

# Influence of Experimental Diabetes on the Microcirculation of Injured Peripheral Nerve

## Functional and Morphological Aspects

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**Regeneration of diabetic axons has delays in onset, rate, and maturation. It is possible that microangiopathy of vasa nervorum, the vascular supply of the peripheral nerve, may render an unfavorable local environment for nerve regeneration. We examined local nerve blood flow proximal and distal to sciatic nerve transection in rats with long-term (8 month) experimental streptozotocin diabetes using laser Doppler flowmetry and microelectrode hydrogen clearance polarography. We then correlated these findings, using in vivo perfusion of an India ink preparation, by outlining the lumens of microvessels from unfixed nerve sections. There were no differences in baseline nerve blood flow between diabetic and nondiabetic uninjured nerves, and vessel number, density, and area were unaltered. After transection, there were greater rises in blood flow in proximal stumps of nondiabetic nerves than in diabetic animals associated with a higher number, density, and caliber of epineurial vessels. Hyperemia also developed in distal stumps of nondiabetic nerves but did not develop in diabetic nerves. In these stumps, diabetic rats had reduced vessel numbers and smaller mean endoneurial vessel areas. Failed or delayed upregulation of nerve blood flow after peripheral nerve injury in diabetes may create a relatively ischemic regenerative microenvironment. *Diabetes* 51:2233–2240, 2002**

**P**atients with diabetes are susceptible to peripheral nerve injury from entrapment and other causes. Regeneration in peripheral nerves can be complicated by relative ischemia, as may occur in cases of nerve infarction (1). The intact peripheral nerve endoneurial vascular nutritive compartment is well supplied by extrinsic “feeding” vessels arising from its epineurial plexus. Ischemia after nerve injury may be “normally” averted by increasing endoneurial nerve blood flow to address the increased nutrient and oxygen con-

sumption of regenerating axons and cellular elements during repair. For example, rises in blood flow, or hyperemia, may be mediated by vasodilation of the extrinsic vasa nervorum (2) by neuropeptides (notably calcitonin gene-related peptide [CGRP], substance P, and vasoactive intestinal peptide [VIP]) and mast cell-derived histamine. Macrophage- and endothelial-derived nitric oxide (NO) also likely augments nerve blood flow at the injury site through vasodilation. At later stages of regeneration and repair, extensive neoangiogenesis may maintain a persistent hyperemic state (3). Revascularization from angiogenesis into a peripheral nerve graft often precedes regenerating axons (4), with fibers near blood vessels having the fastest regeneration rates (5).

Regenerative success in diabetes is impaired as a result of defects in the onset of regeneration (6), in elongation rate of axonal sprouts (7), and subsequently in nerve fiber maturation of experimental (8,9) and human (10) diabetes. The regenerative program could be compromised by a failed upregulation of neurotrophins (11), defective transport of cytoskeletal elements (12,13), or inadequate microvascular support. Diabetic nerve microvessels exhibit basement membrane thickening (as a result of accumulation of type IV collagen), endothelial cell hyperplasia (14), as well as intimal and smooth muscle cell proliferation (15) that all increase with the severity of diabetic polyneuropathy (16) and may impair vasoreactivity. Diabetes has been noted to impair vascular hyperemic responses to agents such as heat and injury (17,18), to surgical exposure of nerve (19), and to epineurial application of capsaicin (20). A diminished supply of vasoactive peptides such as CGRP, substance P, and VIP in intact diabetic nerve and dorsal root ganglion (21), along with excessive scavenging of NO (22), results in blunted vasodilation and hyperemia. Increased intervascular distance in diabetic rats after nerve injury may further predispose the regenerating nerve to ischemia (23).

In this work, we explored the response of vasa nervorum to sciatic nerve injury in rats with chronic experimental diabetes. We examined physiological measures of nerve blood flow by two approaches—laser Doppler flowmetry (LDF) and microelectrode hydrogen clearance (HC) polarography—to address selectively flow dominated by the epineurial plexus and flow within the endoneurial vascular compartment, respectively. These measures were correlated with quantitative morphometric studies of nerve microvessels using in vivo perfusion of an India ink preparation and study of luminal profiles from unfixed

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ANOVA, analysis of variance; CGRP, calcitonin gene-related peptide; HC, hydrogen clearance; LDF, laser Doppler flowmetry; MAP, mean arterial pressure; MR, microvascular resistance; NO, nitric oxide; RBC, red blood cell; STZ, streptozotocin; VEGF, vascular endothelial growth factor; VIP, vasoactive intestinal peptide.

TABLE 1  
Physical characteristics and electrophysiological properties of rats

Property	Weight (g)	Blood glucose (mmol/l)	Motor CV (m/s)	Sensory CV (m/s)
Diabetic	309.1 ± 11.5	31.2 ± 1.2	36.3 ± 1.4	44.9 ± 1.7
Nondiabetic	648.9 ± 16.7	7.9 ± 0.3	54.4 ± 1.4	58.5 ± 1.5
	*	*	*	*

Data are means ± SE (*n* = 48/group). Diabetic and nondiabetic rats were compared on each parameter using a one-way ANOVA with Bonferroni post hoc Student's *t* tests. Motor CV, motor conduction velocity in sciatic-tibial nerve; Sensory CV, caudal sensory conduction velocity. \**P* ≤ 0.001 (*N* = 96).

nerve sections. Assessment of two time points (48 h and 2 weeks) after injury allowed us to examine two distinct but sequential and related stages of injury-induced hyperemia. The findings suggest that there are substantial alterations in how diabetic vasa nervorum respond to injury and how they may support regeneration.

