The Effect of C-Peptide on Cognitive Dysfunction and Hippocampal Apoptosis in Type 1 Diabetic Rats

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Primary diabetic encephalopathy is a recently recognized late complication of diabetes resulting in a progressive decline in cognitive faculties. In the spontaneously type 1 diabetic BB/Wor rat, we recently demonstrated that cognitive impairment was associated with hippocampal apoptotic neuronal loss. Here, we demonstrate that replacement of proinsulin C-peptide in this insulinopenic model significantly prevented spatial learning and memory deficits and hippocampal neuronal loss. C-peptide replacement prevented oxidative stress–, endoplasmic reticulum–, nerve growth factor receptor p75–, and poly(ADP-ribose) polymerase–related apoptotic activities. It partially ameliorated apoptotic stresses mediated via impaired insulin and IGF activities. These findings were associated with the prevention of increased expression of Bax and active caspase 3 and the frequency of caspase 3–positive neurons. The results show that several partially interrelated apoptotic mechanisms are involved in primary encephalopathy and suggest that impaired insulinomimetic action by C-peptide plays a prominent role in cognitive dysfunction and hippocampal apoptosis in type 1 diabetes. Although these abnormalities were not fully prevented by C-peptide replacement, the findings suggest that this regime will substantially prevent cognitive decline in the type 1 diabetic population. *Diabetes* 54:1497–1505, 2005

Primary diabetic encephalopathy is recognized as a late complication of both type 1 and type 2 diabetes (1–3). Impairments in learning, memory, problem solving, and mental and motor speed are more common in type 1 diabetic patients than in the general population (4,5). A diabetes duration–dependent decline in cognitive function occurs independently of hypoglycemic episodes (6), and impaired intellectual and cognitive developments in type 1 diabetic children correlate with diagnosis at young age, male sex, and metabolic status (7).

Cognitive deficits (8,9) and poor performances in abstract reasoning and complex psychomotor functioning (10,11) occur in type 2 diabetes. Learning and memory dysfunctions are more prominent in elderly type 2 diabetic patients (9,10). It has not been determined whether this is because of potentiation of the normal aging process, a function of diabetes duration, or both. Notably, Alzheimer's disease is twice as prevalent in the diabetic population as in nondiabetic subjects (12,13). Several recent studies have implicated abnormal function of the insulin/IGF axis in the early pathogenesis of Alzheimer's disease (14,15). Insulin and IGF-1 are believed to regulate β-amyloid levels (14,16) and tau phosphorylation (16).

Impaired spatial learning and memory occur in animal models of both type 1 and type 2 diabetes (17–22). In the hippocampus of streptozotocin-induced diabetic rats, long-term potentiation is impaired, whereas long-term depression is enhanced (19,23,24), indicating altered hippocampal synaptic plasticity, which is associated with deficits in spatial learning and memory (19,24), which are corrected by insulin treatment (18,25).

In the type 1 BB/Wor rat, impaired spatial learning and memory, as assessed by the Morris water maze paradigm (26), occur with duration of diabetes (21). These changes are associated with impaired insulin and IGF-1 action and neuronal apoptotic stress in the hippocampus and frontal cortex, resulting in significant progressive neuronal losses in the CA_1 and CA_2 regions of the hippocampus (21). The hyperglycemia- and duration-matched type 2 BBZDR rat reveals, by comparison, a milder neuronal loss of the hippocampal CA_1 region (22). In a recent study, various apoptotic activities in these two models were compared. Fas/tumor necrosis factor receptor family and low-affinity nerve growth factor receptor (NGFR) p75 activation, indexes of oxidative stress, and endoplasmic reticulum dysfunction were identified in type 1 BB/Wor rats but not in type 2 BBZDR rats, whereas perturbations of the IGF system and poly(ADP-ribose) polymerase (PARP) activation were demonstrated in both models (22). These findings suggest that partially different apoptotic mechanisms are activated, underpinning hippocampal neuronal loss and cognitive dysfunction in the two types of diabetes, and that the combined apoptotic stress is greater in type 1 diabetes. To explore the relative contributions by hyperglycemia and impaired insulinomimetic effects by C-peptide deficiency, we examined the effects of proinsulin

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8-OHdG, 8-hydroxyl-2′-deoxyguanosine; AIF, apoptosis-inducing factor; NF-κB, nuclear factor-κB; NGF, nerve growth factor; NGFR, nerve growth factor receptor; PARP, poly(ADP-ribose) polymerase; PI-3, phosphatidyinositol-3; ROS, reactive oxygen species; TBS, tris-buffered saline; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling.

TABLE 1
Body weight, blood glucose, HbA_{1c}, plasma insulin, C-peptide, and IGF-1 levels in control, BB/Wor and C-peptide–replaced BB/Wor rats

	n	Body weight (g)	Glucose (mmol/l)	HbA_{1c} (%)	Plasma insulin (pmol/l)	Plasma C-peptide (ng/ml)	Plasma IGF-1 (ng/ml)
Control	10	501.2 ± 10.2	5.0 ± 0.2	3.1 ± 0.2	430.2 ± 20.1	732.8 ± 45.4	1,188.0 ± 31.8
BB/Wor rats	10	383.0 ± 6.5*	23.9 ± 1.3*	11.6 ± 0.7*	52.3 ± 5.8*	<25.0*	770.8 ± 84.5*
BB/Wor rats + C-peptide	10	382.0 ± 21.8*	22.7 ± 1.3*	12.7 ± 1.2*	40.0 ± 7.2*	710.3 ± 65.5	859.8 ± 50.1*

Data are means ± SD. *$P < 0.001$ vs. control rats.

