Effects of Proinsulin C-Peptide in Experimental Diabetic Neuropathy

Vascular Actions and Modulation by Nitric Oxide Synthase Inhibition

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Proinsulin C-peptide treatment can partially prevent nerve dysfunction in type 1 diabetic rats and patients. This could be due to a direct action on nerve fibers or via vascular mechanisms as C-peptide stimulates the nitric oxide (NO) system and NO-mediated vasodilation could potentially account for any beneficial C-peptide effects. To assess this further, we examined neurovascular function in streptozotocin-induced diabetic rats. After 6 weeks of diabetes, rats were treated for 2 weeks with C-peptide to restore circulating levels to those of nondiabetic controls. Additional diabetic groups were given C-peptide with NO synthase inhibitor N^G-nitro-L-arginine (L-NNA) co-treatment or scrambled C-peptide. Diabetes caused 20 and 16% reductions in sciatic motor and saphenous sensory nerve conduction velocity, which were 62 and 78% corrected, respectively, by C-peptide. L-NNA abolished C-peptide effects on nerve conduction. Sciatic blood flow and vascular conductance were 52 and 41%, respectively, reduced by diabetes ($P < 0.001$). C-peptide partially (57–66%) corrected these defects, an effect markedly attenuated by L-NNA co-treatment. Scrambled C-peptide was without effect on nerve conduction or perfusion. Thus, C-peptide replacement improves nerve function in experimental diabetes, and the data are compatible with the notion that this is mediated by a NO-sensitive vascular mechanism. *Diabetes* 52:1812–1817, 2003

It has become clear that proinsulin C-peptide is not biologically inert but has important physiological actions (rev. in 1) whose absence could contribute to the development of diabetic neuropathy. Thus, there are direct effects on neural and other cell types to elevate Na^+,K^+-ATPase activity (2–4). Whole-body glucose utilization under euglycemic clamp conditions is also improved by C-peptide in streptozotocin-induced diabetic rats (5). In addition, there are vascular-related actions noted in studies on patients and experimental models. These include stimulation of nitric oxide (NO) synthesis, reduction of leukocyte-endothelium interactions, and protection against cardiac ischemia-reperfusion damage (3,6–8). Given that impaired nerve perfusion contributes to diabetic neuropathy in patients and experimental models (9), it is plausible that vascular effects may potentially mediate C-peptide effects in neuropathy. Recent studies have shown short-term benefits of C-peptide treatment on autonomic and sensory neuropathy in patients (10–12) and protection and improvement of motor nerve conduction velocity (NCV) and morphometry in type 1 diabetic BB Wistar rats (2). Motor NCV deficits were also partially prevented in streptozotocin-induced diabetic rats (13).

The aims of this investigation were *1*) to assess whether C-peptide treatment could reverse established sensory and motor NCV abnormalities, *2*) to ascertain whether there were parallel changes in nerve blood flow, and *3*) to establish whether the NO system participated in any functional restoration by examining the effects of co-treatment with the NO synthase (NOS) inhibitor N^G-nitro-L-arginine (L-NNA). To this end, diabetes was induced by streptozotocin in mature male rats, which provides a model of a relative rather than an absolute deficit in proinsulin C-peptide.

RESEARCH DESIGN AND METHODS

Experiments were performed in accordance with regulations specified by the U.K. Animal Procedures Act, 1986, and the National Institutes of Health Principles of Laboratory Animal Care, 1985 revised version.

Experimental animals, diabetes induction, and drug treatment. Male Sprague-Dawley rats (Aberdeen University colony) that were 19 weeks old at the start of the study were used. Nondiabetic animals acted as onset controls. Diabetes was induced by intraperitoneal administration of streptozotocin (Astra-Zeneca, Macclesfield, Cheshire, U.K.) at 40–45 mg/kg, freshly dissolved in sterile saline. Diabetes was verified 24 h later by hyperglycemia and glycosuria (Visidex II and Diastix; Ames, Slough, U.K.). Diabetic rats were tested weekly and weighed daily. Animals were rejected when the plasma glucose concentration was <20 mmol/l or when body weight consistently increased over 3 days. Samples were taken from a carotid cannula at the end of the experiments for plasma glucose concentration measurement (GOD-Perid method; Boehringer Mannheim, Mannheim, Germany) and the determination of C-peptide levels.

