Original Article

C-Peptide Reverses Nociceptive Neuropathy in Type 1 Diabetes

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We examined the therapeutic effects of C-peptide on established nociceptive neuropathy in type 1 diabetic BB/Wor rats. Nociceptive nerve function, unmyelinated sural nerve fiber and dorsal root ganglion (DRG) cell morphometry, nociceptive peptide content, and the expression of neurotrophic factors and their receptors were investigated. C-peptide was administered either as a continuous subcutaneous replacement dose via osmopumps or a replacement dose given once daily by subcutaneous injection. Diabetic rats were treated from 4 to 7 months of diabetes and were compared with control and untreated diabetic rats of 4- and 7-month duration. Osmopump delivery but not subcutaneous injection improved hyperalgesia and restored the diabetes-induced reduction of unmyelinated fiber number ($P < 0.01$) and mean axonal size ($P < 0.05$) in the sural nerve. High-affinity nerve growth factor (NGF) receptor (NGFR-TrkA) expression in DRGs was significantly reduced at 4 months ($P < 0.01$). Insulin receptor and IGF-I receptor (IGF-IR) expressions in DRGs and NGF content in sciatic nerve were significantly decreased in 7-month diabetic rats ($P < 0.01$, 0.05, and 0.005, respectively). Osmopump delivery prevented the decline of NGFR-TrkA, insulin receptor ($P < 0.05$), and IGF-IR ($P < 0.005$) expressions in DRGs and improved NGF content ($P < 0.05$) in sciatic nerve. However, subcutaneous injection had only marginal effects on morphometric and molecular changes in diabetic rats. We conclude that C-peptide exerts beneficial therapeutic effects on diabetic nociceptive neuropathy and that optimal effects require maintenance of physiological C-peptide concentrations for a major proportion of the day. *Diabetes* 55:3581–3587, 2006

Painful or nociceptive diabetic neuropathy is a common and debilitating condition in neuropathic patients (1). The underlying mechanisms are complex and not completely understood (2). However, it is generally accepted that degeneration of unmyelinated C-fibers and Aβ-fibers is an initiating factor (2,3). Damage to C-fibers causes increased excitability via upregulation of Na^+ channels and α-adrenergic receptors (4–6). Pain sensation is further enhanced in the dorsal horn by excitotoxic stimulation of nociceptive interneurons by collaterals from Aβ-fibers, so-called central sensitization (2,7).

Painful diabetic neuropathy can be an early symptom and may already be manifested in patients and animals with pre-diabetes (8–10). In previous studies, we have demonstrated a relationship between impaired insulin action and suppressed neurotrophic support, decreased expression of nociceptive neuropeptides, and degeneration of their parent dorsal root ganglion (DRG) cells (11–13).

C-peptide exerts insulinomimetic effects and, like insulin, is lacking in type 1 diabetes (14,15). Although C-peptide has no immediate effect on hyperglycemia, it signals partly through the insulin signaling pathway (14–19). It inhibits insulin signaling at high concentrations and synergizes it at low concentrations (15–17). Previous studies have shown that it enhances the gene expression of nerve growth factor (NGF), IGF-I, and their receptors and that of the insulin receptor itself (11,18,20).

In this study, we examined the therapeutic effects of full C-peptide replacement on nociceptive neuropathy in the spontaneously type 1 diabetic BB/Wor rat, which provides a close model of human type 1 diabetes.

RESEARCH DESIGN AND METHODS

Thirty-five pre-diabetic male BB/Wor rats and 17 age- and sex-matched nondiabetes-prone BB 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 (Keto-Diastrix; Bayer, Elkhart, IN) were monitored daily to ascertain onset of diabetes and were then used as the basis for daily titration of insulin doses. After onset of diabetes at 73 ± 6 days of age, all diabetic rats were supplemented with daily titrated insulin doses of protamine zinc insulin ranging from 1.6 to 2.6 units/day (Novo Nordisk, Princeton, NJ) to maintain blood glucose levels ~25 mmol/l and to prevent ketoacidosis from occurring. Blood glucose levels were measured every 2 weeks and at death. After onset of diabetes, rats were randomly assigned to four groups and two age-matched control groups. At 4 months, eight diabetic and eight control rats were killed. The remaining three diabetic groups received from 4 months of diabetes either saline subcutaneously via osmopumps (Alza, Palo Alto, CA) or synthetic rat C-peptide II (>95% purity by high-performance liquid chromatography; Multiple Peptide System, San Diego, CA). C-peptide was dissolved in PBS and

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CGRP, calcitonin gene–related peptide; DRG, dorsal root ganglion; IGF-IR, IGF-I receptor; NGF, nerve growth factor; NGFR, NGF receptor.

