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Centrally and peripherally injected nesfatin-1-evoked respiratory responses

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ABSTRACT

Nesfatin-1, which is an anorexiogenic peptide, plays a crucial role as a neurotransmitter and/or neuromodulator in the central nervous system for cardiovascular control and energy balance *etc.* It is expressed abundantly in multiple brain nuclei including central respiratory control areas such as nucleus tractus solitarius, nucleus ambiguous, dorsal vagal complex, dorsal motor nucleus of the vagus nerve, and hypothalamus. To date, no previous studies have been found to report nesfatin-1-evoked respiratory effects. Therefore, the present study was designed to investigate the possible impacts of centrally and/or peripherally injected nesfatin-1 on respiratory parameters in either 12h-fasted or fed-*ad libitum* rats.

Intracerebroventricular (ICV) administration of nesfatin-1 provoked significant hyperventilation by increasing tidal volume (TV), respiratory rate (RR) and respiratory minute ventilation (RMV) in both the 12h-fasted and the fed-*ad libitum* Spraque Dawley rats in dose- and time- dependent manner. Moreover, the hyperventilatory effects of centrally injected nesfatin-1 were more potent in the fed-*ad libitum* rats. Intravenous injection of nesfatin-1 induced a significant rise in RR and RMV, but not in TV, in the fed-*ad libitum* rats.

In conclusion, these findings plainly report that both centrally and/or peripherally injected nesfatin-1 induces significant hyperventilatory effects in the 12h-fasted and the fed-*ad libitum* rats. These hyperventilatory effects of nesfatin-1 might show a discrepancy according to the food intake of the rats and the delivery method of the peptide.

1. Introduction

Nesfatin-1 is an 82-amino acid neuropeptide produced by the proteolytic processing of nucleobindin-2 (NUCB2) (Oh-I et al., 2006). NUCB2/nesfatin-1 is widely distributed throughout the central nervous system including the cardiovascular and respiratory control areas such as the hypothalamus, dorsal vagal complex (DVC), the nucleus of the solitary tract (NTS), nucleus ambiguous (NAmb) and the dorsal motor nucleus of the vagus (DMNX) (Goebel et al., 2009; Goebel-Stengel et al., 2011; Oh-I et al., 2006). Initial experimental evidence suggests that NUCB2/nesfatin-1 performs a role in the modulation of feeding behavior, control of the cardiovascular system and also neuroendocrine control of the reproductive axis (Garcia-Galiano et al., 2010a, 2010b; Oh-I et al., 2006; Stengel et al., 2011). Moreover, previous reports have also asserted that the peptide plays a potential role in central cardiovascular control. In fact, intracerebroventricular (ICV) administration of nesfatin-1 has been reported to execute pressor responses by stimulating sympathetic activity in both conscious and anesthetized rats (Tanida and Mori, 2011; Yosten and Samson, 2009). However, when microinjected into NTS, nesfatin-1 produced pressor response along with tachycardia (Mimee et al., 2012). In concert to these results, our group has also previously reported that central administration of nesfatin-1 exerts pressor and bradycardiac effects in normotensive animals and pressor and tachycardiac effects under hypotensive conditions produced by severe hemorrhage (Yilmaz et al., 2015). In the same study, it was also reported that ICV injection of nesfatin-1 increases plasma catecholamine, vasopressin, and renin concentrations, and that these hormones contribute to the pressor effects of the peptide in both conditions (Yilmaz et al., 2015). Furthermore, in multiple previous reports, it has been observed that central melanocortin and oxytocin systems, as well as central corticotropin-releasing hormone (CRH), contribute to the hypertensive action of nesfatin-1 in normotensive animals (Tanida and Mori, 2011; Yosten and Samson, 2009, 2010, 2014). In line with these findings, recently we have also recently reported that central cholinergic system is involved in nesfatin-1-modulated cardiovascular functions as well (Aydin et al., 2018).

