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Expression of Progranulin in a Mouse Model of Newborn Hypoxic-ischemic Brain Damage

Xuxin Ren[1, 2, †], Qiaoli Ren[3, †], Yilin Liu[1, 2], Wenyan Zhao[1, 2], Wei Liu[1, 2], Xing Tu[4], Junhua Yang[4], Jing Liu[4], Guoying Li[4], Mengxia Wang[5, *], Li Luo[4, *]

[1]School of Clinical Medicine, Guangdong Pharmaceutical University, Guangzhou, People's Republic of China

[2]School of Clinical Medicine, The First Affiliated Hospital of Guangdong Pharmaceutical University, Guangzhou, People's Republic of China

[3]Guangzhou Women and Children's Medical Center, Guangzhou, People's Republic of China

[4]School of Biosciences & Biopharmaceutics, Guangdong Pharmaceutical University, Guangzhou, People's Republic of China

[5]Intensive Care Unit, Guangdong No. 2 Provincial People's Hospital, Guangzhou, People's Republic of China

Email address:

josephluoli@hotmail.com (Li Luo), gracemengxia@hotmail.com (Mengxia Wang)

[*]Corresponding author

[†]Xuxin Ren and Qiaoli Ren are co-first authors.

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Abstract: Neonatal hypoxic ischemic encephalopathy (HIE) is one of the main reasons of death and disability in neonatal, for lack of blood and oxygen during the time of birth. Progranulin (PGRN) as a neurotrophic factor is extensively expressed in the brain can regulate neurite growth and promote neuronal survival. The mutations of PGRN gene may contribute to frontotemporal dementia (FTD). However, the role of PGRN in neonatal HIE remains unclear. We designed this study to investigate the changes of PGRN expression in the brain of newborn mice at different time points after hypoxic -ischemic brain damage (HIBD). Postnatal 7day (P7) mouse pups were induced HIBD model by the method of Rice with some improvement. TTC was used to detect the ischemic lesion volume. The localization of PGRN brain cells was detected by immunofluorescence. We also used Western blotting to measure the expression level of PGRN at different days (1, 3, 7 days) following HIBD. The results showed that we established the HIIBD model successfully. PGRN was primarily expressed in neurons and microglia, but rarely in astrocytes. In addition, PGRN expression in the brain of HIBD mice markedly increased at 1 day and 3 days and was restored at 7 days after HIBD. The results indicated that increased PGRN levels may be involved in the pathological mechanism and neural repair process of HIBD.

Keywords: Progranulin, Hypoxia-Ischemic Brain Damage, Neonatal Mice

1. Introduction

PGRN, also acknowledged as Granulin-Epithelin Precursor (GEP), is a 68~88-kDa multifunctional protein, which can be found in epithelial and immune cells, neurons and adipocytes [1]. Research in recent years have demonstrated that PGRN as a growth factor has a conspicuous effect on wound repair, tissue remodeling, cancer growth and survival [2]. Besides, in the brain, the frontotemporal dementia (FTD) and neuronal ceroid lipofuscinosis (NCL) are associated with the loss of the PGRN protein, due to a mutation of the PGRN gene (GRN) [3]. PGRN is capable of protecting neurons from premature death [4] and escalating neurogenesis in the hippocampus of adult mice [5] and engendering neurite outgrowth [6]. Van Damme P reported that PGRN could upgrade the survival of neurons, promote neural progenitor proliferation in vitro [7]. In brief, these studies show that PGRN was a neurotrophic factor, especially during the early development of neural and long-term neuronal survival.

The most prevailing reason of perinatal brain damage is neonatal hypoxic-ischemic (HI), which ensued from the neonatal brain lack of oxygen and blood. According to statistics, 60% of premature and low birth weight newborns suffer from HI brain injury, and the mortality rate is high at ~20% to 50% [8]. Additionally, survivors are generally accompanied with long-term neurological disorders, such as cerebral palsy and motor and cognitive deficits. [9] Up to now, hypothermia therapy has been the most effective therapy to HIBD. In the past decades, numerous researches focus on the new treatment strategies which aim to improve HIBD, but few of these have been used in the clinical practice. [10-11]. Patients treated with hypothermia often require lifelong rehabilitation, which places a heavy financial burden on families [8]. Thus, it has great implication to explore the pathogenesis of neonatal HIE, especially in neuronal protection and regeneration.

