Evaluation of α_1-adrenoceptor antagonist on diabetes-induced changes in peripheral nerve function, metabolism, and antioxidative defense

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ABSTRACT **The role for nerve blood flow (NBF) vs. other factors in motor nerve conduction (MNC) slowing in short-term diabetes was assessed by evaluating α_1-adrenoceptor antagonist prazosin on NBF, MNC, as well as metabolic imbalances and oxidative stress in the neural tissue. Control and diabetic rats were treated with or without prazosin (5 mg·kg^{-1}·d^{-1} for 3 wk). NBF was measured by hydrogen clearance. Both endoneurial vascular conductance and MNC velocity were decreased in diabetic rats vs. controls, and this decrease was prevented by prazosin. Free NAD$^+$:NADH ratios in mitochondrial cristae, matrix, and cytosol assessed by metabolite indicator method, as well as phosphocreatine levels and phosphocreatine/creatine ratios, were decreased in diabetic rats, and this reduction was ameliorated by prazosin. Neither diabetes-induced accumulation of two major glycation agents, glucose and fructose, as well as sorbitol and total malondialdehyde plus 4-hydroxyalkenals nor depletion of *myo*-inositol, GSH, and taurine or decrease in (Na/K)-ATP-ase activity were affected by prazosin. In conclusion, decreased NBF, but not metabolic imbalances or oxidative stress in the neural tissue, is a key mechanism of MNC slowing in short-term diabetes. Further experiments are needed to estimate whether preservation of NBF is sufficient for prevention of nerve dysfunction and morphological abnormalities in long-standing diabetes or whether the aforementioned metabolic imbalances closely associated with impaired neurotropism are of greater importance in advanced than in early diabetic neuropathy.—Obrosova, I. G., Van Huysen, C., Fathallah, L., Cao, X., Stevens, M. J., Greene, D. A. Evaluation of α_1-adrenoceptor antagonist on diabetes-induced changes in peripheral nerve function, metabolism, and antioxidative defense. *FASEB J.* 14, 1548–1558 (2000)**

Key Words: motor nerve conduction · peripheral diabetic neuropathy · nerve blood flow · prazosin

THE PATHOGENESIS OF peripheral diabetic neuropathy (PDN) remains an area of conceptual disagreement. A number of reports indicate that PDN is a hypoxic neuropathy and that a decrease in nerve blood flow (NBF) with resulting endoneurial hypoxia is a key mechanism responsible for nerve conduction (NC) slowing in the diabetic nerve (1–4). This view is supported by studies of different antioxidants (1, 2, 5–7) and metal chelators (8, 9), aldose reductase inhibitors (3, 10, 11), protein kinase C inhibitors (12), inhibitor of nitric oxide synthase (12, 13), potassium channel openers (14), aminoguanidine (15, 16), and various kinds of vasodilators (13, 17, 18), all of which demonstrate a perfect correlation between NBF and NC. Other studies, however, suggest that blood flow is unchanged (19) or even increased (20, 21) in the diabetic nerve and that diabetes-induced NC deficit is independent of NBF, but due to metabolic (20, 22–25) and other (26, 27) changes in the neural tissue. Among these changes, different investigators have implicated accumulation of sorbitol (28–30), depletion of *myo*-inositol (22) and taurine (24), a decrease in (Na/K)-ATP-ase activity (23, 25, 31–33), NAD-redox imbalances (20), alterations in fatty acid metabolism (32, 34) as well as deficits of insulin-like growth factor (IGF) (35) and nerve growth factor (NGF) (36) to be associated at least in part with nerve oxidative injury (36).

Vasodilators represent a valuable tool for addressing this controversy. The purpose of the present study was to assess the role of NBF vs. other factors in motor (M) NC slowing in short-term diabetes by evaluating the vasodilator, α_1-adrenoceptor antagonist prazosin, on diabetes-induced neurovascular dysfunction, motor nerve conduction (MNC), as well as metabolic imbalances and oxidative stress in the neural tissue.

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MATERIALS AND METHODS

The experiments were performed in accordance with regulations specified by the National Institutes of Health *Principles of Laboratory Animal Care, 1985 Revised Version,* and University of Michigan Protocol for Animal Studies.

Animals

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, Ind.), body weight 250–300 g, were fed a standard rat chow diet (ICN Biomedical, Cleveland, Ohio) and had access to water *ad libitum.* Diabetes was induced by a single intraperitoneal (i.p.) injection of streptozotocin (55 mg/kg body weight) to animals fasted overnight. Blood samples for measurements of glucose were taken from the tail vein 48 h after the streptozotocin injection and the day before the animals were killed. The rats with blood glucose of 13.8 mM or more were considered diabetic. The experimental groups comprised control and diabetic rats treated with or without α_1-adrenoceptor antagonist prazosin (5 mg · kg body weight^{-1} · day^{-1} in the drinking water). The treatment of diabetic rats was started ~48 h after streptozotocin injection; the duration of the experiment was 3 wk.