Respiration is a vital homeostatic neural process, controlling levels of oxygen (O_2) and carbon dioxide (CO_2) in blood and tissues, which are crucial for life. Regulation of respiratory system involves coordinated activities within the central respiratory control centers. Central respiratory areas are chiefly located in brain stem nucleus including NTS, NAmb, DVC, and DMNX (Ikeda et al., 2017). It has long

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been reported that hypothalamic nuclei play crucial role in respiratory control (Fukushi et al., 2018). These observations were further consolidated upon the finding that brain regions involved in respiratory control such as hypothalamus, NTS, NAmb, DVC, and DMNX express NUCB2/nesfatin-1 (Goebel et al., 2009; Goebel-Stengel et al., 2011; Oh-I et al., 2006). Moreover, respiratory control is closely related to cardiovascular control and energy metabolism, which nesfatin-1 has a role in. But there is no report showing the effect of nesfatin-1 on respiratory control. Therefore, in the current study, experiments were carefully designed to determine if the nesfatin-1 exerts an impact on respiratory parameters in both 12h-fasted and fed-*ad libitum* rats.

2. Methods

2.1. Animals

In the experiments, 56 adult, male Sprague Dawley rats (3 months old, 275–300 g) (Experimental Animals Breeding and Research Center, Uludag University, Bursa, Turkey) were employed. Before the experiments, they were housed five/cage at temperatures of 20–22 ^{0}C and humidity of 60–70% in a controlled room set to a 12-h light:12-h dark cycle and had access to standard rat pellet and water *ad libitum*. The Animal Care and Use Committee of Uludag University, in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, approved all experimental procedures (NAC, 2011).

During the experiment, each animal was studied separately in a single experimental protocol, and each experimental group consisted of seven rats. The experimental animals and control animals were studied on the same experiment day.

2.2. Surgical procedures

Under the mixture of ketamine (70 mg/kg) and xylazine (10 mg/kg) anesthesia, the trachea of rats was catheterized with tracheal cannula (BS4 73–2941, Harvard Apparatus Inc., MA, USA) in order to monitor respiratory parameters. The guide cannula was stationed into the lateral ventricle of the rats, for the purpose of central injections. For implementation of the guide canula, the rats were placed in a stereotaxic frame. According to the coordinates, which were taken from the atlas of Paxinos and Watson (2005), a burr hole was drilled through the skull 1.5 mm lateral to midline and 1.0 mm posterior to bregma for ICV treatment. A 22-gauge stainless steel hypodermic tubing was directed through the hole towards the lateral ventricle. The cannula was lowered 4.2 mm below the surface of the skull and fixed to the skull by using acrylic cement. After surgical process, rats were placed in the supine position on a heated operating platform to maintain a rectal temperature of 37 ± 0.2 ^{0}C. Temperature of animals was monitored using a rectal probe throughout the study.

2.3. Recording respiratory parameters

In order to record respiratory parameters, the tracheal cannula was connected to an airflow head (RX137, BIOPAC Systems Inc., CA, USA) attached with differential pressure transducer (SS40 L, BIOPAC Systems Inc., CA, USA). The findings were recorded and analyzed by using the MP36 system and AcqKnowledge software (BIOPAC Systems Inc., CA, USA). The tidal volume (TV) and respiratory rate (RR) of rats were obtained by an electronic airflow signal. The minute ventilation of rats (RMV) was then computed from TV and RR, and stated in milliliters per minute (ml/min).

2.4. Experimental protocol

In the present study, firstly, the respiratory responses to centrally injected nesfatin-1 were studied in both 12h-fasted and the fed-*ad libitum* rats. For this purpose, after initial respiratory baseline measurements of the rats, nesfatin-1 (100 and 200 pmol; ICV) or saline (5 µl; ICV) was delivered centrally and changes in respiratory parameters of the rats were recorded for the next 60 min.

In the second experimental protocol, the effects of intravenous (IV) -injected nesfatin-1 on the respiratory parameters were investigated in the fed-*ad libitum* rats. After the initial recording of respiratory parameters baseline of the rats, nesfatin-1 (40 and 80 µg/kg; IV) or saline (1 ml/kg; IV) was injected through the tail vein and respiratory parameters of the rats were monitored for the next 60 min.

