

Lipoic Acid Improves Nerve Blood Flow, Reduces Oxidative Stress, and Improves Distal Nerve Conduction in Experimental Diabetic Neuropathy

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OBJECTIVE — To determine whether lipoic acid (LA) will reduce oxidative stress in diabetic peripheral nerves and improve neuropathy.

RESEARCH DESIGN AND METHODS — We used the model of streptozotocin-induced diabetic neuropathy (SDN) and evaluated the efficacy of LA supplementation in improving nerve blood flow (NBF), electrophysiology, and indexes of oxidative stress in peripheral nerves affected by SDN, at 1 month after onset of diabetes and in age-matched control rats. LA, in doses of 20, 50, and 100 mg/kg, was administered intraperitoneally five times per week after onset of diabetes.

RESULTS — NBF in SDN was reduced by 50%; LA did not affect the NBF of normal nerves but improved that of SDN in a dose-dependent manner. After 1 month of treatment, LA-supplemented rats (100 mg/kg) exhibited normal NBF. The most sensitive and reliable indicator of oxidative stress was a reduction in reduced glutathione, which was significantly reduced in streptozotocin-induced diabetic and α -tocopherol-deficient nerves; it was improved in a dose-dependent manner in LA-supplemented rats. The conduction velocity of the digital nerve was reduced in SDN and was significantly improved by LA.

CONCLUSIONS — These studies suggest that LA improves SDN, in significant part by reducing the effects of oxidative stress. The drug may have potential in the treatment of human diabetic neuropathy.

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Received for publication 15 December 1994 and accepted in revised form 2 March 1995.

BP, blood pressure; DRG, dorsal root ganglion; EDN, experimental diabetic neuropathy; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high-pressure liquid chromatography; LA, lipoic acid; NBF, nerve blood flow; PEEK, polyether ether ketone; SCG, superior cervical ganglion; SOD, superoxide dismutase; STZ, streptozotocin.

Oxidative stress is increased in both human diabetes (1) and experimental diabetic neuropathy (EDN) (2,3). Antioxidant defenses in the nerve are selectively reduced relative to the brain (4) and are further reduced in diabetes (5). α -Tocopherol, the main antioxidant in the nerve membrane, is a chain-breaking antioxidant that prevents free radical-mediated damage to lipids via lipid peroxidation (6). Its depletion will not only worsen EDN but will even cause neuropathy in otherwise normal nerves (3).

α -Tocopherol supplementation alone was not found to improve or prevent neuropathy (3). Hence, alternative approaches to prevent EDN by increasing the free radical-scavenging capacity of the peripheral nerve have been sought. The administration of reduced glutathione (GSH) has partially improved EDN (7), as has the use of antioxidants (8), including lipophilic antioxidants (9). Lipoic acid (LA), a powerful lipophilic free radical scavenger (10) that increases glucose entry (11) and improves diabetic neuropathy (12–14), has been used in the treatment of painful diabetic neuropathy. Only limited data on its mechanisms of action in the nerve are available. We therefore examined whether LA supplementation will improve nerve blood flow (NBF), nerve electrophysiology, and indexes of oxidative stress.

RESEARCH DESIGN AND METHODS

Animals

We studied the following groups of rats: 1) controls, normal α -tocopherol (Con); 2) controls, normal α -tocopherol + LA (50 mg/kg, 100 mg/kg) (Con50, Con100, respectively); 3) controls, α -tocopherol-deficient; Con[–]; 4) controls, restricted caloric intake (Con[R]); 5) controls, α -tocopherol-deficient + LA (100 mg/kg) (Con[–]100); 6) diabetic, normal α -tocopherol (streptozotocin [STZ]-induced); 7) diabetic, normal α -tocopherol + LA (20 mg/kg, 50 mg/kg, 100 mg/kg)

(STZ20, STZ50, STZ100, respectively); 8) diabetic, α -tocopherol-deficient (STZ[-]); and 9) diabetic, α -tocopherol-deficient + LA (20 mg/kg, 100 mg/kg) (STZ[-]20, STZ[-]100, respectively). Groups of at least six rats, with initial weights of 250 ± 5 g, were studied after 1 month.

