The Effects of Treatment With α-Lipoic Acid or Evening Primrose Oil on Vascular Hemostatic and Lipid Risk Factors, Blood Flow, and Peripheral Nerve Conduction in the Streptozotocin-Diabetic Rat

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Oxidative stress and defective fatty acid metabolism in diabetes may lead to impaired nerve perfusion and contribute to the development of peripheral neuropathy. We studied the effects of 2-week treatments with evening primrose oil (EPO; n = 16) or the antioxidant α-lipoic acid (ALA; n = 16) on endoneurial blood flow, nerve conduction parameters, lipids, coagulation, and endothelial factors, in rats with streptozotocin-induced diabetes. Compared with their nondiabetic littermates, untreated diabetic rats had impaired sciatic motor and saphenous sensory nerve-conduction velocity (NCV; *P* < .001), reduced endoneurial blood flow (*P* < .001), and increased serum triglycerides (*P* < .01), cholesterol (*P* < 0.01), plasma factor VII (*P* < .0001), and von Willebrand factor (vWF; *P* < .0001). Plasma fibrinogen and serum high-density lipoprotein concentrations were not significantly different. Treatment with either ALA or EPO effectively corrected the deficits in NCV and endoneurial blood flow. ALA was associated with marked and statistically significant decreases in fibrinogen, factor VII, vWF, and triglycerides (*P* < .01, paired *t* tests before *v* after treatment). In contrast, EPO was associated with significant (*P* < .05) increases in fibrinogen, factor VII, vWF, triglycerides, and cholesterol and a significant decrease in high-density lipoprotein. Changes in levels of coagulation factors and lipids, qualitatively similar to those found with EPO, were obtained with a diet containing sunflower oil (to control for calorific and lipid content) or with a normal diet alone. Blood glucose and hematocrit levels were not significantly altered by treatments. These data suggest that although both ALA and EPO improve blood flow and nerve function, their actions on vascular factors differ. The marked effects of ALA in lowering lipid and hemostatic risk factors for cardiovascular disease indicate potential antithrombotic and antiatherosclerotic actions that could be of benefit in human diabetes and merit further study.

IMPAIRED nerve perfusion is an important factor in the development of diabetic peripheral neuropathy.[1-3] Reduced nerve oxygen tension has been demonstrated in patients with mild to moderate diabetic peripheral neuropathy,[1,4] and athough there is some controversy over measurement of nerve blood flow in humans,[5] several technically sophisticated studies have found a reduction in sural nerve blood flow.[1,4] In the streptozotocin-induced diabetic rat model, it has been more clearly shown that reduction in endoneurial blood flow is an early event that precedes the development of decreased nerve-conduction velocity (NCV).[6]

Oxidative stress and defective fatty acid metabolism are important factors in the development of impaired nerve blood flow and function. Thus, treatment of diabetic rats with vasodilators, antioxidants, and omega-6 essential fatty acids corrects both nerve blood flow and NCV deficits.[7,8] Furthermore, clinical neuropathy trials have shown some beneficial effects of treatment with evening primrose oil (EPO), a source of omega-6 γ-linolenic acid,[9] and the antioxidant α-lipoic acid (ALA).[10]

The mechanisms underlying the reduced nerve perfusion in diabetes are the subject of debate. The importance of abnormalities of blood viscosity and the coagulation system have been stressed,[11] as have dysfunction of vascular endothelium and smooth muscle.[12,13] To clarify these mechanisms further, plasma levels of fibrinogen, factor VII, and von Willebrand factor (vWF) were measured before and after 2 weeks of treatment with doses of EPO or ALA that were effective in correcting neurovascular deficits in diabetic rats. Fibrinogen, the precursor of fibrin, is an essential adhesive molecule in platelet activation, a major determinant of blood viscosity, and an important component of the inflammatory response. Elevated plasma fibrinogen is an independent risk factor for vascular disease[14] and is associated with the development of diabetic microvascular and macrovascular complications.[15,16] In patients with diabetic neuropathy, plasma fibrinogen levels correlated with capillary wall thickening in the sural nerve.[17] Factor VII in its proenzyme form interacts with tissue factor and calcium ions to initiate coagulation. In its activated 2-chain form, VIIa, the enzyme's ability to activate factor X is enhanced up to 100-fold. In the general population, increased plasma factor VII levels are associated with mortality from cardiovascular disease.[18] vWF is a multimeric glycoprotein synthesized in endothelial cells and megakaryocytes. It is essential for platelet adhesion to vessel walls at high shear rates, and it also acts as a carrier protein and regulator of factor VIIIc. Elevated plasma levels of vWF reflect vascular activation or damage.[19] Increased vWF levels were found in a population study of patients with type 1 diabetes and neuropathy,[20] and vWF was predictive of deteriorating nerve function in a prospective study of patients with type 1 diabetes.[21] Stehouwer et al[22] found that increases in vWF levels over 3 years were predictive of microalbuminuria and nephropathy but not of the development of retinopathy.[23]

Plasma vWF levels are also elevated in rats with streptozotocin-induced diabetes,[24] and the mesenteric vessels show enhanced immunohistochemical staining for vWF.[25] In contrast,

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there is little published information on changes in plasma fibrinogen or factor VII in the rat model.

MATERIALS AND METHODS

Experiments were performed in accordance with the regulations specified in the United Kingdom Animal Procedures Act, 1986, and the National Institutes of Health Principles of Laboratory Animal Care, 1985 revised version. Animals were housed 2 to a cage and weighed daily, and food intake was monitored on a groupwise basis.

