Iranian Journal of Health and Physical Activity (2013) 4 (2), (1-7)

Effects of Aerobic Training on Tissue Nesfatin-1/Nucleobindin-2 mRNA, Plasma Nesfatin-1 and High-density Lipoprotein Concentration in Female Rats

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Received 22 May 2012 Accepted 30 July 2012

Abstract

Purpose: Nesfatin-1 is a protein derived from a precursor molecule of the nucleobindin-2 gene. Nesfatin-1 has been suggested to act as a novel inhibitor and a potential regulator of food intake and body weight. It has been reported that changes in proteins and peptides that are released from hypothalamus and adipose tissue are related to cardiovascular diseases (CAD). Therefore, the aim of this study was investigate the effects of eight weeks of aerobic training on nesfatin-1/nucleobindin-2 expression, plasma concentration, and plasma high-density lipoprotein (HDL-C) in female rats (Liver, small intestine and kidney tissues).

Material and Methods: In this study ten Wistar rats (6-8 weeks old and 125-135 g weight) were used. Animals were randomly assigned into control and training groups. Training group was given exercise on a motor-driven treadmill at 25 m/min (0% grade), 60 min/day, 5 days/week, for eight weeks. 72 hours after the last training session and after four hours of fasting, rats were sacrificed. Liver, small intestine and kidney were excised, total RNA was extracted, and after PCR procedure, quantitative real-time RT-PCR was performed. Plasma was also collected for plasma variable measurement. Data are expressed as means ± SEM and the significance level was set at $P<0.05$.

Results: Exercise-induced increases in liver ($P<0.001$), small intestine ($P<0.001$) and kidney ($P<0.019$) nesfatin-1/NUCB2 expression were observed. A significant up-regulation in Plasma HDL-C concentration in the training group was also spotted ($P<0.003$) in comparison to the control group. Data analysis revealed no significant differences in plasma nesfatin-1 concentration between the study groups ($P<0.093$).

Discussion and Conclusion: Tissue nesfatin-1/NUCB2 mRNA expression and plasma HCL-C concentration were affected (up-regulated) by physical exercise while plasma nesfatin-1 levels remained unchanged.

Key Words: Food intake, Physical stress, Energy homeostasis, Gene expression

Introduction

Nesfatin-1 was originally identified as a hypothalamic neuropeptide, derived from the precursor NEFA /nucleobindin 2 (NUCB2), with the ability in metabolic regulation and feeding behavior [1-3]. The patterns of nesfatin-1/NUCB2 expression have been thoroughly characterized in different hypothalamic nuclei and brain areas with proven roles in energy homeostasis [3]. Experimental evidence suggests that nesfatin-1/NUCB2 is also expressed in peripheral tissues including the rat's gastric oxyntic mucosa or gastric X/A like cells [4], stomach [5], pancreatic beta cells [6], and adipose tissue [7] that are engaged in metabolic functions and regulation of food intake and body weight [8, 9]. It directly decreases body fat via appetite suppression [10]. It has been shown that intracerebroventricular (ICV) and peripheral injection of nesfatin-1 elicits a dose-depended reduction of 4-h dark phase food intake [11, 12]. It has been reported that several factors (nutrients, fasting, physical stress) may affect proteins and peptides gene expression. Also changes in peptides and proteins that are released from hypothalamus and adipose tissue contribute to the pathogenesis of insulin resistance and its attending detrimental metabolic consequences including diabetes, dyslipidemia, hypertension, and cardiovascular diseases [13, 14]. Expression of nesfatin-1 and its levels

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in serum/plasma could be affected by fasting and refeeding [3, 15, 16], restraint stress [17, 18], abdominal surgery [4], a high-fat diet [7, 19], and a glycaemic state [20] but no researches have examined the effect of exercise on tissue Nesfatin-1/NUCB2 gene expression. Thus, the current study aimed to investigate the effects of eight weeks of aerobic training on nesfatin-1/nucleobindin-2 expression in female rats' tissues including Liver, small intestine and kidney.

