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C-peptide prevents and improves chronic Type I diabetic polyneuropathy in the BB/Wor rat

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Abstract

Aims/hypothesis. Insulin and C-peptide exert neuroprotective effects and are deficient in Type I (insulin-dependent) diabetes mellitus but not in Type II (non-insulin-dependent) diabetes mellitus. These studies were designed to test the preventive and interventional effects of C-peptide replacement on diabetic polyneuropathy in the Type I diabetic BB/Wor rat.
Methods. Diabetic BB/Wor rats were replaced with rat C-peptide from onset of diabetes and between 5 and 8 months of diabetes. They were examined at 2 and 8 months and compared to non-C-peptide replaced BB/Wor rats, Type II diabetic (non-C-peptide deficient) BB/Z rats and non-diabetic control rats. Animals were monitored as to hyperglycaemia and nerve conduction velocity (NCV). Acute changes such as neural Na^+/K^+-ATPase and paranodal swelling were examined at 2 months, morphometric and teased fiber analyses were done at 8 months.
Results. C-peptide replacement for 2 months in Type I diabetic rats prevented the acute NCV defect by 59 % ($p < 0.005$), the neural Na^+/K^+-ATPase defect by 55 % ($p < 0.001$) and acute paranodal swelling by 61 % ($p < 0.001$). Eight months of C-peptide replacement prevented the chronic nerve conduction defect by 71 % ($p < 0.001$) and totally prevented axoglial dysjunction ($p < 0.001$) and paranodal demyelination ($p < 0.001$). C-peptide treatment from 5 to 8 months showed a 13 % ($p < 0.05$) improvement in NCV, a 33 % ($p < 0.05$) improvement in axoglial dysjunction, normalization ($p < 0.001$) of paranodal demyelination, repair of axonal degeneration ($p < 0.01$), and a fourfold ($p < 0.001$) increase in nerve fibre regeneration.
Conclusion/interpretation. C-peptide replacement of Type I BB/Wor-rats partially prevents acute and chronic metabolic, functional and structural changes that separate Type I diabetic polyneuropathy from its Type II counterpart suggesting that C-peptide deficiency plays a pathogenetic role in Type I diabetic polyneuropathy. [Diabetologia (2001) 44: 889–897]

Keywords Diabetic neuropathy, C-peptide, Na^+/K^+-ATPase, nerve conduction velocity, morphometry.

Diabetic polyneuropathy (DPN) occurs in Type I (insulin-dependent) diabetes mellitus and Type II (non-insulin-dependent) diabetes mellitus and is believed to be initiated and fueled by hyperglycaemia. The mechanisms include increased activation of the polyol pathway [1], impaired blood flow, secondary to

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Abbreviations: AGD, Axoglial dysjunction; BB rat, bio-breeding rat, non-diabetes-prone control rat; BB-Wor rat, bio-breeding Worcester rat, diabetes-prone Type I rat; BB/Z rat, bio-breeding rat outbred on Zucker background, insulin resistant Type II diabetic rat; C-2, 2-month non-diabetes-prone control rats; C-5, 5-month non-diabetes-prone control rats; C-8, 8-month non-diabetes-prone control rats; D1–2, 2-month Type I diabetic rats; D1–5, 5-month Type I diabetic rats; D1–8, 8-month Type I diabetic rats; D1CP-2, 2-month C-peptide replaced Type I diabetic rats; D1CP-8, 8-month C-peptide replaced Type I diabetic rats; D1CP-5/8, Type I diabetic rats treated with C-peptide between 5 and 8-months of diabetes; D2–2, 2-month Type II diabetic rats; D2–8, 8-month Type II diabetic rats; DPN, diabetic polyneuropathy; NCV, nerve conduction velocity; P13-kinase, phosphatidylinositol 3-kinase; p85, regulatory domain of P13-kinase.

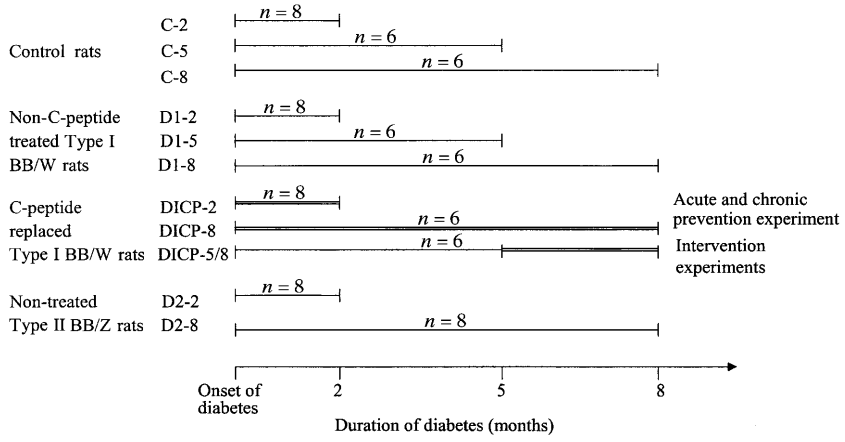

Fig. 1. Flow chart of study design: C-peptide replaced Type I diabetic BB/W rats were examined at 2 months (D1CP-2) and 8 months (D1C-P-8). Double lines indicate periods of C-peptide replacement. These groups were compared with age-, sex- and disease-duration-matched non-C-peptide replaced BB/W rats (D1–2 and D1–8 respectively), non-diabetic controls (C-2 and C-8 respectively), and age-, sex-, hyperglycaemia-, and disease-duration-matched non-C-peptide deficient Type II diabetic BB/Z rats (D2–2 and D2–8 respectively). C-peptide treatment was initiated at 5 months of diabetes for a duration of 3 months (D1C-P-5/8; double line). This group was compared with 5 months diabetic (D1–5) and non-diabetic control animals (C-5)