Reagents

Unless otherwise stated, all chemicals were of reagent grade quality and purchased from Sigma Chemical (St. Louis, Mo.). Methanol (HPLC grade), perchloric acid, hydrochloric acid, and sodium hydroxide were purchased from Fisher Scientific (Pittsburgh, Pa.). Ethyl alcohol (200 proof dehydrated alcohol, U.S.P. punctilious) was purchased from Quantum Chemical Co. (Tiscola, Ill.). Dihydroxyacetone phosphate dilithium salt monohydrate was purchased from Fluka BioChemika (Buchs, Switzerland). β-D-Glucose, sorbitol, N.F., *myo*-inositol, C.P., and D-fructose, U.S.P. were purchased from Pfanstiehl Laboratories, Inc. (Waukegan, Ill.). Kits for malondialdehyde and 4-hydroxyalkenals assay were purchased from Oxis International (Portland, Oreg.).

Experimental procedure

Our pilot experiments revealed that urethane anesthesia distorts the profile of peripheral nerve metabolites whereas rat sedation by a short (~15–20 s) exposure to carbon dioxide with immediate cervical dislocation preserves reduced metabolite and high-energy phosphate levels in the range of those obtained after decapitation without any narcosis (37). For this reason, two different sets of animals were used for functional and metabolic studies. In the first set, the rats were anesthetized by urethane (1–1.2 g/kg, i.p.). Motor nerve conduction velocity (MNCV) measurements were made before the assessment of NBF on the contralateral nerve. In all measurements, body temperature was monitored by a rectal probe and maintained at 37°C with a warming pad. Hind limb skin temperature was also monitored by a thermistor and maintained between 36 and 38°C by radiant heat. In the second set, the rats were sedated by CO_2 in a specially designed chamber (37) and immediately killed by cervical dislocation. The femoral segments of the left sciatic nerve from each rat were rapidly (~30 s) dissected, carefully blotted with fine filter paper to remove any accompanying blood, and frozen in liquid nitrogen for subsequent measurements of β-hydroxybutyrate, acetoacetate, glutamate, α-ketoglutarate, ammonia, phosphocreatine, creatine, and ATP. The remaining part of the left and the right nerve were used for measurements of total malondialdehyde plus 4-hydroxyalkenals, GSH, sorbitol pathway intermediates, *myo*-inositol, taurine, and (Na/K)-ATP-ase activity. The group of prazosin-treated control rats was set up to estimate whether metabolic effects of prazosin, when present, were specific for diabetic rats. Biochemical measurements in this group were confined to the parameters that responded to prazosin treatment in the diabetic group.

Functional studies

Sciatic endoneurial nutritive NBF

NBF was assessed by microelectrode polarography and hydrogen clearance (38). The left carotid artery was cannulated with polyethylene tubing and patency was maintained with heparinized saline (50 U/ml normal saline). The catheter was connected to a transducer and the blood pressure monitored by a MacLab data acquisition system. A tracheostomy was performed and the animal artificially respired with O_2:N_2 (20%:80%) using a small animal ventilator (Harvard Apparatus, South Natick, Mass.). The right sciatic nerve was exposed and gently dissected away from the surrounding tissue. The skin around the incision was positioned to create a reservoir. A ground electrode was inserted subcutaneously into the flank of the rat. Using a micromanipulator, H_2-sensitive platinum electrode (tip diameter 0.2 μm; World Precision Instruments, Sarasota, Fla.) was inserted into the nerve above the trifurcation. Mineral oil at 37°C was used to fill the reservoir and prevent diffusion of gases out of the nerve. The nerve was polarized with 0.25 V; when a stable baseline was achieved, the animal received a gas mixture containing 10% H_2 that was continued until the current change stabilized (10–30 min), when H_2 flow was terminated. Current recordings were made every 30 s until baseline levels were achieved (30–60 min). After the experiment, mono- or biexponential clearance curves were fitted to the data (Graphpad Software, La Jolla, Calif.). Nutritive NBF was taken as the slow component of the curve. An average of two determinations at different sites was used to determine nutritive NBF.

Sciatic MNCV

The left sciatic nerve was stimulated proximally at the sciatic notch and distally at the ankle via bipolar electrodes with supramaximal stimuli (8 V) at 20 Hz. The latencies of the compound muscle action potentials were recorded via bipolar electrodes from the first interosseous muscle of the hind paw and measured from the stimulus artifact to the onset of the negative M-wave deflection. MNCV was calculated by subtracting the distal latency from the proximal latency; the result was divided into the distance between the stimulating and recording electrode.

Metabolic studies

Preparation of perchloric extract

Femoral segments (~20 mg) of the left nerve as well as segments (~20 mg) of a remaining part of the left nerve or the right nerve were weighed, homogenized in 1.5 ml of ice-cold 6% $HClO_4$, and centrifuged at 4000 *g* for 10 min. After centrifugation, the samples were immediately neutralized with 5 M K_2CO_3 to pH 6–7 and centrifuged again at 4000 *g* for 5 min to precipitate insoluble $KClO_4$.

Measurements of glycolytic and tricarboxylic acid cycle intermediates, ketone bodies, glutamate, ammonia, phosphocreatine, creatine, and ATP

The steady-state concentrations of glucose, acetoacetate, β-hydroxybutyrate, glutamate, α-ketoglutarate, ammonia, pyruvate, lactate, phosphocreatine, creatine, and ATP were assayed in perchloric extracts of femoral segments of the left sciatic nerve spectrofluorometrically (Perkin-Elmer LS-5B, Norwalk, Conn.) by enzymatic procedures as described by Lowry and Passonneau (39). The lower limit for all spectrofluorometric procedures in our study including sorbitol, fructose, *myo*-inositol, and GSH was 0.1×10^{-9} M.

