C-peptide corrects endoneurial blood flow but not oxidative stress in type 1 BB/Wor rats

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Submitted 2 February 2004; accepted in final form 24 April 2004


Oxidative stress and neurovascular dysfunction have emerged as contributing factors to the development of experimental diabetic neuropathy (EDN) in streptozotocin-diabetic rodents. Additionally, depletion of C-peptide has been implicated in the pathogenesis of EDN, but the mechanisms of these effects have not been fully characterized. The aims of this study were therefore to explore the effects of diabetes on neurovascular dysfunction and indexes of nerve oxidative stress in type 1 bio-breeding Worcester (BB/Wor) rats and type 2 BB Zucker-derived (ZDR)/Wor rats and to determine the effects of C-peptide replacement in the former. Motor and sensory nerve conduction velocities (NCVs), hindlimb thermal thresholds, endoneurial blood flow, and indicators of oxidative stress were evaluated in nondiabetic control rats, BB/Wor rats, BB/Wor rats with rat II C-peptide replacement (75 nmol C-peptide/kg body wt−1·day−1) for 2 mo, and diabetes duration-matched BBZDR/Wor rats. Endoneurial perfusion was decreased and oxidative stress increased in type 1 BB/Wor rats. C-peptide prevented NCV and neurovascular deficits and attenuated thermal hyperalgesia. Inhibition of nitric oxide (NO) synthase, but not cyclooxygenase, reversed the C-peptide-mediated effects on NCV and nerve blood flow. Indexes of oxidative stress were unaffected by C-peptide. In type 2 BBZDR/Wor rats, neurovascular deficits and increased oxidative stress were unaccompanied by sensory NCV slowing or hyperalgesia. Therefore, nerve oxidative stress is increased and endoneurial perfusion decreased in type 1 BB/Wor and type 2 BBZDR/Wor rats. NO and neurovascular mechanisms, but not oxidative stress, appear to contribute to the effects of C-peptide in type 1 EDN. Sensory nerve deficits are not an inevitable consequence of increased oxidative stress and decreased nerve perfusion in a type 2 diabetic rodent model.

type 1 and type 2 diabetic neuropathy; oxidative stress

The pathogenetic basis for diabetic polyneuropathy (DPN) and its targeted treatment remain enigmatic, despite decades of intense investigations (64, 75, 76, 81). However, several hyperglycemia-induced pathways have been identified, such as J) activation of the polyol pathway, leading to redox imbalances, perturbation of myo-inositol and organic osmolyte imbalances (26, 53, 72); 2) nonenzymatic glycation, yielding advanced glycation end products; and 3) perturbations of neurotrophic homeostasis, particularly affecting NGF and the IGF system (3, 55, 63, 77). Many authors have proposed that these suggested consequences of hyperglycemia eventually come together, causing mitochondrial dysfunction, superoxide overproduction, and oxidative and nitrosative stress, which contribute to depletion of nitric oxide (NO) and impaired nerve perfusion, and that this provides a common mechanism underlying the pathogenesis of DPN (5, 13, 58, 75). These synergistic pathogenetic mechanisms have almost exclusively been identified, however, in streptozotocin-induced diabetes (STZ-D) in rats. Moreover, the vascular hypothesis of DPN remains controversial, and it is unclear whether similar neurovascular deficits exist in other animal models of diabetes.

During the last decade, the concept has emerged that DPN differs metabolically, functionally, and structurally in type 1 and type 2 diabetes in murine models that more closely mimic the human disorders (50, 59, 65–67). Besides hyperglycemia, increasingly the focus has turned to the pathogenetic roles of insulin and C-peptide deficiencies to explain the DPN accompanying type 1 diabetes (67, 84). There is now evidence to suggest that insulin and C-peptide deficiencies are involved in early metabolic perturbations affecting neural Na+–K+-ATPase, as well as the later occurring gene-regulatory deficits involving neurotrophic factors and their receptors (51, 63, 84). This may also be true for apoptotic phenomena afflicting both the peripheral and central nervous system in type 1 diabetes (36, 37, 70). We have previously shown that replacement of insulinomimetic C-peptide in type 1 bio-breeding Worcester (BB/Wor) rats is without any effect on hyperglycemia but nevertheless improves chronic diabetic neuropathy and has preventive effects on suppressed nerve fiber regeneration in longstanding neuropathy (51, 67). However, the mechanism(s) of this effect remains uncertain. In STZ-D rats, C-peptide replacement has been reported to improve nerve function by an NO-sensitive vascular mechanism (20). However, the effects of C-peptide on other glucose-sensitive pathways critically implicated in DPN, such as antioxidant defense systems, were not reported.

The aims of this study were therefore twofold: first, to determine whether neurovascular dysfunction and increased oxidative stress were present in the insulinopenic type 1 BB/Wor rat, and second, to determine the effects of C-peptide replacement on these end points in this animal model. The data were compared with those from isohyperglycemic and hyperinsulinemic C-peptide-replete type 2 BB Zucker-derived (ZDR)/Wor rats and nondiabetic BB rats.