C-peptide replacement on cognitive dysfunction and hippocampal apoptosis in type 1 BB/Wor rats. C-peptide, which is deficient in type 1 diabetes, signals through the insulin signaling pathway (27) but has no effect on hyperglycemia (28). In both human (rev. in 29) and animal (rev. in 30,31) diabetes, it has beneficial metabolic effects on endothelial and inducible nitric oxide synthase and Na^+/K^+ ATPase activities via mitogen-activated protein kinase–dependent transcription (32,33). In vitro and in vivo studies show gene-regulatory effects on the IGF and nerve growth factor (NGF) systems, and cell adhesive molecules mediated via c-fos, c-jun, and nuclear factor-κB (NF-κB) (28,34, rev. in 35). C-peptide facilitates posttranslational modifications of neuronal proteins via phosphorylation or phosphatidylinositol (PI)-3 kinase p85 adducts and inhibits O-linked *N*-acetylglucosamine glycation (28). Effects on neuronal apoptosis include corrective effects on the PI-3 kinase pathway and the bidirectional regulation of p38 mitogen-activated protein kinase and jun NH_2-terminal kinase and on NF-κB via disinhibition by phosphorylated inhibitor of κB, as well as PI-3 kinase–mediated phosphorylation of Bcl_2 (34). These effects are analogous to those induced by insulin and are enhanced synergistically by C-peptide at low insulin levels (36,37).

RESEARCH DESIGN AND METHODS

Pre-diabetic male BB/Wor rats and sex- and age-matched non–diabetes-prone BB rats were obtained from Biomedical Research Models (Worcester, MA). BB/Wor rats developed diabetes spontaneously at 71 ± 3 days of age. They were given small daily substitution doses (0.5–3.0 IU) of protamine zinc insulin (Blue Ridge Pharmaceuticals, Greensboro, NC) to maintain glucose levels at 20.0–25.0 mmol/l and to prevent ketoacidosis (38). Diabetic animals were kept in metabolic cages and monitored daily for body weight, urine volume, glucose, and ketones based on which daily insulin doses were titrated. Blood glucose levels were examined biweekly using glucose test strips (Bayer, Elkhart, IN). The animals had free access to rat diet and drinking water. In 10 diabetic BB/Wor rats, rat II C-peptide replacement doses (75 $mmol \cdot kg^{-1} \cdot day^{-1}$; Sigma Genosys, The Woodlands, TX) were delivered from onset of diabetes via subcutaneously implanted osmopumps (Alzet, Palo Alto, CA) (28,39). At the time of death (after 8 months of diabetes), blood samples were collected for measurements of blood glucose concentrations and HbA_{1c} levels, which were measured using a DCA 2000 Analyzer (Bayer). Plasma levels of insulin, IGF-1, and C-peptide were measured using radioimmunoassays (Linco Research, St. Charles, MO). The animals were cared for in accordance with institutional and National Institutes of Health guidelines (publication no. 85-23, 1995).

Tissue collection. Animals were anesthetized with isoflurane and decapitated. The whole brain was removed and placed on an ice-cooled cutting board. After the meninges were removed, the hemispheres from six animals per group were bisected and hippocampi were dissected, snap-frozen in liquid nitrogen, and stored at −70°C for purification of protein and DNA. For morphometric analyses, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining and immunocytochemical examinations, hippocampi from four animals per group were fixed in 4% paraformaldehyde (PBS buffered, pH 7.4), paraffin embedded, and 6-μm thick sections were prepared as previously described (21).

Morris water maze testing. Water maze testing was performed according to Morris (26) before killing the rats. Rats were placed in a circular pool of water (2.04 m in diameter × 0.40 m in height), in which a platform was hidden 3 cm beneath the water surface, 40 cm away from the edge of the pool. The water temperature was kept at 28°C. The pool area was arbitrarily divided into four quadrants (Q1–Q4). On 2 consecutive days each week for 2 weeks, rats were given three acquisition trials per day. The rats were placed at the periphery of the pool facing the wall of Q1–Q4. The distances to the platform were 148, 124, 84, and 52 cm for Q1–Q4, respectively. Rats were trained to locate the platform and allowed to stay on it for 30 s before being removed for the next trial. Three days after the final day of training, rats were tested and the time was measured in seconds for reaching the platform from the four starting points. Any rat that failed to find the platform within 100 s was taken out of the water and scored as 100.

Measurement of neuronal density in hippocampus. Hemotoxylin-eosin–stained serial paraffin sections were prepared from four hippocampi from individual animals in each group. Regions of hippocampus (CA_1–CA_4) were identified according to Paxinos and Watson (40). Images of 15, 6-μm thick sections 12 μm apart were analyzed using a morphometric analysis system interfaced with Image-Pro Plus 3.0 image analysis software (Media Cybermetics, Silver Spring, MD). Only neurons with identifiable nuclei were counted, and neuronal density was expressed as the number of neurons per millimeter squared.

Ligation-mediated PCR assay. Nucleosomal DNA ladder was detected by the ligation-mediated PCR assay method following the manufacturer's instruction (Clontech, Palo Alto, CA). For each assay, 0.5 μg genomic DNA from four hippocampi per group was extracted (21) and ligated to DNA adapters. The ligated DNA was used as templates for PCR amplification with the adapters as primers. The resultant nucleosomal ladders were visualized by 1.4% agarose gel electrophoresis. A primer set for human GAPDH cDNA (Clontech) was used for amplification of internal control: 5′-ACCACAGTCCATGCCATCAC and 5′-TCCACCACCCTGTTGCTGTA, which yielded a band of 452 bp.

TUNEL staining. TUNEL staining was performed as previously described (21). A Neuro TACS II apoptosis labeling kit (Trevigen, Gaithersburg, MD) was used for labeling apoptotic nuclei on 6-μm thick paraffin sections. Endogenous hydrogenase was quenched by 3% hydrogen peroxide. Biotinylated nucleotides were incorporated into the DNA breaks with TdT in the presence of dNTP mix and Mn^{++}. Incorporated biotinylated nucleotides were incu-

TABLE 2
Latencies in seconds from the four quadrants of the Morris water maze in control, BB/Wor, and C-peptide–replaced BB/Wor rats

		Quadrants and shortest distances to the platform (s)			
Animals	*n*	Q1 (148 cm)	Q2 (124 cm)	Q3 (84 cm)	Q4 (52 cm)
Control rats	10	78.2 ± 8.7	44.1 ± 6.7	26.1 ± 4.5	22.6 ± 5.6
BB/Wor rats	10	88.3 ± 3.1*	61.3 ± 5.9†	50.6 ± 6.2†	35.2 ± 5.6†
BB/Wor rats + C-peptide	10	81.4 ± 8.7‡	47.1 ± 6.5§	38.0 ± 9.3*‖	28.3 ± 5.2‡

Data are means ± SD. *$P < 0.01$, †$P < 0.001$ vs. control rats; ‡$P < 0.05$, §$P < 0.001$, ‖$P < 0.005$ vs. untreated BB/Wor rats.

