyperoxia newborn pig model should not be over-interpreted; however, it should be mentioned that we have recently published the finding that the early stages of STZ-induced diabetes results in tendencies for retinal hyperoxia in rats and mice (Wright et al., 2010). Although not investigated in the

current study, it is possible that 20-HETE, known to be an oxygen-dependent vasoconstrictor (Harder et al., 1996; Lombard et al., 1999; Frisbee et al., 2000), may provide a link between hyperoxia and vasoconstriction in the diabetic retina. However, other diabetes-induced mechanisms of vascular alterations cannot be excluded.

The constriction of 20-HETE is related to other constriction pathways, including those of angiotensin II, vasopressin, endothelin, serotonin, and thromboxane (Miyata and Roman 2005; Roman et al., 2006). Previous studies from our own lab have used inhibitors of several of these pathways to attenuate diabetes-induced decreases in retinal perfusion, including inhibitors of thromboxane synthase (Lee and Harris 2008; Lee et al., 2008; Wright and Harris 2008), the thromboxane-prostanoid receptor (Wright et al., 2009), the angiotensin II type 1 receptor (Lee and Harris 2008), and the endothelin receptor A (Wang et al., 2010). Due to the overlapping mechanisms of these pathways, it might not be surprising that multiple interventions can demonstrate success in attenuating the diabetes-induced decreases in retinal perfusion.

The decrease in retinal perfusion is likely due to local changes in vascular resistance, since we have previously determined that 4 weeks of streptozotocin-induced diabetes does not alter the retinal perfusion pressure (Wright and Harris 2008). The most influential sites of local retinal vascular resistance have not been determined in diabetic rodents, but we have previously found approximate 5–10% decreases in the diameters of primary feed arterioles at a time point of 3–4 weeks, with these decreases sometimes, but not always reaching statistical significance (Lee and Harris 2008; Wright and Harris 2008; Wright et al., 2009; Wang et al., 2010). In the current study, the tendency ($p=0.08$) for a 6% reduction in diameter (diameter = 94% of controls) might not completely explain the 37% decrease in flow, with resistance to flow inversely proportional to the 4th power of diameter for a given pressure gradient ($0.94^4 = 0.78$; only a 22% decrease). Therefore, it could be speculated that even larger increases in vascular resistance could occur downstream of the primary arterioles.

In this study, we found that the largest STZ-induced decreases in RBC velocity and wall shear rate occur in the smaller feed arterioles, as can be seen in the regression analyses of Figures 2 and 5. Overall flow and average shear rates decrease by 30–40%, but these changes are even more dramatic in the pathways fed by the smaller feed arterioles. The mechanisms for this differential response are not clear, but the 20-HETE inhibitor HET0016 was partially able to attenuate the hemodynamic effects seen in the smaller arterioles. These results show a high degree of similarity to our recent study with the endothelin receptor A antagonist atrasentan, which also had its most dominant influence on the smaller vessels (Wang et al., 2010). It is possible that the expression and actions of endothelin-1 and/or its receptor A are more dominant in the smaller arterioles (Polak et al., 2003; Hein et al., 2009), and that endothelin-1 and 20-HETE are acting in a cooperative manner.

We injected eight non-diabetic mice with HET0016 (2.5 mg/kg/day), but unfortunately, we could not collect data from these mice since one died prior to the day of the experiment, and five died following anesthesia on the day of the experiment. We chose this dose based on it being used effectively by Yousif et al. (Yousif et al., 2009) and by Benter et al. (Benter et al., 2005). Although control rats were given HET0016 in the study by Benter et al., no data were presented for this group, and no mention of mortality issues. In the study Yousif et al., HET0016 was not administered to control rats, only to diabetic rats. We are not aware of any studies describing the mortality rates of mice receiving HET0016. In a study using the inhibitor in transgenic hypertensive mice, HET0016 was also provided to controls, with no mention of mortality (Tornavaca et al., 2009). However, the mice of that study were not anesthetized as in our experiments. Since we did not have a mortality problem with

HET0016 injected to diabetics, we can speculate that blocking 20-HETE synthesis is only appropriate in a situation in which the synthesis may be upregulated, which is the case for STZ-induced diabetes (Yousif et al., 2009). 20-HETE has several important biological functions, as reviewed elsewhere (Kroetz and Xu 2005), including a role in the myogenic response, oxygen sensing, regulation of renal blood flow and tubuloglomerular feedback, and mitogenic actions of growth factors and angiogenesis.

