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Changes in the expression of the B subunit of vacuolar H+-ATPase, in the hippocampus, following transient forebrain ischemia in gerbils

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ABSTRACT

Objective(s): Vacuolar H+-ATPase is a highly conserved enzyme that plays an important role in maintaining an acidic environment for lysosomal function and accumulating neurotransmitters in synaptic vesicles. In the present study, we investigated the time-dependent changes in the expression of vacuolar H+-ATPase V_1B_2 (ATP6V1B2), a major neuronal subtype of vacuolar H+-ATPase located in the hippocampus, after 5 min of transient forebrain ischemia in gerbils. We also examined the pH and lactate levels in the hippocampus after ischemia to elucidate the correlation between ATP6V1B2 expression and acidosis.

Materials and Methods: Transient forebrain ischemia was induced by occlusion of both common carotid arteries for 5 min and animals were sacrificed at various time points after ischemia for immunohistochemical staining of ATP6V1B2 and measurements of pH and lactate levels in the hippocampus.

Results: ATP6V1B2 immunoreactivity was found to be transiently increased in the hippocampal CA1 region and dentate gyrus 12–24 hr after ischemia when the pH and lactate levels were decreased. In addition, ATP6V1B2 immunoreactivity significantly increased in the hippocampal CA3 and dentate gyrus, regions relatively resistant to ischemic damage, 4 days after ischemia, when the NeuN-positive, mature neuron numbers were significantly decreased in the hippocampal CA1 region.

Conclusion: These results suggest that ATP6V1B2 expression is transiently increased in the hippocampus following ischemia, which may be intended to compensate for ischemia-related dysfunction of ATP6V1B2 in the hippocampus.

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Introduction

Stroke is a major neurological disorder that leads to death or reduced quality of life worldwide (1). Transient obstruction of blood vessels or cardiac arrest increases the concentration of reactive oxygen species that destabilize lysosomes and activate the apoptotic signal (2, 3). In addition, transient forebrain ischemia causes morphological changes in astrocytes and microglia in the early stages before neuronal death (4, 5). Furthermore, a review paper suggests a relationship between lysosomal dysfunction and reactive astrocytosis following ischemia (6). Lysosomal membrane permeabilization is increased after ischemia (7), and redistribution of lysosomal proteins, such as cathepsin D and prosaposin, takes place in the hippocampus following ischemia in gerbils (8) and hypoxia-ischemia in rats (9).

The vacuolar H+-ATPase plays a critical role in cell function by enabling transmembrane movement of protons for maintaining an acidic environment (pH 4.2 – 5.3) for lysosomal function, with free energy from ATP hydrolysis (10, 11). In addition, vacuolar H+-ATPase consists of several large subunits with two domains: a transmembrane V_0 domain and a cytosolic V_1 domain (12). Among these vacuolar H+-ATPase subunits, subunit B has two isoforms. While the B_1 isoform is mainly expressed in the kidney and epididymis, B_2 is found in the brain (13-15). However, genetic inhibition of V_1B_1 in mice up-regulates the expression of the V_1B_2 isoform

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to promote vacuolar H^+-ATPase assembly (16). In contrast, patients with mutant vacuolar H^+-ATPase V_1B_2 (ATP6V1B2) show dominant deafness-onychodystrophy syndrome and Zimmermann-Laband syndrome characterized by deafness and digital abnormalities, respectively (17). In addition, the ATP6V1B2 mutation impairs lysosomal acidification due to reduced vacuolar H^+-ATPase function (18). Several recent studies also demonstrated that ATP6V1B2 is involved in various diseases, including neurological disorders (19-22). However, the association between ATP6V1B2 expression and neurodegeneration is controversial. Fibroblasts from patients with Alzheimer's disease showed an increase in ATP6V1B2 expression, although lysosomal acidification was found to be impaired (19). According to another study, ATP6V1B2 expression in the cortex decreased with age and Alzheimer's disease in mouse models (21). In a previous study, we observed that pyridoxine deficiency significantly decreased novel object recognition memory and differentiated neuroblast and ATP6V1B2 expressions in the hippocampus (23), suggesting that ATP6V1B2 plays an important role in hippocampal function in animals. However, few studies have been conducted on the changes in the expression of ATP6V1B2 in the hippocampus following transient forebrain ischemia.