perturbed nitric oxide and prostanoid metabolism [2–4], aberrant regulation of neurotrophic influences [5, 6], non-enzymatic glycation [7], and generation of radical oxygen species [8, 9]. These abnormalities occur sequentially and are interrelated, resulting in a complex series of pathogenetic components [10].

Recent data show differences between DPN in the two types of diabetes. The Diabetes Control and Complications Trial (DCCT), designed to achieve normoglycaemia in Type I patients, showed only partial prevention of DPN [11]. Comparisons between DPN in rat models of the two types of diabetes show less severe axonal degeneration in Type II models [12–13]. Furthermore, both human and rodent Type II DPN lack the nodal pathology characteristic of Type I DPN [13–15], suggesting that factors besides hyperglycaemia contribute to Type I DPN. These data are consistent with the Rochester Diabetic Neuropathy Study Report [16] showing that Type I diabetes is associated with more severe DPN.

Since both insulin and C-peptide exert neuroprotective effects [17, 18], their deficiencies could contribute to Type I DPN. Short time studies have reported beneficial effects of C-peptide on renal, sensory and autonomic abnormalities in Type I diabetic patients [19, 20].

We examined the preventive effects of C-peptide replacement on acute and chronic changes of DPN in the C-peptide deficient Type I BB/Wor rat. The effects of C-peptide treatment on chronic functional and structural abnormalities were examined in an intervention study. The data were compared to those of non-diabetic BB rats, non-C-peptide replaced diabetic BB/Wor rats and non-C-peptide deficient Type II BB/Z rats matched for hyperglycaemia and duration of disease.

Materials and methods

Animals. Forty pre-diabetic male BB/Wor rats and 20 age-matched non-diabetes prone BB rats were used (Biomedical Research Models, Worcester, Mass., USA). They were maintained in metabolic cages with free access to water and rat chow. Body weight, urine volume and glucosuria were monitored daily to ascertain onset of diabetes. After onset of diabetes at 71 ± 3 days of age, diabetic rats were supplemented with titrated doses (0.5–3.0 U/day) of protamine zinc insulin (Novo Nordisk, Princeton, N.J., USA) [4]. Blood glucose was measured every 2 weeks and glycated haemoglobin (DCA 2000 Analyser, Bayer, and Elkhart, Ind., USA) every 2 months.

One week after onset of diabetes, two groups of diabetic rats were started on C-peptide replacement (prevention groups) for 2 ($n = 8$) and 8 months ($n = 6$) (D1CP-2 and D1CP-8). They were matched with an equal number of non-C-peptide replaced diabetic rats (D1–2 and D1–8) and age-matched non-diabetic control rats (C2 and C8 resp) (Fig. 1). A group of six diabetic rats was started on C-peptide treatment at 5 months and killed at 8 months of diabetes (intervention group; D1CP-5/8). Six non-C-peptide-treated diabetic BB/Wor rats (D1–5) and 6 age-matched control rats (C-5) were killed at 5 months for baseline controls (Fig. 1). Furthermore, 16 pre-diabetic BB/Z rats, with spontaneous onset of Type II diabetes at approximately 70 d of age, were used as hyperglycaemic non-C-peptide deficient controls for the 2 and 8 month groups (D2–2 and D2–8) (Fig. 1). The animals were cared for in accordance with guidelines of the Animal Investigation Committee, Wayne State University and those of NIH [publication No. 85–23, 1995].

C-peptide replacement. Synthetic rat C-peptide II, with a purity of more than 98% by HPLC (Genosys, Cambridge, UK) was dissolved in saline (12 mg/ml). Osmopumps (Alzet Corporation, Palo Alto, Calif., USA) delivered a minimum subcutaneous dose of 75 nmol C-peptide/kg body weight a day. Control rats and non-C-peptide replaced diabetic rats received osmopumps with saline alone.

