Protective effects of NUCB2 after cerebral ischemia via modulation of Bcl-2/Bax ratio and reducing GFAP expression

Running title: NUCB2 protects hippocampus after cerebral ischemia

Authors: Sohaila Erfani, Ali Moghimi, Nahid Aboutaleb, Mehdi Khaksari

aDepartment of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran
bRayan Center for Neuroscience and Behavior, Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran
cPhysiology Research Center and Department of Physiology, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran
dDepartment of Physiology, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran
eAddiction Research Center, Shahroud University of Medical Sciences, Shahroud, Iran

*Corresponding author:
Dr. Ali Moghimi, Rayan Center for Neuroscience and Behavior, Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran Tel: +98- 05138793912, Email: moghimi@um.ac.ir

** CO-corresponding author:
Dr. Nahid Aboutaleb, Physiology Research Center and Physiology Department, Iran University of Medical Sciences, Tehran, Iran, Tel: +98-21-88622709, Fax: +98-21-88622709, Email: dr.nabotaleb@gmail.com

To appear in: Basic and Clinical Neuroscience

Received date: 2018/05/31

Revised date: 2018/12/3
Accepted date: 2018/12/3

This is a “Just Accepted” manuscript, which has been examined by the peer-review process and has been accepted for publication. A “Just Accepted” manuscript is published online shortly after its acceptance, which is prior to technical editing and formatting and author proofing. Basic and Clinical Neuroscience Journal provides “Just Accepted” as an optional and free service which allows authors to make their results available to the research community as soon as possible after acceptance. After a manuscript has been technically edited and formatted, it will be removed from the “Just Accepted” Web site and published as a published article. Please note that technical editing may introduce minor changes to the manuscript text and/or graphics which may affect the content, and all legal disclaimers that apply to the journal pertain.

Please cite this article as:
http://dx.doi.org/10.32598/bcn.9.10.325
DOI: http://dx.doi.org/10.32598/bcn.9.10.325
Highlight
1- Nesfatin-1 treatment found for the first time, significantly reduced the Bax expression induced by cerebral ischemia
2- Nesfatin-1 treatment increased Bcl-2 protein level after cerebral ischemia
3- Nesfatin-1 treatment reduced GFAP proteins level after cerebral ischemia

Plain Language Summary
Cerebral ischemia reperfusion (I/R) produces complex pathological conditions that leading causes of mortality and disability worldwide. During ischemia the blood supply to an organ is limited, and then perfusion and reoxygenation are renewed simultaneously. The returned blood during reperfusion can transfer oxygen to the cells, so there is a potential for damage to the protein, DNA and membrane of the plasma. Nesfatin-1 is the amino-terminal fragment of nucleobinding-2 (NUCB2) peptide, which was identified recently as anorexigenic factor in hypothalamus and it also has wide distribution in the central nervous system. In addition recently, it has been reported anti-apoptotic and anti-inflammatory properties of nesfatin-1 in both model subarachnoid hemorrhage and traumatic brain injury in rats that created by diminution of caspase-3 activity and proinflammatory cytokines secretion. In this study, we tried to find out the effect of nesfatin-1 on apoptosis an astrogliosis after cerebral ischemia. Transient global cerebral I/R injury model was induced in four groups including sham, ischemia/reperfusion, ischemia/reperfusion+nesfatin-1 and nesfatin-1, which the two last groups received nesfatin-1 (20 µg/kg) at the beginning of reperfusion intraperitoneally. Seven days after ischemia, immunofluorescence and immunohistochemical staining was used for identifying Bax and/or Bcl-2 and GFAP activation. Result of this demonstrated nesfatin-1 inhibits apoptosis and neuroinflammation following cerebral ischemia reperfusion via reduced the Bax and GFAP proteins expression.
Abstract

Introduction: NUCB2/Nesfatin-1, a newly identified anorexigenic peptide, has antioxidant, anti-inflammatory and anti-apoptotic properties. Brain ischemia/reperfusion induces irreversible damage especially in the hippocampus area. Since the until now therapeutic effects of NUCB2/Nesfatin-1 has not been well investigated in cerebral ischemia, this study for the first time was designed to investigate the protective effects of NUCB2/Nesfatin-1 on expression of apoptosis-related proteins and reactive astrogliosis level in CA1 area of hippocampus in an experimental model of transient global cerebral ischemia.

Methods: The male Wistar rats were randomly allocated into 4 groups (sham, NUCB2/nestatin-1, ischemia/reperfusion, ischemia/reperfusion+NUCB2/nestatin-1), (n=7). The model of cerebral ischemia prepared by common carotid arteries occlusion for 20 minutes. Nesfatin-1 (20 µg/kg) and saline (as a vehicle) were injected (intraperitoneally) at the beginning of reperfusion period. Assessment of the proteins expression levels was performed by immunofluorescence and immunohistochemical staining.

