CaSR and Calpain contribute to the ischemia reperfusion injury of spinal cord

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Running title: CaSR and calpain in SCIRI

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Abstract

Spinal cord ischemia reperfusion injury (SCIRI) can cause spinal cord dysfunction and even devastating paraplegia. Calcium-sensing receptor (CaSR) and calpain are two calcium related molecules which have been reported to be involved in the ischemia reperfusion injury of cardiomyocytes and the subsequent apoptosis. Here, we studied the expression of CaSR and calpain in spinal cord neurons and tissues, followed by the further investigation of the role of CaSR/calpain axis in the cellular apoptosis process during SCIRI. The results of in vitro and in vivo studies showed that the expression of CaSR and calpain in spinal cord neurons increased during SCIRI. Moreover, the CaSR agonist $GdCl_3$ and antagonist NPS-2390 enhanced or decreased the expression of CaSR and calpain respectively. The expressions of CaSR and calpain were also consistent with the cellular apoptosis in spinal cord. Taken together, CaSR-calpain contributes to the SCIRI apoptosis, and CaSR antagonist might be a helpful drug for alleviating SCIRI.

Key words: Spinal cord ischemia reperfusion injury (SCIRI), Calcium-sensing receptor (CaSR), calpain, apoptosis

Introduction

Spinal cord ischemia reperfusion injury (SCIRI), a severe complication of thoracoabdominal aortic aneurysm or repair surgeries, can cause spinal cord dysfunction and even devastating paraplegia that will impose huge burdens on patients [1]. The indispensable aorta blocking during these surgical operations induces ischemia, whereas the subsequent reperfusion aggravates the injury instead of mitigating it. Multiple factors and mechanisms are involved in the onset and progression of SCIRI such as lipid peroxidation, elevated production of reactive

oxygen species, glutamate excitotoxicity, and activation of matrix metalloproteinase, all of which are related to calcium to various extents [2].

Calcium is a classical second messenger inside cells. However, accumulating evidences support that extracellular calcium also performs diverse functions as the first messenger [3]. The center of the extracellular calcium signal transduction is calcium-sensing receptor (CaSR) situating across plasma membrane. The CaSR, whose primary ligand is extracellular calcium, belongs to the G protein coupled receptor family [4]. The expression of CaSR is reported to be elevated during the pathophysiological process of ischemia reperfusion injury (IRI) in heart and kidney [5,6]. In contrast, whether CaSR performs significant functions in the central nervous system (CNS) IRI remains to be clarified. CaSR is found to be present in various brain areas, particularly with strong expression in olfactory bulb and hypothalamus, indicating its involvement in the regulation of cellular function in brain [7]. Moreover, brain ischemia can induce cellular apoptosis through CaSR [8]. Even so, whether CaSR is present or plays important roles in SCIRI is not known yet.

Calpain, also known as calcium dependent protease system, is one of the major effect molecules reacting to intracellular calcium signal [9]. When calpain is activated, it will cleave a plethora of proteins, resulting in cellular apoptosis. We thus hypothesized that the CaSR/calpain axis might be responsible for the neuronal apoptosis occurred during SCIRI.

This study first explored the expression of CaSR and calpain in spinal cord neurons and tissues. After that we further investigated the role of CaSR/calpain axis in the cellular apoptosis process during SCIRI.

Materials and Methods

1. Animals and culture of spinal cord neurons

The animal procedures complied with the guidelines issued by Jiangsu University Animal Care and Ethics Committee. One day post-partum SD rats were purchased from the animal center of Jiangsu University. Spinal cord neurons were isolated and cultured in vitro. Briefly, after euthanasia, these neonates were dissected

to harvest spinal cords. Then, both spinal pia mater and blood vessels were removed. The collected tissues were minced and digested in 0.125% trypsin under 37°C for 20 min. The products of digestion were collected by centrifugation and re-suspended with DMEM/F12 medium (containing 10% FBS, 100 U/ml ampicillin, and 100 U/ml streptomycin). Twenty-four hours later, the culture media were replaced with neurobasal media containing 10% FBS and 2% B27 (NFB media). The half of the media was routinely changed every day.