RESEARCH DESIGN AND METHODS

**Animals.** Procedures were approved by the Animal Care Committee of the University of Calgary and carried out in accordance with the "Guide to the Care and Use of Experimental Animals" by the Canadian Council on Animal Care. Adult male Sprague-Dawley rats (300–350 g, *n* = 96) were housed two per plastic cage on a 12–12 h light-dark cycle with food and water available ad libitum. Rats were assigned randomly to either a diabetic or control group and equally to physiological or morphometric studies. Diabetes was initiated by three consecutive injections of streptozotocin (STZ; Zanosar [50 mg/ml]; UpJohn) in citrate buffer (pH 4.8) during the fasting state (one intraperitoneal injection per day; day 1, 100 mg/kg; day 2, 83 mg/kg; day 3, 66 mg/kg). Control animals received equivalent doses of the citrate buffer solution. Hyperglycemia was verified 1 week after the final STZ injection by sampling from a tail vein. A fasting whole-blood glucose ≥16 mmol/l (normal 5–8 mmol/l) was the criterion for experimental diabetes. Whole-blood glucose tests were carried out using a One Touch FastTake (Lifescan Canada; Burnaby, BC), whereas plasma glucose was measured with a glucose oxidase method (Ektachem DT-II Analyzer; Eastman Kodak, Rochester, NY).

**Preparation.** Diabetic and control rats 8 months after STZ or citrate injection were anesthetized with sodium pentobarbital (65 mg/kg, intraperitoneal). Left

sciatic nerves were exposed by blunt dissection, using aseptic techniques, and transected at mid-thigh level using a pair of microscissors. A gap of 5 mm was left between proximal and distal stumps. Once injuries were created, muscle and skin were resutured in layers and the animals were allowed to recover. Sham animals underwent nerve exposure alone without nerve injury.

**Electrophysiology.** Electrophysiological recordings were made under anesthesia (Nicolet Viking I EMG machine; Nicolet, Madison, WI) as reported elsewhere (24). Motor conduction in sciatic tibial fibers was assessed by stimulating at the sciatic notch and knee while recording the M-wave (compound muscle action potential) from the tibial-innervated dorsal interosseus foot muscles. Caudal sensory conduction was measured in the tail by stimulating distally and recording compound nerve action potentials proximally. Recordings were carried out immediately before nerve transection or exposure. All stimulating and recording used platinum subdermal needle electrodes (Grass Instruments; Astro-Med, West Warwick, RI) with near nerve temperature kept constant at 37°C using a subdermal thermistor connected to a temperature controller and heating lamp.

**Nerve blood flow.** LDF and HC polarography were used to measure nerve blood flow in experimental diabetes. With the rats under anesthesia, the trachea was cannulated for artificial ventilation (model 683, Harvard rodent respirator; South Natick, MA) and a polyethylene cannula (PE50) was inserted into the left common carotid artery. The arterial line allowed mean arterial pressure (MAP) to be sampled continuously and arterial blood gases (Radiometer ABL 330; Copenhagen, Denmark) to be monitored periodically (acceptable levels were Po<sub>2</sub> ≥90 mmHg; Pco<sub>2</sub> 35–45 mmHg). The level of anesthesia was maintained by recording MAP, and supplementary pentobarbital (20 mg/kg) was administered every 2 h, as needed.

**LDF.** LDF was measured before HC polarography. Exposed tissues surrounding the sciatic nerve (skin and muscle edges) were covered with a pool of mineral oil maintained at 37°C using a thermistor probe connected to a control

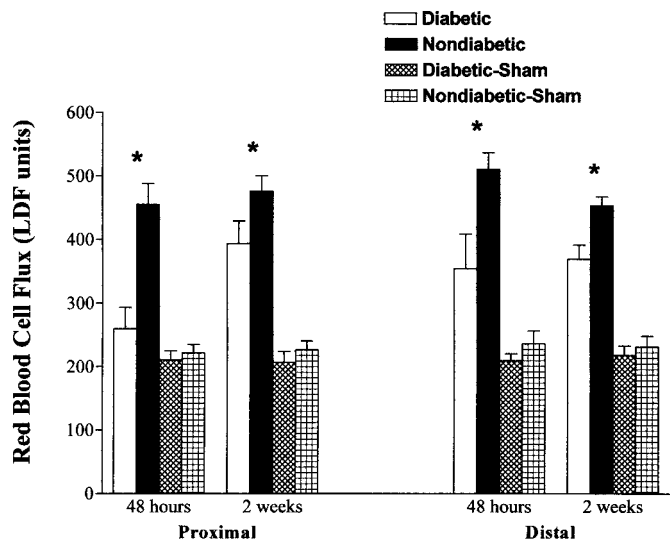


FIG. 1. Mean erythrocyte flux from intact sham-exposed nerves, as well as proximal (5 mm from transection site) and distal (7–10 mm from transection site) stumps of diabetic and nondiabetic rats after sciatic nerve transection. Note that diabetes was initiated 8 months before injury. Shams consist of animals in which the nerve was merely exposed but not transected. Laser Doppler recordings were averaged in each rat from 10 independent measurements. Data are presented as means ± SE. Groups were compared at each time point using a one-way ANOVA with Bonferroni post hoc Student's *t* tests. \**P* ≤ 0.05 (*n* = 6–8).