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TABLE 1
Body weight, blood glucose, serum insulin, and C-peptide levels of control, untreated, and C-peptide–replaced BB/Wor rats

Diabetes duration (months)	Group	n	Body wt (g)	Blood glucose (mmol/l)	Serum insulin level (pmol/l)	Serum C-peptide level (pmol/l)
4	Control	8	458 ± 16	5.0 ± 0.4	418 ± 48	
	BB/Wor	8	374 ± 16*	24.4 ± 1.9*	117 ± 46*	
7	Control	9	505 ± 37	5.3 ± 0.3	526 ± 108	727 ± 24
	BB/Wor	9	370 ± 18*	25.2 ± 1.7*	153 ± 55*	<25*
	OS	9	370 ± 14*	25.6 ± 2.0*	144 ± 48*	696 ± 26
	SC	9	380 ± 16*	24.3 ± 2.1*	135 ± 32*	<25*

Data are means ± SD. OS, 7-month BB/Wor rats replaced with 75 nmol rat C-peptide · kg^{-1} · day^{-1} by osmopump; SC, 7-month BB/Wor rats replaced with 75 nmol rat C-peptide/kg by subcutaneous injection once daily. *$P < 0.001$ vs. corresponding control rats.

administered via either subcutaneously implanted osmopumps delivering 75 nmol · kg^{-1} · 24 h^{-1} or once-daily subcutaneous injections of 75 nmol/kg. These groups were killed at 7 months of diabetes and were compared with untreated diabetic rats at 4 and 7 months and age- and sex-matched nondiabetes-prone control rats. The animals were cared for in accordance with the guidelines of the Animal Investigation Committee, Wayne State University (Detroit, MI), and those of the National Institutes of Health (publication no. 85-23, 1995).

Insulin levels and C-peptide bioavailability and concentrations. Insulin plasma levels were measured at death 20 h after the last insulin injection using ELISA kits (Linco Research, St. Charles, MO). Serum C-peptide levels were measured 20 h after the once-daily subcutaneous injection using radioimmunoassay kits (Linco Research). To examine the bioavailability of C-peptide in the circulation, C-peptide levels in serum were measured at 0.5, 1, 2, 3, 6, and 24 h after the subcutaneous injection of 75 nmol C-peptide/kg body wt.

Thermal plantar test. Latencies of hind paw withdrawal to thermal stimulation (42°C; 152 mW/cm^2) were used as measures of thermal hyperalgesia and were measured at 4 and 7 months using a UGO Biological Research apparatus (Comerio, Italy) (11,12). The time from heat source activation to the animal's self-withdrawal in seconds was measured six times in alternating hind paws. The mean of these measurements was calculated and used as the measure of latency withdrawal.

Tissue collection. After 4 and 7 months of diabetes, diabetic and age-matched control rats were killed with an intraperitoneal overdose of sodium pentobarbital (120 mg/kg body wt). Three control and diabetic rats per group were perfused with ~500 ml 4% paraformaldehyde fixative. L4 and L5 DRGs were fixed by immersion in 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4) overnight at 4°C, rinsed in PBS, dehydrated, immersed in xylene, and embedded in paraffin.

L4 and L5 DRGs and sciatic nerves were collected from six animals per group for protein extraction. Tissues were snap frozen in liquid nitrogen and kept in −80°C until use.

Sural nerves were exposed in diabetic and control rats and fixed in situ for 10 min with 1% cacodylate buffered (pH 7.4) 2.5% glutaraldehyde and then excised and immersed in the same fixative for 2 h at 4°C. They were postfixed in 1% cacodylate buffered (pH 7.4) osmium tetroxide, dehydrated, and embedded in Epon for morphometric analysis.

Morphometric analyses. For analyses of unmyelinated fibers, ultrathin cross sections of sural nerves were obtained with the aid of an LKB ultramicrotome (Marviac Limited, Halifax, Canada) and were stained with uranyl acetate and lead citrate. They were examined in a Zeiss 12AS electron microscope (Carl Zeiss, Oberkochen, Germany). Systematically selected frames representing ~30% of the sural nerve cross-sectional area were obtained. Photographs were enlarged 10,000 times, scanned, and downloaded to a computerized image analysis system (Image-1; Universal Imaging, West Chester, PA). The following morphometric parameters of unmyelinated fibers were obtained: unmyelinated fiber number, fiber density (n/mm^2), mean fiber size (μm^2), axon numbers per Schwann cell unit, and the frequencies of type 2 Schwann cell/axon relationship, collagen pockets, denervated Schwann cell profiles, and regenerating C-fibers (12).