Experimental diabetes. STZ was administered by intraperitoneal injection at 8 weeks of age. All STZ groups received STZ in 0.05 mol/l citrate buffer, pH 4.5 (65 mg/ml; dose 1.32 ml/kg). All control groups received citrate buffer alone. Rats were accepted as diabetic if fasting blood glucose concentrations exceeded 16.7 mmol/l 3 days after STZ administration and remained >16.7 mmol/l at the time of death. All rats were housed in cages with plastic floors covered with wood shavings and had access to an unrestricted supply of water.

Diet-restricted animals. We maintained two groups of diet-restricted animals. α -Tocopherol deficiency was produced by feeding weanling rats a diet deficient in α -tocopherol (no. 904640, ICN, Irvine, CA) (15) for 1 month at the time of entry into the study, and rats received this diet until the time of killing.

To evaluate the role of weight loss on the peripheral nerve, the calories of one group (Con[R] group) were restricted. These rats were fed a reduced number of pellets so that their weight matched those of undernourished rats (STZ and α -tocopherol-deficient animals).

LA supplementation. We administered LA in concentrations of 20, 50, and 100 mg/kg. The powder was mixed with saline, 2 N NaOH was added to dissolve the suspension, and the pH was then brought to 7.4 with 2 N HCl. Saline was used to bring the solution to the final concentration, and the solution was injected intraperitoneally five times per week.

Biochemical measurements

α -Tocopherol. Plasma, sciatic nerve, dorsal root ganglion (DRG), and superior

cervical ganglion (SCG) α -tocopherol content was determined by the reverse-phase high-pressure liquid chromatography (HPLC) method of Ikenoya et al. (16), modified for the peripheral nerve. In brief, an aliquot of tissue homogenized in water was mixed with 2 volumes ethanol and 5 volumes *n*-hexane. The extraction was repeated on the aqueous layer. The *n*-hexane layers were evaporated under N_2 , reconstituted in methanol, and injected into an HPLC column. Separation was obtained on an Ultremex 3 μ m C_{18} column (150 \times 2 mm; Phenomenex, Torrance, CA) using a mobile phase consisting of 3.5 g sodium perchlorate in 500 ml ethanol, methanol, and 70% $HClO_4$ (700:300:1) at a 0.3 ml/min flow rate. An LC-4C amperometric detector (Bioanalytical Systems, West Lafayette, IN) equipped with a dual glassy-carbon electrode in parallel was set at 0.7 V versus Ag/AgCl. Samples were compared with a standard curve using \pm - α -tocopherol (Sigma, St. Louis, MO).

Glutathione. GSH and oxidized glutathione (GSSG) were measured using reverse-phase HPLC and electrochemical detection (17). The mobile phase consisted of 98.5 parts 0.1 mol/l chloroacetic acid, pH 2.6, containing 2.0 mmol/l octyl sulfate, 1 part methanol, and 0.5 parts *N,N*-dimethylformamide at a flow rate of 0.55 ml/min on an α -Chrom 5 μ m C_{18} column (150 \times 2 mm, Upchurch Scientific, Oak Harbor, WA). All tubing consisted of stainless steel or polyether ether ketone (PEEK) (Upchurch) to prevent oxygen from entering the system. PEEK tubing was used from the column to the detector, eliminating the need for critical positioning of stainless steel tubing previously described. The mobile phase was sparged continuously with helium and refluxed to remove oxygen and maintain composition of the mobile phase. Electrochemical detection was performed using dual gold-mercury electrodes in series with the LC-4C amperometric detector. The first was maintained at -1.0 V to reduce the disulfides, and the detecting

electrode was set at $+0.15$ V. Samples were compared with a standard curve using GSH and GSSG (Sigma).