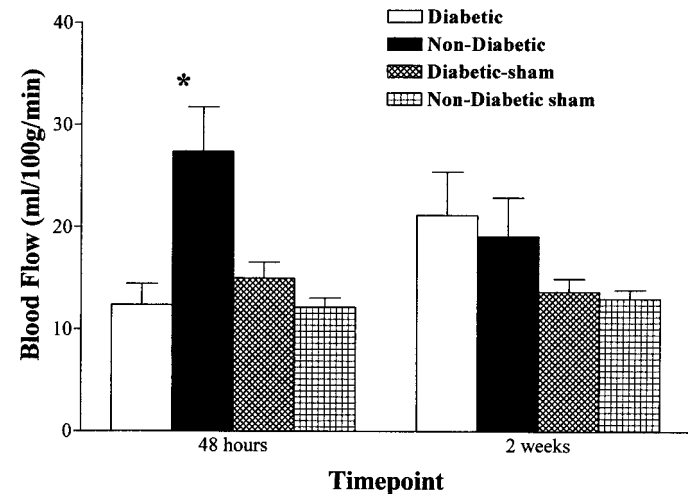
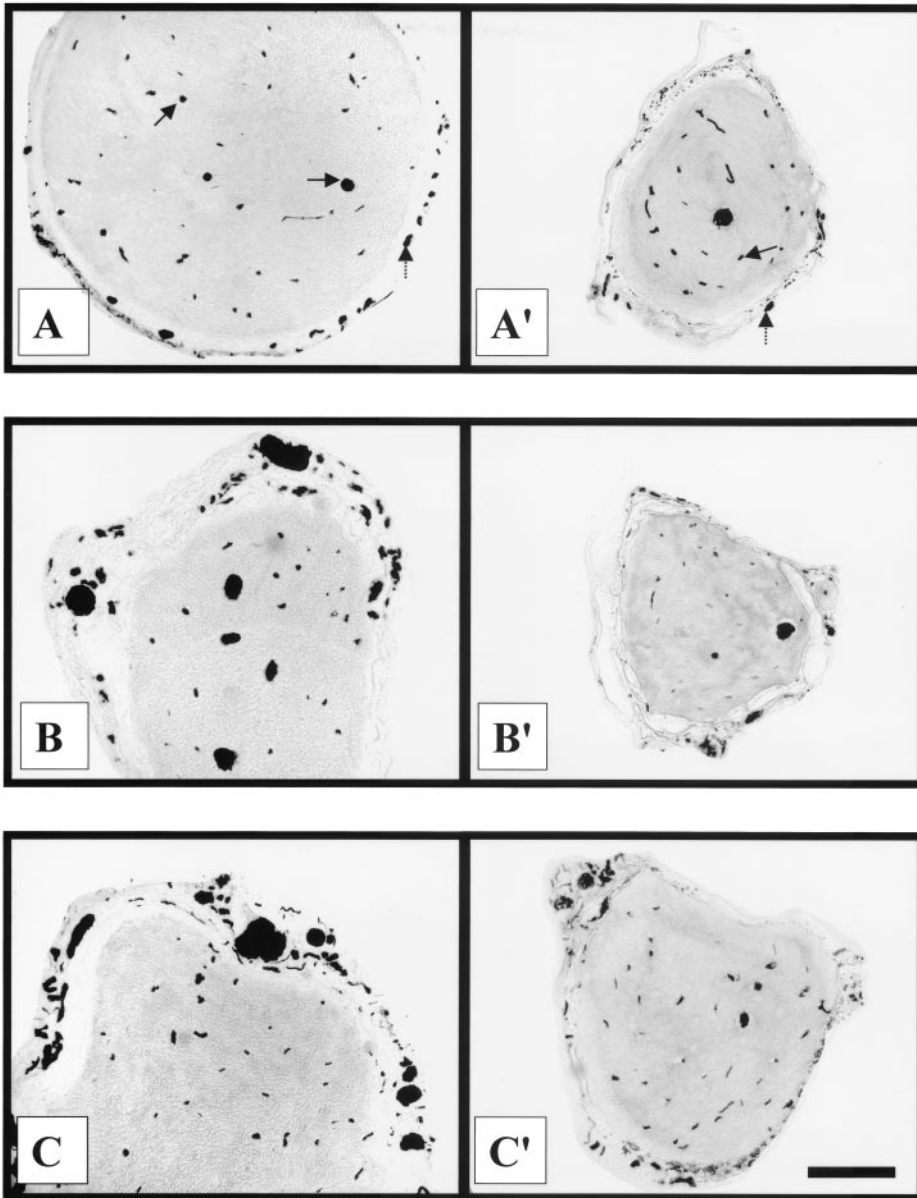


FIG. 2. Quantitative HC polarography measurements of endoneurial perfusion in distal stumps (7–10 mm from transection site) at 48 h and 2 weeks after sciatic nerve transection. Note that diabetes was initiated 8 months before injury. Shams consist of animals in which the nerve was merely exposed but not transected. Six to eight animals were used per group for the blood flow experiments. Three washout curves were taken per animal and averaged for presentation. Data represent means ± SE. Groups at both time points were compared using a one-way ANOVA with Bonferroni post hoc Student's *t* tests. \**P* ≤ 0.05. (*n* = 6–8).



**FIG. 3.** Sciatic nerves 10 mm distal to nerve injury in nondiabetic (*A*, *B*, and *C*) and diabetic rats (*A'*, *B'*, and *C'*) at two time points after injury (*A* and *A'*, uninjured sham nerves; *B* and *B'*, 48 h; *C* and *C'*, 2 weeks). Note the overall small size of the diabetic nerve and smaller vessel caliber compared with nondiabetic rats after injury. Endoneurial (solid arrows) and epineurial (dotted arrows) vessels are shown. Bar = 1 mm;  $n = 5-6$ .