Table 1. Biochemical, functional and morphometric data from 2 month Type I (D1–2), Type II (D2–2), C-peptide replaced Type I (D1CP-2) and control rats (C-2)

Animal groups	Na^+/K^+-ATPase (μmol ADP/mg weight per hour)	Nerve Conduction velocity (m/sec) (95% Conf.)	Paranodal swelling (%) (95% Conf.)	Axonal Area (μm^2) (95% Conf.)	Axon:myelin ratio (95% Conf.)
C-2 ($n = 8$)	149 ± 23	61.8 ± 1.4 [0.74]	1.4 ± 0.3 [0.82]	11.1 ± 1.8 [1.18]	0.64 ± 0.07 [0.05]
D1–2 ($n = 8$)	42 ± 19[a]	44.0 ± 3.1[a] [1.56]	8.0 ± 0.9[a] [1.25]	12.9 ± 1.5 [1.51]	0.81 ± 0.06[c] [0.13]
D1CP-2($n = 8$)	101 ± 14[a,d]	52.6 ± 2.0[b,e] [1.01]	3.9 ± 0.5[b,d] [1.21]	11.3 ± 1.5 [1.67]	0.71 ± 0.06 [0.06]
D2–2($n = 8$)	82 ± 7[a,d]	55.0 ± 2.1[b,e] [1.11]	2.4 ± 0.6[c,d] [1.24]	11.3 ± 1.1 [1.42]	0.60 ± 0.12 [0.09]

[a] $p < 0.001$; [b] $p < 0.01$; [c] $p < 0.05$ vs C-2 rats,
[d] $p < 0.001$; [e] $p < 0.005$ vs D1–2 rats

Electrophysiological studies. Baseline nerve conduction velocity (NCV) was measured within 24 h of onset of diabetes. It was measured in the sciatic-tibial nerves under temperature-controlled (35 °C–37 °C) conditions [4, 13].

Tissue collection. Animals were anaesthetized with Na-pentobarbital (50 mg/kg body weight i.p.) and both sciatic nerves were dissected, weighed and snap-frozen in liquid nitrogen for measurements of nerve glucose, sorbitol, fructose and Na^+/K^+-ATPase activity. The right sural nerve was fixed in 2.5% glutaraldehyde in 0.1 mol/l cacodylate buffer at pH 7.40, and post-fixed in 1% osmium tetroxide (pH 7.40). Cross-sections and longitudinal sections of the proximal sural nerve were embedded in Epon for morphometric assessments. The distal sural nerve was used for teased fibre preparations [13, 15]. Cardiac blood was drawn for serum insulin and C-peptide concentrations between 8:00 and 10:00 am, 18 to 20 hours after the last insulin injection.

Insulin and C-peptide concentrations. Serum insulin and C-peptide concentrations were examined using commercially available RIA kits (Linco Research, St. Charles, Mo., USA).

Biochemical analyses. For nerve glucose, sorbitol and fructose, sciatic nerve samples were homogenized in 2 ml of 5% TCA. Aldonitrile derivatives were formed by adding 0.3 ml hydroxylamine in pyridine-methanol 4:1 (vol:vol). Samples were sonicated for 1 min and 1 ml of acetic anhydride and 2 ml of 1,2 dichlorothane were added and samples were washed in 1.0 N HCl. Samples were reconstituted in 2-butanane and analysed by gas-liquid chromatography [21].

For Na^+/K^+-ATPase activity, nerve samples were homogenized in 2 ml of 0.2 mol/l sucrose and 0.02 mol/l TRIS-HCl at pH 7.5. Between 10 and 20μl of the homogenate was assayed enzymatically for total ATPase in 1 ml of 100 mmol/l NaCl, 10 mmol/l KCl, 2.5 mmol/l $MgCl_2$, 1 mmol/l TRIS ATP, 1 mmol/l phosphoenolpyruvate, 30 mmol/l imidazole HCl buffer (pH 7.30), 0.15 mmol/l NADH, 50 μg lactate dehydrogenase and 30μg pyruvate kinase [4]. To measure ouabain-inhibited ATPase, 20μl of 25 mmol/l of ouabain was added. Na^+/K^+-ATPase activity was defined as the difference before and after ouabain and was expressed as μmol ADP formed per gram of wet weight per hour.

Morphometric analysis. Semithin (0.5 μm) cross-sections of sural nerves were used for morphometric analysis. The following measurements of myelinated fibres were obtained: total number, axonal and myelin size (μm^2), fibre density (n/mm^2), coefficient of variance (CV) of fibre densities between image frames, fibre occupancy (% of endoneurial area), and axon to myelin ratio [4].

Teased fibre examinations. A mean of 168 ± 4 myelinated fibres were teased from each sural nerve and scored for specific changes [4]. The temporal sequence and increasing severity are represented by normality, paranodal swelling, paranodal demyelination, excessive myelin wrinkling, intercalated internodes, segmental demyelination, Wallerian degeneration, and regeneration. Changes were expressed as percentages of total fibres.

Assessment of axoglial dysjunction (AGD). The frequency of AGD was examined electron-microscopically from a mean of 18.2 ± 1.7 paranodes in each nerve [22]. The frequency of myelin loops devoid of axoglial junctions was expressed as a percentage of the myelin loops examined.

Statistical analysis. The results are presented as means ± SD and the significance of differences was calculated by analysis of variance (ANOVA). Group differences were assessed by post hoc analysis using the Student-Newman-Keuls test. Tissue samples for biochemical, morphometric and teased fiber analyses were coded to mask animal identity. A p value of less than 0.05 was considered statistically significant.