Adult (age 19 weeks) male Sprague-Dawley rats were used, as in previous studies, because it is known that axonal growth has stopped at this point. This allows us to interpret differences in nerve function between control and diabetic rats as being caused by the hyperglycemic insult and independent of the effects of impaired axonal development.[26] Rats were made hyperglycemic by a single intraperitoneal injection of streptozotocin 45 mg/kg in sterile saline (Zeneca Pharmaceuticals, Macclesfield, Englnd). For 6 weeks after induction of diabetes, the rats received no treatments and were fed on normal diet with free access to drinking water. Average daily food intake was 25 g/d in nondiabetic animals and 50 g/d in diabetic animals. Baseline blood samples were taken just before the beginning of dietary supplementation. Treatment was with dietary supplements of ALA (ASTA-Medica, Frankfurt am Main, Germany) to give a dose of approximately 300 mg/kg body weight per day, or with EPO (containing 8% γ-linolenic acid) to obtain a dose of approximately 10 g/kg body weight per day, or with the same dose of sunflower oil as a calorific and lipid control. Treatment was continued for 2 weeks, and then posttreatment blood samples were collected. A group of untreated diabetic rats was studied at the same time points to control for variability over the 2-week period attributable to aging or progression of disease. For each set of experiments, control nondiabetic groups were studied under the same conditions.

Blood Measurements

Blood for coagulation tests was drawn from halothane-anesthetized rats by cardiac puncture before and after treatment, using a 21-gauge needle, allowing blood to mix directly with trisodium citrate (0.11 mol/L) in a 2-mL polypropylene syringe. After treatment, an additional 10 mL of blood was collected into a plain glass tube, allowed to clot, and centrifuged at 3,000*g* for 10 minutes to facilitate separation of serum. Anticoagulated blood was dispensed into Eppendorf tubes and centrifuged at 11,000*g* for 3 minutes. Plasma was separated into a clean tube, and the centrifugation step was repeated to ensure removal of platelets from the plasma. This double-spun plasma was separated and stored at −70°C.

Plasma fibrinogen was measured by Clauss assay[27] using Fibriquik thrombin reagent (Organon Teknika, Cambridge, England) and detection by automated coagulometer. Human reference plasma (Immuno Coagulation Reference; Technoclone Ltd, Surrey, England) was used as calibrator.

Plasma factor VII was assayed by 1-stage manual clotting assay using factor VII immune-depleted human plasma (Technoclone Ltd, Surrey, England) and rabbit thromboplastin (Manchester reagent; Helena Laboratories, Tyne & Wear, England).

Plasma vWF antigen was assayed by an in-house enzyme-linked immunosorbent assay (ELISA) method using polyclonal antibody and horseradish peroxidase–conjugated antibody from Dako Ltd, Denmark. Pooled normal rat plasma was prepared and used as a standard in each factor VII and vWF assay, and results were expressed as percentages of normal pool values. Serum lipid levels (total triglycerides, total cholesterol, and high-density lipoprotein [HDL] cholesterol) were estimated using kits from Randox Laboratories Ltd, County Antrim, Ireland, on a Cobas-Fara analyzer (Roche Diagnostics, East Sussex, England). Plasma glucose was measured by the glucose oxidase-peroxidase method (Boehringer, Mannheim, Germany). Haematocrit (packed cell volume) was measured in ethylenediaminetetraacetic acid (EDTA)–anticoagulated blood by the microcapillary method.

Tail Bleed Time

Under halothane anesthesia, rats' tails were warmed for 2 minutes in a beaker of water at 37°C. The distal 1 cm of tail was then rapidly removed with a scalpel. With the tail held perpendicular to the supine body of the rat, the time required for 0.5 mL of blood to collect in a sample tube was measured. A shorter bleed time thus indicates a higher rate of blood loss.

NCV and Blood Flow

Rats were anesthetized with thiobutabarbitone by intraperitoneal injection (50 to 100 mg/kg). The trachea was cannulated for artificial ventilation, and a carotid cannula was used to monitor mean systemic blood pressure. Motor NCV was measured as previously described[26] between the sciatic notch and knee in the nerve branch to the tibialis anterior muscle, which is representative of the whole sciatic nerve in terms of susceptibility to diabetes and treatment effects. Saphenous sensory NCV was measured between the groin and ankle as previously described.[26]

Sciatic nerve blood flow was measured by H_2 clearance as previously described.[7,28] in the nerve contralateral to the one used to estimate NCV. Briefly, the nerve was exposed between the sciatic notch and the knee, and the skin around the incision was sutured to a metal ring to form a pool that was filled with mineral oil maintained at 35° to 37°C by radiant heat. Core temperature was monitored by a rectal probe and kept within the range of 37° to 38°C. Rats were given neuromuscular blockade using *d*-tubocurarine (Sigma-Aldrich Chemical Co, Poole, Dorset, England) at a dose of 2 mg/kg via the carotid cannula and were artificially ventilated. The level of anesthesia was monitored by observing any reaction of blood pressure to manipulation, and supplementary anesthetic was given as necessary. A glass-insulated platinum microelectrode was inserted into the middle portion of the sciatic nerve and polarized at 250 mV with respect to a subcutaneous reference electrode. Ten percent H_2 was added to the inspired gas, and the proportions of O_2 and N_2 were adjusted to 20% and 70%, respectively. When the H_2 current recorded by the electrode had stabilized, indicating equilibrium with arterial blood, the H_2 supply was shut off and N_2 delivery was increased appropriately. The H_2 clearance curve was recorded until a baseline was achieved, defined as no systematic decline in electrode current over 5 minutes. 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 nonlinear regression software (Inplot; Graphpad, San Diego, CA). The slow exponent was used to give a measure of nutritive (capillary) blood flow.[29] Endoneurial nutritive vascular conductance was calculated by dividing flow by the mean arterial blood pressure during the recording period. The averages of the 2 H_2 clearance determinations were considered representative of sciatic endoneurial perfusion parameters.

