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Dynamic changes in proprotein convertase 2 activity in cortical neurons after ischemia/reperfusion and oxygen-glucose deprivation☆

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Abstract

In this study, a rat model of transient focal cerebral ischemia was established by performing 100 minutes of middle cerebral artery occlusion, and an *in vitro* model of experimental oxygen-glucose deprivation using cultured rat cortical neurons was established. Proprotein convertase 2 activity gradually decreased in the ischemic cortex with increasing duration of reperfusion. In cultured rat cortical neurons, the number of terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate-biotin nick end labeling-positive neurons significantly increased and proprotein convertase 2 activity also decreased gradually with increasing duration of oxygen-glucose deprivation. These experimental findings indicate that proprotein convertase 2 activity decreases in ischemic rat cortex after reperfusion, as well as in cultured rat cortical neurons after oxygen-glucose deprivation. These changes in enzyme activity may play an important pathological role in brain injury.

Key Words

neural regeneration; brain injury; proprotein convertase 2; cortex; neuron; cerebral ischemia/reperfusion; oxygen-glucose deprivation; *in vivo* study; *in vitro* study; grants-supported paper; photographs-containing paper; neuroregeneration

Research Highlights

(1) Previous studies on proprotein convertase 2 primarily focused on the correlation between protein expression and endocrine secretion, while little attention was given to proprotein convertase 2 enzymatic activity.
(2) Except for our previous study on the role of proprotein convertase 2 and its substrate in cerebral ischemia, there was no report examining changes in proprotein convertase 2 in ischemia. Thus, the novel findings of our present study provide insight into the role of proprotein convertase 2 in the pathology of ischemic brain injury.

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INTRODUCTION

The brain requires a continuous supply of oxygen and glucose to maintain normal function and viability. Loss of this supply for only a few minutes can trigger a cascade of events leading to neuronal death; if the deprivation is sustained, glial and endothelial cells also succumb. Cerebral ischemia leads to the reduction of blood flow that deprives the afflicted brain region of oxygen and nutrients. It is associated with complex biochemical and molecular

mechanisms that impair neurological functions[1]. Brain ischemia leads to multiple cellular changes, including a rapid influx of calcium from the extracellular space and an efflux of calcium from the endoplasmic reticulum, accompanied by tissue acidosis[2-5].

Proprotein convertase is a secretory mammalian serine proteinase related to bacterial subtilisin-like enzymes. Proprotein convertases participate in the processing of a great variety of secreted and membrane proteins. These proteins processed by proprotein convertase are involved in embryogenesis, gene expression, cell cycle, programmed cell death, intracellular protein targeting, and endocrine/neural functions[6]. The family of proprotein convertases comprises nine members, PC1/3, PC2, furin, PC4, PC5/6, PACE4, PC7, SKI-1/S1P and PCSK9. Proprotein convertase 2 is known to process various neuroendocrine precursors, and it is a calcium- and pH-dependent endoprotease. It performs limited cleavage of precursors of a number of important neuropeptides including, but not limited to, enkephalin, cholecystokinin, VGF (nerve growth factor inducible), nociceptin/orphanin FQ, substance P, somatostatin, α- and γ-melanocyte-stimulating hormone, dynorphin, thyrotropin-releasing hormone, corticotropin releasing hormone, neurotensin, neuromedin N and POMC-derived peptides[7-12]. Dynorphin-A (1–8) has a protective role in the ischemic brain[13]. In proprotein convertase 2-null mice, the production of these neuropeptides is severely attenuated. Proprotein convertase 2-null mice also exhibit a lack of response to certain stresses[14]. It is known that the promoter region of the proprotein convertase 2 gene contains several elements that can potentially respond to ischemic stress[15]. We therefore reasoned that cerebral ischemia/reperfusion may interfere with the biosynthesis of proprotein convertase 2, thereby impairing the activity of the enzyme and affecting its ability to process its neuropeptide substrates.

In this study, we aimed to observe the changes in proprotein convertase 2 activity in the cortex following ischemia/reperfusion, as well as in ischemic cultured rat cortical neurons treated to oxygen-glucose deprivation, in a broader attempt to investigate the roles of proprotein convertase 2 on cerebral ischemia/reperfusion.