GSH measurements

We modified the method of Hissin and Hilf (40) and mixed 0.1 ml of neutralized nerve perchloric extract with 0.89 ml of 0.02 M EDTA in 1.0 M tris-HCI buffer, pH 8.1. The reaction was initiated by addition of 0.01 ml of O-phthaldialdehyde (10 mg-1 ml methanol). Initial and final readings were taken at λ excitation: 345 nm, λ emission: 425 nm, slits: 5 and 5. The differences in initial and final readings were compared with those in corresponding GSH standards ($1–10\times10^{-9}$ M) processed in the same run.

Measurements of sorbitol pathway intermediates, myo-inositol, and total MDA plus 4-hydroxyalkenals

Nerve segments (~40 mg) were weighed and homogenized in 1 ml 0.1 M Na-phosphate buffer, pH 6.5. A 100 μl volume of 0.3 M zinc sulfate, followed by an equivalent of barium hydroxide, was then added to 0.4 ml of the homogenate for protein precipitation. The samples were centrifuged at 4000 *g* for 10 min and 100 μl aliquots of the supernatant were taken for spectrofluorometric measurements of sorbitol, fructose, and *myo*-inositol by enzymatic procedures as described previously (41, 42). The rest of the homogenate was centrifuged at 3000 *g* for 5 min. Aliquots (200 μl) of the supernatant were used for measurements of malondialdehyde (MDA) and total malondialdehyde plus 4-hydroxyalkenals (4-HA) using kits LPO-586 from Oxis International (Portland, Oreg.). The method is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA or MDA and 4-HA after their differential extraction under hydrochloric or methanesulfonic acidic conditions at 45°C (43). The absorbance of chromogenic product was measured at 586 nm using a spectrophotometer Beckman DU 640 (Fullerton, Calif.) and was compared with the absorbance in corresponding standards.

Taurine measurements

Nerve segments (~10 mg) were homogenized in 1 ml of 6% trichloroacetic acid and centrifuged at 4000 *g* for 10 min. Taurine was measured by reverse-phase high-performance liquid chromatography (Waters, Milford, Mass.) after precolumn derivatization with O-phthaldialdehyde (41, 42, 44). Glutamine, added after ion exchange chromatography, was used as the internal standard.

Measurements of (Na/K)-ATP-ase activity

Nerve segments (~10 mg) were homogenized on ice in 2 ml of 0.2 M sucrose plus 0.02 M Tris-HCl pH 7.5 by three 10 s bursts with a Polytron model PT 10–35 (Brinkman Instruments, Westbury, N.Y.). Aliquots of homogenate were assayed enzymatically for total and ouabain-insensitive ATPase activity as described (45). Ouabain-sensitive Na/K-ATPase activity is defined as the difference in activity before and after the addition of ouabain and is expressed as μmol ADP formed/g wet weight per hour.

Calculations of free mitochondrial and cytosolic NAD^+:NADH ratios

According to classical publications of Krebs' laboratory (46, 47) and other studies (48), direct measurements of NAD, NADH, NADP, and NADPH do not provide information on compartmentalization of nicotinamide adenine nucleotides between cytosol and mitochondria and do not separate free from protein-bound forms (only free forms determine direction and free-energy changes of dehydrogenase reactions). The same studies proposed an alternative approach for assessment of free $NAD(P)^+$:NAD(P)H ratios in the cytoplasm and mitochondria from ratios of the concentrations of oxidized and reduced metabolites of suitable NAD(P)-linked dehydrogenase systems. Using this approach, free NAD^+:NADH ratios for mitochondrial cristae, matrix, and cytoplasm were calculated from the steady-state metabolite concentrations and the equilibrium constants of β-hydroxybutyrate, glutamate, and lactate dehydrogenase systems as described (46, 48):

$$\frac{[NAD^+]}{[NADH]}\text{mit cristae} = \frac{[\text{acetoacetate}]}{[\beta\text{-hydroxybutyrate}]} \times \frac{1}{K_1},$$

where K_1 is the equilibrium constant of β-hydroxybutyrate dehydrogenase.

$$\frac{[NAD^+]}{[NADH]}\text{mit matrix} = \frac{[\alpha\text{-ketoglutarate}] \times [\text{ammonia}]}{[\text{glutamate}]} \times \frac{1}{K_2},$$

where K_2 is the equilibrium constant of glutamate dehydrogenase.

$$\frac{[NAD^+]}{[NADH]}\text{cytoplasm} = \frac{[\text{pyruvate}]}{[\text{lactate}]} \times \frac{1}{K_3},$$

where K_3 is the equilibrium constant of lactate dehydrogenase.

