

Characterization of Retinal Leukostasis and Hemodynamics in Insulin Resistance and Diabetes

Role of Oxidants and Protein Kinase-C Activation

Toru Abiko,¹ Atsuko Abiko,¹ Allen C. Clermont,² Brett Shoelson,² Naoichi Horio,¹ Junichi Takahashi,¹ Anthony P. Adamis,³ George L. King,^{1,4} and Sven-Erik Bursell^{2,5}

Increases in leukostasis/monocyte adhesion to the capillary endothelium (leukostasis) and decreases in retinal blood flow may be causally associated and are implicated in the pathogenesis of diabetic retinopathy. In this study, we demonstrate that increases in leukostasis are observed in insulin-resistant states without diabetes, whereas decreases in retinal blood flow require diabetes and hyperglycemia. Microimpaction studies using beads mimicking retinal capillary obstruction by leukocytes did not affect retinal blood flow. In diabetic rats, treatment with the antioxidant α -lipoic acid normalized the amount of leukostasis but not retinal blood flow. In contrast, treatment with D- α -tocopherol and protein kinase-C β -isoform inhibition (LY333531) prevented the increases in leukostasis and decreases in retinal blood flow in diabetic rats. Serum hydroxyperoxide, a marker of oxidative stress, was increased in diabetic rats, but normalized by treatment with antioxidants α -lipoic acid and D- α -tocopherol and, surprisingly, PKC β -isoform inhibition. These findings suggest that leukostasis is associated with endothelial dysfunction, insulin resistance, and oxidative stress but is not related to retinal blood flow and is not sufficient to cause diabetic-like retinopathy. Moreover, treatment with PKC β inhibition is effective to normalize diabetes or hyperglycemia-induced PKC β -isoform activation and oxidative stress. *Diabetes* 52:829–837, 2003

Diabetic retinopathy is clinically manifested by multiple microvascular pathologies from microaneurysms, hemorrhages to neovascularization (1). However, histopathologies in the retinal capillary are known to precede the clinical retinal

findings. Early surrogate clinical markers are needed to diagnose and quantitate the presence of preclinical lesions of diabetic retinopathy that would allow the institution of treatments at the beginning stages of the disease. Abnormalities of retinal blood flow and increased leukocyte adhesion to the retinal capillaries have been noted to occur in diabetic patients and animals with short duration of disease and antecedent to any clinical findings in the retinal fundus (2–5). In addition, both findings have been suggested to be important for the development of diabetic retinopathy, since both reduced retinal blood flow and increased leukocyte adhesion may contribute to the formation of nonperfused capillaries, which are believed to be major contributors for the progression of diabetic retinopathy with increases in capillary permeability and angiogenesis (2–4).

The major metabolic risk factor for the development of diabetic retinopathy has been identified to be persistent and chronic hyperglycemia (6,7). In addition, intensive glycemic control by either insulin or hypoglycemic agents can delay the onset or the progression of diabetic retinopathy (6,7). Multiple studies have also reported that hyperglycemia can increase the expression of vasoconstrictors, such as endothelin-1 (ET-1) and angiotensin or their actions (8,9). The overexpression of vasoconstrictors that have been associated with the progression of diabetic retinopathy results in increased vascular resistance and decreased retinal blood flow (8,10). Similarly, activation of leukocytes and monocytes with increased adhesion to endothelium is observed in the retina as well as in micro- and macrovessels in the peripheral circulation due to the diabetic state (11). The increased leukocyte and monocyte adhesion has been attributed to increased expression of adhesion molecules on the circulating cells and vascular endothelial cells (5,11). Because decreases in retinal blood flow and increases in leukocyte and monocyte adhesion occur in parallel and are regulated by several metabolic factors in common, it is possible that these two early retinal microvascular findings in diabetes may be interrelated with respect to their development. One potential factor associated with both of these abnormalities is endothelial dysfunction, which can be induced by the elevation of free fatty acids as in insulin-resistant states. (12,13) There are many potential pathways through which hyperglycemia can induce endothelial dysfunction, including increases in oxidative stress and nonenzymatic glycation (14), impaired action of endothelium-derived

From the ¹Research Division, Joslin Diabetes Center, Harvard Medical School, Boston, Massachusetts; the ²Beetham Eye Institute, Joslin Diabetes Center, Harvard Medical School, Boston, Massachusetts; the ³Department of Ophthalmology, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, Massachusetts; the ⁴Department of Medicine, Harvard Medical School, Boston, Massachusetts; and the ⁵Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts.

Address correspondence and reprint requests to George L. King, Joslin Diabetes Center, One Joslin Place, Boston, MA 02215. E-mail: george.king@joslin.harvard.edu.

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AOLF, acridine orange leukocyte fluorography; AT, appearance time; ET-1, endothelin-1; MCT, mean circulation time; PKC, protein kinase C; SLO, scanning laser ophthalmoscope; STZ, streptozotocin; VFA, video fluorescein angiography.

relaxation factors (13), and activation of the diacylglycerol (DAG)/protein kinase C (PKC) pathway (5,14). However, endothelial dysfunction can also occur in insulin-resistant states without hyperglycemia. Insulin resistance occurs much more frequently than diabetes and is associated with cardiovascular diseases, but it does not have an established relation to diabetic microvascular complications, such as retinopathy (15).

In the present study, we have characterized the onset of abnormalities in retinal blood flow and leukostasis in insulin-resistant, nondiabetic, and diabetic rats in order to determine whether hyperglycemia is necessary for the onset of these retinal abnormalities. In addition, we have also evaluated the role of increased microimpaction at the retinal capillary level, a potential mechanism by which increased leukostasis can cause changes in retinal blood flow. Lastly, we have compared the effects of antioxidants and inhibitors of PKC because both increases in oxidative stress and PKC activation have been demonstrated in the leukocytes, monocytes, and the vascular endothelium of diabetic animals and patients and are postulated to mediate many of hyperglycemia's adverse effects in micro- and cardiovascular tissues (14,16,17).

RESEARCH DESIGN AND METHODS

Animals. All experiments followed the guideline of the Association for Research in Vision and Ophthalmology and were approved by the Animal Care and Use Committees of the Joslin Diabetes Center. Male Long-Evans (L-E) rats (Taconic Farms, Germantown, NY) with initial weights between 180 and 250 g and male Zucker fatty and lean rats were used for these experiments. Because the L-E rats are pigmented, it facilitates visualization and measurement of the retinal microcirculation and static fluorescent leukocytes and microspheres. Diabetes was induced in L-E rats by intraperitoneal injection of 65 mg/kg streptozotocin (STZ) (Sigma, St. Louis, MO) in 10 mmol/l citrate buffer (pH 4.5) after an overnight fast. The rats with blood glucose levels >250 mg/dl 24 h after STZ injection were considered diabetic. A day before the retinal hemodynamic measurements, all rats were underwent catheterization with a polyvinyl catheter inserted into the right jugular vein as described previously (18). After the hemodynamic measurements, the catheter was flushed and repositioned subcutaneously. The measurement of retinal leukostasis was performed a day after retinal hemodynamic measurements. Blood samples were obtained from the L-E rats in order to perform serum assays associated with the different treatment protocols being performed for these studies. The blood samples were drawn from the descending vena cava using heparinized syringes on the day following the hemodynamic measurements.

