Brain ischemia causes delayed neuronal death. The damage is augmented by pre-existing hyperglycemia or diabetes. The mechanisms responsible for diabetes-enhanced ischemic brain damage are not fully defined. Oxidative stress is implicated in a number of neurological disorders including stroke. Selenium is an essential component for antioxidative enzyme, glutathione peroxidase. Selenoprotein P (SelP), which transports selenium, has been reported to protect cells against oxidative stress.

We hypothesized that diabetic ischemia increases neuronal death by augmenting the production of reactive oxygen species, such as \( \cdot \text{O}_2^{-} \) and by suppressing the antioxidant SelP. To test our hypothesis, we first tested for \( \cdot \text{O}_2^{-} \) production by injecting hydroethidine, a fluorescent probe, into rats before they were subjected to a 5-min ischemia under non-diabetic and diabetic conditions. Brains were sectioned after 6h, 1d and 3d recovery. Production of \( \cdot \text{O}_2^{-} \) was detected under fluorescent microscope. We also performed immunohistochemistry and Western blot analysis of SelP.

Our results show that \( \cdot \text{O}_2^{-} \) was increased in the cortical neurons after 3d of recovery in diabetic compared to non-diabetic rats. Double labeling revealed that \( \cdot \text{O}_2^{-} \) was co-localized with a mitochondria marker, suggesting \( \cdot \text{O}_2^{-} \) is produced in the mitochondria. Immunohistochemistry demonstrated that SelP was decreased in the cortex after 3d in diabetic rats. This finding was further verified by Western analysis showing that SelP protein levels were reduced after 3d recovery in diabetic rats, but not in non-diabetic rats. We concluded that hyperglycemia-enhanced ischemic brain damage is mediated by increased production of \( \cdot \text{O}_2^{-} \) and by decreased levels of antioxidative SelP.

**INTRODUCTION**

Stroke is the third leading cause of mortality in the United States and affects about 700,000 Americans each year. In addition, an estimated $40 billion in health-care cost and lost productivity are exhausted annually due to strokes. Ischemic stroke and hemorrhagic stroke are the two main types of strokes. Approximately 80% of all strokes are ischemic strokes, which are the subject of this study. Ischemic strokes occur when a carotid artery is blocked. The blockage of oxygen flow usually leads to neuronal cell damage and death. Once a patient has suffered from an ischemic stroke, doctors usually try to restore blood flow back to the affected part through reperfusion. Although oxygen and blood are re-circulated, reperfusion can result in increased oxygen levels within the brain that cannot be fully utilized by the mitochondrial electron transport chain. As a result, reactive oxygen species (ROS), also known as free radicals, are produced. The overproduction of these free radicals has been known to cause lipid peroxidation and DNA damage, eventually leading to cell death. In normal physiological conditions, free radicals are usually removed by molecular antioxidants such as superoxide dismutase and glutathione peroxidase (GSHPx). During reperfusion however, these antioxidants are depleted because of the overproduction of free radicals.

GSHPx has been proven to contain selenium, an antioxidant trace element that is required for the amino acid, selenocysteine. Selenocysteine is incorporated into selenoproteins and functions as stop codons. The roles of selenium are therefore completed by selenoproteins. There are many types of selenoproteins located in the body. This study focuses on selenoprotein P (SelP), which is synthesized in tissues and secreted into the plasma. What distinguishes SelP from other selenoproteins is that SelP contains 10–17 selenocysteine residues, while other selenoproteins only contain one. High levels of SelP are produced in the liver and SelP is one mode of transportation of selenium to other tissues. SelP is especially known to be a major supplier of selenium to the brain. Even though it is known to deliver selenium to the brain, the accurate overall function of SelP is still vague. Some studies have demonstrated that SelP may contain antioxidant functions; for example, SelP has expressed to protect against liver and kidney necrosis in rats depleted of glutathione. Therefore, SelP has been postulated to contain antioxidant properties as well as being the transporter of selenium.