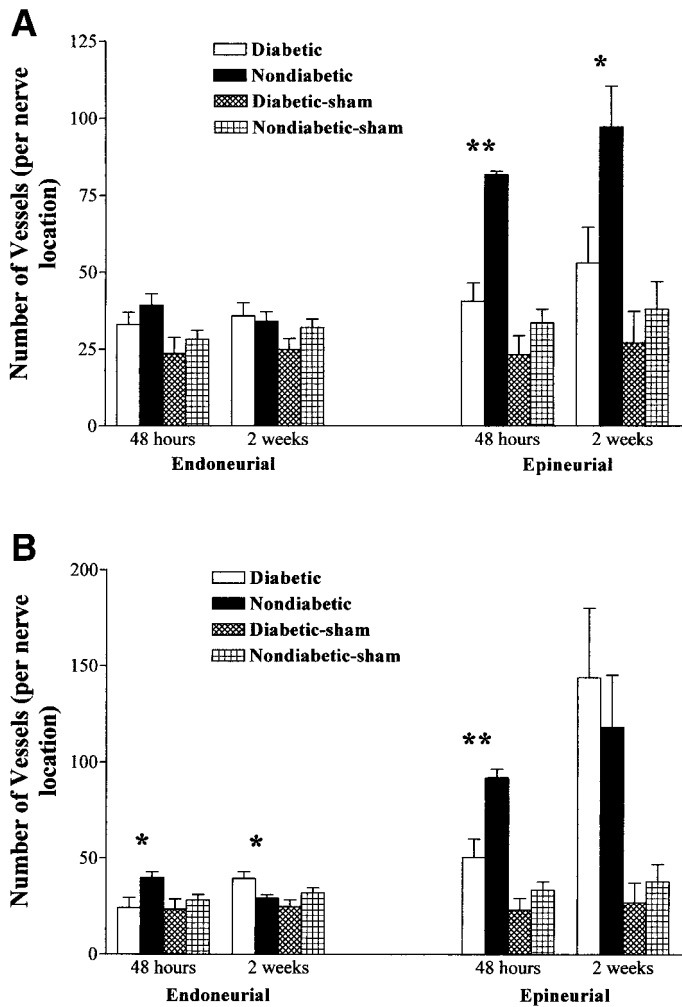
feedback unit (T-CAT 1; Bailey, Saddle Brook, NJ). A laser Doppler probe (1 mm diameter; 250  $\mu\text{m}$  fiber separation attached to a Periflux monitor, Perimed, Sweden) was positioned approximately perpendicular to the nerve (with a micromanipulator) in the liquid pool to achieve maximum signal-to-noise ratio of red blood cell (RBC) flux. Ambient lighting was turned off, and 10 separate measurements were taken in a 1- to 2-mm segment of nerve, avoiding surface vessels. The mean of the 10 measurements was used to calculate mean RBC flux (arbitrary units) for each rat.

**HC polarography.** After anesthesia, animals were paralyzed with tubocurarine (1.5 mg/kg) and ventilated. Supplementary administration of pentobarbital (20 mg/kg) and tubocurarine (0.8 mg/kg) were given two hourly, as needed. The HC method was carried out as previously described (25,26). A small opening was made in the epineurium of the distal stump of the sciatic nerve (7–10 mm distal to nerve section) and bathed in mineral oil maintained at  $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$  with the aid of a thermistor and temperature controller (Sensortek; Clifton, NJ). A glass-insulated platinum microelectrode ( $\sim 3-5 \mu\text{m}$  diameter) was inserted through the epineurial window into the endoneurium with a micromanipulator. A reference electrode (2 mol/l KCl) was also inserted into the subcutaneous tissue of the abdominal wall. Both electrodes were connected to a microsensor (Diamond General, Ann Arbor, MI) with output sent to a polygraph recorder.  $\text{H}_2$  ( $\sim 10\%$ ) was added to the inspiratory gas mixture and  $\text{O}_2$  and  $\text{N}_2$  concentrations were adjusted to maintain a  $\text{Po}_2$  of  $\geq 90$  mmHg. When the  $\text{H}_2$  current recorded by the electrode stabilized (20–30 min), indicating saturation of arterial blood, the  $\text{H}_2$  supply was shut off and  $\text{N}_2$  concentration was adjusted once again. The  $\text{H}_2$  clearance curves were

recorded until the current reached baseline. Three consecutive washout curves were obtained with the first discarded, and endoneurial blood flow was calculated from an average of the two subsequent curves.

Clearance curves were fitted to mono- or biexponential curves using the least squares method for optimizing goodness of fit:  $y = a \cdot \exp(b \cdot x) + c \cdot \exp(d \cdot x)$ , where  $y$  = hydrogen current (arbitrary units),  $x$  = time,  $b$  and  $d$  = fast and slow washout components, respectively, with  $a$  and  $c$  = curve weighting constants (27). Endoneurial blood flow was calculated from the slow washout component as  $-d \cdot 100$ . Endoneurial microvascular resistance (MR) was calculated as  $\text{MAP}/\text{flow}$ .

**Microvessel morphometry.** The procedure was a modification of that by Bray et al. (28). At 48 h and 2 weeks postinjury, experimental and sham-exposed animals (five to six per group) underwent catheterization of the distal descending aorta through the right femoral artery (PE 50). Rats were perfused with 40 ml of a solution of 4% gelatin and 5% mannitol in 100% India ink maintained at  $37^{\circ}\text{C}$  (2 ml/min). Rats were then killed, and their carcasses were maintained at  $-20^{\circ}\text{C}$  for 1 h. Nerve specimens (5 mm proximal and 10 mm distal to nerve transection and sham-exposed intact sciatic nerves) were removed, fast-frozen in OCT (Optimal Cutting Temperature, Miles Laboratories) compound, and sectioned at 20  $\mu\text{m}$  in a cryostat (Microm, Walldorf, Germany). For each nerve specimen, four to six sections underwent quantitative analysis under light microscope (Axioplan; Zeiss, North York, ON, Canada) and a mean value/rat was calculated. Images were captured using a digital camera (AxioCam; Zeiss), and vessel parameters were measured using an image analysis program (Axiovision 2.05; Zeiss). Nerve (total; epineurial



**FIG. 4.** Mean number of endoneurial and epineurial vessels per nerve in stumps at 48 h and 2 weeks after sciatic nerve transection. **A:** Proximal stump (5 mm from transection site). **B:** Distal stump (10 mm from transection site). Shams consisted of nerve exposure but no injury. Data are presented as means  $\pm$  SE. Five to six animals were used per group in the experiment. Diabetic and nondiabetic rats were compared at each time point using a one-way ANOVA with Bonferroni post hoc Student's *t* tests. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$  ( $n = 5-6$ ).

and endoneurial) area, vessel number, vessel density, vessel luminal area, and total vascular area were assessed for each section. All counting was performed with the microscopist masked to the identity of the experimental group.