Statistical analysis

The results are expressed as mean ± standard deviation. Data were subjected to equality of variance F test and then to log transformation, if necessary, before one-way analysis of variance. Where overall significance ($P<0.05$) was attained, individual between group comparisons were made using the Student-Newman-Keuls multiple range test. Significance was defined at $P\leq0.05$. When between-group variance differences could not be normalized by log transformation (data sets for body weights, plasma glucose, and some metabolic parameters), the data were analyzed by the nonparametric Kruskal-Wallis one-way analysis of variance, followed by the Fisher's PLSD test for multiple comparisons.

RESULTS

The final body weights were lower in diabetic rats compared with those in control rats (**Table 1**). The initial body weights were similar in these two groups. No significant difference was found between body

TABLE 1. *Initial and final body weights (g) and blood glucose concentrations (mM)*[a]

	Body weight		Blood glucose concentration	
	Initial	Final	Initial	Final
Control (34)	250 ± 19	390 ± 17	3.7 ± 0.5	2.9 ± 0.5
Control + prazosin (10)	269 ± 20	384 ± 18	4.0 ± 0.5	3.2 ± 0.3
Diabetic (35)	242 ± 21	269 ± 51**	13.4 ± 1.2**	18.9 ± 3.3**
Diabetic + prazosin (32)	253 ± 19	256 ± 39**	14.0 ± 1.1**	19.6 ± 2.3**

[a] The number of observations is indicated in parentheses. ** Significantly different compared to those in controls ($P < 0.01$).

weights in control and diabetic rats treated with prazosin and the corresponding untreated groups.

Blood glucose concentrations were increased by 540% in diabetic rats compared with those in control rats. Prazosin treatment had no effect on blood glucose concentrations in either control or diabetic rats.

Sciatic NBF, mean systemic blood pressure (BP), and endoneurial vascular conductance were 52%, 11.5%, and 40% lower in diabetic rats compared with those in control rats ($P<0.01$ for NBF and VC and < 0.05 for BP, **Fig. 1**). Prazosin treatment of diabetic rats further decreased BP ($P<0.05$ vs. the untreated diabetic group and < 0.01 vs. controls). For this reason, prazosin's effect on NBF was modest (a 36% increase vs. the untreated diabetic group, $P<0.05$ vs. untreated diabetic and < 0.01 vs. control groups) although a decrease in VC, i.e., NBF:BP ratio, was completely prevented ($P<0.01$ vs. the untreated diabetic group).

Sciatic MNCV was decreased by 26% in diabetic rats compared with controls ($P<0.01$, **Fig. 2**). MNCV was 27% higher in the prazosin-treated diabetic group compared with the untreated diabetic group ($P<0.01$). No statistically significant difference was found between MNCVs in prazosin-treated diabetic group and nondiabetic controls.

Concentrations of acetoacetate, β-hydroxybutyrate, glutamate, α-ketoglutarate, ammonia, lactate, and pyruvate were similar in the sciatic nerves of control rats treated with and without prazosin (**Table 2**). Concentrations of acetoacetate and β-hydroxybutyrate were increased 3.4- and 20-fold in diabetic rats compared with those in control rats. Whereas acetoacetate concentration was further increased by prazosin treatment (by 55% vs. the untreated diabetic group), β-hydroxybutyrate concentration was 38% lower in prazosin-treated diabetic rats compared with the untreated diabetic group. Glutamate concentration was increased by 26% by diabetes, and this increase was prevented by prazosin treatment. Ammonia concentrations were similar in diabetic rats treated with or without prazosin. α-Ketoglutarate concentrations were similar in control and diabetic rats and were increased by 27% in prazosin-treated diabetic rats vs. the untreated diabetic group. Pyruvate concentration was increased by 23% in diabetic rats vs. controls and this increase was not prevented by prazosin treatment. Lactate concentration was increased by 58% in diabetic rats compared with controls and tended to decrease with prazosin treatment, although the difference with the untreated diabetic group did not achieve statistical significance.

Free NAD^+:NADH ratios in nerve mitochondrial cristae and matrix (**Fig. 3*A***) as well as cytosol (Fig. 3*B*) were similar in control rats treated with or without prazosin. The three ratios were decreased by 77%, 32%, and 27% in diabetic rats compared with those in control rats ($P<0.01$ for all three ratios). Diabetes-induced NAD-redox changes in mitochondrial cristae were partially prevented by prazosin treatment. Free NAD^+:NADH ratio in prazosin-treated diabetic group was 150% higher than in the

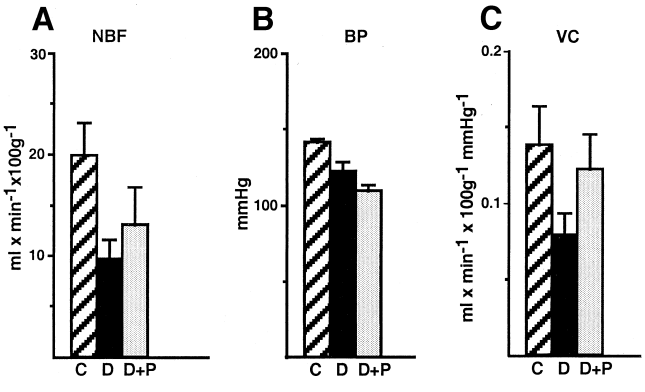

Figure 1. Sciatic NBF (*A*), systemic BP (*B*), and endoneurial VC (*C*) in control and diabetic rats treated with or without prazosin (mean ±SD, n=12–15).