Statistical Analysis

The Student paired *t* test was used to compare data from rats before and after treatments. Results were considered significant at the 5% level. Other data were subjected to 1-way analysis of variance (ANOVA), followed by Student-Newman-Keuls multiple-comparison tests to estimate the significance of differences for individual between-group comparisons. Where inequalities of variance were encountered (Bartlett's test), the data were first given a log transformation to equalize variances.

RESULTS

Plasma glucose was measured in a sample of 12 diabetic rats (4 from each of 3 treatment groups) before and after treatment to verify hyperglycemia. Plasma glucose levels did not change significantly between 6 and 8 weeks of diabetes (before, 31.3 ± 1.0 mmol/L; after, 32.3 ± 1.0 mmol/L; mean ± SEM). There was a mean weight loss of 22.9% (SEM 1.02) from the onset of diabetes to the start of treatment experiments. There was no further significant weight change in untreated rats or rats treated with EPO or sunflower oil, but the rats treated with ALA continued to lose weight (mean weight loss 4.3% in diabetic, 5.5% in nondiabetic; $P < .001$ *v* pretreatment; Table 1). Hematocrit was unchanged by diabetes or treatments.

Hemostatic Factors in Diabetic and Nondiabetic Rats

When rats after 6 weeks of diabetes were compared with their nondiabetic littermates, there was no significant difference in plasma fibrinogen levels, but levels of factor VII and vWF antigen were both significantly elevated ($P < .001$) by diabetes (Fig 1). In time-course experiments, factor VII and vWF were already significantly higher at 1 week after streptozotocin injection (factor VII, 164.75% ± 12.9%; vWF, 154.75% ± 14.66%; mean ± SEM) than in nondiabetic rats (factor VII, 115.2% ± 5.45%; vWF, 104.0% ± 4.0%; $P < .01$) and continued to increase progressively over time (Fig 2).

Effects of Treatment on Hemostatic Factors

In a group of 16 diabetic rats, after supplementation with ALA for 2 weeks, there was a marked decrease in plasma levels of fibrinogen ($P < .01$), factor VII ($P < .001$), and vWF ($P < .001$; Fig 3A). In contrast, over 2 weeks with normal diet alone, there was a nonsignificant trend toward increases in factor VII and vWF (Fig 3B). After dietary supplementation with sunflower oil (Fig 3C), there was a marked increase in factor VII ($P < .001$), a small increase in vWF ($P = .04$), and no significant change in plasma fibrinogen. Treatment of diabetic rats with EPO (Fig 3*D*) was associated with significant increases in plasma factor VII ($P < .001$), fibrinogen ($P < .01$), and vWF ($P = .02$) levels.

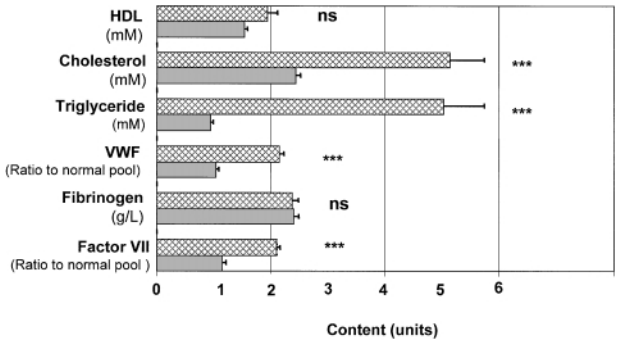

Fig 1. Plasma factor VII (% of normal pool), fibrinogen (g/L), vWF (% of normal pool); and serum triglyceride, cholesterol, and HDL (mmol/L) in rats with streptozotocin-induced diabetes (▨) compared with nondiabetic rats (■). ***$P < .001$; ns, not significant ($P > .05$). Mean ± SEM.

Because of the potentially procoagulant changes resulting from EPO/sunflower oil treatment of diabetic rats, the effects were examined in nondiabetic animals (Table 2). There was a small increase in factor VII after EPO supplementation in nondiabetic rats ($P = .04$) but no other significant changes after treatment with either EPO or sunflower oil. ALA supplementation was associated with significant decreases in factor VII ($P < .001$), fibrinogen ($P < .01$), and vWF ($P < .01$) in nondiabetic as well as in diabetic rats.

To rule out interference from lipemic plasma samples on in vitro coagulation tests, normal rat plasma was spiked with

Table 1. Body Weights

	Before Treatment (19 weeks old)	Start of Treatment (25 weeks old)		End of Treatment (27 weeks old)	
	Mean Weight (SEM) (g)	Mean Weight (SEM) (g)	% Change	Mean Weight (SEM) (g)	% Change
Diabetic					
EPO	488.6	371.6	−23.9*	371.1	−0.1
(n = 16)	(8.00)	(7.24)		(8.29)	
Sunflower oil	481.5	371.5	−22.8*	374.2	+0.7
(n = 15)	(9.57)	(12.05)		(12.31)	
ALA	474.0	368.2	−22.3*	352.4	−4.3†
(n = 16)	(5.62)	(7.50)		(8.20)	
No treatment	481.7	357.5	−25.8*	353.3	−1.2
(n = 6)	(9.33)	(9.76)		(12.02)	
Nondiabetic					
EPO	473.0	477.2	+0.9	483.0	+1.2
(n = 6)	(9.43)	(11.70)		(11.44)	
Sunflower oil	442.8	450.3	+1.7	456.2	+1.3
(n = 14)	(7.30)	(6.76)		(6.82)	
ALA	442.5	438.5	−0.9	414.2	−5.5†
(n = 8)	(1.40)	(2.83)		(4.36)	

* $P < .0001$ *v* before treatment, paired *t* test.