After 6 weeks of untreated diabetes, rats were anesthetized (2.5% halothane in air) and received a subcutaneously implanted osmotic minipumps (Alzet 2ML2; Alza, Palo Alto, CA) filled with either rat C-peptide II or

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EDHF, endothelium-derived hyperpolarizing factor; L-NNA, N^G-nitro-L-arginine; NCV, nerve conduction velocity; NO, nitric oxide; NOS, nitric oxide synthase.

TABLE 1
Body weights and plasma glucose concentrations for the groups of rats used in the investigation

Group	*n*	Body weight (g)		Plasma glucose (mmol/l)
		Start	End	
Nondiabetic group	10		465 ± 8	6.9 ± 0.6
Diabetic group				
Untreated	10	458 ± 8	364 ± 8	40.3 ± 2.2
Scrambled peptide	6	460 ± 6	392 ± 14	44.8 ± 3.7
C-peptide	9	458 ± 7	360 ± 12	44.8 ± 1.6
C-peptide + L-NNA	6	462 ± 11	352 ± 10	40.0 ± 2.3

Data are means ± SE.

scrambled (a peptide with the residues arranged in random order) C-peptide (Sigma, Genosys, Cambridge, U.K.) dissolved in sterile PBS. The output of the pump was fed to a jugular vein cannula. Rats were treated for 14 days with a dose of ~50 pmol · kg^{-1} · min^{-1}, which was chosen to give plasma C-peptide levels within the physiological range. An additional group of rats were given C-peptide treatment and co-treated with the NOS inhibitor L-NNA, dissolved in the drinking water such that they received a dose of ~10 mg · kg^{-1} · day^{-1}. This dose abolished the effects of antioxidants, aldose reductase inhibitors, and aminoguanidine on NCV and blood flow in diabetic rats (14–16).

Nerve conduction studies. At the end of the treatment period, rats were anesthetized with thiobutabarbital (Astra-Zeneca) by intraperitoneal injection (50–100 mg/kg). The trachea was cannulated for artificial ventilation, and a carotid cannula was used to monitor systemic blood pressure. Motor NCV was measured between sciatic notch and knee in the nerve branch to tibialis anterior muscle, which is representative of the whole sciatic nerve in terms of susceptibility to diabetes and treatment effects (17,18). Saphenous sensory NCV was measured between groin and ankle (18). Rectal and nerve temperatures were regulated between 36.5 and 37.5°C.

Nerve blood flow studies. Sciatic endoneurial blood flow was measured in the contralateral leg by microelectrode polarography and H_2 clearance (19). Briefly, core temperature of the rat was 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 to form a pool, which was filled with mineral oil. Pool temperature was maintained between 35 and 37°C by radiant heat. Rats 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 H_2-sensitive platinum polarographic microelectrode was inserted into the middle portion of the sciatic nerve, above its trifurcation. Ten percent H_2 was substituted for N_2 in the inspired gas. When the electrode H_2 current had stabilized, the H_2 supply was shut off and clearance was recorded until baseline. This procedure was repeated at another nerve site. Clearance curves were digitized, and monoexponential or biexponential curves were fitted to the data using nonlinear regression software, the Marquardt algorithm, and the least-squares method for optimizing goodness of fit (Prism, Graphpad, San Diego, CA). The slow exponent was taken to reflect nutritive (capillary) flow (20). Composite (nutritive plus nonnutritive) flow was calculated using the weighted sum of slow and fast exponents (16). Vascular conductance was determined by dividing flow by the mean arterial blood pressure. The average of the two determinations was taken to represent the sciatic endoneurial perfusion measures.

Plasma C-peptide levels. Plasma C-peptide was measured by radioimmunoassay using a commercial kit (Linco Research, St. Charles, MO).

