Up-expression of GAD65 in the Amygdala of the Rat Model of Choric Immobilization Stress with elevated blood glucose

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Abstract

It is believed that hypothalamus is the regulating center for the blood glucose levels, but how chronic stress leads to hyperglycemia is not known. In this experiment, we use chronic immobilization stress rat as a model, and found that only rats with increased expression of GAD65 in amygdala have an elevated level of blood glucose. Considering there are fiber tracks between amygdala and hypothalamus, including GABAergic projection, this result suggested that the changes of GAD65 expression in amygdala may correlate with the changes of blood glucose levels, and point to the importance of the amygdala in blood glucose regulation.

Keywords GAD65; Amygdala; Choric Immobilization Stress; blood glucose; rat

1. Introduction

Type 2 diabetes is a common disease, but its pathogenesis is not clear till now. Modern medical research has revealed that people have a higher incidence of hyperglycemia and type 2 diabetes (Surwit et al., 1992, Karen et al., 2001) under chronic stress. Hypothalamus is the regulating center of the blood glucose, which has two different glucose-sensing neurons, including glucose-inhibited neurons and glucose-excited neurons. They participate in the regulation of homeostasis of blood sugar (Rory et al., 2006), and possibly regulate the releasing concentration of GABA by controlling the opening and closing state of K_{ATP} channel, thereby affecting the transmission of blood glucose information (Chan et al., 2007). Amygdala is one of the basic locations to regulate the stress response in the loop of the nerve center. A study has found that the expression of GABA in the amygdala was increased after stress (Cook, 2004). There is a wide range of fiber tracks between amygdala and hypothalamus, including GABAergic projection. It is not yet clear whether changes of GABA in amygdala correlate with rising of the blood glucose. In choric immobilization stress model, it was observed that, the rat with elevated blood glucose has an increased expression of GABA synthesis enzyme - GAD65 in the amygdala, while rats without elevated blood glucose didn't have this phenomenon. The results primarily suggest that there may be correlation between the change of GAD65 expression in the amygdala and the change of blood glucose levels.

2. Materials and methods

2.1 Animals

Forty-eight male Sprague-Dawley rats (9 ~ 10 weeks of age, weighing 270±26 g when the experiments started) were obtained from the Animal Experiment Center of Sichuan University, and housed for 1 week prior to the experiment under a constant temperature (21℃ ~ 24℃) and Humidity (50% ~ 60%). All experimental procedures complied with National Institute of Health Guide for

the Care and Use of Laboratory Animals (NIH), USA.

2.2 Experimental Design

Rats were randomly divided into two subgroups: the chronic immobilization stress (CIS) group (n = 30), and a control group (n =18). Samples of blood and brain tissues were collected at the 7, 14 and 21 day respectively. There were 10 rats in CIS group at each time point, while 6 rats in control group at each time point.

Choric immobilization stress model is according to method of Chen (Chen et al., 2009). Mineral water bottles were changed into restraint cylinder (length of 15cm, diameter of 7cm). There are numbers of holes at the top and bottom of the cylinder to ensure their breath. CIS group were manacled in the restraint cylinder to limit exercises for 3 hours each day from 6:00 pm to 9:00 pm (water and food is not provided). Control group were feed freely, and did not impose any stimulations.

2.3 Blood glucose testing

Blood samples were collected at 9:00 am at the 7, 14 and 21 days (banning water and fasting before night). The animals were anesthetized by intraperitoneal injection of chloral hydrate solution (3.6%, 1.0 ml/100g body weight). Blood was Collected from the right atrium, then tested blood glucose levels (automatic biochemical analyzer, Hitachi 7600). Rats with blood glucose concentrations higher than 9 mmol/L were considered to be hyperglycemia (Fuhlendorff et al., 1998).

2.4 Sample Preparation

Samples of brain tissue were collected after perfusion with 4% paraformaldehyde PBS solution, embedded in paraffin,　and were cut into 6 µm sections. Brain section (interaural 6.60mm, bregma -2.40mm) were chose to process for immunohistochemistry.

2.5 Immunohistochemistry

Immunohistochemical staining performed followed the SABC procedure, to detect the expression of BDNF in the cerebral cortex and GAD65 in the amygdala. Primary antibody were rabbit anti-BDNF and GAD65 serum (1:150, Beijing Boisynthesis), the second antibody was goat anti-rabbit serum (1:200, Beijing Boisynthesis). For the control of immunostaining specificity, the primary antibody was omitted. Primary antibody pre-adsorbed with BDNF and GAD65 was also detected. Both of theses experiments did not exhibit any specific immunostaining.

2.6 Image Analysis

6 rats were randomly selected in each group. The microscopic photographs of BDNF and GAD65 positive cells were analyzed respectively in the cortex and the amygdala region, and the average optical density (OD) were measured.

