Original Article

SCG10 protein maintenance in superior cervical ganglia promotes sympathetic hyperinnervation after myocardial infarction

Junyi Zhang1,2, Kaidi Zhao2, Yan Li1,3, Yugen Shi1,3, Wenju Yan1, Qian Liu1, Suhua Yan1,3

1Department of Cardiology, Shandong Qianfoshan Hospital, Cheelu College of Medicine, Shandong University, Shandong, China; 2Department of Cardiology, Qingdao Municipal Hospital, Qingdao, Shandong, China; 3Department of Cardiology, Shandong Provincial Qianfoshan Hospital, The First Hospital Affiliated to Shandong First Medical University, Shandong, China

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Abstract: Objective: Our research aimed to investigate whether superior cervical ganglia (SCG) neuron expressing SCG10, and whether SCG10 is involved in sympathetic nerve remodeling after MI. Methods: Adult male Sprague-Dawley rats were divided into a control group, MI group, and MI+SCG10 degeneration inhibitor group. Western blotting and immunofluorescent staining were used to detect SCG10 expression level and location in this study. The time course of SCG10 level variances of SCG and cardiac tissue were obtained by protein analysis on day 0, 1, 3 and 7 using western blotting. Results: From the third day of myocardial infarction, we found that the number of SCG10 cells increased significantly, not only the number of positive cells, but also more expression in the cytoplasm, and there was a significant difference compared with the sham group (P < 0.01). SCG10 positive cells of the MI7D group increased significantly compared with the MI3D group (P < 0.01). The content of SCG10 in the MI7D group was significantly lower than that in the MI7D+SP600125 group in the SCG (P < 0.01). The expression of TH in MI7D group was significantly higher than that in the sham group (P < 0.01). The content of TH in MI7D group was significantly lower than that in the MI7D+SP600125 group (P < 0.01). The SCG10 level in the MI3D was significantly higher than that in the sham group (P < 0.01). MI7D group increased significantly compared with the MI3D group (P < 0.01). The content of SCG10 in MI7D group was significantly lower than that in MI7D+SP600125 group in the SCG (P < 0.01). Conclusion: SCG10 overexpression in sympathetic neurons of SCG might lead to cardiac sympathetic nerve hyperinnervation, and SCG10 could be a new target for regulating cardiac sympathetic activity after MI.

Keywords: Superior cervical ganglion-10, superior cervical ganglion, myocardial infarction, sympathetic hyperinnervation

Introduction

Malignant arrhythmia after myocardial infarction (MI) is an important cause of death and disability. Many previous studies have shown that excessive hyperplasia and imbalance of sympathetic nerve distribution after MI are the most important factors in the pathogenesis of malignant arrhythmia [1]. Research on the mechanism of sympathetic hyperproliferation has attracted much attention at present. Recent studies have focused on γ-aminobutyric acid (GABA) signaling system and inflammation pathway in sympathetic nerve remodeling and susceptibility to malignant arrhythmias after MI [2, 3]. Relevant conclusions have been drawn, but the mechanism is still unclear.

Superior cervical ganglion-10 (SCG10), also known as stathmin 2 (STMN2), a neuron-specific member of the stathmin family of proteins, is an important marker of regenerating sensory axons [4]. It has been reported that SCG10 can not only be used as a marker of nerve injury, but also can affect the survival time of the severed distal nerve [5]. In addition, some investigators have found that the levels of SCG10 are increased in the end bulbs of the proximal stump in both the central and peripheral nervous systems [6]. Furthermore, the binding of
SCG10 to tubulin regulates the polymerization and depolymerization of microtubules, which is a key in axonal regeneration [7].

Clinical research demonstrates that removal of the superior cervical ganglion (SCG) results in the disappearance of symptoms of angina pectoris and the increase of blood pressure and cardiac sympathetic activity [8]. Our experimental group also confirmed that some signal systems in SCG regulates NE release in cardiac sympathetic nerves and intracellular Ca2+ levels after MI. Therefore, we envisage that after MI the level of SCG10 in the SCG may increase and promote the regeneration of sympathetic nerves around the infarct area [9]. To confirm the idea above, we made a rat model of MI to examine whether SCG10 increased in the SCG and the infarct periphery after the onset of MI. Further, rats were injected with SP600125 intraperitoneally three times a week, so as to observe whether the changes in SCG10 content are significantly correlated with the changes in the secretion of TH in the nerve endings.