**Statistical analysis.** All data are presented as means  $\pm$  SE. When multiple recordings (as in LDF and HC) or sections were taken, a mean value/rat was calculated before analyzing results for the entire group. Data were analyzed by a one-way analysis of variance (ANOVA) with appropriate Bonferroni corrected post hoc Student's *t* test comparisons. Statistical significance was set at  $\alpha = 0.05$ .

## RESULTS

**Animals and diabetes.** Diabetic animals demonstrated polydipsia, polyuria, and a decrease in activity associated with hyperglycemia beginning 1 week after STZ injection. Diabetic animals also gained less weight than their nondiabetic counterparts (Table 1).

**Electrophysiology.** After 8 months of diabetes, baseline motor conduction velocity and M-wave amplitudes in the sciatic-tibial nerve territory were measured before nerve transection. Caudal sensory conduction and sensory nerve action potentials were also recorded at this time. There

was slowing of motor and sensory conduction velocity in the diabetic rats (Table 1) without changes in amplitudes (data not shown).

**Blood flow.** During blood flow procedures, recordings of MAP,  $P_{O_2}$ , and  $P_{CO_2}$  were made to ensure physiological stability. No differences were noted between groups.

**Intact sham-exposed nerves.** In the uninjured sham nerves, there was no difference in RBC flux recorded by LDF or blood flow measured by HC between diabetic and nondiabetic animals (Figs. 1 and 2). Arterial  $P_{O_2}$ ,  $P_{CO_2}$ , and MAP levels were also comparable between groups (diabetic  $186.0 \pm 23.3$ ,  $39.0 \pm 1.3$ ,  $99 \pm 2$  mmHg; nondiabetic  $194.1 \pm 20.0$ ,  $42.3 \pm 1.2$ ,  $112 \pm 9$  mmHg). Similarly, endoneurial MR, calculated from HC, did not differ between diabetic ( $9.1 \pm 2.4$  mmHg  $\cdot$  ml $^{-1}$  100 g  $\cdot$  min) and nondiabetic rats ( $8.4 \pm 0.6$  mmHg  $\cdot$  ml $^{-1}$  100 g  $\cdot$  min).

**Injured nerve physiological parameters.** Arterial  $P_{O_2}$  (torr),  $P_{CO_2}$  (torr), and MAP were similar among groups at 48 h (diabetic  $202.3 \pm 14.2$ ,  $39.9 \pm 1.0$ ,  $120 \pm 7$  mmHg; nondiabetic  $204.0 \pm 20.7$ ,  $40.0 \pm 1.1$ ,  $109 \pm 6$  mmHg) and 2 weeks after transection (diabetic  $229.7 \pm 22.2$ ,  $38.5 \pm 0.8$ ,  $117 \pm 6$  mmHg; nondiabetic  $202.8 \pm 18.9$ ,  $39.6 \pm 0.8$ ,  $120 \pm 5$  mmHg).

**RBC flux (LDF).** After injury, RBC flux was elevated above sham levels in both proximal and distal stumps of all animals. This rise, however, was attenuated in diabetic stumps, and they did not reach RBC flux levels observed in nondiabetic nerves at either 48 h or 2 weeks posttransection ( $P \leq 0.05$ ; Fig. 1).

**Endoneurial blood flow (HC).** Blood flow increased above sham levels in the distal stump at 48 h posttransection in nondiabetic animals. This hyperemia lessened in the stumps by 2 weeks postinjury but still remained above levels in sham-exposed uninjured nerves. Diabetic distal stumps failed to develop any hyperemia at 48 h and remained at values observed in sham-exposed uninjured nerves (compared with nondiabetic,  $P \leq 0.05$ ). However, diabetic flow values recovered 2 weeks postinjury to match nondiabetic levels (Fig. 2). Endoneurial MR was significantly higher in diabetic animals 48 h after transection (diabetic  $13.2 \pm 2.6$  mmHg  $\cdot$  ml $^{-1}$  100 g  $\cdot$  min; nondiabetic  $4.8 \pm 1.0$  mmHg  $\cdot$  ml $^{-1}$  100 g  $\cdot$  min;  $P \leq 0.01$ ), whereas nondiabetic MR decreased below sham exposed levels (see INTACT SHAM-EXPOSED NERVES). However, diabetic ( $7.0 \pm 1.2$  mmHg  $\cdot$  ml $^{-1}$  100 g  $\cdot$  min) and nondiabetic ( $9.2 \pm 2.0$  mmHg  $\cdot$  ml $^{-1}$  100 g  $\cdot$  min) endoneurial MR levels were similar 2 weeks posttransection.

### Microvessel quantification

**Intact sham nerves.** Nondiabetic sciatic nerves were slightly larger than those of diabetic rats, largely accounted for by an increased endoneurial compartment ( $P < 0.05$ ; Table 2). Total number of vessels (representing a sum of endoneurial and epineurial/perineurial vessels) showed a trend toward higher numbers in nondiabetic rats (diabetic  $46 \pm 15$ ; nondiabetic  $74 \pm 14$ ), but it was not significant. Vessel densities and vessel areas were also similar between the two groups for both epineurial/perineurial and endoneurial vessels.

