J Pharmacol Sci **106**, 28 – 37 (2008)

Journal of Pharmacological Sciences

Full Paper

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

Lu Tie[1], Ying Xu[1], Yan-Hua Lin[1], Xiao-Hao Yao[1], Hong-Li Wu[1], Yu-Hua Li[1], Zhu-Fang Shen[2], He-Ming Yu[3], and Xue-Jun Li[1,*]

[1]*Department of Pharmacology, School of Basic Medical Sciences and State Key Laboratory of Natural & Biomimetic Drugs, Peking University, Beijing 100083, China*
[2]*Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, 1 Xiannongtan Street, Beijing 100050, China*
[3]*National Research Institute for Family Planning, Beijing 100081, China*

Received July 7, 2007; Accepted October 31, 2007

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

*Corresponding author. xjli@bjmu.edu.cn
Published online in J-STAGE
doi: 10.1254/jphs.FP0071092

"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 with a radioimmunoassay kit (China Atomic Energy Research Institute, Beijing, China). The experimental procedures were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Immunohistochemistry

Rats were anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg body weight) followed by perfusion fixation and the brains were removed. Coronal slices of 3-mm thickness were cut and fixed with 4% formaldehyde in 0.1 M phosphate buffer (PB, pH 7.3) for 24 h. The slice posterior to the infundibular stalk was embedded in paraffin. Coronal sections (6 μm) including the hippocampus were prepared and then mounted onto 3-aminopropyltriethoxysilane (APES)–coated slides. Paraffin sections were deparaffinized with xylene and rehydrated in a graded series of ethanol. Slides were submerged in 3% hydrogen peroxide to quench any endogenous peroxidase activity; washed with distilled water; heated at 95°C – 98°C in 0.01 M citrate buffer, pH 6.0, for 15 min; then cooled at ambient temperature for 40 min; and then washed with PBS. An aliquot of 10% non-immune goat serum was applied to eliminate nonspecific staining. Sections were incubated overnight at 4°C with anti-rat BPRP antibody (1:1000 dilution). The sections were washed with PBS and incubated with biotinylated goat anti-rabbit IgG antibodies for 40 min, rewashed with PBS and incubated with peroxidase-conjugated streptavidin for 40 min. The peroxidation activity was visualized by incubating the sections with a peroxidase substrate solution after sufficient washing. The sections were counterstained with hematoxylin and mounted. Appropriately diluted solutions of non-immune rabbit serum were used as controls in the immunohistochemical localization of the BPRP.

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% CO_2 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 isothiocynate (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-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

Table 1. General characteristics of diabetic and control rats

	Control	Diabetic
Body weight (g)		
4 wks post-injection	318 ± 8	226 ± 14***
8 wks post-injection	401 ± 13	227 ± 15***
Blood glucose levels (mg/dl)		
4 wks post-injection	55.3 ± 5.7	365.2 ± 20.4***
8 wks post-injection	59.6 ± 6.6	475.4 ± 39.4***
Blood insulin levels (μIU/ml)		
4 wks post-injection	20.01 ± 6.03	12.09 ± 1.71
8 wks post-injection	24.18 ± 7.13	9.15 ± 0.48*
Brain weight (g)		
4 wks post-injection	1.92 ± 0.08	1.87 ± 0.05*
8 wks post-injection	2.30 ± 0.07	1.71 ± 0.07**
Brain weight / body weight		
4 wks post-injection	0.0061 ± 0.0004	0.0084 ± 0.0006**
8 wks post-injection	0.0057 ± 0.0001	0.0076 ± 0.0006*

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).