Considering the crucial role of PGRN in neurogenesis, further understanding of the relationship between PGRN and HIE may help clarify the pathogenesis of PGRN in neurodegenerative diseases and provide a basis for future new therapies. In this study, HIBD mice model were induced and the expression of PGRN in neonatal mice brain were detected at different days.

2. Materials and Methods

2.1. Hypoxic-Ischemic Brain Damage Model

C57BL/6 mice were purchased from experimental animal center of Sun Yat-sen University (Guangzhou, China). Mice were bred in‐house and pups of both sexes used for the injury model. All animal related experiments were conducted in accordance with the guidelines of the Experimental Laboratory Animal Committee of Guangdong Pharmaceutical University (permit no: gdpulac2017175), and under the approval of the principles of the National.

The HIBD model used in this study was based on the Rice method [12] with some modifications. Newborn mice pups (age, 7days) were randomly divided into HIBD group (n=20) and sham operation group (n=20). For HIBD group, pups were anesthetized with 2% isoflurane. Then the left common carotid artery (CCA) was exposed with microsurgical instrument. The CCA was ligated and cut off from the middle and completed by closing the incision. The pups were recovered in an incubator at 37°C for 10 minutes and then returned them into their dams for 1h. Afterwards, an airtight container mixed with 8% oxygen and 92% nitrogen were used for accommodated these pups for 120 min (flow velocity, 2L/min) and maintained at 37°C. Ultimately, HIBD pups were returned to their dams. The sham group also underwent anesthesia and exposed their left CCA, but no ligation or hypoxia treatment was performed.

2.2. TTC Staining

After hypoxic and ischemic for 24 hours, a part of the pups in HI groups and sham group were sacrificed respectively and their brains were immediately extracted. These removed brains were coronally cut into 2mm sections promptly by a brain slicer, which were imbued in 2% 2, 3, 5-triphenyl tetrazolium chloride (TTC; Sigma, St. Louis, MO, USA) then stored in an incubator in the dark and maintained at 37°C (20-30 minutes). After that, the slices were fixed with 4% paraformaldehyde and stored overnight for photographing. After that, the stained slices were scanned and the infarct areas were calculated. Then we used the image analysis system to quantify the infarct areas and non-infarct areas of each hemisphere.

2.3. Double Immun of Luorescent Staining

The brain tissues of neonatal mice (after HI 3 days) from HI groups were carefully removed and made into frozen sections and stored at －80°C until next use. We did the double immunofluorescent staining as usual. First day, these frozen sections were heated for 10 minutes in 56°C thermostat. Then using the 10% normal goat serum to block these sections at room temperature for 1 hour. Ultimately, the sections were washed three times with PBS (5 minutes each time), then the primary antibodies against NeuN for neurons (dilution, 1:1000; Sigma), glial fibrillary acidic protein (GFAP) for astrocytes (dilution, 1:1000; Abcam, UK), Iba-1 for microglial cells (dilution, 1:1,000; Abcam) and Anti-PGRN (dilution,1:500, Abcam, UK) in PBS which mixed 0.3% Triton X-100 were applied to incubate them overnight at 4°C. The second day, we washed the sections three times with PBS. Then we used the Alexa Flour 488 and Alexa Flour 594 ([Alexa Fluor®594 goat anti-mouse IgG (H+L), A-11005; and Alexa Fluor®594 goat anti-rabbit IgG (H+L), A-11012; Invitrogen Life Technologies)]) as the secondary antibodies to incubated the sections for 2 hours at 37°C. Afterwards, PBS was used to wash the sections thoroughly. Staining the Nuclei using DAPI for 5 minutes and be rinsed by PBS-T (PBS with 1% Tween 20). At last, we put the coverslips on the slides with Fluor-Save reagent and pictures by fluorescence microscope (BX51WI; Olympus, Tokyo, Japan).