One week later, immunofluorescent staining for neurofilament 200 (NF-200, a marker of neurons), CaSR and calpain was performed to characterize the neurons. Briefly, cells were fixed, blocked, and incubated in primary antibodies solutions overnight at 4°C. Primary antibodies included NF-200, CaSR and calpain (all 1:100, from Abcam). After rinse, cells were incubated with secondary antibodies at 37°C for 1 hour. Negative controls were also performed.

2. Ischemia/ Reperfusion in vitro

Seven days after the culture of neurons, these cells were randomly divided into four groups. (1) control group, which would be continuously cultured with NFB media; (2) IR group, which would be through the ischemia/reperfusion procedures; (3) IR+Gd group, after the ischemia/reperfusion procedures, the neurons would be treated with CaSR agonist- GdCl₃; (4) IR+NPS group, after ischemia/reperfusion, the neurons would be treated with CaSR antagonist-NPS 2390.

As to ischemia/reperfusion procedures, the culture media of the IR, IR+Gd and IR+NPS groups were changed to DMEM without glucose. Then the neurons were put into a hypoxic incubator which was supplied with 5% CO₂/95% N₂ at 37°C for 2 hours. Then the culture media in IR, IR+Gd and IR+NPS groups were immediately changed to NFB media, NFB containing 300 µM GdCl₃, and NFB containing 10uM NPS 2390 respectively. At last, these neurons were put into an incubator containing 5% CO₂/95% filtered air for 12 hours to finish reperfusion.

After the construction of IR model, Western blotting was applied to detect the expression levels of CaSR, calpain, and bax among the four groups. Primary antibodies included CaSR, calpain, bax and β -actin.

3. Immunohistochemistry staining

The spinal cords of SD rats weighted about 250 g were harvested to detect the expression and localization of CaSR and calpain. In brief, the rats were anaesthetized with diethyl ether and 4% paraformaldehyde solution was perfused via the heart to fix the spinal cord. Then paraffin sections were prepared, deparaffinized and rehydrated before the incubation with primary antibodies (CaSR and calpain, both 1:100). After that, an immunohistochemistry detection kit (Boster, China) was used to develop the color.

4. Ischemia/ reperfusion in vivo

Twenty-eight SD rats weighted about 250 g were supplied by the animal center of Jiangsu University. The rats were randomly and equally divided into four groups: (1) control group, only the sham operation were performed; (2) IR group, the ischemia/reperfusion operation were performed; (3) IR+Gd group, after ischemia/reperfusion operation, the animals would be treated with GdCl_3 ; (4) IR+NPS group, after ischemia/reperfusion operation, the animals would be treated with NPS 2390.

The ischemia/reperfusion operation was performed according to the report with slight modifications [10]. The aorta at the level of right renal arteries was isolated and clutched with a Scoville-Lewis aneurysm clip, and the incision partially closed with the clip tail left outside. Thirty minutes after the clipping of aorta, the clip was withdrawn and the incision was closed. Subcutaneous injection of penicillin was given to prevent infection. The animals in control group underwent the same procedures except the clipping of aorta. The animals in the IR+Gd and IR+NPS groups were subjected to the intravenous injection of GdCl_3 (30 $\mu\text{mol/kg}$) and NPS-2390 (10 $\mu\text{mol/kg}$) through tail once a day for 2 days before the operation.

Twenty-four hours after the end of operation, BBB scoring was applied to evaluate the hind-limb function of each group. Then, the spinal cords of four euthanized rats from each group were isolated and subjected to Western blotting detection of CaSR and calpain. The spinal cords of the left three animals were isolated and sectioned. The sections were subjected to immunohistochemistry staining for CaSR and calpain or TUNEL.

5. Assessment of neurologic function

Basso – Beattie - Bresnahan (BBB) open-field locomotor scale was a common and sensitive method to evaluate the neurologic function of rats after IR [11]. It assessed hind limb locomotor functions from complete paralysis (score 0) to normal (score 21). The BBB score of each rat was calculated by averaging the scores determined by three observers who were blinded to the group treatment.

6. TUNEL

The segments of L2-3 lumbar spinal cord were harvested from animals after euthanasia. Sections from these tissues were prepared and subjected to TdT-Mediated dUTP Nick-End Labeling (TUNEL) staining using a commercial detection kit (Roche, Switzerland). The percentage of positive cell number over total cell number in one visual field was defined as apoptotic index. The average of apoptotic indexes of three visual fields in one section, and five sections from the same individual was calculated as the apoptotic index of this individual.