Statistical analysis. Results are expressed as means ± SE. Data were subjected to Bartlett's test for homogeneity of variances, followed by log transformation when necessary (C-peptide levels, vascular conductance, and composite blood flow) before one-way ANOVA. When significance ($P < 0.05$) was reached, between-group differences were established by the Student-Neuman-Keuls post hoc multiple comparison test.

RESULTS

Body weights and blood glucose concentrations are given in Table 1. Diabetes caused an approximate sixfold elevation in plasma glucose and ~20% weight loss after 8 weeks. These parameters were not significantly affected by C-peptide or any of the other treatments used.

Plasma C-peptide levels (Fig. 1) were ~85% reduced by diabetes ($P < 0.001$), and this was unaffected by scrambled peptide treatment. In the C-peptide–treated groups, without or with L-NNA co-treatment, plasma levels were increased ($P < 0.01$) to values within the nondiabetic range.

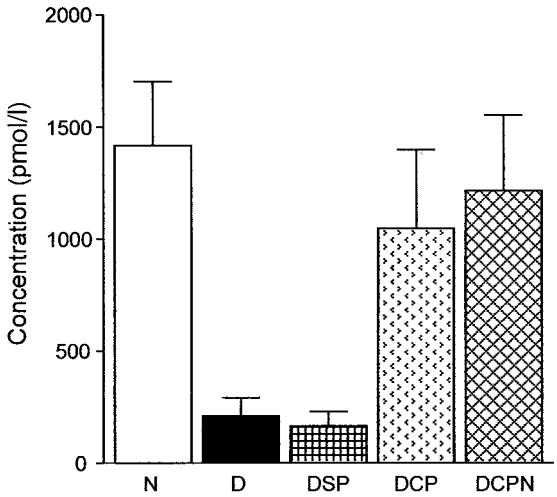

FIG. 1. Plasma C-peptide levels in the experimental groups. Data are group means + SE. N, nondiabetic control group (n = 8); D, 8-week untreated diabetic group (n = 11); DSP, diabetic group untreated for 6 weeks and then given scrambled peptide treatment for 2 weeks (n = 5); DCP, diabetic group untreated for 6 weeks and then given C-peptide treatment for 2 weeks (n = 9); DCPN, C-peptide–treated diabetic group co-treated with 10 mg · kg^{-1} · day^{-1} L-NNA (n = 6). Statistics: N vs. D, $P < 0.001$; N vs. DSP, $P < 0.01$; D vs. DCP, DCPN, $P < 0.01$; all other comparisons, NS.

Sciatic motor NCV (Fig. 2*A*) and saphenous sensory NCV (Fig. 2*B*) were reduced by 19.8 ± 0.8% and 15.7 ± 0.9%, respectively, after 8 weeks of diabetes (both $P <$ 0.001). Scrambled peptide treatment for 2 weeks had no effect, motor and sensory deficits ($P < 0.001$) of 19.9 ± 1.6% and 15.1 ± 0.7% being noted. In contrast, C-peptide treatment for 14 days corrected motor NCV by 61.7 ± 2.9% ($P < 0.001$), although a deficit remained ($P < 0.001$) compared with the nondiabetic control group. The diabetic sensory NCV defect was corrected to the extent of 77.6 ± 6.8% ($P < 0.001$), the resultant value being just below that of the nondiabetic control group ($P < 0.05$). The degree of correction of sensory NCV was somewhat greater than for motor NCV ($P = 0.049$, paired Student's t test). The corrective effects of C-peptide treatment on motor and sensory NCV were completely abolished ($P <$ 0.001) by co-treatment with the NOS inhibitor L-NNA.