Video fluorescein angiography. The measurement of video fluorescein angiography (VFA) has been described previously (18). Briefly, a fundus camera (NFC-50; Nikon, Tokyo, Japan) interfaced to a video camera (SIT; Dage-MTI, Michigan City, IN) was used for the VFA as we have previously reported (3,8,18). A scanning laser ophthalmoscope (SLO) (Rodentstock Instrument, Munich, Germany) was used for VFA measurements in the experiments investigating the effects of microsphere impaction and in the experiments performed using the Zucker rats. The video output from the fundus camera and SLO is stored on videotape, digitized at 30 frames/s through a video digitizer (Targa 2000; Pinnacle Systems, Mountain View, CA) and converted to a sequence of .TIFF images ($640 \times 480 \times 8$ bit) to facilitate image processing. The retinal mean circulation time (MCT) was calculated as the time difference between the retinal arterial and venous mean circulation times for corresponding artery and vein pairs. Retinal vessel diameters were also determined at the retinal vessel sites used to determine the dye dilution curves. Retinal blood flow can be determined from the segmental MCTs and the associated retinal artery and vein diameters. Retinal blood flow is proportional to the sum of the squares of the retinal artery and vein diameters divided by the segmental MCT. We also measured the time between injection of the dye bolus in the jugular vein and the dye appearance in the retinal artery. This appearance time (AT) was used as a systemic indicator to ensure that the systemic deliver of the dye to the eye remained unchanged during the course of the experiments (18).

Acridine orange leukocyte fluorography. Acridine orange fluoresces in the green wavelengths when excited by blue light upon interacting with DNA. When acridine orange solution is injected intravenously, staining occurs in

leukocytes among the circulating blood cells as well as the vascular endothelial cells. Video recordings of the retina are obtained with the SLO using the argon blue laser for illumination with the filter setting for regular fluorescein angiography in the 40° field. Stationary leukocytes in the capillary bed are observed 20 min after the injection of acridine orange. At this time only stationary leukocytes still retain the acridine orange dye. Stationary leukocytes were recognized as fluorescent dots. These images are recorded on videotapes, digitized through the video digitizer, and stored on the computer. The resolution of the digitized images is 640×480 pixels with an eight-bit intensity resolution (256 steps). The average densities of leukocytes for each rat were calculated by averaging the value of the density in each of eight to 10 observation regions. The average density is in units of $(\text{cells}/\text{pixel}^2) \times 10^{-5}$ (19).

VFA procedure. Each rat was anesthetized using pentobarbital sodium before VFA. Right and left eyes were dilated with 1% tropicamide (Mydracil; Alcon, Fort Worth, TX) in the experiments with fluorescent microspheres and only left eyes were dilated in VFA for MCT determination. A 5- μ l bolus of 10% sodium fluorescein was rapidly injected into the jugular vein catheter after the VFA recording was initiated. The time of fluorescein-injection was marked on the video recording for measuring arterial AT of the dye bolus.

Acridine orange leukocyte fluorography procedure. A day after VFA measurements, each rat was anesthetized by intramuscular injection of 50 mg/kg ketamine hydrochloride (Ben Venue Labs, Bedford, OH) and 10 mg/kg xylazine hydrochloride (Sigma) before acridine orange leukocyte fluorography (AOLF) measurements. Both eyes or the left eyes were dilated as described previously. We modified the reported acridine orange fluorography procedure. Acridine orange was dissolved in sterile saline (1.0 mg/ml), and 4 mg/kg was injected through the jugular vein catheter at a rate of 1.5 ml/min. Twenty minutes after the injection, the fundus was observed with the SLO and the images were recorded onto videotapes for subsequent analysis.

Microsphere impaction. Polystyrene microspheres (FluoSpheres; Molecular Probes) with excitation and emission wavelengths of 490 and 515 nm, respectively, were used. For these experiments, microspheres of 15 μ m in diameter were chosen to ensure entrapment in the retinal capillaries. Before microsphere injection, a polyurethane catheter (0.84-mm outside dimension) was inserted from the right external carotid artery into the junction of the common, external, and internal carotid arteries. The internal carotid artery branches into the ophthalmic artery and continues into the central retinal artery branch. Microspheres injected into the right external carotid through the catheter impact only in the retina of the right eye. Retinal images were observed with the SLO and recorded onto videotapes during injection of the microspheres. The number of microspheres that appeared in the retinal arteries were counted. Two weeks after microsphere injection, a heparin-lock catheter was surgically implanted in the right jugular vein. Fluorescein angiography was performed 24 h later using the SLO. MCT of the right and left eyes were measured using our video fluorescein angiography methodology. The relation between microsphere numbers and relative MCT of right eyes against left eyes was evaluated. At the beginning of this study, the difference in retinal circulation between right eyes and left eyes was determined.

Treatment with vitamin E. D- α -tocopherol (40 mg/kg) or placebo vehicle (HCO, polyethylene 60-hydrogenated castor oil) provided by the Eisai Company (Tokyo, Japan) was injected intraperitoneally every other day into diabetic and nondiabetic rats for 2 weeks after the onset of diabetes. The dose of D- α -tocopherol was chosen based on previous reports (12,18,20).

Treatment with antioxidant and PKC β -inhibitor. Diabetic and nondiabetic rats were treated orally with the antioxidant, DL- α -Lipoic Acid (Sigma) (21) at a dose of $200 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ or PKC β -inhibitor (LY333531) at a dose of $10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ for 2 weeks from the onset of diabetes. The doses of α -lipoic acid and LY333531 were chosen based on previous reports (22).

Measurement of plasma hydroperoxides. After the AOLF measurements, the blood of each rat was collected into heparinized tubes. The plasma was obtained by centrifugation of the blood at 2000g for 10 min at room temperature. The plasma hydroperoxides were determined by the ferrous oxidation in xylenol orange assay version two (FOX2 assay) using PeroXO-quant quantitative peroxide assay kit (Pierce, Rockford, IL) (23) by the method described in the kit insert. The difference of hydrogen peroxide values between the sample with and without tris (2-carboxyethyl) phosphine hydrochloride (TCEP) determined as hydroperoxide concentration. Each sample was measured in duplicate, and the mean value was used for statistical analysis.

Statistical analysis. Each value is reported as a mean \pm SD. Paired *t* test was used for comparisons between right eyes and left eyes in same rats. One-way ANOVA was used for group comparisons. Population normality was tested using the Kolmogorov-Smirnov test, and equality of variances was tested using the Levene median test. If either test failed, then the Kruskal-Wallis ANOVA on ranks was performed. All pairwise multiple comparisons were performed

TABLE 1
Physical characteristics of Zucker lean, fatty, and diabetic fatty rats for studies in Figure 2

	Zucker lean	Zucker fatty	Zucker diabetic
<i>n</i> (1)	9	10	6
Final body weight (g)	305.0 ± 22.6	424.0 ± 29.8*	422.0 ± 25.9*
Blood glucose (mg/dl)	96.1 ± 11.2	110.2 ± 9.0*	513.0 ± 52.0*
<i>n</i> (2)	4	6	6
Appearance time (s)	1.43 ± 0.12	1.23 ± 0.19	1.89 ± 0.39
Artery diameter (pixels)	12.7 ± 1.1	13.9 ± 0.8	14.2 ± 1.3
Vein diameter (pixels)	17.6 ± 1.2	18.2 ± 0.7	17.9 ± 1.1
MCT (s)	0.98 ± 0.08	1.03 ± 0.11	1.26 ± 0.20†

Data are means ± SD. **P* < 0.01 compared with nondiabetic control rats; †*P* = 0.036 compared with nondiabetic Zucker fatty rats. 1) Physical characteristics of all Zucker lean, fatty, and diabetic fatty rats from retinal leukostasis and microcirculation studies; 2) retinal vascular parameters of Zucker rats from microcirculation studies alone.

using the Student-Newman-Keuls test. *P* < 0.05 was considered to be statistically significant.