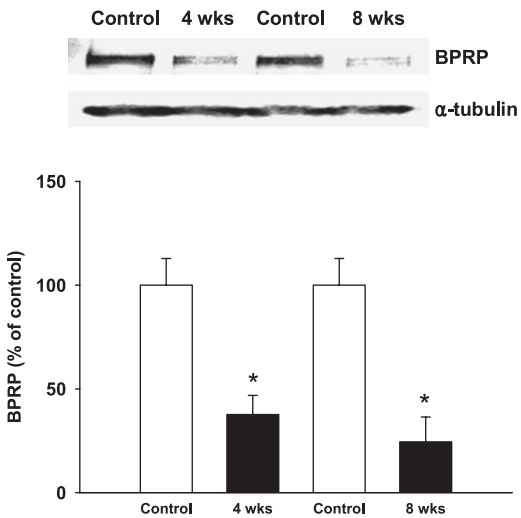

Fig. 1. Effect of diabetes on BPRP levels in rat brain at 4 and 8 weeks duration of diabetes. Rats were treated with a single dose of alloxan at 50 mg/kg, i.v. The protein contents of brain BPRP and α-tubulin were determined by Western blotting. Immunoblots of representative samples are shown; statistical 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).

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 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).

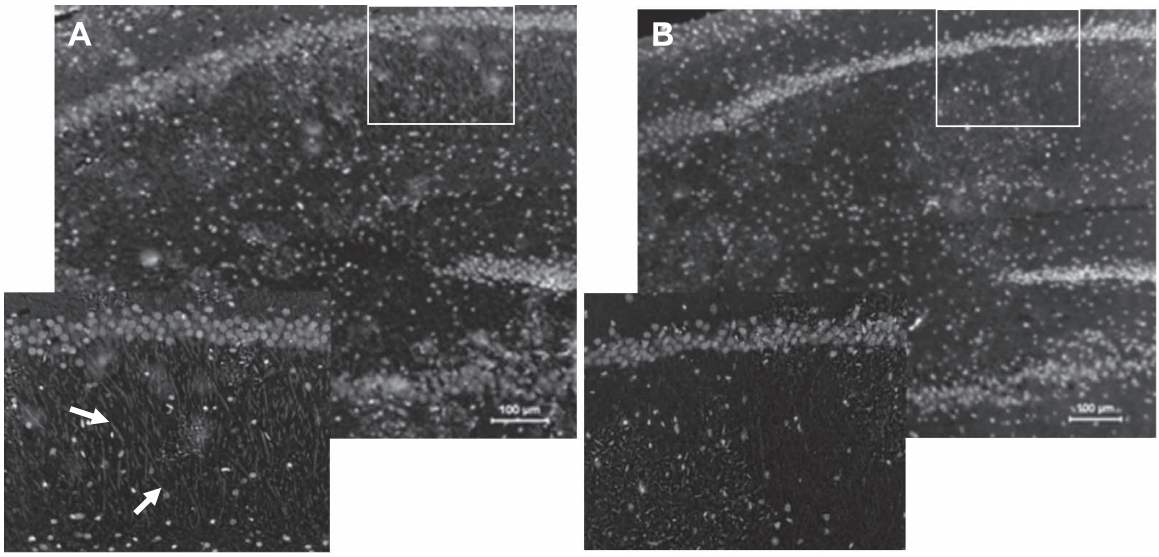

Fig. 2. Immunohistochemistry of BPRP in hippocampus of control (A) and diabetic rats at 8 weeks of diabetes duration (B). Representative sections are shown with arrows indicating typical immunoreactive BPRP; the gray stripes in the figure represented the positive immunoreactive BPRP. In the panel below, the general view of hippocampi at high (100×) magnifications of the CA1 pyramidal cell body layers are presented. Notice that the diabetes resulted in significant decrease of BPRP immunoreactivity in the hippocampus CA1. Bar indicates 100 μm.