Identification and morphometry of substance P and calcitonin gene–related peptide–positive DRG neurons. Six-micrometer-thick sections were immunostained with rabbit polyclonal anti–substance P or rabbit polyclonal anti–calcitonin gene–related peptide (CGRP) antibody (both from Peninsula Laboratories, San Carlost, CA) using an avidin biotin complex kit (Vector Laboratories, Burlingame, CA). Quantifications of positive neurons were performed using the same image analysis system as above. Images of three serially sectioned DRGs (sections 60 μm apart) were captured and assessed using a binary scale. In each ganglion, 200–300 ganglion cells with visible nuclei were captured. The frequencies and areas of substance P– and CGRP-positive neurons were determined in each section (13).

Measurement of substance P and CGRP contents of DRGs. Enzyme immunometric assays were used to assess substance P (Cayman Chemical, Ann Arbor, MI) and CGRP (SPIbio, Massy Cedex, France) in DRGs. Results are expressed as picograms per ganglion (13).

Western blotting. DRGs were lysed in detergent lysis buffer (50 mmol/l Tris-HC1, pH 7.4, 150 mmol/l NaC1, 1 mmol/l EDTA, 1% Triton X-100, 1 mmol/l phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). The lysates were centrifuged at 14,000 rpm for 20 min at 4°C and protein concentrations were measured using bicinchoninic acid protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as standard. Ten to 40 μg was separated by 7.5–12% SDS-PAGE, and transferred to polyvinylidine fluoride membranes (Bio-Rad, Hercules, CA). Membranes were blocked with Tween-20–Tris-buffered saline (10 mmol/l Tris-HC1, pH 7.5, 100 mmol/l NaC1, and 0.1% Tween 20) containing 5% nonfat dry milk (Bio-Rad) before incubation with rabbit polyclonal anti–IGF-I receptor (IGF-IR)-α (dilution 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti–insulin receptor-α (dilution 1:500; Santa Cruz Biotechnology), rabbit polyclonal anti–NGFR-TrkA (dilution 1:500; Santa Cruz Biotechnology), rabbit polyclonal anti-p75 (dilution 1:500; Sigma Chemical, St. Louis, MO), and mouse monoclonal anti-actin (dilution 1:5,000; Chemicon International, Temecula, CA). Antigen detection was performed using chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) with the appropriate horseradish peroxidase–conjugated secondary antibodies. Membranes were exposed to Biomax film (Kodak, Rochester, NY), and images were scanned and densities were determined by the Bio-Rad Fluoro-S multiimager (Bio-Rad). Expression of proteins was corrected for by actin expression in control animals, which was arbitrarily set to 1.0.

NGF analyses. NGF contents of sciatic nerves and DRGs were determined with a quantitative two-site enzyme immunoassay (Emax Immunoassay System; Promega, Madison, WI). Assays were performed according to manufacturer's protocol. Preweighed sciatic nerve segments were homogenized in lysis buffer (137 mmol/l NaC1, 20 mmol/l Tris HC1, pH 8.0, 1% NP40, 10% glycerol, 1 mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.5 mmol/l sodium vanadate) and centrifuged (14,000 rpm for 20 min). The supernatants were loaded on MaxiSorp 96-well plates (Nalge Nunc International, Rochester, NY), and measurements were performed as previously described (12,13). Results were expressed as picograms per milligram protein in sciatic nerve and as picograms per ganglion in DRGs.

Statistical analysis. All values are expressed as means ± SD. Significance of differences was analyzed by ANOVA. Group differences were assessed by Scheffes test. Significance was defined as a P value <0.05. All analyses were performed by personnel unaware of the animal identities.

