Abstract

Seizures are among the most common neurological sequelae of stroke, and ischemic insult in diabetes notably increases the incidence of seizures. Recent studies indicated that autophagy influences the outcome of stroke. However, the association of autophagy and post-ischemic seizures in diabetes remains unclear. The present study aimed to reveal the connection of autophagy in the seizures following cerebral ischemia in diabetes. Diabetes was induced in adult male Wistar rats by intraperitoneal injection of streptozotocin (STZ). The diabetic rats were subjected to transient forebrain ischemia. The neuronal damage was assessed using hematoxylin-eosin staining. Western blotting and immunohistochemistry were performed to investigate the alteration of autophagy marker microtubule-associated protein light chain 1B (LC3B). The results showed that all diabetic animals developed seizures after ischemia. However, no apparent cell death was observed in the hippocampus of seizure rats afterwards. LC3B levels was significantly enhanced in animals post ischemia, and even further enhanced in diabetic animals post ischemia. Furthermore, immunofluorescence double-labeling showed that LC3B mainly increased in neurons. For the first time, our study demonstrated that autophagy activity is significantly increased in diabetic animals with ischemia-induced seizures. Further studies are needed to explore the role of autophagy in seizure generation after ischemia in diabetic conditions.
Autophagy Enhancement in Diabetic Rats with Ischemia-Induced Seizures

I. Introduction

Diabetes mellitus, or more commonly known as diabetes, is a long-term condition affecting millions people worldwide based on the report of American Diabetes Association. The condition can occur when the amount of glucose present in the bloodstream is irregularly high, resulting in high blood pressure levels (hyperglycemia). Depending on the type, this can be caused by the body not producing enough insulin or the cells developing insulin resistance. Because of the lack of insulin or development of insulin resistance, the energy from the glucose cannot be harvested properly, leading to the cells not receiving its needed energy from the sugar. This metabolism disorder is a major risk factor and a major cause of ischemic strokes. Diabetic patients have a larger risk for ischemic strokes and usually suffer more severe ischemic brain damage than nondiabetic patients {Biller, 1993 #6}. Diabetic patients are 2.9 times more likely to have an ischemic stroke than a nondiabetic patient {Air, 2007 #1}. According to the National Stroke Association, 5% of people will have a seizure a couple weeks after having a stroke. However, those chances increase dramatically when the person is diabetic. The chances of having a seizure after a stroke are also drastically higher in diabetic patients.

Autophagy is a process in which the lysosome consumes damaged organelles and protein aggregates in order to recycle obsolete cellular constituents {Wolfe, 2013 #4}. The process is used to balance sources of energy at critical points during development, as well as in response to lack of nutrients. Autophagy can be thought of as a survival mechanism for the cell {Glick, 2010 #3}. There are multiple reports stating the physiological processes of autophagy, including response to
starvation, quality control of intracellular proteins and organelles (mitophagy), embryonic development, cellular defense against bacterial invasion, antigen presentation, tumor suppression, homeostasis of axons, and neurodegeneration {McMahon, 2012 #7}.

According to recent studies, there is data suggesting that autophagy influences exacerbated brain injury under diabetic conditions {Wei, 2013 #5}. Given that diabetes increases the chance of having a seizure, it is logical to assume that autophagy and epileptic seizures are associated with one another.

This study aims to investigate the changes in autophagy levels within the hippocampus and cortex of the rat brain after ischemic strokes under diabetic conditions. By discovering if autophagy and epilepsy are intertwined with one another, we can further discover the role of autophagy in post ischemic seizures under diabetic conditions.

II. Materials and Methods

Adult male Wistar rats with an average mass of 120 grams were used for this experiment. These rats were housed in pairs and triplets, depending on what study group the rats were in, with unrestricted access to rat food and water unless otherwise noted.

2.1 Diabetic Rat Model

The rats were injected with streptozotocin (STZ) to induce diabetes. The streptozotocin damages the insulin-producing beta cells of the rats’ pancreas, disabling the ability to control glucose levels in the blood. We considered the rats diabetic if the glucose levels were above 300 mg/dL. The male rats received an injection of STZ at a dose of 50 mg/kg dissolved in a mol/l
citrate buffer, with a final concentration of 7.5 mg/ml per day. The purpose of the STZ injection is to create a group of diabetic rat models, in which the rats had been diagnosed with diabetes due to the lack of insulin in the rat's system after STZ injection. After the initial STZ injection, it was necessary to wait a week for glucose levels to increase before euthanizing the rats.