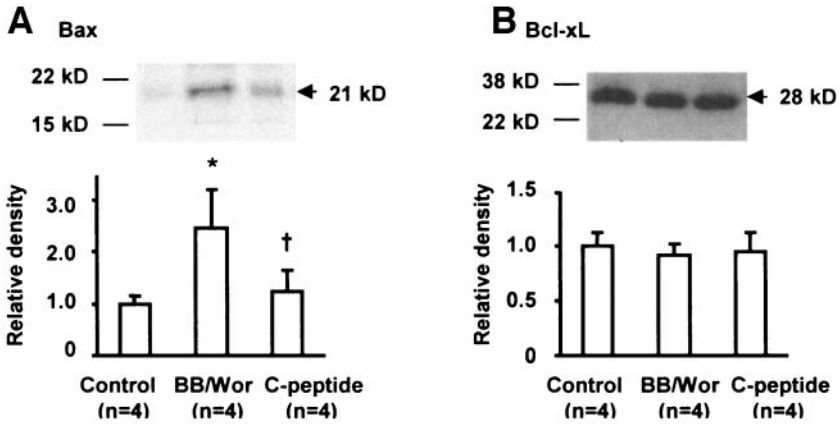

FIG. 1. The protein expression of Bax (*A*) and Bcl-xL (*B*) in hippocampi in four individual animals per group. Bax was significantly ($P < 0.01$) increased in BB/Wor rats. C-peptide replacement significantly ($P < 0.05$) prevented this increase. Bcl-xL was not altered in any of the diabetic groups. *$P < 0.01$ vs. controls; †$P < 0.05$ vs. BB/Wor rats.

bated with (strep)-avidin horseradish peroxidase, and 3,3′-diaminobenzidine tetrahydrochloride was used as chromogen. Positive controls were generated by using DNase-1 to "nick" the DNA, and for negative controls, TdT was omitted. The frequencies of TUNEL-positive nuclei were calculated in each of the four hippocampal sectors (CA_1–CA_4).

Immunoblotting. Dissected hippocampi from four animals per group were used. Protein lysates (40 μg per lane) were resolved by SDS-PAGE under reducing conditions and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were stained with 1% Ponseau S to control for equal loading of protein. The blots were incubated with blocking buffer, 0.1% Tween 20 and 5% nonfat milk (Bio-Rad, Hercules, CA), in Tris-buffered saline (TBS) (pH 7.4) and incubated with the same blocking buffer containing the primary antibody. The primary antibodies were mouse anti-Bax and anti–Bcl-xL antibodies (Trevigen); rabbit anti–active caspase 3, anti–caspase 12, and anti–NGFR p75 antibodies (Chemicon, Temecula, CA); rabbit anti–89 kDa-PARP antibody (Roche Diagnostics, Mannheim, Germany); mouse anti-Fas antibody (BD Biosciences, Lexington, KY); goat anti–IGF-1 and anti–IGF-2 antibodies; and rabbit anti–IGF-I receptor (IGF insulin receptor), anti–insulin receptor β-subunit, anti–NGF high-affinity receptor (NGFR-TrKA), and anti-NGF antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). After four washings, blots were incubated with the appropriate horseradish peroxidase–conjugated secondary antibody (Sigma Chemical, St. Louis, MO) and exposed to an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunocytochemistry. Hippocampi from four animals per group were examined. Deparaffinized 6-μm sections were incubated with the primary antibody for 30 min at room temperature, washed with three changes of TBS, and incubated with the peroxidase-conjugated secondary antibody for 30 min at room temperature. The immunoreactive products were visualized with 3,3′-diaminobenzidine tetrahydrochloride as chromogen. The primary antibodies used were rabbit anti-caspase 12 and anti–apoptosis-inducing factor (AIF) antibodies (Chemicon), mouse anti–8-hydroxyl-2′-deoxyguanosine (8-OHdG) antibody (Genox, Baltimore, MD), rabbit anti-PARP (Roche Diagnostic), and mouse anti-Fas antibody (BD Biosciences) sections were counterstained. The frequencies of Fas- and 8-OHdG–positive cells were obtained from each hippocampal sector.

In vitro studies. Human neuroblastoma cells (SH-SY5Y) were grown as previously described (34). Cells were serum starved for 1 day and then incubated with 5 or 50 mmol/l glucose with or without 3.0 nmol/l human C-peptide and/or 4 nmol/l insulin for 2 and 24 h. For Western blot analyses, protein was extracted as previously described (34). The blots were blocked overnight at 4°C with TBS saline and 0.1% (vol/vol) Tween 20 containing 5% nonfat dry milk. They were then incubated for 1 h at room temperature with blocking solution containing rabbit anti-PARP antibody (Roche Diagnostic), followed by incubation with a secondary antibody conjugated to horseradish peroxidase (Santa Cruz) for 1 h at room temperature. After washing three times in TBS Tween at room temperature, signals were detected by electrochemiluminescence (Amersham Pharmacia Biotech) and exposed to Kodak X-OMAT blue film. Four experiments were performed at each time point.

RESULTS

The clinical data are summarized in Table 1. Eight-month diabetic BB/Wor rats showed a 24% ($P < 0.001$) reduction in body weight. Blood glucose and HbA_{1c} levels were significantly (both $P < 0.001$) increased in untreated BB/Wor rats. C-peptide replacement had no effect on body weight, blood glucose, or HbA_{1c} values. Plasma insulin and C-peptide levels were severely depleted ($P < 0.001$) in BB/Wor rats. C-peptide replacement had no effect on plasma insulin but completely restored plasma C-peptide levels. Systemic IGF-1 levels were significantly ($P < 0.001$) decreased in BB/Wor rats with no C-peptide effects.