2.5. Drugs and ICV injections

Nesfatin-1 (Sigma-Aldrich Co.,Deisenhofen, Germany) solution was prepared freshly in 0.09% saline on the day of the experiment. Therefore 0.09% saline was used as a vehicle in control groups. The central doses of nesfatin-1 were chosen our previous studies (Yilmaz et al., 2015; Aydin et al., 2018). The IV dose of nesfatin-1 was selected by testing. Lower dose of nesfatin-1 (40 µg/kg; IV) did not produce any respiratory responses (data was not shown).

Central injections were executed with a 28-gauge stainless-steel injection cannula connected to a 10 µl microsyringe with polyethylene tubing, which was filled with saline or saline solution of the drug under consideration. For the ICV injection, 5 µl volume of the solution was infused within 60 s. During the injection, an air bubble moving in the polyethylene tubing was closely watched to ensure the drug was delivered in its entirety.

2.6. Data and statistical analysis

All values were given as mean ± standard error of mean (S.E.M.) with $p < 0.05$ considered as the level of significance. Statistical evaluation was performed by analysis of variance (ANOVA) and repeated-measures analysis of variance (RM-ANOVA; two-way) and the post-ANOVA test of *Bonferroni* by using Sigma Stat 3.5 software (CA, USA).

3. Results

3.1. Effects of centrally administrated nesfatin-1 on respiratory parameters

Before the injection of the nesfatin-1 (100 and 200 pmol; ICV) or saline (5 µl; ICV), the baseline TV, RR, and RMV of the 12h-fasted rats ($n = 21$) were recorded to be at 3.016 ± 0.04 ml, 71.02 ± 2.8 breaths/min and 212.7 ± 5.3 ml/min (Fig. 1), respectively. The central nesfatin-1 treatment (100 and 200 pmol) induced dose- and time-dependent rise in TV (Fig. 1A), RR (Fig. 1B) and RMV (Fig. 1C) of the 12h-fasted rats. The maximum increase in respiratory parameters (Fig. 1) was detected at 20th min after nesfatin-1 treatment and continued for almost 50–60 min. Doses of 100 and 200 pmol of nesfatin-1 significantly ($p < 0.05$) induced approximately 0.18 and 0.37 ml rise in TV (Fig. 1A), approximately 1.7 and 2.1 breaths/min rise in RR (Fig. 1B) and approximately 19.2 and 30.4 ml/min rise in RMV (Fig. 1C) in 12h-fasted rats.

The fed-*ad libitum* rats had baseline TV, RR, and RMV recordings at 3.08 ± 0.03 ml, 71.28 ± 2.4 breaths/min and 219.9 ± 2.5 ml/min (Fig. 2), respectively before the start of treatment. ICV administration of 100 and 200 pmol nesfatin-1 produced dose- and time-dependent hyperventilatory responses by significantly ($p < 0.05$) inducing a rise of 0.31 and 0.52 ml in TV (Fig. 2A), 2.6 and 3.8 breaths/min in RR (Fig. 2B) and 31.2 and 52.8 ml/min in RMV (Fig. 2C) in the fed-*ad libitum* rats. Again centrally injected nesfatin-1-evoked maximum hyperventilation effect was observed at 20th min of the injection and the response lasted up to 50-60th min of the injection (Fig. 2).

