**Suppression of glutamate carboxypeptidase II ameliorates neuronal apoptosis
from ischemic brain injury**

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Abstract

Ischemia stroke is a destructive cerebrovascular disease and a major cause of death and life-long neurological disability. N-Acetyl-L-aspartyl-L-glutamate (NAAG) is a neuro-transmitter in the mammalian brain and involves a variety of physiological and pathological functions including ischemia brain injury. Fully understanding the functions of NAAG peptidase (GCP II) in the pathogenesis of ischemia brain injury are extremely valuable for effective therapies to ischemia stroke. In this study, we found that expression of GCP II and NAAG agonist metabotropic glutamate receptor (mGluR3) was up regulated in CoCl₂ induced hypoxia environment in neuronal cells. More, knockdown of GCP II in neuronal cells ameliorated apoptosis from hypoxia-ischemic induced injury through suppressing expression of caspase 3 and caspase 9. In a rat model of middle cerebral artery occlusion (MCAO), double immunofluorescence staining showed increased GCP II levels in the neuronal cells in penumbra area after MCAO. *In vivo* knockdown of GCP II significantly reduced the neuronal apoptosis in the penumbra area. Thus, our results highlighted the roles of NAAG peptidase (GCP II) in the ischemia brain injury, and might provide an important target in therapeutic implications.

Key words: ischemia stroke, hypoxia, NAAG peptidase, metabotropic glutamate receptor, neuronal apoptosis

1. Introduction

Ischemia stroke is a destructive cerebrovascular disease and a leading cause of death worldwide. Most of ischemia stroke patients suffer from physical and mental disabilities [1]. These impairments will be accompanied with a tremendous impact on the patient's life. Yet, to date, few available therapies to ischemia strokes in clinical are far from satisfying [2]. Deep understanding the mechanisms underlying cerebral ischemic injury will provide effective therapeutic interventions that may minimize ischemic brain injury and enhance recovery for this vulnerable patient group.

N-Acetyl-L-aspartyl-L-glutamate (NAAG) is a neuron-transmitters in the mammalian brain, first discovered in the nervous system in the mid-1960s [3]. NAAG peptidase (GCPII) mediates the hydrolysis of NAAG into glutamate and N-acetylaspartate. Glutamate is the primary excitatory neuron-transmitter in the human nervous system and plays an important role in a variety of physiological functions including learning and memory. However, accumulating evidences suggested that excessive glutamate transmission induced neurons damage, and implicated in neurological disorders, such as ischemia stroke [4]. Inhibition of GCPII has been shown to attenuate excitotoxicity associated with enhanced glutamate transmission under pathological conditions. Modulation of GCPII activity with small-molecule inhibitors has implications for the therapy of cancer [5] and inflammatory pain [6] or neuropathic pain [7]. NAAG also acts as an agonist at group II metabotropic glutamate receptors (mGluR3) on neurons and mGluR3 has been reported to mediated the efficacy of NAAG peptidase inhibitor in model of schizophrenia and traumatic brain injury [8, 9]. Yet, the precise function and mechanism of NAAP peptidase in cerebral ischemic injury still need further studied.

We previously found mice knockout of GCPII were less susceptible to traumatic brain injury [9]. In this study, we further study the functions of GCPII in the pathogenesis of ischemic brain injury, and in the hope that the small-molecule inhibitors of GCPII may have implications for the therapy of ischemia stroke.

2. Methods

2.1 Cell Lines and Cell Culture

Rat neuroblastoma B35 cells were purchased from American Type Culture Collection (ATCC) and cultured in DMEM supplemented with 10% fetal bovine serum (PAA, Linz, Austria). All the cells were cultured at 37 °C in a humidified atmosphere with 5 % CO₂.

For the simulating the hypoxia environment, dissociated cells were seeded in 10cm dish with or without CoCl₂ treatment as indicated time. And the cells were observed and analyzed.

2.2 middle cerebral artery occlusion (MCAO)/reperfusion animal model

Sprague-Dawley rats (from the Experimental Animal Center, Shanghai Jiaotong University School of Medicine) weighing 250–300 g were housed in a climate-controlled room with five rats per cage and food and water provided ad libitum on a 12:12 h light/dark cycle. The animal study protocol used in this research was approved through the Ethics Committee for Animal Experimentation and was conducted according to the Guide lines for Animal Experimentation of Shanghai Jiaotong University School of Medicine.