Material and Methods

Animals

All the experiments involving the animals were conducted according to the policy of the Iranian convention for the protection of vertebrate animals used for experimental and other scientific purposes; and the protocol was approved by the Ethics Committee of the Sciences, University of Mazandaran (UMZ) and Babol University of Medical Sciences (BUMS), Mazandaran, Iran. Twenty female, wistar rats (6-8 weeks old, 125-135 g weight) were acquired from Pasteur's Institute (Amol, Mazandaran) and maintained in the Central Animal House of Faculty of Physical Education and Sports Science of UMZ. Five rats were housed per cage (46-L volume) with a 12-h: 12-h light-dark cycle. Temperature was maintained at 22°C ± 1.4°C. Food (a pellet form) and water were provided ad *libitum*. Animals were randomly assigned into control (n = 10) and training (n = 10) groups. The control group remained sedentary, whereas the training group underwent a moderate running exercise program.

Exercise training protocol

At first, the animals were familiarized with the rat treadmill apparatus, every day and for 4 days (the 14-lane motorized-driven treadmill was designed by the primary author,UMZ, Babolsar, Mazandaran, Iran). The exercise group trained for 8 weeks, using the same training methods previously described [21]. The rats ran at 25 m/min, 60 minutes, 5 d/week. The animals were scarified 72 hours after the last exercise session. Food but not water was removed from the rats' cages 4 hours before the sacrifices. The estrous cycle was determined in intact female rats by taking vaginal smears every morning using vaginal lavage. Smears were analyzed under a microscope to determine the type of cells present and the stage of the estrous cycle [22, 23]. Only female rats showing at least two consecutive 4- or 5-day estrous cycles were used. The established estrous cycle in each female was used to select the day of the experiment, during which the estrous cycle stages were confirmed through vaginal smear.

Tissue biopsies

Seventy-two hours after the last training session, rats were anesthetized with intra peritoneal administration of a mixture of ketamine (30– 50 mg / kg body weight) and xylazine (3– 5 mg / kg body weight). Liver, small intestine and kidney were excised, cleaned, divided into two pieces, washed in ice-cold saline, were immediately frozen in liquid nitrogen and were stored at − 80 ° C until RNA extraction. blood samples were also collected in EDAT test tubes as anticoagulant and were immediately processed for plasma preparation, during 10min of centrifugation at 3000rpm. Plasma too was stored at -80C, for future analysis.

RNA extraction, cDNA synthesis and Real-time PCR

Total RNA was extracted from 80 to 100 mg of tissue using RNA purification kits (AccuZol, Bioneer, Cat.No: k3090). Complementary DNA (cDNA) was extended from 1µl oligo-$(dt)_{18}$ primers (0.25 µg per reaction) using cDNA synthesis kit (AccuPower RT PreMix, Bioneer, Cat.No: k2041-B) according to the manufacturer's instructions. Real-time quantitative PCR was performed using Quanti Fast SYBR Green PCR Kit (Cat. No. 204052; Qiagen, GmbH, Germany) using 15 µl reaction containing 0.5µl single-strand cDNA,7.5µl Master Mix, 1µl of the each forward and reverse primers (5 pmol/µl) and 5µl dH_2O.

Nesfatin-1/NUCB2 sense and antisense primers were: 5'- TTTGAACACCTGAACCACCA- 3' and 5'- TGCAAACTTGGCTTCTTCCT -3' (211bp; accession no: Q9JI85), respectively. The β-actin sense and antisense primers were: 5'-TATCGGCAATGAGCGGTTCC-3' and 5'-AGCACTGTGTTGGCATAGAGG-3' (145bp; accession no: NM_031144), respectively [24].

Real-time PCR reactions were performed using the Rotor Gene 3000 real time PCR system from Corbett through following procedures: step1: 95 °C for 5 min and step2: 40 cycle of 95 °C for 10 sec and 60°C for 30 sec. The last heating step in phase 2 was carried out for generation of a melting curve of the product. The amplicons were melted at the rate of 0.1°C/s to generate the high resolution melting profile.