† $P < .001$ *v* start of treatment, paired *t* test.

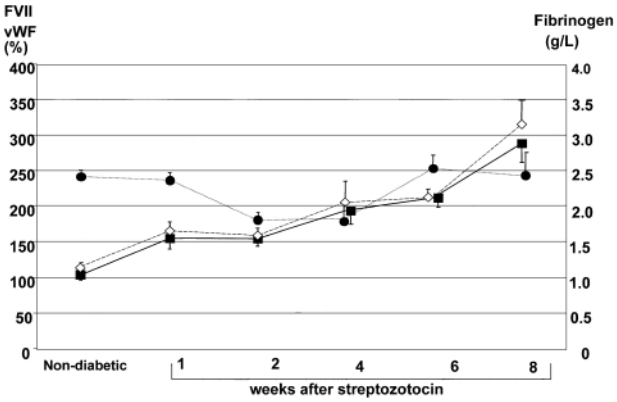

Fig 2. Time course of plasma factor VII (◇), fibrinogen (●), and vWF (■) in rats before and 1, 2, 4, 6 and 8 weeks after a single injection of streptozotocin. Mean ± SEM.

porcine cholesterol at concentrations from 5 to 20 mmol/L. Sonication was used to suspend the cholesterol in plasma. There were no differences between fibrinogen and factor VII levels measured in spiked plasma and in the original normal plasma. In addition, serial dilutions of 2 lipemic diabetic rat plasma samples and 2 normal rat plasma samples were found to produce linear and parallel curves for factor VII, fibrinogen, and vWF levels.

Lipids

Serum cholesterol and triglyceride levels were significantly higher in diabetic rats ($P < .001$) than in their nondiabetic littermates (Fig 1). There was no significant difference in HDL. After ALA treatment of diabetic rats, serum triglyceride was significantly lower ($P < .01$) than in a control group of untreated diabetic rats, but cholesterol levels and HDL did not change significantly. Sunflower oil supplementation was associated with increased cholesterol ($P < .001$) and decreased HDL ($P < .001$) levels. With EPO, both triglyceride ($P < .001$) and total cholesterol ($P < .01$) levels were significantly higher and HDL was significantly lower ($P < .001$) than in the untreated group (Table 3).

Tail Bleed

The times taken for a 0.5-mL tail bleed are shown in Fig 4. Diabetes was associated with a 5-fold prolongation of bleed time compared with that of nondiabetic rats ($P < .001$). EPO and ALA treatments both markedly reduced the 0.5-mL bleed time compared with the diabetic group ($P < .01$), although times remained prolonged compared with those of the nondiabetic group ($P < .05$). There was no significant difference between the effects of EPO and ALA treatment.

NCV and Endoneurial Blood Flow

Data for NCV and sciatic endoneurial blood flow parameters are shown in Table 4. Diabetes was associated with 20% and 16% reductions in sciatic motor and saphenous sensory NCV ($P < .001$). The motor deficit was 96% ± 6% corrected by ALA and 91% ± 8% corrected by EPO; corresponding values for sensory NCV were 92% ± 12% and 79% ± 6%, respectively (all $P < .001$). These posttreatment NCVs did not differ significantly from nondiabetic control values. In contrast, sunflower oil did not significantly alter NCV in diabetic rats. A 47% reduction in endoneurial blood flow ($P < .001$) with diabetes was completely corrected by ALA treatment and 88% corrected by EPO ($P < .01$); sunflower oil had no effect. There were between-group variations in mean systemic blood pressure during the flow recordings, with a tendency toward reduced pressure in diabetic rats, although this difference reached statistical significance only for the ALA-treated group ($P < .05$). Because vasa nervorum does not show pressure autoregulation of flow, the perfusion data are also expressed as vascular conductance. This parameter was reduced 38% ($P < .001$) by diabetes but returned to within the nondiabetic range after EPO treatment. For the ALA-treated diabetic group, conductance was 49% ± 22% supernormal ($P < .05$).

