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Changes in secretory pathway Ca^{2+}-ATPase 2 following focal cerebral ischemia/reperfusion injury☆

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Abstract

This study aimed to investigate changes in secretory pathway Ca^{2+}-ATPase 2 expression following cerebral ischemia/reperfusion injury, and to define the role of Ca^{2+}-ATPases in oxidative stress. A rat model of cerebral ischemia/reperfusion injury was established using the unilateral middle cerebral artery occlusion method. Immunohistochemistry and reverse transcription-PCR assay results showed that compared with the control group, the expression of secretory pathway Ca^{2+}-ATPase 2 protein and mRNA in the cerebral cortex and hippocampus of male rats did not significantly change during the ischemic period. However, secretory pathway Ca^{2+}-ATPase 2 protein and mRNA expression reduced gradually at 1, 3, and 24 hours during the reperfusion period. Our experimental findings indicate that levels of secretory pathway Ca^{2+}-ATPase 2 protein and mRNA expression in brain tissue change in response to cerebral ischemia/reperfusion injury.

Key Words

neural regeneration; brain injury; cerebral infarction; secretory pathway Ca^{2+}-ATPase 2; Golgi apparatus; Ca^{2+} oscillations; manganese; focal cerebral ischemia; oxidative damage; Ca^{2+}-ATPase; grants-supported paper; photographs-containing paper; neuroregeneration

Research Highlights

(1) Expression of secretory pathway Ca^{2+}-ATPase 2 in the rat brain cortex and hippocampus was down-regulated following ischemia/reperfusion injury.
(2) Levels of secretory pathway Ca^{2+}-ATPase 2 in brain tissue changed in response to cerebral ischemia/reperfusion injury.

Abbreviations

SPCA, secretory-pathway Ca^{2+}-ATPase; MCAO, middle cerebral artery occlusion

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INTRODUCTION

The Golgi apparatus is a Ca^{2+} and Mn^{2+}-containing intracellular organelle that plays a major role in intracellular Ca^{2+} and Mn^{2+} homeostasis. Accumulation of Ca^{2+} in the Golgi apparatus is mediated by the sarco (endo) plasmic reticulum Ca^{2+}-ATPase and secretory-pathway Ca^{2+}-ATPase (SPCA)[1]. SPCA pumps mainly contribute to the decrease in cytoplasmic Ca^{2+} concentration though the removal of the activator Ca^{2+} from the cytoplasm after stimulation[2]. In addition to Ca^{2+}, the SPCA pumps and the high affinity Ca^{2+}/Mn^{2+} P-type ATPase, Pmr1p, can also transport Mn^{2+}, which is involved in post-translational modification and vesicle packaging in the secretory pathway[3-4]. There are two types

of SPCAs: SPCA1 and SPCA2. SPCA1 is widely expressed in all tissues as a housekeeping enzyme, and is highly expressed. Recent experiments demonstrated that cerebral ischemia/ reperfusion injury induced a reduction in SPCA1 activity and lipid protein oxidation in rat hippocampal membranes, and that ischemia/reperfusion injury also activated the induction of SPCA1 gene expression in the reperfusion period[5].

However, there has been no research to date that has investigated whether the ischemic insult affects mRNA and protein expression levels of SPCA2. There is also no information available as to whether SPCA2 is present in the cortex of the brain. The answer to these questions may provide further insights into the role of Ca^{2+}-ATPases in the mechanism of oxidative stress. The objective of this research was to investigate the mechanism of oxidative stress in the brain following an ischemic insult by examining SPCA2 mRNA expression in the cortex of the brain, and to analyze protein expression levels of SPCA2 after an ischemic insult. mRNA expression in the cortex was examined using quantitative reverse transcription-PCR, while SPCA2 protein levels were analyzed by immunohistochemistry.