RESULTS

Clinical data. Four-month diabetic animals showed significant weight loss, hyperglycemia, and insulin deficiency (all $P < 0.001$; Table 1). At 7 months, both untreated and C-peptide–treated BB/Wor rats showed severe hyperglycemia ($P < 0.001$) and significant reductions in body weight ($P < 0.001$). Serum insulin level was significantly ($P < 0.001$) decreased in both untreated and C-peptide–treated diabetic rats (Table 1). C-peptide levels were decreased in untreated BB/Wor rats ($P < 0.001$) and were fully restored in osmopump-treated rats but not in subcutaneous injec-

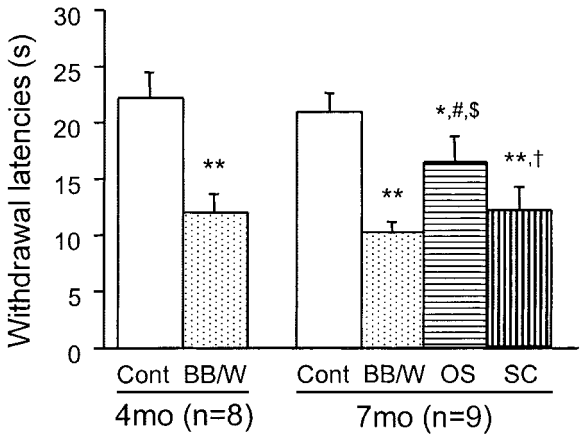

FIG. 1. Withdrawal latencies to thermal stimuli in 4- and 7-month rats. Data are analyzed between six groups: 4-month control rats (Cont), 4-month untreated BB/Wor rats (BB/W), 7-month control rats (Cont), 7-month untreated BB/Wor rats (BB/W), 7-month BB/Wor rats replaced with 75 nmol rat C-peptide · kg^{-1} · day^{-1} by osmopump from 4 months of diabetes (OS) and 7-month BB/Wor rats replaced with 75 nmol rat C-peptide/kg by subcutaneous injection once daily from 4 months of diabetes (SC). Results are means ± SD. *P < 0.005, **P < 0.001 vs. respective control rats; #P < 0.005 vs. 4-month untreated BB/Wor rats; \$$P$ < 0.001 vs. 7-month untreated BB/Wor rats; †P < 0.01 vs. OS.

tion–treated rats (Table 1). The concentrations of C-peptide in the circulation after various time points after the subcutaneous injection of 75 nmol C-peptide/kg body wt were 0.5 h, 13,616 ± 2,142 nmol/l; 1 h, 7,069 ± 965; 2 h, 901 ± 131; 3 h, 169 ± 57; 6 h, 34 ± 7; and after 24 h, <25. The equation for the decay was $y = 3.8204x^{-2.56}$ with $r = 0.9829$.

Thermal plantar test. The latencies to thermal stimuli were measured in 4- and 7-month control and diabetic BB/Wor rats (Fig. 1). The withdrawal latencies were significantly reduced in both 4- and 7-month diabetic rats ($P < 0.001$). Osmopump delivery improved significantly ($P < 0.005$) the reduction in latencies to thermal stimuli in diabetic rats, whereas subcutaneous injection had no significant effect (Fig. 1).

Morphometric analyses. Unmyelinated fiber number of the sural nerve in 4- and 7-month untreated BB/Wor rats was significantly decreased (both $P < 0.001$) compared with control rats (Table 2). Osmopump delivery but not subcutaneous injection significantly improved this decrease ($P < 0.005$ vs. 4-month untreated BB/Wor rats, $P < 0.01$ vs. 7-month untreated BB/Wor rats) (Table 2). Unmyelinated fiber density was significantly decreased in 4- and 7-month untreated BB/Wor rats (both $P < 0.05$), which was prevented by osmopump delivery (Table 2). There was a significant decrease in mean unmyelinated fiber size in 4-month ($P < 0.05$) and 7-month ($P < 0.005$) untreated BB/Wor rats, which was prevented by osmopump delivery but not by subcutaneous injection (Table 2). The frequency of denervated Schwann cells was increased four-fold ($P < 0.005$) in 4-month untreated BB/Wor rats and 2.6-fold ($P < 0.001$) in 7-month untreated BB/Wor rats, which was fully prevented by both osmopump delivery