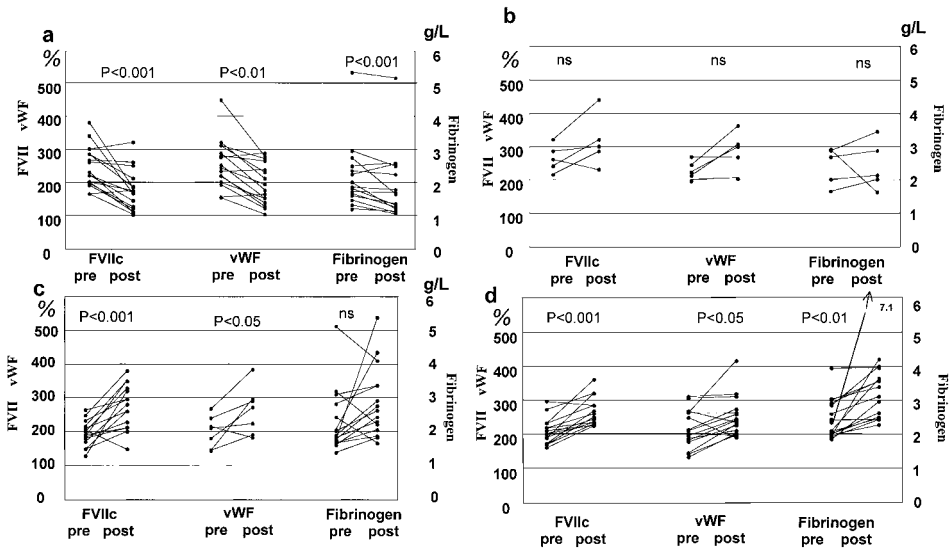

Fig 3. Plasma factor VII, fibrinogen and vWF in (a) individual diabetic rats (n = 16) before treatment (6 weeks after induction of diabetes) and after 2 weeks of ALA treatment (300 mg/kg/d); (b) diabetic rats (n = 5) at 6 and 8 weeks after induction of diabetes, with normal diet; (c) diabetic rats (n = 15) before and after 2 weeks of supplementation with sunflower oil; and (d) diabetic rats (n = 16) before and after supplementation with EPO. Values before and after treatment were analyzed by paired *t* test; ns, not significant.

Table 2. Streptozotocin-Diabetic Rats and Nondiabetic Rats Before and After 2 Weeks of Dietary Supplement or Control Diet

	Factor VIIc Pre (%)	Factor VIIc Post (%)	Fibrinogen Pre (g/L)	Fibrinogen Post (g/L)	Pre vWF (%)	Post vWF (%)
Diabetic						
ALA	243.6	175.34*	2.13	1.85†	255.9	197.2*
(n = 16)	(15.55)	(15.24)	(0.25)	(0.26)	(21.52)	(17.96)
EPO	207.25	267.9*	2.44	3.39†	215.1	250.1‡
(n = 16)	(9.34)	(10.62)	(0.14)	(0.30)	(13.95)	(14.59)
Sunflower oil	193.9	274.7*	2.28	2.88	200.3	262.7‡
(n = 15)	(9.68)	(16.95)	(0.25)	(0.27)	(17.67)	(26.84)
No treatment	264.0	315.0	2.43	2.43	228.8	288.0
(n = 5)	(18.12)	(34.64)	(0.25)	(0.33)	(12.73)	(26.20)
Nondiabetic						
ALA	109.6	76.1*	2.35	1.91†	87.5	62.75†
(n = 8)	(3.32)	(4.62)	(0.03)	(0.08)	(5.25)	(5.48)
EPO	95.3	114.0‡	2.30	2.21	112.1	112.7
(n = 6)	(5.63)	(3.93)	(0.15)	(0.08)	(3.73)	(4.72)
Sunflower oil	123.7	136.6	2.46	2.55	95.9	98.3
(n = 14)	(6.19)	(4.20)	(0.09)	(0.08)	(5.54)	(7.64)

NOTE. Data represent mean (SEM).

* $P < .001$, paired *t* test, pre *v* post.

† $P < .01$, paired *t* test, pre *v* post.

‡ $P < .05$, paired *t* test, pre *v* post.

DISCUSSION

In this study, the hyperglycemia and lipid abnormalities found in rats with streptozotocin-induced diabetes were accompanied by endothelial activation and increased plasma levels of coagulation factor VII. These early changes could have contributed to impaired nerve perfusion because they were found within a week of diabetes induction.[6,30]

Several studies have shown increased vWF, fibrinogen, and factor VII levels in diabetes in humans.[15,16,20] Furthermore, there is a relationship between plasma fibrinogen and the vascular lesion in diabetic nerve,[17] and vWF levels predict the subsequent development of neuropathy.[21] The present study shows similar vWF changes in streptozotocin-diabetic rats, in agreement with an earlier report.[24] We also show the novel finding of an increase in factor VII levels, although plasma fibrinogen was not significantly elevated by streptozotocin-induced diabetes in rats. The results indicate a relationship between coagulation factors and nerve perfusion/function as noted in studies of diabetic patients. However, the contrasting effects of ALA and EPO treatments suggest that the situation is complex.

Treatment of diabetic rats with the antioxidant ALA for 2 weeks was associated with significant reductions in plasma fibrinogen, factor VII, and vWF and a highly significant reduction in serum triglyceride. ALA was also associated with decreases in factor VII, fibrinogen, and vWF in nondiabetic animals. These are novel findings with implications for treatment of cardiovascular risk factors. In contrast, EPO did not induce similar changes in vWF or coagulation, despite benefi-

Table 3. Serum Lipids

	n	Triglycerides (mmol/L)	Cholesterol (mmol/L)	HDL (mmol/L)
Nondiabetic untreated	14	0.93 (0.06)	2.43 (0.08)	1.53 (0.05)
Diabetic untreated	14	5.04 (0.70)	5.14 (0.59)	1.93 (0.18)
Diabetic + ALA	16	2.41† (0.29)	3.77‡ (0.34)	1.98‡ (0.16)
Diabetic + EPO	16	10.26† (0.54)	9.14* (1.00)	0.52† (0.14)
Diabetic + sunflower oil	15	6.31‡ (1.03)	11.99† (1.47)	0.93† (0.17)

NOTE. Data represent mean (SEM). ANOVA and Newman-Keuls multiple-comparison test.

* $P < .01$ compared with diabetic untreated.

† $P < .001$ compared with diabetic untreated.

‡ Not significant compared with diabetic untreated.