RESULTS

Quantitative analysis of experimental animals

Sixty male Sprague-Dawley rats were randomly divided into five groups: sham-surgery group, 90-minute ischemia group, ischemia and reperfusion groups (90-minute ischemia followed by 1-, 3-, 24-hour reperfusion), with 12 rats in each group. In addition, a control group (n = 6) was also included. Six rats in each group were used for the detection of real-time fluorescence quantitative PCR, while the remaining six rats in each group were used for immunohistochemistry. In total, 60 Sprague-Dawley rats were involved in the final analysis, without any dropout or loss.

Successful middle cerebral artery occlusion (MCAO) model confirmed by 2,3,5-triphenyltetrazolium chloride staining

2,3,5-Triphenyltetrazolium chloride staining revealed that transient MCAO after 24-hour ischemia (no reperfusion) produced large apparent ischemic lesions in the basal ganglia and adjacent cerebral cortex (Figure 1).

mRNA levels of SPCA2 Ca^{2+} pump in the cerebral cortex decreased after ischemia/reperfusion injury

Quantitative PCR was used to analyze SPCA2 Ca^{2+} pump mRNA levels. SPCA mRNA expression was detected in areas with ischemia/reperfusion injury using reverse transcription- PCR (Figure 2). Compared with the sham-surgery group, the level of SPCA2 mRNA expression was not different after 90 minutes of ischemia with no reperfusion (P > 0.05). However, cells in the cortex reacted to ischemic injury as observed by a reduction in SPCA2 gene expression in the reperfusion period. mRNA levels of SPCA2 decreased in a time-dependent manner, with a decrease of 44% when compared to the sham control after 1 hour reperfusion (P < 0.05), a decrease of 33% after 3 hours of reperfusion (P < 0.05), and a decrease of 31% after 24 hours of reperfusion (P < 0.05). In the ischemia and of reperfusion groups, SPCA2 mRNA expression levels gradually decreased as reperfusion proceeded, and the minimum value was observed at 24 hours of reperfusion (P < 0.05; Figure 2).

Figure 1 The infarction region visualized by 2,3,5-triphenyltetrazolium chloride staining.

Photographs are shown at 24 hours after cerebral ischemia. Arrow indicates the region of cerebral infarction.

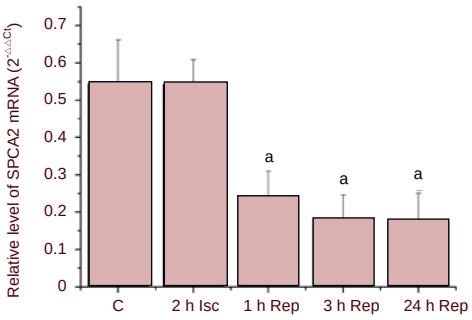

Figure 2 Expression of secretory pathway Ca^{2+}-ATPases 2 (SPCA2) mRNA in the rat brain after 120 minutes of ischemia followed by 0 (ischemia only), 1, 3, and 24 hours of reperfusion.

[a]P < 0.05, *vs*. sham-surgery group (C group). Data are expressed as mean ± SEM of six rats in each group. One-way analysis of variance followed by Fisher's least significant difference test was used for statistical analysis. Isc: Ischemia; Rep: reperfusion; h: hours.

Protein levels of SPCA2 Ca^{2+} pump in the cortex and hippocampus decreased after ischemia/reperfusion injury

Immunohistochemical analysis was used to evaluate whether mRNA expression changes induced by ischemia

also affected SPCA2 protein levels. Expression of SPCA2 protein in the cerebral cortex and hippocampus appeared similar to that of mRNA levels and were not different compared with the sham-surgery group (P > 0.05). SPCA2 protein expression also decreased in the later reperfusion period, with a significant decrease of 30% compared with the sham-surgery group after 1 hour of reperfusion (P < 0.05), a 38% decrease after 3 hours of reperfusion (P < 0.05), and a 33% decrease after 24 hours of reperfusion (P < 0.05). A small but not significant increase was observed during reperfusion in the 3-hour group (P > 0.05). In the ischemia and reperfusion groups, the level of SPCA2 protein expression gradually decreased as reperfusion proceeded (P < 0.05; Figures 3, 4).