Plasma nesfatin-1 and HDL-C measurement

Plasma nesfatin-1 level was measured using a commercially available ELISA kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA) according to the manufacturer's protocol. Plasma high density lipoprotein cholesterol (HDL) was determined through direct Immuno method (HDL-C Immuno FS, Pars Azmoun, Tehran, Iran). the Intra-assay coefficient of variation and sensitivity of the method were 1.2% and 0.03 mmol/L, respectively.

Statistical analysis

The data were analyzed using the comparative threshold cycle method (CT). Briefly, Δ-CT value was calculated by taking the CT of the nesfatin-1 gene and subtracting it from CT of β-actin. The ΔΔ-CT was calculated by subtracting the Δ-CT(sample) values from the Δ-CT(control). The relative quantification was then calculated by the expression $2^{-\Delta\Delta CT}$[25]. The Kolmogorov-Smirnov test was used to determine the normality of the distribution, and variables were found to be normally distributed. All results are expressed as means ± SEM. All variables were compared by unpaired *t*-test. Correlation was calculated using the Pearson Product Moment correlation. All statistical analyses were performed using SPSS (Version 13). All *P*-values <0.05 were considered as significant.

Results

Data analysis revealed a significant difference in liver relative mRNA expression of nesfatin-1/NUCB2 at the end of treadmill running program (F= 273.226, P<0.001). Liver nesfatin-1/NUCB2 was up-regulated in the trained group (as compared to the control group (Fig.1). Data analysis revealed a significant difference in intestine relative mRNA expression of nesfatin-1/NUCB2 at the end of treadmill running program (F=30.635, P<0.001). Small intestine nesfatin-1/NUCB2 increased in the trained group as compared to the control group, at the end of program (Fig.2). In the kidney tissue, exercise-induced increase in nesfatin-1/NUCB2 expression than control group (F= 9.254, P<0.01) (Fig.3). Data analysis revealed no significant differences in plasma nesfatin-1 concentrations at the end of treadmill running program (F=3.640, P<0.09) (Fig.4). There was no significant correlation between tissues' nesfatin-1 expression and plasma nesfatin-1 concentrations (Table.1). Exercise-induced increase in plasma HDL-C concentration (F= 18.312, P<0.003) was not related to tissue nesfatin-1/NUCB2 expression (Fig. 5, Table.1).

Table1: Correlation between tissue (Liver, small intestine and kidney) nesfatin-1 expression and plasma nesfatin-1 and HDL-C concentration.

Variables	Liver nesfatin-1		Small intestine nesfatin-1		Kidney nesfatin-1	
	***r* Value**	***P* Value**	***r* Value**	***P* Value**	***r* Value**	***P* Value**
Plasma nesfatin-1	0.276	0.473	0.015	0.966	0.125	0.749
Plasma HDL-C	-0.292	0.446	0.201	0.577	-0.376	0.347