7. Statistical analysis

All the data were expressed as mean $\pm$ standard deviation (SD). The BBB scores and the expression levels of proteins were compared by ANOVA with SPSS 12.0 software. The test was considered to be statistically significant when $P < 0.05$.

Results:

1. Expression and localization of CaSR and Calpain in neurons in vitro

Figure 1 panel I demonstrated the morphology of neurons cultured in vitro and the expression of NF-200, CaSR and calpain in these cells. The neurons initially were round and attached to the bottom of flask 12 hours after plating. Then long protrusions appeared one day after culture. These protrusions elongated and formed a complex network in the following week. Immunofluorescent staining indicated that CaSR was distributed on the cell membrane, cytosol and protrusions. The expression of calpain was weaker than that of CaSR in neurons.

2. Expression of CaSR and calpain in the IR neurons

Figure 1 panel II showed that after 12 hours of reperfusion which was followed by 2 hours of anoxia, the neuron bodies shrank accompanied with ruffled outlines while the protrusions diminished. Compared with IR group, neurons in IR+Gd group had fewer protrusions. The protrusions of neurons in IR+NPS group were more abundant than those in IR group. It indicated the protective effect of CaSR inhibitor NPS-2390 on IR injury.

Figure 1 panel III part A showed the representative Western blotting results of CaSR, Calpain, bax and β -actin in the four groups including control, IR, IR+Gd, and IR+NPS. ANOVA analysis and LSD-t test revealed that while the expression of CaSR in IR group was significantly higher than that in control group, it was significantly lower than that in IR+Gd group (part B). While the expression of CaSR in IR+NPS group was significantly lower than that in IR group, there was no significant difference between the expression of CaSR in IR+NPS group and the control, which indicated the complete block of up-regulation of CaSR in neurons caused by IR.

As to the expressions of calpain and bax in the spinal cords of animals among the four groups, similar patterns to the expression of CaSR could be found. The expression levels of calpain in IR and IR+Gd groups were significantly higher than those in control and IR+NPS groups, whereas the levels of calpain in IR+NPS group were still higher than those in control group. The expression levels of bax in IR+Gd group was highest followed by those in IR group. Both of the bax expression levels in IR and IR+Gd groups were higher than those in control and IR+NPS groups. Besides,

there was no significant difference between the expression levels of bax in control and IR+NPS groups.

3. Expression of CaSR and calpain in rat spinal cord

Figure 2 demonstrated the expression of CaSR and calpain in different parts of normal spinal cords. In the front horn of spinal cord, the CaSR and calpain positive motor neurons could be observed. The CaSR and calpain positive cells could also be found in the posterior part of gray matter and the boundary areas between gray and white matter as well, implicating the widely distribution of them in spinal cord.

4. The involvement of CaSR and calpain in SCIRI

4.1 Neurological functions of animals

As shown in figure 3 panel II, the BBB scores indicated that neurological function after IR decreased significantly when compared with that of control group, while the neurological function of IR+NPS group was significantly better than that of IR or IR+Gd groups.

4.2 The cellular apoptosis in SCIRI

Figure 3 panel I demonstrated the representative TUNEL results. The specimen from control group was almost negative. In contrast, the specimen from IR group had a plethora of positive cells containing brown nuclei. In addition to the positive large cells reminiscent neurons, small round cells were also positive for TUNEL staining. More apoptotic cells could be found in the spinal cord of the rats from IR+Gd. Compared with IR group, the specimen from IR+NPS group had less positive cells, and most of the positive cells were small, suggesting the damage to large neurons were protected by NPS-2390.

As shown in figure 3 panel III, there was significant difference between any two groups. Importantly, the apoptotic index in IR group was significantly higher than those in control and IR+NPS groups, but was lower than that in IR+Gd group. Obviously, the apoptotic index in IR+NPS was significantly lower than that in IR

group, but was higher than that in control group.

4.3 The expression of CaSR and calpain in SCIRI

Figure 4 consistently showed that the expression of CaSR and calpain in IR group increased when compared with that in control group. The expression of CaSR in IR+Gd group was significantly higher than that in IR group, whereas the expression of calpain in IR+Gd group was merely insignificantly higher than that in IR group. With the pretreatment with NPS-2390 in IR rats, the expression levels of CaSR and calpain decreased when compared to those in IR group. In addition, there was no significant difference between the expression level of CaSR in IR+NPS and control group, which suggesting the complete blocking of CaSR upraise during IR. In contrast, the blocking of calpain increase by NPS-2390 during IR was incomplete which manifested as the significant increase of calpain expression level in IR+NPS group than that in control group.