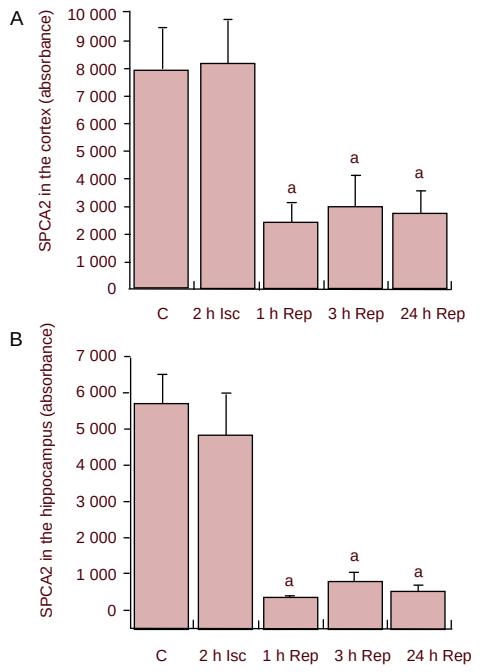

Figure 3 Protein levels of secretory pathway Ca^{2+}-ATPases 2 (SPCA2) in the rat brain cortex (A) and hippocampus (B) after 90-minute ischemia and 0, 1, 3, and 24 hours of reperfusion.

[a]P < 0.05, *vs*. sham-surgery group (C group). Data are presented as mean ± SEM of six rats in each group. One-way analysis of variance followed by Fisher's least significant difference test was used for statistical analysis. Isc: Ischemia; Rep: reperfusion; h: hours.

DISCUSSION

Intracellular Ca^{2+} stores play an important role in cytosolic Ca^{2+} signaling[6-8]. SPCA represents a recently recognized family of phosphorylation-type ATPases that supply the lumen of the Golgi apparatus with Ca^{2+} and Mn^{2+}, ions needed for normal organelle functioning[9-10].

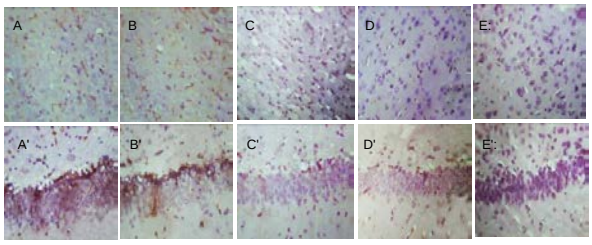

Figure 4 Expression of secretory pathway Ca^{2+}-ATPases 2 in the cortex and hippocampus of the rat brain following cerebral ischemia reperfusion (DAB staining, light microscope, × 200).

(A, A') Sham-surgery group; (B, B') 2-hour ischemia group; (C, C') ischemia + 1 hour of reperfusion, (D, D') 3 hours of reperfusion; (E, E') 24 hours of reperfusion. Upper: Brain cortex; lower: hippocampus. Secretory pathway Ca^{2+}-ATPases 2 protein expression decreased during the reperfusion period compared with the sham-surgery group.

SPCA pumps contribute to decreasing the cytoplasmic Ca^{2+} concentrations through removing the activator Ca^{2+} from the cytoplasm to the Golgi apparatus after stimulation[2]. In addition to Ca^{2+}, SPCA pumps can also transport Mn^{2+}, which is involved in posttranslational modification and vesicle packaging in the secretory pathway[11-12]. Also, ATP2C1 knockout mice showed an increase in apoptosis and Golgi apparatus structural changes[13]. There is evidence that cell death occurs a few days after transient ischemia follows from a Ca^{2+} overload, however, the mechanisms underlying this selective neuronal damage are not understood[14-16]. This hypothesis of Ca^{2+} involvement in neuronal cell death has received support from experiments conducted during the period of ischemia and immediately afterwards[17-18].

