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Effects of Glucose, Insulin, and Supernatant from Pancreatic β -cells on Brain–Pancreas Relative Protein in Rat Hippocampus

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Abstract Brain–pancreas relative protein (BPRP) is a novel protein that mainly expresses in brain and pancreas. In our previous study, we found that various stressors significantly decreased the expression of BPRP in pancreas *in vivo*, accompanied by changes in insulin and glucose levels, and that expression of BPRP in pancreas also decreased significantly in diabetic rats induced by Streptozocin (STZ). All these findings suggest that BPRP may be a glucose or insulin-sensitive protein. However, how the changes in insulin or glucose levels influence the expression of BPRP in hippocampus requires further study. Here, we investigated the effects of insulin or glucose on the expression of BPRP in primary cultured hippocampal neurons. We supplied hippocampal neurons with glucose, insulin, or supernatant from pancreatic β -cells, which

secrete insulin into the supernatant. Our data showed that insulin had beneficial effect on the viability while no significant effect on the expression of BPRP in hippocampal neurons. On the contrary, 40 mM glucose or free glucose culture significantly decreased the expression of BPRP, while had no significant effect on the viability and apoptosis of hippocampal neurons. Further study showed that levels of insulin in the supernatant collected from pancreatic β -cells medium changed over days, and that supernatant increased the viability of hippocampal neurons, while it had no obvious effect on the expression of BPRP in hippocampal neurons. These results suggest that BPRP may be a glucose-sensitive protein.

Keywords Brain–pancreas relative protein · Pancreatic β -cells · Hippocampal neurons · Insulin · Glucose · Diabetes

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Introduction

Using SDS–PAGE analysis, we discovered a dramatic decrease of a novel 260 kDa protein in ischemic brain tissue following experimental occlusion of the middle cerebral artery in rats [1]. Using a polyclonal antibody, we found that expression of this protein primarily occurred in hippocampal neurons and pancreatic β -cells, with lesser amounts found in podocytes of the renal glomeruli and endothelial cells of venous sinuses in the spleen. For that reason, we named the protein brain–pancreas relative protein (BPRP) [1]. In our previous study, we found that chronic mild stressors decreased the expression of BPRP in pancreas accompanied by changes in insulin and glucose levels [2], and

that expression of BPRP in pancreas also decreased significantly in diabetic rats induced by Streptozocin (STZ) (data not published). These findings suggest that BPRP in pancreas is regulated by insulin and/or glucose, and that BPRP may be a diabetes-related protein. At the same time, in view of that diabetes causes a variety of functional and structural disorders in the central and peripheral nervous systems [3], it is interesting and necessary to further study how the changes in insulin or glucose levels influence the expression of BPRP in hippocampal neurons.

It has been known for more than 30 years that insulin is present in the central nervous system (CNS) [4–7]. By use of fluorescent staining, Dorn et al. demonstrated the presence of insulin in neurons of the hypothalamus, thalamus, amygdala, and hippocampus of the murine brain [8]. The presence of insulin and insulin receptors in the brain suggests that the brain is a target organ for insulin [9]. Nevertheless, how insulin affects the hippocampal neurons and the expression of BPRP in vitro remains unknown.

Glucose supplies energy for the maintenance and activities of the CNS. Deterioration in glucose homeostasis including hypoglycemia and hyperglycemia can trigger neuronal injuries [10]. Hypoglycemia is considered to kill neurons by depriving them of glucose [11]. A conspicuous feature of hypoglycemic brain damage in the rat is neuronal necrosis in the dentate gyrus of the hippocampus [12]. In diabetic rodents, structural abnormalities including synaptic and neuronal degeneration, neuron loss, glycogen accumulation, dilated and fragmented endoplasmic reticulum, increased microtubuli, and irregular nuclei have been demonstrated [13–17]. However, how glucose affects the expression of BPRP in hippocampal neurons deserves further study.