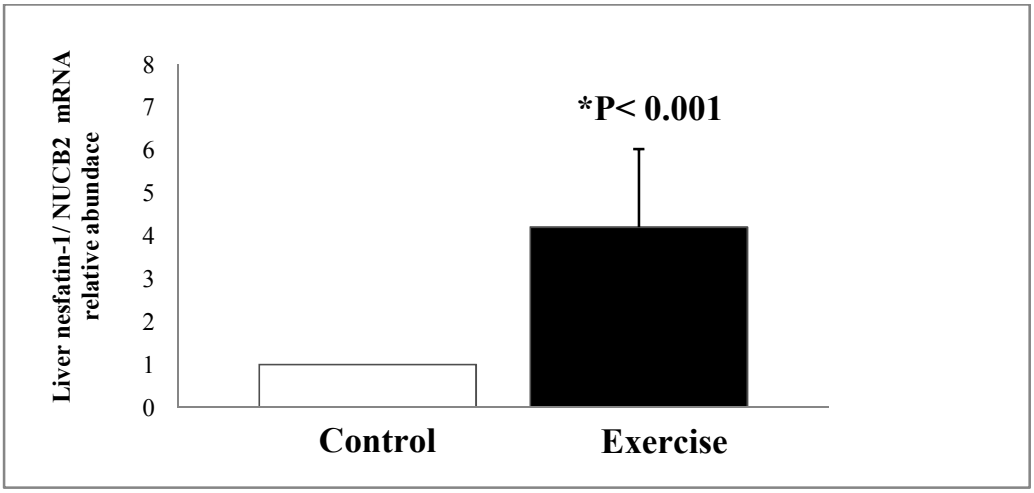

Figure 1: Real-time PCR of liver nesfatin-1/ NUCB2 relative mRNA expression in the control and training groups. Data are expressed as mean ± SEM. Each column represents one group with 5 rats .

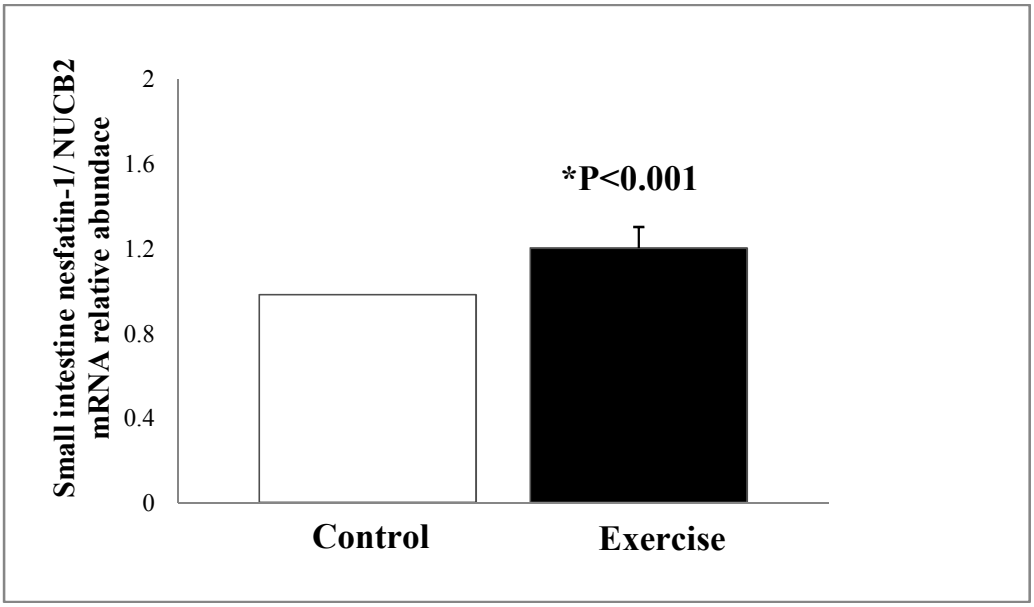

Figure 2: Real-time PCR of small intestine nesfatin-1/ NUCB2 relative mRNA expression in control and training groups. Data are expressed as mean ± SEM. Each column represents one group with 5 rats.