Our current research seeks to determine whether ischemia/reperfusion injury can affect the physical and functional properties of hippocampal and cortical membrane vesicles including the membrane of the Golgi apparatus. Neuronal microsomes are vulnerable to functional and physical oxidative stress damage[19-21], and the nature of the free radical effect on the SPCA2 protein is not yet known.

We show here for the first time that SPCA2 exists in the brain cortex[22]. The SPCA2 gene in the brain cortex was amplified by quantitative reverse transcription-PCR, and the expression of the SPCA2 protein in the rat brain was observed using immunohistochemical techniques. According to our research, the activity of the SPCA2 gene was selectively damaged by free radicals *in vitro*, a property that is similar to other P-type ATPases such as

PMCA and sarco (endo) plasmic reticulum Ca^{2+}-ATPase[23]. Although SPCA2 mRNA and protein levels were not altered during the ischemic period ($P > 0.05$), focal ischemia/reperfusion injury in rats *in vivo* caused a significant reduction in SPCA2 mRNA and protein levels during the reperfusion period ($P < 0.01$). It is possible that during the reperfusion periods, lower SPCA2 activity reduced Ca^{2+} uptake and thereby overloaded cytoplasmic [Ca^{2+}], which is one of the consequences of oxidative stress[2, 21, 24]. The Golgi apparatus Ca^{2+}/Mn^{2+} ATPases have been shown to be important players in ion regulation, and oxidative stress affects cell ion homeostasis considerably; therefore, Golgi apparatus Ca^{2+}/Mn^{2+} dyshomeostasis, in response to oxidative stress, is likely to occur. In addition, other studies have demonstrated that reactive oxygen species contribute to neuronal cell injury secondary to ischemia and reperfusion[25]. Reactive oxygen species generated by the mitochondrial electron transport chain escape cellular antioxidant defenses and promote highly damaging hydroxyl radical activity after transient brain ischemia in the rat[26]. This may also explain the results observed in this study.

SPCA2 can transport 1 Mn^{2+} into the Golgi apparatus per ATP consumed. During ischemia/reperfusion, the role of Golgi apparatus-mediated Mn^{2+} dyshomeostasis is biphasic. Intracellular Mn^{2+} is known to suppress oxidative stress[27-29], but Mn^{2+} not only functions as a cofactor for antioxidant enzymes such as superoxide dismutases, catalases, and peroxidases, but also acts as a scavenger of superoxide radicals[30-32]. Therefore, the decrease in SPCA2 observed in our experiments likely protects against oxidative stress by reducing Mn^{2+} uptake, and thereby increases the levels of Mn^{2+} in the cytoplasm, although this explanation may have overshadowed the otherwise protective function conferred by SPCA2. Under these conditions, Mn^{2+} can be referred to as an antioxidant. However, high concentrations of cytosolic Mn^{2+} are toxic[33]. At the subcellular level, Mn^{2+} may accumulate or increase nitric oxide production in mitochondria, where it disrupts oxidative phosphorylation and increases the generation of reactive oxygen species[34-35]. Exposure to excessive Mn^{2+} levels is known to play a role in the development of Parkinson's disease and neurotoxicity to the extrapyramidal system[33, 36].

In addition, the level of SPCA2 protein increased slightly after 3 hours of reperfusion, although the changes were not statistically significant. One possible explanation comes from a study describing the upregulation of defense mechanisms (antioxidant enzymes) against oxidative stress due to the preconditioning challenge[5].

Our research provides useful information for determining the pattern of SPCA2 expression in the brain cortex. Lower SPCA2 activity during reperfusion periods reduces Ca^{2+} uptake and increases cytoplasmic [Ca^{2+}], producing overload conditions, which is one of the consequences of oxidative stress. Our results suggest a molecular mechanism by which damage occurs due to oxidative stress.