Results: NUCB2/nestatin-1 significantly reduced the Bax and GFAP proteins level in the CA1 area after ischemia (P<0.05), also NUCB2/nestatin-1 increased Bcl-2 protein level (P<0.05). NUCB2/nestatin-1 exerts protective effects against ischemia injury by inhibition of astrocytes activation as an inflammatory response and decrease neuronal cell apoptosis.

Conclusion: Our study for the first time provides the possible neuroprotective view of nestatin-1 in the treatment of ischemia injury model in rat hippocampus.

Key words: NUCB2/nestatin-1, Apoptosis, Astrogliosis, Hippocampus, Ischemia
1. Introduction
Nesfatin-1 is the amino-terminal fragment of nucleobinding-2 (NUCB2) peptide, which was identified recently as anorexigenic factor in hypothalamus and it also has wide distribution in the central nervous system (Kolgazi et al., 2015). It was shown that the neurons of the central nesfatinergic system that expressing nesfatin-1 respond to peripheral inflammatory signals and it may has a coordinated role of nesfatinergic system for response to infection or inflammation (Bonnet et al., 2009). In addition recently, it has been reported anti-inflammatory and anti-apoptotic properties of nesfatin-1 in both model subarachnoid hemorrhage and traumatic brain injury in rats that created by diminution of caspase-3 activity and proinflammatory cytokines secretion (Özsavcı et al., 2011, Tang et al., 2012).

Ischemic stroke and the following reperfusion that can happen after acute therapeutic intervention, by creating irreversible brain damages, leading cause of disability and the second leading mortality in developed countries (Shi et al., 2017). Numerous recent studies have been conducted that the development of many neuroprotective treatment strategies can be reducing brain damage following cerebral ischemia in animal models (Majid, 2014). It seems the possible neuroprotective mechanisms are to inhibit from local inflammation, excitotoxicity, free radical damage, neuronal apoptosis, and calcium influx into cells, that those leading in both improvement of functional outcomes and decrease of infarct size (Tuttolomondo et al., 2015).

The CA1 neurons of the hippocampus are selectively vulnerable to transient global cerebral ischemia, and delayed cell death occurs in this area of the brain in few days after reperfusion (Nishijima et al., 2015). It has been well demonstrated that in ischemic stroke, inhibition of apoptosis can be done with decreasing of the proapoptotic proteins (cleaved caspase-3, caspase-9, and Bax) expression and increasing of the antiapoptotic protein (Bcl-2) expression (Aboutaleb et al., 2015, Zheng et al., 2017). In addition, survival of CA1 pyramidal neurons after transient global ischemia was enhanced in transgenic mice with overexpressing of Bcl-2 protein (MacManus and Linnik, 1997).

The neuroinflammation responses that occur by immune mediators following brain ischemia have a major role in creating of neuronal cell death. Astrocytes are the important mediators of brain that have been reported to release various pro-inflammatory factors after ischemic injury, such as intermediate-filament, glial fibrillary acid protein (GFAP), (Kim et al., 2016). In mice lacking GFAP, a lack of GFAP protein increases the susceptibility to brain damage. Furthermore the previous evidence has shown that reactive astrocytes up regulates of the intermediate-filament, glial fibrillary acidic protein (GFAP) in many neurodegenerative
conditions such as ischemia. Thus it was extensively applied as an alternative marker of neuronal injury in brain ischemia (Cordeau et al., 2008). However, the role of NUCB2/nesfatin-1 in cerebral ischemia/reperfusion has been poorly investigated. According to the brain ischemia mechanisms and the protective effects of nesfatin-1, in this study, we investigate the effects of nesfatin-1 administration on number of GFAP positive cells and apoptotic-related proteins (Bax/Bcl-2) following transient global cerebral ischemia/reperfusion.

2. Materials and Methods
2.1. Animals and drugs
Male Wistar rats (250–300g) were prepared. The animals were placed in standard cages with controlled room temperature (22-24°C), humidity (45-50%) and light exposure conditions 12:12 h light–dark cycle. All animals had free access to food and water. Total experiments and animal handling were accomplished according to the Helsinki Declaration and FUM (Ferdowsi University of Mashhad) committee for biological ethics. Nesfatin-1 (Sigma-Aldrich, Germany) stored at -20°C and was dissolved in saline when injection.