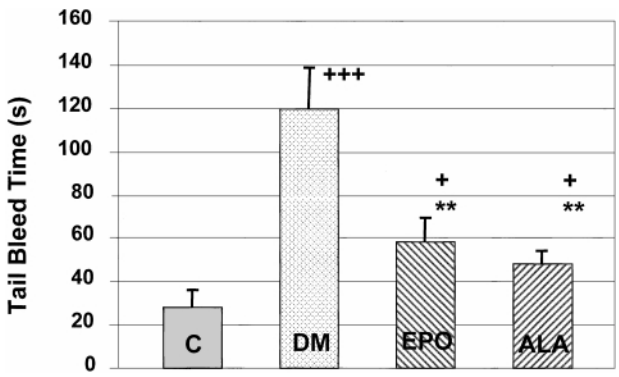

Fig 4. Time (seconds) to bleed 0.5 mL from the tail at 37°C in nondiabetic control rats (C), untreated diabetic rats (DM), and diabetic rats after 2 weeks of treatment with EPO or ALA. $^{+}P < .05$; $^{+++}P < .001$ *v* nondiabetic controls; $^{**}P < .01$ *v* untreated diabetic rats. Mean ± SEM.

Table 4. NCV and Endoneurial Blood Flow

		NCV		Sciatic Nerve Perfusion		
Group	n	Motor (m/s)	Sensory (m/s)	Blood Flow (mL/min/100 g)	Blood Pressure (mm Hg)	Vascular Conductance (mL/min/100 g per mm Hg)
Nondiabetic control	8	63.8 (0.5)	61.0 (0.9)	17.7 (1.3)	136.8 (4.3)	0.13 (0.01)
Diabetic untreated	8	50.9 (0.6)*	51.0 (0.9)*	9.3 (0.8)*	117.8 (6.7)	0.08 (0.01)*
Diabetic + ALA	6	63.3 (0.8)§	60.2 (1.2)§	18.7 (2.3)§	100.2 (8.4)‡	0.19 (0.03)‡§
Diabetic + EPO	6	62.5 (1.0)§#	58.9 (0.6)§,#	16.7 (1.3)§¶	126.6 (7.7)	0.14 (0.02)‖¶
Diabetic + sunflower oil	6	51.1 (0.9)*	51.4 (0.6)*	9.2 (0.6)*	113.8 (5.3)	0.08 (0.01)†

NOTE. Data represent mean (SEM).
* $P < .001$.
† $P < .01$.
‡ $P < .05$ *v* nondiabetic control group.
§ $P < .001$.
‖ $P < .01$ treatment effect *v* untreated diabetic group.
# $P < .001$.
¶ $P < .01$, *v* sunflower oil–treated groups.

cial effects on nerve perfusion and NCV of similar magnitude to those found with ALA treatment. Indeed, EPO treatment was associated with significant increases in levels of fibrinogen, factor VII, and vWF, as well as in cholesterol and triglyceride levels. Furthermore, sunflower oil control treatment was associated with changes qualitatively similar to those associated with EPO, but there was no effect on NCV and blood flow. Both EPO and sunflower oil contain approximately 70% linoleic acid, which argues for the importance of a lesser component of EPO with a vasodilator action. γ-Linolenic acid, in the form of the triglyceride dilinolein mono-γ linolein, appears to be the vasoactive component of EPO and is not found in sunflower oil.[31] In diabetic rats, the deficit in vasa nervorum synthesis of the vasodilator prostacyclin depends largely on reduced availability of the omega-6 substrate arachidonic acid, which is itself a metabolite of γ-linolenic acid.[32,33] Treatment of diabetic rats with prostacyclin analogues mimics the effect of EPO on nerve blood flow and NCV.[34,35]

The pattern of drug treatment effects on NCV and blood flow was roughly paralleled by alterations in bleed time. The time taken for 0.5 mL of blood to be shed was prolonged in rats with streptozotocin-induced diabetes compared with controls and was largely normalized by ALA or EPO treatments. This finding is consistent with the conclusion that this measurement is influenced mainly by the degree of vasodilatation of tail vessels, and therefore blood flow, rather than by hemostatic parameters.

The relationship between factor VII and lipids is well recognized,[36,37] and changes in cholesterol and triglyceride levels could therefore account for some of the changes in factor VII found in this study. Thus, factor VII, triglycerides, and cholesterol were increased by EPO treatment, whereas ALA treatment reduced triglycerides and factor VII. However, increases in fibrinogen and vWF would not be expected to follow directly from changes in dietary lipids. Consistent with this, factor VII and cholesterol levels also increased significantly in the present study after dietary supplementation with sunflower oil of equivalent lipid content and calorific value, whereas changes in fibrinogen did not reach statistical significance. There was also a trend toward increased factor VII in untreated diabetic animals over the equivalent 2-week period, which may not have reached statistical significance because of the small numbers in this group. When animals were studied in a time course from 1 week to 8 weeks after induction of diabetes by streptozotocin, we found gradual increases in plasma factor VII and vWF levels, which were already significant after 1 week, compared with nondiabetic controls (Fig 2). Against this background of increasing procoagulant tendency that accompanies the progression of disease alone, the changes observed after ALA treatment were all the more striking.

ALA is a naturally occurring antioxidant. In addition to direct free radical scavenger and metal chelating actions, in its reduced form (dihydrolipoic acid) it may also participate in the regeneration of the major antioxidants ascorbate and vitamin E. ALA is also capable of modulating transcription factors, especially NF-κB.[38] Cell culture studies have shown that increased oxidative stress in endothelial cells, stimulated for example by advanced glycosylation end products, induces NF-κB activation. Furthermore, this is suppressed by incubation with ALA.[39] NF-κB regulates transcription of many biologic molecules, including endothelial tissue factor. Because activation of factor VII is dependent on tissue factor, inhibition of NF- κB by ALA could provide a possible explanation for the observed changes in factor VII. With the method used in this study, it is not possible to elucidate whether altered factor VII levels were caused by changes in synthesis or in activation status, ie, an increase in the 2-chain form, VIIa, caused by activation by tissue factor. However, because we used a functional coagulation assay, we can be sure that the increase described was in functional factor VII.

