Effects of Acetyl- and Proprionyl-L-Carnitine on Peripheral Nerve Function and Vascular Supply in Experimental Diabetes

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L-Carnitine metabolism is abnormal in diabetes mellitus, and treatment with acetyl-L-carnitine (ALC) improves the function of cardiac muscle, retina, and peripheral nerve in experimental models. The aim was to compare the effects of ALC and proprionyl-L-carnitine (PLC) on motor and sensory nerve conduction in streptozotocin-diabetic rats and to ascertain whether their action could be mediated by a vascular mechanism. ALC and PLC treatment for 2 months after diabetes induction attenuated the development of sciatic motor nerve conduction velocity (NCV) deficits by 59.4% ± 4.4% and 46.9% ± 3.2%, respectively. There was a similar level of protection for sensory saphenous NCV (42.9% ± 6.6% and 47.8% ± 6.0%, respectively). Neither ALC nor PLC prevented the development of resistance to hypoxic conduction failure (RHCF) in sciatic nerve from diabetic rats. A 46.5% ± 3.4% deficit in sciatic endoneurial blood flow, measured by microelectrode polarography and hydrogen clearance, in diabetic rats was partially prevented by both ALC (48.7% ± 6.4%) and PLC (69.4% ± 10.1%). ALC had no significant effect on blood flow in nondiabetic rats. Thus, the data show that these L-carnitine derivatives have a similar efficacy in preventing nerve dysfunction, which depends on a neurovascular action.

REDUCED PERIPHERAL NERVE conduction velocity (NCV) and an increase in resistance to ischemic or hypoxic conduction failure (RHCF) are early abnormalities in patients with diabetes mellitus and in animal models.[1-4] In the long term, axon and Schwann cell degeneration may be sufficiently severe to cause clinical neuropathy. A neurochemical hypothesis, based on activity of the polyol pathway and impaired phosphoinositide metabolism leading to a reduction in neuronal Na^+-K^+–adenosine triphosphatase (ATPase), has been suggested to explain the etiology of diabetic neuropathy.[5] However, recently, evidence has accumulated to support the notion that the main target of metabolic changes in diabetes is nerve vascular supply rather than the neurons themselves. Thus, reduced blood flow and consequent endoneurial hypoxia may be a major factor in the pathogenesis, being responsible for early and late neuronal abnormalities.[6-10]

Carnitine metabolism is aberrant in diabetes, and plasma and myocardial concentrations of free L-carnitine are diminished.[11] L-Carnitine or acetyl-L-carnitine (ALC) treatment corrects some changes in cardiac biochemistry and function in diabetic rats.[12,13] More recently, ALC was found to prevent electroretinographic abnormalities,[14] reduced peripheral NCV,[15,16] and increased albumin permeability in ocular tissues and nerve[17] of diabetic rats. In nondiabetic rats, ALC improves nerve regeneration after crush injury[18] and attenuates age-related neuromuscular dysfunction.[19] The mechanism(s) of ALC effects are not known in detail, although there have been several suggestions. Cellular energy production may be improved as carnitine increases the transport of long-chain fatty acids into mitochondria for β-oxidation. The acetate moiety could also act as an energy source and may further enhance mitochondrial lipid handling.[20] Increasing the metabolism of long-chain acyl derivatives, which accumulate in diabetes, could be beneficial, since these amphipathic molecules interfere with many membrane processes, for example, sarcoplasmic reticulum Ca^{2+} ATPase activity.[12] ALC is naturally occurring and can act as a precursor for acetylcholine synthesis by neurons.[21] It also has neurotrophic properties that enhance responses to and synthesis of growth factors.[22]

The aim of this study was to compare the effects of ALC and proprionyl-L-carnitine (PLC) in preventing changes in NCV and RHCF in diabetic rats and, by measuring sciatic nerve blood flow, to determine whether these compounds have a neurovascular action.