RESULTS

Characterization of leukostasis and microcirculation in the retina. Endothelial dysfunction and activation has been observed in the arterial system of diabetic and nondiabetic insulin-resistant states (12,13). Thus, we de-

termined whether retinal leukostasis and reduced retinal blood flow are only observed in the diabetic state with hyperglycemia or are also present in the insulin-resistant state with and without the presence of hyperglycemia. For these studies, Zucker lean *fa/x*, insulin-resistant obese Zucker *fa/fa* (fatty), and insulin-resistant spontaneous-diabetic Zucker *fa/fa* (ZDF) were used. The Zucker fatty rats were specifically selected because they have been characterized with endothelial dysfunction in the absence of diabetes (24). Physical characteristics for the Zucker fatty, ZDF, and control Zucker lean rats are summarized in Table 1. Random blood glucose levels for the fatty rats (109.4 ± 9.0 mg/dl) were slightly higher compared with those measured in the lean rats (96.1 ± 11.2 mg/dl) but were not in the diabetic range. ZDF rats had much higher blood glucose levels (513.2 ± 52.2 mg/dl) than either the fatty or lean groups. The body weight for both the fatty and ZDF rats were significantly greater than in the Zucker lean rats. Retinal MCTs measured in the Zucker fatty, ZDF, and lean rats are shown in Fig. 1A. There were no significant differences in retinal MCTs between the Zucker lean (0.98 ± 0.08 s) and Zucker fatty rats (1.03 ± 0.11 s). However, the retinal MCTs of the ZDF rats was significantly (*P* = 0.036) prolonged (1.26 ± 0.20 s) compared with nondiabetic Zucker fatty rats.

In contrast, the density of stationary leukocytes/monocytes in the retinas of Zucker fatty rats (12.14 ± 4.26 ×

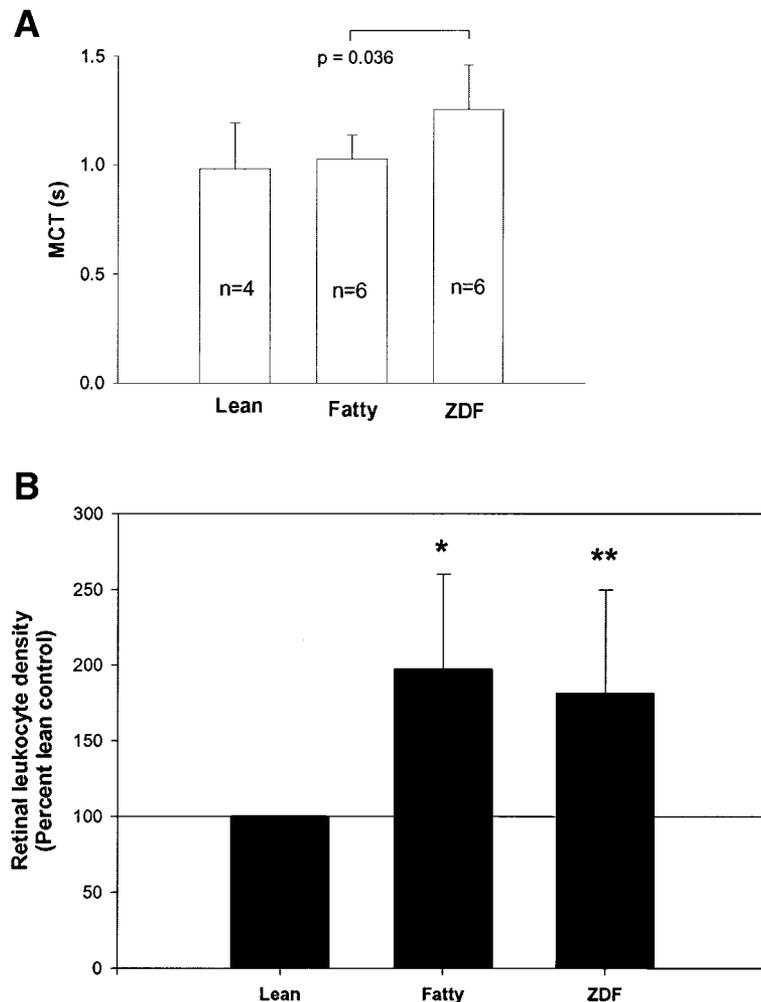


FIG. 1. Retinal vascular function tests in Zucker lean (Lean), Zucker fatty (Fatty), and Zucker hyperglycemic diabetic (ZDF) rats. A: Retinal MCT was obtained by VFA. B: Retinal leukostasis obtained by SLO. Retinal MCT and leukostasis were measured on consecutive days as described in RESEARCH DESIGN AND METHODS. Data are expressed as means ± SD. **P* < 0.01, ***P* = 0.02.

TABLE 2
Physical properties for nondiabetic and diabetic L-E rats used in microsphere impaction studies

	Nondiabetic rats		Diabetic rats	
	Right eyes	Left eyes	Right eyes	Left eyes
<i>n</i>	10		5	
Initial body weight (g)	226.0 ± 15.2		229.0 ± 18.2	
Final body weight (g)	255.0 ± 28.7		245.0 ± 41.2	
Blood glucose (mg/dl)	106.5 ± 9.7		364.6 ± 84.2*	
Appearance time (s)	1.49 ± 0.30	1.31 ± 0.28	1.52 ± 0.38	1.43 ± 0.22
Artery diameter (pixels)	15.5 ± 1.4	14.9 ± 1.3	15.2 ± 1.5	15.1 ± 1.3
Vein diameter (pixels)	20.6 ± 2.7	19.8 ± 2.1	19.8 ± 2.1	19.0 ± 1.7

Data are means ± SD. **P* < 0.01 compared with nondiabetic rats.

10⁻⁵ cells/pixel² was significantly higher (98 ± 62%, *P* = <0.01) than in Zucker lean rats (6.18 ± 2.35 × 10⁻⁵ cells/pixel² (Fig. 1B). Similarly, the density of stationary leukocytes/monocytes in the ZDF rats was also significantly higher (81 ± 69%, *P* = 0.02) than in Zucker lean but not different from Zucker fatty rats.

Effect of microsphere impaction on retinal circulation. The results from Zucker insulin-resistant (fatty) and -sensitive (lean) rats suggested that the increase in leukostasis is related more to insulin resistance rather than to hyperglycemia, whereas MCT prolongation and concomitant decreased retinal blood flow is only observed with hyperglycemia in diabetic rats. However, it may be possible that the decrease in retinal blood flow is a direct result of enhanced leukostasis, which precedes both diabetes and diabetes-related retinal blood flow reductions. Thus, we explored the possibility of whether the increase in retinal leukostasis can physically contribute to the decrease in retinal blood flow using capillary microimpaction with 15-μm diameter fluorescent microspheres.

