Proinsulin C-Peptide Replacement in Type 1 Diabetic BB/Wor-rats Prevents Deficits in Nerve Fiber Regeneration

CHRISTOPHER R. PIERSON, MD, PhD, WEIXIAN ZHANG, MD, and ANDERS A. F. SIMA, MD, PhD

Abstract. We recently reported that early gene responses and expression of cytoskeletal proteins are perturbed in regenerating nerve in type 1 insulin-dependent diabetes but not in type 2 hyperinsulinemic diabetes. We hypothesized that these differences were due to impaired insulin action in the former type of diabetes. To test this hypothesis, type 1 diabetic BB/Wor-rats were replaced with proinsulin C-peptide, which enhances insulin signaling without lowering blood glucose. Following sciatic nerve crush injury, early gene responses such as insulin-like growth factor, c-fos, and nerve growth factor were examined longitudinally in sciatic nerve. Neurotrophic factors, their receptors, and β-tubulin and neurofilament expression were examined in dorsal root ganglia. C-peptide replacement significantly normalized early gene responses in injured sciatic nerve and partially corrected the expression of endogenous neurotrophic factors and their receptors, as well as cytoskeletal protein in dorsal root ganglia. These effects translated into normalization of axonal radial growth and significantly improved axonal elongation of regenerating fibers in C-peptide-replaced BB/Wor rats. The findings in C-peptide replaced type 1 diabetic rats were similar to those previously reported in hyperinsulinemic and iso-hyperglycemic type 2 BB/Z-rats. We conclude that impaired insulin action may be more important than hyperglycemia in suppressing nerve fiber regeneration in type 1 diabetic neuropathy.

Key Words: C-peptide; Diabetic neuropathy; Early gene responses; Neurotrophic factors; Regeneration.

INTRODUCTION

Diabetic polyneuropathy (DPN) differs significantly in the 2 major types of diabetes (1). Type 1 DPN in humans and the spontaneously diabetic BB/Wor-rat is characterized by progressive nerve fiber loss, nodal degeneration, and impaired nerve fiber regeneration (2–4). The latter is believed to contribute to the net fiber loss. The failure of early gene and neurotrophic factor responses as well as impaired synthesis of cytoskeletal proteins in injured nerve underlies impaired nerve regeneration in type 1 DPN (5, 6). Type 2 DPN differs functionally and structurally from type 1 DPN in that it shows milder axonal atrophy and nerve conduction defects, no nodal degeneration, focal nerve fiber loss, and more severe Wallerian degeneration and segmental demyelination (1, 3, 7, 8). Nerve regeneration in type 2 DPN in the BB/Z-rat is relatively preserved compared to type 1 DPN, which is attributed to relatively intact early gene responses following nerve injury (9), and close to normal expression of cytoskeletal proteins (10). The almost normal nerve regeneration is likely to substitute for fibers lost through Wallerian degeneration, thereby accounting for preserved fiber number even after prolonged diabetes in the type 2 diabetic BB/Z-rat (7). The reasons for these differences in nerve fiber regeneration between the 2 major types of diabetes is not known, but we have suggested that deficiencies in insulin and insulinomimetic C-peptide in type 1 DPN may play decisive roles (1, 8).

The biologic role of C-peptide was once thought to be limited to the biosynthesis of insulin, but recent investigations indicate that it has a number of physiologic effects (11). C-peptide has insulinomimetic effects and in rat L6 myoblasts and human neuroblastoma cells, it autophosphorylates the insulin receptor (IR), and promotes a variety of insulin signaling intermediaries (12, 13). C-peptide deficiency plays a role in diabetic complications as its administration in type 1 diabetic patients improves renal function (14, 15), normalizes heart rate variability (16, 17), and improves sensory nerve conduction (18). C-peptide replacement in type 1 BB/Wor-rats leads to dose-dependent increases in the number of sural nerve regenerating fibers (19, 20). Human neuroblastoma cells co-treated with C-peptide and insulin show increased neurite elongation in excess of that produced by either hormone alone (21).

Peripheral nerve regeneration is an intricately orchestrated, multistep process that requires participation by Schwann cells, macrophages, fibroblasts, and neurons (22). Nerve damage induces early gene responses, which include the first wave of neurotrophic factor responses by Schwann cells (6, 23), followed by macrophage recruitment and Wallerian degeneration (5, 23), interleukin induction, Schwann cell proliferation (24), and the second wave of neurotrophic factor (25, 26). This cascade of interdependent events induces the synthesis of cytoskeletal elements, which initiate and sustain axonal regrowth and subsequent myelin synthesis by Schwann cells (10, 27, 28).

Diabetes perturbs peripheral nerve regeneration in a number of ways. Insulin-like growth factor 1 (IGF-1) and
nerve growth factor (NGF) mRNA and protein levels are decreased in type 1 diabetes (6, 9, 10, 29, 30) and are likely to account for a component of impaired regeneration in DPN (31–34). IGF-1 levels are significantly higher in type 2 BB/Z-rats compared to iso-hyperglycemic type 1 BB/Wor-rats (9). The decrease in NGF availability in streptozotocin-induced diabetes is compounded by a downregulation of the expression of TrkA and p75, the high- and low-affinity NGF receptors (30). These alterations are less severe in type 2 BB/Z-rats (9). Altered expression of NGF, p75, TrkA, IGF-1, and insulin-like growth factor 1 receptor (IGF-IR) has negative effects on the synthesis of tubulin and neurofilament proteins in type 1 insulinopenic BB/Wor-rats (6, 10, 33).