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MATERIALS AND METHODS

Animals

Sixty-seven type 1 prediabetic male BB/Wor rats, 37 age- and sex-matched non-diabetes-prone BB rats, and 11 type 2 sex-matched BBZDR/Wor rats were obtained from Biomedical Research Models (Worcester, MA). All animals were maintained in metabolic cages with free access to water and rat chow. Body weight, urine volume, and glucosuria were monitored daily to determine onset of diabetes. After onset of type 1 diabetes in BB/Wor rats at 72 ± 4 days of age, they were supplemented with titrated doses (0.5–3.5 U/day) of pro-tamine zinc insulin (Novo Nordisk, Princeton, NJ). Blood glucose levels were measured every 2 wk. Onset of type 2 diabetes in BBZDR/Wor rats occurred at 75 ± 3 days. BBZDR/Wor rats do not require insulin supplementation but maintain spontaneously hyperglycemic levels between 20 and 25 mmol/L, which is similar to the levels at which type 1 BB/Wor rats are maintained.

At onset of diabetes, 30 type 1 BB/Wor rats were started on rat II C-peptide (Genosys, Cambridge, UK) with a purity of >98% by HPLC. It was dissolved in saline (12 mg/ml) and administered via subcutaneously implanted osmopumps (Alzet, Palo Alto, CA), which delivered a minimum dose of 75 nmol of C-peptide·kg body wt−1·day−1.

Experiment 1

To characterize changes in nerve oxidative defense enzymes, evaluate the effect of type 1 and type 2 diabetes on nerve blood flow, motor and sensory nerve conduction velocities (NCVs), and thermal pain thresholds, and to explore the effects of C-peptide replacement on these deficits in type 1 diabetes, BB/Wor and BBZDR/Wor rats were studied after 60 days of hyperglycemia, a time point when motor and sensory NCV slowing is well established (67). The experimental groups consisted of 1) nondiabetic control BB rats (n = 8); 2) untreated type 1 diabetic BB/Wor rats (n = 8); 3) diabetes BB/Wor rats with C-peptide replacement (n = 8); and untreated type 2 diabetic BBZDR/Wor rats (n = 7). After 2 mo, diabetes was reconfirmed, and motor and sensory NCVs, thermal thresholds, and endoneurial nerve blood flow were measured. The rats were killed, and sciatic nerves were rapidly excised and cleaned for biochemical measurements.

Experiment 2

To explore whether the effects of C-peptide replacement were mediated by NO or a cyclooxygenase (COX) product, nondiabetic control BB rats (n = 29) and untreated (n = 29) and C-peptide-replaced (n = 22) BB/Wor rats were treated with L-nitroarginine (L-NAME), flurbiprofen, or L-NAME + flurbiprofen administered in drinking water. L-NAME and/or flurbiprofen was administered for the final 2 wk of the 2-mo experimental period in both nondiabetic control BB rats and untreated or C-peptide-treated BB/Wor rats. Nondiabetic animals were given drinking water containing 0.685 mM L-NAME, whereas in diabetic animals the concentration was reduced to 0.137 mM to compensate for the fivefold increase in water consumption. Flurbiprofen [a non-selective COX 1 and COX 2 inhibitor, relative COX-2 vs. COX-1 IC50, 5–10 (23, 41)] was administered at a dose of 6 mg·kg−1·day−1, with the concentration of flurbiprofen reduced to 20% in diabetic animals (5:1) to compensate for their increased water consumption.

After 2 wk, diabetes was reconfirmed, and NCVs, thermal thresholds, and nerve blood flow were measured. The rats were killed, and both sciatic nerves were rapidly excised and cleaned for biochemical measurements.

NCVs

NCV measurements were performed noninvasively under temperature-controlled conditions (36–38°C). Animals were sedated by inhalation of Metofane (Pitman-Moore, Mundelein, IL). For motor NCV (MNCV), the left sciatic nerve was stimulated with square pulses (2 Hz) at the sciatic notch, and the tibial nerve was stimulated by supramaximal (8V) stimulations at the ankle. The evoked motor responses were recorded from the first interosseous muscle. Eight to sixteen recordings were computer-averaged. MNCV was calculated by dividing the distance between the two stimulating points by the difference between proximal and distal latencies (67). Sensory NCV (SNCV) was recorded in the right hindlimb. The digital nerves of the second toe were stimulated with square pulses of 0.05-ms duration by supramaximal current intensities. Sensory nerve action potentials were recorded from posterior to the medial malleolus. The distance between stimulating and recording electrodes was 25 mm and that between the active recording and indifferent electrodes was 10 mm. Eight to sixteen responses were averaged. The distance between the stimulating and active recording electrodes was measured and divided by the latency to the peak of the initial negative deflection (68).

Thermal Plantar Test

Hyperalgesia to thermal stimulation was measured using a UGO Basile Biological Research Apparatus (Comerio, Italy) and performed according to Hargreaves et al. (30). Animals were placed in a clear plastic chamber with an elevated plastic bottom and allowed to acclimate for 5 min. A mobile thermal stimulator, generating infrared energy of constant heat intensity, was positioned under the posterior plantar area of a hind paw. The time from heat source activation to the animal’s self-withdrawal of the hind paw was recorded in seconds. This was repeated six times, with 5-min intervals between measurements of alternating hind paws. The mean of these measurements was used as the measure of the latency (83).

