Molecular Alterations Underlie Nodal and Paranodal Degeneration in Type 1 Diabetic Neuropathy and Are Prevented by C-Peptide

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To explore the molecular abnormalities underlying the degeneration of the node of Ranvier, a characteristic aberration of type 1 diabetic neuropathy, we examined in type 1 BB/Wor and type 2 BBZDR/Wor rats changes in expression of key molecules that make up the nodal and paranodal apparatus of peripheral nerve. Their post-translational modifications were examined in vitro. Their responsiveness to restored insulin action was examined in type 1 animals replenished with proinsulin C-peptide. In sciatic nerve, the expression of contactin, receptor protein tyrosine phosphatase β, and the Na+ channel β1 subunit, paranodal caspr and nodal ankyrinG was unaltered in 2-month type 1 diabetic BB/Wor rats but significantly decreased after 8 months of diabetes. These abnormalities were prevented by C-peptide administered to type 1 BB/Wor rats and did not occur in duration- and hyperglycemia-matched type 2 BBZDR/Wor rats. The expression of the α-Na+ channel subunit was unaltered. In SH-SY5Y cells, only the combination of insulin and C-peptide normalized posttranslational O-linked N-acetylglucosamine modifications and maximized serine phosphorylation of ankyrinG and p85 binding to caspr. The beneficial effects of C-peptide resulted in significant normalization of the nerve conduction deficits. These data describe for the first time the progressive molecular aberrations underlying nodal and paranodal degenerative changes in type 1 diabetic neuropathy and demonstrate that they are preventable by insulinomimetic C-peptide. Diabetes 53:1556–1563, 2004

Hyperglycemia and its metabolic consequences are believed to underlie the microvascular complications of diabetes, whereas the role of impaired insulin action has been largely overlooked (1). Diabetic polyneuropathy (DPN) affects type 1 diabetic patients disproportionately (1–3) and differs significantly in the two major types of diabetes in humans and animal models (1,4,5). A key difference is the progressive nodal and paranodal degeneration, which accounts for the more severe clinical neuropathy in type 1 diabetes (1,3,4,6). Insulinomimetic proinsulin C-peptide ameliorates these differences between type 1 and type 2 DPN (7), which is why we have suggested that impaired insulin action plays an additional pathogenetic role in type 1 DPN (1,7).

Axoglial dysjunction, the disruption of the paranodal ion-channel barrier, is the earliest ultrastructural abnormality of paranodal degeneration and results in paranodal demyelination and eventually intercalated internodes in type 1 DPN (1,4,6). This initial lesion in the sequence of paranodal degeneration has been a contentious issue (8–11). It is not specific for type 1 DPN but occurs in a variety of clinical and experimental neuropathies summarized by Yamamoto et al. (12).

The molecular components of the node of Ranvier and the paranodal apparatus are complex (13,14). Voltage-gated Na+ channels are confined to the nodal axolemma and are responsible for ion permeation during the depolarizing phase of action potential initiation and propagation of nerve conduction. They consist of the pore-forming α-subunit and two auxiliary subunits, β1 and β2, which act as cell adhesive anchors (15). Interactions between contactin, ankyrinG, and the β-subunits are critical to Na+ channel α-subunit enrichment and localization at the node (16–18) (Fig. 1). The β1-subunit of the Na+ channel complex and ankyrinG interact with the L1-family cell adhesion molecule (CAM) neurofascin, Nr-CAM, N-cadherin, and L1 at the node (14,19,20), thereby mediating contacts with Schwann cell microvilli (18). Posttranslational modifications of ankyrinG are of regulatory importance for its interaction with other molecules. AnkyrinG receives posttranslational O-linked N-acetylglucosamine (O-GlcNAc) adducts in response to hyperglycemia (21), which transiently inhibit phosphorylation of serine residues, leading to the so-called “yin-yang” relationship (22) (Fig. 1).
β-subunits interact with receptor protein tyrosine phosphatase β (RPTP-β), a ligand for the neuronal receptor contactin (23–25). The signaling of RPTPs is mediated via tyrosine phosphorylation sites, which for the neuron-specific RPTP-β type, are regulated via nerve growth factor and insulin signaling (25) (Fig. 1).