To investigate the role of proinsulin C-peptide deficiency in nerve regeneration following nerve injury, type 1 BB/Wor-rats were replaced with rat II C-peptide and subjected to sciatic nerve crush. In addition to examining the expression of early gene responses in injured nerve, neurotrophic factor and neuroskeletal protein synthesis were studied in dorsal root ganglia (DRGs) and nerve morphometry was used to quantitate axonal regrowth. The findings were compared to age- and sex-matched non-C-peptide-replaced BB/Wor-rats, non-diabetic BB/Wor-rats, and earlier reported data in the type 2 iso-hyperglycemic BB/Z-rat (9, 10).

MATERIALS AND METHODS

Animals

One hundred and eight male prediabetic type 1 BB/Wor- and 54 diabetes-resistant male BB/Wor-rats were obtained from Biomedical Research Models (Worcester, MA). Diabetes-prone animals were monitored daily for glucosuria to ascertain onset of diabetes, which occurred at 74 ± 3 days of age. Diabetes resistant BB/Wor-rats served as non-diabetic control rats. The animals were maintained in metabolic cages and provided ad libitum access to rat chow (Wayne Lab Blox F-6, Wayne Feed Division, Chicago, IL) and water. To maintain blood glucose levels at approximately 20 mmol/L, diabetic BB/Wor-rats were treated with daily doses (1.3±3.0 U/day) of protamine zinc insulin and C-peptide measurements using RIA-kits (Linco Research Inc., St. Charles, MO). One and one half-cm-long portions of sciatic nerves immediately distal to the crush sites were harvested. A laminectomy was performed over the lumbar region and L4 and L5 DRGs from both sides were collected. Sciatic nerves and DRGs used for RNA and protein extraction were snap frozen in liquid nitrogen and stored at −80°C.

Sciatic Nerve Crush Injury

Under isoflurane anesthesia, the lateral aspects of the hind legs were shaved and swabbed with 70% ethanol and 2% iodine. Incisions were made bilaterally along the lateral thighs and 2 cm of the sciatic nerves were exposed. Each sciatic nerve was crushed twice at the same site at the mid-thigh level by eye forceps for 10 seconds each, as previously described (10). The crush site was marked by a 4.0 silk suture placed in the epineurium and the wound was closed by surgical staples.

Tissue Collection

Animals with 6 weeks of diabetes and age-matched control rats with no crush injury (time point 0 h) were used to obtain baseline values. Thereafter, animals were killed at various time points after crush injury: 0.5 h, 2 h, 6 h, 24 h, 2 d, 4 d, 6 d, and 20 d. Four animals per group were used for each time point. They were killed by ventricular puncture and exsanguination under isoflurane anesthesia. Blood was collected for plasma insulin and C-peptide measurements using RIA-kits (Linco Research Inc., St. Charles, MO). One and one half-cm-long portions of sciatic nerves immediately distal to the crush sites were harvested. A laminectomy was performed over the lumbar region and L4 and L5 DRGs from both sides were collected. Sciatic nerves and DRGs used for RNA and protein extraction were snap frozen in liquid nitrogen and stored at −80°C.

Morphometry

For regenerating fiber morphometry, 4 animals per group were killed by Nembutal overdose (100 mg/kg; Fisher Scientific, Pittsburgh, PA) at 20 d post crush injury. Sciatic nerves were fixed in situ for 5 min in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), dissected, and placed in the same buffer. A laminectomy was performed over the lumbar region and L4 and L5 DRGs from both sides were collected. Sciatic nerves and DRGs used for RNA and protein extraction were snap frozen in liquid nitrogen and stored at −80°C.

Nerve Conduction Velocity

Motor nerve conduction velocity (MNCV) was measured at baseline in uninjured 6-week diabetic animals and age-matched control rats under isoflurane (Baxter Pharmaceuticals, Deerfield, IL) inhalational anesthesia. Body temperature was maintained at 37°C by a heating pad and monitored by a rectal probe. The left sciatic-tibial conduction system was stimulated at the sciatic notch and at the ankle via bipolar electrodes with supra-maximal stimuli (8V) at 2 Hz with a pulse width of 100 μs using a Cadwell 5200A electromyographer (Cadwell Lab, Kennewich, WA). The action potentials were recorded via bipolar electrodes from the first interosseous muscle. MNCV was calculated by subtracting the distal from the proximal latency and the result was divided into the distance between the 2 stimulating electrodes. Each measurement represented the averaging of 8 or 16 recordings.

Protein Extraction

Sciatic nerves distal to the crush site and corresponding DRGs (L4+L5) were homogenized (Homogenizer model 125, Fisher Scientific) in a buffer consisting of 1% Triton X-100, 50
TABLE 1

<table>
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<th>Antibody</th>
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**Extraction of Total mRNA**

RNA was extracted from sciatic nerves and DRGs by homogenization in 1 ml TRizol (Life Technologies, Rockville, MD). The samples were placed in 200 μl of chloroform and centrifuged at 14,000 rpm for 15 min at 4°C. The aqueous phase was removed and placed in 200 μl of phenol and chloroform and then centrifuged as before. The aqueous phase was placed in 500 μl of isopropanol and precipitated at −20°C overnight. The samples were then centrifuged at 14,000 rpm for 20 min at 4°C and the resulting RNA pellet was washed in 75% ethanol and air-dried. The RNA was dissolved in 25 μl of DEPC-H2O and the RNA concentration was determined spectrophotometrically (Pharmacia LKB Ultrospec III, Pharmacia, Uppsala, Sweden) by absorbency at 260 nm. The RNA purity was determined by the A260/A280 ratio.

**Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

Total cellular RNA was incubated in DNase (1 U/sample) for 15 min to prevent any DNA contamination. Total RNA was transcribed into cDNA that was used for PCR with primers specific for the gene of interest. GAPDH served as internal control, whose primers were purchased from Clontech (Palo Alto, CA) and yielded a 450 bp fragment (sense: 5'-ACC ACA GTC CAT GCC ATC AC-3'; antisense: 5'-TCC ACC ACC CTG TTG CTG TA-3'). The primers were synthesized by Bio-Synthesis (Lewisville, TX). Their sequences are given in Table 2. To ensure no DNA contamination, PCR was conducted with RNA that was not subjected to reverse transcription.

Superscript II reverse transcriptase (Life Technologies) was used to prepare first strand cDNA as per the manufacturer’s instructions. The reaction mix was prepared as follows: 1 ml random hexamer at 500 μg/ml (Life Technologies), 1 μl total RNA (1 μg/μl), and 10 μl DEPC-H2O. It was incubated at 70°C for 10 min and then quickly chilled on ice. After brief centrifugation, 4 μl of 5× first strand buffer, 2 μl 0.1M DTT, 1 μl 10 mM dNTP mix were added and incubated for 2 min at 42°C. Two hundred units or 1 μl of superscript II was added and reverse transcription was performed for 1 h at 42°C. Incubation at 70°C for 15 min terminated the reaction.

PCR was performed using the Perkin Elmer Gene Amp PCR System 2400 (Norwalk, CT). Two μl of the reverse transcription products were subjected to an initial denaturation for 2 min at 94°C, followed by denaturation at 94°C for 30 s, annealing at 55° to 59°C for 30 s and amplification for 30 s at 72°C (Table 2). This cycle was repeated 20 to 30 times and finished with an elongation at 72°C for 7 min. The number of cycles was selected to permit the PCR amplification to take place in the linear range for both GAPDH and the cDNA of interest.

PCR products were loaded onto a 1% agarose gel impregnated with ethidium bromide and separated at 80 V for 45 min. The gel was analyzed under UV illumination and photographed using Polaroid type 667 film (Polaroid Corp., Cambridge, MA). The negative films were scanned (Hewlett Packard Scan Jet 6200C, Boise, ID) and subjected to densitometric analysis using the Fluoro-S multiimager (Bio-Rad).
TABLE 2

<table>
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<td>p75</td>
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<td>55</td>
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Statistical Analysis

Data are reported as means ± SEM of 4 animals at each time point in each group. One-way ANOVA with Scheffes post hoc test was used for comparison of baseline values, which are expressed as means ± SD. For comparisons of various time points and for group comparisons at the various time points of protein and mRNA expressions a 2-way ANOVA was applied. Statistical significance was defined as p < 0.05.

RESULTS

Clinical Data

Clinical data are summarized in Table 3. Prior to crush injury (baseline), blood glucose levels were significantly (p < 0.001) increased in diabetic animals compared to control rats, but without differences between the 2 diabetic groups (p = 0.4). C-peptide replacement did not influence the daily insulin requirement to maintain equal hyperglycemic levels in the 2 diabetic groups (data not shown). Fasting plasma insulin levels in both groups of BB/Wor-rats were 13% (p < 0.001) of control values. C-peptide plasma levels in non-C-peptide-replaced BB/Wor-rats were 6% (p < 0.001) and in C-peptide-replaced rats were 76% (p < 0.001) of control values. MNCV was significantly decreased to 77% (p < 0.001) of control values in diabetic BB/Wor-rats and to 92% (p < 0.001) in C-peptide-replaced BB/Wor-rats. MNCV was 20% (p < 0.001) faster in C-peptide-replaced BB/Wor-rats as compared to BB/Wor-rats.

IGF-1 and IGF-1R Protein and mRNA Expression in Sciatic Nerve

IGF-1 protein levels at baseline were decreased by 6% (NS) in C-peptide-replaced and by 38% (p < 0.001) in non-replaced type 1 BB/Wor-rats (Fig. 1A). Crush injury lead to an increase in IGF-1 protein expression in all animal groups. Control animals exhibited an early peak at 0.5 h that was 11% higher than baseline, which decreased to 80% of baseline values at 6 h with a recovery to baseline values at 2 d. C-peptide-replaced animals achieved a peak in IGF-1 protein expression at 2 h, which then stabilized at control baseline levels throughout the observation period. Non-C-peptide-replaced diabetic rats showed a delay in IGF-1 protein expression after crush injury with a first peak occurring only at 24 h followed by a decline at 2 d, and a second peak at 4 d.

The pattern of IGF-1 mRNA expression was similar to that of the protein levels (Fig. 1B). Control animals showed significantly higher (p < 0.02) IGF-1 mRNA levels at baseline than non-replaced- but not greater (NS) than C-peptide-replaced BB/Wor-rats. Control rats developed a biphasic expression pattern with an initial peak at 0.5 h that was 2-fold that of baseline values, followed by a decline to 40% of baseline, and a second peak at 2 d that was comparable to baseline levels. C-peptide-replaced BB/Wor-rats exhibited an early peak at 6 h that was 2.9-fold that of baseline values and then maintained...
relatively stable levels with a decline occurring at 6 d. In non-replaced type 1 BB/Wor-rats the first peak in IGF-1 mRNA expression was markedly delayed with a large peak occurring only at 24 h that was 5 times that of baseline, and a second smaller peak occurring at 4 d.