The retinal circulation in both eyes of L-E rats was evaluated before microsphere injection. No significant differences were noted in the baseline values between right eyes (MCT 1.05 ± 0.13 s; AT 1.35 ± 0.24 s; artery

diameter 14.4 ± 1.36 pixels; vein diameter 20.0 ± 1.2 pixels) and left eyes (MCT 1.07 ± 0.19 s; AT 1.40 ± 0.21 s; artery diameter 15.0 ± 1.2 pixels; vein diameter 20.3 ± 1.8 pixels). Because no differences were observed between left and right eyes, the contralateral eye was used as the control for the microsphere-impacted eye. Physical characteristics of the rats that underwent microsphere injection are listed in Table 2. After injection of the microspheres through the right external carotid artery catheter, microspheres appeared only in the vasculature of the right retina. No microspheres were observed in the vasculature of the left retina after injection. No microspheres were observed in the retinal veins indicating that all microspheres that came into retinal arteries and were impacted in the retinal capillaries. VFA images of the retina of a nondiabetic rat 2 weeks after microsphere injection are shown in Fig. 2. Figures 2A and B are representative images of the right retina during fluorescein angiograms midphase. Figure 2A illustrates the stationary microspheres observed in the retinal capillaries. No moving microspheres are observed in the primary vessels. Arrow heads in Fig. 2B indicate retinal areas where microspheres occlude the microcirculation of the retinal capillaries in localized regions distal to the impaction

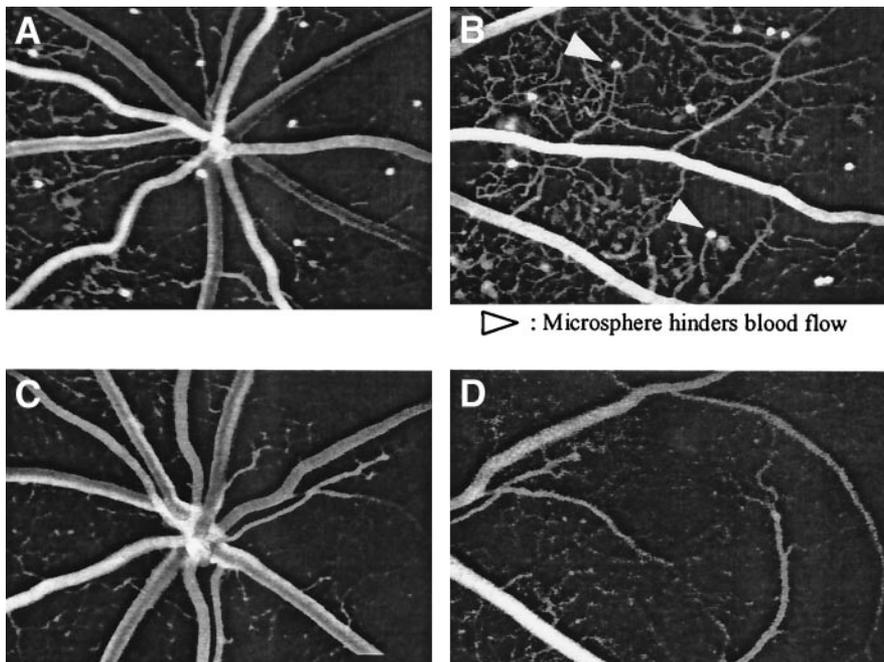


FIG. 2. VFA images of the retinas from a representative nondiabetic rat at 2 weeks after injection of 15 μm microspheres. A: Stationary microspheres were observed in the retinal capillaries, whereas no dynamic microspheres were observed in the primary vessels of the right eye. B: Arrow heads indicate retinal sites where microspheres occlude the microcirculation of the retinal capillaries within the right eye. Areas of nonperfusion can be observed in the downstream vascular beds. No stationary or moving microspheres were observed in the left retina of the same rat during late arterial phase (C) or midvenous phase (D).

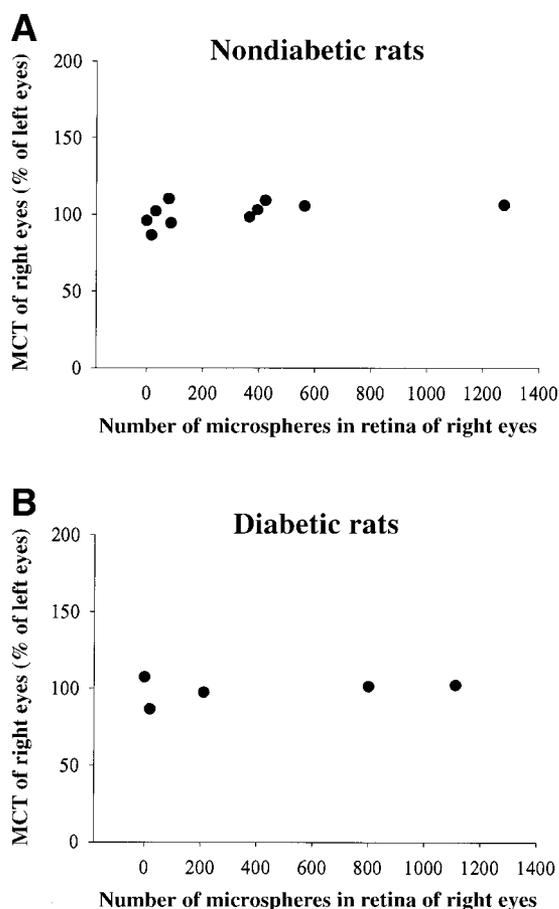


FIG. 3. Effect of microsphere injection on retinal MCT. At 2 weeks following the injection of 15 μm microspheres, the occlusion of capillary beds by impacted microspheres does not alter retinal mean circulation time in the affected right eye as compared with the left. The relation between microsphere numbers in the right retina and the relative retinal MCT of right eyes compared with left eyes in nondiabetic rats (A) ($P = 0.19$, $r = 0.45$, $n = 10$) and diabetic rats (B) ($P = 0.6$, $r = 0.32$, $n = 5$).

point. VFA images of the left eye in the same animal are shown in Figs. 2C and D. No stationary or moving microspheres were observed in the left retinas of the rats. The relation between microsphere numbers in the right retina and the relative MCT of right eyes against left eyes in nondiabetic rats are shown in Fig. 3A and that of the diabetic rats are shown in Fig. 3B. The number of impacted microspheres had no significant correlation with the relative MCT of right eyes in both nondiabetic rats ($P = 0.19$, $r = 0.45$) and diabetic rats ($P = 0.6$, $r = 0.32$).

TABLE 3
Physical properties for control and vitamin E treatment in L-E rats

	Nondiabetic rats		Diabetic rats	
	Control	Vitamin E	Control	Vitamin E
<i>n</i>	9	6	8	10
Initial body weight (g)	205.6 \pm 20.5	200.0 \pm 22.1	206.3 \pm 19.2	202.0 \pm 10.6
Final body weight (g)	289.4 \pm 19.1*	288.3 \pm 14.7*	233.1 \pm 19.1*†	245.0 \pm 27.6*†
Blood glucose (mg/dl)	100.0 \pm 9.5	96.0 \pm 9.5	373.3 \pm 38.7*	333.9 \pm 61.6†
AT (s)	1.21 \pm 0.20	1.12 \pm 0.13	1.26 \pm 0.53	1.27 \pm 0.37
Artery diameter (pixels)	7.2 \pm 0.6	6.7 \pm 0.6	6.5 \pm 0.3	6.9 \pm 0.7
Vein diameter (pixels)	8.5 \pm 1.2	8.5 \pm 0.9	8.4 \pm 0.6	8.3 \pm 0.6

Data are means \pm SD. * $P < 0.01$ compared with initial body weight (paired *t*-test); † $P < 0.01$ compared with nondiabetic control.