Materials and methods

Animal and experimental protocol

Adult male Sprague-Dawley rats (220-250 g, Vital River, Beijing, China) were used. They were housed in cages (3-5 rats per cage) with wood shaving bedding and maintained in a temperature-controlled room (22±2°C) with daily exposure to a twelve-hour light-dark cycle. All procedures were based on the ethical standards of animal research approved by Shandong University Institutional Animal Care and Use Committee. All efforts were made to minimize their suffering. Two separate experiments were conducted. Protocol 1: 40 rats were enrolled and randomly divided into four groups: the sham surgery group (sham group, n=8), 1 day of cardiac infarction group (MI1D group, n=18), 3 days of MI group (MI3D group, n=18), and 7 days of MI group (MI7D group, n=17). Protocol 2: 30 rats were enrolled and randomly divided into three groups: the sham surgery group (sham group, n=8), rats with MI for 7 days (MI7D group, n=17), and rats with MI for 7 days treated with JNK inhibitor SP600125 (Sigma) (MI7D+SP600125 group, n=18). JNK can phosphorylate some sites on the SCG10 domain, thus accelerating the decomposition of SCG10. SP600125 is a reversible and ATP-competitive JNK inhibitor, which can reduce the effect of JNK on SCG10 phosphorylation and slow down the degradation of SCG10. SP600125 was intraperitoneally injected at 5 mg/kg three times a week. The rats were sacrificed and the tissue was frozen at -80°C for further biochemical analysis or embedded in OCT for histological examination.

Experimental myocardial infarction

SD rats were anesthetized by an intra-peritoneal injection of 30 mg/kg of 3% sodium pentobarbital (Sigma-Aldrich, St. Louis, MO, USA). Then we placed the rats in a supine position on a homeothermic heating pad in order to maintain their body temperature. We shaved their fur from the neck and chest areas with an electric shaver. Endotracheal intubation was performed to maintain the breathing by making a 5 mm mid-neck incision and retraction of muscle tissue. The endotracheal tube was inserted into the trachea and connected to a small-animal respirator (HX-300S, TME, Chengdu, China) at 70 breaths/min and a tidal volume of 1.2 ml/100 g body weight. The chest was incised at the left fourth intercostal space, the tissue layers were divided, and a small incision was made in de pleura so that the heart was exposed. Then we ligated the area between the left atrial appendage and the pulmonary artery cone with a 6-0 prolene suture. The infarction was confirmed by ST segment elevation with electrocardiograph. Then we sutured the muscle and skin and removed the respiratory. The rats were closely monitored and taken care of in the recovery period. At the end of surgery, they were injected with penicillin (800,000 U, NCPC).

Harvesting the superior cervical ganglion

The preparatory work was the same as that of the rat myocardial infarction model. After incision of the skin and soft tissue of the rat's neck, we saw the sternohyoid muscle with a median field of vision and the sternomastoideus muscle on both sides of the subdivision. After blunt separation of these two muscles, the common carotid artery was bent out of shape. After the superior thyroid artery and the common carotid artery were bifurcated, the oval superior cervical ganglion could be seen above the vagosympathetic chain. Once the rat's myocardial infarction was confirmed, removal of the superior cervical ganglion was carried out.
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**Immunofluorescence**

SCGs and hearts were immersed in 30% sucrose in PBS, embedded in OCT compound (Tissue-Tek), and frozen in a freezer whose temperature was kept at -80°C. For tissue processing, samples were incubated with primary antibodies anti-STMN2 (1:400; Proteintech) or anti-tyrosinase (1:200; Millipore) overnight at 4°C followed by a two-hour incubation with Alexa Fluor 546 donkey anti-rabbit (1:400; Peprotech) and FITC-conjugated rabbit anti-sheep (1:200; Bethyl) secondary antibodies. In order to identify the nuclei, we used DAPI (Life Technologies) counterstain in the sections. We used Image-Pro Plus 5.0 to calculate and measure the area of stained nerves divided by the total area examined (μm²/mm²). The selected fields are as we previously described.