MATERIALS AND METHODS

Experimental Groups and Diabetes Induction

Male Sprague-Dawley rats (Aberdeen University breeding colony) aged 19 weeks at the start of the study were used. Diabetes was induced by streptozotocin freshly dissolved in sterile 154-mmol · L^{-1} NaCl solution (40 to 45 mg · kg^{-1} intraperitoneally [IP]), and was verified 24 hours later by estimating hyperglycemia and glycosuria (Visidex II and Diastix; Ames, Slough, UK). Animals were tested weekly and weighed daily. They were rejected if blood glucose level was less than 20 mmol · L^{-1} or if they showed a consistent increase in body weight over 3 days. Samples for plasma glucose measurement using a standard test kit (GOD-Perid method; Boehringer, Mannheim, Germany) were taken on the day of final experiments.

Four groups of rats were used: nondiabetic controls, 2-month untreated diabetic controls, and 2-month diabetic groups treated from induction with ALC or PLC (Sigma Tau, Rome, Italy) in the drinking water at an approximate dose of 500 mg · kg^{-1} · d^{-1}. The dose of ALC was chosen after pilot studies using 250 mg · kg^{-1} · d^{-1} showed a motor NCV–prevention effect that was approximately 1 to 2 m · s^{-1} less than with 500 mg · kg^{-1} · d^{-1}. Separate groups were used for NCV, RHCF, and blood flow studies. A nondiabetic group was also treated with 500 mg · kg^{-1} · d^{-1} ALC for 2 months, primarily for blood flow measurements.

NCV and RHCF

In final experiments (1.0 to 1.5 g · kg^{-1} urethane anesthesia IP), NCV was measured in vivo between the sciatic notch and the knee

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Submitted September 17, 1994; accepted January 24, 1995.

Supported by a Wellcome Trust Research Leave Fellowship (N.E.C.) and Scotia Pharmaceuticals Research Studentship (K.C.D.).

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0026-0495/95/4409-0019$03.00/0

for sciatic motor branches supplying tibialis anterior (peroneal division) and gastrocnemius (tibial division) muscles. Sensory NCV was measured in the saphenous nerve between the groin and ankle. Rectal and nerve temperatures were monitored and regulated between 36.5° and 37.5°C. Methods have previously been described in detail.[23]

Sciatic nerve hypoxic resistance was measured in vitro for nerves from the contralateral leg as previously described.[24] The contralateral sciatic trunk was removed and mounted on bipolar stimulating (proximal end) and recording (distal end) electrodes in a chamber containing Krebs-Ringer solution at 35°C, with 5.5 mmol/L glucose for nerves from nondiabetic rats and 40 mmol/L glucose for diabetic rats. A previous study showed that short-term exposure to bath glucose concentrations between 5.5 and 40 mmol/L did not have a significant effect on RHCF in diabetic or nondiabetic rats (N.E. Cameron, M.A. Cotter, and D. Cox, unpublished observations February 1989). Bathing fluid was gassed with 95% O_2–5% CO_2. Nerves were equilibrated for 30 minutes, and then the chamber was refilled with mineral oil pregassed for 1 hour with 100% N_2. Nerves were stimulated with just supramaximal pulses (1 Hz, 0.05 ms width, 10 mA), and compound action-potential amplitude was monitored at 2-minute intervals until it was less than 10% of its initial value.

Sciatic Nerve Blood Flow

Rats were anesthetized with inactin (50 to 100 mg · kg^{-1} IP), the trachea was cannulated for artificial ventilation, and a carotid cannula was used to monitor mean systemic blood pressure. Core temperature of the animal was monitored and regulated between 37° and 38°C using a rectal probe and radiant heat. The skin around the sciatic nerve incision was sutured to a metal ring and used to form a pool that was filled with mineral oil at 37°C to a depth of at least 1 cm to minimize diffusion of gasses directly to or from the nerve. Rats were given neuromuscular blockade using D-tubocurarine (Sigma, Poole, Dorset, UK) 2 mg · kg^{-1} via the carotid cannula and artificially ventilated. The level of anesthesia was monitored by observing any reaction of blood pressure to manipulation, and supplementary inactin was given as necessary. Nerve blood flow was measured by microelectrode hydrogen polarography as previously described.[7,25] Briefly, a glass-insulated platinum microelectrode (tip diameter, 2 to 8 μm) was inserted into the middle portion of the sciatic nerve, above its trifurcation, and polarized at 0.25 V with respect to a reference electrode inserted subcutaneously in the flank of the rat. H_2 10% was added to the inspired gas, with the proportions of O_2 and N_2 being adjusted to 20% and 70%, respectively. When the H_2 current recorded by the electrode had stabilized (20 to 30 minutes), indicating equilibrium with arterial blood, H_2 supply was shut off and N_2 delivery was increased appropriately. The H_2 clearance curve was recorded until baseline (30 minutes to 1 hour). This procedure was then repeated at another nerve site. After the experiment, clearance curves were digitized and monoexponential or biexponential curves were fitted to the data by computer using appropriate software (Inplot; Graphpad, San Diego, CA). The slow exponent, representing nutritive flow, was accepted.[7] The average of the two determinations was taken to represent sciatic endoneurial blood flow. Vascular conductance was calculated by dividing blood flow by mean arterial blood pressure during the recording period.