Discussion

The major objective of our study was to analyze the role of CaSR in the apoptosis during SCIRI. To this end, we first studied the expression of CaSR and its potential effector calpain in neurons and spinal cord, and then we used the agonist and antagonist of CaSR (GdCl_3 and NPS-2390 respectively) to probe the function of them during SCIRI.

Although accumulating evidences have demonstrated the widely expression and important functions of CaSR in brain regions [12,13], whether CaSR expresses in spinal cord and its cellular location remain elusive. Based on the pivotal role of calcium in the physiology of nervous system, it makes sense to hypothesize that CaSR also extensively exists in spinal cord like in brain [14]. Hence, our study determined the expression of CaSR protein in neurons cultured in vitro from the spinal cord of neonatal rat, and then explored the distribution of CaSR in spinal cords. Both of the results consistently indicated the ubiquitous presence of CaSR in neurons of spinal cord. Moreover, CaSR could be easily spotted in the small round cells in spinal cord,

which might indicate the existence of CaSR in glial cells of spinal cord. This was in line with previous report which suggested that in rat oligodendrocytes, CaSR had a potential role in regulation of cellular proliferation through sensing the extracellular calcium and regulation of potassium channel [15].

The expression of CaSR in spinal cord implicated the possibility of its involvement in SCIRI because CaSR was reported to contribute to the injury caused by IR in heart and brain. More importantly, a recent study has reported that in focal cerebral ischemia-reperfusion CaSR activation promotes apoptosis [8]. Our results confirmed the role of CaSR in SCIRI. The results of neuron IR experiment indicated that IR might cause increase in CaSR expression since CaSR antagonist NPS-2390 completely inhibited the increase of CaSR accompanying IR. The enhancement of CaSR expression by calcimimics was also found in BRL cells [16]. In accordance with this, the results of animal experiments also showed the increase of CaSR expression in IR than that in control, and this increase was completely inhibited by NPS-2390 as well. Intriguingly, there was no significant effect of CaSR agonist $GdCl_3$ on the CaSR levels in IR neurons in vitro, but there was a significant increase of the CaSR expression in the IR+Gd rats than that in the IR rats. This discrepancy might be due to the different extent of apoptosis caused by IR in vitro and in vivo. The regulation of glial cells or blood vessel might contribute as well.

Although apoptosis is indispensable for normal development and tissue homeostasis, it is regarded as essential cellular processes in the injury caused by ischemia reperfusion. In rat spinal cord, 45 minutes of ischemia followed by four hours of reperfusion are reported to be adequate for the induction of change of proteins and proteases involved in apoptosis pathway [17]. One of the crucial proteins during apoptosis is bax, which increases the permeability of mitochondria membrane resulting in the release of cytochrome C and activation of caspases [18]. Accordingly, our study also showed the significant increase of bax increase in IR neurons when compared with the control. Despite the insignificant increase of bax in IR+Gd neurons comparing with IR neurons was detected, there was significant decrease of bax in IR+NPS neurons when compared with IR neurons. Combined with the change of

CaSR expression in IR, this result suggested that the increase of CaSR might be one cause of neuronal apoptosis as described in cardiomyocyte [19]. At present, accumulating evidences have displayed the close relationship between CaSR and apoptosis [6, 20-22]. For instance, CaSR is reported to promote apoptosis by modulating the JNK/p38 MAPK pathway in focal cerebral ischemia reperfusion in mice [8]. To further confirm the promoting effect of CaSR on cellular apoptosis during SCIRI, TUNEL staining was applied in the present study. The results demonstrated the significant increase of apoptotic index in IR group comparing with control group. More importantly, Gd significantly enhanced the apoptosis promotion effect of IR, whereas NPS significantly inhibited that of IR. This was consistent with the discoveries of Hayashi et al. that, in a rabbit model of catheter induced SCIRI, around half of spinal cord motor neurons were positive for TUNEL two days after fifteen minutes of ischemia [23]. Also, during cardiac IR and cardiac hypertrophy, increment of CaSR expression was found to be involved in the consequent calcium overload-induced cardiomyocyte apoptosis [24]. Furthermore, the fact that NPS inhibiting apoptosis during IR here agreed with the protective effect of NPS on brain tissues in mice afflicted with controlled cortical impact injury [25].