At the paranode, the myelin loops adhere to the axolemma via tight junctions, which constitute the paranodal ion-channel barrier, separating juxtaparanodal K+ and nodal Na+ channel clusterings (12,13,26). Caspr participates in tight junctions and is coupled to contactin, acting as a neuronal receptor for RPTP-β (27,28) (Fig. 1). Caspr itself has in its cytoplasmic tail domains that mediate protein-protein interactions, such as protein 4.1 binding domains and SH3 domains (28). The latter bind with p85, the regulatory subunit of phosphatidylinositol 3-kinase, possibly mediated by insulin signaling. The neural high-affinity insulin receptor localizes to the nodal axolemma and axoglial junctions in peripheral nerve (29), suggesting that insulin action has a regulatory role on these functionally relevant structures (Fig. 1). Progressive axoglial dysjunction, the initial change of paranodal degeneration in type 1 human and murine DPN, is not detectable at 2 months of diabetes but reaches 35% at 8 months of type 1 diabetes in BB/Wor rats. In 8-month type 2 diabetic BBZDR/Wor rats, the extent of axoglial dysjunction is not different from that in control rats (7).

To explore the molecular basis initiating the progressive degeneration of the node and paranode in type 1 DPN, we examined the expression and posttranslational modifications of the molecular constituents of these structures. We used two rat models with spontaneous onset of diabetes at ~70 days of age. Type 1 BB/Wor rats develop insulinopenic diabetes as a result of an immune-mediated loss of pancreatic β-cells, whereas type 2 BBZDR/Wor rats develop insulin-resistant and hyperinsulinemic diabetes, preceded by obesity (7,30). Hence, these models closely mimic the human disorders. For examining the effects of insulin action, one group of type 1 rats was replenished from onset of diabetes with insulinomimetic proinsulin C-peptide, which enhances insulin signaling without affecting blood glucose levels (1,31–33). Posttranslational modifications of key molecules were examined in vitro in a human neuroblastoma cell line.

**RESEARCH DESIGN AND METHODS**

Forty pre-diabetic male type 1 BB/Wor rats, 20 pre-diabetic male type 2 BBZDR/Wor rats, and 20 non-diabetes-prone male BB rats were obtained from Biomedical Research Models (Worcester, MA). Spontaneous onset of diabetes occurred at 73 ± 2 days in BB/Wor rats and at 76 ± 4 days in BBZDR/Wor rats. All diabetic rats were kept individually in metabolic cages. Type 1 BB/Wor rats were monitored daily as to body weight, urine volume, glucose, and ketone contents, based on which titrated daily insulin doses (0.3–3.0 IU protamin zinc insulin; Novo Nordisk, Bagsvaerd, Denmark) were given to maintain the desired hyperglycemic levels (7). Twenty type 1 BB/Wor rats were given rat II C-peptide (Multiple Peptide Systems, San Diego, CA) from onset of diabetes via subcutaneously implanted osmopumps (ALZA, Palo Alto, CA) delivering 75 nmol·1·kg⁻¹·day⁻¹ per day. C-peptide replacement did not alter the daily insulin doses for maintenance of desired hyperglycemic levels (Table 1). All animals were monitored continuously with respect to blood glucose levels, body weight, and nerve conduction velocities (NCVs), as previously described (7). At the time that the rats were killed, HbA₁c (DCA 2000 Analyser; Bayer, Elkhart, IN), plasma insulin, and C-peptide levels were measured. Animals were killed after 2 and 8 months of diabetes, chosen to represent one time point before the development of axoglial dysjunction (2 months) and one (8 months) at which the breach in the paranodal ion-channel barrier is well established (6,7,34,35). All institutional and National Institutes of Health guidelines for use of animals were followed.