Results

Acute preventive effects of 2 months C-peptide replacement (Table 1). C-peptide replacement of acutely diabetic rats (DICP-2) restored serum C-peptide concentrations to 74% ($p < 0.001$) of normal (C-2, 710 ± 52; D1–2, 43 ± 12; D1CP-2, 527 ± 40; and D2–2, 741 ± 21 pmol/l). It had no effect on serum insulin (C-2, 455 ± 52; D1–2, 57 ± 7; DlCP-2, 63 ± 11; and D2–2, 579 ± 69 pmol/l) blood glucose, insulin requirements or body weight (data not shown) compared to D1–2 rats. Blood glucose concentrations were: C-2, 5.0 ± 0.4; D1–2, 19.3 ± 3.1; DlCP-2, 20.3 ± 3.8; and D2–2, 25.8 ± 0.8 mmol/l. Nerve glucose, sorbitol or fructose concentrations were not effected (data not shown). Compared with D1–2 rats, C-peptide significantly ($p < 0.005$) prevented the NCV slowing and partially ($p < 0.001$) prevented the Na^+/K^+-ATPase defect (Table 1). Na^+/K^+-ATPase was not different from D2–2 rats (Table 1) nor was NCV in DICP-2 rats different from D2–2 rats, suggesting that parts of the acute Na^+/K^+-ATPase and NCV defects are not related to hyperglycaemia but are C-peptide responsive. Paranodal swelling was

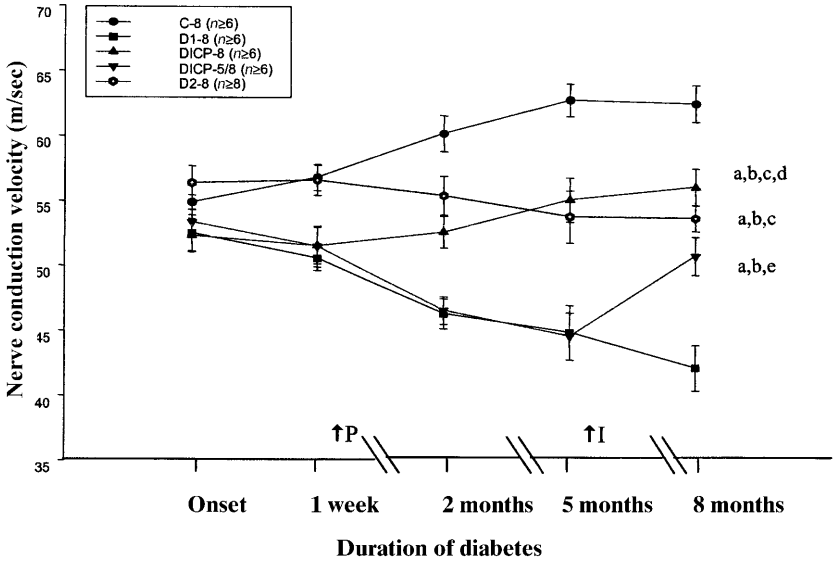

Fig. 2. Nerve conduction velocities (NCV's): Type I diabetic rats showed a severe progressive NCV slowing. C-peptide replacement initiated at 1 week (↑P), not only prevented further NCV slowing but improved NCV ($p < 0.05$). Intervention with C-peptide initiated at 5 months (↑I), resulted in a significant ($p < 0.05$) improvement of NCV at 8 months. This was slower than in D1CP-8 rats ($p < 0.05$). Type II diabetic rats showed a milder slowing of NCV, which at 8 months was not different from that in D1CP-8 rats; ↑P, initiation of C-peptide replacement in the prevention groups; ↑I, initiation of C-peptide treatment in the intervention group; [a] $p < 0.001$ vs age-matched control rats; [b] $p < 0.001$ vs D1–8 rats; [c] $p < 0.05$ vs DICP-5/8 rats; [d] $p < 0.05$ vs C-peptide replacement starting point (↑P) at 1 wk; [e] $p < 0.05$ vs C-peptide treatment starting point at 5 months (↑I)

partially, but significantly ($p < 0.001$) prevented by C-peptide and was not different from D2–2 rats (Table 1). Paranodal swelling in D1–2 rats was reflected by a significant ($p < 0.05$) increase in axon to myelin ratio (Table 1). Other parameters of axonal degeneration were not affected by diabetes or by C-peptide at this stage.

The preventive effect of C-peptide on chronic functional and structural changes. Over the subsequent 6 months, nerve conduction velocity improved ($p < 0.05$) in D1CP-8 rats compared to the values at 1 week (Fig. 2). Compared to D1–8 rats, there was a significant ($p < 0.001$) but partial prevention of NCV slowing. The residual NCV defect ($p < 0.001$) was not different from D2–8 rats (Fig. 2), suggesting a hyperglycaemic and a C-peptide responsive component of the NCV defect [13].