2.2. Experimental design and protocols
Immunofluorescence and immunohistochemical staining were done 7 days after cerebral ischemia induction in 4 groups including sham (n=7), ischemia/reperfusion (n=7), ischemia/reperfusion+nesfatin-1 (n=7) and nesfatin-1 (n=7) which the two last groups received nesfatin-1 (20 µg/kg) at the beginning of reperfusion intraperitoneally. Animals in sham group were operated under the same surgical procedures, except that the common carotid arteries were not obstructed. Animals in nesfatin-1 group weren’t undergo surgery for induction of ischemia. 7 days after ischemia induction, all rats were anesthetized then for staining transcardiac perfusion was performed.

2.3. The model of transient global cerebral ischemia
The model of transient global cerebral ischemia was induced with a procedure that was previously described (Shamsaei et al., 2015). Rats were anesthetized by using ketamine/xylazine (40 mg/kg) intraperitoneally, then were subjected to ischemia model surgery.

In the beginning, both common carotid arteries were exposed and separated from their carotid sheet and vagus nerves carefully. Then both common carotid arteries was occluded by using Yashargil Aneurism micro-clips for 20 min. At the end of the occlusion time period, the clips were expelled for quick reperfusion and rebuilding of blood stream was visually affirmed. The
feedback controlled warming system was kept up their rectal temperature at 36.5±0.5°C during the experimental time. After surgery, rats were put in home confines with free access to food and water and kept independently for seven days. At the start of reperfusion, nesfatin-1 (20 µg/kg) was dissolved in saline and was infused intraperitoneally.

2.4. Tissue preparation for staining

Seven days after ischemia, the animals were anesthetized with ketamine deeply, and transcardiac perfusion was performed with 0.9% saline, trailed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Afterwards their brains were expelled and post fixed in a similar fixative for 3 days interims and brains were embedded in paraffin. Then coronal sections as indicated by the Paxinos atlas (somewhere in the range of 3.3mm and 4.2mm back to bregma) at 7 µm thickness were cut by a microtome for various staining techniques (Erfani et al., 2015a).

2.5. Measurement of Bcl-2 and Bax immunoreactivity and GFAP Immunohistochemical staining

Immunofluorescence staining was utilized for distinguishing Bcl-2 and Bax activation and immunohistochemical staining utilized for GFAP activation recognizing identifying was performed on tissue sections. The sections were incubated at 62°C for 20 min, then rehydrated through a descending alcohol series, and treated with 10% hydrogen peroxide in methanol for 10 min to reduce endogenous peroxidase activity. Subsequent to being washed in Tris buffer (pH 7.4) antigens were recovered via autoclaving for 11 min in Citrate buffer (pH 6). Subsequent to washing, the sections were blocked with 10% typical goat serum for 60 minutes. Sections were then incubated with anti- Bcl-2 and Bax antibody (rabbit antibody against rat, Abcam, UK) as a primary antibody, at 4°C temperature overnight. After wash in PBS, sections were incubated with anti-rabbit secondary antibody conjugated with a fluorochrome (Abcam, UK) for 2 hours in the dark to accomplish visualization of the antigen.

At that point, the sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) in PBS for 5 min for labeling the nucleus. Also, for GFAP polyclonal secondary antibody (HRP) (Abcam, UK) incubated for 30 min at room temperature by adding 3,3′-diaminobenzidine (DAB, Sigma, USA) to detection of the antigen. At long last, counterstaining with hematoxylin (Sigma) was performed for visualization under the microscope (Aboutaleb et al., 2016). After a washing step, the fluorescence signals from the CA1 area of the right hippocampus fields that readied from each slide related per rat were detected with a fluorescence microscope (LABOMED USA, 400× magnification). The number of positive cells and the total number of cells was counted blindly (Image tools software). The results were expressed as the percentage of Bax, Bcl-2 and GFAP-positive cells/total cells.
2.6. Statistical analysis
All data are reported as mean±SD. The Kolmogorov–Smirnov test was demonstrated that the normality of the distribution. One-way analysis of variance (ANOVA) test was used to compare the groups’ differences. When there is a significant difference, the Scheffe’s or Dunnett’s T3 post hoc test was applied to specify where the difference occurred. When the homogeneity of variance was discovered, Scheffe’s post hoc test was utilized, else, we used Dunnett’s T3 post hoc test. The significance level was set at \( P \leq 0.05 \).