The aim of the study is to investigate the changes in the expression of BPRP in hippocampal neurons exposed to different levels of insulin or glucose in vitro. We also examined the effects of the supernatant of pancreatic β -cells during different days on the expression of BPRP in hippocampal neurons. Changes in the expression of BPRP induced by glucose, supernatant, or insulin were measured with flow cytometer (FCM).

Experimental procedure

Pancreatic β -cells culture

Male Sprague–Dawley (SD) rats (weighing 180–200 g) were obtained from the Animal Center of Peking University Health Science Center. The local animal

committee at Peking University approved all experiments. Pancreatic β -cells were isolated by collagenase digestion. In brief, rats were anesthetized by i.p. injection of 3.5% chloral hydrate in normal saline (1 ml/100 g body wt.), the abdominal cavity was opened and the entire pancreas was carefully dissected and collected in a Petri dish with cold Hanks' balanced salt solution (HBSS). Fat and connective tissues were removed and the pooled pancreases were minced into pieces of ~2 mm each and washed several times with cold HBSS to remove cell debris, blood, and remaining fat tissue. The pancreas was digested for 10–12 min under continuous shaking (150 strokes/min) at 37°C in 2.5–3.0 ml HBSS containing 6–8 mg collagenase V (Sigma, St. Louis, MO, USA). After digestion, 15–20 ml cold HBSS was added into the incubation medium. Undigested tissue was removed using a 500 μ m screen, and the recovered tissue was washed twice with cold HBSS followed by centrifugation at 250 g for 4 min. The sediment was washed several times with cold HBSS. Pancreatic β -cells were cultured in RPMI medium 1640 (GIBCO, Grand land, NY, USA) supplemented with 10% heat-inactivated FBS. The supernatant of pancreatic β -cells was collected every 2 days and stored at –80°C. The supernatant was used to culture hippocampal neurons or for insulin measurement.

Insulin measurement

Insulin in supernatant was measured by radioimmunoassay (RIA) using a commercially available kit (China Atomic Energy Research Institute) within a period not exceeding 7 days [18].

Hippocampal neurons culture

Primary hippocampal neurons culture was prepared from 1-day-old SD rats. Briefly, brains were removed, and the hippocampus was dissected and placed in cold HBSS containing 15 mM Hepes. The tissue was incubated with 0.25% trypsin for 10 min at 37°C and then rinsed several times in Dulbecco's modified Eagle's medium (DMEM, 1 g glucose/l) (GIBCO) containing 20% heat-inactivated fetal bovine serum and 100 U/ml penicillin, 100 μ g/ml streptomycin. Neurons were seeded into 16 mm² culture wells containing poly-L-lysine-coated glass coverslips, and routinely cultured at 37°C in a humidified 95% air–5% CO₂ chamber. After 24 h, the medium was changed to DMEM medium containing 10% fetal bovine serum. On the third day, 10 μ M Cytosine (Sigma) was added into the medium. The neurons were used for immunofluorescence assay,

viability assay and BPRP expression study 7 days after plating. For different purposes, we supplied the neurons with different concentrations of insulin or glucose or we replaced the medium with supernatant deriving from pancreatic β -cells for 2 days.

Identification of BPRP with confocal laser scanning microscopy assay

Cells were seeded on the glass coverslips. Before detecting protein levels by immunofluorescence, cells were permeabilized in phosphate buffered saline (PBS)/0.1% Triton X-100 for 5 min, then preincubated with 10% goat serum for 5 min. BPRP antibody [1] was diluted at 1:1,000 in PBS/1.5% BSA and incubated for 2 h, FITC-conjugated goat anti-rabbit IgG (Santa Cruz, California, CA, USA) was diluted at 1:2,000 in PBS/1.5% BSA and incubated for 1.5 h at room temperature. To distinguish the nucleus and cytosol, propidium iodide (PI) (Sigma), which can specifically stain nucleus to be red was incubated (0.625 μ g/ml, dissolved in PBS) for 20 min. After being washed three times, the coverslips were sealed and cells were analyzed with the confocal laser scanning microscopy (CLSM) (LEICA, TCS SP2, Germany). Negative controls were set without the presence of the primary antibody.