Diabetes caused a 51.5 ± 3.6% reduction ($P < 0.001$) in sciatic nutritive endoneurial blood flow (Fig. 3*A*). This was unaffected by scrambled peptide treatment, whereas C-peptide corrected the defect by 56.9 ± 7.5% ($P < 0.001$), although a deficit remained compared with the nondiabetic control group ($P < 0.001$). Co-treatment with L-NNA completely abolished ($P < 0.001$) the effects of C-peptide on endoneurial nutritive perfusion. Compared with the nondiabetic controls, mean systemic blood pressure (Fig. 3*B*) tended to be lower in the control ($P < 0.001$), scrambled peptide–treated ($P < 0.01$), and C-peptide–treated ($P < 0.05$) diabetic groups. As expected (16) as a result of the pressor effect of L-NNA, systemic pressures were in the nondiabetic range for the co-treatment group. Peripheral nerve shows negligible pressure autoregulation (20); thus, variations in systemic pressure tend to obscure the true effects of diabetes and/or drug treatment on the

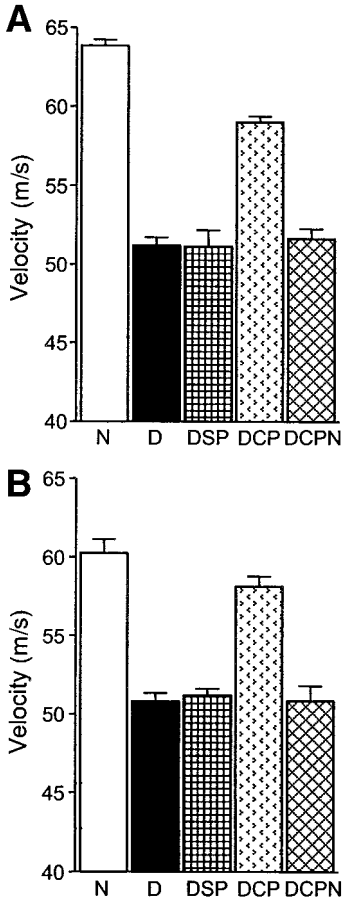

FIG. 2. Conduction velocity of the sciatic nerve branch supplying tibialis anterior muscle (*A*) and sensory saphenous nerve (*B*). Data are group means + SE; group *n* values are given in Table 1. N, nondiabetic control group; D, 8-week untreated diabetic group; DSP, diabetic group untreated for 6 weeks and then given scrambled peptide treatment for 2 weeks; DCP, diabetic group untreated for 6 weeks and then given C-peptide treatment for 2 weeks; DCPN, C-peptide–treated diabetic group co-treated with L-NNA (10 mg · kg^{-1} · day^{-1}). Statistics: for both nerves, N vs. D, DCP, DSP, $P < 0.001$; DCP vs. D, DSP, DCPN, $P < 0.001$; N vs. DCP, $P < 0.001$ for motor and $P < 0.05$ for sensory nerves; all other comparisons, NS.