TABLE 2
Morphometric data of unmyelinated fibers of sural nerves

	Control	BB/Wor	OS	SC
n	5	5	5	5
4 months				
Unmyelinated fibers (n)	5,065 ± 374	3,622 ± 218*		
Unmyelinated fiber density (n/mm^2)	115,375 ± 9,434	103,859 ± 3,998†		
Mean unmyelinated fiber area (μm^2)	0.74 ± 0.09	0.61 ± 0.04†		
Axon numbers/Schwann cell unit (n/Schwann cell)	5.26 ± 0.64	4.22 ± 0.29†		
Denervated Schwann cell profiles (% total unmyelinated fibers)	0.24 ± 0.18	0.97 ± 0.26‡		
Collagen pockets (% total unmyelinated fibers)	0.62 ± 0.42	1.59 ± 0.57†		
Type 2 Schwann cell/axon relationship (% total unmyelinated fibers)	1.18 ± 0.13	6.45 ± 2.00*		
Regenerating fibers (% total unmyelinated fibers)	0.56 ± 0.43	2.07 ± 0.36*		
7 months				
Unmyelinated fibers (n)	6,195 ± 740	3,505 ± 293*	4,865 ± 602§¶‖	4,022 ± 343*
Unmyelinated fiber density (n/mm^2)	122,212 ± 10,540	97,928 ± 7,466†	108,487 ± 11,134	100,622 ± 9,933†
Mean unmyelinated fiber area (μm^2)	0.71 ± 0.07	0.53 ± 0.04‡	0.64 ± 0.06**	0.59 ± 0.04†
Axon numbers/Schwann cell unit (n/Schwann cell)	5.29 ± 0.26	4.17 ± 0.11*	4.83 ± 0.21†††	4.54 ± 0.23*
Denervated Schwann cell profiles (% total unmyelinated fibers)	1.00 ± 0.32	2.61 ± 0.57*	0.93 ± 0.18‡‡	1.10 ± 0.24‡‡
Collagen pockets (% total unmyelinated fibers)	1.65 ± 0.32	3.15 ± 0.89§	1.59 ± 0.51¶	2.06 ± 0.41
Type 2 Schwann cell/axon relationship (% total unmyelinated fibers)	3.30 ± 0.54	14.45 ± 3.02*	6.57 ± 1.49‡‡	8.22 ± 2.16† ††
Regenerating fibers (% total unmyelinated fibers)	1.69 ± 0.51	2.49 ± 0.46	5.48 ± 0.99*‡‡§§	3.94 ± 0.49* **¶¶§§

Data are means ± SD. OS, 7-month BB/Wor rats replaced with 75 nmol rat C-peptide · kg^{-1} · day^{-1} by osmopump; SC, 7-month BB/Wor rats replaced with 75 nmol rat C-peptide/kg by subcutaneous injection once daily. *$P < 0.001$, †$P < 0.05$, ‡$P < 0.005$, §$P < 0.01$ vs. respective control rats; ¶$P < 0.01$, **$P < 0.05$, ††$P < 0.005$, ‡‡$P < 0.001$ vs. 7-month untreated BB/Wor rats; ‖$P < 0.005$, §§$P < 0.001$ vs. 4-month BB/Wor rats; ¶¶$P < 0.05$ vs. OS.

TABLE 3
Substance P and CGRP neuronal content and morphometry

	n	Control	BB/Wor	OS	SC
4 months					
SP content (pg/ganglion)	5	85.5 ± 13.8	74.3 ± 21.0		
SP-positive neurons (%)	3	24.0 ± 0.5	22.1 ± 1.7		
Mean area of SP-positive neurons (μm^2)	3	448.8 ± 11.8	380.6 ± 56.3		
CGRP content (pg/ganglion)	5	717.6 ± 160.1	628.1 ± 85.1		
CGRP-positive neurons (%)	3	29.1 ± 0.7	27.5 ± 2.6		
Mean area of CGRP-positive neurons (μm^2)	3	519.2 ± 18.9	439.2 ± 28.2*		
7 months					
SP content (pg/ganglion)	6	87.6 ± 11.4	60.6 ± 20.5*	73.4 ± 14.8	68.9 ± 12.6
SP-positive neurons (%)	3	23.8 ± 1.2	18.3 ± 0.9†	21.1 ± 1.3	18.6 ± 0.6†‡
Mean area of SP-positive neurons (μm^2)	3	448.1 ± 14.3	346.7 ± 8.4§	412.2 ± 10.5*¶	360.5 ± 9.7§‖
CGRP content (pg/ganglion)	6	739.4 ± 109.0	488.3 ± 80.1†	580.3 ± 104.9	529.0 ± 92.4*
CGRP-positive neurons (%)	3	29.4 ± 3.0	22.7 ± 1.5*	25.2 ± 2.9	22.8 ± 0.9*‡
Mean area of CGRP-positive neurons (μm^2)	3	536.9 ± 27.3	431.0 ± 30.0*	510.7 ± 24.0‡	471.1 ± 46.0