IGF-1R protein expression in non-replaced diabetic rats showed higher baseline levels than control rats (p < 0.05) and C-peptide-replaced BB/Wor-rats showed intermediate levels (NS) (Fig. 2A). After crush injury a biphasic pattern of IGF-1R protein expression developed in control and C-peptide-replaced diabetic animals that was roughly synchronous to the undulations of IGF-1 protein levels. Control rats showed an early peak at 0.5 h, followed by a decrease at 6 h, and a subsequent increase at 4 and 6 d. However, this latter increase in C-peptide-replaced animals was less than in non-diabetic controls. In contrast, non-replaced type 1 BB/Wor-rats showed a continuous decline of IGF-1R protein to 18% of baseline values at 6 d, with a profile that was asynchronous to that of IGF-1 protein expression.

IGF-1R mRNA levels (Fig. 2B) showed a pattern similar to that of the protein levels. Non-C-peptide-replaced type 1 BB/Wor-rats showed a significantly higher level of IGF-1R mRNA at baseline compared to control and C-peptide-replaced BB/Wor-rats (p < 0.01). This was followed by a marked decline throughout the observation period. In contrast, control and C-peptide-replaced BB/Wor-rats exhibited a peak in IGF-1R expression occurring...
at 2 h that was about 2-fold that of baseline values, followed by a decline at 6 h, and then a small increase in control animals at later time points.

**c-fos Protein and mRNA Expression in Sciatic Nerve**

Protein expression of c-fos did not differ significantly between the 3 groups at baseline (Fig. 3A). An increase in c-fos protein was evident in all animals after crush injury. A biphasic response was seen in control animals with peaks at 6 h and 4 d. C-peptide-replaced BB/Wor-rats did not exhibit a distinct biphasic pattern, but the expression levels were similar to those of control animals. In contrast, non-C-peptide-replaced BB/Wor-rats showed a significant (p < 0.001) suppression of c-fos expression during the first 24 h, with a peak developing only at 24 h.

All groups of animals showed comparable levels of c-fos mRNA at baseline (NS) (Fig. 3B). Following crush injury, c-fos expression increased in control and C-peptide-replaced BB/Wor-rats with initial peaks developing at 6 h and 24 h, respectively, which were approximately 3-fold (p < 0.001) of respective baseline values. C-fos mRNA levels then dropped in all animal groups with a small peak developing at 4 d post-crush in the control animals. Non-C-peptide-replaced type 1 BB/Wor-rats exhibited a significantly (p < 0.001) smaller initial increase in c-fos mRNA, during the first 0.5 to 24 h, and showed a peak that was only 1.3-fold at control baseline values at 2 d.

**NGF and p75 Protein and mRNA Expression in Sciatic Nerve**

NGF protein expression at baseline was barely detectable in any of the animal groups (Fig. 4A). After crush injury, NGF protein expression increased in all groups and a biphasic pattern developed in control animals, which was less marked in C-peptide-replaced BB/Wor-rats, whereas non-C-peptide-replaced BB/Wor-rats developed a monophasic pattern. In control animals an early peak was noted at 6 h that was 2.4-fold that of baseline. C-peptide-replaced BB/Wor-rats exhibited a first peak of NGF expression at 6 h that was 2-fold that of baseline values. Non-C-peptide-replaced BB/Wor-rats developed a peak occurring at 2 d, which was 2.2-fold that of baseline values.

NGF mRNA expression showed a pattern that was similar to that of NGF protein (Fig. 4B), although the magnitude of changes was more dramatic. NGF mRNA levels were comparable at baseline in all animal groups and increased during the first 6 h. Control animals showed a distinct biphasic expression pattern with peaks occurring at 6 h and 4 d, which were 12-fold and 9-fold those of baseline levels. C-peptide-replaced BB/Wor-rats developed 1 NGF mRNA peak that was 10-fold that of baseline at 24 h, followed by stabilization in expression. Non-C-peptide-replaced rats exhibited only 1 peak at 2 d that was 15-fold that of baseline with a subsequent rapid decrease.

All animal groups exhibited comparable levels of p75 protein expression at baseline, which remained unchanged for the first 24 h after crush (Fig. 5A). After 2 d, control and C-peptide-replaced BB/Wor-rats showed an upregulation of p75 protein that was greater (p < 0.02) than that seen in non-replaced type 1 BB/Wor-rats at 4 d and 6 d.

The pattern of p75 mRNA expression was similar. At baseline all groups showed similar levels of p75 mRNA.
Sciatic nerve NGF Protein and mRNA expression and message localization. No differences in NGF protein were detectable between groups at baseline (0 h). All groups showed a progressive increase, which in control and C-peptide-replaced diabetic rats peaked at 6 h post-crush, whereas this occurred at 2 d in non-replaced diabetic BB/Wor-rats. Two-way ANOVA showed significant differences between groups for time ($p < 0.001$; $F = 141$) and for group $\times$ time ($p < 0.001$; $F = 109$). The NGF mRNA messages were not different between groups at baseline ($B$). The mRNA messages were similar to the protein expression, although their magnitudes were far greater. For each time point 2-way ANOVA showed a significance of $p < 0.001$ ($F = 277$) and for group $\times$ time $p < 0.001$ ($F = 40$).

IGF-1 and IGF-1R Expression in DRGs after Sciatic Nerve Crush Injury

IGF-1 protein expression at baseline was significantly higher in control rats than in C-peptide-replaced and non-replaced BB/Wor-rats ($p < 0.01$) (Fig. 6A). After crush injury, IGF-1 protein levels increased achieving a peak at 6 h that was approximately 1.3-fold that of control baseline. Expression stabilized in both groups at 2 d at levels that were about 1.4-fold that of control baseline. In contrast, non-replaced BB/Wor-rats showed a significant pattern of IGF-1 mRNA expression with an initial decrease at 0.5 h, followed by an increase at 2 h and a second decline in expression at 6–24 h, followed by another increase at 2 d and subsequent decrease at 4 d.