2.2 Global Cerebral Ischemic Stroke Model

In order to create a global cerebral ischemic stroke model, it was necessary for authorized personnel to perform four-vessel occlusion (4-VO) of global ischemia model in the rats. During this procedure, the rats were anesthetized with isoflurane (1-2%). Both vertebral arteries of the rat were then electro cauterized. Silicone tubing (0.025-in. I.D., 0.047-in. O.D.) was loosely placed around the common carotid arteries and passed through two holes in a small Teflon button before being tied in a loop. A suture line was tied at the end of the loop. The silicone tubing was then threaded and drawn through a 2-cm plastic cylinder, compressing the artery against the Teflon button. During occlusion, the rectal temperature was maintained at about 37°C with a heating lamp. At the termination of occlusion, the silicone tubing, sutures, and the Teflon buttons were removed, the incision and wound sewed back together. The rats were then placed back into a standard cage to recover from the surgery for 24 hours.

2.3 Hematoxylin Eosin Staining

After slicing the rat brains provided by the principal investigator, it was necessary for the brain slices to undergo a hematoxylin eosin staining process before being able to study the slices. Six sets of coronal sections containing the hippocampus were cut (50 μm) with a vibratome (VT 1000; Leica, Nussloch, Germany) and collected in phosphate-buffered saline (0.01 mol/L PBS,
PH 7.4). The brain slices are put onto clear glass slides and soaked in distilled water, hematoxylin, Eosin solution, alcohol, and xylene solutions. After dried, the slides were examined with a microscope.

2.4 Western Blotting

The macromolecule proteins were separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred to a polyvinylidenedifluoride (PVDF) membrane. Then the membrane was blocked in blocking buffer used to block nonspecific bindings of antibodies for 1 hour. After that, microtubule-associated protein 1 light chain 3 (LC3B, 1:1000, Cell Signal Technology) antibody was used to incubate with the membrane overnight. The next day, the membrane was washed with TBST (25 mM Tris-HCl, pH 7.6, 1.5 M NaCl, and 0.05% Tween20, PH 7.6) three times and incubated with an anti-rabbit HRP-conjugated secondary antibody. The membrane was constantly washed with (TBST). The signals were visualized using chemiluminescence and exposed to X-ray films.

2.5 Immunohistochemistry

The final procedure needed to be done to the slides prior to examining the brain slices is immunohistochemistry (IHC). The sections from the brain were blocked and permeabilized in permeabilization solution (5% goat serum, 0.1% Triton X-100 in PBS) for 1 hour at room temperature. Afterwards, the sections were incubated with an antibody against microtubule-associated proteins 1B (LC3B 1:2000, Cell Signal Technology) in permeabilization solution overnight at 4°C. After being washed, the sections were incubated with biotinylated goat anti-rabbit IgG (1:100; Vector Laboratories, Burlingame, CA,) in blocking solution (5% goat serum in
PBS) for 1 hour at room temperature. After three washes, the sections were processed with ABC and diaminobenzidine (DAB) reaction. All sections within the reaction were exposed to DAB for the same amount of time. Following this, the sections were mounted onto slides, air dried, dehydrated in graded series of ethanol, and infiltrated in xylene. The slides were then examined to identify in autophagy increase levels in different locations of the brain.

2.6 Data Analysis

The values were presented as mean ± SEM. The results were analyzed using one-way ANOVA followed by post hoc Scheffé’s test, Student’s t-test, paired t-test, Chi-square test, or Mann-Whitney U-test (StatView 5.0; Abacus Concepts, Berkeley, CA, USA). Changes were considered significant if P < 0.05.

III. Results

3.1 Glucose levels after streptozotocin injection

After injections of streptozotocin were administered, it took approximately one week for blood glucose levels in the injected rats to rise to approximately 500 mg/dl. The control groups maintained the same blood glucose level.

Figure 1: Graph demonstrates blood glucose levels in rats one week after streptozotocin injection. Control group maintained an average glucose level of 100 mg/dl, while STZ rats averaged 500 mg/dl.
3.2 Increased chance of epileptic seizure after ischemia in diabetic conditions

All 12 rats injected with STZ experienced some form of seizure after a 15 minute ischemia. Of the 11 rats not injected with STZ, none exhibited any signs of epileptic seizures (Figure 2).

![Graph showing seizure rate](image)

**Figure 2**: Graph demonstrates all the rats (12) injected with STZ had some form of seizure. All rats not injected with STZ displayed no signs of epileptic seizure. Ischemia duration for all rats was 15 minutes.