The animals were randomly divided into the following groups (n = 6-8 for each group): a sham-operated control group (operation but no occlusion), and a 24h post MCAO/reperfusion, misRNA group with 24h post MCAO/reperfusion (misRNA intraventricular injection) and siRNA group with 24h post MCAO/reperfusion (GCPH-siRNA intraventricular injection). Rats anesthetized with i.p. injection of 50 mg/kg pentobarbital were placed in a stereotaxic instrument (ASI Company, USA). A 28-gauge stainless steel injection cannula was paved into the right lateral ventricle as previously described [10]. Then the rats received a single injection of Id2-siRNA or misRNA at a rate of 0.5 µl/min using amcor infusion pump and a 10µl microsyringe. Once the infusion was done, the cannula remained in place for 5min, then withdrawn at a rate of 1 mm/min. One day after the injection, all the rats were performed with the operation of MCAO as previously described [11]. Briefly, a 3–0 monofilament nylon suture (Beijing Shadong Industrial Corp., China) with a heat-treated rounded tip was introduced into the right internal carotid artery (ICA) via the ECA until a slight resistance was achieved. The suture was maintained in place for 90 min and then

withdrawn to facilitate reperfusion.

At the desired time after the onset of reperfusion, the animals were deeply anesthetized using sodium pentobarbital (100 mg/kg,i.p.). For GCPII mRNA and protein analysis, the rats were transcardially perfused with 4°C normal saline, and regions corresponding to the ischemic core and penumbra were dissected on ice using previously described methods [11]. For TUNEL staining and immunostaining, the rats were transcardially perfused with 4 °C normal saline, followed by 4% paraformaldehyde (PFA; pH = 7.4). The brains were removed and post-fixed in 4% PFA and subsequently cryoprotected in 20% sucrose solution, followed by 30% sucrose solution. Serial coronal sections (25 µm) were collected between −1.80 and −4.80 mm bregma levels. Every fifth section for a total of 24 sections was selected for staining.

2.3 Real-time-PCR (RT-PCR)

Cellular RNA was isolated by Trizol-Reagent according to the manufacturer's instructions. Briefly, the DNA was removed from the samples using DNase treatment (DNA-free kit; Ambion Applied Biosystems) and cDNA was synthesized from the purified RNA using Moloney murine leukemia virus reverse transcription kit (Promega). Gene-specific primer sets are listed in **Table1** and Actin primer sets are used to produce a normalization control. Real-time PCR was carried out in triplicate with the SYBR Green PCR Master Mix (Applied Biosystems) and a 7900HT Fast Real-Time PCR machine (Applied Biosystems).

2.4 Western blot analysis

RIPA buffer in the presence of protease inhibitor cocktail and phosphorylation inhibitor cocktail were used to extract total protein. Appropriate amount protein was loaded into 10–15% SDS–polyacrylamide gel and transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA). Primary antibodies were incubated overnight and secondary antibodies were incubated for 1 h at the appropriate dilutions. The signal was observed and developed with Kodak film by exposure to Enhanced Chemiluminescence (ECL) plus Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ, USA). Western blot was performed with antibodies

against GCPII, mGluR3, Tgfb1, apoptosis associated Bax, Caspase3, Caspase9, Bcl2, Bax and actin is used as control.

2.5 Lentivirus mediated shRNA gene knockdown GCPII

The stable knockdown GCPII cell lines were generated by transduction a lentiviral-mediated expression siRNA specific target of GCPII. These vectors were constructed by Hanyin Co. (Shanghai, China). The recombinant lentiviruses (Hanyin Co. Shanghai, China) were prepared and titered to 10^9 transfection units (TU)/ml. The targeted knockdown sequence was 3'-GCCTGGATTGTTGAGTTAT-5'. The virus transfected B35 cell line with 8 μ g/ml polybrene. After 48hours, the cells are harvested, and the knockdown efficiency was tested by real-time- PCR and western blot.

2.6 TUNEL assay

Cells were fixed in 4% PFA for 20 min before incubated in 0.1%Triton-X, 0.1%sodium citrate. And then rinse with PBS for three times, 10 minutes each. Incubate slides in 0.3% H₂O₂ for 30 minutes to inhibit endogenous peroxidase activity followed by rinsing with PBS. Add 50 μ l TUNEL reaction mixture (In Situ Cell Death Detection Kit, Fluorescein, Roche, USA) on sample. Incubate slide in a humidified atmosphere for 60min at 37°C in the dark. Then rinse 3 times with PBS. Observe the slides under a fluorescence microscope (Nikon TE300, Japan). Use an excitation wavelength in the range of 450-550 nm and detection in the range of 515-565 nm.