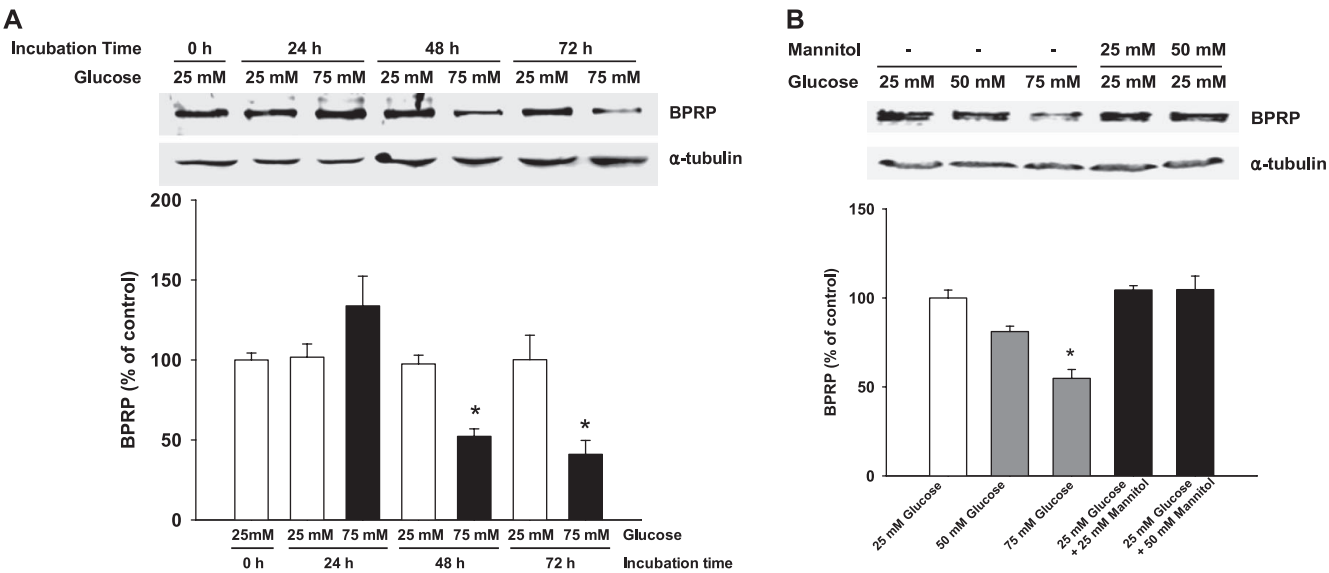

Fig. 3. Effect of high glucose on BPRP protein levels in PC12 cells. A: PC12 cells were incubated in medium containing 25 mM glucose (low glucose) or 75 mM glucose (high glucose) for 24, 48, and 72 h; then they were harvested and analyzed by Western blotting. B: PC12 cells were incubated with various concentrations of glucose or mannitol for 48 h; then they were harvested and analyzed by Western blotting. Immunoblots of representative samples are shown; statistical data are shown as histograms in the bottom panels. "Control" refers to cells treated with 25 mM glucose. Data are each expressed as the mean ± S.E.M. and are shown as a percentage of the control, **P*<0.05, *vs* the cells incubated with 25 mM glucose.