The effect of C-peptide on cognitive function. Morris water maze latencies in diabetic BB/Wor rats were significantly prolonged in all four quadrants (Table 2). In C-peptide–replaced BB/Wor rats, the latencies in quadrants Q1, Q2, and Q4 were not significantly different from control animals, whereas the latency in quadrant Q3 was improved ($P < 0.005$) but remained prolonged ($P < 0.01$) compared with control rats.

Hippocampal neuronal apoptosis, nucleosomal DNA laddering, and neuronal loss. No TUNEL-positive neu-

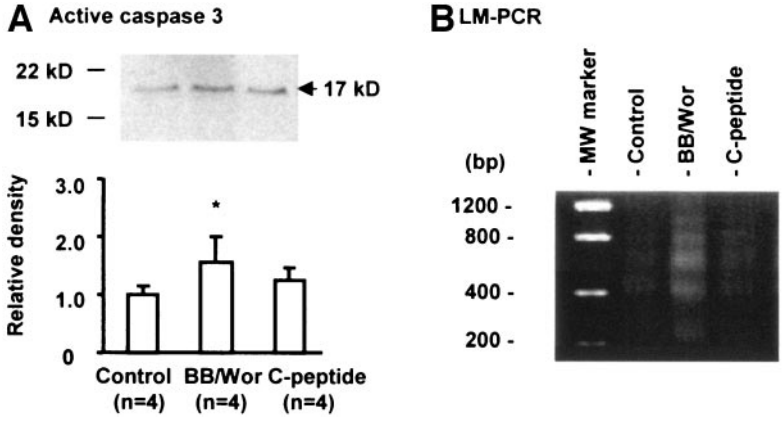

FIG. 2. *A*: The expression of active caspase 3 was significantly ($P < 0.05$) increased in BB/Wor rats but not in C-peptide–replaced BB/Wor rats. Data represent four experiments per animal group. *B*: DNA laddering is clearly visible in hippocampi of BB/Wor rats but not in those of control or C-peptide–replaced BB/Wor rats. *$P < 0.05$ vs. controls.

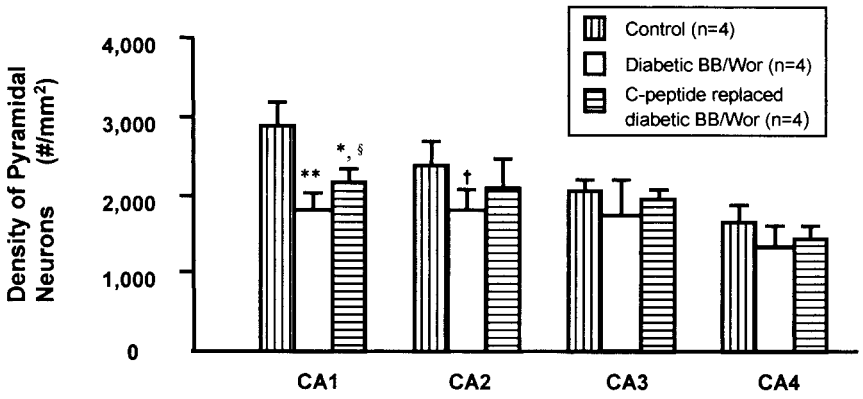

FIG. 3. Neuronal densities in CA_1–CA_4 in four hippocampi from each control, BB/Wor, and C-peptide–replaced BB/Wor rat. In CA_1 and CA_2 of BB/Wor rats neuronal densities were significantly ($P < 0.001$ and $P < 0.05$, respectively) decreased. C-peptide replacement resulted in a significant ($P < 0.05$) protection against neuronal loss in CA_1, which, however, was still significantly greater ($P < 0.01$) than in control rats. In CA_2, it was not significantly greater than in control rats. No significant differences existed between the groups in CA_3 and CA_4. **$P < 0.001$; *$P < 0.01$, †$P < 0.05$ vs. controls; §$P < 0.05$ vs. BB/Wor rats.

rons were detected in either hippocampal sector in control rats. TUNEL-positive neurons in untreated 8-month BB/Wor rats accounted for 3.9 ± 1.0% ($P < 0.001$) of CA_1 neurons and 0.6 ± 0.4% ($P < 0.05$) in CA_2, whereas CA_3 and CA_4 contained no TUNEL-positive neurons. In C-peptide–replaced rats, 0.9 ± 0.4% CA_1 neurons were TUNEL-positive ($P < 0.001$ vs. BB/Wor rats) and 0% were TUNEL-positive in CA_2–CA_4.

The proapoptoic protein Bax was increased 2.5-fold ($P < 0.01$) in hippocampi of untreated BB/Wor rats and by an insignificant amount (20%) in C-peptide–replaced BB/Wor rats. The Bax expression in the latter was significantly ($P < 0.05$) less than in non–C-peptide–replaced BB/Wor rats (Fig. 1*A*). Bcl-xL expression, on the other hand, was not altered in either experimental group (Fig. 1*B*).

Caspase 3 is activated via several apoptotic pathways, including perturbed tyrosine kinase signaling, Fas activation via caspase 8, mitochondrial and endoplasmic reticulum dysfunction, and caspase 12. The expression of active caspase 3 was increased by 50% ($P < 0.05$) in BB/Wor rats but was unchanged in C-peptide–replaced diabetic rats (Fig. 2*A*). These findings corresponded to nucleosomal DNA laddering in 8-month BB/Wor rats, which was not evident in control or C-peptide–replaced BB/Wor rats (Fig. 2*B*).

The densities of pyramidal cell neurons were significantly decreased in CA_1 ($P < 0.001$) and CA_2 ($P < 0.05$) but not in CA_3 and CA_4 in untreated BB/Wor rats (Fig. 3). C-peptide replacement resulted in a significant ($P < 0.05$) protection of neuronal loss in CA_1, which, however, was still significantly ($P < 0.01$) greater than in control rats. In CA_2, it was not significantly different from the density in control rats (Fig. 3).