Statistical Analysis

Data were subjected to Bartlett's test for homogeneity of variances, and where necessary (blood pressure and vascular conductance results) they were normalized by log transformation before being tested using one-way ANOVA. Where significance was attained ($P < .05$), between-group differences were established using the Student-Neuman-Keuls multiple comparison test (Instat; Graphpad). Data are expressed as the mean ± SEM.

RESULTS

Body weights and plasma glucose levels for the groups are listed in Table 1. Diabetes caused a high level of hyperglycemia, and body weight was reduced by approximately 35% over 2 months. Treatment with ALC or PLC did not significantly affect these parameters.

Data for motor (Fig 1A and B) and sensory (Fig 1C) NCV showed 19.4% ± 0.7% ($P < .001$) and 14.3% ± 1.1% ($P < .001$) reductions after 2-month untreated diabetes, respectively. Attenuation of the mean sciatic motor deficit was 59.4% ± 4.4% for ALC ($P < .001$) and 46.9% ± 3.2% for PLC ($P < .001$). For both treatments, motor NCV to tibialis anterior (Fig 1A) and gastrocnemius (Fig 1B) muscles remained significantly ($P < .001$) reduced as compared with nondiabetic rats, and the slightly lower protection provided by PLC as compared with ALC for the gastrocnemius branch was statistically significant ($P < .05$). Saphenous sensory NCV (Fig 1C) deficits were also partially prevented by ALC (42.9% ± 6.6%, $P < .001$) and PLC (47.8% ± 6.0%, $P < .001$). ALC treatment of the group of nondiabetic rats used for blood flow measurements did not significantly affect motor NCV to tibialis anterior muscle (65.6 ± 0.9 *v* 63.2 ± 0.7 m · s^{-1} for the onset control group).

RHCF (Fig 2) was markedly increased by 2 months of diabetes. Thus, the duration of hypoxia necessary to reduce sciatic compound action-potential amplitude by 50% was 15.2 ± 0.5 minutes in nondiabetic rats, and was increased to 25.8 ± 1.2 ($P < .001$) by untreated diabetes. Values for ALC (24.5 ± 1.0 minutes) and PLC (25.8 ± 1.4) were similarly elevated as compared with nondiabetic controls ($P < .001$) and were not significantly different from values for untreated diabetes. There was a small (34%, $P < .05$) reduction in the initial sciatic nerve compound action-potential amplitude (Fig 2, inset) with diabetes, which was not significantly affected by treatment.

The nutritive (capillary) component of endoneurial blood flow (Fig 3A) was reduced 46.5% ± 3.4% ($P < .001$) by untreated diabetes. ALC and PLC treatments had similar effects in diabetic rats and partially prevented the decrease in perfusion by 48.7% ± 6.4% and 69.4% ± 10.1%, respectively (both $P < .001$), although the flow remained significantly reduced ($P < .001$ for ALC and $P < .05$ for

Table 1. Body Weights and Plasma Glucose Concentrations of Rat Groups

Group	Body Weight (g)		Plasma Glucose (mmol/L)
	Start	End	
Nondiabetic (n = 20)	479 ± 7	—	8.0 ± 0.3
Nondiabetic ALC-treated (n = 10)	466 ± 6	506 ± 6	7.1 ± 0.3
2-mo diabetic (n = 18)	449 ± 9	293 ± 9	41.2 ± 1.7
2-mo diabetic ALC-treated (n = 17)	451 ± 8	294 ± 11	42.1 ± 2.0
2-mo diabetic PLC-treated (n = 18)	486 ± 7	299 ± 8	40.2 ± 3.1

NOTE. Mean ± SEM.