**Injured proximal stumps.** Proximal stumps of both groups enlarged in area to values greater than those of sham-exposed intact nerves, but nondiabetic stumps continued to remain larger, again because of a greater endo-

TABLE 2  
Morphometric properties of proximal and distal stumps

Time after injury property	Sham		48 h		2 weeks	
	Diabetic	Nondiabetic	Diabetic	Nondiabetic	Diabetic	Nondiabetic
<b>Proximal stump</b>						
Area (mm <sup>2</sup> )						
Total nerve	0.87 ± 0.04	1.14 ± 0.03*	0.88 ± 0.04	1.19 ± 0.11*	0.96 ± 0.12	1.47 ± 0.23*
Epi	0.24 ± 0.03	0.29 ± 0.05	0.28 ± 0.01	0.34 ± 0.06	0.32 ± 0.07	0.56 ± 0.0
Endo	0.57 ± 0.02	0.73 ± 0.13*	0.59 ± 0.03	0.86 ± 0.05*	0.66 ± 0.06	0.95 ± 0.10*
Vessel density (#/mm <sup>2</sup> )						
Total Nerve	54 ± 18	51 ± 6	107 ± 24	108 ± 7	94 ± 7	93 ± 8
Epi	140 ± 41	169 ± 35	141 ± 20	325 ± 55*	168 ± 16	245 ± 47
Endo	42 ± 8	47 ± 6	57 ± 9	47 ± 2	57 ± 7	35 ± 3*
<b>Distal stump</b>						
Area (mm <sup>2</sup> )						
Total nerve	0.78 ± 0.02	1.04 ± 0.09*	1.03 ± 0.13	1.14 ± 0.06	1.56 ± 0.32	1.78 ± 0.14
Epi	0.30 ± 0.03	0.27 ± 0.05	0.41 ± 0.08	0.39 ± 0.03	0.58 ± 0.11	0.68 ± 0.09
Endo	0.55 ± 0.04	0.78 ± 0.10	0.62 ± 0.09	0.77 ± 0.09	0.71 ± 0.09	1.09 ± 0.07*
Vessel density (#/mm <sup>2</sup> )						
Total nerve	52 ± 14	50 ± 11	75 ± 13	119 ± 12*	135 ± 18	86 ± 12*
Epi	137 ± 41	145 ± 31	135 ± 37	231 ± 12*	227 ± 49	170 ± 35
Endo	35 ± 10	43 ± 8	51 ± 10	54 ± 7	59 ± 10	30 ± 6*

Data are means ± SE ( $n = 5-6$ ) Sham consisted of animals that received nerve exposure but no transection. Diabetic and nondiabetic rats were compared on each parameter using a one-way ANOVA with Bonferroni post hoc Student's  $t$  tests. Epi, epineurial; Endo, endoneurial, Lu, luminal. \* $P \leq 0.05$  diabetic versus nondiabetic.

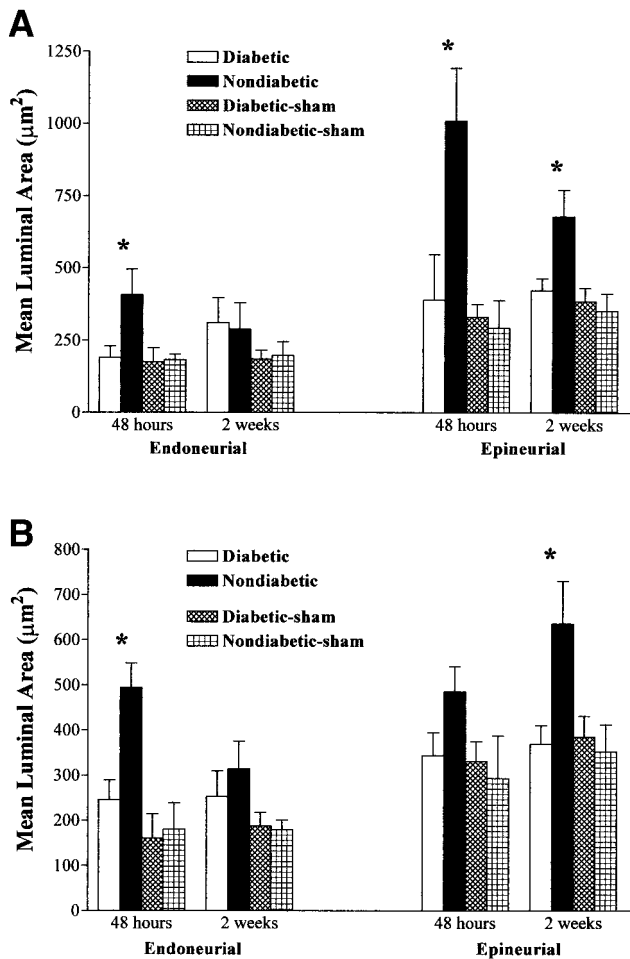
neurial area ( $P \leq 0.05$ ). There was an increase in total vessel number per nerve for both groups at 48 h (diabetic  $72 \pm 6$ ; nondiabetic  $120 \pm 5$ ;  $P < 0.01$ ) and 2 weeks after transection (diabetic  $89 \pm 10$ ; nondiabetic  $133 \pm 13$ ;  $P < 0.05$ ) that largely reflected an elevation in numbers of epineurial and perineurial vessels, most notably in nondiabetic compared with diabetic rats (48 h  $P \leq 0.01$ ; 2 weeks  $P \leq 0.05$ ; Fig. 4A). At 48 h postinjury, epineurial vessel densities increased and nondiabetic rats had higher densities than diabetic rats ( $P \leq 0.05$ ); the latter were not different than levels in sham-exposed but intact nerves (Table 2). Diabetic rats had a higher endoneurial vessel density than nondiabetic rats at 2 weeks ( $P \leq 0.05$ ). Nondiabetic mean vessel luminal areas were enlarged to a greater extent than diabetic vessels at 48 h (epineurial and endoneurial  $P \leq 0.05$ ) and 2 weeks (epineurial  $P \leq 0.05$ ; Fig. 5A). Total vascular luminal area per nerve was also significantly higher in nondiabetic endoneurial (48 h  $P \leq 0.05$ ) and epineurial (48 h and 2 weeks  $P \leq 0.01$ ) compartments (Fig. 6A).