IGF-1R protein expression at baseline was higher in non-C-peptide-replaced type 1 BB/Wor-rats than in control ($p < 0.01$), but not in C-peptide-replaced BB/Wor-rats (NS) (Fig. 7A). In control and C-peptide-replaced BB/Wor-rats, IGF-1R protein levels increased after crush achieving levels at 6 h that were approximately 1.5-fold that of control baseline. Expression stabilized in both groups at 2 d at levels that were about 1.4-fold that of control baseline. IGF-1R mRNA expression at baseline was significantly higher in non-replaced BB/Wor-rats compared to control and C-peptide-replaced BB/Wor-rats ($p < 0.01$) (Fig. 6B). IGF-1 mRNA expression then fell to levels that were slightly above baseline at 6 h after crush in C-peptide-replaced BB/Wor- and control rats. IGF-1 mRNA in non-replaced BB/Wor rats stabilized at significantly lower levels (Fig. 6B).

IGF-1R protein expression at baseline was higher in non-C-peptide-replaced type 1 BB/Wor-rats than in control ($p < 0.01$), but not in C-peptide-replaced BB/Wor-rats (NS) (Fig. 7A). In control and C-peptide-replaced BB/Wor-rats, IGF-1R protein levels increased after crush achieving levels at 6 h that were approximately 1.5-fold that of control baseline. Expression stabilized in both groups at 2 d at levels that were about 1.4-fold that of control baseline. In contrast, non-replaced BB/Wor-rats showed an undulating pattern of IGF-1R protein expression with an initial decrease at 0.5 h, followed by an increase at 2 h and a second decline in expression at 6–24 h, followed by another increase at 2 d and subsequent decrease at 4 d.

IGF-1R mRNA expression at baseline was significantly higher in non-C-peptide-replaced type 1 BB/Wor-rats than in control ($p < 0.01$), but not in C-peptide-replaced BB/Wor-rats (NS) (Fig. 7A). In control and C-peptide-replaced BB/Wor-rats, IGF-1R protein levels increased after crush achieving levels at 6 h that were approximately 1.5-fold that of control baseline. Expression stabilized in both groups at 2 d at levels that were about 1.4-fold that of control baseline. In contrast, non-replaced BB/Wor-rats showed an undulating pattern of IGF-1R protein expression with an initial decrease at 0.5 h, followed by an increase at 2 h and a second decline in expression at 6–24 h, followed by another increase at 2 d and subsequent decrease at 4 d.

IGF-1 and IGF-1R Expression in DRGs after Sciatic Nerve Crush Injury

IGF-1 protein expression at baseline was significantly higher in control rats than in C-peptide-replaced and non-replaced BB/Wor-rats ($p < 0.01$) (Fig. 6A). After crush injury, IGF-1 protein levels increased achieving a small peak in all 3 groups at 2 h. Following this peak, IGF-1 protein levels decreased and stabilized at baseline levels in control animals, whereas the decline continued in the diabetic groups. However, at 4 to 6 d, an increase in expression developed in C-peptide-replaced animals, whereas that in non-replaced BB/Wor-rats declined.

At baseline, IGF-1 mRNA levels were significantly ($p < 0.03$) lower in non-replaced BB/Wor-rats compared to control rats (Fig. 6B). C-peptide-replaced rats did not differ significantly from controls. After crush injury, IGF-1 mRNA levels rose in all animal groups, achieving a peak at 2 h that was approximately 2-fold that of the respective baseline values. The peak in non-C-peptide-replaced BB/Wor-rats at this time was significantly lower ($p < 0.001$) than in control and C-peptide-replaced BB/Wor-rats. IGF-1 mRNA expression then fell to levels that were slightly above baseline at 6 h after crush in C-peptide-replaced BB/Wor- and control rats. IGF-1 mRNA in non-replaced BB/Wor rats stabilized at significantly lower levels (Fig. 6B).

p75 Protein and mRNA Expression in DRGs after Sciatic Nerve Crush Injury

At the protein level, p75 expression was comparable at baseline in all 3 animal groups (Fig. 8A). After crush injury a decrease in expression developed that was approximately 80% of baseline in all 3 animal groups. An increase in p75 protein developed in control and C-peptide-replaced BB/Wor-rats that reached baseline levels at
Fig. 5. Sciatic nerve p75 protein and mRNA expression and protein localization. Both protein (A) and mRNA (B) expression were not different between groups at baseline and remained unchanged for the first 24 h. Following this time point there were moderate increases in p75 protein expression in control and C-peptide-replaced diabetic rats. These were smaller in non-C-peptide-replaced diabetic rats. The p75 mRNA pattern was similar to that of p75 protein expression in the 3 groups, but as with NGF mRNA expression the magnitude of mRNA increases was greater than that of p75 protein (B). For p75 protein (A), 2-way ANOVA (each time point) showed $p < 0.001$ ($F = 591$) and $p < 0.001$ for group $\times$ time ($F = 23$). For (C) 2-way ANOVA showed $p < 0.001$ ($F = 258$) for time and a $p < 0.001$ ($F = 12$) for group $\times$ time.

BB/Wor-rats exhibited a slight downregulation in $\beta$-tubulin protein expression after injury to levels 80% of baseline at 20 d. At baseline no significant differences in $\beta$II-tubulin mRNA were evident between the 3 groups (Fig. 10B). $\beta$II-tubulin mRNA in C-peptide-replaced BB/Wor- and control rats exhibited a decrease in expression 6 h after crush, which was followed by an increase at 24 h that continued achieving maximal values of 1.3-fold and 1.5-fold of baseline values, respectively, at 20 d post-crush. Non-C-peptide-replaced BB/Wor-rats failed to show the initial downregulation. Instead, the levels remained stable throughout the observation period (Fig. 10B).