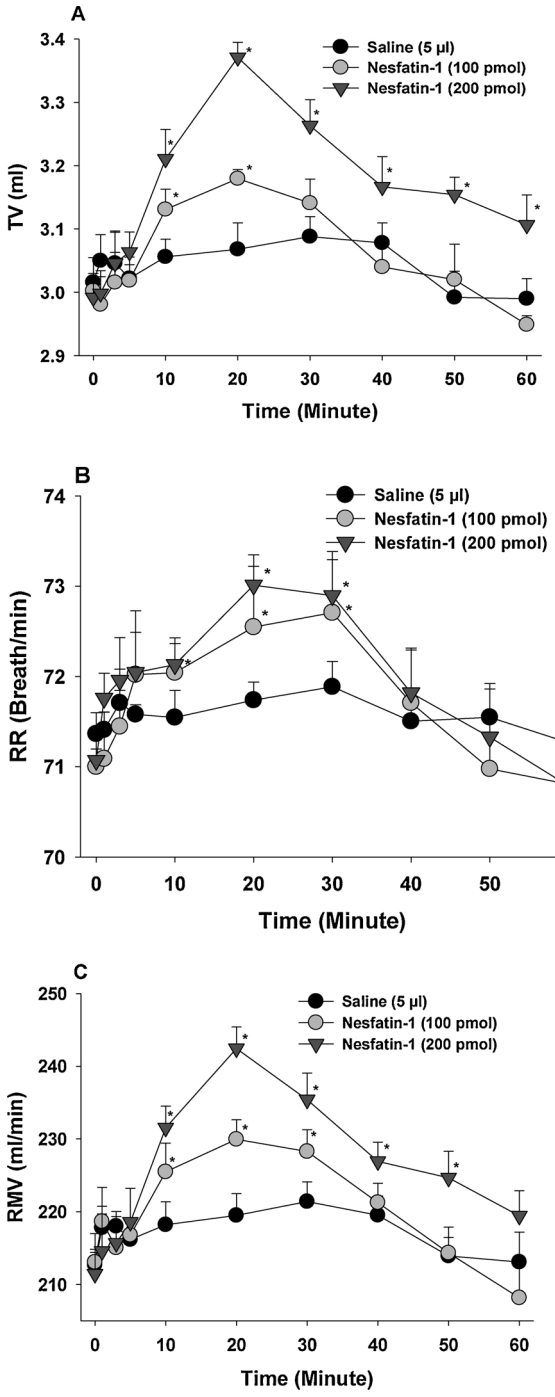

Fig. 1. Respiratory effects of centrally administrated nesfatin-1 in 12h-fasted rats.

The rats fasted for 12 h before the experiment, were treated with nesfatin-1 (100 and 200 pmol; ICV) or saline (5 µl; ICV) after baseline TV (A), RR (B) and RMV (C) measurements had been obtained. Then these respiratory parameters were monitored for the next 60 min. Data are presented as means ± S.E.M. of seven measurements. "0″ shows time of different dose of nesfatin-1 or saline injection. Statistical analysis was performed using two-way RM-ANOVA with post hoc *Bonferroni* test. * shows significant difference ($p < 0.05$) from the value of the "Saline" group.

3.2. Effects of peripherally administrated nesfatin-1 on respiratory parameters

The baseline TV, RR, and RMV of the fed-*ad libitum* rats (n = 10) were 3.03 ± 0.03 ml, 71.02 ± 2.8 breaths/min and 215.1 ± 3.0 ml/