Therefore, in the present study, we investigated the spatial and temporal changes in ATP6V1B2 immunoreactivity and its association with parameters related to acidic environments, such as pH and lactate levels, in gerbil hippocampus after ischemia.

Matrials and Methods

Experimental animals

Male and female Mongolian gerbils were purchased from Japan SLC, Inc. (Shizuoka, Japan) and mated. The animals were housed and cared for based on the Guide for the Care and Use of Laboratory Animals (8th edition, 2011). The experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Seoul National University. An effort was made to reduce the number of animals and minimize the suffering caused by procedures employed in the present study.

Ischemic surgery

Six-month-old male gerbils were anesthetized using a mixture of 2.5% isoflurane (Baxter, Deerfield, IL, USA) in 33% oxygen and 67% nitrous oxide. Transient ischemic surgery was conducted, as described in previous studies (24, 25). Briefly, both common carotid arteries were isolated and occluded for 5 min with non-traumatic aneurysm clips, and the blood circulation in the brain was assessed from the central artery of the retinae using an ophthalmoscope (HEINE K180®; HEINE Optotechnik, Herrsching, Germany). During and after ischemic surgery, the body temperature was strictly controlled using a thermometric blanket (37 ± 0.5 °C) with a rectal temperature probe (TR-100; Fine Science Tools, Foster City, CA, USA) and isothermal incubator.

Immunohistochemistry

Control (n = 5) and ischemic animals (n = 5 in each time point) were anesthetized using a mixture of alfaxalone (Alfaxan, 75 mg/kg; Careside, Seongnam, South Korea) and xylazine (10 mg/kg; Bayer Korea, Seoul, South Korea) at 1, 2, 4, and 10 days after ischemia/reperfusion and perfused transcardially, as described in previous studies (23, 24). The brain was removed, and serial 30-μm-thick sections were cut from an area 2.0–2.7 mm caudal to the bregma, based on a gerbil atlas (26). Five sections (located 120-μm apart from each other) were selected and incubated with mouse anti-NeuN antibody (1:1000; Merck Millipore, Temecula, CA, USA) or rabbit anti-ATP6V1B2 (1:2000; Abcam, Cambridge, UK). Brain sections were sequentially incubated with anti-rabbit or anti-mouse IgG and peroxidase-conjugated streptavidin (Vector, Burlingame, CA, USA). NeuN or ATP6V1B2 immunoreactive structures were visualized with reaction to 3,3-diaminobenzidine tetrachloride (Sigma, St. Louis, MO, USA) in 0.1 M Tris-HCl buffer (pH 7.2). Sections were dehydrated and mounted on gelatin-coated slides in Canada balsam (Kanto Chemical, Tokyo, Japan).

Five sections located 120-μm apart from each other located 2.0–2.7 mm caudal to the bregma, based on a gerbil atlas (26), were used for calculating the immunodensity of ATP6V1B2 in the hippocampal CA1, CA3, and dentate gyrus. The midpoints of the CA1, CA3, and dentate gyrus were captured with a BX51 light microscope (Olympus, Tokyo, Japan) equipped with a digital camera (DP72, Olympus), and the images were converted to grayscale. The unlabeled structures were removed using Photoshop CC software (Adobe Systems Inc., San Jose, CA, USA), the pixels and intensity of stained structures were measured using ImageJ v. 1.80 software (National Institutes of Health), and the optical density was added up by each pixel × intensity for each image. The data are expressed as percentage of the sham-operated group values (set to 100%).

pH and lactate levels

Control (n = 5) and ischemic animals (n = 5 in each time point) were sacrificed using a mixture of 75 mg/kg alfaxalone and 10 mg/kg xylazine at 3 hr, 12 hr, 1 day, 2 days, and 4 days after ischemia/reperfusion. Both hippocampal tissues were quickly frozen with liquid nitrogen. Hippocampal pH and lactate levels in the hippocampus were measured by a spectrophotometric method, as described in a previous study (27). Commercially available kits were used to measure intracellular pH (Merck, Darmstadt, Germany) and lactate levels (Abcam, Cambridge, UK) according to the manufacturer's protocol.

Statistical analysis

The data obtained represent the mean with standard deviation and differences among means were statistically analyzed using the one-way analysis of variance (ANOVA) test, followed by Bonferroni *post-hoc* tests using GraphPad Prism 5.01 software (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was set at $P<0.05$.