RESULTS

Quantitative analysis of experimental animals

This experiment is composed of an *in vivo* study and an *in vitro* study. For the *in vivo* study, a total of 18 Sprague-Dawley rats were initially included in the experiment, and were equally and randomly divided into three groups: control, and 8 and 24 hours following reperfusion. Rats in the 8 and 24 hours ischemia/reperfusion groups were subjected to transient focal cerebral ischemia by performing middle cerebral artery occlusion with a 3-0 silk suture. After 100 minutes of middle cerebral artery occlusion, the suture was withdrawn to allow reperfusion for a period of 8 or 24 hours. All rats were included in the final analysis. For the *in vitro* study, about 30 rat pups that were born within 3 days were used for cell cultures.

Decreased proprotein convertase 2 activity in the cortex of rats with cerebral ischemia/reperfusion injury

In the ischemic cortex after 8 or 24 hours of reperfusion, proprotein convertase 2 activity was significantly lower than in the control cortex ($P < 0.05$). Proprotein convertase 2 activity decreased gradually with increasing reperfusion time in the ischemic cortex ($P < 0.05$; Figure 1).

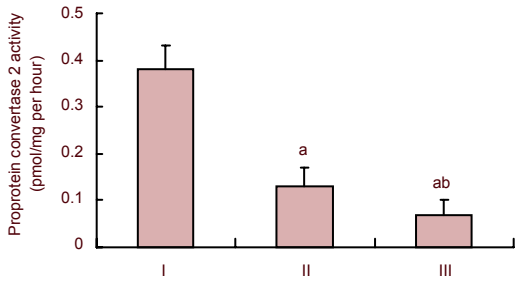

Figure 1 Proprotein convertase 2 activity in rat cortex following cerebral ischemia/reperfusion.

Proprotein convertase 2 activity was significantly decreased after 8 and 24 hours of reperfusion compared with the controls. Data are expressed as mean ± SEM ($n = 6$). $^{a}P < 0.05$, *vs*. control group; $^{b}P < 0.05$, *vs*. 8 hours, by least significant difference *t*-test.

I: Control; II, III: 8, 24 hours after reperfusion.

Increased number of apoptotic neurons in the rat cortex following oxygen-glucose deprivation

In rat cortical neuron cultures, the number of terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate-biotin nick end labeling (TUNEL)-positive neurons increased significantly after 60, 120 and 180 minutes of oxygen-glucose deprivation, relative to the control ($P < 0.05$; Figure 2).

Decreased proprotein convertase 2 activity in cultured rat cortical neurons following oxygen-glucose deprivation

Proprotein convertase 2 activity was significantly lower in

cultured rat cortical neurons after 30, 60, 120 and 180 minutes of oxygen-glucose deprivation, compared with the control ($P < 0.05$). Proprotein convertase 2 activity decreased gradually with increasing duration of oxygen-glucose deprivation in cultured rat cortical neurons ($P < 0.05$; Figure 3).

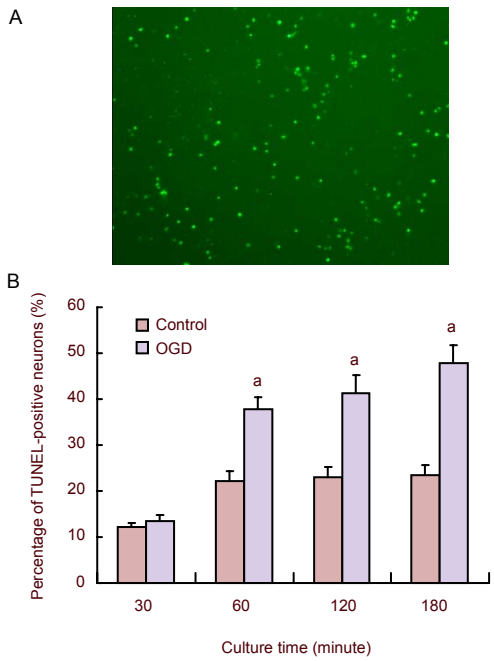

Figure 2 Apoptotic neurons in rat cortex following oxygen-glucose deprivation (OGD).

(A) Rat cortical neurons after 120 minutes of OGD (TUNEL staining, × 100). The cells showing green fluorescence are TUNEL-positive neurons.

(B) Percentages of TUNEL-positive neurons. Data are expressed as mean ± SEM. $^aP < 0.05$, *vs*. control group, by paired *t*-test. TUNEL: Terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate-biotin nick end labeling.