2.4. Western Blot

Western blot analysis was performed to semi-quantitatively measure PGRN expression in the pups' brain after HI injured. Brains from the HI groups (1, 3, 7 days) were respectively separated from the olfactory bulb to the cerebellum using a brain slicer and immediately placed on ice (<1 min relative to initial handling). And put these samples at −80 °C until further analysis. The brain tissues were lysed with tissue lysis buffer（Beyotime Institute of Biotechnology, Shanghai, China） and homogenized by ultrasonic wave in ice-cold. Through the bicinchoninic acid (BCA) method (Beyotime Institute of Biotechnology, Shanghai, China) to determine the protein concentrations. Protein samples were separated by 12% sodium dodecyl sulfate-polyamide gel electrophoresis (SDSPAGE) and transferred to polyvinylidene difluoride membranes (PVDF, Millipore, Schwalbach, Germany). The PVDF membranes were blocked in blocking buffer [30 ml TBS-T (0.1% Tween

20) containing 1.5g fat-free milk powder] 1 hour and then incubated with mouse monoclonal anti-PGRN antibodies (1:3000), mouse monoclonal anti-β-actin antibodies (1:10000) overnight at 4 °C. After this, washing these membranes three times (Ten minutes each time) with TBS-T. HRP (1:5000) were as the secondary antibodies to incubate the protein at 37°C 2 hours and washed these membranes three times (Twenty minutes each time) with TBS-T. Using β-actin as a control for protein loading. In the end, Immunoreactive stripes were analyzed using the ECL system (Millipore) and imaging software. (Image Quant Las 4000; GE Healthcare, Pittsburgh, PA, USA).

2.5. Data Analysis

Data is expressed as mean ± standard error. Statistical differences between groups were analyzed using a one-way analysis of variance followed by Duncan's multiple range test. $P< 0.05$ was considered statistically significant.

3. Results

3.1. HIBD Model

TTC-staining assay was performed to detect the formation of ischemic foci in pups. The dramatical infarction were observed. Compared with the non-ischemic brain tissue, the infarction was white and the other side was red (Figure 1). The results showed that the HIBD model had been successfully established.

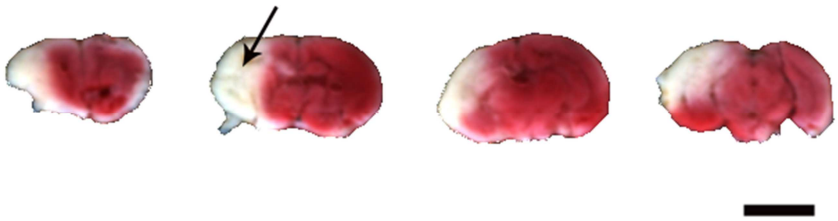

Figure 1. *The results of TTC staining.*

Whole brains of HIBD mice were collected quickly and sectioned coronally into 2 mm slices for TTC staining. TTC staining showed that the infarction site of the brain was white, and the normal tissue staining was red. The arrow indicates that the white area is the cerebral infarction. Bar=1 cm

3.2. Cell Location of Pgrn in the Brains of the Hibd Mouse Model

With the aid of double immunofluorescent staining, the specificity expression of PGRN in the brain of HIBD mice model were revealed. Neuron, astrocytes, microglial cells were performed immunofluorescent stained by their specific markers (NeuN, GFAP, Iba-1 and) respectively. Meanwhile, PGRN were also did the same stained by Anti-PGRN. Finally, we found that cells labeled by NeuN and iba-1 were also labeled by PGRN through the fluorescence microscope (Figure 2). This staining indicated that PGRN was primarily expressed in neurons and microglia but rarely in astrocytes after HIBD. Previous studies [21, 36] have confirmed that the human brain-derived PGRN are mainly derived from microglia cells rather than astrocytes. Furthermore, the expression of PGRN in microglia depends on its activation state, while in astrocytes is affected by pro-inflammatory factors. But the expression in neurons is related to its maturity [36, 37]. Our results confirmed that the expression location of PGRN in the brain of mice was not affected by HIBD.