Results show that the localized reduction of regional microcirculation in the retina has no significant effect on retinal MCTs measured at the level of the major retinal vessels even with >1,000 beads impacted in the retina. This number of microsphere impactions was much greater than the total numbers of stationary leukocytes generally measured in the retinas of diabetic rats (~ 100 stationary leukocytes/retina).

Effect of vitamin E on retinal leukostasis and circulation. Multiple mechanisms have been postulated to cause endothelial dysfunction and mediate hyperglycemia's adverse effects including oxidative stress and activation of PKC (12–16). To test whether increases in oxidative stress or activation of PKC may cause the increased leukostasis and reduction of retinal blood flow in diabetic rats, the effect of D- α -tocopherol, an antioxidant that has been shown to have an inhibitory effect on PKC activity, was examined. Table 3 shows initial body weights and characteristics of STZ-induced diabetic and nondiabetic rats in the experimental groups (treatment with D- α -tocopherol or placebo) that were used for the leukostasis measurements. There was no significant difference in AT, artery diameter, and venous diameter among these four groups of rats. Thus, a prolonged MCT would reflect a reduction in retinal blood flow. The MCT of placebo-treated diabetic rats (1.19 ± 0.21 s) was significantly longer than that of the placebo-treated nondiabetic rats (0.73 ± 0.12 s) ($P < 0.001$) by 1.7-fold (Fig. 4A). The MCT of the D- α -tocopherol-treated diabetic rats (0.86 ± 0.17 s) was significantly shorter than that of control or placebo-treated diabetic rats ($P < 0.001$). There was no significant difference in MCT for the placebo-treated nondiabetic rats (0.73 ± 0.12 s) and the D- α -tocopherol-treated nondiabetic rats (0.75 ± 0.10 s). These results show that vitamin E treatment normalized the prolonged MCT observed in diabetic rats as previously reported (20).

The effect of D- α -tocopherol on leukostasis in the retina was also characterized (Fig. 4B). Leukostasis in the placebo-treated diabetic rats ($11.03 \pm 3.67 \times 10^{-5}$ cells/pixel²) (2) was significantly higher than that of placebo-treated nondiabetic rats ($4.90 \pm 1.73 \times 10^{-5}$ cells/pixel², $P < 0.01$) by 2.3-fold and the nondiabetic rats treated with D- α -tocopherol ($6.39 \pm 1.93 \times 10^{-5}$ cells/pixel²) ($P < 0.02$) by 1.7-fold. Leukostasis in diabetic rats treated with D- α -tocopherol ($6.40 \pm 2.56 \times 10^{-5}$ cells/pixel²) was significantly lower than that of diabetic rats treated with placebo ($P < 0.01$) and was not significantly different from those measured in the nondiabetic rat groups.

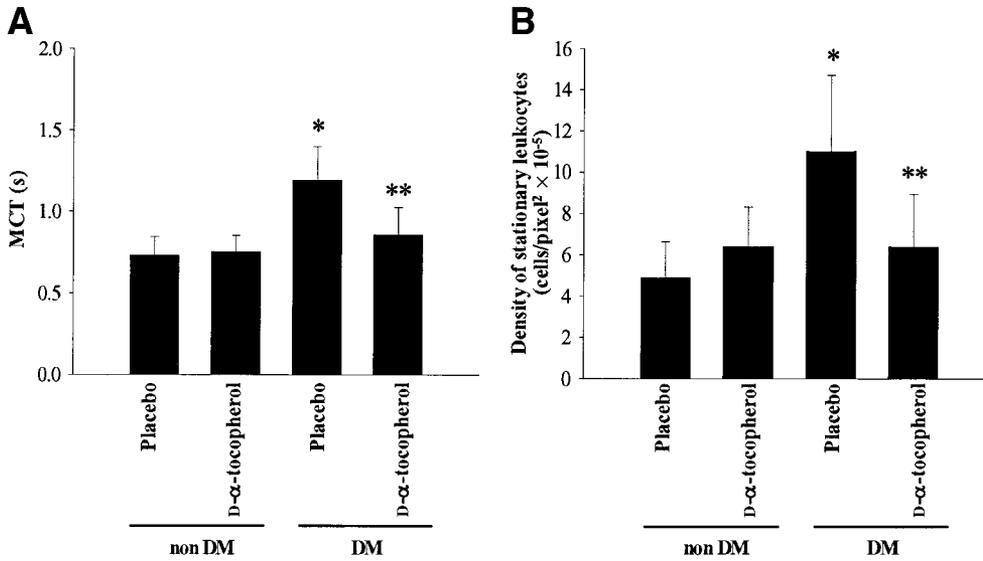


FIG. 4. Effect of D-α-tocopherol treatment on retinal MCT and leukostasis. Retinal MCT was measured by VFA (A), and retinal leukostasis was measured by SLO (B). Groups are shown as nondiabetic (nonDM) treated with placebo (n = 9) or D-α-tocopherol (n = 6) and diabetic (DM) treated with placebo (n = 8) or D-α-tocopherol (n = 10). Data are expressed as mean ± SD *P < 0.05 vs. nondiabetic (nonDM) groups. **P < 0.01 vs. diabetic (DM) placebo group.

Effect of antioxidant and PKC β-inhibitor on retinal circulation. Because vitamin E has both an antioxidant effect and PKC β-inhibitory effect as previously reported (25,26), we attempted to separate these two effects of vitamin E by using a specific PKC β-isoform inhibitor LY333531 and an antioxidant lacking PKC inhibitor action, α-lipoic acid (21,27). Table 4 summarizes the initial body weights and characteristics of diabetic and nondiabetic rat groups that were treated with α-lipoic acid (antioxidant), LY333531 (PKC β-inhibitor), or normal chow at the time of leukostasis measurements. There was no significant difference in body weights and blood glucose levels among three diabetic groups and among three nondiabetic groups. The MCT (Fig. 5A) of untreated diabetic rats fed normal chow (1.32 ± 0.26 s) and rats treated with α-lipoic acid (1.29 ± 0.53 s) was significantly longer than that of untreated nondiabetic rats fed normal chow (0.86 ± 0.13 s) by 1.5-fold ($P < 0.01$). There was no significant differences in MCT among nondiabetic groups. The MCT of diabetic rats treated with the PKC inhibitor LY333531 (1.02 ± 0.22 s) was significantly shorter than that of untreated diabetic rats fed normal chow ($P < 0.05$). This result shows that PKC β-inhibition normalized the diabetes-induced prolongation of MCT as previously reported (27), but α-lipoic acid had no significant effect on retinal MCT.

The measurements of retinal leukostasis in these animals (Fig. 5B) showed that the retinas of untreated diabetic rats fed normal chow ($9.62 \pm 2.51 \times 10^{-5}$ cells/pixel²) was significantly higher than that of untreated

nondiabetic rats fed normal chow ($4.60 \pm 2.35 \times 10^{-5}$ cells/pixel², $P < 0.001$) by 2.1-fold. There were no significant differences in leukostasis among the nondiabetic groups. The retinal leukostasis in diabetic rats treated with α-lipoic acid ($5.94 \pm 2.08 \times 10^{-5}$ cells/pixel²) or LY333531 ($5.62 \pm 2.41 \times 10^{-5}$ cells/pixel²) was significantly lower than that of untreated diabetic rats fed normal chow ($P < 0.001$ and $P < 0.001$, respectively). These results showed that both the antioxidant α-lipoic acid and the PKC β-inhibitor were effective in preventing the increases in retinal leukostasis induced by diabetes.