NF-L Protein and mRNA Expression in DRGs after Sciatic Nerve Crush Injury

NF-L protein expression in non-diabetic animals showed a continuous downregulation throughout the observation period to levels that reached 30% of baseline.
Fig. 6. IGF-1 protein and mRNA expression in DRGs. IGF-1 protein expression (A) was significantly greater (p < 0.01) in control rats than in diabetic and C-peptide-replaced diabetic BB/Wor-rats. All groups showed minor peaks at 2 h, after which the levels decreased and then increased in control and C-peptide-replaced BB/Wor-rats, whereas in diabetic BB/Wor-rats there was a progressive decrease to ~60% of baseline at 6 d. Two-way ANOVA for (A) showed p < 0.001 (F = 303) for time and p < 0.001 (F = 60) for group × time. mRNA expression showed a similar pattern in all 3 groups (B). Diabetic BB/Wor-rats showed substantially lower levels compared to control rats. C-peptide-replaced diabetic rats showed intermediate levels. Two-way ANOVA (for each time point) showed p < 0.001 (F = 354) and for group × time p < 0.001 (F = 12). Each data point represents the mean ± SEM of 4 experiments.

at 20 d (p < 0.05) (Fig. 11A). On the other hand, non-C-peptide-replaced BB/Wor-rats had the lowest baseline levels of NF-L protein (p < 0.01 vs control and C-peptide-replaced) and exhibited no downregulation in NF-L protein. C-peptide-replaced BB/Wor-rats showed baseline levels comparable to non-diabetic animals, however the downregulation that resulted after crush was slower compared to non-diabetic animals, reaching 40% (p < 0.01) of baseline values only at 20 d.

NF-L mRNA expression in C-peptide-replaced BB/ Wor- and non-diabetic rats were comparable at baseline and remained stable until 24 h after injury when a decrease in their expression ensued to levels that were 25% (both p < 0.01) of baseline at 20 d (Fig. 11B). Non-C-peptide-replaced BB/Wor-rats showed baseline levels that were 70% of control levels (p < 0.003). The levels of

NF-L mRNA remained relatively stable throughout the observation period and were at no time point significantly different from baseline.

Sciatic Nerve Morphometric Analysis 20 d Post-Crush Injury

At 20 d following sciatic nerve crush, mean axonal area in C-peptide-replaced BB/Wor-rats was 94% (NS) and in non-replaced BB/Wor-rats 76% (p < 0.01) of that in non-diabetic rats (Fig. 12A). At the same time point, diabetic BB/Wor-rats showed a fiber density that was 63% (p < 0.001) of that in control rats (Fig. 12B). C-peptide-replaced BB/Wor-rats were significantly (p <
Fig. 8. p75 protein and mRNA expression in DRGs following sciatic nerve crush injury. Protein levels (A) of p75 were the same at baseline in the 3 groups. They all showed an initial decline followed by an initial peak at 6 h and a second peak at 4 d in control and C-peptide-replaced diabetic rats. On the other hand, non-C-peptide-replaced diabetic rats showed a single peak only at 2 d. Two-way ANOVA (each time point) showed p < 0.001 (F = 142) and p < 0.001 (F = 222) for group × time. p75 mRNA expression levels were similar at baseline. Control and C-peptide-replaced diabetic rats showed a progressive increase during the first 24 h, which was less robust in non-C-peptide-replaced diabetic rats. In all groups, a peak occurred at 24 h followed by a decline and a renewed increase at 6 d. Two-way ANOVA showed p < 0.001 (F = 288) for time and p < 0.001 (F = 123) for group × time.

Fig. 9. DRG TrkA protein and mRNA expression. Both protein (A) and mRNA (B) expression levels were higher (A, p < 0.01; B, p < 0.05) in control and C-peptide-replaced diabetic rats than in non-C-peptide-replaced diabetic rats. Protein levels showed initially a small decline followed by a peak at 24 h in control and C-peptide-replaced rats. This was markedly attenuated in non-C-peptide-replaced rats. Two-way ANOVA showed p < 0.001 (F = 72) for time and p < 0.001 (F = 51) for group × time. The TrkA mRNA levels declined in all 3 groups after crush injury throughout the observation period. Non-C-peptide-replaced diabetic rats showed consistently lower levels than control and C-peptide-replaced diabetic rats. Two-way ANOVA analyses revealed p < 0.001 (F = 69) for time and p < 0.05 (F = 5) for group × time.

0.01) protected against retarded elongation of regenerating fibers, although this was not normalized (80%; p < 0.05 vs controls). Myelinated fiber size histograms of regenerating fibers showed a similar size distribution in non-diabetic control and C-peptide-replaced BB/Wor-rats, whereas non-replaced BB/Wor-rats showed a shift of fibers toward smaller sizes (p < 0.05 by chi-square) (Fig. 12C).

DISCUSSION
We have previously shown that early gene responses and subsequent expression of trophic factors and cytoskeletal protein in DRGs are severely perturbed following sciatic nerve injury in type 1 diabetic BB/Wor-rats (6, 33), and that these abnormalities are substantially milder in iso-hyperglycemic hyperinsulinemic type 2 BB/Z-rats (9, 10). These findings led us to suggest that insulin deficiency may be a more important factor in suppressed nerve regeneration in type 1 DPN than hyperglycemia per se (1, 4, 9, 10). Proinsulin C-peptide demonstrates insulinomimetic effects and enhances insulin signaling without effecting blood glucose levels (12, 13) and it prevents and improves type 1 DPN in both patients (18) and the BB/Wor-rat (7).

