Hyperglycemia Decreased Medial Amygdala Projections to Medial Preoptic Area in Experimental Model of Diabetes Mellitus

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Abstract - In Wistar rats, reproductive behavior is controlled in a neural circuit of ventral forebrain including the medial amygdala (Me), bed nucleus of the stria terminalis (BNST) and medial preoptic area (MPOA) via perception of social odors. Diabetes Mellitus (DM) is a widespread metabolic disease that affects many organs in a variety of levels. DM can cause central neuropathies such as neuronal apoptosis, dendritic atrophy, neurochemical alterations and also causes reproductive dysfunctions. So we hypothesized damage to the nuclei of this circuit can cause reproductive dysfunctions. Therefore in this project we assessed diabetic effect on these nuclei. For this purpose neuron tracing technique and TUNEL assay were used. We injected HRP in the MPOA and counted labeled cells in the Me and BNST to evaluate the reduction of neurons in diabetic animals. Also, coronal sections were analyzed with the TMB histochemistry method. Animals in this study were adult male Wistar rats (230 ± 8g) divided to control and 10-week streptozotocin-induced diabetic groups. After data analysis by SPSS 16 software, a significant reduction of HRP-labeled neurons was shown in both Me and BNST nuclei in the diabetic group. Moreover, apoptotic cells were significantly observed in diabetic animals in contrast to control the group. In conclusion, these alterations of the circuit as a result of diabetes might be one of the reasons for reproductive dysfunctions.

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Introduction

Diabetes mellitus (DM) is a chronic endocrine disease that described as a disorder in carbohydrate metabolism. Two factors are mentioned as leading causes of DM including destruction of pancreatic β-cells (type 1 diabetes) and insulin insensitivity (type 2 diabetes) (1). Over 171 million people in the world are suffering from diabetes, and it is estimated to grow up to rate of 366 million people in 2030 (2). In Iran, 7.7% of adult people had diabetes in 2008 (3).

Peripheral and central neuropathies caused by diabetes are well known (4). For example, DM results in stress oxidative (5), morphological plasticity such as decrease of neuron diameter (6), neuroaxonal dystrophy (7),dendritic atrophy and decrease in spine density in CA1 pyramidal neurons (8), neuronal apoptosis (9), behavioral problems (10,11), finally electrophysiological and neurochemical alterations (12).

Numerous studies reported reproductive dysfunctions as another important complication of diabetes (13-15). Although peripheral organs perform reproductive behavior such as spermatogenesis, ejaculation, fertility and androgenic hormone secretion, but it controls in central nervous system.

In many rodent species, proper reproductive behaviors depend on reception and perception of social odors (16), then these chemosensory signals merge with hormonal signals and process in a neural circuit between three nuclei in forebrain including medial amygdala (Me), bed nucleus of stria terminalis (BNST) and medial preoptic area (MPOA) (17,18). Particularly BNST and Me both receive chemosensory signals (19) and are densely connected to each other and to MPOA (18, 20, 21). In addition, according to studies conducted on male hamsters, damage of Me (22) and MPOA (23)
Hyperglycemia decreased medial amygdala projections eliminates copulatory behavior and causes severe deficits in preference for volatile opposite-sex odors (23,24). However, in lesion of BNST in male hamsters, the elimination just was observed in odor preference (25) and deficits in copulatory behavior were temporal and more subtle (26,27). Consequently, it is concluded that MPOA, Me and BNST act as a neural network in the regulation of reproductive behavior. According to these findings and this fact that these nuclei contain glucose-sensing neurons which express glucokinase (that plays an important role in regulation of glucose level through insulin secretion) mRNA and respond directly to availability and changes of blood glucose levels (28,29), in this project we hypothesize diabetes causes reproductive dysfunction through damage to this circuit as a controlling center for reproductive functions.

Materials and Methods

Animals
24 adult male Wistar rats (230 ± 8 g) are used in this study. The animals were holding on 12 hours light/dark cycle. Water and food were available all the time. Animals were assigned into diabetic and control groups randomly. Type 1 diabetes (n=12) was induced by a single intraperitoneal injection of 60 mg/kg b.w. Streptozotocin (Sigma-Aldrich, St. Louis, MO, The USA). A week after the induction, glucose level was assessed using blood-glucose monitoring test strips. Blood glucose level was >300 mg/dl in all STZ-injected animals. Diabetic animals were maintained for 10 weeks before the study.

Neuron is tracing, and TUNEL staining was used to study the effects of diabetes on the Me and BNST. Stereotaxic injection of HRP
After anesthesia using a single intraperitoneal injection of ketamine and xylazine (80 mg/kg and 8 mg/kg respectively), the animal head was placed into a stereotaxic apparatus using ear bars. The skull was exposed, and lambda and bregma were aligned by regulating the incisor bar. 200 nl HRP (Sigma, type VI) was injected into MPOA (by 1 μl syringe, Hamilton, Reno, NV, USA) according to coordinates in Paxinos and Watson Atlas (2007). Following injection, HRP is transported retrogradely via axonal endings to perikarya of Me and BNST projections to the injection site. Subjects were recovered for 48 hours. After deep anesthesia with ketamine and xylazine perfusion was performed transcardially with 200 ml normal saline followed by 300 mL 4% paraformaldehyde and 200 ml sucrose buffer 10% and soaking in 10% glycerin overnight at 4°C. The brains were removed and cut in coronal sections (40 μm) on a freezing microtome (Cryocut 1800, ELICA). The sections were reacted with tetramethyl benzidine (Sigma. St Louis. Mo, The USA) following the procedure of Mesolam et al., (30) to distinguish HRP-labeled neurons. After mounting and counterstaining with 1% neutral red, the slides were studied by light microscope and digital photos were taken. The injection site was evaluated, and optika software counted diffusion of labeled cells in Me and BNST. SPSS 16.0 software was used for data analysis.