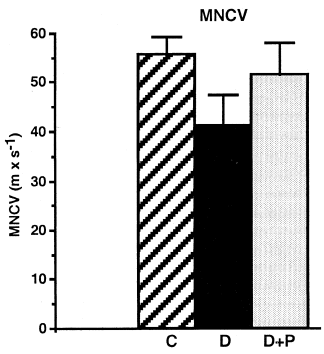

Figure 2. Sciatic MNCV in control and diabetic rats treated with or without prazosin (mean ±SD, n=8).

TABLE 2. *Steady-state concentrations (μmol/g wet weight) of metabolites of β-hydroxybutyrate dehydrogenase, glutamate dehydrogenase, and lactate dehydrogenase systems in control and diabetic rats treated with or without prazosin (n = 7-20)*

	Control	Control + prazosin	Diabetic	Diabetic + prazosin
Acetoacetate	0.088 ± 0.025	0.079 ± 0.020	0.389 ± 0.060**	0.602 ± 0.069**,##
β-Hydroxybutyrate	0.061 ± 0.021	0.064 ± 0.013	1.282 ± 0.163**	0.801 ± 0.065**,##
Glutamate	1.75 ± 0.19	1.78 ± 0.36	2.20 ± 0.27**	1.61 ± 0.12##
α-Ketoglutarate	0.163 ± 0.016	0.181 ± 0.017	0.154 ± 0.024	0.195 ± 0.016**,##
Ammonia	1.06 ± 0.16	0.90 ± 0.18	0.95 ± 0.14	0.98 ± 0.16
Pyruvate	0.188 ± 0.031	0.163 ± 0.023	0.232 ± 0.038*	0.245 ± 0.042**
Lactate	1.787 ± 0.399	1.50 ± 0.140	2.82 ± 0.396**	2.503 ± 0.396**

*,** Significantly different compared to those in controls ($P < 0.05$ and < 0.01, respectively); ## significantly different compared to those in untreated diabetics ($P < 0.01$).

untreated diabetic group ($P<0.01$) and 44% lower than in nondiabetic controls ($P<0.01$). A diabetes-induced decrease in free NAD^+:NADH ratios in mitochondrial matrix and cytosol was prevented by prazosin treatment ($P<0.01$ and < 0.05 vs. the untreated diabetic group). Free NAD^+:NADH ratios in either mitochondrial matrix or cytosol were not different between diabetic rats treated with prazosin and nondiabetic controls.

Nerve ATP concentrations were similar in control and diabetic groups treated with and without prazosin (**Table 3**). PCr concentrations and PCr:Cr ratios were not different in control rats treated with or without prazosin. PCr concentrations and PCr:Cr ratios were reduced in diabetic rats (by 13% and 22.5% vs. control group), and the decrease in both parameters was prevented by prazosin treatment. Cr concentrations were not different among control and diabetic rats treated with and without prazosin.

Nerve glucose, sorbitol, and fructose concentrations were 852%, 456%, and 175% higher in diabetic rats compared with those in control rats (**Table 4**); none of these parameters were affected by prazosin treatment.

Nerve *myo*-inositol and taurine concentrations (**Fig. 4*A***) were reduced by 24% and 39% in diabetic rats vs. controls ($P<0.05$ and < 0.01, respectively), and depletion of either osmolyte was not prevented by prazosin treatment. (Na/K)-ATP-ase activity (Fig. 4*B*) was 34% lower in diabetic rats compared with the control group ($P<0.01$); this decrease was not affected by prazosin treatment.

Nerve GSH concentration (**Fig. 5*A***) was decreased by 29% in diabetic rats vs. controls ($P<0.01$), and this decrease was not prevented by prazosin treatment. Nerve MDA concentrations were similar in control, diabetic untreated, and prazosin-treated diabetic rats (Fig. 5*B*). Nerve MDA plus 4-HA concentration was increased by 40% in diabetic rats vs. controls ($P<0.05$); this increase was not prevented by prazosin treatment.

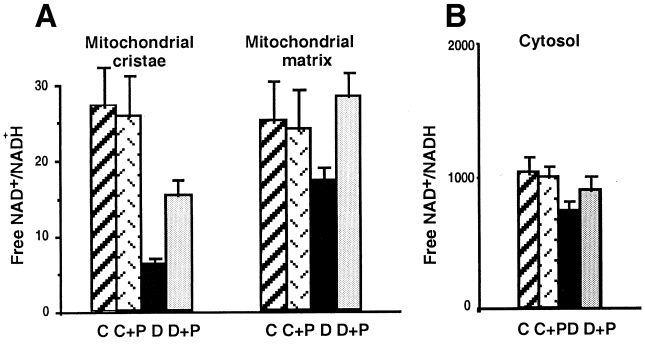

Figure 3. Free NAD^+:NADH ratios in sciatic nerve mitochondrial cristae and matrix (*A*) and cytosol (*B*) in control and diabetic rats treated with or without prazosin (mean ±SD, n=7–18).