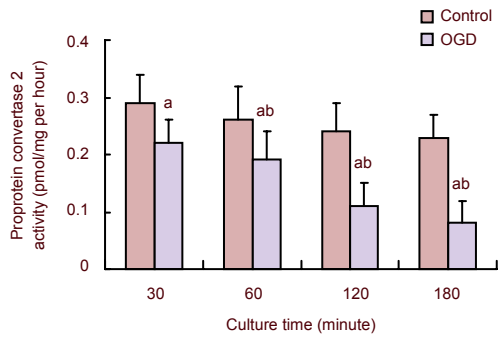

Figure 3 Proprotein convertase 2 activity in cultured rat cortical neurons following oxygen-glucose deprivation (OGD).

Proprotein convertase 2 activity was significantly decreased after 30, 60, 120 and 180 minutes of OGD, compared with the control group. Data are expressed as mean ± SEM. $^aP < 0.05$, *vs*. control group; $^bP < 0.05$, *vs*. previous time point, by least significant difference *t*-test.

DISCUSSION

Most small peptides and proteins, including many peptide hormones and neuropeptide neurotransmitters, are initially synthesized as larger precursors and require several posttranslational processing steps, including proteolytic cleavage, for the formation of the bioactive species. Prohormone convertases are responsible for the enzymatic maturation of these peptides and protein precursors[16-17]. The cell biology of these proteases within the regulated secretory pathway of neuroendocrine cells is complex, and they are themselves initially synthesized as inactive precursor molecules[18].

Proprotein convertase 2 is a subtilisin-related proteolytic enzyme that performs neuroendocrine-specific cleavages. Proprotein convertase 2 is found exclusively in neural and endocrine cells equipped with a regulatory secretory pathway[19-20]. Rat pituitary contains high levels of proprotein convertase 2 enzymatic activity, while the hypothalamus and other brain regions contain moderate levels. Proprotein convertase 2 is mostly localized within immature and dense core secretory granules in neural and endocrine cells, and as such are poised to process most polypeptide prohormones within the regulated secretory pathway[21]. Li *et al* [22] found that proprotein convertase 2 enzymatic activity and mRNA levels are somewhat discordant, suggesting that proprotein convertase 2 mRNA levels do not always reflect enzymatic activity.

Preceding the action of carboxypeptidase E or other carboxypeptidases, proprotein convertase 2 performs limited cleavage of precursors for a number of important neuropeptides. Many of these are not fully processed in proprotein convertase 2-null mice. Proprotein convertase 2-null mice appear normal at birth, but they exhibit retarded growth. Proprotein convertase 2-null mice exhibit chronic fasting hypoglycemia and a deficiency in circulating glucagon[23], and are significantly less (rather than more) responsive to stimuli, compared with wild-type mice[24]. Thus, proprotein convertase 2 has very important physiological functions, and has multiple roles in the central nervous system[25].

In this study, proprotein convertase 2 activity decreased

gradually at 8 and 24 hours of reperfusion after 100 minutes of middle cerebral artery occlusion in the rat cortex. The 75- and 64-kDa forms of proprotein convertase 2 presumably represent the pro-enzyme and the mature protein, respectively[26-29]. These results suggest that cerebral ischemia decreases proprotein convertase 2 activity in rat cortex. Thus, the biosynthesis of active proprotein convertase 2 may be impaired during cerebral ischemia, thereby reducing processing of the enzyme's neuropeptide substrates.

Cultured rat primary cortical neurons are extensively used in neuroscience research. In this study, with increasing duration of oxygen-glucose deprivation, proprotein convertase 2 activity decreased steadily after oxygen-glucose deprivation in cultured rat cortical neurons. This decrease in enzyme activity was accompanied by an increase in the number of TUNEL-positive neurons. These results suggest that oxygen-glucose deprivation decreases proprotein convertase 2 activity and increases apoptosis in cultured rat cortical neurons.

Proprotein convertase 2 plays a critical role in the production of many neuropeptides and other secretory pathway peptides[30]. Some of the secretory pathway peptides detected in different brain regions of wild-type mice are undetectable in proprotein convertase 2 knockout mice[31]. The decrease in proprotein convertase 2 activity in rat cortex and cultured rat cortical neurons under ischemia and oxygen-glucose deprivation will attenuate proprotein convertase 2-mediated neuropeptide processing. This attenuated neuropeptide processing will lead to the accumulation of some substrate neuropeptide precursors and a decrease in active neuropeptide levels. Consequently, the functions of these neuropeptides will be affected. Some of these neuropeptides, such as dynorphin-A (1–8), play neuroprotective roles in ischemia[13]. Thus, the decreased proprotein convertase 2 activity may ultimately reduce the survival of neurons under cerebral ischemia.