2.7 Statistical Analysis

All data were expressed as means ± standard deviation. A one-way ANOVA test with SPSS 17.0 were used for Statistical analysis, t-tests were performed between individual groups. The level of significance was set at $P<0.05$.

3. Results

In this experiment, It was found that Adrenal gland index increased in CIS group by weighing the fresh tissue ($p < 0.05$) (table 1), indicated that the the CIS model was successful. Compared with control group, 9 rats in the CIS group showed hyperglycemia ($p < 0.05$), the remaining 21 did not show an increase of blood glucose levels (table 2).

The average optical density of GAD65 was not significantly different in each control group of ($p> 0.05$) (this paper do not show the data). Compared with control group, the expression of GAD65 of CIS group was significantly

higher in rats with elevated blood glucose ($p < 0.05$), while it was not significant different among rats without elevated blood glucose ($p > 0.05$) (table 3, figure 1).

The average optical density of BDNF was not significantly different in each control group ($p > 0.05$) (this paper do not show the data). The expression of BDNF in the CIS group (no matter whether there was an increase of blood glucose levels) were significantly lower than that of the control group ($P < 0.05$) (table 3, figure 2).

**Table 1** The comparison of the adrenal gland index in each group

|	control group（n=18）	7d CIS（n=10）	14d CIS（n=10）	21d CIS（n=10）
Adrenal wt(mg)×100/ body wt (g)	10.3±0.6	13.0±2.6*	11.2±1.0*	12.7±1.5*

* p<0.05, compared with control group

Table 2 Comparison of fasting blood glucose in each group

	control group（n=18）	CIS & Blood glucose increased group（n=9）	CIS & Blood glucose Not increased group（n=21）
FPG	7.24 ± 0.84	9.89 ± 0.62*	7.37 ± 0.89

* p<0.05, compared with control group

A   B   C
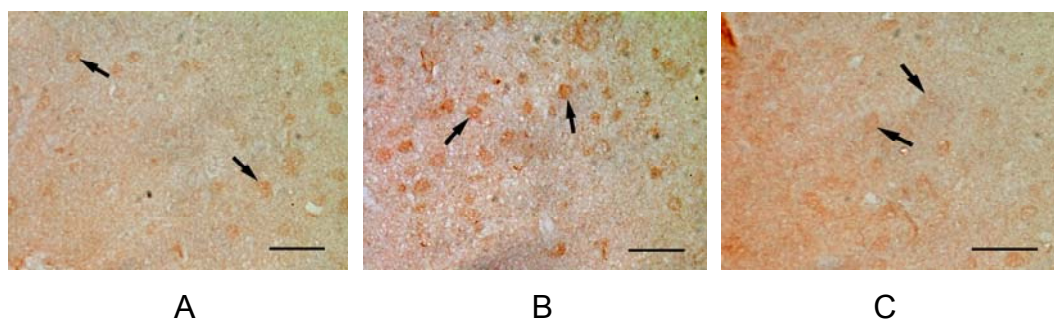
Fig. 1 GAD65 immunopositive neurons in the amygdala in control group(A), CIS & blood sugar increased group (B) and CIS & blood sugar not increased group(C). Positive neurons indicated by "↗". Bar＝100 μm. (10 × 40)
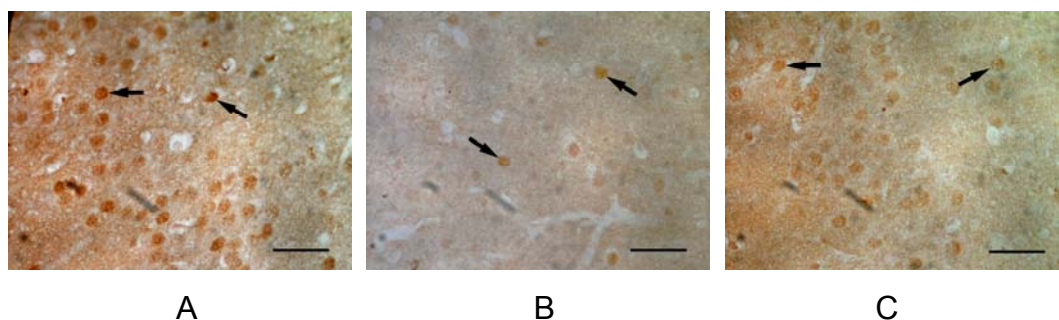
Fig. 2 BDNF immunopositive neurons in the cerebral cortex in control group (A), CIS & blood glucose increased group (B) and CIS & blood glucose not increased group(C). Positive neurons indicated by "↗". Bar＝100 μm. (10 × 40)

Table 3 Comparisons of the blood glucose level (mmol/L), the OD of BDNF and GAD65 in each group