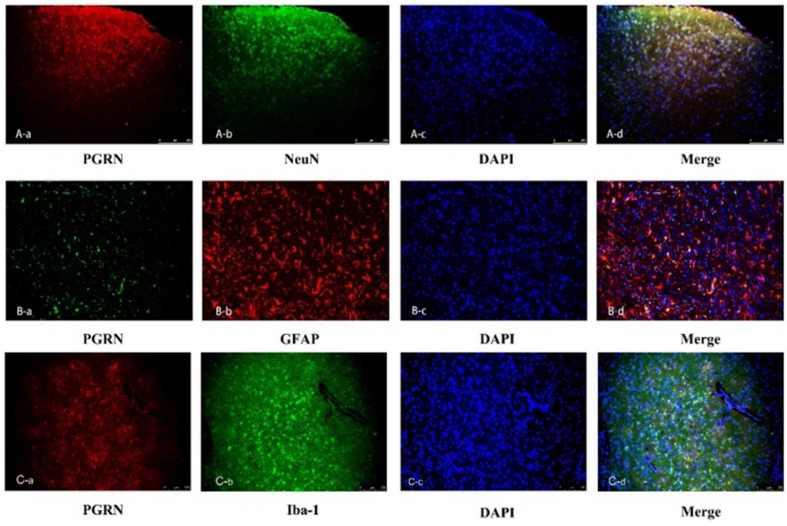

Figure 2. *Cell localization of PGRN in the cortex of HIBD mice. PGRN was expressed in neurons (A: a-d), and microglia (C: a-d) but rarely in astrocytes (B: a-d). Bar=250 um..*

3.3. Western Blot Analysis of PGRN in the HIBD Mouse Model

The results of western blot analysis revealed that PGRN have a dynamic change in the HI groups. Contrasted with the control group, the expression level of PGRN has noticeable increased on the first day and 3 day after HIBD but was recovered at 7 day. Compared with the first day, the expression level of PGRN in the third and seventh day decreased significantly. But it was still higher than the sham group. This information manifests that PGRN may be involved in the repair process of neurons in the early stage of HIBD (Figure 3).

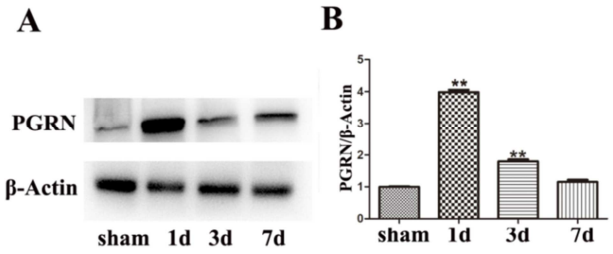

Figure 3. *The expression of PGRN following neonatal HIBD mice A.*

The PGRN expression levels in the brain following exposure to HIBD at different day. B. Western blot analysis demonstrating that PGRN expression was significantly increased in HI group compared with the sham group at 1 day and 3 day after HIBD, but it returned to a near-normal level on the seventh day. (***P<0.01*).

4. Discussion

At present, by utilizing neurotrophic factor to treat ischemic brain injury have received the most attention at the international level [13]. We designed this study to explore the expression of neurotrophic factor PGRN in the brain of mouse pups after HI injury. In the present study, we proved that, (a) HIBD mouse model can be produced successfully by Rice method [12] with some modifications. (b) PGRN were widespread expressed in the Neuron and microglial of HIBD mouse model. (c) Compared with the sham group, the expression of PGRN increased markedly on the first day and third day after HIBD. But it returned to a near-normal level on the seventh day.

The damage caused by HIE is thought as a change to adapt to the external environment which made by the injured brain [14]. Brain injury always appeared when the brain tissues at a low oxygen environment. At this time, the anaerobic breathing will increase, and the energy supply is deficient [15]. Nonetheless, as a disease with highly mortality and disability, HIE still doesn't have enough efficient therapy to improve the neonatal brain injury except mild hypothermia treatment currently. Fortunately, some scholars have found that some neurotrophic factors, such as erythropoietin (EPO) [16-17], vascular endothelial growth factor (VEGF) [18] and brain - derived neurotrophic factor (BDNF) [19] can improve the ischemic hypoxic brain damages in the animal model. In addition, Nikan h. Khatibi et al. [15] found that both high and low doses of capsaicin pretreatment improved myogenic tone and decreased apoptotic changes in the distal MCA and significantly reduce the infarcts of neonatal HIE in rats. Furthermore, C-type natriuretic peptide (CNP) and its cognate receptor NPR2 possess the innate protective mechanism in neonatal HI brain injury and other neurological disorders have been proven [20]. Despite all the above factors have certain positive effects in animal models have been proven, we still not clear which growth factors have the best brain protection and repair functionThe previous research shown that PGRN as a neurotrophic factor exerts a enormous function on the early development of neurons and the mutational of the PGRN gene (GRN) resulting in partial or complete loss of the PGRN protein were reported to associate with frontotemporal dementia (FTD) [23-24]. Beyond that, a growing evidence showed that PGRN is overexpressed early in neuron development and acts as neurotrophic to regulate neurite growth and promote neuronal survival at the same time [7-25]. Suh HS et al. [36] published an important report about the expression of human brain-derived PGRN in 2012, they found the PGRN were mainly come from microglia cells rather than astrocytes. In addition, in microglia cells, they proved some cytokines such as lipopolysaccharide (LPS) or cytokines IL-1β/interferon-gamma (IFN-γ) can downregulate the PGRN expression and secretion, but others like IL-4 and IL-13 can upregulate its expression. On the other hand, PGRN expression in astrocytes was stimulated by pro-inflammatory agents, including cytokines IL-1β/IFN-γ or the toll-like receptor 3 ligand polyinosinic: polycytidylic acid (poly I: C). It is worth noting that the expression of PGRN in microglia cells is influenced by its activation degree, while the expression in neurons is correlated with the maturity of neurons [37]. Under normal circumstances, PGRN is less expressed in microglia. However, the expression of PGRN in microglia cells increased significantly when traumatic brain injury (TBI), toxin-induced brain injuries, spinal cord injury and nerve axotomy occurred [37, 38, 39, 40, 41] In the focal cerebral ischemia-reperfusion injury, the absence of PGRN will promote the destruction of the blood-brain barrier (BBB) [26] and giving exogenous recombinant-PGRN (R-PGRN) can significantly reduce the size of the infarct by inhibiting recruitment of neutrophils [27]. All the above results confirm that PGRN pushes forward immense influence on neuroprotective after brain injury by regulating phagocytes to playing an anti-inflammatory role and promoting neuron regeneration.