TUNEL assay
After deep anesthesia, perfusion was performed with 200 ml of normal saline followed by 10% formalin. Paraffin-embedded brains were cut coronally (7 μm) by microtome. After deparaffinized, the sections were incubated with proteinase K (Sigma, St. Louis, MO, and The U.S.A.) to strip nuclei of tissue of proteins. Endogenous peroxidase activity was quenched with 2% H2O2 in phosphate- buffered saline (PBS). The slices were incubated in a humidified chamber at 37°C for 1 h with terminal deoxynucleotidyl transferase (TdT) and dUTP-digoxigenin to label 3’-OH DNA ends in apoptotic nuclei. Incubation with the stop/wash buffer at 37°C for 30 min was done to stop the reaction. The slices were colorized with diaminobenzidine /H2O2 solution after incubation with anti-digoxigenin-peroxidase. Finally, coronal sections were counterstained with hematoxylin and studied by light microscopy.

Results
Blood glucose level and body weight
Animal weight and blood glucose level were recorded during the study in both control and diabetic groups. Body weight normally increased in control animals (from 228 ±10 to 334 ±10) and blood glucose level was 80-110 mg/dl with no change. In contrast, body weight of animals in the diabetic group was decreased (230 ±7 to 171 ±4) and blood sugar was >300 mg/dl during the disease (Figure 1).

Neuron tracing
HRP was injected in MPOA (Figure 2) and labeled cells were seen in Me and BNST. The cells were counted in both nuclei ipsilateral to the injection site. Based on Paxinos Atlas, 6 sections per nucleus in each animal were selected to study by light microscope.
Labeled neurons in these sections were counted. In both Me and BNST, number of labeled neurons in first and sixth sections was less in contrast to other sections (19%). In the control group the total mean of labeled neurons of Me and BNST were 33.83±2/13 and 41.66±1/96 respectively. These numbers in the diabetic group were 26.83±2/31 and 33.83±1.16 in Me and BNST respectively. These data show a significant reduction (20.69% in Me and 18.79% in BNST) of labeled cells in 10-week diabetic group ($P\leq0.05$) (Figure 3 and 4).

**Apoptosis**

Apoptosis (DNA fragmentation) was investigated with the ApopTag kit staining. A neuron with a wrinkled and condensed nucleus (due to the DNA fragmentation) was considered as an apoptotic cell. We counted apoptotic neurons of the Me and BNST in two groups. In control animal’s brain sections, a considerable amount of apoptosis did not appear in any nuclei. Cell death was approximately equal in Me and BNST (~0-3 per section) in this group. In case animals, the diabetes had affected on neuron survival, and the number of apoptotic cells had increased significantly ($P\leq0.05$). Nearly 20 apoptotic neurons per section were seen in the both nuclei. In this group, a number of apoptosis in the Me and BNST was equivalent (Figure 5).

![Figure 1. Changes of body weight in diabetic and control groups in duration of the study](image1)

![Figure 2. Injection site](image2)
Hyperglycemia decreased medial amygdala projections

Figure 3. HRP-labeled cells
A: Me and B: BNST in the control group. C: Me and D: BNST in diabetic group (∗×400)

Figure 4. The number of labeled cells of Me (the left chart) and BNST (the right chart) in control and diabetic groups
*Significant differences between control and diabetic groups

Figure 5. Apoptotic activity in the control group
(A: Me ×200, B: BNST ×200, C: Me ×400) and in the diabetic group (D: Me ×200, E: BNST ×200, F: Me ×400)
Discussion

This study shows that the STZ-induced diabetes decreases the retrogradely axonal transport of HRP in the Me and BNST projections to the MPOA. Other studies showed this reduction as well as cell death in peripheral nerves following DM (31). Also, cell death was confirmed in our study by TUNEL assay. Here the apoptosis was detected in the Me and BNST after 10 weeks diabetes in agreement with Rizk and Li research that observed apoptosis in hippocampus and cortex in 6-week diabetic rats (32,33). However, Li had not observed the apoptosis in the hippocampus after 2 months diabetes in 2002 (9). In both control and diabetic groups labeled, neurons in BNST were more than of those in Me. This fact shows more projections to MPOA from BNST than Me. So we realize a dense interconnection between MPOA and BNST. Although the reduction of retrogradely transport has usually be seen at the late stages of diabetes after four months (34, 35) but we observed this change in 10-week diabetic rats in this study. However, the reduction was be seen in raphe nucleus-projecting neurons to striatum after two months diabetes although it was not significant (36). On the other hand marked decreased retrogradely transport of neurotrophic protein nerve growth factor (NGF) was observed in the sciatic nerve in diabetic rats after 2 months (37). The mechanisms of these reductions are not known, but some reasons can be alterations in access to transportable agent and alterations in agent receptors as the significant decrease of NGF-receptor saturation was observed in Hellweg research (37). These findings suggest that the diabetes can decrease transport of each biological agent by similar mechanisms.

We think that the reduced number of neurons and their apoptosis in our study were due to the sensitivity of them to changes of blood glucose level (28,29). In addition to these changes, some researchers have shown that diabetes affects the Me and BNST at physiological and neurobiological level (38-40). Many studies show these nuclei contribute and control reproductive functions via reception and merging of chemosensory and hormonal signals (17,18). Therefore, deficits in this network can cause reproductive dysfunctions that reported as a complication of diabetes in many studies (13,41). For this reasons, we suspect diabetic effects on these nuclei can be a neural mechanism for reproductive dysfunctions. In conclusion, our findings demonstrate that the diabetes induce apoptosis and affect retrogradely axonal transport in projecting neurons from Me and BNST to medial preoptic area.

References


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