Results

Transient forebrain ischemia induces neuronal death in the hippocampus

In the sham-operated control group, NeuN-positive

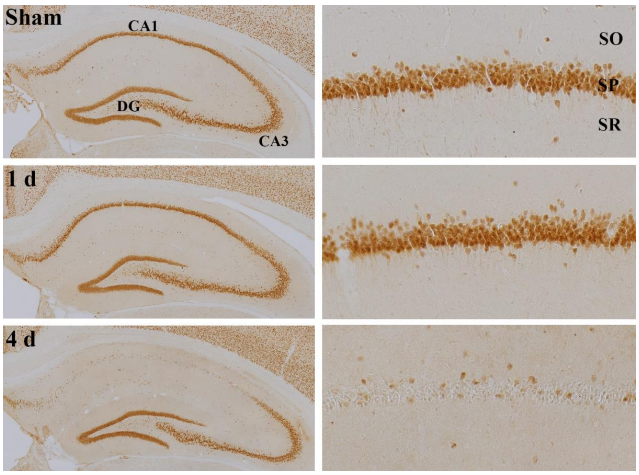

Figure 1. Microphotographs of NeuN immunoreactivity in the whole hippocampus and magnified hippocampal CA1 region in sham- and ischemia-operated groups. In the sham-operated group, NeuN-positive nuclei were found in the hippocampal CA1, CA2, CA3, and dentate gyrus (DG). In the hippocampal CA1 region, NeuN-positive nuclei were abundantly found in the stratum pyramidale (SP), but only a few NeuN-positive nuclei were detected in the stratum oriens (SO) and radiatum (SR). Note that NeuN immunoreactive structures were also found in the hippocampus 1 day after ischemia, but only a few NeuN-positive nuclei were found in the SP of hippocampal CA1 region 4 days after ischemia. Scale bar = 400 μm (left), 50 μm (right)

nuclei were abundantly observed in all hippocampal regions including CA1, CA3, and dentate gyrus. In the hippocampal CA1 region, most neurons were found in the stratum pyramidale, but a few neurons were also found in the stratum oriens and radiatum. One day after ischemia, the distribution pattern of NeuN-positive nuclei was similar to that observed in the sham-operated group. Four days after ischemia, many NeuN-positive nuclei were found in the hippocampal CA3 region and dentate gyrus, while in the CA1 region, a few NeuN-positive nuclei were found in the stratum pyramidale (Figure 1).

Transient forebrain ischemia transiently increases ATP6V1B2 in the hippocampus

In the sham-operated control group, ATP6V1B2 immunoreactivity was mainly found in the stratum pyramidale of the hippocampal CA1 and CA3 regions. In addition, ATP6V1B2 immunoreactivity was found in the stratum radiatum of CA1 and CA3 regions and the inner molecular layer of the dentate gyrus. The distribution pattern of the ATP6V1B2 immunoreactivity did not show significant changes after ischemia, but ATP6V1B2 immunoreactivity was found to be significantly increased in the hippocampus after ischemia. One day after ischemia, ATP6V1B2 immunoreactivity was significantly increased in the hippocampal CA1 region and dentate gyrus and returned to its control levels 2 days after ischemia in all regions. Four days after ischemia, ATP6V1B2 immunoreactivity was markedly increased in the CA3 region and dentate gyrus compared with that in the control group. Ten days after ischemia, ATP6V1B2 immunoreactivity was dramatically decreased in the hippocampal CA1 region, but not in the CA3 or dentate gyrus (Figure 2).