The effect of PKC inhibitor and α-lipoic acid on oxidative stress. To investigate the effect of the various treatments on oxidative stress in vivo, plasma hydroperoxide levels were measured. Plasma hydroperoxide levels (Fig. 5C) in untreated diabetic rats (5.65 ± 2.45 μmol/l) were significantly ($P < 0.05$) higher than in untreated nondiabetic controls (2.68 ± 1.43 μmol/l) by 2.1-fold. There was no significant difference in plasma hydroperoxide levels among nondiabetic groups. Treatment with α-lipoic acid and LY333531 diabetic rats significantly ($P < 0.05$ and $P < 0.05$, respectively) lowered the plasma hydroperoxide levels (3.06 ± 1.35 and 3.37 ± 2.16 μmol/l, respectively) compared with untreated diabetic rats.

DISCUSSION

In this study, we have characterized two of the early changes in the retinal microcirculation that have been

TABLE 4
Physical properties of L-E rats used for studies with lipoic acid and LY333531

	Nondiabetic rats			Diabetic rats		
	Control	Lipoic acid	LY333531	Control	Lipoic acid	LY333531
n	15	14	15	20	17	16
Initial body weight (g)	223.7 ± 20.9	237.1 ± 17.0	227.0 ± 15.9	224.8 ± 11.2	227.4 ± 13.1	216.9 ± 19.4
Final body weight (g)	305.3 ± 18.9*	291.8 ± 11.5*	307.7 ± 22.9*	247.3 ± 21.9*†	235.6 ± 15.9*†	242.8 ± 27.6*†
Blood glucose (mg/dl)	96.9 ± 11.0	94.1 ± 8.2	101.1 ± 8.7	355.3 ± 51.4†	351.8 ± 48.6†	380.3 ± 54.8†
AT (s)	1.55 ± 0.44	1.42 ± 0.24	1.40 ± 0.36	1.58 ± 0.38	1.55 ± 0.27	1.51 ± 0.59
Artery diameter (pixels)	6.5 ± 0.4	7.1 ± 0.4	7.0 ± 0.5	6.7 ± 0.5	6.9 ± 0.6	6.6 ± 0.5
Vein diameter (pixels)	8.7 ± 0.4	8.7 ± 0.5	8.5 ± 0.5	8.3 ± 0.8	8.8 ± 0.7	8.3 ± 0.4

Data are means ± SD. *P < 0.05 compared with initial body weight (paired t-test); †P < 0.01 compared with nondiabetic control rats.

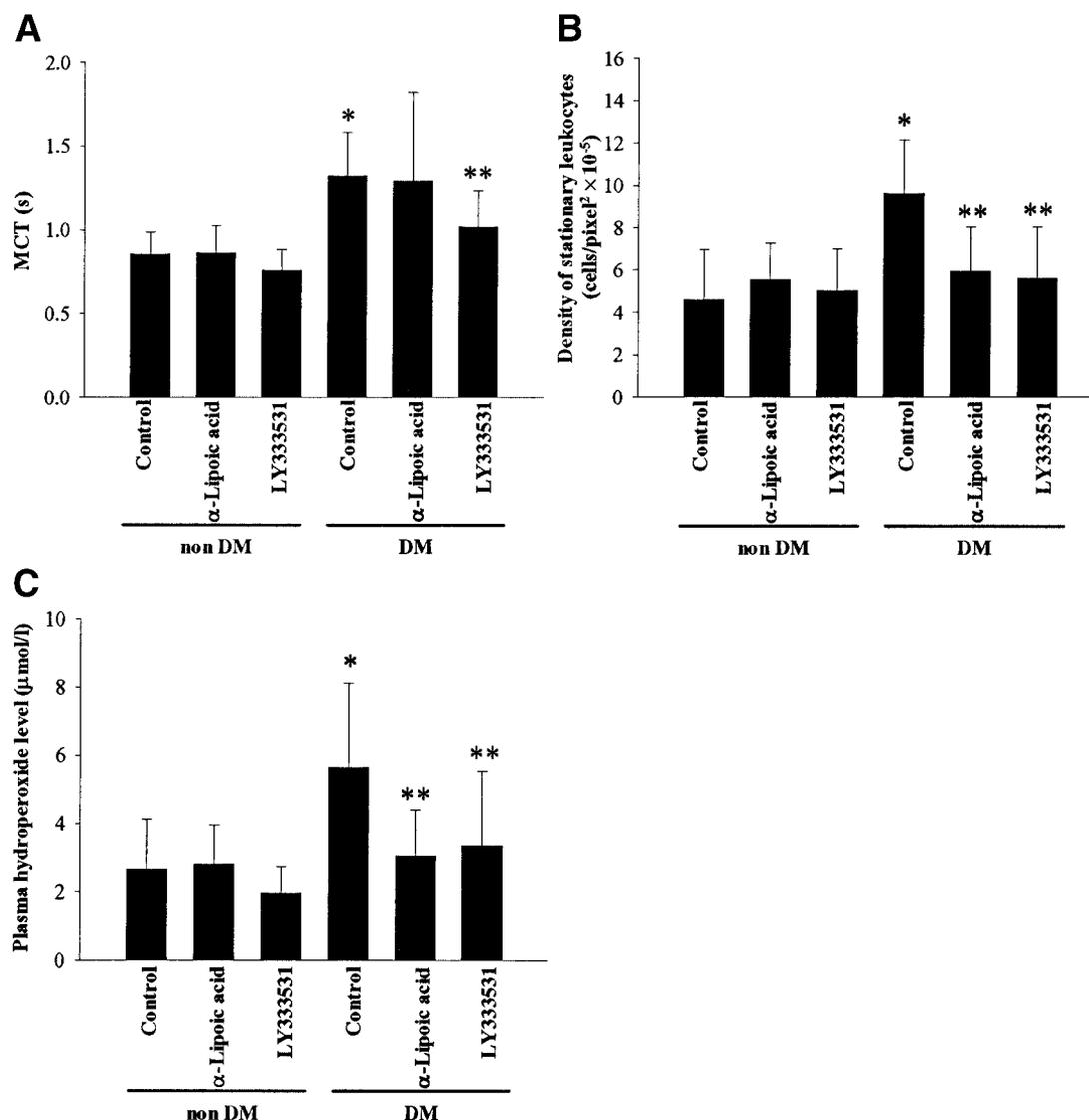


FIG. 5. Effect of α -lipoic acid and LY333531 treatment on MCT (A), retinal leukostasis (B), and plasma hydroperoxide level (C). Groups are shown as nondiabetic (nonDM) treated with control ($n = 15$), lipoic acid ($n = 14$), or LY333531 ($n = 15$) and diabetic (DM) treated with control ($n = 20$), lipoic acid ($n = 17$), or LY333531 ($n = 16$). Data are expressed as means \pm SD * $P < 0.01$ vs. nondiabetic (nonDM) groups. ** $P < 0.05$ vs. diabetic (DM) control group.

