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EXPERIMENTAL STUDY

The effect of nesfatin-1 on heart L-type Ca^{2+} channel α1c subunit in rats subjected to chronic restraint stress

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Abstract: *Background:* Elevated L-type Ca^{2+} channel expression level increases Ca^{2+} influx. This can cause hypertrophy and pathological remodeling of the heart especially under stress conditions. Nesfatin-1 can activate hypothalamic L, P and Q type Ca^{2+} channels and increase insulin secretion in pancreatic islet beta cells via activation of L-type Ca^{2+} channels. On the other hand, the effect of nesfatin-1 on cardiac L-type Ca^{2+} channels has not been studied yet.
Objectives: We aimed to identify the effect of peripheral chronic nesfatin-1 application on cardiac L-type Ca^{2+} channel α1c subunit expression level in normal rats and those subjected to chronic restraint stress.
Methods: Three-month aged *Wistar albino* rats were randomly divided into 4 groups (n = 7) as Control, Stress, Control+Nesfatin-1, and Nesfatin-1+Stress. Rats in groups subjected to restraint stress were placed in a specially built size-manipulable cabin for 2 h/day (between 10:00 and 12:00 a.m.) for 10 consecutive days without allowing water and food intake. Nesfatin-1 segment (0.25 nmol/g bw intraperitoneally) was applied during the 10 consecutive days. Western blot analyses were performed to determine the expression level of L-type Ca^{2+} channel α1c subunit protein in rat cardiac extracts.
Results: Cardiac L-type Ca^{2+} channel α1c subunit protein expression levels were increased significantly after chronic peripheral nesfatin-1 application in rats subjected to restraint stress ($p = 0.032$).
Conclusion: We can conclude that nesfatin-1 can cause cardiac failures during clinical treatments by elevating cardiac L-type Ca^{2+} channel α1c subunit protein expression level *(Fig. 2, Ref. 26)*. Text in PDF *www.elis.sk*.
Key words: nesfatin-1, chronic restraint stress, cardiac failure, L-type Ca^{2+} channel α1c subunit, Western blot.

Introduction

Cardiac L-type Ca^{2+} channels determine physiological functions of cardiac myocytes. These channels have an important role for many cell functions such as membrane excitability, Ca^{2+} homeostasis, protein phosphorylation and gene regulation (1). L-type Ca^{2+} channels initiate the pacemaker potential by Ca^{2+} influx in cardiomyocytes and also cause the plateau phase of action potential. That is why they are also important for excitation-contraction coupling process in the heart (2, 3). L-type Ca^{2+} channels are pore-structured proteins and contain alfa-1c (α1c), alfa2/delta (α2/δ) and beta (β) subunits. The α1c subunit is the part sensitive to voltage and contains receptor regions for different classes of Ca^{2+} channel agonists and antagonists. Thus, α1c subunit is accepted as the main physiological regulator part of L-type Ca^{2+} channels (4). There should be a possible relationship between the level or function of L-type Ca^{2+} channels alterations and several heart diseases such as atrial fibrillation, and heart failure. Acute and chronic restraint stress applications can elevate the expression level of L-type Ca^{2+} channel (5, 6).

Nesfatin-1 is a peptide composed of 82 amino acids and derived from the larger protein nucleobindin-2 (NUCB2). It regulates feeding and is distributed in the central nervous system (CNS) including the hypothalamic paraventricular nucleus (PVN), arcuate nucleus (ARC), lateral hypothalamic area and supraoptic nucleus (SON) (7). Under stress conditions the level of nesfaitn-1 in the brain gets increased. This is accepted as a possible link between nesfatin-1 and stress (8). Intracerebroventricular (icv) and peripheral administrations of nesfatin-1 can increase blood pressure (9, 10, 11). Recently, nesfatin-1 has been described as a cardiac peptide (12). It can activate hypothalamic L, P and Q type Ca^{2+} channels independently from blood pressure activation (13) and can also increase insulin secretion by activation of L- type Ca^{2+} channels in pancreatic islet beta cells (14). Consequently nesfatin-1 receives close attention, especially for its potential of becoming a novel therapeutic agent for diseases such as obesity and diabetes mellitus. If we consider that these diseases are chronic stress factors for human body, we believe that it is necessary to know more about nesfatin-1 effects under chronic stress conditions. Thus, we have applied chronic restraint stress to mimic stress condition created by diseases. On the other hand, the effect of nesfatin-1 on cardiac L- type Ca^{2+} channel has not been studied yet. Due to these rea-

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sons, in present study we aimed to determine the effect of peripheral chronic nesfatin-1 application on cardiac L-type Ca^{2+} channel α1c subunit expression level in normal rats and those subjected to chronic restraint stress.