Although the effects of EPO and ALA on NCV and blood flow were indistinguishable, confirming our previous findings,[3,7] only ALA was able to reverse the effects of streptozotocin-induced diabetes on coagulation and endothelium. This suggests that either the pathogenesis of the nerve defects is dependent on other factors, or the modes of action of the 2 agents differ. It has previously been shown that synergism occurs between low doses of EPO and antioxidants in their effects on NCV and blood flow,[7,40] which would argue for independent mechanisms of action for the 2 agents. Whereas

EPO increases endothelial prostacyclin synthesis, antioxidants such as ALA and vitamin E improve endothelial function, particularly in relation to the vasodilatory nitric oxide system.[41-44] This probably results from scavenging of superoxide to prevent its reaction with nitric oxide. Thus, antioxidant treatment potentially would have 3 consequences: preservation of vasodilatation by nitric oxide; prevention of the formation of peroxynitrite, which can decompose to liberate the cytotoxic hydroxyl radical; and scavenging of any hydroxyl radicals formed, thus protecting endothelium from damage. These actions would be sufficient to account for the observed effect of ALA on vWF levels, as well as on nerve blood flow and NCV in diabetic rats. Both of these last parameters can also be improved by treatment with a nitric oxide donor.[45]

It is of potential interest that ALA markedly decreased plasma triglycerides, and this could also contribute to better endothelial function. The results of this study provide further evidence for the importance of impaired blood flow in the pathogenesis of experimental diabetic neuropathy. They favor etiologic arguments based on reduced endothelial production of vasodilators over those that stress the importance of increased coagulation, although both probably contribute to neuropathy. ALA has entered clinical trials for treatment of diabetic neuropathy.[10] Moreover, the marked effects of ALA on risk factors for cardiovascular disease, both lipid and hemostatic, are of particular importance because they indicate potential antithrombotic and antiatherosclerotic actions that could prove beneficial in large vessel disease and merit further study.

REFERENCES

1. Tesfaye S, Malik RA, Ward JD: Vascular factors in diabetic neuropathy. Diabetologia 37:847-853, 1994
2. Dyck PJ: Pathologic alterations in the diabetic neuropathies of humans: A review. J Neuropathol Exp Neurol 55:1181-1193, 1996
3. Cameron NE, Cotter MA: Metabolic and vascular factors in the pathogenesis of diabetic neuropathy. Diabetes 46:S31-S37, 1997 (suppl 2)
4. Ibrahim S, Harris ND, Radatz M, et al: A new minimally invasive technique to show nerve ischaemia in diabetic neuropathy. Diabetologia 42:737-742, 1999
5. Thierault M, Dort J, Sutherland G, et al: Local human sural nerve blood flow in diabetic and other polyneuropathies. Brain 120:1131-1138, 1997
6. Cameron NE, Cotter MA, Low PA: Nerve blood flow in early experimental diabetes in rats: Relation to conduction deficits. Am J Physiol 261:E1-E8, 1991
7. Cameron NE, Cotter MA, Horrobin DH, et al: Effects of α-lipoic acid on neurovascular function in diabetic rats: Interaction with essential fatty acids. Diabetologia 41:390-399, 1998
8. Cotter MA, Cameron NE: Effects of dietary supplementation with arachidonic acid rich oils on nerve conduction and blood flow in streptozotocin-diabetic rats. Prostaglandins Leukot Essent Fatty Acids 56:337-343, 1997
9. Keen H, Payan J, Allawi J, et al: Treatment of diabetic neuropathy with gamma-linolenic acid. The gamma-Linolenic Acid Multicentre Trial Group. Diabetes Care 16:1309-1310, 1993
10. Ziegler D, Gries FA: Alpha-lipoic acid in the treatment of diabetic peripheral and cardiac autonomic neuropathy. Diabetes 46: S62-S66, 1997 (suppl 2)
11. Simpson LO: Altered blood rheology in the pathogenesis of diabetic and other neuropathies. Muscle Nerve 11:725-744, 1988
12. Kihara M, Low PA: Impaired vasoreactivity to nitric oxide in experimental diabetic neuropathy. Exp Neurol 132:180-185, 1995
13. Maxfield EK, Cameron NE, Cotter MA: Effects of diabetes on reactivity of sciatic vasa nervorum in rats. J Diabet Complications 11:47-55, 1997
14. Meade TW, Mellows S, Brozovic M, et al: Haemostatic function and ischaemic heart disease: Principal results of the Northwick Park Heart Study. Lancet 2:533-537, 1986
15. Christe M, Fritschi J, Lammle B, et al: Fifteen coagulation and fibrinolysis parameters in diabetes mellitus and in patients with vasculopathy. Thromb Haemost 52:138-143, 1984
16. Fuller JH, Keen H, Jarrett RJ, et al: Haemostatic variables associated with diabetes mellitus and its complications. Br Med J 2:964-966, 1979
17. Ford I, Malik RA, Newrick PG, et al: Relationships between haemostatic factors and capillary morphology in human diabetic neuropathy. Thromb Haemost 68:628-633, 1992
18. Meade TW: Factor VII and ischaemic heart disease: Epidemiological evidence. Haemostasis 13:178-185, 1983
19. Greaves M, Pickering C, Knight G, et al: Changes in the factor VIII complex in diabetic ketoacidosis: Evidence of endothelial damage. Diabetologia 30:160-165, 1987
20. Greaves M, Malia RG, Goodfellow K, et al: Fibrinogen and von Willebrand factor in IDDM: Relationships to lipid vascular risk factors, blood pressure, glycaemic control and urinary albumin excretion rate: The EURODIAB IDDM complications study. Diabetologia 40:698-705, 1997
21. Plater ME, Ford I, Dent MT, et al:Elevated von Willebrand factor antigen predicts deterioration in diabetic peripheral nerve function. Diabetologia 39:336-343, 1996
22. Stehouwer CDA, Andreas Fischer HR, VanKuijk AWR, et al: Endothelial dysfunction precedes development of microalbuminuria in IDDM. Diabetes 44:561-564, 1995
23. Stehouwer CDA, Zellenrath P, Polack BCP, et al: Von Willebrand factor and early diabetic retinopathy: no evidence for a relationship in patients with type 1 (insulin-dependent) diabetes mellitus and normal urinary albumin excretion. Diabetologia 35:555-559, 1992
24. Winocour PD, Lopes-Virella M, Laimins M, et al: The time course of changes in in vitro platelet function and plasma von Willebrand factor activity (VIIIR:WF) and factor VIII-related antigen (VIIIR:Ag) in the diabetic rat. J Lab Clin Med 102:795-804, 1983
25. Cooper ME, Rumble J, Komers R, et al: Diabetes-associated mesenteric vascular hypertrophy is attenuated by angiotensin-converting enzyme inhibition. Diabetes 43:1221-1228, 1994
26. Cameron NE, Cotter MA, Robertson S: The effect of aldose reductase inhibition on the pattern of nerve conduction deficits in diabetic rats. Q J Exp Physiol 74:917-926, 1989
27. Clauss A: Gerinnungsphysiologische Schnellmethode zur Bestimmung des Fibrinogens. Acta Hematol 17:237-240, 1957
28. Cameron NE, Cotter MA, Hohman TC: Interactions between essential fatty acid, prostanoid, polyol pathway and nitric oxide mechanisms in the neurovascular deficit of diabetic rats. Diabetologia 39: 172-182, 1996
29. Day TJ, Lagerlund TD, Low PA: Analysis of H_2 clearance curves used to measure blood flow in rat sciatic nerve. J Physiol 414:35-54, 1989
30. Wright RA, Nukada H: Vascular and metabolic factors in the pathogenesis of experimental diabetic neuropathy in mature rats. Brain 117:1395-1407, 1994
31. Dines KC, Cameron NE, Cotter MA: Comparison of the effects of evening primrose oil and triglycerides containing gamma-linolenic acid on nerve conduction and blood flow in diabetic rats. J Pharmacol Exp Ther 273:49-55, 1995
32. Ward KK, Low PA, Schmelzer JD, et al: Prostacyclin and