2.7 Statistical analysis

Statistical analysis was performed using the Student's *t* test. A *P* value less than or equal to 0.05 was chosen to be statistically significant difference.

3. Results

3.1. Upregulated expression of GCPII in response to hypoxia stimuli in neuronal cells.

The pathogenesis of ischemia induced brain injury due to the cellular apoptosis in the oxygen and energy failure environment. First, we examined the expression of GCPII and mGluR3 in hypoxia environment. CoCl₂ is a widely used agent to induce oxygen failure. Neuronal B35 cells were treated with CoCl₂ for 12, 24, 36 and 72 hours respectively and the expression of GCPII were detected through

real-time-PCR. We found that the expression of GCPII were increased in a time dependent manner in B35 cells. There were more than two fold increases for GCPII in B35 cells compared to untreated cells (**Figure 1**). Meanwhile, NAAG activated metabotropic glutamate receptors (mGluR3) also upregulated with fivefold increase in response to hypoxia (**Figure 1**).

Further, the protein expression of GCPII and mGluR3 were detected through western blot after CoCl₂ treatment for 12, 24, 36 and 72 hours. Compared to the untreated cells, both GCPII and mGluR3 were up regulated in B35 cells (**Figure 2**). Thus, from mRNA and protein level, we showed that the NAAG peptidase (GCPII) and its agonist metabotropic glutamate receptors (mGluR3) were both upregulated suffered from hypoxia environment in neurons, suggesting that NAAG peptidase and mGluR3 may play important roles in hypoxia-ischemia.

Accumulating data have shown that TGF- β has the neuron-protective effects response to acute ischemic damage. Therefore, we tested the expression of TGF- β , with and without CoCl₂ treatment, to find out whether this protective factor will have function in hypoxia-ischemia. We found that after CoCl₂ treatment, TGF- β expression was decreased both in mRNA and protein level in B35 cells. Those results suggested that CoCl₂ treatment could eliminate the TGF- β associated neuron-protective effects, thus deteriorate the progress of hypoxia-ischemia brain injury (**Figure1 and 2**).

3.2. Knockdown of GCPII expression in neuronal cells.

Previously, we have shown that the expression of GCPII was up regulated after CoCl₂ treatment in both mRNA and protein level, so, we want to find out if inhibiting the GCPII expression could ameliorate neuronal apoptosis. Using lenti-virus mediated shRNA gene knockdown system, we knocked down GCPII expression in B35 cells. From the mRNA level, more than 80% of GCPII expression was decreased. The protein level of GCPII was also decreased suggesting the high efficiency of siRNA knock down system (**Figure 3**).

3.3. Suppression of GCPII ameliorates neuronal cell apoptosis under hypoxia stress.

Then, we treated GCPII knocked-down B35 cells with CoCl₂. Using TUNEL

assay, we found that loss of GCPII expression reduced hypoxia-ischemia induced brain injury. As shown in **Figure 4**, the TUNEL positive cells were greatly increased compared to GCPII knock down cells after CoCl₂ treatment in B35 cells.

We further analyzed classic cellular apoptotic markers in the CoCl₂ induced hypoxia environment. Indeed, after 48 hours CoCl₂ treatment, the cellular apoptotic markers Bax, Caspase3 and Caspase9 were greatly increased in B35 cells in mRNA level (**Figure 5**). In addition, the anti-apoptosis marker Bcl2 was decreased. We also detected the protein level of Bax, Caspase3, Caspase9 and Bcl2. Similar to mRNA level, the BAX, Caspase3, Caspase9 protein were activated after 48 hours CoCl₂ treatment, and BCL2 was down regulated (**Figure 6**). The changes in BAX, Caspase3, Caspase9 and BCL2 expression suggesting the apoptosis progress in the hypoxia environment, and those apoptosis progresses contribute to the cognitive and motor disorders suffered from hypoxia-ischemia brain injury.

We also found that the up regulation of Bax, Caspase3 and Caspase9 were partially reversed by loss of GCPII expression in both mRNA and protein level (**Figure 5 and 6**). Also, the anti-apoptosis expression of BCL2 was reversed. These results highlighted the importance of GCPII in the hypoxia-ischemia induced brain injury and suggested that inhibition of GCPII functions may have implications for the therapy of ischemia stroke.