DISCUSSION

The diabetic state in humans is associated with marked abnormalities of microvascular regulation (49). Diabetes-induced decrease in NBF in the present study is consistent with other reports for both exposed (1–6, 8–12, 50–52) and unexposed (53, 54) diabetic nerve. Prevention of both diabetes-induced neurovascular dysfunction and MNCV deficit by α_1-adrenoceptor antagonist prazosin is consistent with previous studies by Cameron's group with prazosin (55), another α_1-adrenoceptor antagonist, doxazosin (13), and vasodilators such as the β_2-adrenoreceptor agonist salbutamol (13), angiotensin-converting enzyme inhibitor lisinopril (17), and the calcium antagonist nifedipine (18).

A decrease in free NAD^+:NADH ratios in nerve mitochondrial cristae and matrix in diabetes as well as partial (cristae) or complete (matrix) prevention of the NAD-redox imbalances by the vasodilator therapy is indicative of diabetes-induced endoneurial hypoxia. The effect of prazosin on both ratios is specific for diabetic animals and therefore is not due to some unidentified intrinsic properties of the compound, not related to its vasodilator activity. The findings of decreased free mitochondrial NAD^+:NADH ratios in the diabetic peripheral nerve are consistent with a diabetes-induced decrease in NBF and peripheral nerve oxygen tensions (10, 56, 57).

TABLE 3. *Nerve energy status in control and diabetic rats treated with or without prazosin (n = 10-20)*[a]

	Control	Control + prazosin	Diabetic	Diabetic + prazosin
ATP	1.103 ± 0.309	0.979 ± 0.244	1.150 ± 0.364	0.982 ± 0.158
PCr	2.697 ± 0.373	2.705 ± 0.405	2.359 ± 0.416*	2.574 ± 0.418[#]
Cr	3.951 ± 0.639	3.365 ± 0.632	4.339 ± 0.559	3.854 ± 0.958
PCr/Cr	0.703 ± 0.092	0.818 ± 0.145	0.545 ± 0.084**	0.723 ± 0.109[##]

[a] Concentrations of ATP, PCr, and Cr are expressed in μmol/g wet weight. *,** Significantly different compared with those in controls ($P < 0.05$ and < 0.01, respectively); #,## significantly different compared with those in untreated diabetics ($P < 0.05$ and < 0.01, respectively). Abbreviations: PCr, phosphocreatine; Cr, creatine.

Furthermore, the assessment of free mitochondrial NAD^+:NADH ratios in our study has been performed in femoral segments of the sciatic nerve sampled within 30 s after euthanasia, i.e., without prolonged surgical exposure of the nerve. Thus, our results argue against the premise that the findings of decreased NBF and oxygenation in the diabetic nerve is an artifact of nerve surgical exposure during NBF measurements by hydrogen clearance or laser Doppler procedures (21), and confirm that the diabetic peripheral nerve is truly hypoxic. The findings of a 77% decrease in free NAD^+:NADH ratio in the mitochondrial cristae of the diabetic nerve vs. ~40–50% decrease of nerve oxygen tensions in other reports (10, 56, 57), as well as of incomplete prevention of the NAD-redox changes despite preservation of normal VC in prazosin-treated diabetic rats, indicate that, in addition to decreased NBF/endoneurial hypoxia, other factors affect mitochondrial oxidative capacity of the diabetic nerve. These may include osmotic stress with resulting disturbances in Ca^{2+} homeostasis and oxidative stress leading to inhibition of respiratory chain enzymes, e.g., cytochrome C oxidase (58). The role for these nonvascular mechanisms affecting mitochondrial oxidative capacity in diabetes-induced nerve conduction slowing is minor as MNCV deficit was prevented by prazosin treatment. Despite a decrease of nerve mitochondrial oxidative capacity to about half that of normal, a free NAD^+:NADH ratio in mitochondrial matrix of prazosin-treated diabetic rats was preserved in the range of nondiabetic animals. This ratio controls the activity of tricarboxylic acid cycle, the major source of energy in aerobic tissues. Therefore, it is not surprising that PCr levels as well as the PCr/Cr ratio, the most sensitive parameter of the peripheral nerve energy state (59, 60) in the diabetic rats treated with prazosin, are in the range of those in controls. The responsiveness of nerve PCr concentration to vasodilator treatment in our experiments is consistent with reports (61, 62) of the sensitivity of this parameter to changes in perfusion/oxygenation. Two aforementioned studies (61, 62) as well as our findings suggest that of a variety of metabolic parameters, nerve energy state correlates best with MNC.

Decrease in free cytosolic NAD^+:NADH ratio in the diabetic nerve is also prevented by the vasodilator treatment despite the absence of any effect of prazosin on the sorbitol pathway intermediates. Therefore, our findings do not support the concept of 'diabetic pseudohypoxia', suggesting that diabetes-induced cytosolic NAD-redox imbalances are due to increased oxidation of sorbitol to fructose, coupled to reduction of NAD to NADH, by sorbitol dehydrogenase (SDH) (63). The conclusion of the independence of the shift toward a more reduced state of free cytosolic NAD-couple from increased SDH activity is supported by another study from our laboratory (37). We have found that an SDH inhibitor (SDI) at a dose resulting in 91% inhibition of the increased flux through SDH failed to prevent either cytosolic or mitochondrial NAD-redox imbalances in the diabetic nerve. Taking into consideration, that mitochondrial and cytosolic pools of nicotinamide adenine dinucleotides are linked through dicarboxylate carriers and that the peripheral nerve strongly depends on oxidative (aerobic) metabolism (64), it is reasonable to suggest that diabetes-associated cytosolic NAD-redox imbalances have a mitochondrial origin, i.e., reflect a metabolic response of the cytoplasm to endoneurial hypoxia developing due to decreased NBF.