Material and method

Animals and experimental conditions

In this study, 28 three-month old *Wistar Albino* male rats were used, weighing 200–250 g. They were reared under the supervision of a veterinarian, kept in a well-ventilated, noiseless environment, and allowed free access to food and water. The rats were housed in a room with controlled temperature (23 ± 1 °C) and relative humidity (50 ± 5 %), and kept in transparent plastic cages (42 x 26 x 15 cm), each containing three or four rats exposed to a 12:12 light/dark cycle. All experimental protocols conducted on animals were consistent with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85–23) and approved by the Pamukkale University Ethics Committee of Animal Care and Usage.

Experimental design

Rats were randomly divided into four experimental groups (n = 7) including; control (C) rats without any treatment, rats subjected to chronic restraint stress (S) without any injection, control+nesfatin-1 applied (C+N) rats, and nesfatin-1 applied + subjected to chronic restraint stress (N+S). Each time, nesfatin-1 was applied just before chronic restraint stress application in N+S group. Rats were placed in a specially built size-manipulable cabin and to be able to create the animal model of restraint stress (15, 16) in stress groups, they were not allowed to water and food intake for 2 h/day (between 10:00–12:00 a.m.) for 10 consecutive days. Totally 14 rats in the C+N and N+S groups were treated with rat nesfatin-1 segment (0.25 nmol/g bw intraperitoneally) during 10 consecutive days (17).

Collection of heart samples

At the end of the experimental period, all animals were anesthetized with ketamin/xylazine HCl (75 mg/kg/10 mg/kg intraperitoneally). Heart tissues of each rat were carefully cleaned from fat and connective tissues and placed in liquid nitrogen to freeze immediately. Heart samples were stored at - 80ºC until Western-Blot analysis.

Western blot analysis of L-type Ca^{2+} channel α1c subunit protein expression

To isolate proteins, heart tissues were homogenized in radioimmunoprecipitation (RIPA) lysis buffer with a protease-inhibitor and centrifuged at 4 °C. Protein concentrations were measured by Lowry assay with bovine serum albumin (BSA) to load each sample in equal concentration to the gel. Proteins were denatured by loading buffer with sodium dodecyl sulfate (SDS), and run on 10 % SDS-polyacrylamide gel using a mini-Protean cell (Bio-Rad, Hercules, CA, USA). Obtained bands were transferred to polyvinylidine difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) using a Trans-Blot SD cell (Bio-Rad). For blocking step, the membrane was incubated with 1 % BSA in Tris at 4 °C overnight. For immunodetection, membranes were first incubated with primary antibody (sc-25686, anti-cardiac α1c subunit, 1:500, Santa Cruz) for 2 hours at room temperature (RT) and after that they were incubated with secondary antibody (sc-2030, horseradish-peroxidase-conjugated goat anti-rabbit IgG, 1:5000, Santa Cruz) for 1 h at RT. Immunoreactive bands were visualized by densitometry by UVP BioSpectrum Imaging System (Upland, California, USA) using the chemiluminescence method. Density of each band was quantified by the same system (Modified protocol from 18).

Statistical analysis

Statistical analyses were done by SPSS (Statistical Package for Social Sciences, Chicago, IL, USA) 16.0 package program. All data were given as mean ± standard deviation (SD). Statistical significances among all groups and between two groups were analyzed by Kruskal–Wallis and Mann–Whitney U tests, respectively. Differences were considered significant at $P<0.05$.

Results

Expression levels of heart L-type Ca^{2+} channel α1c subunit

We observed no statistically significant difference in expression levels of heart L-type Ca^{2+} channel α1c subunit among the C (15.06 ± 4.71), S (21.17 ± 7.63), C+N (19.23 ± 6.74) and N+S (25.73 ± 5.52) groups ($p = 0.178$). The level of heart L-type Ca^{2+} channel α1c subunit protein was significantly increased in N+S compared to C group ($p = 0.032$) (Fig. 1). The increase in heart L-type Ca^{2+} channel α1c subunit protein expression level in N+S group compared to C group has been demonstrated also by Western Blot analyses in which samples from rats selected randomly from each group were applied (Fig. 2).

Discussion

Cardiac L-type Ca^{2+} channels play an important role for characteristic physiological functions of cardiac myocytes (1). The

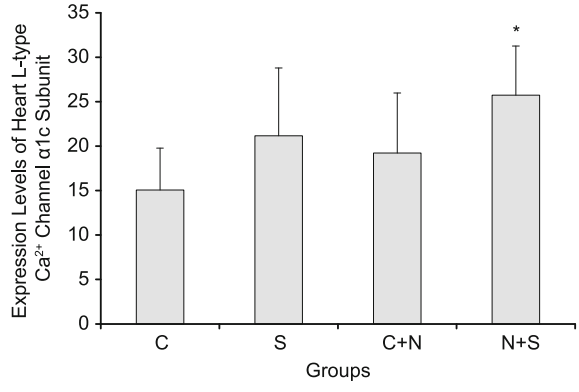

Fig. 1. The expression levels of heart L-type Ca^{2+} channel α1c subunit in C, S, C+N and N+S groups. * The significance between C and the other groups, $p < 0.05$ (Mann-Whitney U test).