**Injured distal stumps.** Whole-nerve transverse area was elevated in distal stumps, attributable to an increase in both epineurial and endoneurial areas. Nondiabetic endoneurial areas were larger than those of diabetic rats (2 weeks  $P \leq 0.05$ ). Total vascular luminal area was elevated in endoneurial ( $P \leq 0.01$ ) and epineurial ( $P \leq 0.05$ ) sections at 48 h in nondiabetic animals (Fig. 6B). There was an increase in the total number of vessels per nerve after injury for both nondiabetic and diabetic groups at 48 h (diabetic  $75 \pm 8$ ; nondiabetic  $132 \pm 3$ ;  $P < 0.01$  between groups) and 2 weeks (diabetic  $178 \pm 49$ ; nondiabetic  $151 \pm 30$ ) posttransection. Despite an increase in epineurial vessel number (but below nondiabetic numbers,  $P \leq 0.01$ ), diabetic rats did not demonstrate an increase in endoneurial vessel number at 48 h postinjury ( $P \leq 0.05$ ), unlike nondiabetic rats. Diabetic endoneurial vessel numbers did increase later at the 2-week time point

once nondiabetic vessel numbers were already declining ( $P \leq 0.05$ ; Fig. 4B). The increase in endoneurial vessel number resulted in diabetic rats actually having a higher endoneurial (and total) vessel density at the 2-week time point ( $P \leq 0.05$ ; Table 2). After injury, nondiabetic vessels (epineurial and endoneurial) had larger mean luminal areas at 48 h, but diabetic vessels did not dilate, especially in the endoneurial compartment ( $P \leq 0.05$ ; Fig. 5B). In fact, diabetic vessel mean luminal area by 2 weeks remained smaller than that of nondiabetic rats (total  $P \leq 0.05$ ; epineurial  $P \leq 0.05$ ; Fig. 5B).

## DISCUSSION

The major findings of the present work were as follows: 1) long-standing experimental diabetes of rats had slowed motor and sensory conduction velocity in uninjured nerves, resembling human diabetic neuropathy (although differing from human disease in the absence of overt axon loss); 2) nerve blood flow was not compromised in uninjured diabetic nerves, a finding matching previous results from this laboratory; 3) after nerve transection, there was attenuated hyperemia of the epineurial plexus in diabetic proximal stumps, as measured by LDF, that correlated with a blunted rise in vessel numbers (total and epineurial) and a smaller mean luminal area (total and epineurial) at 48 h and 2 weeks postinjury; 4) similar attenuation of epineurial plexus hyperemia was observed in diabetic distal stumps associated with a blunted rise in vessel number and density (total and epineurial) at 48 h with a smaller mean luminal area (total and epineurial) at 2 weeks; and 5) injury-related hyperemia was absent in the endoneurial vasculature of diabetic distal stumps at 48 h by HC, corresponding to reduced vessel numbers (total and endoneurial) and mean luminal areas (total and endoneurial) compared with injured nondiabetic nerves but with subsequent recovery of hyperemia at 2 weeks, asso-

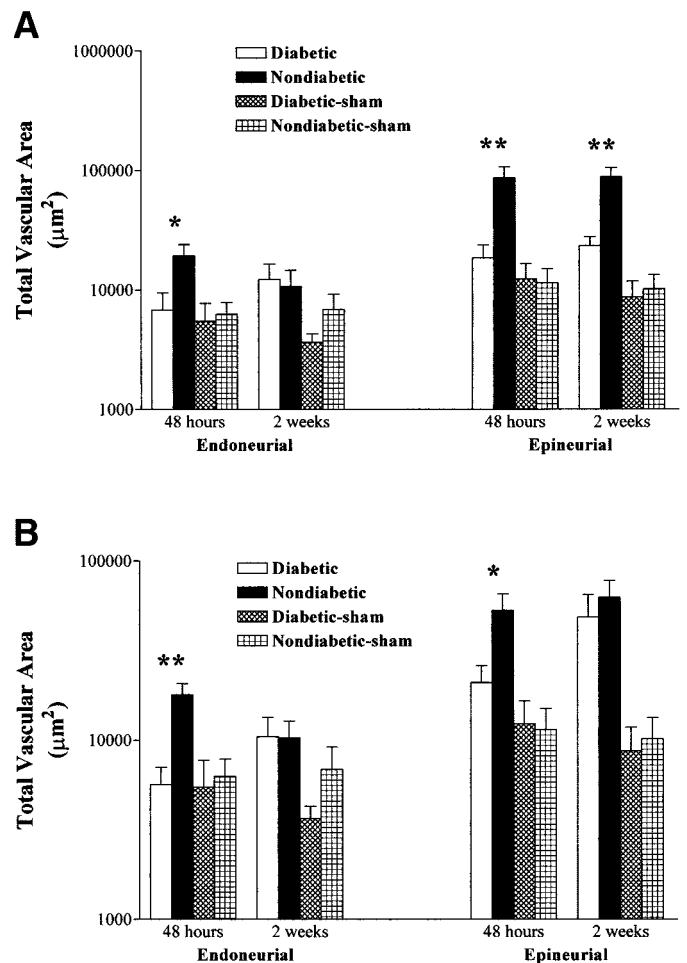


**FIG. 5.** Mean vessel area of endoneurial and epineurial vessels in nerve stumps at 48 h and 2 weeks after sciatic nerve transection. **A:** Proximal stump (5 mm from transection site). **B:** Distal stump (10 mm from transection site). Shams consisted of nerve exposure but no injury. Data are presented as means  $\pm$  SE. Five to six animals were used per group in the experiment. Diabetic and nondiabetic rats were compared at each time point using a one-way ANOVA with Bonferroni post hoc Student's *t* tests. \* $P \leq 0.05$ . ( $n = 5-6$ ).

ciated with an increased number and density of endoneurial vessels.

Intact or increased blood flow of uninjured nerves in rats with varying durations of experimental diabetes has been consistently found in our laboratory (24,26), as well as in others (29,30). In the present work, near-normal blood flow correlated with preserved microvessel caliber and density in diabetic animals. This finding contrasts with our previous observation of angiogenesis in earlier (12 week) experimental diabetic nerves (31). Two physiological measures of blood flow were used in the present set of experiments to assess different subcompartments of the peripheral nerve. The LDF signal is largely influenced by the epineurial plexus, whereas HC was used to address selectively endoneurial blood flow.