High glucose decreased half-life of BPRP in PC12 cells

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

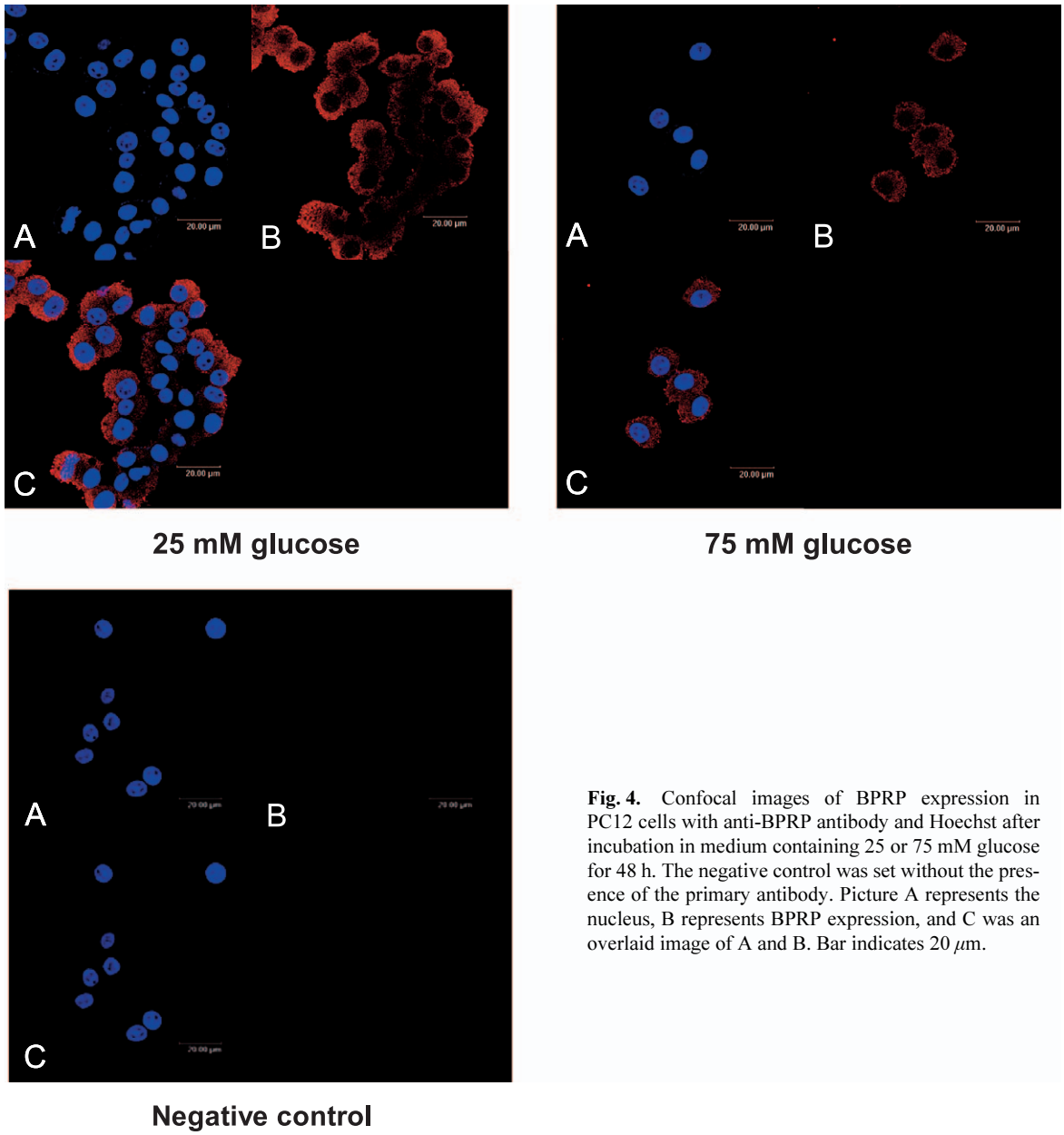

Fig. 4. Confocal images of BPRP expression in PC12 cells with anti-BPRP antibody and Hoechst after incubation in medium containing 25 or 75 mM glucose for 48 h. The negative control was set without the presence of the primary antibody. Picture A represents the nucleus, B represents BPRP expression, and C was an overlaid image of A and B. Bar indicates 20 μm.

with that in cells treated with CHX alone. These data demonstrated high glucose induced down-regulation of BPRP was at the protein level.