Measurement of Endoneurial Nutritive Nerve Blood Flow by H2 Clearance

Nitrergic nerve blood flow (NBF) was assessed by H2 clearance, as previously reported (52). Animals were anesthetized with an injection of thiobarbituratal (Inactin, 65–85 mg/kg ip). The left carotid artery was cannulated with polyethylene tubing and patency maintained with heparinized saline (50 U/ml normal saline). The catheter was connected to a transducer and the blood pressure monitored by a MacLab data acquisition system. A tracheostomy was performed, and the animals respiried artificially with O2–N2 (21.79) with a small animal ventilator (Harvard Apparatus, South Natick, MA). Core body temperature was monitored by a rectal probe and hindlimb muscle temperature by a needle probe inserted into the muscle layer; both were maintained at 37°C by a Homeothermic Blanket Control Unit (Harvard Apparatus). The right sciatic nerve was exposed and gently dissected away from the surrounding tissue. The skin around the incision was positioned to create a reservoir. A ground electrode was inserted subcutaneously into the flank of the rat. Using a micromanipulator, a hydrogen-sensitive platinum electrode (tip diameter 1–2 μm; World Precision Instruments, Sarasota, FL) was inserted into the nerve above the trifurcation. Mineral oil at 37°C was used to fill the reservoir and prevent diffusion of gases out of the nerve. Oil temperature was monitored and maintained at 37°C by radiant heat. The nerve was polarized with 0.25 V, and when a stable baseline was achieved, the animal received a gas mixture containing 10% hydrogen, which was continued until the current change stabilized (10–30 min), at which time hydrogen flow was terminated. Current recordings were made every 30 s until baseline levels were achieved (30–60 min). After the experiment, mono- or biexponential clearance curves were fitted to the data (GraphPad Software, La Jolla, CA). Nitrergic endoneurial NBF was taken as the slow component of the curve. An average of two determinations at different sites was used to determine nitrergic endoneurial nerve perfusion.
Nerve Biochemical Measurements

Malonyldialdehyde plus 4-hydroxyalkenal. Measurements of total malonyldialdehyde (MDA) and 4-hydroxyalkenal (4-HA) levels were performed using commercially available kits [Oxis International, Portland, OR (LPO-586 assay)]. The method is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA and 4-HA at 45°C (47). The samples were prepared by homogenization of preweighed nerve segments (~40 mg) in 1 ml of 20 mM Tris buffer, pH 7.4, containing 5 mM butylated hydroxytoluene. Two hundred microliters of homogenate were used for measurements of total MDA and 4-HA levels, according to the procedure described in detail in the kit. The absorbance of chromogenic product was measured at 586 nm (spectrophotometer Beckman DU 640) and was compared with the absorbance in corresponding 4-HA standards.

GSH. Approximately 15–20 mg of the sciatic nerves were weighed, homogenized in 1 ml of ice-cold 6% HClO4, and centrifuged at 4,000 g for 10 min. After centrifugation, the samples were immediately neutralized with 5 M K2CO3 to pH 6–7 and centrifuged again at 4,000 g for 5 min to precipitate insoluble KClO4. Reduced glutathione (GSH) levels were assayed in perchloric extracts spectrophotometrically. Briefly, 0.1 ml of extract was mixed with 0.89 ml of 20 mM EDTA in 1.0 M Tris-HCl buffer (pH 8.1), and the reaction was initiated by the addition of 0.01 ml of 1% methanol solution of O-phthalaldehyde.

Catalase. Catalase enzyme activity was measured fluorometrically by using a kit (cat. no. A-22180; Molecular Probes, Eugene, OR). Samples were allowed to react with H2O2, followed by reaction with the chromagen Amplex Red. The chromagen is oxidized by any enzyme activity when compared against a catalase standard curve. Samples were allowed to react with H2O2, followed by reaction with Amplex Red. The amount of catalase enzyme activity was calculated by comparing unknowns against a catalase standard curve.

Superoxide dismutase. Cu/Zn-superoxide dismutase (SOD) enzyme activity was measured by using a kit (no. 21010; Oxis International). Briefly, tissues were rapidly dissected, washed in ice-cold PBS, and snap-frozen in liquid nitrogen until ready for assay. Tissues were then thawed and homogenized in 50 mM NaH2PO4 pH 7.4 buffer on ice and clarified by centrifugation (10,000 g, 4°C, 15 min). Supernatants were reacted with H2O2 followed by Amplex Red. The amount of catalase activity was measured fluorometrically at absorption/emission (Abs/Em) 563/587. Absolute activity was calculated by comparing unknowns against a catalase standard curve.

RESULTS

Effects of Diabetes and C-Peptide on Plasma Glucose Values and Body Weights (Experiment 1)

After 2 mo of diabetes, body weights were 17% lower in BB/Wor rats vs. control rats (P < 0.001) and 54% higher in BBZDWR/Wor rats vs. control rats (P < 0.001; Table 1). Body weights were unaffected by C-peptide treatment. Blood glucose levels were elevated about four- to fivefold in all diabetic rat groups. Serum insulin levels were markedly decreased in BB/Wor rats (P < 0.001) and increased in BBZDWR/Wor rats (P < 0.01). C-peptide replacement of BB/Wor rats did not alter weight, hyperglycemia, required daily insulin dosing, or plasma insulin levels, but it completely normalized circulating C-peptide levels.