3.4 Expression of GCPII in the penumbra area in a rat model of MCAO/reperfusion.

From the immunofluorescent analysis, GCPII-positive cells were increased at 24 h post MCAO/reperfusion (**Figure 7**). To investigate the location of up-regulated GCPII, double immunofluorescence labeling for GCPII and NeuN was performed. As shown in **Fig. 7**, the obvious co-localization of GCPII and NeuN were detected at 24 h post MCAO/reperfusion group, whereas weak GCPII immunofluorescence was observed in neurons from the sham-operated control group, indicating that GCPII was increased after ischemia in vivo, including neuronal and non-neuronal cells.

3.5 Knockdown of GCPII ameliorated apoptosis in the penumbra area of the rats with MCAO.

Given the observation that GCPII modulates hypoxia induced neuronal apoptosis

in vitro, we sought to find out whether GCPII would also have an effect on neurons suffering from ischemic stroke as mimicked in MCAO model. To this end, *in vivo* knockdown of GCPII in brain tissues was performed by injecting GCPII-siRNA2 into the left side of the lateral ventricle in Sprague-Dawley rats. To confirm effectiveness of the delivered GCPII-siRNA in knocking down GCPII expression, the cortex of penumbra area was subjected to immunofluorescent analysis. As shown in Fig.8A, GCPII staining was found to be much lower in the siRNA group, compared with control group (**Figure 8A**). There was a significant decrease in the number of TUNEL-positive cells in the GCPII-siRNA group compared to misRNA group (**Figure 8B**). These results verified the importance of GCPII in the hypoxia-ischemia induced brain injury *in vivo*.

4. Discussion

Ischemia-induced brain injury is a major cause of morbidity and mortality in infants and children, and few effective therapies to this disease are not available [12-14]. Glutamate is the primary excitatory neuron-transmitter in the human nervous system with pathological functions in diseases. The importance of upstream regulation of glutamate in hypoxia-ischemia is unknown. In the present study, we found (GCPII and mGluR3 were increased after CoCl₂ treatment in both mRNA and protein level, suggesting glutamate associated pathway may participate in the pathology of hypoxia-ischemia disease.

Several previous studies have demonstrated that the pathogenesis of ischemia brain injury may due to the neuronal apoptosis in the oxygen and energy failure environment [15, 16]. Indeed, we observed great cell apoptosis after CoCl₂ treatment. More importantly, those cell apoptosis markers induced by CoCl₂ treatment could be blocked by knock down GCPII expression in both mRNA and protein level, and thus ameliorates the neuronal apoptosis progress in TUNEL assay.

In summary, our results highlighted the important of GCPII in the ischemia brain injury and suggested that inhibition of GCPII may have implications for the therapy of hypoxia-ischemia.

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Conflict of interests

The authors declare no conflict of interests.

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Figure legends

Figure 1. Detection of the mRNA expression of GCPII, mGluR3 and TGF- β through real-time-PCR assay. B35 cells were seeded in 10cm dish, and after CoCl_2 treatment with indicated time, cells were harvested and extracted RNA. The expression of GCPII, mGluR3 and TGF- β was detected through real-time-PCR assay. The mRNA expression levels are relative to actin. Results were representative of three independent experiments.

Figure 2. Detection of the protein expression of GCPII, mGluR3 and TGF- β through western blot assay. B35 cells were treated with CoCl_2 . After indicated time, protein was extracted and detected through western blot. B-actin was used as loading control. Similar results were repeated three times.

Figure 3. Detected the efficiency of siRNA knock down GCPII expression system.

(A) Quantitative real-time PCR analysis of GCPII mRNA levels in B35 cells stable transfected with control RNA or GCPII-siRNA. (B) Western blot analysis of GCPII protein levels in B35 cells transfected with GCPII-control RNA or GCPII-siRNA.

Figure 4. Detected the apoptosis through TUNEL assay in hypoxia environment.

B35 cells stably transfected with control RNA or GCPII-siRNA were treated with CoCl_2 for 48 hours, and the apoptotic cells were detected by TUNEL assay. Representative image of TUNEL staining were shown. Statistical number of apoptotic cells was from three experiments.

Figure 5. Detected the mRNA expression of apoptosis associated BAX, Caspase3, Caspase9 and BCL2 through real-time-PCR assay.

B35 cells stably transfected with control RNA or GCPII-siRNA were treated with CoCl_2 for 48hours, and the mRNA expression of BAX, Caspase3, Caspase9 and Bcl2 were detected through real-time-PCR. Results were representative of three independent experiments.

Figure 6. Detected the protein expression of apoptosis associated BAX, Caspase3, Caspase9 and BCL2 through western blot assay.