3.3 No apparent neuronal cell death after diabetic ischemia

There were no apparent neuronal cell death after ischemia in diabetic conditions, as shown by almost no change in color nor density after H&E staining. The control group, diabetic group, ischemic group and diabetic ischemic group all seem identical regarding cell death. There were no noticeable changes within the CA1, CA3 or dentate gyrus of the diabetic ischemia group.
Figure 3: Representative microphotographs showing cell neuronal damage in the hippocampus and cortex in the control group, STZ group, ischemic group, and diabetic ischemic group. Photomicrographs of coronal sections (uppermost panels) taken at 4x magnification. Cortex, CA1, DG, and CA3 (lower panels) all taken at 20x magnification.
3.4 Autophagy activity increased in diabetic ischemic group.

Western Blotting was performed in order to detect protein density. The primary antibody LC3B indicates increase or decrease in autophagy levels; an increase in LC3B determines an increase in autophagy levels, while a decrease in LC3B means a decrease in autophagy levels. Figure 3 demonstrates the diabetic or ischemic rats had slight increase in protein expression. However, the diabetic ischemia group exhibited a significant increase in autophagy levels. Immunohistochemistry was also utilized in order to determine protein location within the brain and to confirm the results of the Western Blotting.
**Figure 4** (above): Western Blotting process used in order to detect protein density. A, B: Actinin was used as a loading control to evaluate the increases or decreases in objective protein expression. Left graph depicts protein levels in rat cortex, while right graph depicts the levels in the hippocampus. LC3B levels significantly increased in diabetic ischemic rats. Ratio formed by LC3B-II/LC3B-1 dictates protein expression.
Figure 5 (above): Immunohistochemistry visualizes protein location. C: All photomicrographs above show the LC3B antibody staining. Uppermost panels were taken at 4x magnification; lower panels taken at 40x magnification. Noticeable LC3B staining were observed in ischemia and diabetic ischemia groups in all regions. The dentate gyrus has the most distinct rise in protein amount.

IV. Discussion

Treatments for post-ischemic epileptic patients with diabetes have been limited because of the lack of knowledge regarding the brain’s reaction to diabetic ischemia as well as its pathological mechanisms. The result of this investigation is that while there is a difference in protein densities in within the different groups, there is little to no change when it comes to neuronal cell damage within the different groups. While further investigation in this field is required before making this result a stable finding, this finding does input knowledge into why autophagy and neuronal cell death after ischemia differ depending on diabetic and epileptic seizure conditions.

4.1 Notable Seizures in Diabetic Ischemia

One of the most differentiating results determined from this experiment can be seen in the result of seizures after ischemia in the diabetic ischemic rat group, while no seizures occurred in ischemic rats without diabetes. The seizures occurred at different times, ranging from 1.5 hours to 24.5 hours following ischemia. It can be hypothesized that the lack of insulin in the animal models’
system plays a major role in how the model reacts to the loss of blood in the brain, therefore impacting the chances of seizures.

4.2 Increase in Autophagy Levels

Western blotting in the experiment enabled the identification of the levels of autophagy in each of the four groups. In Figure 3, while it is possible to see that while the diabetic and ischemic experienced a slight increase in autophagy levels, the diabetic ischemic rats experienced the largest increase in autophagy levels. It is necessary to confirm this finding from western blotting with immunohistochemistry (IHC) to identify if the largest increase of protein levels is also located in the diabetic ischemic group. Figure 4 shows the LC3B antibodies at work in the photomicrographs, revealing that the protein densities are greatest in the diabetic ischemia group. This result may also be directly correlated with why rats with diabetes experienced epileptic seizures 15 minutes after ischemia, while rats with only ischemia did not experience any epileptic seizures.

4.3 Neuronal Cell Damage is Dispensable with Autophagy

It was originally hypothesized that the level of cell damage would also increase with the levels in autophagy and protein densities, particularly in the diabetic ischemic rats. However, the result of this experiment shows that there is virtually no noticeable damage in any of the four groups (Figure 2). This result contradicts the results from the western blotting, as the levels of autophagy in the cells were dispensable with cell damage in early on-set of seizures. While one can infer that autophagy activity was increased in diabetic ischemic rats, it is necessary to expand
this experiment to a larger sample size along with reproduction of this experiment before making a factual statement.

Conclusion and Future Work

The present study has examined the possible link between the difference of autophagy levels and neuronal cell death in animal models with diabetes, ischemia, and diabetic ischemia. It was possible to perform this experiment using the four-vessel occlusion (4-VO) model to create the ischemic groups as well as the drug streptozotocin (STZ) to decrease insulin levels in the rats to create a diabetic model group. When comparing the results of the group, it is possible to see that the autophagy levels increased the most in the diabetic ischemic group, despite no apparent cell death in each of the groups. Using this data, it is possible to hypothesize that the difference in glucose levels in the brain alter the way the animal responds to lack of blood flow to the brain. Further research will need to be conducted to understand the role of autophagy in seizure generation after ischemia in diabetic conditions.