Nerve injury likely requires heightened metabolic nutrients and oxygen to support the outgrowth of neurites and the activities of the cell populations supporting regeneration. For instance, Foster (32) initially noted high metabolic demands of Schwann cells, but this was amplified by a 13-fold increase in their population during axonal degeneration (33). Neurogenic inflammation results in enhanced



**FIG. 6.** Total endoneurial and epineurial vascular luminal area in nerve stumps at 48 h and 2 weeks after sciatic nerve transection. **A:** Proximal stump (5 mm from transection site). **B:** Distal stump (10 mm from transection site). Shams consisted of nerve exposure but no injury. Means  $\pm$  SE are presented on a  $\log_{10}$  scale for clarity. Five to six animals per group were used in the experiment. Diabetic and nondiabetic rats were compared at both time points using a one-way ANOVA with Bonferroni post hoc Student's *t* tests. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$  ( $n = 5-6$ ).

local blood flow, leading to an increased resting oxygen consumption of the nerve (33), and subsequent recruitment of immunomodulatory cells. An immediate (transient) increase in blood flow may result from a release of vasoactive substances in response to stretching of vessels, analogous to shearing induced release of NO by endothelial cells (34). In nerve, such actions may in particular be mediated by NO (35).

Beyond such immediate effects, nerve injury creates a two-phased response of the microcirculation in response to tissue degeneration and regeneration, resulting in enhanced blood flow. The early phase (peaking within the first week) consists of an increase in vessel caliber but not number. The changes in caliber reflect vasodilation mediated by the synthesis, release, and transport of neuropeptides such as CGRP, substance P, neurokinin A, and neurokinin B from perivascular nerve terminals or injured axons themselves, histamine from mast cells, and NO from axons or other cells (2,35). The peak action of the neuropeptides, observed by antagonism of  $\alpha$ -CGRP receptors, was 48 h after injury (26). Diabetic rats in our experiment

demonstrated an early blunted hyperemic response that may prove detrimental for early as well as later axonal sprouting, regeneration, and maturation in the model. Hyperemia is noted to be blunted in patients with diabetic foot ulceration (36). Zochodne and Ho (20) observed impaired hyperemia in response to capsaicin-induced peripheral nerve trunk neurogenic inflammation in diabetic nerve. By not upregulating blood flow, the injured diabetic nerve may prove to be relatively ischemic at a time when enhanced delivery of metabolic substrate is required. As a result, Schwann cells may not rapidly proliferate, aid in clearance of cellular debris, or form bands of Bungner to guide regenerating axons. Axonal clearance mediated by macrophages may also be compromised. A limited delivery of substrate may explain the delayed onset of regeneration and rate that has been observed in previous reports of experimental diabetes (6,10). A recovery of endoneurial blood flow by 2 weeks postinjury may allow regeneration to resume, but there may be delayed maturation of new fibers as compared to nondiabetic rats (9).

A reduction in CGRP and substance P has been noted in nerve terminals of diabetic animals (21) and may contribute to the lack of a peripheral nerve injury-induced hyperemic response observed early after injury. Substance P, CGRP, and histamine mediate dilation through intact endothelium by releasing NO (37), a mechanism impaired by diabetes-induced defects at the endothelial level (38). Free radicals that are normally released at the injury site from several sources, including activated neutrophils, intracellular xanthine, and mitochondria (39), combined with elevated levels of advanced glycosylation end products in the diabetic condition, may also quench NO (22). Furthermore, the percentage of vessels innervated by peptidergic perivascular fibers that supply vasoactive molecules may be decreased and there may be a reduced capacity to store and maintain neurotransmitters in those that remain (40). Diabetic microvessels may further be resistant to dilation through a combination of basement membrane thickening and glycation of collagen (41). Additional nonspecific alterations in the smooth muscle contractile apparatus (38) may create "rigid" arterioles.

The second phase response of the microcirculation consists of an increase in the number of vessels and their density, thus maintaining earlier rises in blood flow (42). The recovery of endoneurial blood flow in diabetic distal stumps at 2 weeks postinjury may similarly reflect higher number and density of vessels at this time point. Vascular endothelial growth factor (VEGF) is an attractive candidate mediating angiogenesis after an injury (43) but is also noted to be a potent mitogen for Schwann cells (44). VEGF is also thought to exert a survival function on neurons and satellite cells (44,45). A delayed rise in VEGF in the diabetic condition, marked by a blunted early increase in total vessel density, may limit the number of neurons that actually survive axotomy and are able to mount a regenerative response.

Hypoxia is noted to enhance expression of VEGF in tissues, whereas the presence of elevated VEGF levels generating new vessels may prevent ischemia. Because diabetic DRG neurons and axons express VEGF protein (46), it is likely that in the intact state hypoxia is present, as has been demonstrated using direct measurements (24).

However, Sone et al. (47) and Williams et al. (48) demonstrated that exposure to high glucose concentrations, independent of changes in oxygen tension, also increase VEGF expression in cultured retinal and smooth muscle cells, respectively. Recently, intramuscular VEGF gene transfer was applied to experimental diabetes (49). Furthermore, recombinant human VEGF administration increases blood flow in granulation tissue, whereas neutralizing antibodies to VEGF can reverse glucose-induced hyperemia in this same system (50). As a result, greater numbers of diabetic vessels late in the regenerative process may reflect hypoxia-mediated angiogenesis through VEGF, at a time when blood flow is simultaneously normalized.

Our results indicate that there is an altered peripheral nerve hyperemic response in diabetic animals after injury. The abnormal response corresponds with closely related alterations in microvessel morphological properties. The diabetic regenerative program is not only impaired but also significantly delayed in many respects, including its associated changes in vasa nervorum. An impaired upregulation of blood flow may create a short-term microenvironment with relative ischemia resulting in delayed mitogenicity of support and migratory cells and a slowed regeneration rate.

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