D1–8 rats showed loss of myelinated fibres not evident in D1CP-8 rats (Table 2). Axonal size and axon-to-myelin ratios were decreased ($p < 0.001$ and $p < 0.05$) in D1–8 compared to C-8 rats. C-peptide prevented ($p < 0.01$) axonal atrophy, which was not different from that in D2–8 rats (Table 2). These findings correlate with teased fibre scoring of axonal degeneration. In D1–8 rats 17% of sural nerve fibres showed axonal degeneration (Fig. 3), which was significantly ($p < 0.001$) prevented by C-peptide to 4.8%. This was greater ($p < 0.01$) than 1.8% seen in C-8 rats (Fig. 3) but not different from 6.7% seen in D2–8 rats (Fig. 3).

Axoglial dysjunction and subsequent paranodal demyelination are repaired by remyelination forming intercalated internodes [4]. Axoglial dysjunction was increased 3.5-fold ($p < 0.001$) in D1–8 but not in

Table 2. Morphometric data from 5 month control rats (C-5) and Type I diabetic (D1–5), 8 month control (C-8), Type I (D1–8) and Type II (D2–8) diabetic, and C-peptide replaced (D1CP-8) diabetic and treated (D1CP-5/8) Type I diabetic rats

Animal groups	Fiber number (n)	Fiber density (n/mm^2)	Axonal area (μm^2)	Axon/myelin ratio (μm^2/μm^2)
C-8 ($n = 6$)	810 ± 59	16268 ± 1314	14.7 ± 0.3	0.62 + 0.2
D1–8 ($n = 6$)	589 ± 56[b]	13981 ± 775[c]	12.0 ± 0.4[a]	0.55 ± 0.03[b]
DICP-8 ($n = 6$)	751 ± 84[f]	15335 ± 687	13.9 ± 0.5[d]	0.60 ± 0.03
C-5 ($n = 6$)	789 ± 76	15736 ± 776	14.9 ± 0.3	0.59 ± 0.02
D1–5 ($n = 6$)	731 ± 92	16411 ± 611	14.1 ± 0.7	0.55 + 0.02[b]
DICP-5/8 ($n = 6$)	730 ± 69	16357 ± 906[e]	14.2 ± 0.5[d]	0.58 ± 0.03
D2–8 ($n = 6$)	801 ± 71[e]	16491 ± 657[e]	14.1 ± 0.5[d]	0.59 ± 0.03

[a] $p < 0.001$; [b] $p < 0.05$; [c] $p = 0.07$ vs C-8 rats;
[d] $p < 0.01$; [e] $p < 0.05$; [f] $p = 0.07$ vs D1–8 rats

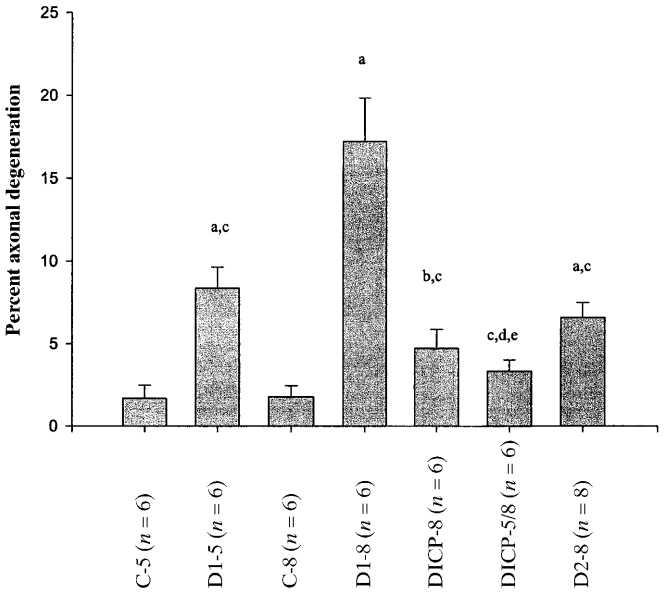

Fig. 3. Teased fiber assessment of axonal degeneration: C-peptide replacement for 8 months (D1C-peptide-8) showed a marked protection ($p < 0.001$) against axonal degeneration, and C-peptide treatment (D1CP-5/8) resulted in a significant ($p < 0.001$) repair of axonal degeneration compared to D1–5 rats. Axonal degeneration in D1C-peptide-8 rats was not different from that in D2–8 rats, and it was less frequent ($p < 0.05$) in D1C-peptide-5/8 compared to D2–8 rats. [a] $p < 0.001$; [b] $p < 0.01$ vs age-matched control rats; [c] $p < 0.001$ vs D1–8 rats; [d] $p < 0.001$ vs D1–5 rats; [e] $p < 0.05$ vs D2–8 rats

D2–8 rats (Fig. 4A). C-peptide prevented ($p < 0.001$) axoglial dysjunction so that no differences could be shown between D1CP-8, D2–8 and C-8 rats (Fig. 4A). Paranodal demyelination was increased more than eightfold in D1–8 rats ($p < 0.001$) and was prevented by C-peptide ($p < 0.001$) and was not different from C-8 or D2–8 rats (Fig. 4B). Compared to C-8 rats, D1–8 rats showed a threefold increase in intercalated internodes ($p < 0.001$) (Fig. 4C). This was