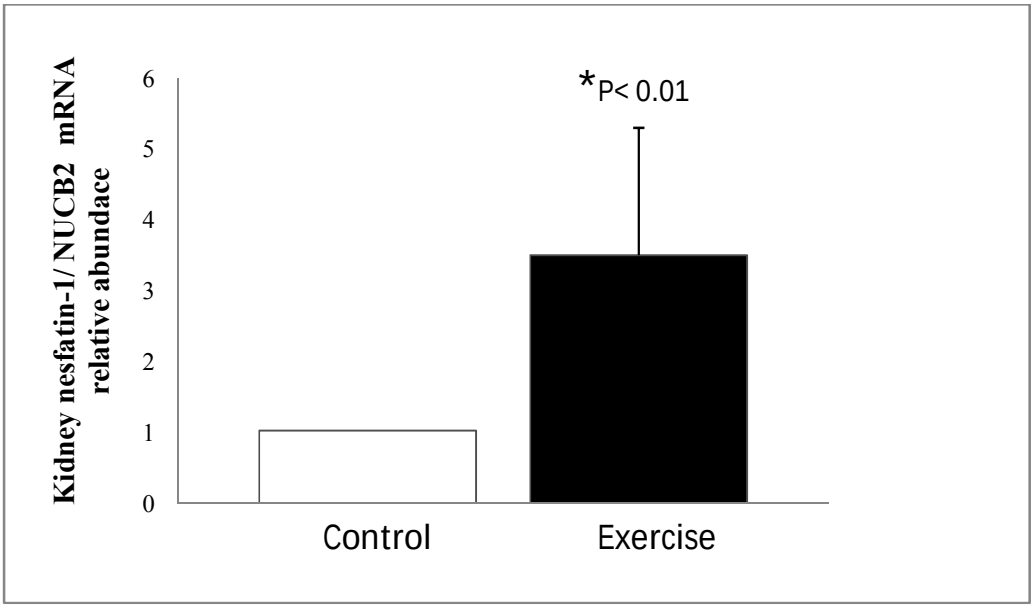

Figure 3: Real-time PCR of kidney nesfatin-1/ NUCB2 relative mRNA expression in control and training groups. Data are expressed as mean ± SEM. Each column represents one group with 5 rats

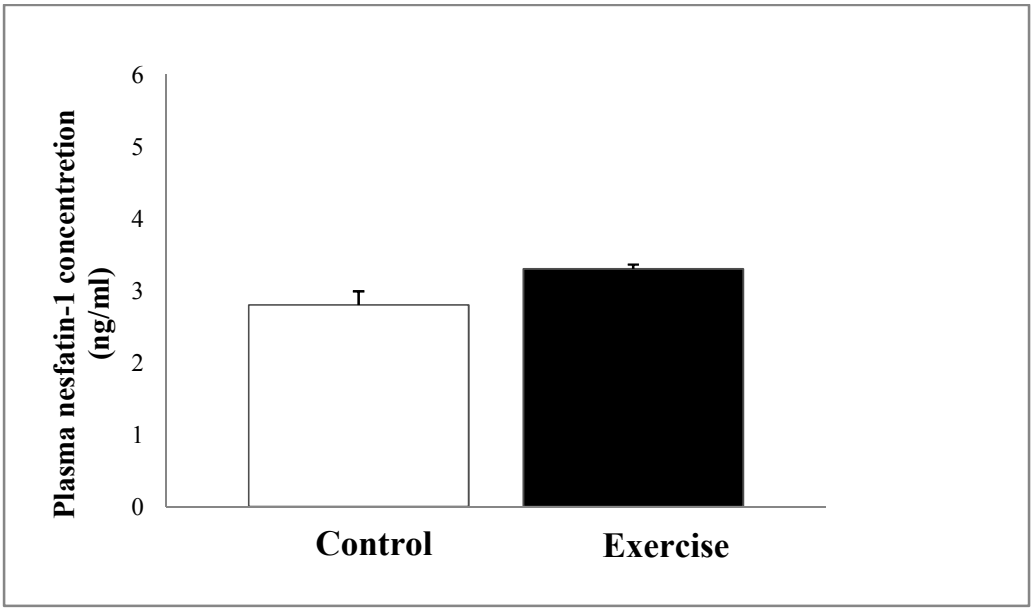

Figure 4: Plasma nesfatin-1 concentration in control and training groups. Data are expressed as mean ± SEM. Each column represents one group with 5 rats