3. Results

3.1. Nesfatin-1 reduced the Bax protein levels after cerebral ischemia
The results of Bax immunofluorescence staining showed that, in the CA1 area of hippocampus, there were statistically significant differences among groups with respect to the percentage of Bax-positive cells. Bax were weakly expressed in sham-operated (10.1%±1.24) and nesfatin-1 (13.2%±1.78) groups. Also, the percentage of Bax-positive cells increased in ischemia group (62.5%±4.74) compared to the sham group (\( P<0.01 \)). In the nesfatin-1 treatment group, the percentage of Bax-positive cells decreased (49.10%±1.81) compared to the ischemia group (\( P<0.05 \)), (Figures 1, 2).

3.2. Nesfatin-1 increased Bcl-2 protein level in the CA1 area
In accordance with the results of Bcl-2 immunofluorescence staining, there was a significant difference in the percentage of Bcl-2-positive cells among groups. The expression of Bcl-2 protein was higher in the sham (59%±1.58) and nesfatin-1 (57.4%±1.34) groups. The cerebral ischemia decreased the percentage of Bcl-2-positive cells (21.4%±1.55) compared to the sham group (\( P<0.001 \)). In the nesfatin-1 treatment group, the percentage of Bcl-2-positive cells increased (37.1%±3.31) compared to the ischemia group (\( P<0.05 \)), (Figures 1, 3).

3.3. Nesfatin-1 reduced the GFAP protein levels after ischemia
The results of GFAP immunohistochemical staining demonstrated that there was significant difference in the percentage of GFAP-positive cells among groups in the hippocampal CA1 area. GFAP was expressed in sham group weakly (23.8% ± 2.94) and nesfatin-1 group (27% ± 3.8). Additionally, the percentage of GFAP-positive cells was increased in ischemia group (81.4% ± 2.3) compared to sham and nesfatin-1 groups (\( P<0.001 \)). In the nesfatin-1 group, the percentage of GFAP-positive cells was decreased (69.4% ± 3.04) compared to the ischemia group (\( P<0.05 \)), (Figure 4).

4. Discussion
This study for the first time demonstrated that neuroprotective effects of nesfatin-1 against I/R injury by GFAP immunohistochemical staining that presented the number of active GFAP-positive cells was significantly increased following transient cerebral ischemia in the hippocampal CA1 area. This provided the evidence that cerebral ischemia causes neuroinflammation intermediary activity of astrocytes in the CA1 area of the hippocampus. On the other hand, treatment with NUCB2/nesfatin-1 considerably reduces the ischemia/reperfusion-induced GFAP expression.

The peptide therapeutic strategy has been applied in incremental form in recent years. This has revealed that peptides have an important role in the treatment of many diseases such as infectious and autoimmune diseases (Xiao et al., 2015, Thundimadathil, 2012). On the other hand, the available clinical pharmacological tools to reduce brain injury and treatment for patients with stroke are very limited (Herson and Traystman, 2014), so it seems using of novel peptide such as Nesfatin-1 is a suitable method for reduction of brain ischemia injury.

Previous studies well established that neuroinflammatory mediators play a crucial role in the brain ischemia pathophysiology by contribution to ischemic tissue damage (Amantea et al., 2009). Reactive astrogliosis accompanies with many pathological conditions that affect the CNS, such as trauma, neuroinflammation and ischemic damage. Reactive astrocytes increase the expression their structural proteins including GFAP and vimentin (Chen and Swanson, 2003).

Moreover, it was reported reactive astrocytes following brain ischemia plays an important role in the regulation of inflammation that is provided by a major source of the proinflammatory cytokines and chemokines (Morizawa et al., 2017, Kudabayeva et al., 2017, Li et al., 2017). Then the major proinflammatory products that are including interleukin-6 (IL-6), interleukin 1 beta (IL-1β), monocyte chemotactic protein-1 (MCP-1/CCL2), and etc, and chemokines induces the cross-talk between activate astrocytes and CNS-infiltrating immune cells thereby activating infiltrating lymphocytes (Sofroniew, 2015). It was shown that infiltrating leukocytes such as lymphocytes are the main factor of cerebral ischemic inflammation (Li et al., 2017).

One of the possible neuroprotective mechanisms of nesfatin-1 can be results from its inflammation suppression properties. Consistent with our observation, the recent studies of Chong-Hui Tang et al and Derya Özsavcıl et al identified treatment with nesfatin-1 reduces concentrations of inflammatory mediators such as interleukin-1 beta, tumor necrosis factor alpha, and interleukin-6 after traumatic brain injury and subarachnoid hemorrhage brain damage (Tang et al., 2012, Özsavcıl et al., 2011).
The results of the present study also show that treatment with novel peptide nesfatin-1 at the 20 µg/kg dose, decreases the ischemia/reperfusion-induced pro-apoptotic protein Bax expression and increases the anti-apoptotic protein Bcl-2 expression that was markedly reduce under ischemia conditions.