Although advanced glycation end-products accumulate in the peripheral nerve at a later stage of

TABLE 4. *Nerve glucose, sorbitol, and fructose concentrations (μmol/g wet weight) in control and diabetic rats treated with or without prazosin (n = 7-9)*

	Control	Diabetic	Diabetic + prazosin
Glucose	1.88 ± 0.44	17.89 ± 6.80**	19.24 ± 3.51**
Sorbitol	0.329 ± 0.079	1.83 ± 0.38**	2.36 ± 0.55**
Fructose	2.31 ± 0.72	6.36 ± 2.07**	6.51 ± 1.30**

** Significantly different compared to those in controls ($P < 0.01$).

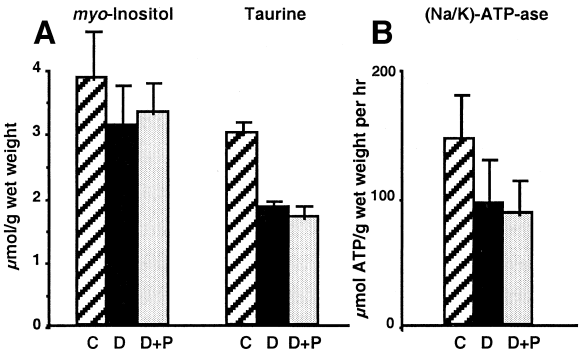

Figure 4. Sciatic nerve *myo*-inositol and taurine concentrations (*A*) and (Na/K)-ATP-ase activities (*B*) in control and diabetic rats treated with or without prazosin (mean ±SD, n=7–8).

PDN (65), the Amadori products recently implicated in the pathogenesis of diabetic complications (66) form in early diabetes. The fact that the vasodilator treatment prevented MNCV deficit without affecting the concentrations of two most abundant glycation agents, glucose and fructose (67), suggests that nonenzymatic glycation of neural tissue macromolecules is not implicated in MNC slowing in short-term diabetes. This assumption is consistent with the absence of increased levels of methylglyoxal, the α-dicarbonyl compound that reacts with proteins with formation of imidazolium cross-linking AGEs (68) in the sciatic nerve at the early stage of PDN (69). There are no reports of the presence of another potent glycation agent, 3-deoxyglucosone (70, 71), in the neural tissues. Furthermore, this reactive dicarbonyl is formed by nonenzymatic degradation of glucose-derived Amadori products (70–72) and, in some tissues (73), by nonenzymatic decomposition of sorbitol pathway-originated fructose 3-phosphate. Therefore, it is inconceivable that 3-deoxyglucosone levels, if present, are modulated by the agent (prazosin) that does not affect either glucose or fructose levels in the diabetic nerve. The blockade of a preventive effect of aminoguanidine on diabetes-induced nerve conduction slowing by cotreatment with the nitric oxide synthase inhibitor NG-nitro-L-arginine (16) is consistent with the important role of AGE in *vasa nervorum*, but not neural tissue, in NCV deficit in early diabetes.

In a similar fashion, prevention of diabetes-induced MNCV deficit despite the absence of any effect of prazosin on nerve sorbitol levels implies that sorbitol accumulation is not a major culprit in the early phase of PDN. This conclusion is consistent with observations of others. In particular, Cameron et al. (11) did not find any MNC and sensory NC slowing in nondiabetic rats treated with a SDI that increased nerve sorbitol accumulation to the level found in the diabetic rats. Ng et al. (74) reported similar MNCV deficits in the diabetic mice with normal SDH levels and SDH-deficient diabetic mice with 8.9-fold higher nerve sorbitol concentration. Song et al. (75) found no difference in MNCV deficits between diabetic mice, overexpressing aldose reductase in Schwann cells, and nontransgenic diabetic mice. However, despite these and our findings, one should keep in mind that nerve sorbitol accumulation could be of much greater importance in advanced PDN. Dyck et al. (76) have reported that nerve sorbitol content in the diabetic patients is inversely related to the number of myelinated fibers. Of particular interest is the study by Schmidt et al. (77), who found a dramatic increase in ileal mesenteric nerve axonal dystrophy with SDH inhibition by the dose of SDI not affecting NBF in the diabetic model of lesser duration (11). Long-term experiments with a SDI are needed to estimate whether similar axonopathy will develop in the peripheral nerve in response to persistent excessive nerve sorbitol accumulation.