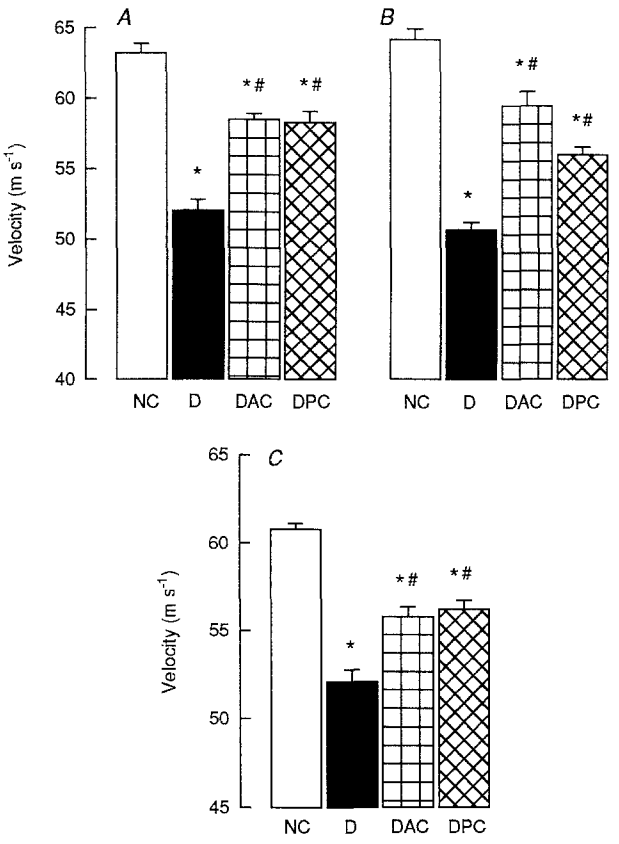

Fig 1. Effects of diabetes and ALC and PLC treatment on sciatic motor NCV to tibialis anterior (A) and gastrocnemis (B) muscles and sensory NCV in saphenous nerve (C). NC, nondiabetic control group (n = 10); D, 2-month diabetic group (n = 10); DAC, 2-month diabetic group treated with ALC 500 mg/kg/d (n = 9); DPC, 2-month diabetic group treated with PLC 500 mg/kg/d (n = 10). Mean ± SEM. *P < .001 *v* NC group; #P < .001 *v* D group.

PLC) as compared with nondiabetic control levels. Flow was not significantly altered in nondiabetic rats treated with ALC for 2 months. Systemic arterial blood pressure was lower in treated and untreated diabetic rats than in nondiabetic rats: 142.3 ± 8.2 and 140.7 ± 4.5 mm Hg for untreated and ALC-treated nondiabetic groups, respectively, as compared with 118.1 ± 2.3 (P < .01) for untreated, 109.0 ± 2.8 (P < .001) for ALC-treated, and 120.3 ± 5.0 (P < .05) for PLC-treated diabetic groups. When this reduction in perfusion pressure was taken into account by expressing the data as vascular conductance (Fig 3B), there remained a 37.6% ± 3.1% deficit with diabetes (P < .001), which was largely prevented by ALC (P < .001) and PLC (P < .001) treatments, with the resultant values being within the nondiabetic range.

DISCUSSION

Both ALC and PLC partially prevented NCV deficits in diabetic rats. Motor effects agree with reports for ALC,[15-17] but saphenous sensory NCV changes have not previously been described. The degree of prevention of NCV deficits was comparable to other investigations. Sixteen weeks' ALC treatment (150 mg/kg) prevented reduced motor NCV to the interosseous muscles of the foot by 69%,[15] and 6 weeks' treatment (200 to 250 mg/kg) produced 46%[16] or complete[17] protection for caudal nerve. Although ALC was slightly more effective than PLC for gastrocnemius NCV, the overall degree of protection was similar (~50%) for both compounds. This emphasizes the importance of the L-carnitine moiety, in line with a recent report that reduced caudal NCV was prevented by L-carnitine treatment.[17] The ALC effect is unlikely to depend on acetylcholine synthesis[21] from an "activated" acetyl group, since PLC would not fulfill this role. Previous studies have shown that ALC does not affect nerve polyol or *myo*-inositol concentration.[15,17]