The pathophysiology mechanisms of cerebral ischemia/reperfusion are complex. Several studies have revealed that reperfusion plays an important role in the brain ischemia injury and when blood flow is returned to the tissue, lead to a series of processes including oxidative stress, inflammation, energy failure, excitotoxicity, calcium dysregulation, the activation of several cell-signaling pathways of neuronal death and apoptosis (Erfani et al., 2015b, Wang et al., 2017). That seems any factor that prevents those processes can be used for treatment of brain ischemia. Cellular apoptosis that related to cerebral IR, accompanied with the expression of apoptosis-related genes (Cregan et al., 2002). In addition several studies showed that expression of the key antiapoptotic protein Bcl-2, is decreased and also the Bax expression as a proapoptotic proteinis increased at different times of reperfusion (Xing et al., 2008). The correlation between Bcl-2 expression and resistance to apoptosis has been seen that it results from Bcl-2 properties for example, Bcl-2 sensitivity to redox changes and antioxidant functions of Bcl-2 during calcium stress thus lead to attenuate of cell death (Doyle et al., 2008).

It seems the neuroprotection of nesfatin-1 in the CA1 area of hippocampus after transient global cerebral ischemia can cause by its antiapoptotic and antioxidant activity. Like that the recent experiment showed the protective effects of nesfatin-1 in intestinal ischemia-reperfusion by decrease endothelial nitric oxide synthases (eNOS) level and oxidative stress index (OSI), (Ceylan Ayada et al., 2015). Moreover the evidence of Guanjun Jiang et al showed that nesfatin-1 treatment ameliorates acute renal ischemia-reperfusion injury by malondialdehyde (MDA) level reduction, andincrease the superoxide dismutase (SOD) and catalase (CAT) activities, as well as nesfatin-1 makes less apoptotic tubular cells, by caspase-3 activity decrease and an increase in the bcl-2/Bax ratio (Jiang et al., 2015).

The previous observation indicated that transient cerebral ischemia causes the degeneration of vulnerable neurons such as those in the hippocampus, neocortex and striatum. The neuronal death was happen selectively and slowly that is named ‘delayed neuronal death’ (DND), (Hye Kim et al., 2016). Cognitive performance such as learning and memory are disrupted following cerebral ischemia. It has been shown loss of hippocampal CA1 neurons is related with memory function impairment (Erfani et al., 2015c).

**Conclusion:** Due to the importance and sensitivity of CA1 area of hippocampus and the pathophysiology mechanisms of cerebral ischemia, this study provided the novel therapeutic
window of nesfatin-1 by inhibition of astrocytes activation as an inflammatory response and increase expression of the antiapoptotic protein Bcl-2 and also lessen Bax mediated neuronal cell apoptosis after transient cerebral ischemia in rats, however further experiments are required to clarify these believes.

**Ethical Considerations**

Compliance with ethical guideline:
Each animal was used only once. Rats got familiar with their new environment prior to starting experimental process. All tests were executed according to the guide for the care and use of laboratory animals (National Institutes of Health Newsletter No. 80-23, revised 1996).

**Funding**

This study was sponsored by the Ferdowsi University of Mashhad research affairs by grant No.3/42964.

**Conflict of interest**

There is no conflict exists of interest for all authors.


Figures:

**Fig.1.** The percentage of active Bax positive cells (A) and active Bcl-2 positive cells (B) in the CA1 area of hippocampus following the cerebral ischemia in different groups.

* Significantly different compared with sham and nesfatin-1 groups (P<0.001).
# Significantly different compared with ischemia group (P<0.05).

**Fig.2.** Photomicrographs of immunofluorescence staining of Bax in the hippocampus. Immunofluorescence staining was used for identifying Bax activation in the hippocampal CA1 area following the transient cerebral ischemia. Representative Bax stained (green) and DAPI-stained (blue) in the sections (×400 Magnifications).
Immunofluorescence staining was used for identifying Bcl-2 activation in the hippocampal CA1 area following the transient cerebral ischemia. Representative Bcl-2 stained (green) and DAPI-stained (blue) in the sections (×400 Magnifications).

Fig.3. Photomicrographs of immunofluorescence staining of Bcl-2 in the hippocampus.

Fig.4. The percentage of GFAP positive cells in different groups.
**Left:** Photomicrographs of immunohistochemical staining of GFAP in the hippocampal CA1 area after transient cerebral ischemia (×400 Magnifications).

**Right:** Effects of nesfatin-1 on the GFAP levels in the CA1 area following the cerebral ischemia.
* Significantly different compared with sham and nesfatin-1 groups (P<0.001).
# Significantly different compared with ischemia group (P<0.05).