The role for *myo*-inositol depletion in diabetes-induced NC deficit remains controversial. Two groups (78–80) have reported amelioration of MNCV deficit in the diabetic rats fed 1% *myo*-inositol diet or receiving 500 mg/kg per day of *myo*-inositol in the drinking water, whereas others did not find any effect of dietary *myo*-inositol supplementation on either neurovascular dysfunction or nerve conduction deficits in the intervention study with a ~2.5-fold higher dose of *myo*-inositol (81). It is unclear whether different observations regarding efficacy of *myo*-inositol on diabetes-induced NC slowing are due to the difference in dose and whether 2.5% *myo*-inositol supplementation (81) is associated with adverse side effects, preventing preservation of MNCV and not characteristic of the 1% diet. In addition, clinical studies have revealed the absence of *myo*-inositol depletion in the diabetic patients (76, 82, 83). Sundkvist et al. (83), but not Dyck et al. (76), found an association between nerve *myo*-inositol depletion and the presence of PDN. The present study, demonstrating that MNCV deficit is prevented by prazosin despite the absence of any effect of the

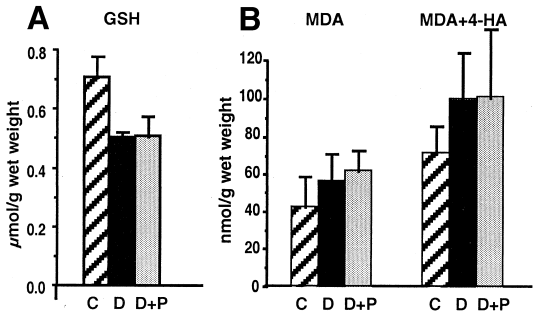

Figure 5. Sciatic nerve GSH (*A*) and MDA and MDA plus 4-HA (*B*) concentrations in control and diabetic rats treated with or without prazosin (mean ±SD, n=12–15).

vasodilator on nerve *myo*-inositol and taurine concentrations, indicates that preservation the two osmolytes in the neural tissue is not essential for prevention of NC slowing in short-term diabetes. Taking into consideration that taurine prevents diabetes-induced changes in NBF (84) and that Na^+/taurine cotransporter is localized in *vasa nervorum* (85), it is reasonable to suggest that the preventive effect of taurine on MNC and sensory NC in diabetes (84) is of vascular origin. The latter would be consistent with vasodilator properties of taurine (86) and its inhibitory effect on protein kinase C (87).

Despite the lack of prevention of diabetes-induced decrease in (Na/K)-ATP-ase activity, preservation of MNCV by prazosin treatment indicates that down-regulation of (Na/K)-ATP-ase is not implicated in early MNCV deficit in diabetes. This conclusion is supported by other studies demonstrating the absence of any up-regulation of this enzyme by vasodilators that effectively prevent MNC slowing (55) or, conversely, an up-regulation of (Na/K)-ATP-ase without any improvement of MNCV by NGF (M. J. Stevens, unpublished results) or DL-α-lipoic acid (88). The role for (Na/K)-ATP-ase in advanced PDN remains unclear.

One of the most interesting findings of the present study is an apparent dissociation between accumulation of lipid peroxidation products in the neural tissue and early MNCV deficit in diabetes despite reports of prevention (1, 2, 36) and reversal (57) of diabetes-induced changes in NC by different antioxidants and metal chelators. These observations and our findings demonstrating that prazosin preserves MNCV without preventing diabetes-induced lipid peroxidation in the neural tissue lead to the conclusion that effects of antioxidants and metal chelators on NC in short-term diabetes are mediated through vascular mechanisms. Preservation of GSH and prevention of lipid peroxidation in nerve vasculature is probably more important in short-term diabetes than preservation of nerve GSH concentration and arrest of lipid peroxidation in the neural tissue (1, 89, 90). This conclusion is consistent with report of Cameron and Cotter (91) indicating that the beneficial effect of the free radical scavenger BM 15.0639 on diabetes-related NCV deficit is largely abolished by cotreatment with nitric oxide synthase inhibitor. However, it is important to remember that oxidative stress is implicated in impaired neurotropism (36) and mitochondrial dysfunction (92), associated with Schwann cell injury (93, 94), and is a powerful activator of three subfamilies of mitogen-activated protein kinases (MAPKs), i.e., stress-activated protein kinase/c-Jun-terminal kinases, the extracellularly responsive kinases and p38-MAPK (95–97), glucose transducers for diabetic complications (98), and particularly axonopathy (99). Both Schwann cell injury and axonopathy, developing gradually and becoming manifested in long-standing diabetes, exacerbate nerve functional deficits acquired in the initial phase of PDR. Thus, oxidative stress, acting through both vascular and nonvascular mechanisms, contributes to both onset and progression of PDR. In addition, oxidative injury affects neurotransmission (100) contributing to diabetes-induced endothelial dysfunction (101).

In conclusion, a decrease in NBF with resulting endoneurial hypoxia is a key mechanism of MNC slowing in the early phase of PDN. The question regarding the importance of vascular vs. nonvascular mechanisms in advanced PDN remains open. Long-term experiments with vasodilators are needed to establish whether preservation of NBF is sufficient for prevention of functional and morphological abnormalities in the peripheral nerve in long-standing diabetes. On the other hand, chronic experiments with coadministration of nitric oxide synthase inhibitor and one of the following agents, i.e., aldose reductase inhibitors, *myo-in*ositol, taurine, antioxidants, IGF or NGF, or SDI administration, can clarify the role for metabolic imbalances and enhanced oxidative stress in the neural tissue as well as impaired neurotrophic support in advanced PDN. FJ

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