reported to occur in diabetic retinopathy, increases in leukocyte/monocyte adhesion (leukostasis) and changes in MCT/retinal blood flow (2–5). Increases in leukostasis have been shown to occur in animal models of diabetes with short duration of disease (19). Multiple reports have suggested that the increases in leukostasis are associated with leukocyte or endothelial cell activation and increased expression of intracellular adhesion molecules (5,28). The consequences of increases in retinal vessel leukostasis have not been established, but potentially the immobilization of leukocytes and monocytes can hinder retinal microvascular blood flow physically. Several reports have suggested that increases in leukocyte/monocyte adhesion is a critical factor in early retinopathy causing decreases in retinal blood flow and increases in cytokine expression, such as vascular endothelial growth factor (4,29). However, increases in leukocyte/monocyte adhesion to the vascular endothelium have also been reported to occur systemically in association with endothelial dysfunction and have been reported in insulin-resistant states with or

without diabetes (30,31). The results from the present study on lean and fatty Zucker rats provided clear evidence that the increases in leukostasis can also occur in retinal microcirculation when only insulin resistance is present without diabetes or overt hyperglycemia, indicating that leukostasis in the retina can occur before the development of diabetes. In contrast, increases in MCT and decreases in retinal blood flow appear to require more than the presence of insulin resistance. The data demonstrates that the presence of diabetes and hyperglycemia are necessary for a measurable effect on retinal blood flow reduction. The finding that increases in retinal leukostasis are correlated to endothelial dysfunction rather than hyperglycemia confirms studies in systemic circulation reporting that endothelial dysfunction may play an important role in the increased binding of leukocyte/monocyte to endothelial cells in insulin-resistant states (32). Endothelial dysfunction, as observed systemically as a consequence of insulin resistance alone or with diabetes can also be induced by elevation of the free fatty acid and is

believed to contribute to the increased risk of cardiovascular disease but not to the microvascular disease observed in patients with these conditions (33).

The finding that increases in leukostasis may precede the decreases in retinal blood flow and diabetes suggests that the decrease in retinal blood flow observed in a diabetic state of short duration could be the results of leukostasis impeding retinal microcirculation. However, the results of studies using the fluorescent microspheres (15 $\mu\text{mol/l}$ diameter), which were large enough so that they would be impacted into multiple retinal capillaries, suggested that the blockage of capillaries even with >1,000 microspheres/retina did not alter MCT in either control or diabetic rats. Thus, it is unlikely that increases in leukocyte/monocyte adhesion alone are responsible for observed decreases in retinal blood flow. This conclusion is supported also by the findings that the magnitude of the increases in leukostasis in the insulin-resistant state was comparable with the magnitude of leukostasis measured in the presence of diabetes. Thus, it is very likely that endothelial dysfunction, which has been documented in both animal models and in patients with insulin resistance alone and/or with overt diabetes, is responsible for the development of leukostasis (5,34). The inducing factor of endothelial dysfunction in the insulin-resistant state is likely to be elevated free fatty acid or hyperlipidemia, both of which are metabolic consequences of the loss on insulin's actions on adipose tissues (35). In contrast, increases in MCT and decreases in retinal blood flow require the presence of hyperglycemia and diabetes, possibly through induction of increased expression of ET-1, a vasoconstrictor that we have previously reported to be elevated in the retina of diabetic animals (8). These findings also indicate that increases in leukostasis in the retina is not sufficient to decrease retinal blood flow or to induce the development of clinical diabetic retinopathy, which is not observed in the insulin-resistant state without diabetes or hyperglycemia. However, it is possible that increases in leukocyte/monocyte adhesion may enhance the adverse effects of hyperglycemia to accelerate diabetic retinal pathologies.

The potential mechanisms causing the increases in leukostasis and reduction of retinal blood flow in diabetic rats were also characterized using inhibitors of oxidative stress, α -lipoic acid, PKC β -isoform inhibitor, LY333531, and an antioxidant (D- α -tocopherol) that also has PKC inhibitory effects (3,22,26,27). A great deal of evidence has suggested that increases in oxidant production or PKC activation, especially the PKC β -isoforms induced by hyperglycemia, are partly responsible for the various retinal and other vascular dysfunction in diabetes (36). It has been reported that α -lipoic acid treatment can improve microcirculation in the peripheral nerves and is a well-documented antioxidant (37). These results in the retinal circulation suggested that increases in leukostasis are related to oxidative stress, since both α -lipoic acid and D- α -tocopherol were able to prevent the increase in plasma lipid peroxide and leukostasis. Surprisingly, however, inhibition of PKC β -isoform LY333531 was also able to prevent the increases in lipid peroxidation (oxidative stress) systemically and leukostasis. The apparent inhibitory effect of PKC β -isoform to prevent increases in lipid

peroxidation could potentially be explained by the inhibition of NADP oxidases, which have been reported to be activated by hyperglycemia via PKC β -isoform activation (36,38,39).

Unlike leukostasis, manifestation of decreased retinal blood flow requires the presence of diabetes and hyperglycemia as described above. Further, only PKC β -isoform inhibitor (LY333531) and D- α -tocopherol but not α -lipoic acid normalized the decrease in retinal blood flow (22). These results suggested that oxidative stress is not the major cause of abnormalities of retinal blood flow, as retinal blood flow change is probably not associated directly to increases in leukostasis. This is consistent with the microsphere results indicating that microspheres trapped in the capillary beds at >1,000 capillaries per retina had no significant affect on retinal blood flow measured from the major retinal vessels. These results support our previous reports showing that the decreased retinal blood flow is related to hyperglycemia and PKC activation, especially the β -isoforms (27). Because treatments with insulin or PKC β -isoform-specific inhibitor normalized retinal blood flow in diabetic animals, the decreases in retinal blood flow are likely to be the result of increases in the expression of ET-1, which we have reported can be induced by elevation of glucose via PKC activation. In addition, inhibitors of the endothelin A receptor, such as BQ123, were effective in normalizing retinal MCT and blood flow in diabetic rats. (8)

In summary, these results have identified increased leukocyte/monocyte adhesion in the retina, as associated with endothelial dysfunction, and are observed in insulin resistance and oxidative stress but not require hyperglycemia and may thus not be an obligatory step in the development of diabetic retinopathy. In contrast, the manifestation of decreased retinal blood flow in diabetes requires hyperglycemia and PKC activation. Inhibition of PKC β -activation appears to be effective both to prevent hyperglycemia-induced oxidative stress and PKC activation, systemically, suggesting that PKC β -isoform activation could be an important regulator of several pathways by which hyperglycemia mediates its adverse effects in micro- and cardiovascular tissues of diabetes.

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REFERENCES

1. Aiello LP, Cahill M, Wong JS: Systemic considerations in the management of diabetic retinopathy. *Am J Ophthalmol* 132:760-776, 2001
2. Kohner EM, Patel V, Rassam SM: Role of blood flow and impaired autoregulation in the pathogenesis of diabetic retinopathy. *Diabetes* 44: 603-607, 1995
3. Bursell SE, Clermont AC, Aiello LP, Aiello LM, Schlossman DK, Feener EP, Laffel L, King GL: High-dose vitamin E supplementation normalizes retinal blood flow and creatinine clearance in patients with type 1 diabetes. *Diabetes Care* 22:1245-1251, 1999
4. Miyamoto K, Ogura Y: Pathogenetic potential of leukocytes in diabetic retinopathy. *Semin Ophthalmol* 14:233-239, 1999
5. Miyamoto K, Khosrof S, Bursell SE, Rohan R, Murata T, Clermont AC, Aiello LP, Ogura Y, Adamis AP: Prevention of leukostasis and vascular leakage in streptozotocin-induced diabetic retinopathy via intercellular adhesion molecule-1 inhibition. *Proc Natl Acad Sci U S A* 96:10836-10841, 1999
6. The Diabetes Control and Complications Trial Research Group: The effect

