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Ozagrel reverses streptozotocin-induced constriction of arterioles in rat retina

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Abstract

Retinal blood flow decreases early in the progression of diabetic retinopathy; however, the mediators and mechanisms responsible for this decrease have yet to be determined. In this study, diabetes was induced by streptozotocin in rats, and retinal blood flow was measured via intravital microscopy one or three weeks following the induction of hyperglycemia. Additionally, retinal arteriolar diameters and flow were measured prior to and following acute administration of the thromboxane synthase inhibitor ozagrel to investigate the potential role of thromboxane in the observed constriction. Minimal changes in the retinal diameters and flow were observed at one week of diabetes; however, at three weeks of diabetes, arteriolar constriction and decreases in blood flow were significant. Notably, the constriction occurred only in the arterioles that were in closer proximity to the venules draining the retina. Acute administration of ozagrel reversed the constriction of the closely venule-paired arterioles. In summary, the results suggest that thromboxane mediates localized, venule-dependent arteriolar constriction induced by streptozotocin-induced diabetes in rats.

Keywords

microcirculation; thromboxane; diabetes; microvascular flow

Introduction

Retinal microvascular abnormalities that accompany diabetes include arteriolar constriction (Klein *et al.*, 2003; Moss *et al.*, 2003; Wong *et al.*, 2002; Wong *et al.*, 2005), capillary dropout, and localized areas of ischemia, the last of which is implicated in the eventual vision problems associated with the pathological growth of new blood vessels (Kim *et al.*, 2004). The decreases in retinal blood flow associated with human diabetes, which have been reported to be ~33% (Bursell *et al.*, 1996; Clermont *et al.*, 1997), occur early in the progression of the disease. The mechanisms of the decrease in blood flow have yet to be determined.

Animal models of diabetes are potentially valuable tools in the investigation of early changes in the diabetic retina. However, existing reports of diabetes-induced changes in retinal blood flow have shown a variety of responses, possibly due to the differences in the experimental animals, duration of hyperglycemia, and measurement techniques. While the use of animal

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models should allow investigations of the vasoactive mediators potentially responsible for the decreased flow observed in human diabetic patients, to date, there is little information available on the identity of these vasoconstrictors, even in animal models.

In the streptozotocin model of diabetes in rats, De La Cruz et al. have reported that the vasoconstrictor thromboxane is responsible for decreases in capillary density (De La Cruz *et al.*, 1997). However, their reports did not evaluate the potential role of this mediator in arteriolar constriction. Hypothesizing such a role for thromboxane is reasonable, inasmuch as the vasoactive molecule has been reported to constrict arterioles in other models of inflammation. For example, in the reperfusion response following ischemia in rat skeletal muscle, a thromboxane receptor antagonist (ONO-3708) was able to inhibit the development of arteriolar constriction (Mazolewski *et al.*, 1999). Additionally, in a model of intestinal inflammation induced by dextran sodium sulfate, an acute administration of a thromboxane synthase inhibitor (ozagrel) was able to dilate constricted arterioles (Harris *et al.*, 2005). In both of these models, the thromboxane-induced constriction followed an interesting pattern of being more severe in arterioles in closer proximity to postcapillary venules (Harris *et al.*, 2005; Zamboni *et al.*, 1993).

Based on this background, we hypothesize that diabetes will induce retinal arteriolar constriction that is 1) more severe in the proximity of venules, and 2) mediated by thromboxane. Recently, we have noted evidence of this retinal phenomenon in two separate models of diabetic mice (Lee & Harris, 2008; Wright & Harris, 2008). In this study, diabetes in rats was induced by streptozotocin, and thromboxane-induced arteriolar constriction was studied using intravital microscopy, by which its relationship with retinal venules could be evaluated.

Methods

Diabetic Animals and Surgery

Male Wistar rats (Harlan, Indianapolis, IN, USA) aged 2–3 months were given an intraperitoneal injection of streptozotocin (Sigma Chemical, St. Louis, MO, USA) at a dose of 65 mg/kg (in 10 mM citrate buffer at pH 4.5) to induce diabetes. Non-fasting blood glucose levels were determined 2 days after injection using a One Touch Ultra blood glucose monitoring system (Lifescan, Milpitas, CA, USA). The tail veins were punctured with 23 G needles to obtain blood samples and animals with blood glucose levels greater than 300 mg/dl were considered diabetic. Experiments were performed on the rats following 1 or 3 weeks of hyperglycemia; age-matched rats that did not receive an injection of streptozotocin were used as controls. All animals were provided standard laboratory chow and water ad libitum.