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

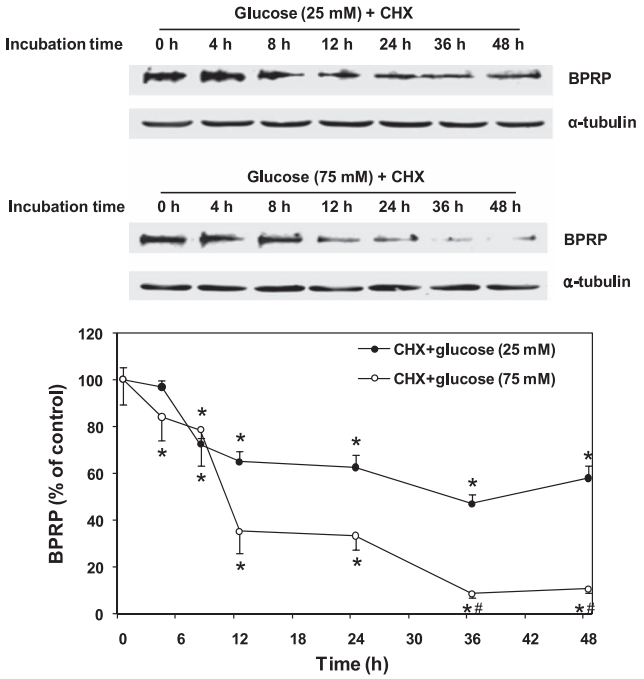

Fig. 5. Effect of high glucose and CHX on BPRP protein levels in PC12 cells. PC12 cells were incubated in medium containing 10 μg/ml CHX and 25 or 75 mM glucose for various time intervals, and then cells were harvested and analyzed by Western blotting. Immunoblots of representative samples are shown; statistical data are shown as line diagrams in the bottom panels. "Control" refers to cells at the 0 h time point. Data are each expressed as the mean ± S.E.M. and are shown as a percentage of the control. *P<0.05, *vs* the cells at the 0 h time point; $^{\#}P$<0.05, *vs* the cells incubated with 10 μg/ml CHX and 25 mM glucose.