The deficit in endoneurial nutritive perfusion with diabetes is in good agreement with other studies using hydrogen clearance.[7,25,26-30] Both ALC and PLC were similarly effective in partially preventing this neurovascular deficiency, consistent with the degree of NCV protection. Thus, the main action of L-carnitine derivatives was probably on nerve vascular supply rather than a direct neuronal effect. However, ALC is not a general vasodilator, since nerve perfusion was unaffected in nondiabetic rats. In contrast, noradrenergic blockade and nitrovasodilators elevate nerve blood flow similarly in nondiabetic and diabetic rats.[7,31,32] A vascular effect from the prevention by ALC/PLC of specific

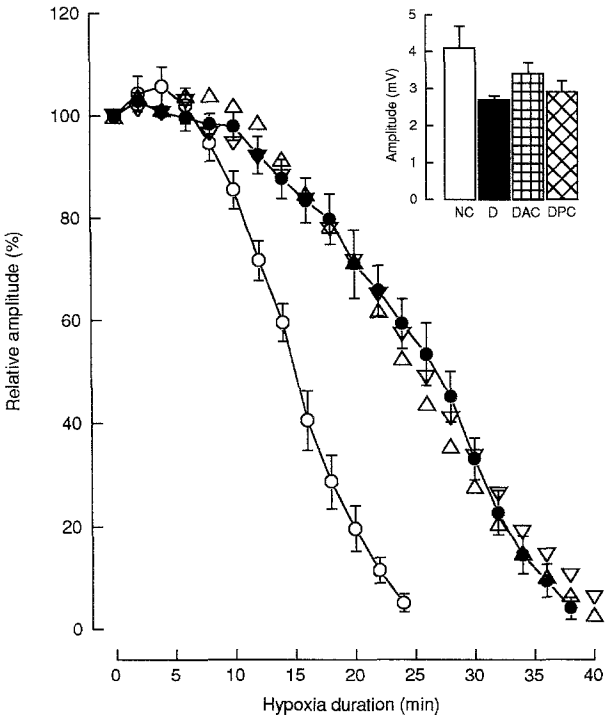

Fig 2. Sciatic nerve RHCF in vitro for nondiabetic rats and diabetic rats with and without ALC or PLC treatment. (○) Nondiabetic control group, n = 7; (●) 2-month diabetic group, n = 10; (△) 2-month diabetic group treated with ALC 500 mg/kg/d, n = 8; (▽) 2-month diabetic group treated with PLC 500 mg/kg/d, n = 8. Error bars are SEM and have been omitted from the treated diabetic groups for clarity. Inset, initial compound action-potential amplitude. After 8 minutes' hypoxia, relative compound action-potential amplitude was greater in diabetic and treated diabetic rats than in the nondiabetic group (P < .05). There were no significant differences between diabetic and ALC- and PLC-treated diabetic groups.