Blood glucose. Blood glucose was determined using a glucose oxidase method (Sigma kit no. 510). Measurements were done in duplicate at 450 nm on an LKB spectrophotometer (Ultraspec II, Pharmacia, Piscataway, NJ).

Physiological measurements

Nerve preparation. Rats were fasted overnight, then weighed and anesthetized with an intraperitoneal injection of either pentobarbital or Inactin (5-sec-butyl-5-ethyl-2-thiobarbituric acid, 100 mg/kg) and atropine sulfate (10 μ g). Rats were paralyzed with curare (2 U intravenously and 6 U intraperitoneally) and ventilated with a rodent respirator supplying a mixture of nitrogen and oxygen. Adequate analgesia was maintained by monitoring the corneal reflex and the continuous recordings of blood pressure (BP) and heart rate. Supplemental barbiturate was added when the level of anesthesia lightened. A polyethylene catheter was placed in the left common carotid artery to monitor mean arterial BP and blood gases. One end of a polyethylene tube filled with 2 mol/l potassium chloride in 3% agar was inserted into the abdominal subcutaneous tissue, and the other end was connected to the reference terminal of a current-sensitive amplifier (Chemical Microsensor, Diamond Electrotech, Ann Arbor, MI). The left sciatic nerve was exposed, and a pool was formed with the surrounding muscle and skin. The pool was filled with mineral oil maintained at $33.5 \pm 0.1^\circ C$ with a servo-controlled infrared lamp.

NBF. NBF was measured by microelectrode hydrogen polarography (tip size 2–5 μ m) (18). The signal was inputted into a computer via an analog-to-digital converter for simultaneous display and storage. A curve was fitted to the data using a nonlinear regression program based on the Marquardt algorithm (19).

Nerve electrophysiology. We used techniques that are standard for our labora-

Table 1—Weight, blood glucose, and tissue α -tocopherol concentrations of normal, diabetic, α -tocopherol-deficient, and LA-supplemented rats at 1 month

Group	n	Weight (g)	Glucose (mmol/l)	α -Tocopherol concentration (ng/mg wet wt)		
				Sciatic nerve	DRG	SCG
Con	8	383 \pm 5	8.3 \pm 0.7	13.8 \pm 1.3	16.0 \pm 0.9	23.4 \pm 2.2
Con[R]	8	217 \pm 4†	6.5 \pm 0.2*	13.7 \pm 0.7	14.4 \pm 1.0	18.4 \pm 1.8
Con100	8	329 \pm 4†	6.2 \pm 0.3*	13.8 \pm 1.4	14.6 \pm 1.1	20.6 \pm 1.5
Con[-]	8	178 \pm 8†	6.0 \pm 0.4*	8.3 \pm 1.2†	4.1 \pm 0.4†	1.2 \pm 0.3†
Con[-]100	8	169 \pm 5†	6.0 \pm 0.3*	7.5 \pm 0.9†	4.0 \pm 0.4†	1.2 \pm 0.2†
STZ	9	201 \pm 14†	29.3 \pm 0.7†	18.3 \pm 1.4*	21.8 \pm 0.9†	27.7 \pm 4.4
STZ20	9	230 \pm 9†	24.6 \pm 1.4†	19.5 \pm 2.5	18.6 \pm 1.3	30.2 \pm 2.8
STZ50	9	229 \pm 15†	27.3 \pm 1.9†	17.5 \pm 1.7	18.4 \pm 0.8	26.2 \pm 2.6
STZ100	9	243 \pm 15†	30.2 \pm 1.2†	20.3 \pm 2.5*	19.5 \pm 1.3*	27.5 \pm 3.5
STZ[-]	8	149 \pm 10†	28.7 \pm 1.8†	8.2 \pm 1.2†	4.5 \pm 0.4†	0.7 \pm 0.2†
STZ[-]20	9	141 \pm 12†	31.6 \pm 1.4†	9.9 \pm 1.3*	4.7 \pm 0.5†	1.1 \pm 0.2†
STZ[-]100	8	125 \pm 10†	29.5 \pm 1.8†	10.8 \pm 1.9	8.3 \pm 2.1†	1.4 \pm 0.5†