In mice lacking active proprotein convertase 2, exacerbated brain injury is observed after an otherwise non-lethal focal ischemia. Brain ischemia attenuates proprotein convertase 2 levels and impairs neuropeptide processing mediated by the enzyme. This attenuation may play a role in the pathology of ischemic/reperfusion brain injury[13].

In our previous study, we found that there is a transient upregulation of proprotein convertase 2 mRNA in rat cortex early during reperfusion following 100 minutes of middle cerebral artery occlusion, which gradually decreases over time[7]. In this study, proprotein convertase 2 activity decreased gradually after 100 minutes of middle cerebral artery occlusion. This suggests that both proprotein convertase 2 mRNA and enzymatic activity decreases after cerebral ischemia. This decrease in proprotein convertase 2 activity will diminish the processing of its substrate neuropeptides. In turn, this will affect the functions mediated by the neuropeptides after cerebral ischemia.

MATERIALS AND METHODS

Design

A randomized controlled animal study focusing on cytobiology.

Time and setting

The experiment was performed at the Robert S. Dow Neurobiology Laboratories, Legacy Clinical Research and Technology Center, Portland, OR, USA from March 2006 to July 2007.

Materials

A total of 18 male Sprague-Dawley rats, weighing 250–300 g, of clean grade, aged 3 months, were purchased from Charles River Laboratories (Wilmington, MA, USA). Timed pregnant rats were also purchased from Charles River Laboratories. About 30 rat pups born within 3 days were used for cell cultures. The rats were housed on a 12-hour light/dark cycle and freely fed at 22 ± 2°C and a relative humidity of 55 ± 5%. All animal experiments were conducted in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

Methods

Modeling of transient focal cerebral ischemia/reperfusion

Focal cerebral ischemia/reperfusion was induced by middle cerebral artery occlusion[32-34]. Briefly, rats were anesthetized with 4% isoflurane in 70% nitrous oxide/30% oxygen, and maintained with 2% isoflurane in 70% nitrous oxide/30% oxygen. Middle cerebral artery occlusion was achieved by introducing a 3-0 silk suture into the lumen of the right internal carotid artery, with the external carotid artery and the extracranial branch of the internal carotid artery ligated. After 100 minutes of middle cerebral artery occlusion, the suture was withdrawn to allow reperfusion up to 24 hours. Control animals were

sham operated. They underwent the same surgical procedure, but the suture was not advanced to the middle cerebral artery. Relative regional cerebral blood flow was monitored with a laser-Doppler flowmetry apparatus (Transonic Systems Inc., Ithaca, NY, USA), and successful occlusion and reperfusion were confirmed using this system. A ≥ 80% reduction in regional cerebral blood flow observed immediately after introducing the silk suture into the lumen of the internal carotid artery was required in the current study[35].

Preparation of cortical tissues

At 8 and 24 hours after reperfusion, animals were decapitated under anesthesia. Tissues from the ischemic cortical regions of the territory supplied by the middle cerebral artery, as illustrated in earlier studies[32-33, 36], were immediately dissected, frozen on dry ice, and stored at –80°C.

Primary rat cortical neuron cultures and in vitro simulated ischemia

Primary rat cortical neurons were cultured as follows. Briefly, the cerebral cortices from about 30 rat pups born within 3 days were anesthetized with isoflurane and dissected and incubated with 0.05% ethylenediaminetetraacetic acid in PBS for 10 minutes at 37°C, followed by trituration with flame-polished glass pipettes. For neuronal cultures, dispersed cortical cells were seeded onto poly-L-ornithine-coated 35-mm dishes at a density of 1 × 10^6 cells/dish. Cells were maintained in Eagle's minimum essential medium supplemented with 10% horse serum (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere containing 5% CO_2. 5-fluoro-2-deoxyuridine (5 μM) and uridine (5 μM) were added 72 hours after seeding to suppress the growth of glial cells. Cells were used for experiments 7 to 8 days after seeding[37].