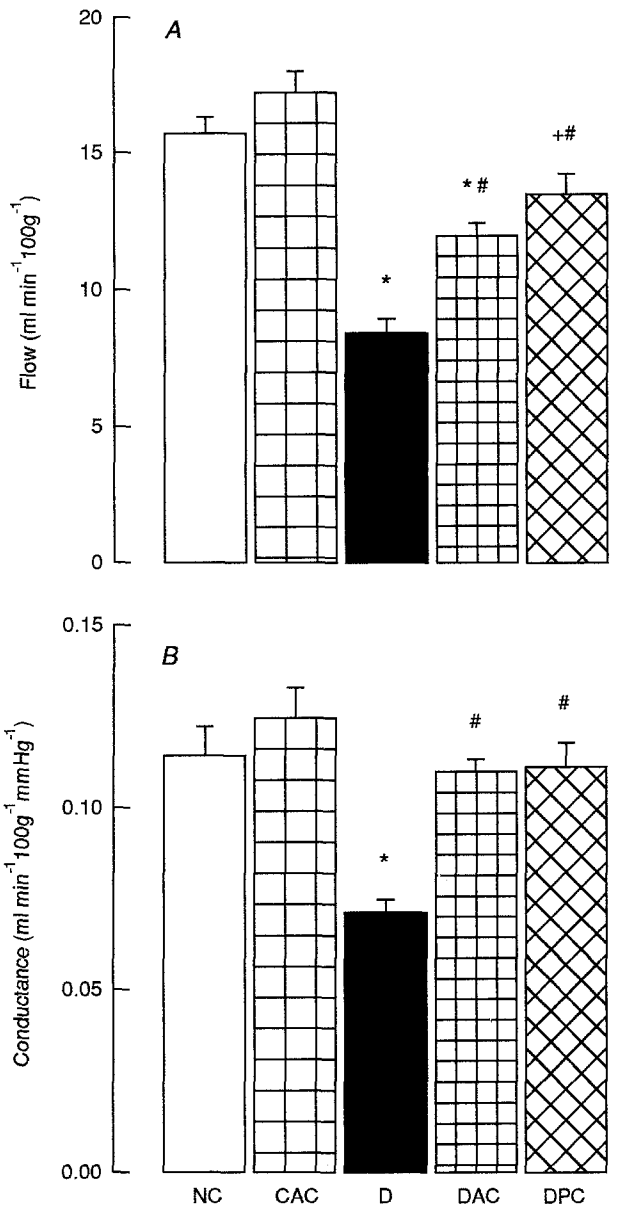

Fig 3. Effects of ALC and PLC on sciatic nutritive endoneurial blood flow (A) and vascular conductance (B). CAC, nondiabetic group treated for 2 months with ALC 500 mg/kg/d (n = 9); D, n = 8; DAC, n = 8; DPC, n = 8. Mean ± SEM. *$P < .001$, +$P < .05$ *v* NC group; #$P < .001$ *v* group.

diabetic abnormalities is consistent with the outcome of other metabolic interventions such as aldose reductase inhibition,[29] antioxidants,[28] ω6 essential fatty acids,[30] and aminoguanidine,[26] which increase nutritive flow in diabetic but not nondiabetic rats.

ALC and PLC did not alter RHCF, in contrast to NCV and blood flow effects. RHCF mainly reflects an adaptation to the hypoxic endoneurium, involving increased reliance on anaerobic energy metabolism.[6] It occurs in nondiabetic rats reared under hypoxic conditions or with chronic manipulations that reduce nerve blood flow.[33,34] ALC- and PLC-mediated increases in nerve perfusion would partially reduce endoneurial hypoxia; however, this may not be sufficient to prevent RHCF development, which is considerably more resistant than NCV to treatments that elevate blood flow.[8] In addition, hyperglycemic exposure can independently stimulate nerve anaerobic metabolism.[35] Thus, the continuing presence of RHCF in ALC/PLC-treated diabetic rats is consistent with an incomplete prevention of nerve perfusion deficits.

This study suggests that L-carnitine effects are mediated largely by a vascular action in nerve; however, the precise mechanism is unclear. One hypothesis emphasizes "hyperglycemic pseudohypoxia," and thus an increased cytosolic NADH/NAD^+ ratio due to enhanced polyol pathway flux may lead to cellular dysfunction via a complex of effects on intermediate metabolism.[36] Parallels have been drawn with changes in NADH/NAD^+ ratio for ischemia, which is pertinent since endoneurial hypoxia is well documented in diabetic rats and patients.[9,25,28-30] Pseudohypoxic and true hypoxic effects may be additive. Vascular abnormalities that indirectly affect nerve function could also have a potential contribution from pseudohypoxia.[36]

A potential role for L-carnitine in this complex metabolic hypothesis has been suggested.[17,36] An increased mitochondrial NADH/NAD^+ ratio inhibits β-oxidation of fatty acids. Together with a reduction in tissue L-carnitine, which is necessary for mitochondrial long-chain fatty acid transport, this may be responsible for accumulation of long-chain fatty acid esters, as noted in the heart.[11-13] These amphipathic molecules modulate the function of enzymes such as Na^+-K^+-ATPase, Ca^{2+}-ATPase, and protein kinase C. Empirically, treatment with L-carnitine, ALC, or PLC attenuates the accumulation of long-chain fatty acid esters and consequent functional effects, for example, in cultured myocytes or granulation tissue vessels.[17,36]