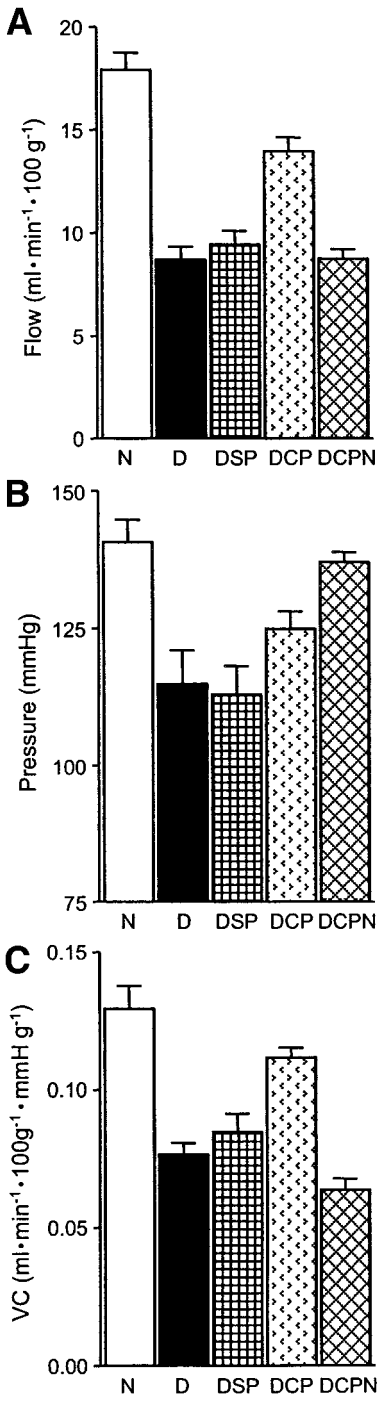

FIG. 3. Effects of diabetes and treatment on sciatic endoneurial nutritive perfusion parameters. Blood flow (*A*), mean systemic blood pressure (*B*), and vascular conductance (VC; *C*). Data are group means + SE; group *n* values are given in Table 1. N, nondiabetic control group; D, 8-week untreated diabetic group; DSP, diabetic group untreated for 6 weeks and then given scrambled peptide treatment for 2 weeks; DCP, diabetic group untreated for 6 weeks and then given C-peptide treatment for 2 weeks; DCPN, C-peptide–treated diabetic group co-treated with L-NNA (10 mg · kg^{-1} · day^{-1}). Statistics: blood flow, N vs. D, DSP, DCPN, DCP, $P < 0.001$; DCP vs. D, DSP, DCPN, $P < 0.001$. Blood pressure, N or DCPN vs. D, DSP, $P < 0.05$; N vs. DCP, $P < 0.05$. Vascular conductance, N vs. D, DSP, DCPN, $P < 0.001$; DCP vs. D, DCPN, $P < 0.001$; DCP vs. DSP, $P < 0.01$. All other comparisons, NS.

vasa nervorum. For overcoming this, the data were also expressed in terms of vascular conductance (Fig. 3*C*). There was a 40.8 ± 3.3% diabetic conductance deficit ($P < 0.001$), which was 66.2 ± 6.8% corrected ($P < 0.001$) by C-peptide treatment such that the resultant value did not differ significantly from that of the nondiabetic control group. Scrambled peptide treatment was without effect on conductance, whereas the effects of C-peptide were negated by L-NNA co-treatment, this group having the lowest values.

Hydrogen clearance curves for peripheral nerve are usually composed of two simultaneously recorded components. A fast component arises as a result of clearance by large vessels (nonnutritive arterial, venous, and particularly arteriovenous flow), and a slow component appears as the result of nutritive (capillary) clearance (2). Data for composite (nutritive plus nonnutritive) flow and conductance (Fig. 4*A* and *B*) show 64.1 ± 5.3% ($P < 0.001$) and 55.5 ± 6.2% ($P < 0.01$) diabetic deficits, respectively. This did not differ significantly in the scrambled peptide or C-peptide plus L-NNA co-treatment groups. With C-peptide alone, there were ~36% improvements in composite flow and conductance, although this did not reach full statistical significance, values remaining not significantly differ-

