Down-Regulation of Brain-Pancreas Relative Protein in Diabetic Rats and by High Glucose in PC12 Cells: Prevention by Calpain Inhibitors

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Abstract. Brain-pancreas relative protein (BPRP) is a novel protein that we found in our laboratory. Previously we demonstrated that it is involved in ischemia and depression. In light of the putative association between diabetes and clinical depression, and the selective expression of BPRP in brain and pancreas, the present study examined whether BPRP levels are affected by induction of diabetes by alloxan injection in rats and exposure to high glucose levels in PC12 cells. Western blot and immunohistochemical analyses revealed that BPRP levels were decreased in the hippocampal CA1 neurons of diabetic rats 4 and 8 weeks post-alloxan injection and in PC12 cells 48 h after exposure to high concentrations of glucose. BPRP protein levels were not affected by osmolarity control treatments with mannitol. Follow-up pharmacological experiments in PC12 cells revealed that glucose-induced BPRP down-regulation was markedly attenuated by the calpain inhibitors N-acetyl-Leu-Leu-norleucinal (ALLN) or calpeptin, but not the proteasome-specific inhibitor carbobenzoxy-Leu-Leu-leucinal (MG132). The ability of calpain inhibitors to specifically counter the effects of high glucose exposure on BPRP levels further suggests that BPRP and calpain activity may contribute to diabetes complications in the central nervous system.

Keywords: brain-pancreas relative protein, diabetes, high glucose, proteasome inhibitor, calpeptin

Introduction

In previous studies we found a marked reduction of a 260-kDa protein in the neurons of ischemic brain following experimental occlusion of the middle cerebral artery in rats (1, 2). Using its polyclonal antibody, we found the protein primarily localized in hippocampal neurons of the brain and islets cells of the pancreas, with lesser amounts being found in podocytes of the renal glomeruli and endothelial cells of venous sinuses in the spleen. For that reason, we named this protein “brain-pancreas relative protein (BPRP)” (3). BPRP was subsequently analyzed by peptide mass fingerprinting (PMF) and matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS). Those results showed that BPRP was a novel protein with a sequence similar to protein kinase A anchoring 6 (AKAP6) and microtubule-associated protein 1A (MAP1A) (2), both of which are cytoskeletal components involved in regulation of neurons. While the localization of BPRP in two widely separate areas of the body strongly suggests it may have some function in these tissues, its physiological and pathophysiological significance needs to be clarified.

Diabetes, an increasingly common metabolic disease, can lead to a number of different complications, such as retinopathy, nephropathy, and peripheral neuropathy (4). Accumulated evidence has showed that it is also associated with gradual development of end-organ damage in the central nervous system, referred to as
“diabetic encephalopathy” (5, 6). Clinically, it is characterized by cognitive impairment, electrophysiological and structural changes, and depression or anxiety disorder (7); the underlying mechanisms remain to be elucidated. In a previous study, we observed that chronic mild stressors decreased the expression of BPRP accompanied by an increase of blood glucose and a decrease of insulin (3). Based on the putative association between diabetes and clinical depression (8), in this study, we asked whether the alteration of BPRP is also involved in the duration of diabetes.

In the present study, we used an animal model of type 1 diabetes and PC12 cells, which have the capacity for high expression of BPRP (9), to evaluate the effect of diabetes or high glucose on the expression of BPRP. For analysis of the molecular mechanism involved, several proteasome inhibitors were used.

### Materials and Methods

#### Experimental animals

Male Sprague-Dawley (SD) rats of initial weight between 180 and 190 g (Animals obtained from animal Center of the Chinese Academy of Medical Sciences) were used in this study. The rats were housed five per cage under standard rat colony conditions, with a 12-h light/12-h dark cycle and provided food and water ad libitum. Diabetes was induced by an intravenous injection of alloxan [Beijing Xizhong Chemical Plant (Beijing, China), 50 mg/kg body weight] freshly dissolved in 0.9% saline. Control rats were injected with saline alone. Seventy two hours after the alloxan or saline injections, blood glucose was measured and animals with fasting blood glucose above 200 mg/dl were considered diabetic. Both control groups and diabetic animals were maintained for 4 or 8 weeks duration. Animals were weighed daily, serum blood glucose was determined weekly using a diagnostic kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and plasma insulin was assayed every 2 weeks and maintained in a humidified atmosphere of 5% CO₂ at 37°C. The medium was changed every other day and cells were plated at an appropriate density according to each experimental scale. The concentration of glucose in DMEM was 25 mM. To determine the effect of high glucose on BPRP expression, PC12 cells were grown in low glucose (25 mM) or high glucose (75 mM) for various time intervals.