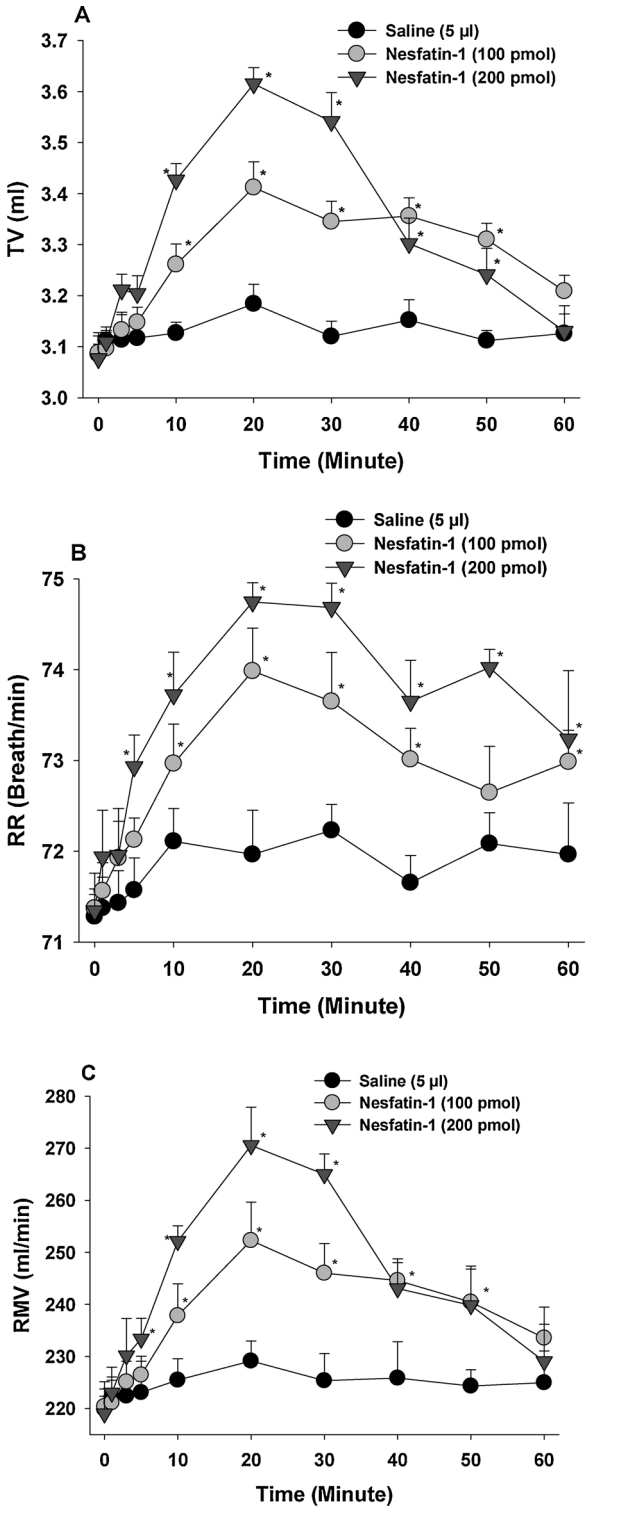

Fig. 2. Respiratory effects of centrally administered nesfatin-1 in rats fed-*ad libitum*.

After baseline TV (A), RR (B) and RMV (C) measurements had been recorded, nesfatin-1 (100 and 200 pmol; ICV) or saline (5 µl; ICV) was injected in the rats, which were fed-*ad libitum* before the experiment. And then these respiratory parameters of the rats were monitored for the next 60 min after the injections. Data are presented as means ± S.E.M. of seven measurements. "0" shows time of different dose of nesfatin-1 or saline injection. Statistical analysis was performed using two-way RM-ANOVA with post hoc *Bonferroni* test. * shows significant difference ($p < 0.05$) from the value of the "Saline" group.