**NCVs.** NCVs were measured under controlled temperature conditions (7). Animals were anesthetized by inhalation of isoflurane (Baxter Pharmaceuticals, Deerfield, IL). Hindlimb skin temperature was maintained between 36 and 38°C by radiant heat and a warming pad. For motor NCV (MNCV), the left sciatic-tibial nerves were stimulated proximally at the sciatic notch and distally at the ankle via bipolar electrodes with supramaximal stimuli (8 volts) at 2 Hz and a pulse width of 100 µs by a Cadwell 5200 A Electromyographer (Cadwell Laboratories, Kennewick, WA) as previously described (7). The latencies of the muscle action potentials were recorded from the first interosseous muscle of the hind paw. MNCV was calculated by subtracting the distal from the proximal latency, and the result was divided into the distance...
**Table 1**

Clinical data at 8 months of diabetes in type 1 BB/Wor rats, C-peptide–replaced BB/Wor rats, type 2 BBZDR/Wor rats, and age-matched nondiabetic control rats.

<table>
<thead>
<tr>
<th>Insulin requirement (IU/d)</th>
<th>Blood glucose (mg/dl)</th>
<th>Body weight (g)</th>
<th>C-peptide Plasma level (pmol/l)</th>
<th>Glucose Plasma level (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 10)</td>
<td>5.8 ± 1.6</td>
<td>22.4 ± 4.1</td>
<td>30.1 ± 12.9</td>
<td>39.0 ± 1.0</td>
</tr>
<tr>
<td>BB/Wor (n = 10)</td>
<td>0</td>
<td>2.3 ± 0.3</td>
<td>11.4 ± 0.7</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>BB/Wor + C-peptide (n = 10)</td>
<td>2.1 ± 0.2</td>
<td>2.1 ± 0.2</td>
<td>12.3 ± 1.0</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>BBZDR/Wor (n = 10)</td>
<td>0</td>
<td>0</td>
<td>13.0 ± 1.2</td>
<td>4.0 ± 0.1</td>
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</tbody>
</table>

Data are means ± SD; *P < 0.001, †P < 0.01 vs. control.

**Sensory NCV (SNCV)** was recorded in the right hind limb. The digital nerves of the second toe were stimulated with square pulses of 0.05-ms duration using supramaximal currents. Action potentials were recorded at the level of the medial malleolus. The distance between stimulating and recording electrodes was 25 mm, and that between the active recording and indifferent electrodes was 10 mm. Eight to 16 responses were averaged. The distance between the stimulating and active recording electrodes was divided by the latency to the peak of the negative deflection (30).

**Insulin and C-peptide concentrations.** Serum insulin and C-peptide concentrations were obtained at the time that the rats were killed using radioimmunoassay kits (Linco Research, St. Charles, MO).

**Tissue collection.** Rats were killed by ventricular exsanguination under isoflurane (Baxter Pharmaceuticals) anesthesia at 2 and 8 months of diabetes. Sciatic nerves or teased sciatic myelinated nerve fibers were snap-frozen in liquid nitrogen and stored at −80°C for extraction of protein or fixed in 4% paraformaldehyde overnight and processed into paraffin blocks for immunohistochemistry.

**Protein extraction and immunoblotting.** For each experiment, the two sciatic nerves from an individual animal were pooled for protein extraction. Protein extraction and Western blotting were performed according to methods described previously (33). Protein lysates (40 μg per lane) were resolved by SDS-PAGE under reducing conditions and transferred onto 0.45-μm polyvinylidene difluoride membranes (Millipore, Bedford, MA). Equal loading of protein was ascertained by Ponceau S-stain. The blots were blocked overnight at 4°C with Tris-buffered saline/0.1% (vol/vol) Tween 20 (TBS-T) containing 5% nonfat dry milk and incubated for 1 h at room temperature with the same blocking solution that contained the primary antibody. Antibody against Na+-channel α-subunit was from Upstate Biotechnology (Lake Placid, NY); N-cadherin and insulin receptor were from Santa Cruz Biotechnology (Santa Cruz, CA); Nr-Cam, L1, and contactin were from Transduction Lab (San Diego, CA); RPTP-β was from BD Bioscience (San Diego, CA); actin was from Chemicon International (Temecula, CA); ankyrin, γ was a gift from Dr. V. Bennett (Duke University, Durham, NC); caspr was provided by Dr. J.-A. Girault (INSERM, Collège de France, Paris, France); and Na+-channel β1- and β2-subunits were given to us by Dr. L.L. Isom (University of Michigan, Ann Arbor, MD). Blots were then incubated with the horseradish peroxidase–conjugated secondary antibodies (Santa Cruz) for 1 h, followed by washing three times with TBS-T at room temperature. The signals were detected using the ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ) and exposed to Kodak X-OMAT blue film. For assessment of protein expression, three to five separate experiments were performed. The immunoblots were quantified using a Fluor-S multi-imager densitometer (Bio-Rad, Hercules, CA).