#### Cell culture

PC12 cells were plated on poly-L-lysine-coated petri dishes and grown in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO, Grand Island, NY, USA) supplemented with 10% horse serum, 5% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin and maintained in a humidified atmosphere of 5% CO₂ at 37°C. The medium was changed every other day and cells were plated at an appropriate density according to each experimental scale. The concentration of glucose in DMEM was 25 mM. To determine the effect of high glucose on BPRP expression, PC12 cells were grown in low glucose (25 mM) or high glucose (75 mM) for various time intervals.

#### Immunofluorescence and confocal imaging

Cells plated on glass bottom dishes (MatTek Corp, Ashland, MA, USA) were washed with PBS (pH 7.4), fixed with 4% paraformaldehyde for 15 min, and permeated with 0.5% Triton X-100 for 10 min at room temperature. Nonspecific binding sites in the cells were blocked with 5% normal goat serum for 30 min and incubated overnight at 4°C with the anti-rat BPRP antibody (1:100 dilution). The following day, the cells were washed with PBS and incubated for 1 h with the tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min in the...
dark at 37°C. For nuclear staining, cells were incubated for 5 min at room temperature with Hoechst (Sigma, St. Louis, MO, USA). The immunofluorescent signal was observed using a Leica TCS SP2 confocal microscope (Leica Co., Wetzlar, Germany).

**Protein extraction and Western blot**

Western blot analysis for BPRP protein was performed as previously described (1, 2). Briefly, the brain tissues were suspended in a solution containing 0.32 M sucrose, 0.3 mM phenylmethylsulfonyl fluoride, 20 mM Tris-HCl, pH 7.4, and homogenized in a Teflon-glass homogenizer. The homogenates were centrifuged at 4°C for 25 min at 800 × g, and the supernatant was collected and centrifuged at 4°C for 1.5 h at 16,000 × g. For cell studies, after incubation with glucose, cells were lysed with high salt lysis buffer [1% Triton X-100, 50 mM Tris-HCl (pH 8.0), 0.25 M NaCl, 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride]. The supernatant was collected following centrifugation at 12,000 × g for 10 min. Protein was quantified by the Bradford method, and equal amounts of protein were analyzed by electrophoresis on a 7.5% SDS-PAGE transferred onto polyvinylidene difluoride (PVDF) immobilion-P membranes of 0.45-µm pore size (Millipore Corp., Bedford, MA, USA). Membranes were incubated at 4°C overnight with the rabbit polyclonal antibody against BPRP (1:1000 dilution) or mouse monoclonal antibody against α-(1:1000 dilution) or mouse monoclonal antibody against β-tubulin (1:1000 dilution; Invitrogen Corp, Carlsbad, CA, USA), and immunoreactivity was visualized with alkaline phosphatase conjugated to the appropriate secondary antibody and bromochloroindolyl phosphate p-toluidine salt (BCIP)/nitroblue tetrazolium (NBT). The staining intensity of the bands was determined by densitometric image analysis with Quantity One software version 4.4.0 (Bio-Rad, Hercules, CA, USA).

**Cytotoxicity assay**

Cells were seeded at a concentration of 1 × 10^5 cells/ml in triplicate on 96-well microtiter plates containing various concentrations of glucose, and then the plates were incubated at 37°C for 24, 48, and 72 h. Following incubation, 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (5 mg/ml) (Sigma) was then added to each well, and plates were incubated at 37°C for 4 h. At the end of incubation period, all culture media were replaced with 100 µl of DMSO and the plates were left in the dark for 3 h at room temperature. The optical density (OD) of the wells was measured at 570 nm with background subtracted at 630 nm.