Data are means ± SD. SP, Substance P; OS, 7-month BB/Wor rats replaced with 75 nmol rat C-peptide · kg^{-1} · day^{-1} by osmopump; SC, 7-month BB/Wor rats replaced with 75 nmol rat C-peptide/kg by subcutaneous injection once daily. *$P < 0.05$, †$P < 0.005$, §$P < 0.001$ vs. respective control rats; ‡$P < 0.05$ vs. 4-month BB/Wor rats; ¶$P < 0.001$ vs. 7-month untreated BB/Wor rats; ‖$P < 0.005$ vs. OS.

and subcutaneous injection (both $P < 0.001$) (Table 2). Similarly, the frequency of collagen pockets within Schwann cells and the frequency of type 2 Schwann cell/axon relationship were increased in 4-month ($P < 0.05$ and 0.001, respectively) and 7-month ($P < 0.01$ and 0.001, respectively) untreated diabetic rats. The incremental increases from 4 to 7 months were fully prevented by osmopump delivery ($P < 0.01$ and 0.001, respectively), whereas these effects were less in subcutaneous injection (Table 2). The frequency of regenerating fiber was significantly ($P < 0.001$) increased in 4-month but not in 7-month diabetic rats. Both osmopump delivery and subcutaneous injection significantly (both $P < 0.001$) enhanced C-fiber regeneration compared with 4-month diabetic rats.

Number of substance P– and CGRP-positive neurons and neuropeptide content in DRGs. The content of substance P/ganglion was decreased by 13% (NS) and 31% ($P < 0.05$) in 4- and 7-month untreated BB/Wor rats, respectively, a decrease that was fully prevented by osmopump delivery and to a lesser extent by subcutaneous injection (Table 3). Osmopump delivery partially prevented the decreased CGRP content in DRGs of 7-month untreated BB/Wor rats (Table 3). The frequencies of substance P– and CGRP-positive neurons were nonsignificantly decreased at 4-month but significantly decreased in 7-month untreated diabetic rats ($P < 0.005$ and 0.05, respectively). The incremental loss of substance P and CGRP neurons was prevented by osmopump delivery but not by subcutaneous injection. Mean substance P– and CGRP-positive neuronal sizes were decreased significantly ($P < 0.001$ and 0.05, respectively) in 7-month untreated BB/Wor rats. The atrophy of substance P neurons was prevented by osmopump delivery ($P < 0.001$), whereas CGRP neuronal size was increased ($P < 0.05$) compared with 4-month diabetic animals and was not significantly different from that of control animals. Subcutaneous injection had no effect on either neuronal size (Table 3).

Expression of neurotrophic receptors in DRGs. The expression of the insulin receptor was significantly ($P < 0.01$) decreased in 7-month untreated BB/Wor rats, a decrease that was significantly ($P < 0.05$) prevented by osmopump delivery and less so by subcutaneous injection (Fig. 2). IGF-IR expression was not altered in 4-month diabetic rats but was decreased by 25% ($P < 0.05$) in 7-month untreated diabetic rats. This decrease was fully