However, it is still not clear whether the change in Ca^{2+}/Mn^{2+} homeostasis or oxidative stress represents the manifestation of another pathological process or if these changes are a primary phenomenon; some models indicate that changes in [Ca^{2+}] are the primary phenomenon and oxidative stress is a consequence of this[37]. The possible physiological processes of SPCA2 are still unclear, and further research is required to understand the role of SPCA2 in ischemia/reperfusion injury in the brain.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

This experiment was performed in the Central Laboratory of the Second Xiangya Hospital in Central South University, China during March 2011 to December 2011.

Materials

Animals were provided by the Central Animal Care of Central South University, Changsha, China (license No. SCXK (Xiang) 2009-0004). Sixty male Sprague-Dawley rats weighing 200–250 g were given free access to food and water and kept in a 12-hour light/dark cycle at 22.0 ± 1.0°C. All rats received humane care in compliance with the guidelines of Central South University of China. The treatment of animals conformed to the *Guidance Suggestions for the Care and Use of Laboratory Animals*, published by the Ministry of Science and Technology of China[38].

Methods

Establishment of MCAO models

The rat model of focal cerebral ischemia-reperfusion was used, and rats were treated according to a previously

published method[39]. Briefly, male Sprague-Dawley rats were anesthetized with 10% (v/v) chloral hydrate (3.5 mL/100 g, intraperitoneal injection). After the right common carotid artery was exposed through a midline neck incision, the common carotid artery, external carotid artery and internal carotid artery were carefully separated from the adjacent tissue and vagus nerve. Next, a 4-0 silk suture was tied loosely around the mobilized external carotid artery stump. A 4-cm length of fish wire was inserted through the incision of the common carotid artery into the internal carotid artery and then into the circle of Willis, effectively occluding the middle cerebral artery. The silk sutures around the external carotid artery were lifted until the suture reached the middle cerebral artery. The silk suture around the common carotid artery stump was then tightened around the intraluminal occluders, which were made of fish wire, to prevent bleeding. The diameter of the tip was 0.26 mm for rats below 250 g and 0.28 mm for rats above 250 g. After 90 minutes of MCAO, the fish wire was withdrawn from the internal carotid artery into the stump of the common carotid artery, stopping as soon as resistance was felt. Reperfusion then started in the ischemic brain area. The animals were then returned to their cages after recovering from anesthesia and allowed to recover, with free access to food and water. Sham control animals (n = 6) were prepared in the same way without carotid occlusion.

The rats underwent 90 minutes of ischemia (n = 6), followed by 1, 3, and 24 hours of reperfusion (n = 6 each). Neurological examinations were performed 90 minutes after ischemia. The neurological findings were scored on a five-point scale: a score of 0 indicated no neurological deficit, a score of 1 (failure to extend left forepaw fully) indicated a mild focal neurological deficit, a score of 2 (circling to the left) revealed moderate focal neurological deficit, and a score of 3 (falling to the left) indicated a severe focal deficit; rats with a score of 4 did not walk spontaneously and had a depressed level of consciousness. Rats scoring 2–3 were chosen for the study.

Identification of models

At 24 hours after ischemia, histological staining was performed using 0.2% (w/v) 2,3,5-triphenyltetrazolium chloride. Normal tissues were red, while ischemic tissue was pale. Brains were cut into approximately 2-mm thick coronal slices starting 1 mm from the frontal pole. The infarct region was visualized by staining with 2,3,5-triphenyltetrazolium chloride solution for 15–30 minutes at 37°C under dark conditions. The stained sections were then photographed.

Real-time fluorescence quantitative PCR assay for SPCA2 gene changes

Real-time PCR and data analysis were performed in a total volume of 20 μL using 48-well micro well plates and a StepOne™ Real-Time PCR System (Applied BioSystems, Foster City, CA, USA). RNA was extracted from the cortex of the experimental animals under RNAse-free conditions. RNA was extracted using Trizol (Beijing CoWin Biotech Co., Ltd., Beijing, China) and phenol-chloroform with subsequent reverse transcription. Reverse transcription was performed using the PrimeScript RT reagent Kit system (TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, Liaoning Province, China) with 1 μg of RNA. Multiplex PCR for SPCA2 and β-actin was carried out with 500 ng of each cDNA in a final volume of 10 μL containing 2 μL of 5 × PrimeScript Buffer (for Real Time), 0.5 μL PrimeScript RT Enzyme Mix 1, 0.5 μL Oligo dT Primer (50 μM), and 0.5 μL Random 6 mers (100 μM). The forward and reverse primers used to amplify SPCA2 and β-actin are shown in Table 1.