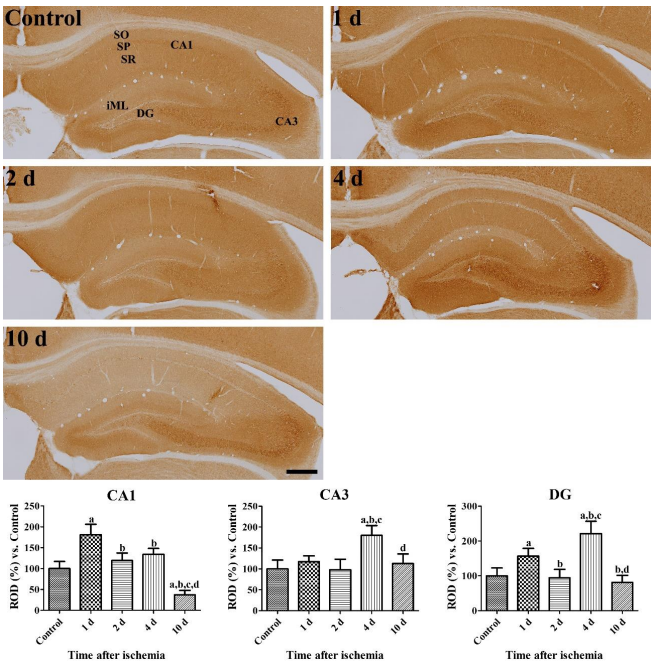

Figure 2. Microphotographs of ATP6V1B2 immunoreactivity in the hippocampus of sham- and ischemia-operated groups. ATP6V1B2 immunoreactive structures were mainly observed in the stratum pyramidale (SP) of hippocampal CA1 and CA3 regions. In addition, ATP6V1B2 immunoreactivity was found in the stratum radiatum (SR) of CA1 and CA3 regions and the inner molecular layer (iML) of the dentate gyrus. Note that strong ATP6V1B2 immunoreactive structures were found in the SR of CA1 region and iML of dentate gyrus 1 day after ischemia and also in the SP of CA3 region and polymorphic layer (PoL) of dentate gyrus 4 days after ischemia. Scale bar = 400 μm. Relative optical densities (ROD) are expressed as a percentage of the value of ATP6V1B2 immunoreactivity in the sham-operated group in CA1, CA3, and dentate gyrus per section of sham-operated and ischemia-operated groups, respectively (n=5 in control or at each time point in the ischemic group; the data were analyzed by one-way analysis of variance followed by a Bonferroni's post-hoc test, aP<0.05, which indicates significant difference from the sham-operated control group, bP<0.05, which indicates significant difference from the 1-day post-ischemic group, cP<0.05, which indicates significant difference from the 2-day post-ischemic group, dP<0.05, which indicates significant difference from the 4 days post-ischemic group). The bars indicate the mean values ± standard deviation

Transient ischemia changes pH and lactate levels in the hippocampus

In the sham-operated group, the pH level in the hippocampal homogenates was 7.17 ± 0.08, and pH levels decreased in a time-dependent manner within 4 days after ischemia/reperfusion. One-way ANOVA indicated that pH levels significantly decreased from 12 hr after ischemia/reperfusion compared with that in the sham-operated group.

The lactate level in the sham-operated group was 0.85 ± 0.21 nmol/mg and lactate levels dramatically increased 3 hr after ischemia to 747.49% of the sham-operated group. Lactate levels were observed to be significantly decreased 12 hr after ischemia (to 399.0% of the control group) compared with those in the 3 hr post-ischemic group. Thereafter, they decreased with time and showed significantly lower levels compared with the control group (Figure 3).

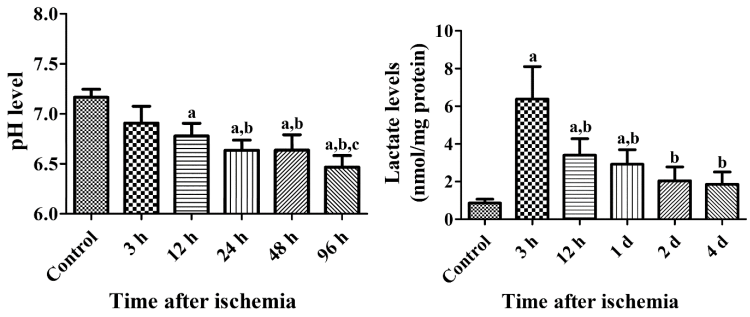

Figure 3. pH and lactate levels in the hippocampus of sham- and ischemia-operated groups. Note that pH gradually decreased with time after ischemia, while lactate levels significantly increased 3 hr after ischemia and thereafter decreased with time. Data were analyzed by one-way analysis of variance followed by Bonferroni's post-hoc test (n = 5 in control or at each time point in the ischemic group; aP<0.05, which indicates significant difference from the sham-operated control group, bP<0.05, which indicates significant difference from the 3 hr post-ischemic group, cP<0.05, which indicates significant difference from the 12 hr post-ischemic group). The bars indicate the mean values ± standard deviation