On the day of experiments, the rats were anesthetized with intraperitoneal injections of pentobarbital (50 mg/kg, Nembutal®, Abbott Laboratories, North Chicago, IL, USA) and ketamine (50 mg/kg, Ketaset®, Animal Health, Fort Dodge, IA, USA). The right femoral vein was cannulated for injections. The eyes under examination (left eyes) were kept moist with a drop of Gonak® (Akorn Inc., Buffalo Grove, IL, USA) after the pupils were dilated using two drops each of tropicamide (1%; Alcon Laboratories Inc., Fort Worth, TX, USA) and phenylephrine (2.5%; Bausch & Lomb, Tampa, FL, USA). Body temperature was maintained near 37°C at all times with an infrared heat lamp over the rat. Procedures complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. At the end of the experiments, animals were euthanized using an overdose of sodium pentobarbital (150 mg/kg) as approved by the Institutional Animal Care and Use Committee.

Intravital Microscopy

After anesthesia was induced, the rat was placed on the microscope stage and a cover slip placed on the eye, which was observed with a 4x objective (N.A. 0.13; 1.71 cm working distance) and 10x objective (N.A. 0.25; 1.05 cm working distance) on a Nikon fluorescence microscope (Eclipse E600FN, Nikon Instruments Inc., Melville, NY, USA). (See Figure 1 for examples of retinal microscopic images.) An autofluorescent image of the retina was used to locate and focus on the vessels extending into and out of the optic disk, using a fluorescein filter. The images were recorded on DVD (DMR-ES10 recorder, Panasonic, Osaka, Japan) through a Sony color camera (DXC-390, Sony, Tokyo, Japan) and a time-date generator (Panasonic WJ-810, Panasonic Industrial Co., Secaucus, NJ, USA) for subsequent analysis.

Platelet/Red Blood Cell Labeling

To obtain platelets, approximately 3 mL of blood were collected from protocol-matched donor rats. The blood was centrifuged at 1200 rpm for 8 minutes and the platelet-rich supernatant transferred to a new centrifuge tube. The supernatant was centrifuged again at 1200 rpm for 3 minutes to separate any remaining red blood cells (RBCs) and leukocytes from platelet-rich plasma. The platelet-rich plasma was transferred to a new tube and centrifuged at 3000 rpm to form a platelet pellet. The platelet-poor plasma was decanted and the pellet resuspended slowly in 0.5 mL phosphate-buffered saline (PBS; pH 7.37). The number of platelets per volume was calculated using a hemacytometer with 5 μ L stained platelets suspended in PBS. Based on these calculations, the required amount of platelets (1×10^8 cells) suspended in PBS was withdrawn and brought to a total volume of 1.5 mL by adding PBS. The platelets were labeled with 9 μ L carboxyfluorescein diacetate succinimidyl ester (CFSE; final concentration 90 μ M; Sigma, St. Louis, MO, USA), recentrifuged at 3000 rpm for 10 minutes and suspended in 0.3 mL PBS again before slow intravenous injection into the recipient over approximately 30 s.

For RBC labeling, approximately 3 mL of blood was collected from a donor rat. The blood was run through a column of packed sterile cotton with 10 mL of PBS and then centrifuged at 3000 rpm for 10 minutes. The supernatant was removed and the pellet resuspended slowly in 1 mL PBS and then centrifuged again for 5 minutes. A preparation of 100 μ L 1,1'-diiodo-3,3',3',3'-tetramethylindocarbocyanine perchlorate (DiI; D3911; Invitrogen Molecular Probes, Eugene, OR, USA) stock solution (5 mg/mL) was diluted with 9900 μ L PBS as a DiI labeling solution. Then 100 μ L packed RBCs were dispersed in the DiI solution, where they incubated for 30 min. The labeled RBCs were centrifuged at 3000 rpm and washed in 1 mL PBS 4–5 times and resuspended slowly in 1 mL PBS. One donor rat supplied enough platelets or RBC for approximately 3 recipients.