**Immunocytochemistry.** Immunocytochemical localization was performed on either formalin-fixed 5-μm-thick deparaffinized sections using the avidin-biotin-peroxidase method with 3,3-diaminobenzidine (Sigma Chemical) as chromogen or immunofluorescent staining of frozen, permeabilized, and teased fiber preparations.

Deparaffinized sections were blocked in goat serum (1:10; Sigma Chemical) for 30 min, then the primary antibody was applied overnight at 4°C. Avidin-biotin-peroxidase kits (Vector Laboratories, Burlingame, CA) were used for immunohistochemical studies. After washing, the secondary antibody (diluted at 1:200 in TBS) was applied for 1 h at room temperature. Streptavidin–horseradish peroxidase was applied as per the manufacturer’s directions after washing and 3,3-diaminobenzidine was used as chromogen. The sections were then washed in TBS, counterstained with hematoxylin, dehydrated through graded ethanol, and mounted with Permount (Fisher Scientific, Pittsburgh, PA).

Immunofluorescent staining was performed on formalin-fixed and frozen sections. They were permeabilized for 15 min at 4°C in TBS with 0.5% Triton X-100, 0.2% Tween-20, and 10 mg/ml BSA and then washed three times at 4°C in TBS with 0.2% Tween-20 and incubated with the primary antibody in TBS containing 0.2% Tween-20 and 10 mg/ml BSA. The sections were washed, and donkey anti-mouse IgG conjugated to rhodamine (Jackson Immuno Research, West Grove, PA) was incubated at a 1:500 dilution for 2 h at 4°C. The sections were washed twice, once for 5 min. The sections were then washed once more, and streptavidin-conjugated Cy2 (Jackson Immuno Research) at a 1:100 dilution was applied for 2 h at 4°C. Sections were washed, mounted in Aqua Poly/Mount (Polysciences, Warrington, PA), and visualized on a Leica DML fluorescent microscope (Wetzlar, Germany) using Spot Imaging software version 3.1 (Spot Diagnostic Instruments, Sterling Heights, MI).

**In vitro studies.** Human neuroblastoma SH-SY5Y cells (American Type Culture Collection, Manassas, VA) were grown in a 1:1 mixture of Dulbecco’s modified Eagle medium and F12 Ham with 10% FCS at 37°C in a humidified
Four experiments in AAgarose was added and incubated for 1 h at 4 °C with aprotinin (0.1 mg/ml) with a total volume of 1 ml. Ten microliters of 50% Protein A: C-peptide was used (Schwarz Pharma AG, Monheim, Germany). Immunoprecipitation. Protein lysate (500 μg) was mixed with 5 μg of anti-GlcNAc antibody (Zymed, San Francisco, CA) or anti-caspr antibody and 500 μl of 2× immunoprecipitation (IP) buffer (2% Triton X-100, 0.32 mol/l NaCl, 40 mmol/l Tris [pH 7.5], 0.2 mmol/l EDTA, 2 mmol/l EGTA [pH 8.0], 2 mmol/l Na3VO4, 2 mmol/l phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 20 μg/ml aprozin) with a total volume of 1 ml. Ten microliters of 50% Protein A: Agarose was added and incubated for 1 h at 4 °C. In the case of anti-GlcNAc, 5 μg of rabbit anti-mouse IgG was added and incubated at 4 °C overnight. Samples were centrifuged for 8 min at 14,000 rpm at 4 °C. The supernatant was removed and washed three times in buffer containing 50 mmol/l Tris (pH 7.5), 150 mmol/l NaCl, 0.1% NP-40, 10% glycerol, and 1 mmol/l Na3VO4, then centrifuged at 14,000 rpm for 8 min at 4 °C. The pellet was resuspended in 10–15 μl of 2× Laemmli sample buffer and boiled for 5 min for SDS-PAGE. Immunoblotting was then performed with anti-ankyrinG, or anti-phosphatidylinositol 3-kinase p85 antibody (Santa Cruz Biotechnologies) as previously described (33).