Discussion

Vacuolar H^+-ATPase is a highly conserved enzyme that acidifies the organelles across the plasma membrane. It produces proton gradients using energy from ATP hydrolysis and helps to regulate cytosolic pH in neurons (28). Among these vacuolar H^+-ATPase enzymes, ATP6V facilitates the utilization of neurotransmitters such as acetylcholine, glutamate, and GABA in coordination with their transporters on synaptic vesicles (29, 30). In contrast, inhibition of ATP6V activity by bafilomycin A1 significantly decreased synaptic transmission of glutamate GABA in rat primary hippocampal neurons (31). In a previous proteomic study, we found that vitamin B_6 deficiency reduced ATP6V1B2 expression in the hippocampus, and pyridoxine deficiency significantly decreased dopaminergic fibers in the hippocampus (23).

In the present study, we examined the spatial and temporal changes in ATP6V1B2 immunoreactivity in the hippocampal CA1 region after 5 min of ischemia to investigate the role of ATP6V1B2 in ischemic damage. ATP6V1B2 immunoreactivity was found to be significantly increased in the hippocampal CA1 and 3 regions, 1 day and 4 days after ischemia, respectively, compared with that in the control group. In addition, ATP6V1B2 immunoreactivity showed significant increases 1 day and 4 days after ischemia, compared with the control group. The significant increase in ATP6V1B2 immunoreactivity may be associated with neuronal death occurring in the CA1 region, based on the reduction in the number of NeuN-positive nuclei. Some studies have demonstrated an increase in ATP6V1B2 expression in an animal model of Alzheimer's disease (32-34). Fibroblasts in patients with Alzheimer's disease impair lysosomal acidification, and Coffey *et al*. (19) suggested that ATP6V1B2 expression is increased to compensate for the impairment in lysosomal acidification. However, ATP6V1B2 levels were found to be decreased in normal aging rats (35) and mice (21), as well as in animal models of Alzheimer's disease (21).

In the present study, we also observed pH and lactate levels in the hippocampus to elucidate the changes in cellular acidification after transient forebrain ischemia. It has been reported that ATP and tissue pH levels were dramatically decreased during ischemia, but similar levels were observed in the control group 2 hr after ischemia-reperfusion (36). In addition, gerbils showed a significant increase in lactate release in the hippocampus after transient forebrain ischemia (27, 37). In the present study, we observed significantly higher lactate levels in the hippocampus 1 day after ischemia, and lower pH levels 12 hr after ischemia. Lysosomal dysfunction has been implicated in ischemia, and maintenance of lysosomal function in neurons is one of the therapeutic approaches used to protect neurons from ischemic damage (38, 39). Mutated *Atp6v1b2* caused cognitive impairments (40, 41), abnormal morphology and acidification in lysosomes (18, 42), and a significant decrease in the number of mature neurons in the hippocampal CA1 region (41). These results suggest that enhanced expression of ATP6V1B2 in the CA1 region and dentate gyrus may be associated with a compensatory mechanism intended to prevent the impairment of lysosome acidification. In addition, we also observed significantly higher levels of ATP6V1B2 immunoreactivity in the CA3 region and dentate gyrus, which are relatively resistant to ischemic damage, 4 days after ischemia/reperfusion.

Conclusion

ATP6V1B2 expression is transiently increased in the hippocampal CA1 region 1 day after ischemia when the pH and lactate levels are significantly lower and higher than the control level. In addition, ATP6V1B2 immunoreactivity was found to be significantly increased in the CA3 and dentate gyrus 4 days after ischemia, when neuronal cell death was detected in the hippocampal CA1 region. These results suggest that transient increases in ATP6V1B2 levels in lysosomes may be associated with compensatory mechanisms employed to prevent lysosomal dysfunction after transient forebrain ischemia.

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conflicts of Interest

None declared

Authors' Contributions

All authors conceived the experiment and manuscript. HYJ and IKH designed the study and wrote the manuscript. HYJ, WK, KRH, and MSK conducted the animal experiments. HJK and DWK conducted biochemical experiments. JHC, YSY, DWK, DYY, and MHW participated in designing and discussing the study. All authors have read and approved the final manuscript.

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