Ischemia was simulated in cultured neurons by oxygen-glucose deprivation, as previously described[32] , by incubating cells in glucose-free, serum-free, and glutamine-free medium in an anaerobic chamber (Forma Scientific, Marietta, OH, USA) containing 85% nitrous oxide/5% carbon dioxide/10% hydrogen for 30, 60, 120 or 180 minutes. Cells in the control group were incubated in control medium (consisting of 25 mM Hepes, pH 7.4; 2 mM $CaCl_2$; 135 mM NaCl; 5 mM KCl; 1 × essential amino acids; 1 × L-glutamine; 20 mM glucose; penicillin/streptomycin) for 30, 60, 120 or 180 minutes. After oxygen-glucose deprivation and control medium treatment, cells were allowed to recover in complete medium (Neurobasal-A with Glutamax-1, B27 supplement, Pen/Strep) under normal conditions overnight.

TUNEL staining for neuronal apoptosis

The oxygen-glucose deprivation-injured cells were identified by detecting DNA fragmentation using the TUNEL method with a commercial kit (Roche Diagnostics GmbH, Roche Applied Science, 68298 Mannheim, Germany), following the manufacturer's instructions. Briefly, cell samples (approximately 360 cells in a field at 100 × magnification) were fixed with freshly prepared 4% paraformaldehyde in PBS (pH 7.4). Samples were rinsed with PBS and incubated in 0.1% Triton X-100 in PBS for 2 minutes on ice (2–8°C). Samples were then rinsed twice with PBS, and a 50-μL aliquot of TUNEL reaction mixture was added to the samples, followed by incubation in a humidified atmosphere for 60 minutes at 37°C in the dark. Samples were rinsed with PBS (three times) and mounted with DAPI-containing mounting solution. After staining, fluorescence signals were examined with a Leica epifluorescence microscope (Leica Microsystems Inc., Bannockburn, IL, USA) attached to a digital camera, and analyzed with the assistance of the Bioquant program (Bioquant Image Analysis, Nashville, TN, USA). TUNEL-positive cells were counted in ten fields at 100 × magnification and summed for each dish.

Proprotein convertase 2 activity assay

Proteins were extracted from brain tissues following standard protocols[38]. The extraction buffer for cortical samples and cells consisted of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% NP-40, 1% TritonX-100, 0.1% sodium deoxycholate, 0.1% SDS, 2 mM edetic acid, and a cocktail of protease inhibitors[39]. Insoluble material was removed by centrifugation at 10 000 × *g* for 10 minutes at 4°C. Analyses of proprotein convertase 2 activity followed protocols described by Berman *et al* [39]. Briefly, 20 mg of proteins from tissue homogenates were incubated with 200 mM L-pGlu-Arg-Thr-Lys-Arg-7-amino-4-methylcoumarin (Peptides International, Louisville, KY, USA) in 100 mM sodium acetate, pH 5.0, and 1 mM $CaCl_2$ at 37°C for 4 hours. In parallel incubations, 1 mM carboxyl-terminal peptide (Sigma-Genosys, The Woodlands, TX, USA), a proprotein convertase 2-specific inhibitor[40], was added to reactions. The release of 7-amino-4-methylcoumarin (AMC) was measured using a Spectra Max GEMINI spectrofluorometer (Molecular Devices, Union City, CA, USA; excitation 360 nm/emission 480 nm). The amount of product formed was calculated using free AMC as a standard. The activity inhibited by the presence of carboxyl-terminal peptide was considered as a

proprotein convertase 2-specific activity.

Statistical analysis

SPSS 12.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis. Data were expressed as mean ± SEM. Homogeneity of variance test was performed. Changes of proprotein convertase 2 activity at multiple reperfusion hours were compared using a randomized design one-way analysis of variance. For *post-hoc* testing, the least significant difference *t*-test was used for comparison between groups. The percentages of TUNEL-positive cells were compared between the control and oxygen-glucose deprivation groups using the paired *t*-test, where a value of $P < 0.05$ was accepted as significant.

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Author contributions: Shuqin Zhan participated in experimental operations, photographs taking, data analysis and manuscript writing. An Zhou participated in study design, technical support, laboratory and experimental equipment supply, experimental control, study guidance and supervision. Chelsea Piper partly participated in cell cultures. Tao Yang participated in the surgeries on rats for middle cerebral artery occlusion. All authors read the paper and agreed to publication.

Conflicts of interest: None declared.

Ethical approval: The protocols were approved by the Institutional Animal Care and Use Committee of Legacy Research Center (Portland, OR, USA).

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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