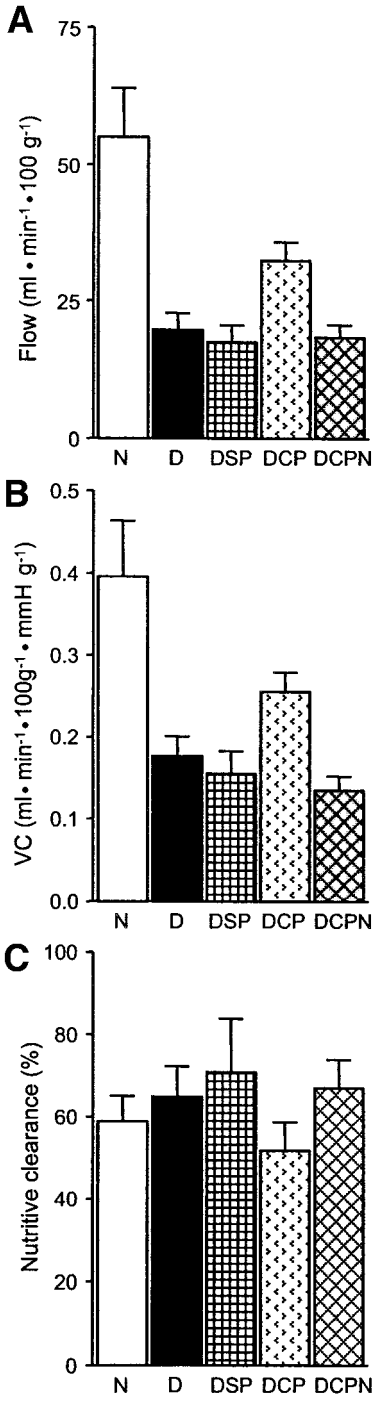

FIG. 4. Effects of diabetes and treatment on sciatic composite (nutritive plus nonnutritive) perfusion parameters. Blood flow (*A*), vascular conductance (VC; *B*), and the percentage of hydrogen clearance carried by the nutritive flow component (*C*). Data are group means + SE; group *n* values are given in Table 1. N, nondiabetic control group; D, 8-week untreated diabetic group; DSP, diabetic group untreated for 6 weeks and then given scrambled peptide treatment for 2 weeks; DCP, diabetic group untreated for 6 weeks and then given C-peptide treatment for 2 weeks; DCPN, C-peptide–treated diabetic group co-treated with L-NNA (10 mg · kg^{-1} · day^{-1}). Statistics: flow and conductance, N vs. D, DSP, DCPN, $P < 0.01$; all other comparisons, NS.

ent from those of nondiabetic or diabetic control groups. There were no statistically significant between-group differences in the proportion of hydrogen clearance carried by the nonnutritive flow component (Fig. 4*C*).

DISCUSSION

The data show that rat C-peptide in replacement doses had marked effects on NCV in diabetic rats. The partial correction of motor NCV is in agreement with and extends previous preventive studies in streptozotocin-induced diabetes and is also in line with preventive and corrective investigations in the type 1 BB/W rat model (2,13). This is the first report of correction of sensory NCV deficits by C-peptide in diabetic rats, an effect that was moderately greater than for motor NCV. A variety of agents correct saphenous sensory better than sciatic motor NCV in diabetic rats, including aldose reductase inhibitors, antioxidants, and some vasodilators; a similar but more exaggerated phenomenon has been noted for comparisons between sciatic-tibial motor and sensory-digital NCV (21–23). The reason is unclear, but these drugs are vasoactive, and it may be that sensory nerve trunk or dorsal root ganglia perfusion is more easily restored by treatment. Saphenous nerve and particularly digital nerve have much smaller diameters and hence are potentially more easily perfused than sciatic nerve. Moreover, saphenous nerve is closely apposed to saphenous artery for most of its length in the leg. Thus, saphenous nerve is likely to benefit from the saphenous artery collateral supply and may have a greater vascular reserve than sciatic nerve, which has three separate vascular inputs along its length, with the possibility of watershed effects between them under conditions in which vascular function is compromised. Other potential explanations could be based on pharmacological differences between the vascular beds of the nerves; saphenous and digital vasa nervorum have not been studied in this regard. A recent clinical trial of C-peptide in patients with type 1 diabetes revealed greater effects on sensory than motor NCV (12).

The nerve blood flow measurements gave clear evidence of vascular actions for C-peptide. There is a strong correlation between sciatic motor NCV and nutritive endoneurial blood flow, based on multiple studies using vasodilators and other vasoactive drugs (9). The degree of correction of the diabetic blood flow deficit by C-peptide is entirely consistent with its effects on NCV; thus, it is likely that the predominant effect of C-peptide on nerve function was indirect via vasa nervorum. Although C-peptide can increase whole-body glucose utilization (5), there is no information as to whether this has an effect on energy metabolism in peripheral nerve that could potentially also have contributed to the NCV effects. C-peptide increased both nutritive and nonnutritive blood flow, although statistically the effect on the latter was unclear. However, there were no between-group differences in the percentage of hydrogen clearance by nutritive flow, suggesting that C-peptide had an overall stimulatory effect on both nutritive and nonnutritive perfusion. This is similar to effects seen with antioxidants and some vasodilators and contrasts with findings for aldose reductase inhibitors, which alter the perfusion pattern by diverting arteriovenous-shunt perfusion to the nutritive circuit (21). A previous study concluded that C-peptide reduced total nerve blood flow in diabetic rats (13), although this estimate was based on a microsphere entrapment technique, which has technical limitations when applied to a small tissue such as rat sciatic nerve (24–26).