Experimental protocols

Fluorescently labeled platelets or RBCs were injected through a femoral vein catheter to determine retinal blood velocity approximately 500 μ m away from the center of the optic disk (see Fig 1B). The fluorescent images of the circulating platelets/RBCs were recorded by the camera onto DVD approximately 5 minutes after the start of the infusion. The movement of a given platelet/RBC appeared as a fluorescent streak whose length was proportional to velocity and camera exposure time (in our case, 16.7 ms) in a frame of video. When necessary to confirm the length of the fluorescent streak, three consecutive frames were used, with the start and end of the streak in the second frame lining up with the end and start of the streak in the first and third frames, respectively. The frames were analyzed using an image grabber (Studio Plus, Pinnacle Systems Inc., Mountain View, CA, USA) and image processor (SigmaScan Pro v 5.0.0, Systat Software Inc., Point Richmond, CA, USA).

Arteriolar diameters were measured (with a 10x objective) as the width of the column of RBCs, or in some experiments, as the width filled with fluorescence following injection of fluorescein

isothio-cyanate (FITC)-dextran (MW = 2,000,000; 25mg/mL in saline; FD2000S; Sigma, St. Louis, MO, USA). With the latter, 25 μ L was infused through the femoral vein catheter. The diameter for each arteriole was measured in three different points along the vessel and averaged (see Fig 1C). The center measuring point was about 550 μ m away from the optic disk along the vessel length and the other two measuring points were 250 μ m away from the center measuring point in either direction. An average of about 15 platelet or RBC velocities (mean velocity, V) was used to estimate flow rate according to the calculation $V\pi D^2/4$, assuming a uniform cylindrical diameter D. In some experiments, the thromboxane synthase inhibitor ozagrel ([E]-3-[4-(1H-imidazol-1-ylmethyl) phenyl]-2-propenoic acid hydrochloride; Sigma Chemical, St. Louis, MO, USA) was injected intravenously (20 mg/kg (Gomi *et al.*, 1989; Gomi *et al.*, 1995)), and after 30 minutes the changes in arteriolar diameter and velocity were repeated and compared with baseline values as described previously (Harris *et al.*, 2005). Ozagrel, the first thromboxane synthase inhibitor released onto the market for human patients, has been described as a selective thromboxane A₂ synthase inhibitor in many models (Dogne *et al.*, 2004; Gentilini *et al.*, 1988; Ichikawa *et al.*, 1999; Ito *et al.*, 2003; Westphal *et al.*, 2005). The reported effective half-life of ozagrel is ~2 hours (Kaku *et al.*, 1996; Kawakatsu *et al.*, 1990; Okumura *et al.*, 2003; Oshima *et al.*, 1984).

Arterio-venular pairing angles

Blood vessels extending into and out of the optic disk are found in a radial arrangement in the rat retina, with an alternating pattern of arterioles and venules as shown in Fig 1. The angle between an arteriole and a neighboring venule can be smaller than 10° (nearly parallel) and as large as 70°. Using the retinal images from our experimental rats, we averaged the two angles that an arteriole made with the neighboring venules: the distribution of angles (with a median of 30.2°) is shown in Figure 2. In some of our data analyses, we used this median to divide the arterioles into closely paired ($\leq 30.2^\circ$) vs distantly paired ($> 30.2^\circ$).

Pressure Measurements

Intraocular pressure (IOP) was measured using TonoPen XL® (Mentor, Norwell, MA, USA). Tonopen has been reported to be an acceptable non-invasive device to measure IOP in rodents (Moore *et al.*, 1993; Reitsamer *et al.*, 2004); the method modified from Reitsamer *et al.* (Reitsamer *et al.*, 2004) was used for this study. Once the rat was put under anesthesia, 10 measurements were made on the right eye with the Tonopen calibrated according to the manufacturer's instructions. Any measurement below 6 mmHg was excluded from analysis, as these readings could represent insufficiently light touches of the cornea with the tip of the Tonopen (Moore *et al.*, 1993). The Tonopen was cleaned between each measurement using a cotton pad and alcohol, and the eye was kept moist with saline solution. For measurements of mean arterial pressure (MAP) in separate animals, a carotid arterial catheter was connected to a Pressure Monitor BP-1 (World Precision Instruments, Sarasota, FL, USA).

Statistics

Minitab software (v 12.22; Minitab Inc., State College, PA, USA) was used for linear regression, with outliers and high residuals (> 2.5) removed from the analyses. Statistical differences between groups were analyzed with ANOVA using the Student-Newman-Keuls multiple comparisons test (GraphPad InStat software v 3.05; GraphPad Software, San Diego, CA, USA). Error bars are presented as $\pm$ standard error (SE), and statistical significance was set at $p < 0.05$.