The effect of C-peptide on tyrosine kinase receptors and their ligands. Expression of hippocampal insulin receptor was decreased by 63% ($P < 0.001$) in BB/Wor rats. C-peptide replacement partially prevented ($P < 0.05$) insulin receptor expression, which, however, remained less ($P < 0.05$) than in control rats (Fig. 4*A*). IGF insulin receptor expression was decreased by 50% ($P < 0.001$) in untreated BB/Wor rats and by 31% ($P < 0.01$) in C-peptide–replaced rats, resulting in a significant ($P < 0.05$) protection (Fig. 4*B*). Endogenous hippocampal IGF-1 and -2 were significantly ($P < 0.05$ and $P < 0.001$, respectively) decreased in BB/Wor rats. C-peptide replenishment prevented the decreased IGF-1 expression and partially decreased ($P < 0.05$) that of IGF-2 expression (Fig. 4*C* and *D*). Total endogenous hippocampal NGF was significantly ($P < 0.01$) decreased in BB/Wor rats but not in C-peptide–replaced rats (Fig. 5*A*). NGFR TrA expression was similarly decreased ($P < 0.05$) in BB/Wor rats but not in C-peptide–replaced rats (Fig. 5*B*).

The effect of C-peptide on Fas and NGFR p75 expression. NGFR p75 is the original member of the Fas/tumor necrosis factor (TNF) receptor family known for its role in apoptosis (41). The NGFR p75 expression was increased 2.2-fold ($P < 0.05$) in the hippocampi of BB/Wor rats. C-peptide replacement fully ($P < 0.05$) prevented this increase (Fig. 6*A*). The expression of Fas was significantly ($P < 0.05$) increased in BB/Wor rats and was not effected

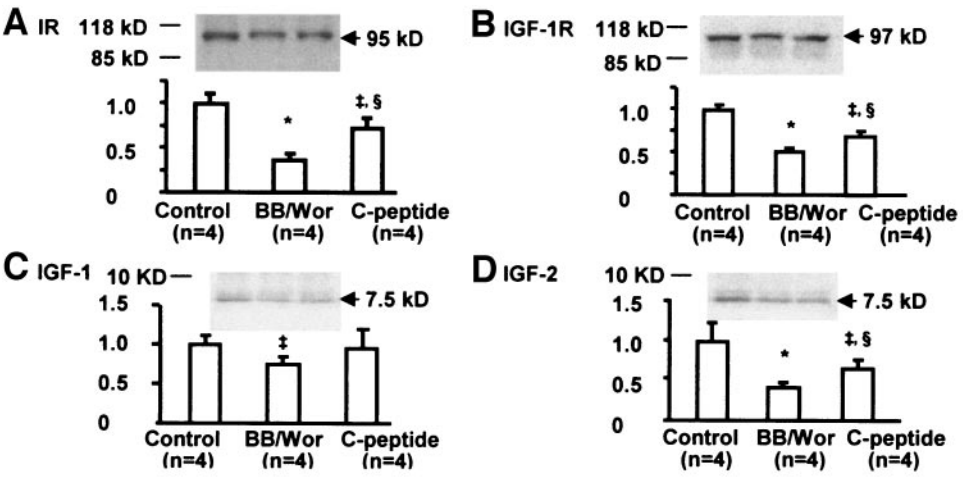

FIG. 4. Protein expression of insulin receptor (*A*), IGF insulin receptor (IGF-IR) (*B*), IGF-1 (*C*), and IGF-2 (*D*) in hippocampi from four animals per experimental group. Insulin receptor (*A*) was significantly ($P < 0.001$) decreased in BB/Wor rats. C-peptide replacement significantly prevented ($P < 0.05$) this decrease, which, however, was still significant ($P < 0.05$) compared with control rats. IGF-1 receptor (*B*) showed a similar decrease ($P < 0.005$) in BB/Wor rats that was partially prevented ($P < 0.05$) by C-peptide replacement. Endogenous IGF-1 (*C*) and IGF-2 (*D*) were both suppressed in hippocampi of BB/Wor rats ($P < 0.05$ and $P < 0.001$, respectively). IGF-1 was completely prevented by C-peptide replacement, whereas this effect was only partial ($P < 0.05$) with respect to IGF-2. *$P < 0.001$, **$P < 0.005$, ‡$P < 0.05$ vs. controls; §$P < 0.05$ vs. BB/Wor rats.

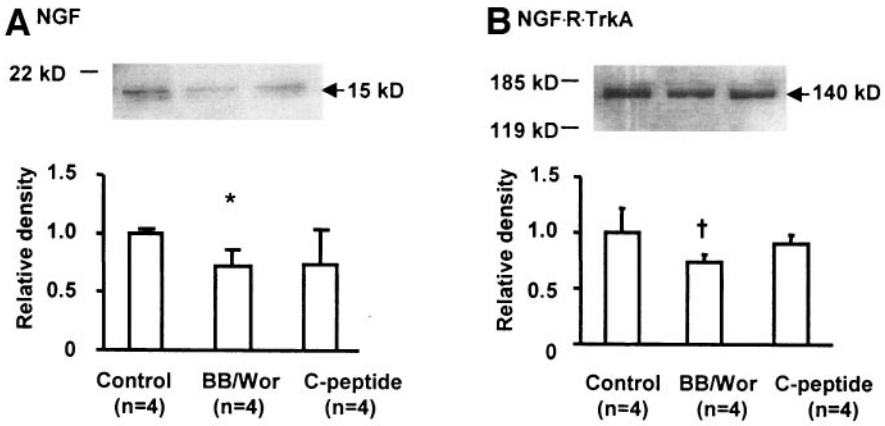

FIG. 5. Hippocampal expression of NGF (*A*) and NGFR tryosine receptor kinase A (TrkA) (*B*) in four control, BB/Wor- and C-peptide–replaced BB/Wor rats. NGF expression (*A*) was significantly ($P < 0.01$) suppressed in BB/Wor rats but not in C-peptide–replenished rats. The high affinity NGFR-tryosine receptor kinase A (*B*) was also suppressed ($P < 0.05$) in BB/Wor rats, which was prevented by C-peptide replacement. *$P < 0.01$, †$P < 0.05$ vs. control rats.

by C-peptide replacement (Fig. 6*B*). However, distinct Fas immunostaining was evident in 4.8 ± 1.1% ($P < 0.001$) of CA_1 neurons in BB/Wor rats and 0.4 ± 0.3% ($P < 0.001$ vs. BB/Wor rats) in C-peptide–replaced diabetic rats. No positive staining was detected in control rats (Fig. 6*C*).