C-PEPTIDE REPLACEMENT AMELIORATES MOTOR AND SENSORY NCV SLOWING AND THERMAL HYPERALGESIA IN BB/WOR RATS (EXPERIMENT 1)

After 2 mo of diabetes, as expected, MNCV and SNCV were reduced by 22 and 14%, respectively (P < 0.001) in BB/Wor vs. nondiabetic control rats (Fig. 1). The administration of C-peptide partially prevented MNCV deficits by 72% (P < 0.05) and completely prevented SNCV deficits. In response to thermal heating, limb withdrawal time was decreased by 42% (P < 0.001) in BB/Wor rats and was partially corrected by C-peptide (39%; P < 0.05).

In contrast, MNCV was decreased by only 5% (P < 0.05) in BBZDWR/Wor rats vs. nondiabetic control animals, and SNCV was unaltered. Plantar heat withdrawal thresholds were not significantly affected in BBZDWR/Wor rats.

Table 1. Clinical data of control, type 1 diabetic BB/Wor rats, type 1 diabetic BB/Wor rats with C-peptide replacement, and type 2 BBZDWR/Wor rats

<table>
<thead>
<tr>
<th>n</th>
<th>Body Weight, g</th>
<th>Blood Glucose, mmol/l</th>
<th>Insulin Dose, U/day</th>
<th>Insulin Levels, pmol/l</th>
<th>C-Peptide, pmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>404±10</td>
<td>5.3±0</td>
<td>0</td>
<td>430±20</td>
</tr>
<tr>
<td>BB/Wor</td>
<td>8</td>
<td>337±9†</td>
<td>22.4±0.5†</td>
<td>2.1±0.1</td>
<td>52±5†</td>
</tr>
<tr>
<td>BB/Wor + C-Peptide</td>
<td>8</td>
<td>336±8†</td>
<td>23.4±0.9†</td>
<td>2.1±0.1</td>
<td>40±7†</td>
</tr>
<tr>
<td>BBZDWR/Wor</td>
<td>7</td>
<td>626±18†</td>
<td>23.4±2.1†</td>
<td>0</td>
<td>586±25*</td>
</tr>
</tbody>
</table>

Clinical values (means ± SE; n, no. of rats/group) of control, type 1 diabetic bio-breeding Worcester (BB/Wor) rats, type 1 BB/Wor rats with C-peptide replacement, and type 2 BB Zucker-derived (ZDR)/Wor rats. C-peptide replacement had no effect on body weight, blood glucose levels, or required daily insulin dose in BB/Wor rats. Type 2 BBZDWR/Wor rats were significantly (P < 0.001) heavier than control and BB/Wor rats but showed hyperglycemic levels equal to those in BB/Wor rats and BB/Wor rats with C-peptide replacement. C-peptide replacement of BB/Wor rats had no effect on plasma insulin levels and normalized fully plasma C-peptide levels. Insulin levels were significantly (P < 0.01) higher in type 2 BBZDWR/Wor rats than in BB/Wor rats. *P < 0.01; †P < 0.001 vs. control group.
C-PEPTIDE CORRECTS ENDOONEURIAL BLOOD FLOW

**A**

![Graph showing MNCV (m/sec) with statistical significance](image)

**B**

![Graph showing SNCV (m/sec) with statistical significance](image)

**C**

![Graph showing Plantar Test (sec) with statistical significance](image)

Fig. 1. Effect of diabetes and C-peptide on nerve conduction velocities (NCVs) and thermal hyperalgesia. Hindlimb motor (M)NCV and sensory (S)NCV and thermal thresholds were determined in untreated and C-peptide-treated type 1 bio-breeding Worcester (BB/Wor) rats and untreated BB/Zucker-derived (ZDR) rats after 60 days of hyperglycemia. MNCV was measured in the left sciatic-tibial conducting system, SNCV in the left foot digital nerves, and hyperalgesia by response to thermal stimulation of a hind paw, as outlined in MATERIALS AND METHODS. Data are means ± SE; n = no. of rats/group. ***P < 0.001, **P < 0.01, *P < 0.05 vs. control (Cont); †P < 0.05 vs. BB/Wor rats.

**Endoneurial Blood Flow is Decreased in BB/Wor and BBZDR/Wor Rats and is Sensitive to C-Peptide Replacement in the Former (Experiment 1)**

In BB/Wor rats, endoneurial blood flow was reduced by 44% (P < 0.01) vs. control rats (Fig. 2). C-peptide treatment of BB/Wor rats prevented the endoneurial perfusion deficits (endoneurial perfusion increased by 58%, P < 0.05, vs. untreated BB/Wor rats). In parallel, endoneurial blood flow was decreased by 33% in BBZDR/Wor rats (P < 0.01). Mean arterial blood pressure was significantly reduced in BB/Wor rats compared with controls (119 ± 16 vs. 142 ± 7 mmHg, respectively, P < 0.05) but was unchanged in BBZDR/Wor animals (142 ± 18 mmHg). The results were not significantly affected after correction for blood pressure and were expressed as vascular conductance.

**Antioxidant Defense is Impaired and Lipid Peroxidation is Increased in BB/Wor and BBZDR/Wor Rats (Experiment 1)**

Nerve MDA plus 4-HA concentration was 2.3-fold (P < 0.01) higher in BB/Wor rats compared with nondiabetic control animals (Table 2). The diabetes-induced increase in lipid peroxidation was unaffected by C-peptide treatment. SOD activity was decreased 49% (P < 0.01) in these rats compared with control animals and was also unaffected by C-peptide treatment. Similarly, catalase activity was decreased by 53% (P < 0.05) in BB/Wor rats and was unaffected by C-peptide.