**Statistical analyses**

Data were expressed as means ± S.E.M. The significance of differences was evaluated with one-way ANOVA followed by the Student-Newman-Keuls test. A probability level of P<0.05 was considered statistically significant.

**Results**

**Confirmation of diabetes in the experimental animals**

Alloxan administration established a definitive diabetic state (10, 11). Diabetes resulted in a significant increase in blood glucose and decrease in blood insulin levels as determined at 4 and 8 weeks. These findings are expected for the alloxan model of diabetes and verified the presence of diabetes in our experimental animals. Diabetes also resulted in a highly significant impairment of body weight gain, decrease in overall brain weight, and an increase in the brain weight/body weight ratio. These results are presented in Table 1.

**Diabetic animals demonstrate lower levels of BPRP expression in the hippocampal CA1 neurons**

Western blot analysis showed a significant decrease of BPRP protein levels in the brain of diabetic rats, compared to the tissue of control groups (Fig. 1). By 4 weeks duration of diabetes, BPRP levels in the diabetic rats were 32.88 ± 8.02% of control values. By 8 weeks duration of diabetes, BPRP levels were 23.95 ± 11.72% of control values for the brain. Pervious study has shown that BPRP primarily localized in the cytoplasm of neurons and the cytoplasm and dendrites of pyramidal cells. As shown in Fig. 2, after 4 weeks of diabetes duration, the immunoreactivity of BPRP was greatly reduced in the hippocampal CA1 neurons which are particularly sensitive to changes in blood-glucose concentrations (12).

**High glucose results in a decrease in BPRP in PC12 cells**

The optimum glucose concentration for PC12 cells is 25 mM. Therefore, 50 and 75 mM glucose were chosen as the high experimental concentrations of glucose to evaluate the effect of various glucose concentrations on BPRP expression (13). PC12 cells were incubated with 50 or 75 mM glucose for various time intervals after reaching confluence and whole-cell lysates were analyzed by Western blotting. As shown in Fig. 3A, BPRP showed a slight decrease at 48 h and declined significantly after 72 h of incubation with high glucose, as compared to control cells (cells incubated in 25 mM glucose).

In addition, the MTT assay was carried out to monitor
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After 24, 48, and 72 h of incubation with high glucose, the MTT assay revealed that 75 mM glucose did not significantly decrease cell viability compared with controls (data not shown), which indicates that high concentration of glucose had no apparent influence on cell viability.

To distinguish the effect of glucose from that of hyperosmolarity, we also examined the effect of mannitol at the same osmolarity as glucose on BPRP protein levels in PC12. As shown in Fig. 3B, neither 25 nor 50 mM mannitol (with 25 mM control glucose-total osmolarity made equal to that of 50 mM or 75 mM glucose) inhibited the BPRP protein levels, suggesting that high-glucose–induced inhibition of BPRP protein levels was not simply associated with osmotic alteration. Immunofluorescence analyses showed high glucose exposure induced the decrease of BPRP fluorescence in the cytoplasm in PC12 cells (Fig. 4).

Insulin has no obvious effect on BPRP expression levels

Insulin plays important roles in the regulation of brain metabolism and neurotrophism. It appears to have neuroprotective effects, and disturbances in insulin and its receptor could adversely affect cognitive function (14). To examine whether insulin could directly cause BPRP overexpression in PC12 cells in vitro, we incubated PC12 cells with 1 – 100 nM insulin for 48 h. BPRP immunoreactivity levels in the medium remained the same during studies of all periods as compared to those in nontreated PC12 cells (data not shown).

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Table 1. General characteristics of diabetic and control rats