Data are means \pm SE. * P < 0.05, † P < 0.01, ‡ P < 0.001 vs. Con.

tory. We measured sensory conduction velocity and nerve action potential amplitude in the caudal nerve (20), sciatic-tibial motor nerve conduction velocity, and the amplitude of the compound muscle action potential (21) using fine stainless steel near-nerve stimulating and recording electrodes. Recordings were done at 35°C and amplified \times 1,000, stored on a computer disk, and analyzed offline using a Nicolet 1170 digital oscilloscope (Nicolet Instruments, Madison, WI) with associated stimulators and stimulus isolation units. We also measured the conduction velocity and amplitude of the hind-limb digital nerve (22). The compound nerve action potential was recorded with near-nerve recording electrodes after stimulation of the nerve trunk proximally. All recordings were done at 1 and 3 months.

Statistical analysis

Values are expressed as means \pm SE. For normally distributed data, statistical analysis was done using the unpaired two-tailed Student's *t* test when each pair-wise comparison was of interest in itself, as recommended by O'Brien (23), and analysis of variance with Bonferonni post hoc analysis when no such interest existed.

RESULTS

Glucose and weights

The weights and blood glucose of rats at 1 month are shown in Table 1. Diabetic rats had significantly reduced weights (P < 0.001). The calorie-restricted animals were well matched with the STZ and α -tocopherol-deficient groups by weight. Supplementation with LA had no significant effect on their weights when compared with the nonsupplemented animals. The α -tocopherol-deficient groups weighed significantly less, with an entry weight of 150 \pm 5 g instead of 250 \pm 5 g for other groups. The STZ groups were all severely hyperglycemic (P < 0.001) and LA supplementation did not affect blood glucose.

NBF

NBF and nerve vascular resistance (which corrects for differences in BP) are shown in Fig. 1. NBF of diabetic rats was significantly reduced (P < 0.001) to about 50% that of controls. LA supplementation did not affect NBF of control rats. In contrast, supplementation increased NBF in a dose-dependent manner beginning at concentrations of 50 mg/kg. NBF for the 100 mg/kg concentration was not significantly different from normal controls.

Nerve vascular resistance showed the same statistical differences (Fig. 1). NBF measurements were done after completion of electrophysiological studies.

α -Tocopherol

Sciatic nerve α -tocopherol concentrations were mildly and significantly increased in most tissues of STZ rats (Table 1) and markedly and significantly reduced in the tissues of α -tocopherol-deficient control and STZ rats. LA supplementation resulted in a dose-dependent but nonsignificant increase in the vitamin in the sciatic nerve and DRG of STZ[-] rats.

GSH

Dietary restriction resulted in a modest but significant reduction in GSH (Fig. 2). An α -tocopherol-deficient diet resulted in a 25% reduction (P < 0.001) in GSH. Diabetes resulted in a significant reduction (38%; P < 0.001) in GSH. LA supplementation resulted in a dose-dependent improvement in GSH, reaching normal values for the largest dose. The reduction in GSH was associated with a corresponding increase in GSSG as indicated by a corresponding increase in the GSSG:(GSH + 2GSSG) ratio (Fig. 2).