Results

Table 1 presents the numbers of rats (and the number of arterioles) in the two different protocols. We included 7–8 rats per group, analyzing an average of 5–6 arterioles per rat.

Glucose levels increased rapidly in the diabetics, to 473 ± 29 mg/dl one week after the streptozotocin injection, and to approximately 550 mg/dl three weeks after streptozotocin. Body weights of the diabetic rats were lower than those of controls as shown in Table 1.

Calculating flow from diameters (width of RBC column) and velocity (of fluorescently labeled platelets), we examined the correlation between arteriolar flow and arterio-venular pairing angle as presented in Figure 3. No correlation was found in control (non-diabetic) or 1-week diabetics, indicating that flow was essentially independent of the pairing arrangement. However, flow decreased after three weeks of diabetes, and as shown in Figure 3C, the decrease in flow occurred preferentially in the more closely venule-paired arterioles ($p=0.002$). This pattern of a decrease in flow was reversed upon acute administration of the thromboxane synthase inhibitor ozagrel (20 mg/kg), as shown in Figure 3D. Figures 3C and 3D are obtained from the same arterioles, before and 30 min after injection of ozagrel, respectively. Regression analyses of diameter vs pairing angle (data not shown) gave similar results, that is, a significant slope was observed only in the untreated three-week diabetic group ($p=0.002$; $r=0.473$; slope = $0.65 \mu\text{m}/^\circ$). No significant correlations were found between velocity and pairing angles, with velocity remaining unchanged by the diabetic protocol (see Table 2).

Figure 4 segregates the data according to the median pairing angle (30.2°). In the 3-week diabetic rats, we found arteriolar constriction in the more closely paired arterioles (diameter = $58 \pm 2 \mu\text{m}$, vs $73 \pm 3 \mu\text{m}$ in the non-diabetic controls; Fig 4A). No significant constriction was observed in the more distantly paired arterioles. Corresponding to the pattern of constriction, flow was significantly lower in the more closely paired arterioles in the 3-week diabetic rats (58 nl/s, vs 88 nl/s in the non-diabetic controls; Fig 4B). Since the decrease in flow was limited to the arterioles in which constriction was observed, it is unlikely that the decrease in flow was a result of potential alterations in perfusion pressure, that is, mean arterial pressure (MAP) minus intraocular pressure (IOP). However, we measured these pressures (IOP in the same animals; MAP in separate animals) to confirm whether its influence should be taken into account. In controls, MAP averaged 102 ± 2 mmHg ($N=5$) and IOP averaged 10.7 ± 0.3 mmHg ($N=7$). In 3-week untreated diabetics, the values were virtually identical: 103 ± 2 ($N=5$; MAP) and 10.9 ± 0.2 mmHg ($N=7$; IOP).

We repeated the experiments with fluorescently labeled RBCs as the flow markers and measured arteriolar diameters as the column filled with an injection of FITC-dextran (data shown in Figure 5 and Figure 6). These data demonstrate the same phenomenon as that shown in Figure 3 and Figure 4, that is, diabetes-induced arteriolar constriction occurs to a greater extent in the more closely venule-paired arterioles. In Figure 5, the arterioles from which baseline data were obtained in controls (panel A) and diabetics (panel C) are also shown in panels B and D after the injection of ozagrel. Although ozagrel did not have a significant effect on controls, it restored flow in the constricted arterioles of the diabetic group (also presented in Fig 6). Regression analyses of diameter vs pairing angle (data not shown) gave similar results to the regressions shown in Fig 5, that is, a significant slope was observed in the untreated three-week diabetic group ($p=0.005$; $r=0.43$; slope = $0.62 \mu\text{m}/^\circ$). Following ozagrel administration, this slope was attenuated but not eliminated ($p=0.027$; $r=0.34$; slope = $0.39 \mu\text{m}/^\circ$). No significant correlations were found between RBC velocity and pairing angles, with velocity remaining unchanged by the diabetic protocol (see Table 2).

As expected, measurements of arteriolar diameters (and flow) were slightly higher using FITC-dextran to fill the vessels than using the RBC column (compare values in Fig 4 vs Fig 6), since the dextran fills the RBC-free zone near the endothelium. However, essentially the same patterns of localized, diabetes-induced arteriolar constrictions were measured independent of flow marker or vascular filling techniques. Whether using platelets or RBCs as the flow markers, Figure 7 demonstrates that inhibition of thromboxane in diabetic rats dilates closely

venule-paired arterioles (y-intercept = 62–96%), while having essentially no effect (~0% dilation) when the arterio-venular pairing angle approaches 40–50°.