BPRP analysis with flow cytometer

Quantification of the expression of BPRP was performed by FCM (Becton Dickson, San Diego, CA, USA). In brief, pancreatic β -cells and hippocampal neurons were collected after being treated with glucose or supernatant, then washed with cold 0.1 M PBS (3×10 min, pH 7.4) and centrifuged at 800 rpm for 5 min at 4°C. After that, cells were fixed with 70% ethanol for at least 18 h at 4°C. The cells were washed with cold 0.1 M PBS (3×10 min, pH 7.4) and centrifuged at 800 rpm for 5 min at 4°C, then incubated with BPRP antibody which was diluted at 1:1,000 in PBS/1.5% BSA for 2 h. After being washed and centrifuged as above, the cells were incubated with FITC-conjugated goat anti-rabbit IgG (Santa Cruz), which was diluted at 1:2,000 in PBS/1.5% BSA for 1.5 h at room temperature. The cells then were analyzed with FCM after being washed with cold 0.1 M PBS (3×10 min, pH 7.4) and centrifuged at 800 rpm for 5 min at 4°C. Negative controls were set without the presence of the primary antibody. The data were expressed as BPRP expression, which was analyzed and compared to the internal standard by the software automatically in FCM.

Apoptosis analysis

Quantification of the apoptotic rate was examined by FCM (Becton Dickson). In brief, cells ($>10^6$ cells/ml) were fixed with 70% ethanol for at least 18 h at 4°C. After washing, cells were incubated with 0.625 μ g/ml PI for 20 min, then apoptotic rate was quantified by means of FCM.

Determination of cell viability

Cells were planted into the 96-well plates and treated with supernatant, insulin or glucose for certain time. After that cells were incubated with 0.5 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) for 4 h at 37°C, subsequently the medium was carefully removed and the formed formazan was dissolved in 100 μ l DMSO per well. The amount of MTT formazan product was determined by measuring optical density with a microplate reader (Thermo Miltiskan MK3) at a test wavelength of 570 nm and a reference wavelength of 655 nm.

Statistical analysis

Data were evaluated for statistical significance with one-way ANOVA (Bonferroni post hoc) with the SPSS software. The number of experimental samples used in each group was presented in the figure legends. All data were represented as the mean $\pm$ SEM. $P < 0.05$ was considered statistically significant.

Results

BPRP expressed in hippocampal neurons

Previously we found that BPRP was expressed in certain brain area such as hippocampus [1], which was further confirmed in our study. As shown in Fig. 1, the green fluorescence represented the expression of BPRP, while the red fluorescence represented the nucleus of a cell. We found that BPPR was expressed in the membrane and cytosol, while not in the nucleus of the hippocampal neurons.

Effect of insulin on viability and expression of BPRP in hippocampal neurons

We added different levels of insulin into normal RPMI medium for 2 days and then observed the effects of insulin on viability and expression of BPRP in hippocampal neurons. As shown in Fig. 2 that although

Fig. 1 Confocal image of BPRP expression in hippocampal neurons. A ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 515 \text{ nm}$) represents the expression of BPRP (green) and B ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 605 \text{ nm}$) represents nucleus (red), and C is an overlaid image of A and B

insulin had no significant effect on the viability of hippocampal neurons compared to normal RPMI medium, it induced an increasing trend in the viability. Also no significant effect of insulin on the BPRP expression in hippocampal neurons was observed.

Fig. 2 Effect of insulin on viability and expression of BPRP in hippocampal neurons. Hippocampal neurons were incubated with different concentrations of insulin for 2 days. A Effect of insulin on the viability of hippocampal neurons; B Effect of insulin on the expression of BPRP in hippocampal neurons. All data are represented as the mean $\pm$ SEM and the values were obtained from six (MTT) or three (FCM) separate cultures

Insulin secretion in pancreatic β -cells

As shown in Fig. 3 that pancreatic β -cells constantly secreted insulin during 10 days, the maximum level of insulin may reach on the second day, up to $5.68 \mu\text{U/ml}$, it decreased stably to $3.52 \mu\text{U/ml}$ on the fourth day, to $2.74 \mu\text{U/ml}$ on the sixth day, and reached nearly the minimum of $2.08 \mu\text{U/ml}$ on the eighth day.