The effect of C-peptide on PARP activities and oxidative stress–induced DNA damage. PARP was expressed in the nuclei of CA_1 hippocampal neurons in BB/Wor rats. This was markedly less in C-peptide–replaced rats and absent in control rats (Fig. 7*A*). The expression of cleaved PARP (89 kDa), a marker of apoptosis (42), was increased 4.3-fold ($P < 0.001$) in diabetic BB/Wor rats vs. 2.0-fold ($P < 0.05$) in C-peptide–replaced rats (Fig. 7*B*), which was significantly ($P < 0.05$) less than in BB/Wor rats.

8-OHdG is a specific marker for reduced oxygen species–induced DNA damage (43). In BB/Wor rats, nuclear 8-OHdG staining was present in 2.7 ± 1.1% ($P < 0.02$) of CA_1 neurons. No nuclear 8-OHdG staining was observed in control rats (Fig. 7*C*), and an insignificant 0.1 ± 0.0% was seen in C-peptide–replaced rats. Immunostaining of AIF showed occasional stippled cytosolic staining of control neurons. In BB/Wor rats, positive AIF staining was common within neuronal nuclei, whereas in C-peptide–replaced rats, AIF positivity was confined to the neuronal cytoplasm (data not shown).

Expression and localization of caspase 12. The expression of caspase 12 emanating from endoplasmic reticulum dysfunction was increased 2.4-fold ($P < 0.05$) in BB/Wor rats, which was prevented in C-peptide–replaced diabetic rats (Fig. 8*A*). This was associated with increased immunostaining of caspase 12 in nuclei of CA_1 neurons in BB/Wor rats. Similar staining was not detectable in control or C-peptide–replaced rats (Fig. 8*B*). Cleaved PARP expression in SH-SY5Y cells.

After 2 h exposure to 50 mmol/l glucose, SH-SY5Y cells showed an insignificant increase in the expression of cleaved PARP (89 kDa) (data not shown). Following 24 h exposure, there was an 80% ($P < 0.01$) increase in cleaved PARP compared with cells incubated in 5 mmol/l glucose (Fig. 9). C-peptide alone had no effect on the elevated expression of cleaved PARP, whereas 4 nmol/l insulin resulted in a significant ($P < 0.05$) protection against increased cleaved PARP expression, a protection which was maximal ($P < 0.01$) following treatment with 3 nmol/l C-peptide and 4 nmol/l insulin (Fig. 9).

DISCUSSION

Apoptosis is common in diabetes and degenerative central nervous system disorders. It is involved in β-cell loss in type 1 diabetes (44,45), occurs in diabetic retinopathy (46), and has been claimed (43,47,48) to be a prominent phenomenon in diabetic neuropathy, which, however, has not been reproduced by other investigators (49).

These discrepancies are due to an overreliance on TUNEL–staining as an indicator of apoptosis in dorsal root ganglion neurons exceeding 30% in streptozotocin-induced diabetic rats (43,47). Even the modest frequencies of TUNEL-positive neurons reported here are not likely to reflect true apoptotic cell death. Instead, additional supportive parameters like morphometric analyses, ligation-mediated PCR assays, and indicators of apoptotic stress

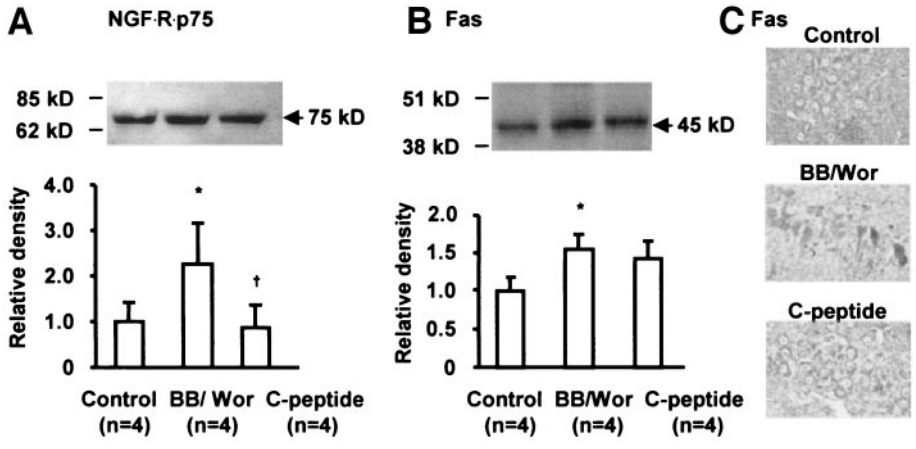

FIG. 6. The expression of NGFR P75 (*A*) and Fas (*B*) were both significantly ($P < 0.05$) increased in BB/Wor rat hippocampi. The increase in NGFR P75 (*A*) was fully ($P < 0.05$) prevented by C-peptide replacement. Fas expression was not effect by C-peptide replacement. Immunostaining of Fas (*C*) in CA_1 revealed occasional (4.8% of neurons) nuclear staining in BB/Wor rats. This was substantially ($P < 0.001$) less (0.4%) in C-peptide–replaced animals and was absent in control rats. Magnification ×150. *$P < 0.05$ vs. controls; †$P < 0.05$ vs. BB/Wor rats.