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

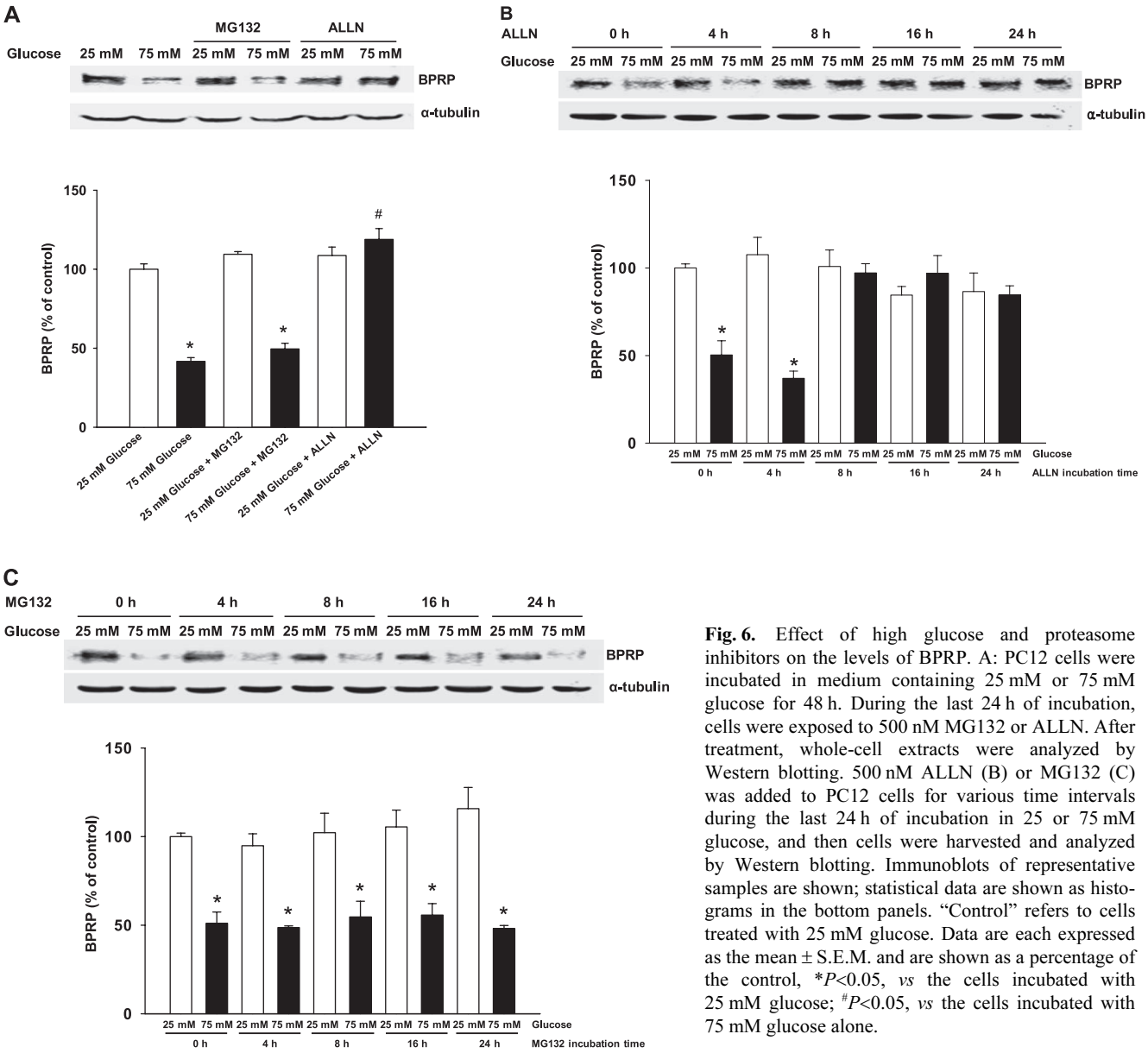

Fig. 6. Effect of high glucose and proteasome inhibitors on the levels of BPRP. A: PC12 cells were incubated in medium containing 25 mM or 75 mM glucose for 48 h. During the last 24 h of incubation, cells were exposed to 500 nM MG132 or ALLN. After treatment, whole-cell extracts were analyzed by Western blotting. 500 nM ALLN (B) or MG132 (C) was added to PC12 cells for various time intervals during the last 24 h of incubation in 25 or 75 mM glucose, and then cells were harvested and analyzed by Western blotting. Immunoblots of representative samples are shown; statistical data are shown as histograms in the bottom panels. "Control" refers to cells treated with 25 mM glucose. Data are each expressed as the mean ± S.E.M. and are shown as a percentage of the control, **P*<0.05, *vs* the cells incubated with 25 mM glucose; #*P*<0.05, *vs* the cells incubated with 75 mM glucose alone.

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.

