# Hyperglycemic Ischemia Increases the Production of Superoxide Free Radicals and Decreases the Level of Antioxidative Selenoprotein P

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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 neuronal disorders including stroke. Selenium is an essential component for antioxidative enzyme, glutathione peroxidase. Selenoprotien 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 $\cdot O_2^-$ and by suppressing the antioxidant SelP. To test our hypothesis, we first tested for $\cdot O_2^-$ 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 $\cdot O_2^-$ was detected under fluorescent microscope. We also performed immunohistochemistry and Western blot analysis of SelP.

Our results show that $\cdot O_2^-$ was increased in the cortical neurons after 3d of recovery in diabetic compared to non-diabetic rats. Double labeling revealed that $\cdot O_2^-$ was co-localized with a mitochondria marker, suggesting $\cdot O_2^-$ 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 $O_2^-$ and by decreased levels of antioxidative SelP.

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## Introduction

Stroke is the third leading cause of mortality in the United States and affects about 700,000 Americans each year. In addiction, 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 selenoprotien 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 selenoprotiens 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 $\cdot O_2$, 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 $\cdot O_2$- 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 $\cdot O_2^-$. At first, HEt gives off a blue fluorescence, but once HEt meets with $\cdot O_2^-$, 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.

### $\cdot O_2^-$

$\cdot O_2^-$ was detected in pre-ischemic conditions as illustrated by red fluorescent shades. A number of $\cdot O_2^-$ stained neurons and $\cdot O_2^-$ 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 $\cdot O_2^-$, 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 nomoglycemic ischemia; diabetes further decreased the level of SelP as seen in the Western blot analysis. The free radical detection using HEt had shown that $\cdot O_2^-$ 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 $\cdot O_2^-$ and by decreased levels of the antioxidant SelP.

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