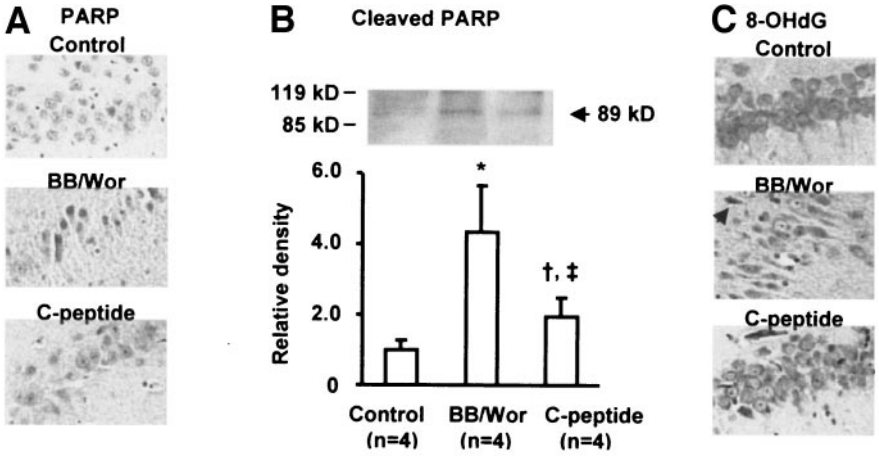

FIG. 7. Immunostaining of PARP (*A*) showed frequent nuclear immunoreactivity in CA_1 of BB/Wor rats. This was less common in CA_1 of C-peptide–replaced BB/Wor rats and was not observed in control rats. Magnification ×180. This corresponded to increased ($P < 0.001$) expression of cleaved PARP in BB/Wor rats, which was significantly ($P < 0.05$) but not fully ($P < 0.05$) prevented by C-peptide. Immunostaining of 8-OHdG (*C*) reflecting ROS-induced DNA damage was present in 2.7% ($P < 0.05$) of CA_1 neuronal nuclei in BB/Wor rats and to a significantly ($P < 0.05$; 0.1%) lesser extent in C-peptide–replaced rats. Magnification ×150. *$P < 0.001$, †$P < 0.05$ vs. control rats; ‡$P < 0.05$ vs. BB/Wor rats.

such as caspase 3 activity and Bax expression need to be considered to obtain an accurate assessment of apoptotic cell death, as outlined by Cheng and Zochodne (49).

We previously described a duration-related apoptotic neuronal loss in the hippocampus of type 1 BB/Wor rats (21) and type 2 BBZDR/Wor rats (22). Apoptosis in diabetes has been ascribed to hyperglycemia and oxidative stress (43,47,48), although the potential role of impaired insulin action has not been addressed (35,37).

Here we demonstrate that the replacement of insulinomimetic C-peptide protects against cognitive dysfunction and hippocampal neuronal loss by preventing several apoptotic stresses. These effects were achieved in the absence of an effect on hyperglycemia, suggesting that diabetic cognitive dysfunction is in part caused by impaired insulin/C-peptide action.

Insulin, proinsulin C-peptide, and IGF provide antiapoptotic effects (34,50). Originally, it was believed that insulin's apoptotic effects were mediated via the IGF-1 receptor, however, recent studies have revealed that insulin exerts its antiapoptotic effects through its own receptor via Raf-1–dependent signaling (50). This finding is consistent with data showing that the antiapoptotic effects of insulin and/or C-peptide are mediated via PI-3 kinase stimulation, p38 activation, disinhibition of inhibitor of κB, translocation of NF-κB, promotion of Bcl_2, and inhibition of jun NH_2-terminal kinase phosphorylation (21,34).

Inactivation of NF-κB will have consequences on gene regulation of the TNF receptor, Bcl_2, and Bcl-xL, which play important roles in apoptosis (34,51,52). In the present study, although C-peptide replacement had significant preventive effects on the suppressed endogenous expression of insulin receptor, IGF insulin receptor, and IGF-2, it did not provide complete protection. In isohyperglycemic and normo–C-peptidemic type 2 BBZDR/Wor rats (22), the endogenous hippocampal insulin receptor is normal, whereas IGF insulin receptor and IGF-2 are suppressed similarly to C-peptide–replaced animals. This suggests that hyperglycemia or associated perturbed lipid metabolism may play contributing roles.

Besides insulin (34), growth hormone and prostaglandin E_2 play regulatory roles on extrahepatic IGF synthesis (53). Prostaglandin E_2 levels are significantly suppressed in neural tissue of the BB/Wor rat (54), hence providing one plausible explanation. Although not known, possible insulin resistance in the central nervous system in type 2 diabetes could account for impaired IGF expression.

Oxidative stress is a major factor in diabetes-related apoptosis (43,48,55). A multitude of mechanisms lead to oxidative stress, such as mitochondrial dysfunction, endoplasmic reticulum stress-activating caspase 12, lipid peroxidation, and receptor for advanced glycation end products activation, as well as impaired glutathion and superoxide dismutase production. Reactive oxygen species (ROS) generation and lipid peroxidation is further facilitated by Fas/TNFβ–mediated increases in apoptotic stress (56,57). Oxidative stress leads to apoptosis via increased ceramide (58), hyperglutamatergic activity (59), or activation of AIF, perpetuating further mitochondrial

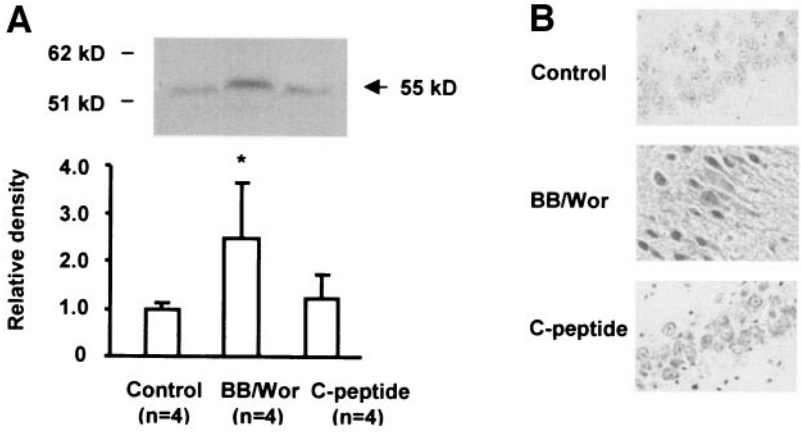

FIG. 8. The expression of hippocampal caspase 12 (*A*) was significantly ($P < 0.05$) increased in BB/Wor rats. This was prevented by C-peptide replacement. In CA_1, this corresponded to frequent immunostaining of caspase 12 (*B*) in neuronal nuclei, a staining pattern that was not obvious in control and C-peptide–replaced animals. Magnification ×150. *$P < 0.05$ vs. control rats.