Here we demonstrate that abnormalities in early gene responses of trophic factors and their respective receptors in type 1 BB/Wor-rats were significantly prevented in BB/Wor-rats given C-peptide substitution from onset of diabetes, despite the fact that C-peptide did not correct hyperglycemia. The findings in C-peptide-replaced BB/Wor-rats are strikingly similar to those previously reported in hyperinsulinemic but iso-hyperglycemic type 2
Fig. 10. DRG β-tubulin protein expression and βII-tubulin mRNA levels. Protein levels (A) of β-tubulin were the same in the 3 animal groups at baseline. Following crush injury, the protein levels in control and C-peptide-replaced diabetic rats showed steady increases, whereas the levels in non-C-peptide-replaced diabetic rats remained unchanged throughout the 20 days. Two-way ANOVA showed for time \( p < 0.001 \) (\( F = 67 \)) and for group × time \( p < 0.001 \) (\( F = 23 \)). βII mRNA levels (B) were similar at baseline in the 3 groups. Control and C-peptide-replaced diabetic rats showed a decline at 6 h followed by progressive increases in mRNA expression, whereas in non-C-peptide-replaced diabetic rats the expression levels remained unchanged throughout the observation period. Two-way ANOVA showed \( p < 0.001 \) (\( F = 298 \)) for time and \( p < 0.001 \) (\( F = 64 \)) for group × time.

Fig. 11. Protein and mRNA expression of NF-L in dorsal root ganglia. Protein levels were at baseline significantly (\( p < 0.01 \)) lower in non-C-peptide-replaced BB/Wor-rats compared to control and C-peptide-replaced rats. Control rats showed a progressive decline in NF-L protein expression. This was slower in the 2 diabetic groups up to 6 d, after which time point C-peptide-replaced BB/Wor-rats showed a decline in NF-L protein expression. A: Two-way ANOVA for each time point showed \( p < 0.001 \) (\( F = 107 \)) and \( p < 0.001 \) (\( F = 18 \)) for group × time. The expression of NF-L mRNA was significantly (\( p < 0.01 \)) lower in diabetic BB/Wor-rats compared to control and C-peptide-replaced BB/Wor-rats. Both control and C-peptide-replaced BB/Wor-rats showed a slowly progressive decline over the observation period to approximately 25% of baseline values. In contrast, non-C-peptide-replaced diabetic rats showed unchanged expression levels throughout the 20 d following crush injury. Two-way ANOVA showed for time \( p < 0.001 \) (\( F = 100 \)) and \( p < 0.001 \) (\( F = 16 \)) for group × time. Each time point represents the mean ± SEM of 4 experiments.

BB/Z-rats (9). In the sequence of early gene responses, the expression of c-fos is induced by IGF-I as a growth factor-induced immediate early gene (6, 34, 35), that in turn mediates the lesion-induced increase in NGF (35, 36).

The expression levels of NGF and its low affinity receptor p75 were significantly normalized in C-peptide-replaced type 1 BB/Wor-rats. The early upregulation of NGF induces macrophage recruitment necessary for subsequent phagocytosis and secretion of interleukins and trophic factors. Previously demonstrated sequential abnormalities such as impaired macrophage recruitment and delayed Wallerian degeneration, Schwann cell proliferation, and initiation of axonal regrowth (5, 37, 38) in the diabetic BB/Wor-rat are all consequent to perturbation of early gene expression. Its partial correction by C-peptide is therefore consistent with increased axonal regrowth as reflected by increased fiber densities. As demonstrated here and by previous data (9), insulin deficiency in type 1 diabetic rats is associated with a compensatory upregulation of both IR and IGF-1R expression in peripheral nerve, abnormalities that are significantly corrected by C-peptide (39). In the present study we demonstrate in non-diabetic control rats and C-peptide-replaced BB/Wor-rats a synchronous regulation of IGF-1 and its receptor, whereas this occurred asynchronously in non-C-peptide-replaced diabetic rats (9). Therefore sustained insulin and C-peptide deficiencies may attenuate early transcription factors regulating neurotrophic factors and their receptors. Recent data from our laboratory have shown that
insulin in the presence of C-peptide activates and translocates NF-κB to the nucleus in human neuroblastoma SY-5YSY cells (21) and may regulate gene responses of trophic factors.

In order for successful nerve regeneration to occur, amplification of neurotrophic factors in the cell somata and the availability of insulin are required for induction of neuroskeletal protein synthesis (40–44). Here we show that the expression of IGF-1, IGF-1R, and TrkA in DRGs are significantly attenuated and delayed in type 1 BB/Wor rats following sciatic nerve injury. Diabetic BB/Wor rats replaced with C-peptide showed a timely regulation of DRG neurotrophic factors, although expression levels were in general somewhat lower than in control rats. In type 1 BB/Wor rats, these abnormalities were associated with an absent upregulation of β-tubulin and an attenuated or absent downregulation of NF in the DRGs. In contrast, C-peptide-treated BB/Wor rats showed near normal upregulation of tubulin and a slower but nevertheless a downregulation of NF. In non-C-peptide-replaced BB/Wor rats, these findings were associated with impaired elongation of regenerating fibers as reflected by decreased fiber densities and with decreased radial axonal growth, and hence a shift of nerve fiber caliber spectra to smaller regenerating fibers. In contrast, C-peptide-treated rats showed more robust nerve fiber regeneration, although this was not completely normalized. These findings are consistent with a C-peptide dose-related increase in nerve fiber regeneration in uninjured sural nerves of diabetic BB/Wor rats (20), and with long-term intervention with C-peptide in the BB/Wor rat, resulting in significantly increased nerve fiber regeneration as well as decreased axonal degeneration (19).