Statistical methods. Results are presented as means ± SD. Significance of differences was analyzed by ANOVA, and group differences were assessed by the Newman-Keuls test. Differences was analyzed by ANOVA, and group differences were assessed by the Newman-Keuls test.

RESULTS

Glucose, HbA1c, insulin, and C-peptide levels and nerve conduction data. Diabetic animals were examined at 2 and 8 months of diabetes and after exposure to the same severity of hyperglycemia (Table 1). Body weights, blood glucose levels, HbA1c, daily insulin requirements, and plasma insulin levels in type 1 BB/Wor rats were not affected by C-peptide replacement (Table 1). In non–C-peptide–treated type 1 rats, MUNCV and SNCV were significantly decreased (P < 0.001) and significantly (P < 0.01 and < 0.001, respectively) but not completely (P < 0.01) prevented by C-peptide replacement (Table 1). C-peptide replacement completely normalized plasma C-peptide levels. Type 2 BBZDR/Wor rats were substantially heavier (P < 0.001) and hyperinsulinemic (P < 0.001) and showed the same high blood glucose and HbA1c levels as the two groups of BB/Wor rats (Table 1). Despite this, their nerve conduction defects were substantially milder (P > 0.01) and not significantly different from those in C-peptide–replaced BB/Wor rats (Table 1).

Western blotting. The expression of the insulin receptor was increased by 40% (P < 0.001) in BB/Wor rats and reduced by 38% (P < 0.01) in BBZDR/Wor rats, whereas rat II C-peptide–replaced type 1 rats showed normal expression of the insulin receptor (Fig. 2A). The expression of contactin, RPTP-β, ankyrinG, caspr, L1, and α- and β-Na+–channel subunits was not altered in any of the animal groups at 2 months of diabetes (data not shown). Nr-Cam and N-cadherin expression was increased (P < 0.01) in both type 1 and 2 diabetic rats and was not affected by C-peptide treatment at 2 months (Fig. 3). At 8 months, the protein expression of ankyrinG was decreased by 73% (P < 0.001) in type 1 rats and by 22% (P < 0.05) in type 2 rats (Fig. 2B). C-peptide replacement corrected the deficit in type 1 rats by 55% (P < 0.01), leaving a significant (P < 0.01) residual deficit compared with control rats (Fig. 2B). Nr-Cam and N-Cam showed increased protein expression in all diabetic groups compared with control rats at 8 months’ duration (P < 0.01), whereas L1 showed no change in any of the diabetic groups (Fig. 3). In type 1 rats, the protein expression of contactin was reduced by 50% (P < 0.001; Fig. 4A), β1-subunit was reduced by 12% (P < 0.01; Fig. 4B), and caspr was reduced by 40% (P < 0.01; Fig. 4C). The expression of the β2 Na+–channel subunit was reduced by 15% (P < 0.01; data not shown) compared with age- and sex-matched control rats. The expression of contactin was significantly (P < 0.05) but not fully (P < 0.05) prevented by C-peptide (Fig. 4A). The expression of the β3 Na+–channel subunit (Fig. 4B) and caspr (Fig. 4C) was fully prevented by C-peptide in type 1 and was not altered in type 2 rats. The decrease in β2 Na+–channel subunit expression was not corrected by C-peptide (data not shown). α-Na+–channel expression was not altered in incubator with 5% CO2 (33). They were serum-starved for 24 h and then exposed to 5 mmol/l glucose, 4 mmol/l insulin, and/or 3 mmol/l human C-peptide and examined after 2 h. For in vitro studies of this human cell line, human C-peptide was used (Schwarz Pharma AG, Monheim, Germany).