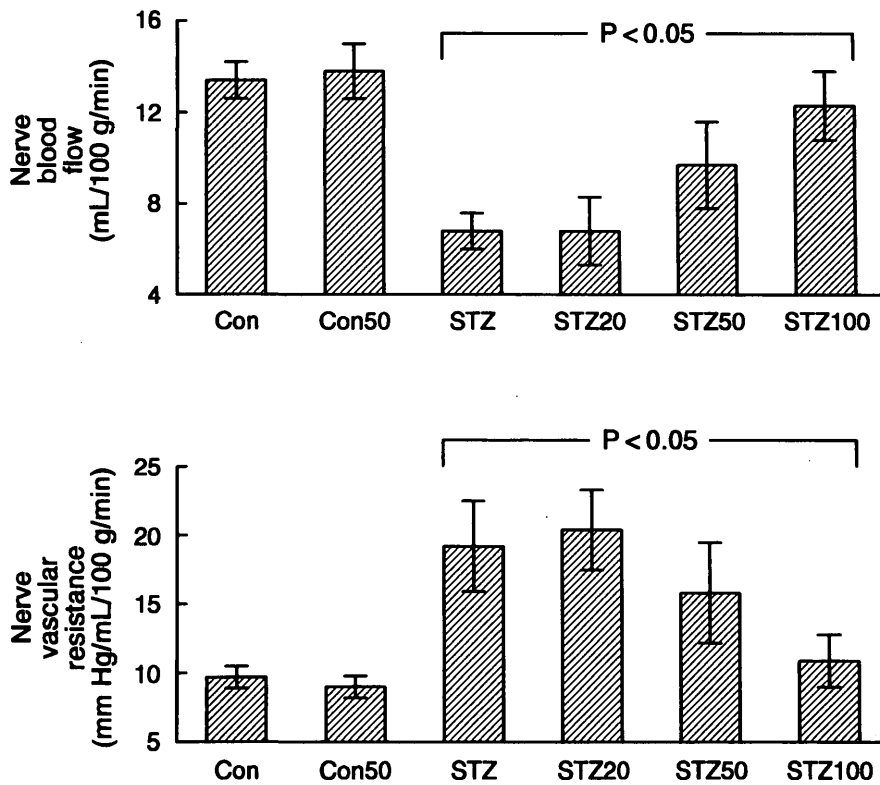


Figure 1—NBF and nerve vascular resistance of control (Con; n = 8), streptozotocin-induced diabetic neuropathy (STZ; n = 5), and animals given LA supplements at doses of 20 mg/kg (STZ20; n = 6), 50 mg/kg (Con50; STZ50; n = 5), and 100 mg/kg (STZ100; n = 8). LA supplementation results in normal NBF and nerve vascular resistance. Significance of difference, STZ-supplemented vs. STZ. Bars, SE.

Nerve electrophysiology

The peripheral nerves of rats with EDN had reduced conduction velocities (Table 2). LA-treated rats had normal digital nerve conduction at all supplemented doses at 3 months (Fig. 3). The electrophysiology of more proximal motor and sensory nerve fibers of sciatic and caudal nerves were not improved by treatment with LA (Table 2).

CONCLUSIONS— Our main findings are that EDN results in a reduction in NBF by 50%, nerve conduction slowing, and a large reduction in GSH and that LA supplementation resulted in a dose-dependent normalization of NBF and GSH and a time- and dose-dependent improvement in digital nerve conduction velocity.

Free radical-mediated damage depends on the balance between oxida-

tive stress and free radical defenses (24). The pattern of defenses for the nerve is unique. Compared with that of brain, nerve superoxide dismutase (SOD) activity is as high (4) but GSH and GSH-related enzymatic scavengers (glutathione peroxidase, transferases, and reductase) are only about 10% that of brain (4). With ischemia, superoxide anion is converted by SOD to H₂O₂, but its further decomposition, mediated by glutathione peroxidase (25), may be compromised if the low content of this enzyme is further reduced. Indeed, our observation that the most consistent and sensitive indicator of oxidative stress is a reduction in GSH (3) supports the critical dependence on GSH. We have thus used GSH as our index of free radical activity because of its high sensitivity and reliability.