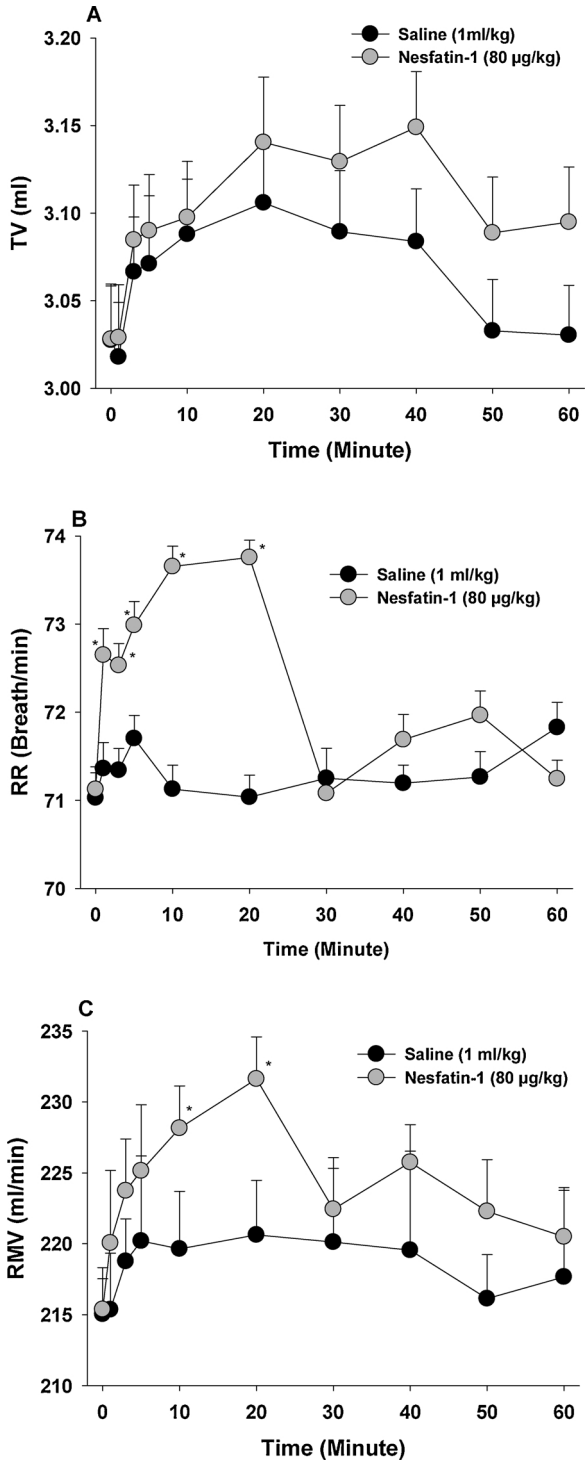

Fig. 3. Respiratory effects of peripherallydelivered nesfatin-1 in rats fed-*ad libitum*.

Rats were offered *ad libitum* feed and water intake before the experiments. Nesfatin-1 (80 µg/kg; IV) or saline (1 ml/kg; IV) was injected in the rats *via* tail vein after baseline TV (A), RR (B) and RMV (C) measurements had been recorded. After the injections the respiratory parameters of the rats were recorded for the next 60 min. Data are presented as means ± S.E.M. of seven measurements. "0" shows time of different dose of nesfatin-1 or saline injection. Statistical analysis was performed using two-way RM-ANOVA with post hoc *Bonferroni* test. * shows significant difference ($p < 0.05$) from the value of the "Saline" group.

min, respectively (Fig. 3). While the peripheral nesfatin-1 (80 µg/kg; iv) treatment did not change the TV of the fed-*ad libitum* rats (Fig. 3A), it significantly ($p < 0.05$) increased RR (Fig. 3B) and RMV (Fig. 3C) of the animals. The maximum increase in RR and RMV was detected at the 20th min after nesfatin-1 treatment and continued for almost 30 min (Fig. 3B,C). Peripherally injected nesfatin-1 caused approximately 2.7 breaths/min rise in RR (Fig. 3B) and 17 ml/min rise in RMV (Fig. 3C) in the rats, compared to the saline (1 ml/kg; IV) treated animals.

4. Discussion

These data report, for the first time, that centrally and peripherally injected nesfatin-1 evoked hyperventilatory effects on the respiratory system of the 12h-fasted and fed-*ad libitum* rats. ICV injected nesfatin-1 provoked a rise in TV, RR and RMV on the respiratory system of the 12h-fasted and fed-*ad libitum* rats, compared to controls. In addition, IV injected nesfatin-1 generated increase in RR and RMV, but not in TV, on respiratory system of the fed-*ad libitum* rats in comparison with control group animals.