Immunoprecipitation. Protein lysate (500 μg) was mixed with 5 μg of anti-GlcNAc antibody (Zymed, San Francisco, CA) or anti-caspr antibody and 500 μl of 2× immunoprecipitation (IP) buffer (2% Triton X-100, 0.32 mol/l NaCl, 40 mmol/l Tris [pH 7.5], 0.2 mmol/l EDTA, 2 mmol/l EGTA [pH 8.0], 2 mmol/l Na3VO4, 2 mmol/l phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 20 μg/ml aprozin) with a total volume of 1 ml. Ten microliters of 50% Protein A: Agarose was added and incubated for 1 h at 4 °C. In the case of anti-GlcNAc, 5 μg of rabbit anti-mouse IgG was added and incubated at 4 °C overnight. Samples were centrifuged for 8 min at 14,000 rpm at 4 °C. The supernatant was removed and washed three times in buffer containing 50 mmol/l Tris (pH 7.5), 150 mmol/l NaCl, 0.1% NP-40, 10% glycerol, and 1 mmol/l Na3VO4, then centrifuged at 14,000 rpm for 8 min at 4 °C. The pellet was resuspended in 10–15 μl of 2× Laemmli sample buffer and boiled for 5 min for SDS-PAGE. Immunoblotting was then performed with anti-ankyrinG, or anti-phosphatidylinositol 3-kinase p85 antibody (Santa Cruz Biotechnologies) as previously described (33).
any of the groups at 2 or 8 months of diabetes (Fig. 5). The expression of the contactin ligand RPTP-β common to both the node and the paranode (Fig. 1) was reduced by 26% \((P < 0.001)\) in type 1 diabetic rats and overexpressed in C-peptide–replaced rats \((134%; P < 0.001)\) and in BBZDR/Wor rats \((138%; P < 0.001; \text{Fig. } 6A)\).

**Immunocytochemistry.** In type 1 BB/Wor rats at 8 months of diabetes, contactin was not dislodged beyond the domains of the node and paranode (Fig. 4A), whereas \(\beta_1\) \(\text{Na}^+\)-channel subunit (data not shown) subunits and caspr were displaced laterally from the paranodal apparatus (Fig. 4B and C). As demonstrated previously, the \(\alpha\)-\(\text{Na}^+\)-channel subunit is displaced into the internodal domain in chronically diabetic BB/Wor rats (26). None of these molecules showed a lateral displacement in C-peptide–treated BB/Wor rats, BBZDR/Wor rats, or control BB rats (Fig. 4).

**In vitro studies.** For testing whether insulin and/or C-peptide can posttranslationally modify ankyrin\(_G\) by mediating addition of O-GlcNAc moieties, IP was carried out in human neuroblastoma cells (SH-SY5Y) grown in 5% glucose. As shown in Fig. 6B, insulin and C-peptide mediate this activity differently. In cells that were treated with 3 nmol/l C-peptide, O-GlcNAc modification was comparable to that of control conditions but significantly \((P < 0.001)\) increased when exposed to 4 nmol/l insulin alone. This effect was abolished when cells were treated with both C-peptide and insulin. Conversely, phosphorylation of precipitated serine residues was unaffected by C-peptide and decreased by insulin alone \((P < 0.01)\), whereas