Increases in oxidative stress, reduced free radical defenses, and altered pro-oxidant status of both human and experimental diabetes have been documented. Changes in plasma and other tissues have been documented and previously reviewed (2,26); this discussion will focus on alterations in the pe-

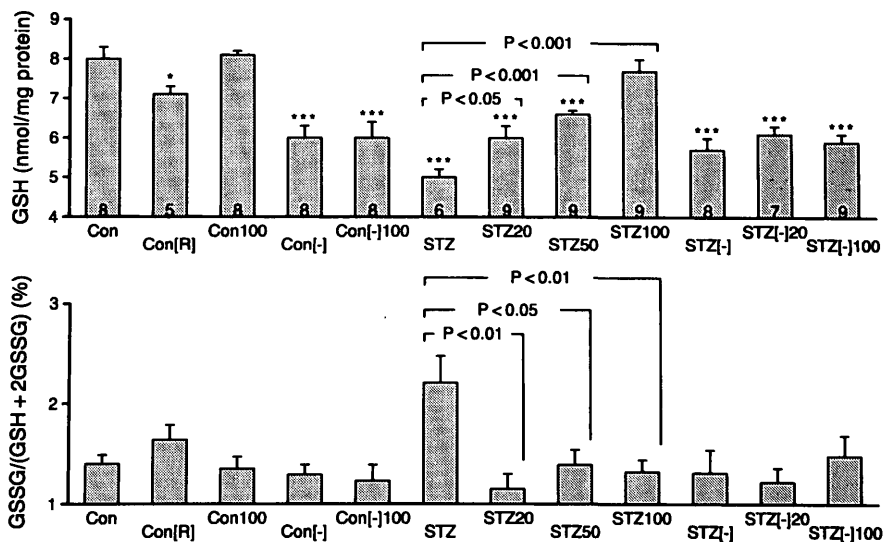


Figure 2—Sciatic nerve GSH concentrations and the GSH:(GSH + 2 GSSG) ratio in controls (Con), restricted caloric intake (Con[R]), STZ-diabetic (STZ), α -tocopherol-depleted [-], and LA supplemented at 20, 50, and 100 mg/kg. LA supplementation resulted in a dose-dependent prevention of GSH oxidation. Significance of difference vs. control, *P < 0.05; ***P < 0.001.

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Table 2—Sciatic-tibial, caudal motor, and caudal sensory conduction velocities of control, STZ-diabetic, and rats supplemented with LA (CON50, STZ20, STZ100)

Group	Time (months)	n	Sciatic-tibial	Caudal motor	Caudal sensory
Con	1	29	42.5 ± 0.6	30.9 ± 0.5	41.7 ± 0.4
	3	15	49.3 ± 0.7	37.8 ± 0.5	52.1 ± 0.7
Con50	1	29	43.3 ± 0.5	31.0 ± 0.4	42.2 ± 0.6
	3	11	49.7 ± 1.4	39.5 ± 1.2	50.9 ± 0.7
STZ	1	31	37.7 ± 0.6*	28.6 ± 0.6	36.2 ± 0.6
	3	19	41.8 ± 1.0*	34.5 ± 1.1	41.9 ± 1.1
STZ20	1	26	37.7 ± 0.7	28.8 ± 0.8	36.4 ± 0.8
	3	13	41.6 ± 1.2	33.7 ± 1.3	41.1 ± 1.3
STZ100	1	35	37.6 ± 0.5	28.6 ± 0.6	36.8 ± 0.7
	3	13	40.9 ± 1.2	31.0 ± 1.3	39.4 ± 1.4

Data are means ± SE. *P < 0.001 vs. control.

ripheral nerve. The diabetic sciatic nerve exhibits altered reducing equivalents (20), reduced norepinephrine (27), reduced SOD (2), increased conjugated dienes (2), reduced GSH (3), and reduced glutathione peroxidase (5). Experimental depletion of tissue α -tocopherol results in depletion in GSH, increased lipid peroxidation, and the development of a sensory neuropathy in previously normal nerves and worsens neuropathy in diabetic nerves (3). GSH has been reported to reduce the effects of oxidative stress in diabetes (7).