Central and peripheral injection of nesfatin-1 produced a rapid hyperventilation response in both the 12h-fasted and the fed-*ad libitum* rats. The hyperventilation effects of nesfatin-1 reached at their peak at 20th min after ICV or IV administration. Time scale of nesfatin-1-induced hyperventilation effect was similar to its cardiovascular effect (Yilmaz et al., 2015; Aydin et al., 2018). Cardiovascular and respiratory systems are two closely related systems and it is well known that nesfatin-1 plays a critical role in the central control of cardiovascular system. In fact, we have recently reported that centrally applied nesfatin-1 exerts a pressor effect on the mean arterial pressure and produces heart rate responses including both bradycardiac and tachycardiac responses in normotensive rats by activating central cholinergic system (Aydin et al., 2018). Moreover, in another study, we have also reported that central administration of nesfatin-1 exerts pressor and bradycardiac effects in normotensive animals, while pressor and tachycardiac effects under hypotensive conditions (Yilmaz et al., 2015). Furthermore, it has been also reported that nesfatin-1 increases mean arterial pressure when injected ICV (Tanida and Mori, 2011; Yosten and Samson, 2009, 2010; 2014). Moreover, microinjection of nesfatin-1 into the NTS induces pressor and tachycardic responses in rats (Mimee et al., 2012). However, microinjection of nesfatin-1 into the NAmb generated a decrease in heart rate, but failed to inflict any change on blood pressure in conscious rats (Brailoiu et al., 2013). These reports clearly show that nesfatin-1 plays a crucial role to modulate the cardiovascular responses.

The initial report by Oh-I et al. explained the anorexiogenic effects of nesfatin-1 and its precursor NUCB2 (Oh-I et al., 2006). In this first report, it was shown that NUCB2 mRNA expression in the hypothalamus was significantly down-regulated after 24-h fasting in rats (Oh-I et al., 2006). On the other hand, re-feeding after a 48-h fast resulted in rise of activated nesfatin-1 immunoreactive neurons in the hypothalamus (Kohno et al., 2008). It was also reported that gastric mucosa expressed NUCB2 mRNA almost 10 times more than in the brain (Stengel et al., 2009). In line with these hypothalamic responses, NUCB2 mRNA expression in gastric mucosal tissue was significantly down-regulated after 24 h fasting in rats. Those reports evidently indicate that the fasting suppresses the central and/or peripheral production of nesfatin-1. Moreover, these reports support our findings stating that central injection of nesfatin-1 in the fed-*ad libitum* rats had more potent hyperventilation effect than the 12h-fasted rats. The tentative explanation could be the facts that the rise in endogenous nesfatin-1 related with satiety may synergistically contribute to the hyperventilation response induced by exogenously injected nesfatin-1.

In the current study, the effect of IV injected nesfatin-1 on respiratory system was tested only in the fed-*ad libitum* rats due to the fact that fed-*ad libitum* rats demonstrated more efficient hyperventilation response than the 12h-fasted rats, when nesfatin-1 was injected ICV.

The hyperventilatory effects of IV injected nesfatin-1 could find its roots from direct effect on peripheral respiratory system or effect on respiratory centers in brain after passing through brain-blood barrier as it has previously been shown that nesfatin-1 is able to cross the blood-brain barrier without saturation (Pan et al., 2007). Moreover it has been reported that lung tissue also highly express nesfatin-1/NUCB2 (Chung et al., 2013; Kim et al., 2014). Those reports suggest that nesfatin-1 might play an important role as a local regulator for breathing. But currently there are no reports explaining the role of nesfatin-1 in lung tissue. Therefore our finding might be the first report explaining the function of nesfatin-1 in lung tissue.

Respiration is a vital behavior, essentially continuous from birth to death. Respiratory rhythm is generated within the central nervous systems. The coordinated motor patterns of the respiratory muscles regulate nuclei within brain stem (Ikeda et al., 2017) and hypothalamus (Fukushi et al., 2018). It was reported that those nucleuses involving in breathing control expressed NUCB2/nesfatin-1 (Goebel et al., 2009; Goebel-Stengel et al., 2011; Oh-I et al., 2006). These neuroanatomical similarities paved the path towards the role of nesfatin-1 in respiratory control and support our current findings showing nesfatin-1-evokes hyperventilation response. Moreover, a number of neurotransmitter and/or neuromodulator have also been reported to play a role in respiratory control in these breath regulatory regions within the brain stem and hypothalamus (Shao and Feldman, 2009; Ikeda et al., 2017; Fukushi et al., 2018). Previously, we have also reported that centrally administered CDP-choline, as a choline donor, induced hyperventilation (Topuz et al., 2014). Recently, we have also reported that ICV injected nesfatin-1 induced a rise in posterior hypothalamic extracellular acetylcholine and choline levels, and furthermore centrally injected nesfatin-1-evoked cardiovascular responses were mediated by central cholinergic nicotinic and muscarinic receptors (Aydin et al., 2018). These reports showing the role of central cholinergic system in respiratory control and interaction between nesfatin-1 and cholinergic system might corroborate our current findings about nesfatin-1-induced hyperventilation effect.