Discussion

Data from diabetic patients indicate a substantial decrease in retinal blood flow early in the progression of the disease (Bursell *et al.*, 1996; Clermont *et al.*, 1997). The mechanisms and mediators of this response have yet to be elucidated; however, the results of this animal study suggest the possibility that the vasoconstrictor thromboxane could be involved. In a model of streptozotocin-induced diabetes in rats, we observed retinal arteriolar constriction and decreased blood flow at three weeks of hyperglycemia, which was reversible upon acute administration of the thromboxane synthase inhibitor ozagrel. With no diabetes-induced alterations in perfusion pressure, the decrease in flow was most likely due to localized arteriolar constriction. The changes in diameter and flow were not equal in all arterioles; instead, the constriction occurred preferentially in arterioles that were more closely paired with venules.

Venular control of arteriolar tone has been described in the literature for approximately the past 15 years. In healthy tissue and in tissue experiencing an enhanced metabolic need, venules can signal arteriolar dilation (Hester & Hammer, 2002). However, in inflammatory conditions, venules can promote vasoconstriction, or attenuate endothelium-dependent dilation. For example, in the intestine of hypercholesterolemic mice, arteriolar dilation induced by bradykinin is inhibited by ~55% when taken as an overall average; however, the inhibition is ~65–70% in more closely venule-paired arterioles compared to only ~35% in more distantly venule-paired arterioles (Kim *et al.*, 2007). In the acute inflammatory response of ischemia-reperfusion (in rat skeletal muscle), only closely venule-paired arterioles constrict (Zamboni *et al.*, 1993), via a mechanism involving thromboxane (Mazolewski *et al.*, 1999). Local control of blood flow could potentially be more important in the eye than in other tissues due to the lack of autonomic innervation. Although arrangements of counter-current arterioles and venules provide substantial length and surface area for diffusive exchange, it should also be mentioned that such diffusion could occur at arterio-venular crossings, which in the retina can be enclosed in a sheath.

Hyperglycemia induces an increase in thromboxane synthesis (Ferroni *et al.*, 2004) that has been observed as an approximate doubling of plasma thromboxane B₂ following streptozotocin injection in rats (De La Cruz *et al.*, 1997). However, the fact that we observed venule-dependent differences in thromboxane-mediated constriction suggests that the concentrations of the vasoconstrictor may differ at the microvascular level. It is possible that thromboxane is being produced in the retinal tissue or microvascular bed, and the levels of the vasoconstrictor in the draining venules produces a concentration gradient from venule to arteriole (Figure 8) that is reflected by the gradual decrease in effect as the arterio-venular pairing angle increases (as seen in Figure 3 and Figure 5). Inhibition of thromboxane synthase in diabetic rats produces a similar venule-dependent pattern of effect (Figure 7), with the more closely paired arterioles experiencing the largest arteriolar dilations in response to the inhibitor ozagrel.

Not all studies using animal models of diabetes have reported a decrease in retinal blood flow, with some of the variability possibly due to differences in species, flow measurement techniques, and duration of diabetes. Flow measured with the hydrogen clearance method was found to be elevated in a STZ-induced diabetic rat model after 5–6 weeks duration of hyperglycemia (Cringle *et al.*, 1993). Also, retinal blood flow measured with microspheres was observed to increase in STZ-injected rats after six weeks of hyperglycemia (Tilton *et al.*, 1989). However, other studies demonstrate the reverse, that is, diabetes-induced decreases in retinal blood flow; in alloxan-injected dogs (5 months of hyperglycemia; flow measured with microspheres) (Small *et al.*, 1987), and in STZ-injected rats (one and three weeks of

hyperglycemia; flow measured with microspheres and with fluorescein angiography) (Bursell *et al.*, 1992; Clermont *et al.*, 1994; Granstam & Granstam, 1999). In our study, the magnitude of STZ-induced decreases in flow at three weeks of diabetes was similar irrespective of flow marker or diameter measurement technique.