FIG. 4. Immunolocalization and protein expression of contactin (A), \(\beta_1\) \(\text{Na}^+\)-subunit (B), and caspr (C) in 8-month diabetic rats compared with age-matched controls. In control (a), BB/Wor (b), C-peptide–treated (c), and type 2 diabetic rats (d), all proteins were localized to the nodal and paranodal areas (arrows). In BB/Wor rats, \(\beta_1\) \(\text{Na}^+\)-channel subunit (B, b) and caspr (C, b) were displaced laterally, whereas contactin (A, b) was confined to the node and the now demyelinated paranode (arrowheads). The expression of contactin, \(\beta_1\) \(\text{Na}^+\)-channel subunit, and caspr showed a decrease in type 1 BB/Wor rats, which was prevented by C-peptide and did not occur in type 2 rats. Each bar represents the means \(\pm\) SD of three experiments in A and five in B and C. \(*P < 0.001; **P < 0.05\) vs. a; \(\dagger P < 0.01\) vs. a and d; \(\ddagger P < 0.05\) vs. c; \(\ddagger\ddagger P = 0.06\) vs. c; \(\# P < 0.001 \) vs. a, c, and d.

FIG. 5. \(\text{Na}^+\)-channel \(\alpha\) protein expression in sciatic nerve of 2-month (A) and 8-month (B) diabetic rats was not altered. a: Control rats. b: BB/Wor rats. c: C-peptide–treated BB/Wor rats. d: BBZDR/Wor rats. Each bar represents five experiments.
the combination of C-peptide and insulin resulted in a 70% ($P < 0.001$) increase in phosphorylation (Fig. 6C). IP for assessment of p85 binding to caspr showed no increase after exposure to either 4 nmol/l insulin or 3 nmol/l C-peptide alone but was maximal ($P < 0.01$) after exposure to both hormones (Fig. 6D).

**DISCUSSION**

The novelty of these results is twofold. One, increasingly, data that make the beneficial effects of C-peptide replenishment in type 1 diabetes irrefutable are accumulating (36). C-peptide exerts a beneficial effect on impaired nerve fiber regeneration in type 1 diabetes by normalizing early gene responses and expression of neurotrophic factors (33,37). It normalizes early metabolic abnormalities such as neural Na⁺/K⁺-ATPase activity and endoneurial endothelial nitric oxide synthase and corrects endoneurial blood flow (38–41), which underlie the early reversible “metabolic” nerve conduction defect (7). However, it does not affect oxidative stress in peripheral nerve (39). Two, here we demonstrate for the first time that the progressive nodal and paranodal structural changes, characteristic of both human and murine type 1 DPN (4,6,7), correlate with progressive decreases in the expression of the principal nodal and paranodal molecules and that these are correctable by C-peptide. The progressive nature of these abnormalities, as demonstrated here, are in keeping with a report by Brown et al. (10) in which they failed to demonstrate any changes of the node of Ranvier in BB/Wor rats with milder type 1 diabetes of 5 weeks’ duration or less. In addition, in vitro studies demonstrate that posttranslational modifications of paranodal caspr and nodal ankyrinG, necessary for protein-protein interaction, are optimized only in the presence of both insulin and C-peptide.

The abnormalities in the expression of caspr, contactin, RPTP-β, and the cell adhesive Na⁺-channel β-subunits are most likely caused by impaired insulin action in insulino-nepic type 1 BB/Wor rats, because they were not observed in isohyperglycemic but hyperinsulinemic BBZDR/Wor rats. This is further supported by the protective effects of insulinomimic C-peptide on these constituents. These perturbations in type 1 diabetes and the possible dysregulatory posttranslational modifications of caspr therefore are likely to underlie the progressive breach of the paranodal ion-channel barrier (“axogial dysjunction”) characterizing type 1 DPN, resulting in more severe functional defects compared with type 2 DPN and ultimately the more severe clinical picture in type 1 diabetes.

At the nodal apparatus, decreased expression of ankyrinG, contactin, RPTP-β, and the Na⁺-channel β-subunits and possible defective posttranslational modifications of ankyrinG are likely to dislodge the otherwise normally expressed α-Na⁺ channels laterally through the now breached paranodal barrier, as demonstrated previously (26). Furthermore, although not examined here, the L1 family of cell adhesive molecules serve as substrates for protein tyrosine kinases, and their phosphorylation abolishes ankyrinG binding (42). Whether the overexpression of some of these adhesive molecules under hyperglycemic conditions may contribute to the dislodgement of α-Na⁺ channels is not known.