<table>
<thead>
<tr>
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<th>Control</th>
<th>Diabetic</th>
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<tr>
<td>Body weight (g)</td>
<td></td>
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<tr>
<td>4 wks post-injection</td>
<td>318 ± 8</td>
<td>226 ± 14***</td>
</tr>
<tr>
<td>8 wks post-injection</td>
<td>401 ± 13</td>
<td>227 ± 15***</td>
</tr>
<tr>
<td>Blood glucose levels (mg/dl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 wks post-injection</td>
<td>55.3 ± 5.7</td>
<td>365.2 ± 20.4***</td>
</tr>
<tr>
<td>8 wks post-injection</td>
<td>59.6 ± 6.6</td>
<td>475.4 ± 39.4***</td>
</tr>
<tr>
<td>Blood insulin levels (µIU/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 wks post-injection</td>
<td>20.01 ± 6.03</td>
<td>12.09 ± 1.71</td>
</tr>
<tr>
<td>8 wks post-injection</td>
<td>24.18 ± 7.13</td>
<td>9.15 ± 0.48*</td>
</tr>
<tr>
<td>Brain weight (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 wks post-injection</td>
<td>1.92 ± 0.08</td>
<td>1.87 ± 0.05*</td>
</tr>
<tr>
<td>8 wks post-injection</td>
<td>2.30 ± 0.07</td>
<td>1.71 ± 0.07**</td>
</tr>
<tr>
<td>Brain weight / body weight</td>
<td></td>
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<tr>
<td>4 wks post-injection</td>
<td>0.0061 ± 0.0004</td>
<td>0.0084 ± 0.0006**</td>
</tr>
<tr>
<td>8 wks post-injection</td>
<td>0.0057 ± 0.0001</td>
<td>0.0076 ± 0.0006*</td>
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Diabetic status induced by a single dose injection of alloxan (50 mg/kg, i.v.) in rats. Values are each the mean ± S.E.M. (Control: n = 5, Diabetic: n = 5 for each time period; *P<0.05, **P<0.01, ***P<0.001, compared with untreated rats).

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Fig. 1. Effect of diabetes on BPRP levels in rat brain at 4 and 8 weeks duration of diabetes. Rats were treated with a single dose of alloxan at 50 mg/kg, i.v. The protein contents of brain BPRP and α-tubulin were determined by Western blotting. Immunoblots of representative samples are shown; statistical data are shown as histograms in the bottom panels. Data are expressed as the mean ± S.E.M. and are shown as a percentage of the control (n = 5 for each time period. *P<0.05, vs untreated rats).

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the toxicity of high glucose. After 24, 48, and 72 h of incubation with high glucose, the MTT assay revealed that 75 mM glucose did not significantly decrease cell viability compared with controls (data not shown), which indicates that high concentration of glucose had no apparent influence on cell viability.
To examine whether high glucose causes BPRP down-regulation at the protein or mRNA level, cycloheximide (CHX), a reversible inhibitor of protein synthesis, was used. As shown in Fig. 5, BPRP degraded in PC12 cells with a half-life of about 30 h. When cells were incubated with both high glucose and CHX, BPRP protein level was markedly decreased compared
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ALLN, but not MG132, prevented the degradation of BPRP induced by high glucose

To investigate the mechanisms whereby high glucose leads to the decrease of BPRP, we evaluated the participation of the proteasome on BPRP degradation at high glucose concentration by treating PC12 cells with MG132 and ALLN, which are proteasome inhibitors. As shown in Fig. 6A, ALLN, an inhibitor of the proteasome and a partial inhibitor of the calpain protease, was able to inhibit the degradation of BPRP induced by high glucose to a significant extent. In contrast, MG132, a specific proteasome inhibitor, was ineffective in increasing BPRP levels. Furthermore, 500 nM ALLN could time-dependently abolish the effects of high glucose on BPRP (Fig. 6B), while 500 nM MG132 failed to prevent the high-glucose–induced loss of BPRP (Fig. 6C). These data show that the high-glucose–induced degradation of BPRP is most likely not mediated by proteasome.

Calpain inhibitor prevented the degradation of BPRP induced by high glucose

To confirm whether the activation of calpain is
attributed to the high-glucose–induced decline of BPRP, a specific inhibitor for calpain (calpeptin) was used. Calpeptin, unlike ALLN, does not inhibit proteasome function (15). Calpeptin interacts with the active site of calpain and is much more selective for inhibition of calpain compared to ALLN. As shown in Fig. 7, to a significant extent, calpeptin exerted a preventive influence against the loss of BPRP induced by high glucose.