Effect of supernatant on viability and expression of BPRP in hippocampal neurons

The medium of hippocampal neurons was removed on the seventh day and cells were incubated with the supernatant of pancreatic β -cells, respectively, for 1, 2 or 4 days, while the normal group's medium was

Fig. 3 Time course of insulin secretion during 10 days. All data are represented as the mean $\pm$ SEM and the values are obtained from three separate cultures

Fig. 4 Effect of supernatant from pancreatic β -cells on the viability and expression of BPRP in hippocampal neurons. The medium of hippocampal neurons was replaced with supernatant of pancreatic β -cells on the seventh day, while the normal group's medium was replaced with complete RPMI 1640 medium. **A** Effect of supernatant on the viability of hippocampal neurons; **B** Effect of supernatant on the expression of BPRP in hippocampal neurons. All data are represented as the mean $\pm$ SEM and the values were obtained from six (MTT) or three (FCM) separate cultures, * $P < 0.05$, # $P < 0.05$ versus normal group

replaced with completed RPMI 1640 medium. As shown in Fig. 4A, hippocampal neurons cultured with normal completed RPMI 1640 medium or supernatant from the eighth day grew slowly and there was no significance compared to the "0" day neurons or the corresponding day neurons in normal RPMI 1640-cultured group. Supernatant from the fourth and six day increased the viability of hippocampal neurons more quickly, whereas there was still no significance compared to the normal RPMI 1640-cultured group. However, supernatant from the second day obviously increased the viability of hippocampal neurons after 2 or 4 days incubation ($P < 0.05$). As shown in Fig. 4B, supernatant, irrespective of the age of the islet cells, including the second day's supernatant had no obvious effect on the expression of BPRP expression in hippocampal neurons after being incubating with supernatant for 2 days.

Effect of glucose on the viability, expression of BPRP, and apoptosis in hippocampal neurons

Hippocampal neurons were treated with different concentrations of glucose for 2 days. As shown in

Fig. 5 Effect of glucose on viability, apoptosis and expression of BPRP in hippocampal neurons. Hippocampal neurons were incubated with different concentration of glucose for 2 days. **A** Effect of glucose on the viability of hippocampal neurons; **B** Effect of glucose on the expression of BPRP in hippocampal neurons; **C** Effect of glucose on the apoptosis of hippocampal neurons. All data are represented as the mean $\pm$ SEM and the values were obtained from six (MTT) or three (FCM) separate cultures, * $P < 0.05$ vs. 5 mM group

Fig. 5A, different concentrations of glucose had no obvious effect on the viability of hippocampal neurons. However, free glucose and 40 mM glucose obviously decreased the expression of BPRP in hippocampal neurons compared to 5 mM glucose group ($P < 0.05$) (Fig. 5B). In order to exclude the possibility that the reduction in the expression of BPRP induced by free glucose or high concentrations of glucose was due to apoptosis, we tested the effect of glucose on the apoptosis and found that although they led to a decreasing trend in apoptosis, free glucose, 20 and 40 mM glucose had no significant effect on the apoptosis compared to 5 mM glucose group (Fig. 5C).

Discussion

There is increasing evidence that insulin has metabolic, neurotrophic, neuromodulatory, and neuroendocrine

effects in the brain [19, 20], and that hippocampus contains substantial amounts of immunoreactive insulin, insulin receptors [21–25], insulin-like growth factor, and its receptor [26]. All these are necessary for neuronal growth, differentiation, and function [27–30]. In our study, although there was no significance compared to normal group, our data showed that insulin induced an increasing trend in the viability. However, no significant effects of insulin on the expression of BPRP in hippocampal neurons were examined. These findings confirm that insulin is necessary for neuronal growth, differentiation, and function.