	Control group（n=6）	CIS & Blood glucose increased group（n=6）	CIS & Blood glucose not increased group（n=6）
Blood Glucose	7.23 ± 0.71	9.63 ± 0.18	7.44 ± 0.98
BDNF	0.35 ± 0.16	0.20 ± 0.07*	0.22 ± 0.10*
GAD65	0.07 ± 0.02	0.18 ± 0.07*	0.08 ± 0.02

* $p<0.05$, Compared with control group

4. Discussion

In this experiment, after choric immobilization stress, activities of rats were decreased, and the adrenal stress index was significantly higher compared to control group, indicating that this stress model was successful (Vyas et al., 2002). In CIS group, only rats with increased expression of GAD65 in amygdala have an elevated level of blood glucose; but if expression of GAD65 did not increase, blood glucose level did not change. It is prompted that changes of blood glucose levels appeared to correlate with increased

expression of GAD65 in the amygdala. Study found that the amygdala increased the expression of GABA under stress (Cook, 2004), so GABA which was synthesized by amygdala GAD65 may also be increased, and GABA neurotransmission to hypothalamus may enhanced accordingly. Enhanced GABAergic projection will inhibit the activity of glucose-sensitive neurons in the hypothalamus. It leads to a result that the neuron ability to apperceive blood glucose information and regulate blood glucose is reduced. If the inhibitory state sustained, blood glucose may eventually out of control.

Why stress leads to increase expression of GAD65? Studies In vitro have proved that BDNF regulates the expression of GAD65 through the Ras / ERKs pathway (Sanchez-Huertas et al., 2010). BDNF is synthesized mainly in the cerebral cortex. Smith found that under stress, BDNF was decreased in the hippocampus, piriform cortex and other brain regions (Smith et al., 1996). In this experiment, the expression of BDNF in CIS group is lower in cortex (consistent with the observation of smith), while the expression of GAD65 appeared two cases: increased and not increased. This result is inconsistent with previous reports related to (Sanchez-Huertas et al., 2010). The following reasons may explain this phenomenon. First, the environment in vivo is more complex than in vitro, so expression of GAD65 may regulated by variety of factors. Second, there are individual differences among animals, and the impact of stress also corresponds with the adaptability of the animal in stress condition. In addition, the animals have a series of biochemical control mechanisms to mitigate the overreaction (Martí et al., 1998). Third, there may be some unknown factors which need further investigation.

In order to explain our results further, information from some documents may be helpful. For example, it was reported that dopamine, another neurotransmitter, can activate its D1 receptor and increase the GAD65 mRNA through the cAMP/PKA signaling (Noriko et al., 2008, Eva et al., 2000). Additionally, 5-Hydroxytryptamine (5-HT) blocks the $GABA_B$ synaptic potential in rat dopamine neurons through its 1B receptor (Johnson, 1992), and 5-HT

itself can be influenced by GABA (Broadbelt et al., 2010). The GAD and 5-HT constitute a closed regulatory circuit. Interestingly, 5-HT promotes the gene expression for BDNF in rat brain (Zetterström et al., 1999). Considering our result and the Ras / ERKs pathway mentioned above, the GAD-5-HT-BDNF may form a larger negative feedback circuit. The remaining question is what's the relationship between dopamine and BDNF, and is there any other mechanism involving? Till now, we cannot answer all of the questions, which need to be investigated in future.

In this study, we chose the time for animal instinctive active exercises peak to limit its exercises for a fixed time. This constraint condition simulated chronic stress caused by sedentary habits and tension in people's real life. Before experiment, animals were breed a week to adapt the condition of our lab. Male rats weighed about 270g and aged 9 to 10 weeks were selected for this experiment. There are two reasons for it. On one hand, the rats having moderate body weight can enter the restraint cylinder smoothly, at the same time they can't turn the corner and withdraw from it. On the other hand, the young rats aged 9 to 10 weeks are equivalent to the Young adults in human, and most people during this period are under stress. There are two subtypes of GABA synthesis rate-limiting enzyme: GAD65 and GAD67. GAD65 was investigated in this experiment, because GAD65 regulates GABA synthesis for synaptic release, while GAD67 regulates GABA synthesis for metabolic functions of the cell (Soghomonian et al., 1998).

Amygdala is the emotional integration center, involved in the regulation of stress response. After chronic immobilization stress, we observed that expression of GAD65 in the amygdala increased only among the blood glucose elevated rats. In contrast, If GAD65 expression did not increase, blood glucose levels did not change. This phenomenon suggested that the changes of GAD65 expression in amygdala may correlate with the changes of blood glucose, and point to the importance of these regions in blood glucose regulation. In Future work, we will study the mechanism how GABA in the

amygdala participates in the regulation of blood glucose.

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