One concern with EDN, always associated with emaciation, is that the changes might be due to undernutrition alone. Rats fed an α -tocopherol-deficient diet also fail to gain weight as well as those fed a normal diet. We suspect that the pellets are less palatable but have been unable to correct it. This study demonstrates that undernutrition alone (group Con[R]), achieved by calorie restriction, resulted in a group that was well matched in weight with STZ and α -tocopherol-deficient groups; Con[R] had only a modest reduction in GSH and normal α -tocopherol concentrations, suggesting that the alterations in EDN are not primarily due to starvation. The modest GSH depletion induced by starvation could result from the increased hydrogen peroxide formation in liver, because of stimulated

β -oxidation (28). Moreover, our results agree with those of Pentieva et al. (29), in which the limited GSH depletion induced by fasting rats does not account for the increased lipid peroxidation induced by ethanol in those rats, and by Wohaieb and Godin (30), who found that starvation caused a mild reduction in both GSH and malondialdehyde.

In a previous study (3) and this

study, the plasma and tissue of EDN have the paradoxical combination of reduced GSH and increased α -tocopherol. Similar increases in α -tocopherol content had been reported to occur in plasma from both insulin-dependent (31) and -independent diabetic (32) patients, as well as in liver mitochondria from STZ-induced diabetic rats (33). The mechanism of this increase is uncertain. We had previously postulated that with increased oxidative stress, α -tocopherol can be increased or decreased (3). A simpler explanation, which we currently favor, is that the increased concentrations reflect the polyphagia that is characteristic of the diabetic state. When α -tocopherol is removed from the diet, its concentrations in EDN resemble those of other rats on a restricted vitamin diet. The alterations in plasma lipoproteins consistently found in the diabetic state (32), together with the increased availability of α -tocopherol due to polyphagia, could result in the retention of this lipophilic vitamin, yielding increased concentrations. One implication of this observation is that α -tocopherol deficiency is deleterious but that its ex-

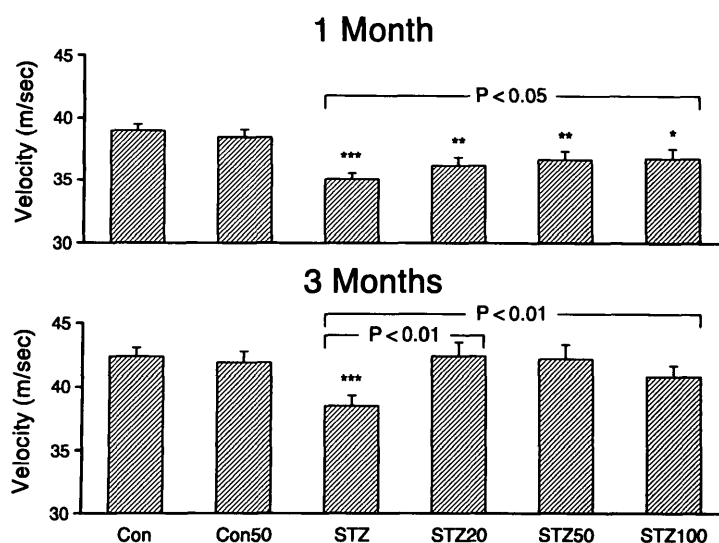


Figure 3—Digital nerve conduction velocity at 1 and 3 months of supplementation with LA at 0 mg/kg (Con, n = 29; STZ, n = 31), 20 mg/kg (STZ20; n = 26), 50 mg/kg (Con50; STZ50; n = 30), and 100 mg/kg (STZ100; n = 33) intraperitoneally. LA supplementation improved conduction velocity. * P < 0.05; ** P < 0.01; * P < 0.001.**

cess does not confer protection. We had previously attempted α -tocopherol supplementation in EDN and did not find any improvement in nerve function (3). Relevant to this discussion is the observation that hyperglycemia reduces the specific binding of α -tocopherol to endothelial cells (34). A similar mechanism in nervous tissue could result in increased levels of improperly located vitamin of reduced function.