**Discussion**

In this present study, we used an animal model of diabetes that was induced by alloxan injection and the PC12 cell line mimicking hyperglycemia when exposed to high glucose in order to clarify the alteration of BPRP in diabetes, which is a novel protein identified in previous studies by our group (1, 2). The animal studies showed that alloxan-induced diabetes resulted in a significant increase in blood glucose and decrease in BPRP levels in the hippocampal CA1 neurons at both 4 and 8 weeks duration of diabetes. The in vitro studies showed that treatment of PC12 cells with various concentrations of glucose significantly decreased BPRP levels in a dose-dependent and time-dependent manner, which indicates that the BPRP may be a glucose-sensitive protein. In addition, the activation of calpain may be involved in high-glucose–induced degradation of BPRP.

CNS complications of diabetes are accompanied by neurophysiological, structural and cognitive changes in the brain (16, 17). Hippocampus neurons mediate some of the elemental cognitive processes (18). Studies have showed that in the animal models of diabetes, neuronal apoptosis occurs in the hippocampus accompanying by the functional cognitive impairments, particularly in CA1 (19). However, the markers of apoptosis, such as DNA fragmentation, positive TUNEL staining, and elevated Bax, became evident in the 8 months diabetic BB/W rats. Diabetes evoked the decrease of BPRP in the hippocampus neurons especially in CA1 at both 1 and 2 months, suggesting the loss of BPRP in the hippocampus occurs before the neuronal apoptosis.

The multifactorial pathogenesis of diabetic cerebral dysfunction is not yet completely defined. Those factors can be divided into three main aspects: direct effects of hyperglycaemia, vascular changes (20, 21), alterations in neurotrophic support (22), and changes of insulin and its receptor in the brain. The present study revealed that the loss of BPRP in PC12 cells was accompanied by increased levels of glucose, but this effect was not observed when mannitol was substituted for glucose. Insulin could not affect the protein levels of BPRP. These results suggest that the decreased levels of BPRP induced by high glucose might be due to the direct effect of hyperglycemia. We previously found that the brain BPRP was not affected in stressed animals accompanied by elevated blood glucose (3). Because the blood glucose level of stressed animals (147.4 ± 13.5 mg/dl) is much lower compared to diabetic ones (365.2 ± 20.4 mg/dl), this would explain the discrepancy.

Protein degradation induced by the ubiquitin-proteasome system/pathway, specific proteases (calcium-dependent proteases calpains), or lysosomal proteases (cathepsins B, H, L) contributes to protein levels decrease (23–25). MG132, a specific inhibitor of proteasome, failed to prevent the high-glucose–induced loss of BPRP. ALLN, an inhibitor of the proteasome and a partial inhibitor of the calpain protease, could time-dependently abolish the effects of high glucose on BPRP (data not shown). Calpeptin, a much more selective inhibitor of calpain, as compared to ALLN (15), showed a protective effect against the down-regulation of BPRP mediated by high glucose. All these data suggest that the degradation of BPRP is likely due to the activation of calpain.

Calpain is a family of cytoplasmic cysteine proteases...
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Activated by calcium ions. It has been implicated in a large number of physiological processes and pathological conditions, including susceptibility to diabetes (26, 27). Increased calpain activity has been associated with neuronal degeneration caused by traumatic events such as excitotoxicity and hypoxia/ischemia (28, 29). The calpain inhibitor was shown to correct penile nitrergic nerve deficit in diabetic mice (30). Numerous cytoskeletal proteins, including tau, microtubule-associated protein 2 (MAP2), neurofilaments, and spectrin, have been shown to be excellent calpain substrates (31–33); cleavage of cytoskeletal proteins leads to programmed cell death. As previously reported, BPRP was shown to have 46% matched peptide sequence with AKAP6, which is an anchor protein (2), suggesting that BPRP may be another substrate of calpain. The degradation of BPRP might act as a biomarker of protease activation and neuron damage in certain pathological situations such as diabetes or high-glucose–induced brain and neuron damages as we observed in this study.

In conclusion, the current research suggests that under the pathological conditions of diabetes or high glucose, the degradation of BPRP is induced, and inhibition of calpain reverses the glucose-induced reduction of BPRP. Under such condition there is the intriguing possibility that the high glucose could activate the calpain, one of the proteases, followed by decreased levels of BPRP.
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