Table 1 Oligonucleotide primers for real-time reverse transcription-PCR

Gene	Sequences (5'–3')
β-actin	Forward: GGA GAT TAC TGC CCT GGC TCC TA
	Reverse: GAC TCA TCG TAC TCC TGC TTG CTG
SPCA2	Forward: CTC TCG GTT GCT GTA ACG TCA T
	Reverse: CCC TTC GCC ACT GTA TCC AAT

The primers were all purchased from Invitrogen, China. The PCR reaction conditions were: 30 seconds of denaturation at 95°C, followed by 45 cycles of 5 seconds of denaturation at 95°C, and 31 seconds of annealing at 62°C. The $2^{-\Delta\Delta Ct}$ method was used to determine the different expression levels.

Immunohistochemistry for SPCA2 protein changes

For immunohistochemical analysis, animals were anesthetized with 10% (v/v) chloral hydrate (3.5 mL/100 g body weight), followed by 4% (v/v) polyoxymethylene solution in 0.1 M PBS (pH 7.2). Brains were removed and fixed in 4% (v/v) polyoxymethylene solution for 8–12 hours at 4°C. Tissue samples were dehydrated and embedded in paraffin following routine procedures[40]. After standard histological processing and embedding in paraffin, 4-mm-thick sections were prepared. All the following steps were carried out in a moist chamber. Deparaffinized and rehydrated sections were processed as follows: 2 × 10 minutes with xylene; 3 × 2 minutes with 100% (v/v) ethanol; 2 minutes with 80% (v/v) ethanol; 2 minutes with 70% (v/v) ethanol; and 3 × 3 minutes with PBS. Sections were then blocked for 10 minutes with 3% (v/v) peroxide at

room temperature for endogenous peroxidase ablation. The method of antigen retrieval is shown below: sections were fixed in citric acid-citrate buffer solution (pH 6.0) for 15–20 minutes at 92–98°C. The sections were incubated with a rabbit anti-SPCA2 polyclonal antibody (1:50, sc-134761; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 6–8 hours at 4°C. The sections were then incubated for 30 minutes at 37°C with goat anti-rabbit IgG (PV-6001; Beijing Zhongshan Golden Bridge Company, China). Diaminobenzidine staining was enhanced by treating the sections for 10 seconds with DAB Enhancing Solution (Beijing Zhongshan Golden Bridge Company). Sections were washed, counterstained with Gill's hematoxylin, cleared, mounted with Aquamount (Polysciences), and then examined by light microscopy with a Motic 5000 microscope (Berlin, Germany).

Protein determination

Five sections of each brain tissue sample were selected for image analysis. Under the optical microscope (magnification × 200), two areas of the frontal cortex and hippocampus were selected for imaging. The locations of the chosen field of vision of each slice were consistent. Image Pro-Plus 6.0 (Media Cybernetics, Silver Spring, USA) software was used for analysis after the photos had been inputted into the computer. The software measured the integrated absorbance of positive cells (part of the yellow) in each picture.

Statistical analysis

Data are expressed as mean ± SEM. All statistical analyses were performed using SPSS Statistics 17.0 (SPSS, Chicago, IL, USA). One-way analysis of variance followed by the Fisher's least significant difference test was used for statistical analysis to determine significant differences between the control, ischemic and reperfusion groups. A *P*-value less than 0.05 was considered significant.

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Conflicts of interest: None declared.
Ethical approval: The experimental procedures were approved by the Experimental Animal Ethics Committee of Central South University in China.
Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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