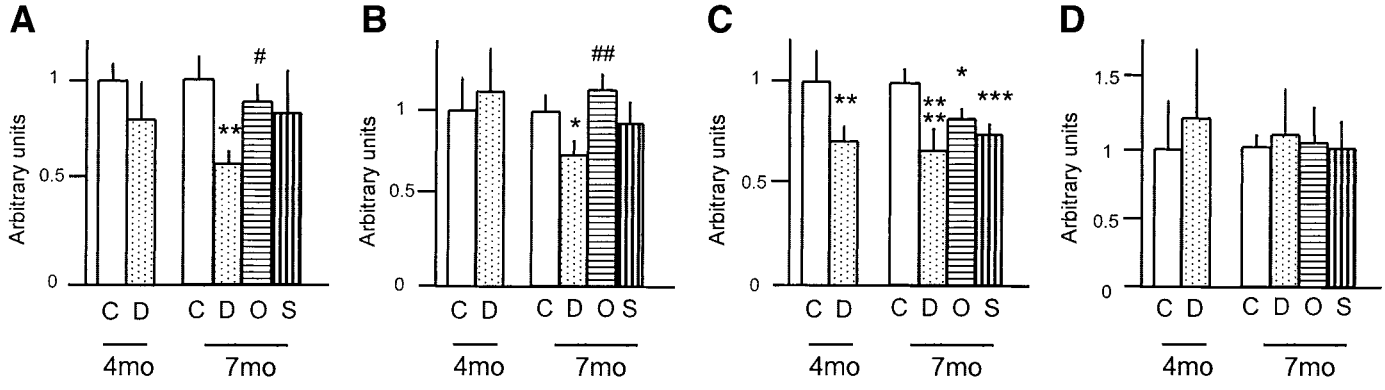

FIG. 2. Comparison of protein expression in DRGs and NGF in sciatic nerve between 4- and 7-month rats. *A*: Insulin receptor ($n = 4$). *B*: IGF-I receptor ($n = 4$). *C*: High-affinity NGFR (NGFR-TrkA) ($n = 4$). *D*: Low-affinity NGFR (NGFR-p75) ($n = 4$) in DRGs. C, control rats; D, untreated BB/Wor rats; O, BB/Wor rats replaced with 75 nmol rat C-peptide · kg^{-1} · day^{-1} by osmopump from 4 months of diabetes; S, BB/Wor rats replaced with 75 nmol rat C-peptide/kg by subcutaneous injection once daily from 4 months of diabetes. Results are means ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, ****$P < 0.001$ vs. respective control rats; #$P < 0.05$, ##$P < 0.005$ vs. 7-month untreated BB/Wor rats.

TABLE 4
NGF content in sciatic nerve

	n	Control	BB/Wor	OS	SC
4 month NGF (pg/mg protein)	5	2,702 ± 356	2,150 ± 376*		
7 month NGF (pg/mg protein)	6	3,518 ± 341	2,207 ± 480†	3,029 ± 494‡§	2,429 ± 511¶

Data are means ± SD. OS, 7-month BB/Wor rats replaced with 75 nmol rat C-peptide · kg^{-1} · day^{-1} by osmopump; SC, 7-month BB/Wor rats replaced with 75 nmol rat C-peptide/kg by subcutaneous injection once daily. *$P < 0.05$, †$P < 0.005$, ¶$P < 0.01$ vs. respective control rats; ‡$P < 0.05$ vs. 7-month untreated BB/Wor rats; §$P < 0.05$ vs. 4-month BB/Wor rats.

prevented by osmopump delivery ($P < 0.005$) and to lesser extent by subcutaneous injection (Fig. 2). The high-affinity NGF receptor (NGFR-TrkA) expression in DRGs was significantly suppressed in both 4- and 7-month BB/Wor rats ($P < 0.01$ and 0.001, respectively). Osmopump delivery partially ameliorated this deficit (Fig. 2), whereas subcutaneous injection had little effect. The low-affinity NGFR (NGFR-p75) (Fig. 2) and actin expressions (not shown) were not altered in any of the groups.

NGF content in sciatic nerve. In the sciatic nerve, the NGF content was decreased by 20% ($P < 0.05$) and 37% ($P < 0.005$) in 4- and 7-month untreated BB/Wor rats, respectively, a decrease that was prevented ($P < 0.05$) by osmopump delivery but not by subcutaneous injection (Table 4).

DISCUSSION

Here, we demonstrate the therapeutic effects of C-peptide replenishment on established nociceptive neuropathy. Continuous supply of full C-peptide replacement improved hyperalgesia and underlying C-fiber loss. This was accompanied by normalization of the expression of insulin receptor and IGF-IR in DRG ganglion cells and promotion of C-fiber regeneration. Osmopump delivery prevented further decline in C-fiber density, size, and degenerative changes. These effects were paralleled by prevention of further loss of nociceptive substance P and CGRP neurons and their trophic support by NGF via NGFR-TrkA. These effects occurred in the presence of unaltered hyperglycemia. On the other hand, once-daily administration of full replacement dose of C-peptide merely prevented further decline in fiber numbers with a lesser effect on C-fiber regeneration and the expression of insulin receptor and IGF-IR. These findings show that continuous C-peptide replacement optimizes its therapeutic effects on nociceptive neuropathy. The differential effects between the two modes of C-peptide delivery can be explained by the differences in C-peptide plasma bioavailability. The half-life of C-peptide in plasma is ~1 h. Hence, once-daily administration will leave most of the 24-h cycle depleted of C-peptide. Continuous infusion is more in keeping with the normal physiological secretion of C-peptide with three major peaks and several intervening peaks during 24 h, hence providing a more sustained C-peptide presence (21). The extremely high systemic C-peptide levels immediately after subcutaneous injection administration may even inhibit insulin signaling (15–17).