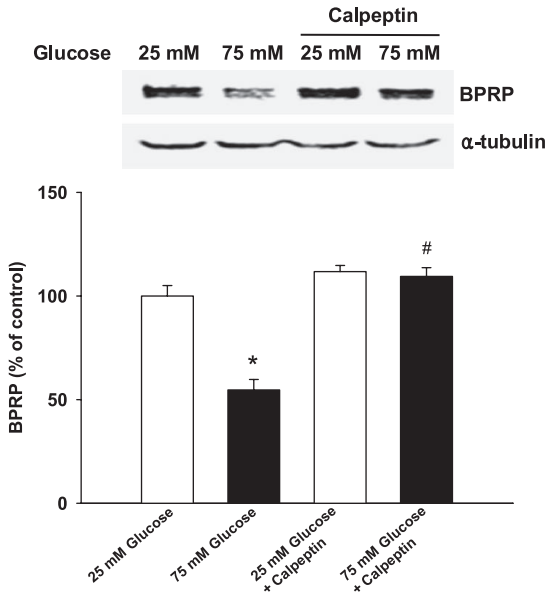

Fig. 7. Effect of high glucose and a calpain inhibitor on the levels of BPRP. PC12 cells were incubated in medium containing 25 or 75 mM glucose for 48 h. During the last 24 h of incubation, cells were exposed to 500 nM calpeptin. After the treatment, whole-cell extracts were analyzed by Western blotting. Immunoblots of representative samples are shown; statistical data are shown as histograms in the bottom panels. "Control" refers to cells treated with 25 mM glucose. Data are expressed as the mean ± S.E.M. and are shown as a percentage of the control, $*P<0.05$, *vs* the cells incubated with 25 mM glucose; $^{\#}P<0.05$, *vs* the cells incubated with 75 mM glucose alone.

Acknowledgments

The authors thank Prof. Michael Allen McNutt for suggestions to improve the quality of this manuscript. This work was supported by the National Nature Science Foundation of China (No. 30270528), 973 Program of the Ministry of Science and Technology (No. 2004CB518902), research fund from Ministry of Education of China No. 20020001082, and 985 Program from Ministry of Education of China.

References

1 Lu A, Yu H, Chen K, Koide SS, Li X. Alteration in brain proteins following occlusion of the middle cerebral artery in rat. Life Sci. 1999;65:493–500.
2 Yao XH, Yu HM, Koide SS, Li XJ. Identification of a key protein associated with cerebral ischemia. Brain Res. 2003;967: 11–18.
3 Lin YH, Liu AH, Xu Y, Tie L, Yu HM, Li XJ. Effect of chronic unpredictable mild stress on brain-pancreas relative protein in rat brain and pancreas. Behav Brain Res. 2005;165:63–71.
4 Association AD. Standards of medical care for patients with diabetes mellitus. Diabetes Care. 2002:25:213–219.
5 Pirart J. [Diabetes mellitus and its degenerative complications: a prospective study of 4,400 patients observed between 1947 and 1973 (3rd and last part) (author's transl)]. Diabete Metab. 1977;3:245–256. (in French)
6 Reske-Nielsen E, Lundbaek K, Rafaelsen OJ. Pathological changes in the central and peripheral nervous system of young long-term diabetics I. Diabetic encephalopathy. Diabetologia. 1966:1:233–241.
7 Kramer L, Fasching P, Madl C, Schneider B, Damjancic P, Waldhausl W, et al. Previous episodes of hypoglycemic coma are not associated with permanent cognitive brain dysfunction in IDDM patients on intensive insulin treatment. Diabetes. 1998;47:1909–1914.
8 Jacobson AM, Samson JA, Weinger K, Ryan CM. Diabetes, the brain, and behavior: is there a biological mechanism underlying the association between diabetes and depression? Int Rev Neurobiol. 2002;51:455–479.
9 Lin YH, Liu AH, Wu HL, Westenbroek C, Song QL, Yu HM, et al. Salvianolic acid B, an antioxidant from Salvia miltiorrhiza, prevents Abeta(25-35)-induced reduction in BPRP in PC12 cells. Biochem Biophys Res Commun. 2006;348:593–599.
10 Szkudelski T. The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. Physiol Res. 2001;50:537–546.
11 Di Giulio AM, Lesma E, Gorio A. Diabetic neuropathy in the rat: 1. Alcar augments the reduced levels and axoplasmic transport of substance P. J Neurosci Res. 1995;40:414–419.
12 Reagan LP. Neuronal insulin signal transduction mechanisms in diabetes phenotypes. Neurobiol Aging. 2005;26 Suppl 1:56–59.
13 Koshimura K, Tanaka J, Murakami Y, Kato Y. Involvement of nitric oxide in glucose toxicity on differentiated PC12 cells: prevention of glucose toxicity by tetrahydrobiopterin, a cofactor for nitric oxide synthase. Neurosci Res. 2002;43:31–38.
14 Sima AA, Kamiya H, Li ZG. Insulin, C-peptide, hyperglycemia, and central nervous system complications in diabetes. Eur J Pharmacol. 2004;490:187–197.
15 Katagiri K, Yokosawa H, Kinashi T, Kawashima S, Irie S, Tanaka K, et al. Ubiquitin-proteasome system is involved in induction of LFA-1/ICAM-1-dependent adhesion of HL-60 cells. J Leukoc Biol. 1999;65:778–785.
16 Gispen WH, Biessels GJ. Cognition and synaptic plasticity in diabetes mellitus. Trends Neurosci. 2000;23:542–549.
17 Brands AM, Kessels RP, de Haan EH, Kappelle LJ, Biessels GJ. Cerebral dysfunction in type 1 diabetes: effects of insulin, vascular risk factors and blood-glucose levels. Eur J Pharmacol. 2004;490:159–168.
18 Eichenbaum H. Hippocampus: cognitive processes and neural representations that underlie declarative memory. Neuron. 2004;44:109–120.
19 Li ZG, Zhang W, Grunberger G, Sima AA. Hippocampal neuronal apoptosis in type 1 diabetes. Brain Res. 2002;946:221–231.
20 Jakobsen J, Nedergaard M, Aarslew-Jensen M, Diemer NH. Regional brain glucose metabolism and blood flow in streptozocin-induced diabetic rats. Diabetes. 1990;39:437–440.
21 Mankovsky BN, Metzger BE, Molitch ME, Biller J. Cerebrovascular disorders in patients with diabetes mellitus. J Diabetes Complications. 1996;10:228–242.
22 Wuarin L, Namdev R, Burns JG, Fei ZJ, Ishii DN. Brain insulin-like growth factor-II mRNA content is reduced in insulin-