**Western blot analysis**

We extracted proteins by using a Protein Extraction Kit (Beyotime Institute of Biotechnology, Jiangsu, China). Extracted proteins from different tissues were measured with the BCA Protein Assay Kit (Pierce). The same amount of protein from each sample underwent electrophoresis on a 12% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in 5% non-fat dry milk in TBST (TBS containing 0.05% Tween-20) incubated overnight at 4°C with primary rabbit anti-STMN2 (1:2000; Proteintech). Then membranes were probed with anti-rabbit secondary antibodies (1:5000; Zhong Shan-Golden Bridge Biotechnology) for 1 h. Enhanced chemiluminescence (ECL) detection kit (Millipore) was used to visualize the membrane. Visualization and quantification of the densities were performed using the FluorChem E Image (Protein-Simple, Santa Clara, CA, USA) and NIH Image software. Protein densities were analyzed relative to individual GAPDH densities.

**Statistical analysis**

Analyses were performed using SPSS 17.0 software (SPSS Inc. Chicago, IL, USA). All statistical analyses were considered significant at P-values < 0.05.

**Results**

**Pathological morphology**

Pathological H&E staining was performed in myocardial infarction rats and operation group rats. The results showed that there was vacuolar degeneration, partial rupture, unclear, disappeared and diffuse inflammatory cell infiltration in the infarct site. The normal myocardial tissue showed that the morphology of myocardial cells was complete and clearly defined, the striation in the cytoplasm was clear, and the nucleus was oval (Figure 1).

![Figure 1. Myocardial histopathology of myocardial infarction rats and sham operated rats. A. Myocardial infarction rats, Vacuolar degeneration, partial rupture, unclear, disappearance and diffuse inflammatory cell infiltration can be seen in myocardial tissue. B. The normal myocardial tissue showed that the morphology of myocardial cells was complete and clearly defined, the striation in the cytoplasm was clear, and the nucleus was oval.](image)

![Figure 2. Western blot showing the expression profiles of SCG10 in the SCG after MI. SCG10 was quantified relative to the GAPDH (37 KD) levels. #P < 0.01 vs. sham; &P < 0.01 vs. MI3D.](image)
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Then we studied the difference of TH content around myocardial infarction border in rats (Figure 4). The expression of TH was very rare in the sham group. In the MI1D group the expression around the infarct area was also rare. There was no significant change between the MI3D group and the sham group (P > 0.05). However, the expression of TH in MI7D group was significantly higher than that in the sham group (P < 0.01). The content of TH in MI7D group was significantly lower than that in MI7D+SP600125 group (P < 0.01) (Figure 5).

SCG10 protein expression in SCG and myocardium

In order to confirm the expression of SCG10 in the superior cervical ganglion and myocardium of rats after MI, we used the western blot method (Figures 6-9). It was found that the content
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In the study of SCG10 content in rat myocardium, we found similar results. There was no significant difference between the MI1D group and the sham group, while the SCG10 level increased more significantly in MI3D group than the sham group ($P < 0.01$) and MI7D group increased significantly compared with the MI3D group ($P < 0.01$). The content of SCG10 in MI7D group was significantly lower than that in MI7D+SP600125 group in the myocardium ($P < 0.01$).

**Discussion**

After myocardial infarction, excessive regeneration of cardiac nerve, especially sympathetic nerve, is the main cause of increased mortality. At present, the mechanism of nerve regeneration is not clear [10, 11].

In normal axons, microtubules require transitions in polymerization and depolymerization to maintain the function of the axon, referred as “dynamic instability”, which is vital to the survival of the normal axons [12, 13]. In mature axons, microtubules exist for hours or days, while in growing axons, the half-life of microtubules is only a few minutes. Taxol increases the stability of microtubule, which leads to the degeneration of the central neurites and causes neurological disease [14, 15]. Therefore, for newborn axons, excessive stability of microtubule cytoskeleton will be detrimental to the extension of axons [16, 17]. Cho’s study found that the level of acetylated tubulin decreased significantly in the proximal stump of the injured nerve and increased the activity of microtubules, suggesting that microtubule instability is essential to axon regeneration [18].