Diabetic neuropathy is caused by several sequential, partially interacting, and dynamic pathogenetic mechanisms that conceptually can be divided into two stages. The early metabolic phase shows reversible nerve dysfunctions following metabolic corrections. This is gradually, with duration of diabetes, overshadowed by a late structural phase that becomes increasingly nonresponsive to metabolic interventions (22).

In this study, the intervention was purposely initiated at a stage of nociceptive neuropathy, when its structural phase is well established (11,12), to explore the reparative effects of C-peptide and to mimic the clinical scenario.

C-peptide, being derived from proinsulin, is secreted in equimolar concentrations to insulin and is therefore depleted in type 1 diabetes. Although a specific C-peptide receptor has not been identified, C-peptide is known to interact with insulin signaling (17,19,23). It possesses neurotrophic effects on its own, similar to insulin, and together with insulin, has gene regulatory effects on NGF, IGF-I, NT-3, and their respective receptors including the insulin receptor via upregulation of NF-κB (18,20). It has a promoting effect on neural Na^+/K^+-ATPase activity and eNOS, which underlie its preventive effects on early diabetic neuropathy (24–29). Like insulin, it also effects phosphorylation and other posttranslational modifications of several neural proteins (30).

Nociceptive DRG neurons are mainly dependent on neurotrophic support by NGF and insulin (31,32). In the present study, these two factors and their respective receptors were either normalized or maintained at pretreatment levels after osmopump delivery. Diabetic rats treated with subcutaneous injection administration, however, showed little effect. The discrepancies between the two groups were also reflected in the differences in nociceptive neuronal numbers, sizes, and neuropeptide contents.

The increase in C-fiber numbers in the osmopump group is most likely accounted for by the increased number of regenerating fibers. Similarly, the maintenance of the pretreatment fiber number in the subcutaneous injection group reflects the lesser effects of C-peptide on regeneration and degenerative changes such as type 2 Schwann cell/axon relationship, collagen pockets, and denervated Schwann cells.

The promoting effect of C-peptide replacement on nerve fiber regeneration is in keeping with earlier findings on early gene regulation of IGF-I, c-fos, and NGF with subsequent initial upregulation of neurotubulin and downregulation of neurofilaments (20). The maintenance of axonal size and integrity is highly dependent on the synthesis and proper phosphorylation of neurofilaments in neuronal somata and their axons (33,34), which in turn are dependent on neurotrophic support (35,36). Impaired synthesis of neuroskeletal proteins secondary to neurotrophic withdrawal and instability of their mRNAs due to insulin deficiency results in impaired axonal transport, axonal atrophy, and dying-back degeneration, the very hallmark of diabetic neuropathy (13,22,37–39). Therefore the corrective effects of C-peptide on neurotrophic factors and

their receptors and on insulin action are in keeping with sustained axonal size and the ameliorating effects on neuronal and axonal degeneration. The present findings provide a mechanistic basis for the clinical observations of improved nerve function in type 1 diabetic patients treated with C-peptide (40,41).

Clinically, painful diabetic neuropathy occurs in patients with pre-diabetes (8,9) and early in the course of diabetic neuropathy in type 2 patients (42,43). This has been linked to deficits in neurotrophic factors such as NGF and impaired insulin action with impacts on neuroeffector peptides like substance P and CGRP (36,38,44). These clinical conditions have been explored experimentally in the glucose-intolerant Stockholm-bred GK rat and in the type 2 diabetic BBZDR/Wor rat (10,12). Compared with type 1 BB/Wor rats, the latter shows a slower progression of functional, molecular, and structural changes of nociceptive neuropathy before becoming increasingly analgesic (12). Such differences in progression rates may explain the higher incidence (up to 32%) of painful diabetic neuropathy in type 2 diabetic subjects (42). Whether C-peptide has the same effects on type 2 diabetic nociceptive neuropathy as shown here in type 1 diabetes is currently being investigated in the BBZDR/Wor rat.

In summary, the present study demonstrates that continuous C-peptide replacement provides a better therapeutic effect on nociceptive diabetic neuropathy than single daily subcutaneous injections. These findings agree with the same effects on myelinated fiber function and pathology (45). Therefore, these studies strongly suggest that optimal therapeutic effects in type 1 diabetic patients will require maintenance of physiological C-peptide concentrations for a major proportion of the day.

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