The in vitro findings with regard to ankyrinG are in keeping with the “yin-yang” relationship between O-GlcNAc and phosphorylation (22). It is interesting that insulin alone exaggerated O-GlcNAc modifications with a reciprocal decrease in phosphorylated serine sites, whereas only the combination of insulin and C-peptide normalized this relationship. This suggests that in addition to increased extracellular levels of glucose (22), insulin in the absence of C-peptide is capable of increasing O-GlcNAc modifications and hence inhibiting serine phosphorylation. Because increased O-GlcNAc glycation of insulin receptor substrate 1 and 2 is believed to play a role in insulin...
resistance (22), one may speculate as to whether insulin treatment alone in type 1 diabetes could lead to progressive insulin resistance, which has been reported (43).

DPN is a chronic progressive disorder with shifting pathogenetic influences that produces increasingly severe degenerative changes, which are not present at the onset of the disorder (1,29,34,44). In view of the present results, one may argue that the decreased expression of several key nodal and paranodal molecules reflects the progressive loss of myelinated nerve fibers described in distal sural nerves of the BB/Wor rat. This amounts to 16% at 8 months of diabetes (7), which is substantially less than the reduction in the expression of most molecules examined.

To this should be added that the proximal sciatic nerve examined here is substantially less affected by myelinated fiber loss, as a result of its length dependence in DPN. From a functional viewpoint, these molecular abnormalities, allowing for lateral displacement of α-Na⁺ channels, are in keeping with the previously described irreversible decrease in maximum peak Na⁺ permeability and initial inward Na⁺ current in single-fiber recordings, resulting in impaired and potentially subthreshold membrane depolarization and conduction block (34).

The protective effects of C-peptide on the molecular integrity demonstrated in type 1 BB/Wor rats are most likely mediated via its synergistic effects on intermediaries of the insulin-signaling cascade (31,32). Although the high-affinity insulin receptor was compensatorily overexpressed in type 1 BB/Wor rats, this did not seem to be sufficient to compensate for the severe insulinopenia. The findings in type 2 BBZRD/Wor rats with sufficient circulating insulin and C-peptide levels are in keeping with this notion. Further support is provided by a previous report that showed that islet cell allotransplantation in type 1 streptozotocin-induced diabetic rats prevents axoglial dysjunction, whereas animals with graft rejection show the characteristic development of nodal and paranodal degenerative changes (45). How these insulin effects are mediated is not known but may reflect regulation of transcription factors for gene induction, as previously shown for neurotrophic factors (33,37), and by optimizing posttranslational modifications of key molecules only in the presence of both insulin and C-peptide, as suggested by the present in vitro studies.

By extrapolation, the present findings indicate that C-peptide may provide an efficacious, simple, safe, and cheap adjunct to insulin treatment in type 1 diabetic patients. Anogous to the rationale for insulin supplementation to control hyperglycemia, adjunct C-peptide replacement seems to be necessary for optimal prevention of DPN even under close to euglycemic conditions (7,46). This notion is in keeping with the results of the Diabetes Control and Complications Trial (47), showing that despite optimal glycemic control with insulin, DPN was prevented in only 57% of patients over 5 years, suggesting that close-to-normal glycemic control is not sufficient to fully prevent DPN from developing. These findings are in keeping with experimental data in type 1 diabetes showing that insulin treatment to euglycemic levels, without C-peptide replenishment, does not fully prevent DPN, including nodal and paranodal changes (6). Recent clinical reports support this contention by demonstrating improved somatic and autonomic nerve function in type 1 diabetic patients who received insulin plus C-peptide versus those who received insulin alone (48,49). For firmly establishing the beneficial clinical effects of C-peptide treatment on type 1 DPN, well-designed Food and Drug Administration–approved clinical trials are urgently needed.

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