In BBZDR/Wor rats, MDA plus 4-HA concentration was increased 2.8-fold (P < 0.01) and SOD activity was unchanged, whereas catalase activity was decreased by 45% (P < 0.05). Nerve GSH levels were not significantly changed in any of the experimental groups.

**Effects of L-NAME and Flurbiprofen on Body Weight and Plasma Glucose (Experiment 2)**

Neither L-NAME nor flurbiprofen alone or in combination affected body weights in nondiabetic, untreated, or C-peptide-treated BB/Wor rats (data not shown).

**Effect of L-NAME and Flurbiprofen on MNCV, SNCV, and Thermal Hyperalgesia (Experiment 2)**

After 2 wk, neither L-NAME nor flurbiprofen, alone or in combination, affected MNCV, SNCV, and thermal hyperalgesia in nondiabetic control rats (Table 3). In non-C-peptide-treated BB/Wor rats, L-NAME improved partially but significantly (18%, P < 0.05) the latency of thermal hyperalgesia. In C-peptide-treated BB/Wor rats, L-NAME eliminated partially the effect of C-peptide on MNCV (48%, P < 0.001) and SNCV (54%, P < 0.01). L-NAME had no effect on thermal hyperalgesia in C-peptide-treated BB/Wor rats (Table 3). Flurbiprofen had no effects on MNCV, SNCV, or thermal hyperalgesia in C-peptide-treated rats (Table 3).

**L-NAME, but not Flurbiprofen, Reverses the Effect of C-Peptide on Endoneurial Nutritive NBF (Experiment 2)**

In L-NAME-, flurbiprofen-, and L-NAME + flurbiprofen-treated nondiabetic control rats, endoneurial blood flow was reduced by 30, 23, and 27%, respectively (P < 0.05 vs. untreated nondiabetic rats; Table 4). In BB/Wor rats, the diabetes-induced deficit of endoneurial perfusion was not affected by either L-NAME or flurbiprofen. In BB/Wor rats, L-NAME, but not flurbiprofen, completely prevented the protective effect of C-peptide on endoneurial blood flow. As expected, mean arterial blood pressure was significantly increased in all the L-NAME-treated animal groups but was unaffected by flurbiprofen (Table 4). These results were not affected after correction for blood pressure; they are expressed as vascular conductance.

**DISCUSSION**

Oxidative stress and neurovascular dysfunction have merged as contributing factors to the development of acute EDN in
STZ-D rodents. However, the vascular hypothesis of DPN remains controversial, and the acute STZ-D rat has been criticized as a model of human DPN (64). Moreover, it is not known whether similar metabolic and neurovascular deficits are present in other diabetic rodent models and to what extent these deficits, if present, may reflect depletion of C-peptide. The aims of this study were, therefore, to explore the effects of diabetes on neurovascular dysfunction and indexes of nerve oxidative stress in type 1 C-peptide-deficient BB/Wor rats and in type 2 C-peptide-replete BBZDR/Wor rats, and to determine the effects of C-peptide replacement in the type 1 animal model. In concert with the STZ-D rat (53), NCV and endothelial conductance were decreased and oxidative stress was increased in type 1 BB/Wor rats. C-peptide replacement prevented NCV slowing and neurovascular deficits but not oxidative stress by a NO-sensitive mechanism. In type 2 BBZDR/Wor rats, neurovascular deficits and increased oxidative stress were unaccompanied by sensory NCV slowing or hyperalgesia. These data therefore implicate NO and neurovascular mechanisms, but not oxidative stress, as mediators of the effects of C-peptide replacement in type 1 BB/Wor rats and dissociate neurovascular deficits and oxidative stress from C-peptide depletion and sensory defects in type 2 BBZDR/Wor rats.

C-peptide treatment has been shown to prevent or reverse experimental DPN in type 1 BB/Wor rats (67) as well as STZ-D animals (20). Additionally, several physiological effects of C-peptide have been identified in patients and animal models with type 1 diabetes (for reviews see Refs. 63, 64, and 80). The studies reported herein, together with another report (20), are consistent with an NO-sensitive neurovascular action, which may complement actions mediated via a G protein-coupled receptor (56) or via interaction with the insulin receptor (28, 37). Indeed, C-peptide has been shown to exert insulin-like effects without affecting blood glucose levels (28, 51, 67) and to synergize the effects of insulin (28, 37). A neurovascular effect is also consistent with the findings that C-peptide stimulates endothelial NOS with release of NO from bovine aortic endothelial cells (35), increases forearm blood flow in type 1 patients (25), and produces a concentration-dependent dilatation of rat skeletal muscle arteries (33).

Decreased endoneurial perfusion has emerged as an important contributing factor to the development of MNCV slowing in experimental diabetes on the basis of the findings in STZ-D rats (53, 75). Decreased NBF and consequent endoneurial hypoxia are thought to contribute to NCV slowing by promoting nerve energy deficits (74, 75). This report is the first to demonstrate in the type 1 BB/Wor rat that endoneurial perfusion is also decreased in concert with NCV slowing. Type 2 hyperinsulinemic BBZDR/Wor rats also exhibited a significant decrease in endoneurial blood flow. However, despite 2 mo of hyperglycemia, motor NCV was only minimally slowed, and sensory NCV and thermal latencies were unaffected in this model, suggesting that reduced endoneurial perfusion does not necessarily result in NCV slowing or hyperalgesia. In this type 2 animal model, longer durations of diabetes are required to develop more profound nerve conduction deficits [a 17% reduction of NCV has been observed after 14 mo of diabetes (68)]. Moreover, in BBZDR/Wor rats, reduced nerve perfusion was observed despite the absence of C-peptide depletion.