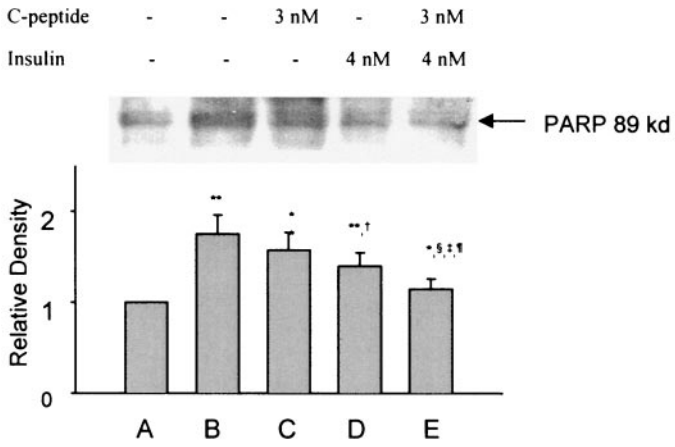

FIG. 9. Expression of cleaved PARP (89 kDa) indicative of apoptosis in human neuroblastoma (SH-SY5Y) cells incubated for 24 h in 5 mmol/l glucose (*A*); 50 mmol/l glucose (*B*); and 50 mmol/l glucose + 3 nmol/l C-peptide (*C*), + 4 nmol/l insulin (*D*), or + 3 nmol/l C-peptide and 4 nmol/l insulin (*E*). Fifty micromoles of glucose significantly increased ($P < 0.01$) 89-kDa PARP expression. This was not effected by C-peptide alone (*C*) but was significantly ($P < 0.05$) prevented by insulin alone (*D*) and maximally prevented ($P < 0.01$) by the combination of C-peptide and insulin (*E*). The data represent the mean ± SD of four experiments.*$P < 0.05$, **$P < 0.01$ vs. A; †$P < 0.05$, §$P < 0.01$ vs. B; ‡$P < 0.05$ vs. D; ¶$P < 0.01$ vs. C.

permeabilization (56, 60) and mediating caspase-independent apoptosis (60–62).

Here we demonstrate that C-peptide replacement prevents increased expression of caspase 12, indicative of endoplasmic reticulum dysfunction, nuclear stainability of 8-OHdG, reflecting oxidative stress-induced DNA damage, and nuclear staining of AIF. Therefore, C-peptide prevents mitochondrial and endoplasmic reticulum dysfunction and downstream oxidative stress–induced DNA damage. This is at odds with the lack of effects by C-peptide on peripheral nerve lipid peroxidation or antioxidant defense enzymes while correcting neurovascular deficits, thereby dissociating the latter from oxidative stress (63). NGF can block ROS induction and stabilize mitochondrial membrane potential (48). Here, C-peptide replacement prevented the decrease in endogenous NGF and NGFR-tryosine receptor kinase A expression. This is not unexpected, since insulin normalizes the expression of NGF (64) and is necessary for its binding to NGFR-tryosine receptor kinase A (65). Furthermore, the effect of C-peptide on NGFR p75 provides a further explanation, since activation of the Fas/TNF receptor family induces ROS via mitochondrial AIF release and lipid peroxidation (57,61,66,67). The beneficial effects on the NGF system therefore contribute to the positive effects on oxidative stress. Apart from the role of the Fas/TNF pathway activation in exacerbating oxidative stress, it perturbs jun NH_2-terminal kinase and p38, thereby fueling the apoptogenic effects caused by aberrations of the PI-3 kinase pathway (68).

PARP is activated by DNA strand breaks and plays a role in DNA repair and in apoptosis (69). Apoptosis induces cleavage of the 113-kDa PARP into 89- and 24-kDa fragments. The presence of cleaved PARP is therefore an indication of apoptosis. PARP participates in caspase-independent apoptosis via the mitochondrial-derived AIF pathway (69). The prevention of 89-kDa PARP expression and nuclear presence of PARP in C-peptide–replaced BB/Wor rats suggest an effect on PARP-mediated caspase-independent apoptosis. These findings were confirmed in human neuroblastoma cells exposed to high glucose, in which C-peptide together with insulin maximized the protective effect on the expression of cleaved PARP.

In summary, C-peptide replacement in type 1 diabetic rats prevents the progressive cognitive dysfunction by preventing apoptosis-induced neuronal loss in the hippocampus and potentially in other brain regions. Several apoptotic mechanisms are involved in this process. C-peptide significantly prevented NGFR P75–mediated apoptosis and oxidative stress-induced apoptotic activity, as well as PARP and AIF-related caspase-independent apoptotic stresses. It had partial but significant effects on PI-3 kinase pathway–related apoptotic mechanisms. These effects were reflected in significant prevention of proapoptotic Bax and active caspase 3 expression. Therefore, the present data demonstrate that impaired insulinomimetic action by C-peptide plays a prominent role in primary diabetic encephalopathy. This is further supported by data from the age-, duration-, and hyperglycemia-matched type 2 diabetic BBZDR/Wor rat. This model shows normal C-peptide levels, significantly milder neuronal loss, and a spectrum of apoptotic activities (22) similar to those reported here in C-peptide–replaced type 1 BB/Wor rats. As this type 2 model becomes increasingly insulin and C-peptide deficient, it shows an acceleration in the development of diabetic polyneuropathy (37). One may speculate that a similar worsening of primary diabetic encephalopathy may occur as they become increasingly insulin and C-peptide deficient, potentially explaining the higher incidence of cognitive deficits in older patients with presumably longer duration of possibly insulin-deficient type 2 diabetes (9,10).

In conclusion, the present data show that impaired C-peptide activity in insulinopenic type 1 diabetes plays a prominent role in primary diabetic encephalopathy. They suggest that proinsulin C-peptide replacement in type 1 diabetic patients is likely to have a protective effect on diabetes duration–related cognitive deficits.

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