Alterations in IGF-1 action are likely to have several consequences. IGF-1 in concert with insulin action enhances the expression of neurofilament and tubulin mRNA (45, 46), which is consistent with the present data. Exogenous IGF-1 facilitates elongation of regenerating fibers by promoting actin reorganization and focal adhesion kinase activation (41, 47). Furthermore, IGF-1 has autocrine functions by which it induces NF-κB (48), and modulates p38 and JNK and exerts antiapoptotic effect (49, 50). Although not examined in the present study, programmed cell death of DRGs has been shown in STZ diabetic rats (51). This anti-apoptotic effect by IGF-1 is consistent with in vitro data (49, 52) and with its role in hippocampal apoptosis in the BB/Wor rat (50). NGF upregulates NF-M and NF-L synthesis in vitro (44, 53) and indirectly upregulates β-tubulin protein by stabilizing its mRNA (43, 46), activities that appear to be facilitated by insulin, since insulin is required for NGF receptor binding (54).

Therefore, the abnormalities seen in the expression of these growth factors and their receptors in DRGs, including that of the insulin-receptor itself (55, 56), as well as the insulinenopenia in type 1 diabetic BB/Wor rats, are likely to underlie the abnormalities in tubulin and NF expression demonstrated here. In contrast, the lesser aberrations of neurotrophic factor expression in C-peptide-replaced BB/Wor rats were not sufficient to significantly alter the expression of cytoskeletal proteins. Although a number

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**Fig. 12.** Morphometric data of regenerating fibers. Axonal size (A) of regenerating fibers, 10 mm distal from the crush site and 20 d after the injury, was significantly (p < 0.01) decreased in non-C-peptide-replaced BB/Wor rats (p < 0.01), whereas axonal size in C-peptide replaced animals was not different from that in control rats. Nerve fiber density at this distance from the crush site is reflective of axonal elongation and was significantly (p < 0.001) decreased in non-replaced BB rats. Rats replaced with C-peptide showed significantly (p < 0.01) improved regenerated fiber density, although it was not normal (p < 0.05). Fiber size distributions 10 mm distal from the crush site were similar in control and C-peptide replaced BB/Wor rats, whereas non-replaced BB/Wor rats showed a fiber size distribution shifted to smaller fiber sizes (p < 0.05) (C).
of tubulin genes have been described, only β-II and β-III increase in DRGs after nerve injury (57–59). Increased synthesis of tubulin protein forms microtubules that initiate growth cone elongation (60). Decreased tubulin expression in BB/Wor-rats is therefore consistent with the impaired fiber elongation as reflected by decreased fiber density, compared to C-peptide-replaced BB/Wor and control rats. NF proteins are important constituents in normal nerve regeneration, since they are determinants of axonal radial growth (53, 61, 62). Of these, NF-L appears to be of importance since it appears to steer the regulation of NF-M and NF-H (63), and it interacts with other intermediate filaments such as peripherin (64). Under normal regenerative conditions, NFs are initially downregulated as tubulins are upregulated. We (33) and others (58) have suggested a negative feedback mechanism by tubulin on NF expression to facilitate axonal transport of tubulin and growth cone elongation. Both tubulin and NFs utilize the slow component of axonal transport, SCa, whereas tubulin only utilizes the faster slow component, SCb. To prevent crossbridge formation between the 2 proteins, a downregulation of NF subunits facilitates a shift of tubulin transport via the faster SCb to reach the growth cone in a timely fashion. The lack of NF downregulation in type 1 diabetic rats may therefore impede tubulin transport contributing further to impaired growth cone elongation (33). This abnormality did not occur in C-peptide-replaced BB/Wor-rats, which showed normal tubulin upregulation associated with NF downregulation. The near normal expression of NF in C-peptide-replaced BB/Wor-rats likely accounts for the normal axonal size and fiber size distribution as shown morphometrically.

The pathogenesis of DPN is complex and multifactorial, with hyperglycemia historically being regarded as the most critical initiating factor (1, 4). More recently, a role for insulin and possibly C-peptide deficiency in DPN has been postulated. The diabetes control and complications trial demonstrated that intensive insulin treatment in type 1 patients reduced the onset of clinical neuropathy by 57% over 5 years (65). However, the fact that DPN still occurred in the DCCT cohort despite near glycemic control suggests that additional pathogenic factors (1, 4) are involved. Among these, insulin and/or C-peptide deficiencies must be considered not only in type 1 patients, but potentially also in type 2 patients deficient of endogenous insulin and C-peptide secretion. This is supported by the fact that human type 1 diabetes is a prominent risk factor for severe DPN (3, 66). Also, more severe structural and functional alterations are evident in humans and animal models of DPN in type 1 diabetes than in type 2 diabetes despite comparable levels of hyperglycemia (3, 7, 66).

We have demonstrated that replacement of insulinomimetic proinsulin C-peptide in the type 1 diabetic BB/Wor-rat significantly prevents aberrations in early neurotrophic gene responses in injured sciatic nerve without correcting blood glucose levels. These corrections translated into partial prevention of DRG trophic responses and expression of neuroskeletal proteins. In turn, morphometric analyses of regenerating nerve fibers showed complete normalization of axonal radial growth and fiber size distribution and partial correction of regenerated fiber elongation. These findings are similar to those demonstrated in iso-hyperglycemic hyperinsulinemic type 2 BB/Z-rats (10). We conclude that impaired insulin action may be more important than hyperglycemia in explaining impaired nerve fiber regeneration in type 1 DPN. This suggests that future therapies in patients with type 1 diabetes should not merely be concerned with achieving glycemic control but also be directed at ameliorating insulin deficiency, perhaps by the supplementation of insulinomimetic C-peptide.

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