B35 cells stably transfected with control RNA or GCPII-siRNA were treated with CoCl_2 for 48hours, and the protein expression of BAX, Caspase3, Caspase9 and Bcl2 were detected through western blot.

Figure 7. Expression of GCPII in the brain tissues of rats with MCAO.

Fluorescent images showed the staining for NeuN (green) and GCPII (red) in the cortical region at 24 h after MCAO.

Figure 8. Knockdown of GCPII ameliorated apoptosis in the penumbra area of the rats with MCAO.

(A). Fluorescent images showed the staining for GCPII (red) in the cortical region at 72 h after the siRNA injection in misRNA or GCPII siRNA group. (B) TUNEL analysis in the cortical region of GCPII siRNA or misRNA group after MCAO.

Figure 1

Figure 1. Relative expression of GCP11, mGluR3 and TGFb1.

Figure 2

Figure 2. WB analyses of GCP II, mGluR3 and TGFb1.

Figure 3

Figure 3. Knockdown of GCP II in B35 cells.

Figure 4

Figure 4. TUNEL analysis of apoptosis in GCPII KD and control B35 cells.

Figure 5

Figure 5. RT-PCR analyses of apoptosis genes in GCPII KD and control B35 cells.

Figure 6

Figure 6. WB analysis of apoptosis genes in GCPII KD and control B35 cells.

Figure 7

Figure 7. IF analysis of GCPII expression in NeuN-positive neuronal cells.

Figure 8

Figure 8. Suppression of GCP II reduced cell death in ischemic animal models.

Table1 Sequences of RT-PCR primers.

GCPII-F: TTGGTTGAGTTATCTGATTATGAT

GCPII-R: GAGAAGGCACTGTATGGT

mGluR3-F: CCGAGTATATGTGTCCTGAT

mGluR3-R: GCTGTATGAACCACCAATG

Tgfb1-F: AATTCCTGGCGTTACCTT

Tgfb1-R: TGTATTCCGTCTCCTTGG

Casp3-F: ATTATGGAATTGATGGATAGTGTT

Casp3-R: GTAGTCGCCTCTGAAGAA

Casp9-F: ACTGCCTCATCATCAACA

Casp9-R: GTTCTTCACCTCCACCAT

Bax-F: GATGAACTGGACAACAACAT

Bax-R: CACGGAAGAAGACCTCTC

Bcl-2-F: GGATGACTGAGTACCTGAA

Bcl-2-R: GCCATATAGTTCCACAAAGG

Actin-F: CGTAAAGACCTCTATGCCAACA

Actin-R: AGCCACCAATCCACACAGAG

Table(s)

	Ct value					delta Ct		
	GCPII	Mglur3	Tgfb1	Actin		GCPII	Mglur3	Tgfb1
B35-NC	25.8	30.9	20.92	15.79		10.01	15.11	5.13
	25.76	31.45	20.83	15.79	15.78333	9.97	15.66	5.04
	25.69	30.69	20.78	15.77		9.9	14.9	4.99
B35-12	28.35	33.98	24.26	19		9.36667	14.99667	5.27667
	28.43	33.96	0	18.97	18.98333	9.44667	14.97667	
	28.39	33.82	24.15	18.98		9.40667	14.83667	5.16667
B35-24	28.52	0	24.96	19.5		9.00667		5.44667
	28.45	33.84	24.98	19.52	19.51333	8.93667	14.32667	5.46667
	28.52	33.94	24.95	19.52		9.00667	14.42667	5.43667
B35-48	27.88	32.85	25.04	19		8.87333	13.84333	6.03333
	27.89	32.79	25.02	19.01	19.00667	8.88333	13.78333	6.01333
	27.87	32.92	25.03	19.01		8.86333	13.91333	6.02333
B35-72	27.01	31.16	25.21	18.51		8.52667	12.67667	6.72667
	27.09	31.28	25.23	18.45	18.48333	8.60667	12.79667	6.74667
	27.13	31.19	25.26	18.49		8.64667	12.70667	6.77667

	GCPII	SD	Mglur3	SD	Tgfb1	SD
0 h	1	0.055678	1	0.392471	1	0.070946
12 h	1.467469	0.04	1.219813	0.087178	0.889868	0.077782
24 h	1.967909	0.040415	1.798333	0.070711	0.759609	0.015275
48 h	2.123833	0.01	2.596677	0.065064	0.510507	0.01
72 h	2.578735	0.061101	5.643773	0.06245	0.308497	0.025166