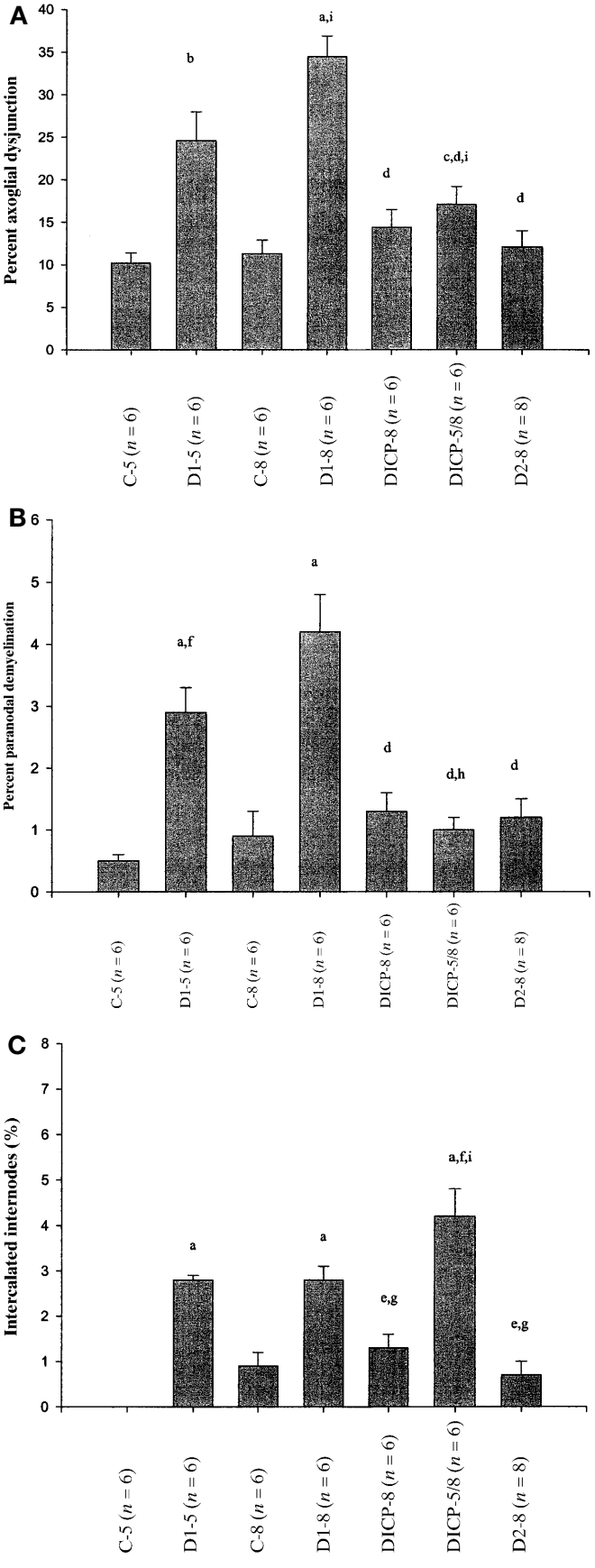

Fig. 4. The effect of C-peptide on nodal changes: C-peptide replacement (D1CP-8) for 8 months prevented AGD which was not different from that in C-8 or D2–8 rats (**A**). Intervention with C-peptide (D1CP-5/8) repaired significantly AGD ($p < 0.05$) (**A**). AGD proceeds to paranodal demyelination (**B**). C-peptide prevented (D1CP-8) completely ($p < 0.001$) paranodal demyelination (**B**). Intervention with C-peptide (D1CP-5/8) not only halted the progressive paranodal demyelination but significantly ($p < 0.001$) improved it compared to D1–5 rats (**B**). Intercalated internodes in D1CP-5/8 rats, were significantly ($p < 0.05$) more frequent than in D1–5 rats (**C**). Intercalated nodes were not increased in D1CP-8 rats (Fig. 4c), probably because of preceding AGD and paranodal demyelination (**A, B**) were fully prevented by C-peptide. These nodal changes do not occur in Type II BB/Z rats (**A–C**). [a] $p < 0.001$; [b] $p < 0.005$; [c] $p < 0.05$ vs age-matched control rats; [d] $p < 0.001$; [e] $p < 0.01$; [f] $p < 0.05$ vs D1–8 rats; [g] $p < 0.001$ vs D1CP-5/8 rats; [h] $p < 0.001$; [i] $p < 0.05$ vs D1–5 rats