In view of the complex effect of insulin and other components *in vivo*, we further cultured pancreatic β -cells and collected the supernatant, which contained different levels of insulin. It showed that supernatant from pancreatic β -cells significantly increased the viability of hippocampal neurons. Our data were in concordance with the data from Hooghe-Peters et al. who found that if nerve cells dissociated from the cerebral cortex of mice were co-cultured with isolated pancreatic islets of Langerhans, the attachment and/or survival and differentiation of these nerve cells were markedly promoted [31]. Furthermore, the hippocampus of rats with uncontrolled insulin-dependent diabetes undergoes retraction and simplification of apical dendrites of the CA3 pyramidal neurons, and peritoneal implantation of macroencapsulated pancreatic islets prevents the dendritic retraction [32]. All these findings suggest that the supernatant contains some neurotropic factors, including insulin, which can stimulate the growth of hippocampal neurons. Although supernatant stimulated the growth of hippocampal neurons, it had no obvious effect on the expression of BPRP in hippocampal neurons. Obviously, insulin was responsible for partial effects on viability of hippocampal neurons according to the former data, which factors else were also responsible for the effects on viability maybe an interesting topic and requires further study due to the fact that we used a supernatant from cell culture and the composition of that solution was unknown.

Disturbances in glucose homeostasis that results from hypoglycemia or hyperglycemia can trigger neuronal injuries [10]. In our study, free glucose or high concentrations of glucose decreased the expression of BPRP. However, it is essential to check the survival of the neurons in presence of such different concentrations of glucose in order to study the possibility that the decrease in expression of BPRP may be due to the presence of fewer surviving cells. The data showed that free glucose and 40 mM glucose, although they induced a decreasing trend,

had no significant effects on the viability of hippocampal neurons. Furthermore, free glucose and 40 mM glucose did not induce significant apoptosis. According to these findings, the reduction of the expression of BPRP was not attributed to less amount of hippocampal neurons. In our other study, we also found that glucose and oxygen deprivation significantly decreased the expression of BPRP mediated by reactive oxygen species (ROS) (submitted), which further indicates that not only hypoglycemia but also hyperglycemia decreased the expression of BPRP in hippocampal neurons. All these findings suggest that a relation exists between expression of BPRP and changes in glucose levels.

We previously found that glucose levels did not influence the expression of BPRP in hippocampal neurons *in vivo* [2]. An explanation for this discrepancy could be that although the glucose level *in vivo* changed, levels were still within the range suitable for hippocampal neurons to survive and subsequently no effect on the expression of BPRP was found. In the present study, we found that 10 mM glucose had no effect on the BPRP expression in hippocampal neurons, which is consistent with the results found *in vivo*. However, once glucose levels increase to a certain extent, it will damage the brain [10] and affect the expression of hippocampal BPRP expression, as was shown in this report. Furthermore, glucose seems to be compartmentalized within the brain, and there is dissociation in glucose concentrations between different brain areas and between the brain and the blood, and brain glucose levels do not readily follow levels in blood [33, 34]. At the same time insulin present in the CNS is a hormonal regulator of central glucose metabolism [35–38]. These are also the reasons for us to choose the *in vitro* model to study the effects of insulin and glucose on BPRP in hippocampus. Nevertheless, the *in vivo* relevance of expression of BPRP in different model of hyperglycemia and hypoglycemia also requires further study.

Based on these results, we concluded that glucose deprivation and high levels of glucose both decreased the expression of BPRP in hippocampal neurons, and that the reduction in BPRP expression was not due to the less number of hippocampal neurons. Although insulin had a positive effect on neuronal growth, it did not affect BPRP expression in cultured hippocampal neurons. Combining the former data and the data in this report, we can draw a conclusion that BPRP is a glucose-sensitive protein both in pancreas and in brain. However, whether the reduction in the expression of BPRP is a marker of disturbances in glucose homeostasis needs more data to substantiate.

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