Table 2. Effects of diabetes and C-peptide on sciatic nerve lipid peroxidation products and antioxidant defense enzymes

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>SOD, μmol/g</th>
<th>Catalase, μmol/g</th>
<th>GSH, μmol/g</th>
<th>MDA + 4-HA, μmol/g</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>78±28</td>
<td>501±210</td>
<td>0.15±0.04</td>
<td>1.2±0.4</td>
</tr>
<tr>
<td>BB/Wor</td>
<td>6</td>
<td>40±11†</td>
<td>233±110†*</td>
<td>0.19±0.03</td>
<td>2.7±0.7†</td>
</tr>
<tr>
<td>BB/Wor + C-peptide</td>
<td>6</td>
<td>42±6†</td>
<td>176±60*</td>
<td>0.20±0.04</td>
<td>2.7±0.3†</td>
</tr>
<tr>
<td>BBZDR/Wor</td>
<td>6</td>
<td>86±10</td>
<td>275±59*</td>
<td>0.21±0.05</td>
<td>3.4±0.9†</td>
</tr>
</tbody>
</table>

Levels of sciatic nerve Cu/Zn-superoxide dismutase (SOD), catalase, GSH, and lipid peroxidation products malondialdehyde and 4 hydroxyalkenal (MDA + 4-HA) were measured spectrophotometrically with freshly prepared sciatic nerve homogenates, as described in MATERIALS AND METHODS. Values are means ± SE of 6 rats/group. *P < 0.05, †P < 0.01 vs. control.
suggesting that endoneurial blood flow regulation is unrelated to C-peptide in type 2 EDN, at least in this animal model. However, the salutary effects of C-peptide replacement on NBF deficits are consistent with a neurovascular action of C-peptide replacement in type 1 diabetes, which may compensate for other diabetes-induced vascular perturbations. The vascular effects of C-peptide are consistent with other reports in diabetic rodent models, which demonstrate that correction of endoneurial perfusion deficits correlates with the prevention or correction of motor NCV slowing in experimental DPN (8, 10, 11, 13, 18, 19, 44, 48, 49, 52, 71, 73, 75, 82).

In nondiabetic control animals, 2 wk of treatment with L-NAME and/or flurbiprofen decreased endoneurial perfusion without affecting NCV, demonstrating that acute reductions in nerve perfusion alone are not sufficient to produce electrophysiological deficits. We have previously reported that more chronic (3-mo) treatment with L-NAME is required to slow NCV in nondiabetic Wistar rats (72). However, the beneficial effects of C-peptide on neurovascular perfusion and NCV slowing were reversed by the simultaneous treatment with L-NAME but not flurbiprofen, implicating an NO, but not COX-mediated, mechanism. We have previously reported the ability of L-NAME specifically to competitively inhibit NOS in experimental type 1 diabetes, because its effects on NCV slowing and blood pressure elevation can be prevented by arginine supplementation (72). The sensitivity of the effect of C-peptide to L-NAME is consistent with other interventions that appear to work via NO, including aldose reductase inhibitors (ARIs) (12, 72), evening primrose oil (12), acetyl L-carnitine (19), salbutamol, and doxazosin (18).

The effects of diabetes on NO metabolism and the precise location of the critical NO effect appear to be complex. NO synthase, the enzyme catalyzing the conversion of L-arginine to citrulline and NO at the expense of NADPH (43), is situated at sites critical for the regulation of neurovascular function, including endothelial cells, vascular smooth muscle cells, and sympathetic ganglia (75). In sympathetic ganglia, immunohistochemical staining has shown a reduction of NO synthase stainability in diabetic rats (60). In the sympathetic autonomic nervous system, NO may inhibit sympathetic tone (61) such that impaired NO synthase activity in diabetes may increase sympathetic outflow, resulting in neurovascular dysfunction. Alternatively, depletion of NO in the vasa nervorum has been invoked as a critical factor in the development of nerve perfusion deficits (12, 75). Impaired synthesis of NO has been linked to polyol pathway activation through an NADPH-mediated mechanism and alterations in protein kinase activation and calcium levels (7, 27, 72). Additionally, increased oxidative stress has been implicated in the depletion of vascular NO (31, 75). Conversely, human studies have demonstrated elevated plasma levels of NO3− in subjects with advanced neuropathy (40), and increased plasma nitrotyrosine, an indirect marker of pro-oxidant peroxynitrite formation (32), has been identified in diabetic patients (15). In animal models of EDN, increased peroxynitrite formation has been implicated in cell death (22). Therefore, the effects of diabetes on NO metabolism appear complex, highly compartmentalized, and critically affected by oxidative stress. Although our data clearly indicate NO repletion in the beneficial effects of C-peptide, which is consistent with other reports (25, 33, 35), the

### Table 3. Nerve conduction velocities and thermal hyperalgesia latencies in animals treated with L-NAME or flurbiprofen or both (experiment 2)