The finding that both NCV and blood flow effects of C-peptide were abolished by co-treatment with a dose of L-NNA that has only modest functional effects in nondiabetic rats (27) provides strong support for a predominantly vascular mode of action and implicates the NO vasodilator system. This likely reflects a wider vascular action of C-peptide; stimulatory effects on whole-body glucose utilization in diabetic rats were 85% attenuated by NOS blockade (5). In experimental diabetic neuropathy, NOS inhibition also blocks the effects of several agents known to improve endothelium-dependent relaxation, including antioxidants, aminoguanidine, and aldose reductase inhibitors (9,14–16). Theoretically, C-peptide could influence the NO system in at least two ways: first, by directly stimulating NOS activity or expression, presumably via a receptor-mediated process, as has been noted in vascular preparations in vivo and in vitro, as well as in endothelial cell tissue culture, and by iontophoresis of acetylcholine in human subjects (3,6,7,28,29); and second, indirectly by increasing blood flow by another mechanism, which would then be augmented by endothelial flow-induced NO production. The latter action has been noted for vasodilators such as doxazosin, which act on vascular smooth muscle (30). For C-peptide, it is plausible that both mechanisms are involved, and in a variety of experimental situations, the vascular effects of C-peptide are abolished by NOS inhibition (3,6,28,29). In diabetic rats, the vasa nervorum NO system is compromised (31,32), and chronic NO-donor treatment resulted in NCV and nerve blood flow improvement that were similar in magnitude to those seen with C-peptide (33). This provides further support for the viability of the hypothesis that the major neural action of C-peptide is via the vasa nervorum NO system.

C-peptide increases intracellular Ca^{2+} concentrations by causing an influx of extracellular Ca^{2+} (4,34), which may contribute to elevated NO output. This could also modulate production of vasodilator prostanoids and perhaps endothelium-derived hyperpolarizing factor (EDHF), both of which are important Ca^{2+}-dependent vasodilators in vasa nervorum (9,35). This Ca^{2+} effect is probably elicited after binding of C-peptide to a G-protein–coupled membrane receptor (36), but non–receptor-mediated mechanisms have also been suggested (13). However, the results with L-NNA co-treatment suggest that the major C-peptide action on vasa nervorum involves the NO system.

One potentially important cellular effect of C-peptide is to enhance Na^+,K^+-ATPase pump activity. This has been noted for renal tubular cells, erythrocytes, and sciatic nerve (2–4,13) and could have functional consequences. However, whereas nerve Na^+,K^+-ATPase activity is reduced in diabetic rats, there is little correlation between this defect and nerve dysfunction. For example, vasodilator treatment improved NCV without any effects on the Na^+,K^+-ATPase deficit (37); protein kinase C inhibition also corrected NCV at doses that had no effect on Na^+,K^+-ATPase, whereas higher doses corrected the Na^+,K^+-ATPase deficit but had deleterious effects on NCV (38). Furthermore, low-dose acetyl carnitine prevented the Na^+,K^+-ATPase defect but had no effect on NCV in diabetic rats (39).

It is possible, however, that enhanced Na^+,K^+-ATPase activity in vascular smooth muscle could contribute to C-peptide's vascular actions. Thus, Na^+,K^+-ATPase activity is reduced in vessels from diabetic animals and under hyperglycemic conditions in vitro (40). Restoring pump activity would promote hyperpolarization and vasorelaxation. Consequently, any improvement in blood flow would stimulate flow-induced NO production. Moreover, EDHF-dependent vasodilation is markedly reduced by diabetes (35,41,42). In some vessels, EDHF action depends on Na^+,K^+-ATPase stimulation (43); thus, enhancement of the Na^+,K^+ pump by C-peptide could augment EDHF-mediated responses.

In conclusion, C-peptide treatment at physiological levels improves sensory and motor NCV in diabetic rats. This primarily depends on a vascular action with an important involvement of the NO system. It is plausible that C-peptide supplementation in conjunction with insulin therapy could give added protection against the neural and vascular complications of type 1 diabetes.

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