dependent and non-insulin-dependent diabetes mellitus. J Neurochem. 1996;67:742–751.
23 Donohue TM Jr, Osna NA. Intracellular proteolytic systems in alcohol-induced tissue injury. Alcohol Res Health. 2003;27: 317–324.
24 Chondrogianni N, Fragoulis EG, Gonos ES. Protein degradation during aging: the lysosome-, the calpain- and the proteasome-dependent cellular proteolytic systems. Biogerontology. 2002;3: 121–123.
25 Goldberg AL. Protein degradation and protection against misfolded or damaged proteins. Nature. 2003;426:895–899.
26 Sorimachi H, Suzuki K. The structure of calpain. J Biochem (Tokyo). 2001;129:653–664.
27 Huang Y, Wang KK. The calpain family and human disease. Trends Mol Med. 2001;7:355–362.
28 Kampfl A, Posmantur R, Nixon R, Grynspan F, Zhao X, Liu SJ, et al. mu-calpain activation and calpain-mediated cytoskeletal proteolysis following traumatic brain injury. J Neurochem. 1996;67:1575–1583.
29 Yamashima T, Saido TC, Takita M, Miyazawa A, Yamano J, Miyakawa A, et al. Transient brain ischaemia provokes Ca^{2+}, PIP2 and calpain responses prior to delayed neuronal death in monkeys. Eur J Neurosci. 1996;8:1932–1944.
30 Nangle MR, Cotter MA, Cameron NE. The calpain inhibitor, A-705253, corrects penile nitrergic nerve dysfunction in diabetic mice. Eur J Pharmacol. 2006;538:148–153.
31 Johnson GV, Jope RS, Binder LI. Proteolysis of tau by calpain. Biochem Biophys Res Commun. 1989;163:1505–1511.
32 Nixon RA, Brown BA, Marotta CA. Limited proteolytic modification of a neurofilament protein involves a proteinase activated by endogenous levels of calcium. Brain Res. 1983; 275:384–388.
33 Siman R, Baudry M, Lynch G. Brain fodrin: substrate for calpain I, an endogenous calcium-activated protease. Proc Natl Acad Sci U S A. 1984;81:3572–3576.