Pre-existing hyperglycemia or diabetes has been shown to enhance cell death compared to non-diabetic conditions. The mechanisms responsible for this detrimental effect are poorly understood. The aim of this study was to detect the production of ROS, such as \( \cdot \text{O}_2^{-} \) and antioxidant SelP level in the brain after ischemia under diabetic (DM) and non-diabetic (ND) conditions. We hypothesized that diabetic ischemia enhances neuronal death by increasing the production of ROS and by suppressing the antioxidant SelP. We expect that SelP levels will decrease and the \( \cdot \text{O}_2^{-} \) levels will increase significantly after 3 days of recovery after ischemia. We also expect further enhanced damage in the DM conditions.
METHODS

Animal Model
For our ischemic model, we used non-diabetic and diabetic rats that had undergone 5 minutes ischemia, 2 vessel occlusion. Subsequently, they went through 6-hour, 1-day, and 3-day reperfusion. To induce diabetes, the rats were previously injected with 40mg/kg Streptozotocin 4 weeks before the induction of ischemia.

Immunohistography
Immunohistography was conducted on vibratome brain sections collected from 6h, 1d, and 3d of recovery in both NG and DM. The sections were incubated with the primary antibody SelP (mouse IgG, 1:500) and then secondary anti-mouse antibody conjugated with Fluorescein isothiocyanate (FITC, 1:300). The sections were then mounted and counter stained with propidium iodide (PI).

Western Blot Analysis
The Western blot analysis was performed on brain homogenates. Protein content was determined by using the Bradford assay. Equal amounts of protein were loaded on a gel for electrophoresis. After the proteins were separated on the gel, they were transferred to an immobilon-polyvinylidine fluoride membrane. The membrane was incubated with primary antibody SelP (mouse IgG 1:500). Next, the membrane was incubated with peroxidase labeled with anti-mouse antibody. The blots were developed using the enhanced chemiluminescence method.

In Vivo Superoxide Radical Detection
Hydroethidine (HEt) was injected intravenously into the rats 10 minutes before ischemia. HEt is a probe used to detect \(-\text{O}_2^-\). At first, HEt gives off a blue fluorescence, but once HEt meets with \(-\text{O}_2^-\), it exhibits a red fluorescence.

Data Analysis
Optical densities of SelP bonds were analyzed with ANOVA followed by Scheffes test. Student t-test was employed when comparison was made between NG and DM groups at identical reperfusion periods.

RESULTS

SelP/PI
From the immunohistochemistry, we were able to locate SelP in the cortex of the brain, viewed as a green color under a fluorescent microscope. Neurons were marked with PI expressed as red fluorescence. Based on morphology, SelP seems to be located in the glial cells (astrocytes), but not in the neurons.

Western Blot Analysis
The Western blot analysis illustrated that SelP had decreased after 3d recovery in non-diabetic conditions. SelP levels were all the more reduced in the diabetic conditions.

\([-\text{O}_2^-]\)
\(-\text{O}_2^-\) was detected in pre-ischemic conditions as illustrated by red fluorescent shades. A number of \(-\text{O}_2^-\) stained neurons and \(-\text{O}_2^-\) fluorescent intensities were significantly increased in DM rats at 3 days of recovery.

CONCLUSION

In this study, our goal was to detect the production of ROS, such as \(-\text{O}_2^-\), and antioxidant SelP level in the brain after ischemia and reperfusion under diabetic and non-diabetic conditions. We were able to locate SelP in the brain from our immunohistochemistry and found that SelP might be located in astrocytes and not in the nucleus. SelP levels were reduced after normoglycemic ischemia; diabetes further decreased the level of SelP as seen in the Western blot analysis. The free radical detection using HEt had shown that \(-\text{O}_2^-\) in the brain is increased significantly after ischemia. Therefore, we can conclude that diabetes-enhanced ischemic brain damage may be mediated by increased production of \(-\text{O}_2^-\) and by decreased levels of the antioxidant SelP.

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