In summary, the 20-HETE inhibitor HET0016 was determined to be partially effective in attenuating the decreases in retinal perfusion found in the initial weeks of STZ-induced diabetes.

Acknowledgments

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- The study is the first to investigate 20-HETE in diabetic retinal blood flow.
- Diabetes reduces retinal blood velocity, vessel shear rate, and blood flow rate in mice.
- Diabetes-induced reductions in retinal hemodynamics are attenuated by a 20-HETE inhibitor.

Figure 1.

In vivo microscopic images of the retinal microcirculation. (A) 4x view of alternating arterioles and venules coming out of the optic disk; hemodynamic data from these superficial vessels are included in the present study. (B) 10x view of the upper left quadrant of panel A; the 10x view was used for measurements of diameter of the FITC-dextran- filled vessels. (C) 10 x view of the upper left quadrant of panel A; the length of the fluorescent streaks demonstrate the distance that the DiI-labeled RBCs travel in 10 ms. The shorter streak is a RBC in an underlying microvessel below the superficial arterioles and venules.

A Controls **B STZ**

Figure 2. Arteriolar red blood cell (RBC) velocity in relation to diameter in the mouse retina, (A) in non-diabetic control mice, (B) in untreated STZ mice, and (C) in HET0016-treated STZ mice. A statistically significant correlation was found only the untreated STZ group ($p < 0.01$).

Figure 3. Retinal arteriolar and venular RBC velocity averages in the three groups of mice. * $p<0.05$ vs controls; *** $p<0.001$ vs controls; +++ $p<0.001$ vs untreated STZ.

Figure 4.
Retinal arteriolar and venular diameters in the three groups of mice.

Figure 5.

Arteriolar wall shear rate in relation to diameter in the mouse retina, (A) in non-diabetic mice, (B) in untreated STZ mice, and (C) in HET0016-treated STZ mice. Statistically significant correlations were found in the control mice ($p < 0.01$) and in the HET0016-treated STZ mice ($p < 0.01$).

Figure 6. Retinal arteriolar and venular shear rate averages in the three groups of mice. * $p < 0.05$ and *** $p < 0.001$ vs controls; ++ $p < 0.01$ and +++ $p < 0.001$ vs untreated STZ.

Figure 7. Retinal blood flow averages in the three groups of mice. ** $p < 0.01$ vs controls; ++ $p < 0.01$ vs untreated STZ.

Table 1

Mean body weights, median plasma glucose values, and mean arterial blood pressure for the three experimental groups. The number of animals is provided in parentheses; the measurement was not collected in a few instances where the value of N is less than the total number of animals.

Group	Prior to injection		On day of experiment		
	Weight (g)	Glucose (mg/dl)	Weight (g)	Glucose (mg/dl)	Blood pressure (mmHg)
Control (7)	24.7±0.4 (7)	195 (6)	25.5±0.4 (7)	207 (7)	83±2 (6)
STZ (7)	24.0±0.5 (7)	192 (7)	23.8±0.3* (7)	470** (7)	82±5 (5)
STZ + HET0016 (9)	23.5±0.6 (9)	201 (9)	21.9±0.6***++ (9)	367* (9)	83±2 (6)

* p<0.05 vs Controls;

** p<0.01 vs Controls;

*** p<0.001 vs Controls;

++ p<0.01 vs untreated STZ.