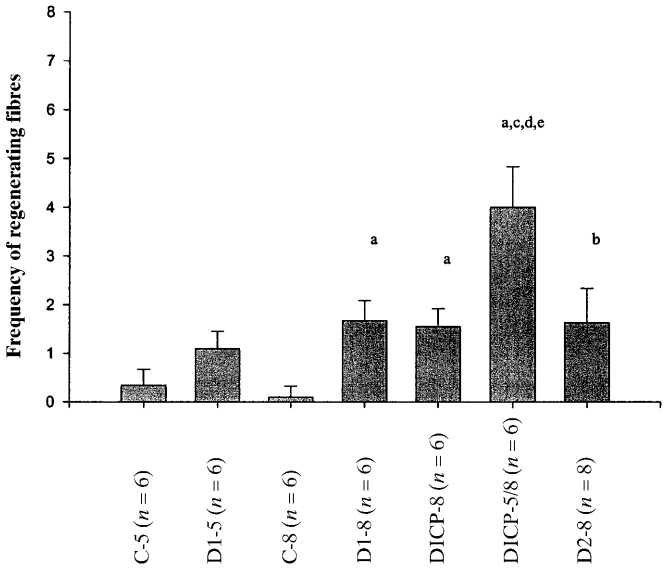

Fig. 5. Nerve fibre regeneration: regenerating fibres in D1CP-8 rats were not different from D1–8 or D2–8 rats. However, C-peptide-treated rats (D1CP-5/8) showed a marked increase ($p < 0.001$) in fibre regeneration compared to D1–5 rats. [a] $p < 0.001$, [b] $p < 0.05$ vs age-matched control rats; [c] $p < 0.001$ vs D1–8 rats; [d] $p < 0.001$ vs D1–5 rats; [e] $p < 0.01$ vs D2–8 rats

prevented ($p < 0.001$) by C-peptide, which was expected, since preceding axoglial dysjunction and paranodal demyelination were prevented by C-peptide. No difference was found between intercalated nodes in D1CP-8, D2–8 and C-8 rats (Fig. 4C).

Regenerating fibres in D1–8 rats was increased ($p < 0.001$) compared to C-8 rats as were those in D2–8 rats ($p < 0.05$) (Fig. 5). C-peptide resulted in an increase ($p < 0.001$) in fibre regeneration compared to C-8 rats but was not different from D1–8 rats (Fig. 5).

The intervention effects of C-peptide treatment. The NCV in D1–8-rats showed a progressive decline. At 5 months there was a 30% decrease ($p < 0.001$) in NCV compared to C-5 rats (Fig. 2). C-peptide treatment initiated at this timepoint resulted in a recovery ($p < 0.05$) of the NCV defect in D1CP-5/8 rats (Fig. 2). It was not normalized ($p < 0.001$) but improved ($p < 0.001$) compared to D1–8 rats but remained slower ($p < 0.05$) than in D1CP-8 and D2–8 rats (Fig. 2).

At 5 months of diabetes, no significant changes were found in myelinated fibre number, density, axonal size or axon-to-myelin ratio (Table 2). However, C-peptide treatment from 5 to 8 months prevented the ensuing decrease in fibre loss ($p < 0.05$) and axonal atrophy ($p < 0.01$) (Table 2). Compared to Type II diabetic BB/Z rats, no significant differences could be shown between D2–8 and D1C-peptide-5/8 rats in fibre number, density, axonal area or axon-to-myelin ratio (Table 2).

The more sensitive teased fibre analyses showed an increase ($p < 0.001$) in axonal degeneration in D1–5 rats compared to controls (Fig. 3). C-peptide resulted in a 60% ($p < 0.001$) repair of axonal degeneration (Fig. 3). Compared to D1–8 rats, axonal degeneration was reduced by 80% ($p < 0.001$) (Fig. 3) and was less than in D2–8 rats ($p < 0.05$).

In D1–5 rats, axoglial dysjunction was increased 2.5-fold ($p < 0.01$) (Fig. 4A), paranodal demyelination sixfold ($p < 0.001$) (Fig. 4B) as well as intercalated internodes ($p < 0.001$) (Fig. 4C). Intervention with C-peptide resulted in a 50% ($p < 0.05$) reduction of axoglial dysjunction, which was not different from that in D1CP-8 or D2–8 rats (Fig. 4A) but was not normalized ($p < 0.05$). Paranodal demyelination showed a complete ($p < 0.001$) recovery, and was not different from C-8, D1CP-8 or D2–8 rats (Fig. 4B). In contrast, intercalated internodes showed a 46% ($p < 0.05$) increase in D1CP-5/8 compared to D1–5 rats and was more common than in D1–8 ($p < 0.05$), D1CP-8 and D2–8 rats (both $p < 0.001$) (Fig. 4C). Because intercalated internodes reflect a reparative change, these findings suggest that C-peptide promotes remyelination of demyelinated nodes.

Nerve fiber regeneration, the ultimate reparative response, was not significantly increased in D1–5 rats compared to controls and not different from D1–8 or D2–8 rats (Fig. 5). However, C-peptide resulted in a fourfold ($p < 0.001$) increase compared to D1–5 rats, and was greater than in D1–8 ($p < 0.001$) or D2–8 rats ($p < 0.01$) (Fig. 5).

Discussion

Until recently, the view was held that C-peptide does not exert biological effects on its own, apart from its role in insulin synthesis [23]. However, C-peptide treatment of Type I diabetic patients improves renal function [20], increases blood flow, augments glucose utilization, and improves autonomic and somatic nerve function [18–20]. These beneficial effects correlate with stimulation of Na^+/K^+-ATPase and endothelial nitric oxide synthase activities (18, 24, 25, 31).