Therefore, there must be some kind of protein and pathway to regulate the activity of microtubules in the neonatal axons [19, 20]. In the study of axonal dissection, the researchers found an interesting phenomenon that the fast consumption of SCG10 of the distal axons after transection of peripheral axons result in the degradation of distal axons, but the levels of...
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SCG10 are increased in the end bulbs of the proximal stump. Our study also found a significant increase in SCG10 content in the peripheral axons of MI. Shin’s study, which demonstrates that JNK can phosphorylate SCG10 in the distal axons of injured nerves, thus making vesicles after being synthesized in neurons [23, 24]. Then the protein binds tubulin dimer and limits the pool of building for microtubule assembly causing microtubule instability [25]. In the process of neurite outgrowth, it especially requires the SCG10 induced “dynamic instability”, which promotes the growth of axons [26, 27]. In conclusion, our study found that the increase of SCG10 content in the nerve growth cone around infarcted myocardium was positively correlated with the expression of TH-positive nerves, which further confirmed that SCG10-induced microtubule instability is an important mechanism for nerve regeneration.

In our research, the content of SCG10 on the axonal growth cone of infarcted myocardium did increase significantly, but we do not know where SCG10 came from. Clinical research demonstrated that removal of the superior cervical ganglion (SCG) results in the disappearance of symptoms of angina pectoris and the increase of blood pressure and cardiac sympathetic activity [28]. In our research group’s previous animal experiments, we found that some signaling systems in the SCG can manage sympathetic nerve activity via NE after MI.
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Therefore, we envisage that SCG is related to the changes of sympathetic activity and function after MI.

Our data show that the amount of SCG10 in the SCG has a significant increase with the progression of MI, and we have obtained consistent results by immunofluorescence and immunoblotting. When we injected JNK inhibitor into MI rats, the expression of SCG10 in SCG and myocardium was significantly higher than that in MI rats only. This indicates that the injury of the autonomic nervous system after MI leads to the massive synthesis of SCG10 in the SCG that transports through the axons to the nerve endings, thus participating in the injured nerve, especially the sympathetic nerve, and the repair and the maintenance of the function. However, we do not know the exact mechanism of SCG10 generation induced by nerve injury, which is the direction of further research.

When MI occurs, the cardiac sympathetic nerve releases a large amount of catecholamine, which is the main substance leading to malignant arrhythmia and further myocardial damage [29]. Tyrosine hydroxylase (TH) is a speed limiting enzyme for the synthesis of catecholamine and also plays a catalytic role [30]. Its positive location can represent the distribution of sympathetic nerves. In recent research, Parrish found that transient denervation of peri-infarct myocardium does not alter arrhythmia susceptibility [31]. Therefore, reducing the remodeling of sympathetic nerves after MI is still the key to reduce the incidence of malignant arrhythmia. In our experiment, immunofluorescence showed that the density of TH-positive nerves around the infarcted area also changed with the content of SCG10 in nerve endings. The results showed that the decrease of SCG10 levels in nerve terminals ameliorated sympathetic hyperinnervation. The decrease of neurotransmitter released by sympathetic nerve will further reduce the incidence of malignant arrhythmia.

In conclusion, our data indicate that MI induced elevated levels of SCG10 in the SCG and infarcted border. The increase of SCG10 levels promotes axonal growth by the instability of tubulin and eventually resulted in hyperinnervation of sympathetic nerve in rats. This discovery may provide a new perspective for study of the mechanism of hyperinnervation and a potential therapeutic target for sympathetic remodeling and malignant arrhythmia after myocardial infarction.

Disclosure of conflict of interest

None.

Address correspondence to: Suhua Yan, Department of Cardiology, Shandong Qianfoshan Hospital, Chee-loo College of Medicine, Shandong University, No. 16766, Jingshi Road, Qingdao 266011, Shandong, China. Tel: +86-0532-89269418; E-mail: yansuhua116@yeah.net

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