The calpains are cysteine proteases family consisting of catalytic and regulatory subunits which mainly have 2 types, μ -calpain and m-calpain (aka calpain-1 and calpain-2) [26]. One of the major calpain activation inducer is intracellular calcium overload which can be caused by CaSR activation [27]. A previous study indicated that during IR in rat heart, the activation of CaSR by GdCl_3 resulted in intracellular calcium overload [5]. The calcium overload might be due to the Ca^{2+} release from the smooth endoplasmic reticulum after the activation of CaSR. In the present study, during IR the expression of calpain in both cultured neurons and animal spinal cords increased significantly. Moreover, the CaSR antagonist NPS-2390 partly inhibited the increase of calpain after IR. It strongly suggested that the activation of CaSR might lead to the increase of calpain. Thus, in spite of the direct promotion of calcium overload on mitochondrial apoptotic pathway, calpain especially m-calpain might also contribute to the apoptotic effect of CaSR [28]. The pro-apoptotic effect of calpain

might be due to its proteolytic activities on a number of substrates such as spectrin and neurofilament [29]. The degradation of neuronal cytoskeleton is pro-apoptotic as well as the mitochondrial pathway [30]. Interestingly, in parathyroid cells m-calpain colocalizes with CaSR in caveolae and participates in degradation of CaSR which manifesting CaSR as the proteolytic substrate of calpain [26]. If the same scenario happens during SCIRI, the proteolytic effect of calpain on CaSR must be compensated by other IR factors.

In summary, the effects of CaSR agonist and antagonist on the change of CaSR, calpain expression and the accompanying apoptosis during SCIRI implicate the involvement of CaSR and calpain in SCIRI. Additionally, CaSR activation exacerbates the ischemia/reperfusion injury in heart, kidney and liver [5, 6, 16]. Furthermore, NPS-2390 shows protective effect on neurological function after IR in this study. Hence, CaSR antagonist might help alleviate SCIRI.

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Conflict of Interests

None.

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Figure Legends

Figure 1. Panel I: NF-200 positive neurons from spinal cord expressed CaSR and calpain. a, cultured neurons from spinal cord of neonatal rats; b, these cells were NF-200 positive; c, d, the expression of CaSR and calpain in cultured neurons respectively. Panel II: The effects of IR, GdCl₃ and NPS-2390 on the morphology of neurons. a, neurons in control group; b, neurons in IR group; c, neurons in IR+Gd group; d, neurons in IR+NPS group. Panel III: The expression of CaSR, Calpain, bax and β -actin in control, IR, IR+Gd, and IR+NPS groups. A, representative Western blotting results; B, quantitative analysis of the expression levels of CaSR, Calpain and bax standardized to β -actin, n=5. *, $P<0.05$; **, $P<0.01$. Bar=50 μ m.

Figure 2. The expression of CaSR and calpain in spinal cords. a,b,c the expression of CaSR in the front horn, posterior area of gray matter and the boundary areas between gray and white matter respectively; e,f,g the expression of calpain in the front, posterior and the boundary areas respectively; d,h the negative controls. Bar=50 μ m.

Figure 3. Panel I: the representative TUNEL staining results of spinal cords from control, IR, IR+Gd, and IR+NPS groups (a,b,c,d respectively), bar=20 μ m. Panel II: BBB scores of animals, n=7. Panel III: The quantitative results of cellular apoptosis from TUNEL staining, n=3. *, $P<0.05$; **, $P<0.01$.

Figure 4. Panel I: The expression of CaSR and calpain in spinal cords from 4 groups. Upper row, the expression of CaSR in spinal cords from control, IR, IR+Gd, and IR+NPS groups (a,b,c,d respectively); lower row, the expression of calpain in spinal

cords from control, IR, IR+Gd, and IR+NPS groups (e,f,g,h respectively), bar=20 μ m. Panel II: The Western blotting results of CaSR and calpain in spinal cords. A, representative Western blotting results; B, quantitative analysis of the expression levels of CaSR and calpain standardized to β -actin, n=4. *, $P<0.05$; **, $P<0.01$.

Figure 1

Figure 2

Figure 3

Figure 4