noradrenaline in peripheral nerve of chronic experimental diabetes in rats. Brain 112:197-208, 1989

33. Cotter MA, Cameron NE: Effects of dietary supplementation with arachidonic acid rich oils on nerve conduction and blood flow in streptozotocin-diabetic rats. Prostaglandins Leukot Essent Fatty Acids 56:337-343, 1997

34. Cotter MA, Dines KC, Cameron NE: Prevention and reversal of motor and sensory peripheral nerve conduction abnormalities in streptozotocin-diabetic rats by treatment with the prostacyclin analogue iloprost. Naunyn Schmeidebergs Arch Pharmacol 347:534-540, 1993

35. Hotta N, Koh N, Sakakibara F, et al: Effects of beraprost sodium and insulin on the electroretinogram, nerve conduction and nerve blood flow in rats with streptozotocin-induced diabetes. Diabetes 45:361-366, 1996

36. Elkeles RS, Chakrabarti R, Vickers M, et al: Effect of treatment of hyperlipidaemia on haemostatic variables. Br Med J 281:973-974, 1980

37. Miller GJ, Martin J, Webster J, et al: Association between dietary fat intake and plasma factor VII coagulant activity—A predictor of cardiovascular mortality. Atherosclerosis 60:269-277, 1986

38. Packer L: Alpha-lipoic acid: A metabolic antioxidant, which regulates NF-κB and protects against oxidative injury. Drug Metab Rev 30:245-275, 1998

39. Bierhaus A, Chevion S, Chevion M, et al: Advanced glycosylation endproduct-induced activation of NF-κB is suppressed by alpha-lipoic acid in cultured endothelial cells. Diabetes 46:1482-1490, 1997

40. Cameron NE, Cotter MA: Interaction between oxidative stress and gamma-linolenic acid in impaired neurovascular function of diabetic rats. Am J Physiol 271:E471-E476, 1996

41. Keegan A, Walbank H, Cotter MA, et al: Chronic vitamin E treatment prevents defective endothelium-dependent relaxation in diabetic rat aorta. Diabetologia 38:1475-1478, 1995

42. Keegan A, Cotter MA, Cameron NE: Effects of diabetes and treatment with the antioxidant, α-lipoic acid, on endothelial and neurogenic functions of corpus cavernosum in rats. Diabetologia 42:343-350, 1999

43. Rosen P, Ballhausen T, Bloch W, et al: Endothelial relaxation is disturbed by oxidative stress in the diabetic rat heart: Influence of tocopherol as antioxidant. Diabetologia 38:1157-1168, 1995

44. Archibald V, Cotter MA, Keegan A, et al: Contraction and relaxation of aortas from diabetic rats: Effects of chronic anti-oxidant and aminoguanidine treatments. Naunyn Schmeidebergs Arch Pharmacol 353:584-591, 1996

45. Cameron NE, Cotter MA: Effects of chronic treatment with a nitric oxide donor on nerve conduction abnormalities and endoneurial blood flow in streptozotocin-diabetic rats. Eur J Clin Invest 25:19-24, 1995