Against arguments in favor of a pseudohypoxia hypothesis applied to peripheral nerve and vasa nervorum, other findings must be considered. Vasodilator treatment corrects NCV and blood flow deficits without altering the main cause of pseudohypoxic changes in the NADH/NAD^+ ratio, polyol pathway activity.[37,38] Therefore, direct polyol/pseudohypoxic effects on nerve fibers are probably unimportant as compared with the endoneurial hypoxia consequent to reduced blood flow. Pseudohypoxia may contribute to changes in RHCF, involving greater anaerobic metabolism, which can result from hyperglycemia.[35] This effect is glucose-specific in vitro, being absent with galactose exposure,[39] which is consistent with dependence on flux through the second half of the polyol pathway; NADH formation is catalyzed by sorbitol dehydrogenase, for which galactitol is a poor substrate. However, galactosemic and diabetic rats show comparable RHCF,[40] and in both cases reduced endoneurial oxygenation appears to be responsible. This is caused by the blood flow defect in diabetes, but mainly results from increased oxygen diffusion distances due to endoneurial edema in galactosemia.[41] Thus, although pseudohypoxia probably does not directly affect nerve fibers, the blood flow data could suggest effects on vasa nervorum, which would indirectly cause neuronal dysfunction.

Increased diacylglycerol levels have been found in the

aorta of chronically galactosemic and diabetic dogs and in rat endothelial and smooth muscle cells cultured under high glucose or galactose concentrations.[42] These data indirectly support a hypothesis involving stimulation of protein kinase C by diacylglycerol, which may account for reductions in retinal blood flow in diabetic rats.[43] Interestingly, ALC treatment reduced diacylglycerol levels in nerves of diabetic rats.[17] Thus, L-carnitine–mediated correction of neurovascular insufficiency may depend on alterations in diacylglycerol metabolism, which requires further investigation.

Studies characterizing potential actions of L-carnitine on vascular smooth muscle or endothelium in diabetes have not been reported, although the blood flow data suggest that effects are likely. Endothelium-dependent relaxation is impaired by diabetes, a phenomenon linked to some of the metabolic changes that alter nerve function: polyol pathway, advanced glycation, and oxidative stress.[44-47] Diminished ATP supply depresses nitric oxide production by endothelial cells.[48] ATP synthesis could be limited by pseudohypoxia or other mechanisms. Increased β-oxidation of fatty acids, stimulated by ALC and PLC,[20] might increase ATP synthesis in the endothelium of resistance vessels, where, unlike nerve endoneurium,[6] oxygen availability would not limit mitochondrial metabolism. Long-chain acyl coenzyme A accumulation in diabetes inhibits mitochondrial transport of adenine nucleotides, which could further compromise ATP production.[49] ALC and PLC treatments prevent the accumulation of these amphipathic molecules. Ca^{2+} is also required for nitric oxide synthesis by endothelium,[50] and Ca^{2+} homeostasis is altered in several diabetic tissues.[12,13,50] Elevated vascular reactivity to norepinephrine in diabetic rats partly depends on increased activity/number of smooth muscle voltage-sensitive Ca^{2+} channels, the result being greater reliance on extracellular Ca^{2+} for contraction.[51] This may be an adaptation to impaired sarcoplasmic reticulum function caused by the inhibitory effect of long-chain fatty acid esters on Ca^{2+}-ATPase pump activity.[49,52] Thus, as in cardiac muscle,[12,13,36] L-carnitine may improve Ca^{2+} handling. Together, L-carnitine effects on ATP synthesis and Ca^{2+} regulation could partially correct endothelium and smooth muscle function and therefore nerve blood flow.

In conclusion, whatever the exact mechanism of ALC and PLC neurovascular action, they both partially prevent nerve dysfunction in diabetic rats. Thus, L-carnitine derivatives could have a potentially important dual action against clinical neuropathy in diabetic patients: namely improvement of endoneurial perfusion and, via a trophic effect,[18,22] stimulation of fiber regeneration.

ACKNOWLEDGMENT

We are grateful to Dr Z. Orfalian and Sigma-Tau for the supply of L-carnitine derivatives.

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