In the present study, we have confirmed, with the GSH data, the previously demonstrated increased free radical activity (2,3) that occurs in EDN. LA is a powerful lipophilic free radical scavenger (10,35,36). In vivo, it is converted to dihydrolipoate (37), the antioxidant mechanisms of which include its action as a chain-breaking antioxidant, acting jointly with α -tocopherol (35,38), or as a metal chelator (39,40). Our results of a dose-dependent improvement in NBF and digital nerve conduction velocity with LA supplementation suggest that the neuropathy is due in significant part to oxidative stress and that improving free radical-scavenging capacity (as indicated by an improvement in GSH) is responsible for the improvement in both NBF and the neuropathy. The mechanism of improvement of NBF is uncertain. One mechanism of reduced NBF is the inhibitory effect of superoxide anion on nitric oxide synthase, with resultant reduced nitric oxide in EDN (41). LA, by reducing oxidative stress, could prevent this inhibition of nitric oxide synthase.

The lack of improvement in more proximal nerves, in spite of normal NBF and GSH, warrants discussion. Lipid peroxidation occurs in diabetic nerves because of endoneurial ischemia (2,42). Oxidative stress also occurs because of hyperglycemia per se (5,43–45). Oxygen free radicals will damage microvessels and are responsible for reduced reperfusion (46). Reduction of oxidative stress by LA presumably is the mechanism of prevention of the reduced NBF and the improvement in nerve conduction. The incomplete improvement seen in this study

and in other studies (7,22) suggests either that there is a selective vulnerability of certain fiber populations or that mechanisms other than lipid peroxidation are involved in the pathogenesis of diabetic neuropathy. An α -tocopherol-restricted diet causes the most dramatic reduction in autonomic neurons (SCG) followed by sensory neurons (DRG) and least of all the sciatic nerve trunk (3) (this study). It is possible that the electrophysiological changes reflect some of this selectivity. Motor conduction abnormalities are not improved. Caudal sensory nerve trunk conduction (strictly mixed nerve) is unimproved, whereas digital nerve, purely sensory, is improved. This selective improvement in digital nerves is of particular interest. In human diabetic patients, a major problem is the troublesome chronic distal complaints of burning feet, a condition often termed distal small-fiber neuropathy (47,48) and for which treatment is unsatisfactory.

The electrophysiological improvement caused by LA is also of interest because it additionally enhances glucose entry into cells, as demonstrated in experimental animals (11,49) and in humans using a glucose clamp (S. Jacob, E.J. Henriksen, H.J. Tritschler, D.E. Clancy, I. Simon, A.L. Schiemann, H. Ulrich, I. Jung, G.J. Dietze, H.J. Augustin, unpublished observations). Although endoneurial glucose is increased in diabetes, hyperglycemia inhibits glucose transport to all tissues studied (51–53), resulting in a relative reduction in cellular glucose. The same situation likely occurs in diabetic nerves, since it is known that the peripheral nerve contains a glucose transporter (54). The dual beneficial effects of LA, reduction of lipid peroxidation and enhancement of glucose entry, might be responsible for the clinical observation of improvement in painful diabetic neuropathy with LA (13,14).

Acknowledgments— This study was supported in part by grants (RO1-NS-22352, NS-14302) from the National Institute of Neuro-

logical and Communicative Diseases and Stroke, Bethesda, MD, Muscular Dystrophy Association, Asta, and Mayo Funds.

P.A.L. is the recipient of a Jacob Javits Neuroscience Investigator Award.

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