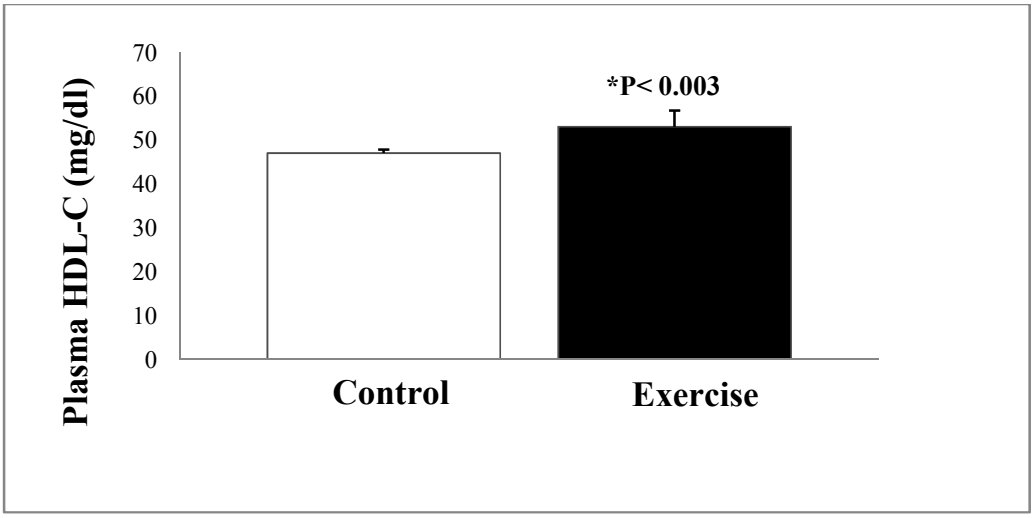

Figure 5: Plasma HDL-C concentration in control and training groups. Data are expressed as mean ± SEM. Each column represents one group with 5 rats

Discussion and Conclusion

To our knowledge, this is the first report to demonstrate alterations of female rats' tissue nesfatin-1/NUCB2 in response to a treadmill running program. Since the molecular weight of nesfatin-1 is very low, (9.7 KD) [5] the extension of distinct piece is not possible. For example, by using immunoreactivity methods, antibodies were not able to identify nesfatin-1, but they recognized the full-length of NUCB2 [26]. As nesfatin-1 is derived from NUCB2, the activated neurons are likely to reflect nesfatin-1/NUCB2 mRNA expression [5, 27]. Nesfatin-1 is expressed in different tissues including the rat's gastric oxyntic mucosa or gastric X/A like cells [4], stomach [5], pancreatic beta cells [6], and adipose tissue [7]. Results of the present study confirmed nesfatin-1/NUCB2 mRNA expression in the liver, small intestine and kidney tissues. Information concerning the effects of exercise training on rat's tissue nesfatin-1/NUCB2 mRNA is lacking. Moreover, only one existing study has focused on the effects of exercise (two different anaerobic exercise sessions) on plasma

nesfatin-1 concentrations in human subjects [28]. In that study, plasma nesfatin-1 concentrations did not change significantly, that is concurrent with findings of the present study..

The mechanisms by which endurance exercise training could change nesfatin-1/NUCB2 mRNA expression are not yet known. However, fasting has been shown to affect nesfatin-1 concentrations in serum and refeeding has been reported to increase the activity of nesfatin-1 neurons in the hypothalamus and nesfatin-1 mRNA expression in the supraoptic nucleus of the hypothalamus [1, 3]. This presented study showed a significant increase in liver, small intestine and kidney nesfatin-1 following a treadmill running program.

In summary, this investigation studied the effects of eight weeks of endurance training on relative tissue expression, plasma concentration of nesfatin-1, and plasma HDL-C concentrations in female rats. This study confirmed nesfatin-1/ NUCB2 expression in liver, small intestine and kidney tissues. The main finding of the present study was the higher and significant relative expression of nesfatin-1/ NUCB2 in the liver, small intestine and kidney tissues of female rats, following a treadmill running program. This is while increase concentration of plasma HDL-C – induced exercise. Nesfatin-1 is a peptide that plays an important role in energy balance and is affected by physical exercise.

Acknowledgements

We wish to thank students of Molecular Genetics lab (Department of Animal Science, Agricultural Sciences and Natural Resources University, Sari, Iran) for their helpful comments and guides.

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