- of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes-mellitus. *N Engl J Med* 329:977-986, 1993
7. U.K. Prospective Diabetes Study Group: Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet* 352:837-853, 1998
 8. Takagi C, Bursell SE, Lin YW, Takagi H, Duh E, Jiang Z, Clermont AC, King GL: Regulation of retinal hemodynamics in diabetic rats by increased expression and action of endothelin-1. *Invest Ophthalmol Vis Sci* 37:2504-2518, 1996
 9. Penno G, Chaturvedi N, Talmud PJ, Cotroneo P, Manto A, Nannipieri M, Luong LA, Fuller JH: Effect of angiotensin-converting enzyme (ACE) gene polymorphism on progression of renal disease and the influence of ACE inhibition in IDDM patients: findings from the EUCLID Randomized Controlled Trial. EURODIAB Controlled Trial of Lisinepril in IDDM. *Diabetes* 47:1507-1511, 1998
 10. Evans T, Deng DX, Chen S, Chakrabarti S: Endothelin receptor blockade prevents augmented extracellular matrix component mRNA expression and capillary basement membrane thickening in the retina of diabetic and galactose-fed rats. *Diabetes* 49:662-666, 2000
 11. Booth G, Stalker TJ, Lefer AM, Scalia R: Mechanisms of amelioration of glucose-induced endothelial dysfunction following inhibition of protein kinase C in vivo. *Diabetes* 51:1556-1564, 2002
 12. Beckman JA, Goldfine AB, Gordon MB, Garrett LA, Creager MA: Inhibition of protein kinase C beta prevents impaired endothelium-dependant vasodilation caused by hyperglycemia in humans. *Circ Res* 90:107-111, 2002
 13. Baron AD: Insulin resistance and vascular function. *J Diabetes Complications* 16:92-102, 2002
 14. Way KJ, Katai N, King GL: Protein kinase C and the development of diabetic vascular complications. *Diabet Med* 18:945-959, 2001
 15. Resnick HE, Howard BV: Diabetes and cardiovascular disease. *Annu Rev Med* 53:245-267, 2002
 16. Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813-820, 2001
 17. Frank RN: Potential new medical therapies for diabetic retinopathy: protein kinase C inhibitors. *Am J Ophthalmol* 133:693-698, 2002
 18. Clermont AC, Brittis M, Shiba T, McGovern T, King GL, Bursell SE: Normalization of retinal blood flow in diabetic rats with primary intervention using insulin pumps. *Invest Ophthalmol Vis Sci* 35:981-990, 1994
 19. Miyamoto K, Hiroshiba N, Tsujikawa A, Ogura Y: In vivo demonstration of increased leukocyte entrapment in retinal microcirculation of diabetic rats. *Invest Ophthalmol Vis Sci* 39:2190-2194, 1998
 20. Kunisaki M, Bursell SE, Umeda F, Nawata H, King GL: Normalization of diacylglycerol-protein kinase C activation by vitamin E in aorta of diabetic rats and cultured rat smooth muscle cells exposed to elevated glucose levels. *Diabetes* 43:1372-1377, 1994
 21. Packer L, Kraemer K, Rimbach G: Molecular aspects of lipoic acid in the prevention of diabetes complications. *Nutrition* 17:888-895, 2001
 22. Kagokawa H, Clermont AC, Bursell SE: Differential effect of vitamin E and lipoic acid in retinal blood flow in diabetic rats (Abstract). *Diabetes* 48 (Suppl. 1): 153A, 1999
 23. Nourooz-Zadeh J, Tajaddini-Sarmadi J, Wolff SP: Measurement of plasma hydro peroxide concentrations by the ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine. *Anal Biochem* 220:403-409, 1994
 24. Unger RH, Orci L: Diseases of liporegulation: new perspective on obesity and related disorders. *FASEB J* 15:312-321, 2001
 25. Frei B: On the role of vitamin C and other antioxidants in atherogenesis and vascular dysfunction. *Proc Soc Exp Biol Med* 222:196-204, 1999
 26. Kunisaki M, Bursell SE, Clermont AC, Ishii H, Ballas LM, Jirousek MR, Umeda F, Nawata H, King GL: Vitamin E prevents diabetes-induced abnormal retinal blood flow via the diacylglycerol-protein kinase C pathway. *Am J Physiol* 269:E239-E246, 1995
 27. Ishii H, Jirousek MR, Koya D, Takagi C, Xia P, Clermont AC, Bursell SE, Kern TS, Ballas LM, Heath WF, Stramm LE, Feener EP, King GL: Amelioration of vascular dysfunctions in diabetic rats by an oral PKC beta inhibitor. *Science* 272:728-731, 1996
 28. Lo SK, Janakidevi K, Lai L, Malik AB: Hydrogen peroxide-induced increase in endothelial adhesiveness is dependent on ICAM-1 activation. *Am J Physiol* 264:L406-L412, 1993
 29. Jousen AM, Poulaki V, Qin W, Kirchoff B, Mitsiades N, Wiegand SJ, Rudge J, Yancopoulos GD, Adamis AP: Retinal vascular endothelial growth factor induces intercellular adhesion molecular-1 and endothelial nitric oxide synthase expression and initiates early diabetic retinal leukocyte adhesion in vivo. *Am J Pathol* 160:501-509, 2002
 30. Matsumoto K, Sera Y, Nakamura H, Ueki Y, Miyake S: Serum concentrations of soluble adhesion molecules are related to degree of hyperglycemia and insulin resistance in patients with type 2 diabetes mellitus. *Diabetes Res Clin Pract* 55:131-138, 2002
 31. Chen NG, Holmes M, Reaven GM: Relationship between insulin resistance, soluble adhesion molecules, and mononuclear cell binding in healthy volunteers. *J Clin Endocrinol Metab* 84:3485-3489, 1999
 32. Bluher M, Unger R, Rassoul F, Richter V, Paschke R: Relation between glycaemic control, hyperinsulinaemia, and plasma concentrations of soluble adhesion molecules in patients with impaired glucose tolerance or type II diabetes. *Diabetologia* 45:210-216, 2002
 33. Licata G, Scaglione R, Dominguez LJ: Early markers of cardiovascular damage in obese subjects. *Nutr Metab Cardiovasc Dis* 9:78-86, 1999
 34. Paques M, Boval B, Richard S, Tadayori R, Massin P, Mundler O, Gaugric A, Vicaute E: Evaluation of fluorescein-labeled autologous leukocytes for examination of retinal circulation in humans. *Curr Eye Res* 21:560-565, 2000
 35. Bergman RN, Mittelman SD: Central role of the adipocyte in insulin resistance. *J Basic Clin Physiol Pharmacol* 9:205-221, 1998
 36. Inoguchi T, Li P, Umeda F, Yu HY, Kakimoto M, Inamura M, Aoki T, Etoh T, Hashimoto T, Naruse M, Sano H, Utsumi H, Nawata H: High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C-dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes* 49:1939-1945, 2000
 37. van Dam PS, van Asbeck BS, Van Oirschot JF, Biessels GJ, Haners FP, Marx JJ: Glutathione and alpha-lipoate in diabetic rats: nerve function, blood flow and oxidative state. *Eur J Clin Invest* 31:417-424, 2001
 38. Gorlach A, Brandes RP, Nguyen K, Amidi M, Dehghani F, Busse R: A gp91phox containing NADPH oxidase selectively expressed in endothelial cells is a major source of oxygen radical generation in the arterial wall. *Circ Res* 87:26-32, 2000
 39. Kowluru RA: Diabetes-induced elevations in retinal oxidative stress, protein kinase C and nitric oxide are interrelated. *Acta Diabetol* 38:179-185, 2001