<table>
<thead>
<tr>
<th>Condition</th>
<th>NMCV, m/s</th>
<th>SNCV, m/s</th>
<th>Plantar Test, s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>58.1 ± 0.7</td>
<td>40.6 ± 0.4</td>
<td>19.8 ± 0.6</td>
</tr>
<tr>
<td>Control + L</td>
<td>59.3 ± 0.4</td>
<td>40.8 ± 0.5</td>
<td>20.0 ± 0.7</td>
</tr>
<tr>
<td>Control + F</td>
<td>58.6 ± 0.4</td>
<td>39.7 ± 0.5</td>
<td>20.4 ± 0.7</td>
</tr>
<tr>
<td>Control + L + F</td>
<td>59.1 ± 0.7</td>
<td>40.9 ± 0.4</td>
<td>19.8 ± 0.8</td>
</tr>
<tr>
<td>BB/Wor</td>
<td>45.1 ± 0.7 †</td>
<td>34.4 ± 0.4†</td>
<td>11.5 ± 0.5†</td>
</tr>
<tr>
<td>BB/Wor + L</td>
<td>46.6 ± 0.7 †</td>
<td>35.3 ± 0.3†</td>
<td>13.0 ± 0.4†</td>
</tr>
<tr>
<td>BB/Wor + F</td>
<td>45.7 ± 0.5†</td>
<td>35.8 ± 0.2†</td>
<td>10.9 ± 0.4†</td>
</tr>
<tr>
<td>BB/Wor + L + F</td>
<td>43.6 ± 1.1†</td>
<td>34.7 ± 0.7†</td>
<td>12.1 ± 0.6†</td>
</tr>
<tr>
<td>BB/Wor + C-peptide</td>
<td>54.6 ± 0.7 †</td>
<td>39.0 ± 0.3 †</td>
<td>14.7 ± 0.7 ††</td>
</tr>
<tr>
<td>BB/Wor + C-peptide + L</td>
<td>50.3 ± 0.7</td>
<td>37.2 ± 0.4</td>
<td>13.7 ± 0.3 ††</td>
</tr>
<tr>
<td>BB/Wor + C-peptide + F</td>
<td>54.0 ± 0.7 ††</td>
<td>39.2 ± 0.3 ††</td>
<td>14.5 ± 0.5 ††</td>
</tr>
</tbody>
</table>

Neither L-NAME (+L) nor flurbiprofen (+F) had any effect on nerve conduction velocities (NCV) and thermal hyperalgesia latencies in control rats. In non-C-peptide-treated BB/Wor rats, L-NAME improved partially but significantly (P < 0.05) the latency of thermal hyperalgesia. In C-peptide-treated BB/Wor rats, L-NAME eliminated partially the effect of C-peptide on motor NCV (MNCV; P < 0.001) and sensory NCV (SNCV; P < 0.01), L-NAME had no effect on thermal hyperalgesia in C-peptide-treated BB/Wor rats. Flurbiprofen had no effects on MNCV, SNCV, or thermal hyperalgesia in C-peptide-treated rats. *P < 0.01, †P < 0.001 vs. control; ‡P < 0.05 vs. BB/Wor.

### Table 4. Effect of L-NAME and flurbiprofen on endoneurial nutritive nerve blood flow (experiment 2)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Endoneurial Blood Flow, mℓ/ℓmin -100 g−1</th>
<th>Mean Arterial Blood Pressure, mmHg</th>
<th>Vascular Conductance (mℓ/ℓmin -100 g−1-mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.3 ± 0.7</td>
<td>142 ± 2.9</td>
<td>0.11 ± 0.004</td>
</tr>
<tr>
<td>Control + L</td>
<td>10.7 ± 0.9*</td>
<td>173 ± 6.1†</td>
<td>0.06 ± 0.01†</td>
</tr>
<tr>
<td>Control + F</td>
<td>11.8 ± 1.0*</td>
<td>147 ± 3.5</td>
<td>0.08 ± 0.01*</td>
</tr>
<tr>
<td>Control + L + F</td>
<td>11.2 ± 1.2*</td>
<td>159 ± 8.1*</td>
<td>0.07 ± 0.01†</td>
</tr>
<tr>
<td>BB/Wor</td>
<td>8.6 ± 0.7†</td>
<td>119 ± 6.2*</td>
<td>0.07 ± 0.003†</td>
</tr>
<tr>
<td>BB/Wor + L</td>
<td>10.6 ± 0.8†</td>
<td>146 ± 3.1†</td>
<td>0.07 ± 0.003†</td>
</tr>
<tr>
<td>BB/Wor + F</td>
<td>10.5 ± 0.8†</td>
<td>128 ± 3.4</td>
<td>0.08 ± 0.01*</td>
</tr>
<tr>
<td>BB/Wor + C-peptide</td>
<td>11.7 ± 0.9*</td>
<td>130 ± 3.6</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>BB/Wor + C-peptide + L</td>
<td>13.6 ± 1.1*</td>
<td>132 ± 4.6</td>
<td>0.10 ± 0.004*</td>
</tr>
<tr>
<td>BB/Wor + C-peptide + F</td>
<td>8.6 ± 0.5†</td>
<td>147 ± 3.1†</td>
<td>0.07 ± 0.01†</td>
</tr>
<tr>
<td>BB/Wor + C-peptide + F + L</td>
<td>13.0 ± 1.3*</td>
<td>127 ± 4.2</td>
<td>0.10 ± 0.01†</td>
</tr>
</tbody>
</table>

Endoneurial nerve blood flow (NBF) was assessed by hydrogen clearance technique, as described in MATERIALS AND METHODS. All flow data were also expressed as vascular conductance, which was calculated by dividing the blood flow by the mean systemic blood pressure over the recording period. Values are means ± SE. *P < 0.05, †P < 0.01 vs. control; ‡P < 0.05 vs. BB/Wor.
mechanism of this effect is unclear, because C-peptide does not alter polyol pathway flux (67) or indexes of oxidative stress.