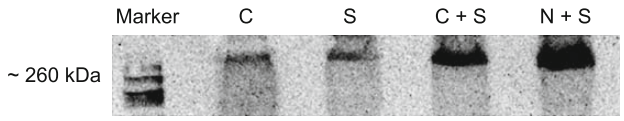

Fig. 2. The Western blot demonstration of heart L-type Ca^{2+} channel α1c subunit expression level.

level or function of L-type Ca^{2+} channels is changing in several heart diseases such as atrial fibrillation, and heart failure. That is why it is believed that there is a possible relationship between cardiovascular diseases and pathophysiological alterations in Ca^{2+} homeostasis that is regulated by L-type Ca^{2+} channels. In addition, dysregulated Ca^{2+} homeostasis has a role in the pathogenesis of cell death (5). The α1c subunit is described as the main physiological regulatory part of L-type Ca^{2+} channels (4). Abnormal cardiovascular responses can occur after acute and chronic stress. Both types of stress can increase risk factors for the development of cardiovascular diseases (19). Chronic restraint stress is in relationship with cardiac dysfunction and structural changes. It can cause elevated expression level of L-type Ca^{2+} channel α1c subunit as well as lead to cardiomyocyte damage (5, 6). Nesfatin-1 activates hypothalamic L, P and Q type Ca^{+2} channels and additionally L type Ca^{+2} channels in pancreatic beta cells which increase insulin secretion (13, 14). It is also very well known that nesfatin-1 inhibits feeding via a pathway that is different from that of leptin (20, 21). That is why it became a very popular therapeutic target. It has been also shown that there could be a relationship between nesfatin-1 and stress conditions (8, 22, 23). Nevertheless there are still knowledge gaps in respect of direct cardiac effects of nesfatin-1. The effects of nesfatin-1 on feeding have been studied on a large scale. We believe that we still need to seek more information about physiological effects of nesfatin-1 and mechanisms of these effects. That is why we hypothesized that nesfatin-1 could have adverse effects on cardimyocytes and using it in treatment procedures for various diseases such as obesity and diabetes mellitus could possibly lead to serious health problems. Thus, in present study, an experimental model was chosen to mimic human body under disease conditions.

In present study, we identified the effect of peripheral chronic nesfatin-1 application on cardiac L-type Ca^{2+} channel α1c subunit expression level in normal and chronic restraint stress created rats. Chronic restraint stress during 21 days of application increases the expression level of α1c subunit of heart L-type Ca^{2+} channels (5). In this study, the applied stress model is described as a chronic stress model (16). Although not significantly, we observed elevated levels of heart L-type Ca^{2+} channels α1c subunit in S group compared to C group. We assume that different characteristics and durations of stress application can change the expression level of heart L-type Ca^{2+} channels α1c subunit in mutually different manners. That is why we can conclude that the applied stress model in the present study may elevate the expression level of heart L-type Ca^{2+} channels α1c subunit. In literature, we failed to observe any report about the effect of peripheral nesfatin-1 application on heart L-type Ca^{2+} channels α1c subunit. Compared to C group, we found the level of heart L-type Ca^{2+} channels α1c subunit in C+N group to be elevated, although not significantly. In addition, a statistically significant increase was found in the level of heart L-type Ca^{2+} channels α1c subunit in N+S group compared to C group. Unfortunately, according to our results it is not possible to claim that this elevation was directly caused by stress conditions or nesfatin-1 administration. Although they both elevate the level of heart L-type Ca^{2+} channels α1c subunit, this effect is not significant. If we consider that this elevation is statistically significant in N+S, we can conclude that chronic peripheral nesfatin-1 administration can increase the expression level of heart L-type Ca^{2+} channels α1c subunit protein especially under chronic stress conditions.

Conclusion

Cardiovascular effects of nesfatin-1 should be in tight relation with direct cardiac effect of nesfatin-1. If nesfatin-1 is thought to be used as an effective therapeutic agent, we believe it is necessary to know more about its effects on different tissues under different conditions. According to the present study, we can conclude that nesfatin-1 application can have adverse effect on cardimyocytes and can lead to serious health problems during clinical treatment by elevating the expression level of heart L-type Ca^{2+} channels α1c subunit protein.

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