A C-peptide receptor has not been identified. However, C-peptide binds specifically to cell surfaces [26] with subsequent activation of Ca^{2+} dependent intracellular signalling pathways [25, 26]. No cross-reactivity with insulin, pro-insulin of IGF-1 and IGF-2 have been observed [25]. We have shown that C-peptide phosphorylates the insulin receptor and IRS-1 and inhibits protein tyrosin phosphatase while stimulating glycogen synthesis in L6 myoblasts [27]. This points to an insulinomimetic effect, without competing with insulin at the receptor level, suggesting a different ligand site.

We show significant effects of C-peptide replacement on DPN in the insulin and C-peptide deficient

Type I diabetic BB/Wor rat. C-peptide showed beneficial effects on the acute NCV, metabolic and structural changes. Long-term prevention and intervention with C-peptide partially prevented and improved the chronic NCV defect and degenerative changes of myelinated fibers, producing a functional and structural DPN similar to that in hyperglycaemia and duration-matched non-C-peptide deficient BB/Z rats [13]. This suggests that in Type I DPN one component can be ascribed to hyperglycaemia as in Type II DPN and that an additional component is linked to C-peptide deficiency.

C-peptide replacement resulted in a partial correction of the acute Na^+/K^+-ATPase defect, consistent with the reported metabolic effects of C-peptide [18, 24]. Decreased Na^+/K^+-ATPase activity is associated with increased inactivation of Na^+-channels and intraaxonal Na^+ accumulation at the node [28], resulting in paranodal swelling [29]. Paranodal swelling was prevented by 61 % in keeping with the partial prevention of the Na^+/K^+-ATPase defect. Impaired NCV in DPN is believed to be caused by impaired blood flow [30], or increased polyol-pathway activity [1], both affecting Na^+/K^+-ATPase [8]. C-peptide has a corrective effect on nitric oxide [31] and it could improve nerve blood flow and the acute NCV defect [3, 8, 9]. The residual NCV and Na^+/K^+-ATPase defects, not responsive to C-peptide replacement, could be accounted for by the unaffected activation of the polyol pathway [1, 32].

One of the most profound effects of C-peptide was the prevention and repair of nodal and paranodal changes in Type I DPN, changes which separate it from Type II DPN. These findings are likely to explain the partial prevention and improvement of the chronic NCV defect, since nodal changes correlate closely with the NCV deficit [15]. These effects could be more than coincidental because the insulin receptor in peripheral nerves co-localizes with axoglial junctions [33]. It is conceivable that C-peptide via its insulinomimetic effects could regulate molecules important for the nodal integrity, such as $ankyrin_G$, caspr and caspr II [34–36]. p85 of P13-kinase binds via its SH3 domains to the proline-rich sequence of the caspr protein, suggesting that insulin signalling intermediaries regulate nodal protein-protein interactions [37]. Beside their involvement in the nodal barrier function in itself, these molecules are responsible for the nodal localization of Na^+/K^+-ATPase, Na^+-channels, and K^+-channels [34–36], which are altered or displaced in Type I DPN [10, 38]. Axoglial dysjunction proceeds to paranodal demyelination, which is repaired by intercalated internodes. In the intervention group, prevention of axoglial dysjunction prevented further paranodal demyelination and residual paranodal demyelination at 5 months was likely repaired as reflected by the increased frequency of intercalated internodes.

Eight months of C-peptide replacement prevented axonal atrophy and degeneration. Progressive axonal degeneration in Type I DPN is in part due to impaired neurotrophic support [39] by NGF and IGF-1, which show reduced expression in diabetic rodents [39–41]. Nerve growth factor, IGF-1 and insulin promote the synthesis of neurofilaments [42, 43], which are structural determinants for axonal size [44]. It is conceivable that the insulinomimetic effects of C-peptide has a normalizing effect on structural protein synthesis, thereby explaining prevention and repair of axonal degeneration.

Nerve fibre regeneration is impaired in Type I DNP and contributes to the progressive net fibre loss. It is a complex series of temporospatial events. Early immediate gene responses involving the sequential up-regulation of IGF-1, c-fos and NGF initiate this progression of events [40, 47]. Their delay and suppression in the diabetic BB/Wor rat [40] probably result in impaired nerve fibre regeneration [46, 47]. In the intervention group, regenerating fibres were fourfold more numerous than at 5 months of diabetes, when axonal degeneration was fivefold greater than in control rats. After C-peptide treatment, these fibres were either repaired (normalization of axonal degeneration) or substituted by regenerated fibers as reflected by a normal fiber number in D1CP-5/8 rats, suggesting a neuroprotective effect by C-peptide.

In summary, replacement doses of C-peptide partially prevent the non-hyperglycaemic-induced metabolic, functional and structural changes in Type I DPN. C-peptide treatment of established Type I DPN results in functional improvement, structural repair, and promotion of fibre regeneration. We conclude that deficiency of insulinomimetic C-peptide plays a pathogenetic role in Type I DPN. While analogous findings in humans remain to be established, the results suggest that C-peptide replacement in Type I diabetic patients could provide a valuable adjunct in preventing DPN.

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