Two months of diabetes resulted in hindlimb thermal hyperalgesia in type 1 BB/Wor rats, consistent with the findings in other diabetic rodent models (1, 2, 21). Hyperalgesia was significantly attenuated by C-peptide replacement. Interestingly, thermal hyperalgesia was not detected in the C-peptide-replete BBZDR/Wor rats despite the presence of decreased nerve blood flow, implicating a nonvascular C-peptide-related pathogenesis. (Hypersensitivity to mechanical stimuli was not tested, and so we cannot exclude a deficit in this sensory modality in this model.) Although there are limitations to the current models of painful diabetic neuropathy, damage to C fibers (1, 6, 17), producing central sensitization (16, 17) as well as small myelinated afferent fibers (34), has been implicated in the development of pain (57). Increased spontaneous activity from cutaneous nociceptors has been demonstrated in STZ-D rats (16, 62) as well as in the proximal stump of the saphenous nerve of BB/Wor rats (6), implicating spontaneous excitation of cutaneous nociceptors in the development of pain and dysesthesia in diabetes.

The precise mechanisms whereby hyperglycemia/insulin/C-peptide depletion contributes to hyperalgesia remain unclear. Oxidative stress has been invoked as a contributing factor to hyperalgesia (14, 78) and abnormal calcium signaling in the dorsal root ganglia (DRG) neurons of diabetic rodents (29, 79), and consequent activation of NO/cGMP/PKG pathways has been invoked in the pathogenesis of experimentally induced pain (42, 69). Our demonstration that L-NAME treatment of BB/Wor rats attenuated hyperalgesia is consistent with other reports implicating NO as a second messenger system contributing to hyperalgesia (2). The persistent effect of C-peptide on hyperalgesia despite L-NAME cotreatment, the absence of a C-peptide effect on oxidative stress, and the lack of hyperalgesia in BBZDR/Wor rats implies that neurovascular deficits, oxidative stress, and NO are not involved in this effect. In BB/Wor rats, degeneration of nociceptive C fibers is associated with decreased expression of NGF, substance P, and calcitonin gene-related peptide in DRG neurons, which is prevented by C-peptide (Murakawa Y and Sima AAF, unpublished data), implicating a neutrophic/neuropeptide-sensitive mechanism. Further studies are required to characterize these effects.

Increased oxidative and nitrosative stress (4, 10, 24, 38, 39, 44, 45, 74) has emerged a leading candidate in the pathogenesis of DPN. A direct relationship has emerged between measures of oxidative stress and the development of NBF deficits (4, 10, 38, 39, 44, 45, 74). C-peptide replacement prevented neurovascular deficits without attenuating oxidative stress, demonstrating that nerve perfusion deficits are not the cause of increased ROS production in this animal model. Consistent with findings in the STZ-D rat model (46, 47), nerve lipid peroxidation products were increased in both BB/Wor and BBZDR/Wor models after 2 mo of hyperglycemia. However, differences emerged in the response of the antioxidant defense enzymes, because GSH was not depleted in either animal model, and SOD activity (but not catalase) was preserved in BBZDR/Wor rats. The presence of increased lipid peroxidation but lack of GSH depletion parallels the findings in some diabetic mouse models of DPN (Stevens M and Obrosova IG, personal observations) but is in contrast to the STZ-D rat (46, 47) and suggests nonuniformity in the diabetic rodent response to oxidative stress. Whether disruption of other antioxidant defense systems, such as the ascorbate/dehydroascorbate or taurine systems, is of greater importance in these animal models, and the precise loci of the critical oxidative insult, remain to be explored. Nevertheless, these heterogeneous responses in different animal models may aid understanding of the mechanisms contributing to and protecting from oxidative stress in diabetes.

In summary, the present study has demonstrated that endoneurial perfusion is decreased and oxidative stress increased in type 1 BB/Wor rats. C-peptide replacement did not affect oxidative stress but, nevertheless, prevented NCV and neurovascular deficits and attenuated thermal hyperalgesia, by NO-sensitive and -insensitive mechanisms, respectively. In type 2 BBZDR/Wor rats, neurovascular deficits and increased oxidative stress were unaccompanied by sensory NCV slowing or hyperalgesia. These data therefore demonstrate that C-peptide replacement can compensate for the downstream consequences of persistent oxidative stress in a model of type 1 DPN, and that sensory nerve deficits are not an inevitable consequence of increased oxidative stress and decreased nerve perfusion in a type 2 diabetic rodent model.

GRANTS

This study was supported in part by the Juvenile Diabetes Research Foundation (Stevens MJ and Sima AAF), the National Institutes of Health Grant RO1-DK-52391 (Stevens MJ), and the Thomas Foundation (Sima AAF and Zhang W).

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