In our study, we through the double immunofluorescence staining found that PGRN was mainly expressed in microglia cells and neurons after HIBD. To further validate the effects of PGRN in the HIE model, we used Western blot to quantify the expression of PGRN. Western blot results showed that PGRN expression dramatically increased on the first day and third day after HIBD.. At this moment, the repair mechanism in the brain is activated, and PGRN is involved in the repair

of neurons. We speculated that the HIBD activated the large amount of microglia cells through stress response, after that, these activated microglia cells upregulate the synthesis and secretion of PGRN by some cytokines. Then PGRN play a protective role by promoting neuronal regeneration and regulating phagocytes to playing an anti-inflammatory role. But with the disappearance of the stress state，the number of activated microglia decreased. At the same time, other repair mechanisms in the brain have been activated, both of these may contribute to the decreased of PGRN on the seventh day. Therefore, it prompted that PGRN was participant in the self-healing mechanisms of brain, in neonatal mice, and continue for a certain period of time. This is consistent with others reasoning. Moreover, Yin et al reported in the model of oxygen glucose deprivation [28] and oxidative injury [29-30], PGRN plays a neurotrophic [31-32] and anti-inflammatory [33-35] role, this indirectly justifies our conclusion. These results also indirectly support our speculation.

5. Conclusion

In general, immunofluorescence staining and western blot analysis demonstrated the expression and cell location of PGRN in the brains of neonatal HIBD mice. (1) Through the double immunofluorescent staining, we demonstrated that PGRN was mainly expressed and secreted by neurons and microglia cells in the brain of newborn mice after HIBD. And the stress reaction after HIBD could not increase the expression and secretion of PGRN in astrocytes, which may cause by the gene regulation. (2) In addition, Western Blot analysis help us confirmed that the PGRN in the brain of mice was dynamically changed after HIBD. PGRN was significantly increased on the first and third days after HIBD, and nearly returned to the normal level on the seventh day (Still higher than the control group). We hypothesized that the stress state after HIBD activated a large number of microglia, the activated microglia raised PGRN expression and secretion by some cytokines. Then these PGRN by promoting regeneration of neurons and regulate phagocytes to exert neuroprotective and anti-inflammatory effects. But with the disappearance of the stress state，the number of activated microglia decreased. At the same time, other repair mechanisms in the brain have been activated, both of these may contribute to the decreased of PGRN on the seventh day. These findings suggest that PGRN is involved in the earliest repair mechanism after HIBD. But there still many we haven't clarify. Therefore, further studies should focus on the mechanism underlying the regulation of the changes in PGRN during HIBD.

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Ethics Approval and Consent to Participate

The animal-related experiments were approved and organized in accordance with the guidelines of the Experimental Laboratory Animal Committee of Guangdong Pharmaceutical University (permit no: gdpulac2017175).

Conflict of Interest Statement

The authors declare that they have no competing interests.

Availability of Data and Materials Statements

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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