Variability of changes in retinal blood flow in human diabetics is also observed, with the differences attributable to a bi-phasic time course and to the severity of disease (Bursell *et al.*, 1996; Clermont *et al.*, 1997). The early decrease in blood flow, which occurs before many of the clinical signs of diabetic retinopathy, could potentially lead to areas of ischemia and a subsequent increase in angiogenic and inflammatory mediators such as vascular endothelial growth factor. Therefore, additional studies into the molecules and mechanisms involved in the early decrease in retinal blood flow are warranted.

In summary, diabetes induced by streptozotocin in rats produces a decrease in retinal blood flow within three weeks of injection. The decrease in flow does not occur equally in all feeding arterioles, but is more severe in the arterioles that are more closely paired with draining venules, as has been observed in other animal models of inflammation. Finally, the venule-dependent arteriolar constriction appears to be mediated by thromboxane, inasmuch as the thromboxane synthase inhibitor ozagrel is able to reverse the localized constrictions.

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1.

(A) Low power (4x) intravital microscopic image of the rat retinal circulation near the optic disk. FITC-dextran has been injected into the rat and is filling the arterioles (a) before reaching the venules (v). One angle of 21° is indicated on the image. (B) Fluorescent streak in a retinal arteriole of a labeled RBC traveling 19.8 mm/s. (C) Image of a retinal arteriole filled with FITC-albumin, where the diameter of 80 μm is an average of three locations along the vessel length.

2.

Distribution of arteriole-venule pairing angles in the rat retina. Values of the angle represent the average between an arteriole and the two neighboring venules.

3.

Relationship between the arteriole-venule pairing angles and blood flow rate in the arterioles of the various groups as indicated in panels A–D. In the group of rats that had been diabetic for three weeks, a statistically significant correlation was found, due to a decrease in flow in the more closely paired arterioles.

4. Arteriolar diameters (A) and flow rate (B) in non-diabetic control rats, rats diabetic for 1 week, and rats diabetic for 3 weeks (before and after ozagrel treatment for the latter). For each group, the data are segregated by pairing angle into closely paired ($\leq 30.2^\circ$) and distantly paired ($>30.2^\circ$) arterioles. * $p<0.05$ and ** $p=0.01$ between the indicated groups.

5.

Relationship between the arteriole-venule pairing angles and blood flow rate in the arterioles of the various groups as indicated in panels A–D. In the group of rats that had been diabetic for three weeks, a statistically significant correlation was found, due to a decrease in flow in the more closely paired arterioles.

6. Arteriolar diameters (A) and flow rate (B) in non-diabetic control rats and rats diabetic for 3 weeks (before and after ozagrel treatment). The data are separated by pairing angle into closely paired ($\leq 30.2^\circ$) and distantly paired ($>30.2^\circ$) arterioles. ** $p < 0.01$ between the indicated groups.

7.

Relationship between the change in arteriolar blood flow rate induced by ozagrel and the arteriole-venule pairing angle. The flow rates in (A) were obtained using fluorescently labeled platelets, and the flow rates in (B) were obtained using fluorescently labeled red blood cells (RBCs).

8. Diagram showing the working hypothesis: an elevated level of vasoconstrictor in a draining venule generates a concentration gradient extending from the venule. This gradient would induce more constriction in an arteriole closer to the venule.

Table 1

Statistical data from the five groups of rats

	Group	# of rats	# of arterioles	Blood glucose level (mg/dl)	Body weight (g)
Protocol using platelets as flow markers	Control	8	46	133 ± 9	415 ± 15
	1-week STZ	8	39	473 ± 29	336 ± 13
	3-week STZ	8	43	550 ± 32	321 ± 8
Protocol using RBCs as flow markers	Control	7	40	110 ± 5	415 ± 5
	3-week STZ	7	43	562 ± 23	320 ± 8

Table 2

Average velocity of closely and distantly paired arterioles from the five groups of rats

	Group	Closely paired velocity (mm/s)	Distantly paired velocity (mm/s)
Protocol using platelets as flow markers	Control	21.92 ± 1.3	21.00 ± 1.5
	3-week STZ	21.02 ± 0.9	20.18 ± 1.5
	3-week STZ	21.70 ± 1.4	19.05 ± 0.9
	3-week STZ after ozagrel	21.39 ± 0.9	21.90 ± 0.6
Protocol using RBCs as flow markers	Control	16.08 ± 0.9	17.42 ± 1.5
	Control after ozagrel	15.88 ± 0.8	17.23 ± 1.3
	3-week STZ	16.67 ± 1.0	17.24 ± 0.6
	3-week STZ after ozagrel	17.10 ± 0.9	16.70 ± 0.6