It is well known that the autonomic nervous system controls cardiorespiratory functions. The increase in renal sympathetic nerve activity (Tanida and Mori, 2011; Yosten and Samson, 2009) and increase in plasma catecholamines levels (Yilmaz et al., 2015) after central nesfatin-1 injection suggest that nesfatin-1 activates the sympathoadrenergic system. Additionally, increases in parasympathetic cardiac tone with microinjection of nesfatin-1 into the NAmb have been reported (Brailoiu et al., 2013). Activation of both sympathetic and parasympathetic systems with nesfatin-1 might also explain the current findings showing nesfatin-1-induced hyperventilation response. Also some previous reports have shown that nesfatin-1 was directly or indirectly involved in control of breathing. Experimental evidence shows that nesfatin-1 is physiologically involved in the regulation of energy balance (Schalla and Stengel, 2018). Furthermore it is well known that energy balance and oxygen consumption are in correlation to each other, as it has been reported that knockout of the transcription factor Yin Yang 1 increased energy expenditure and oxygen consumption in beige and white fat depots along with an increased expression of NUCB2 mRNA in brown adipose tissue (Verdeguer et al., 2015). Mortazavi et al. (2015) have also reported that long-term infusion of nesfatin-1 caused a decrease of oxygen consumption and energy expenditure in the light phase, but no alteration in the dark phase in freely feeding rats. Those findings support our present data by explaining oxygen consumption dependent on nesfatin-1-regulated energy balance. Furthermore an inverse correlation between plasma nesfatin-1 level and the severity of disease was observed in sleep apnea syndrome patients (Aksu et al., 2015; Araz et al., 2015; Shen et al., 2015). Lower plasma nesfatin-1 level in severe sleep apnea syndrome patients might be the evidence that nesfatin-1 is able to be positively affect respiratory control areas as nesfatin-1 can cross the blood-brain barrier and reach the central nervous system and hence may modulate respiratory system (Pan et al., 2007). These reports also consolidate our findings showing peripherally injected nesfatin-1-evoked hyperventilation response.

Anesthesia is ethically desirable for all experimental protocols that are deemed to be highly invasive or difficult to perform in conscious animals, and as such the anaesthetized rat has become a common model used in cardiovascular and respiratory research. However there are limitations associated with anesthesia because all anesthetic agents could exert effects on cardiorespiratory regulation. In the current study the animals were anaesthetized with ketamine and xylazine mixture. Ketamine (70 mg/kg) and xylazine (10 mg/kg) mixture in the doses used in the current study produced an enough time like 90 min to prepare the animals and to complete the experiments. It was showed that ketamine and xylazine mixture had moderate cardiorespiratory depressive effect and acceptable decrease in partial pressure of carbon dioxide and oxygen, oxygen saturation percentage and arterial blood pH in rats (Sumitra et al., 2004). In the present study, respiratory parameters obtained treated rats were compared with saline injected control rats. All rats were used study were anaesthsized with ketamine and xylazine mixture.

In conclusion, the current findings are preliminary data indicating that nesfatin-1 may affect the respiratory system. When nesfatin-1 is injected peripherally or centrally, it can produce hyperventilatory effect. The peptide shows more potent hyperventilation effect when injected centrally. Moreover, food intake is able to influence the hyperventilation effect of the peptide. Hence the rats fed-*ad libitum* demonstrate more potent hyperventilatory response than fasted rats. Although the present data show the exogenously injected nesfatin-1-induced the respiratory response, the data give clue about the physiological role of endogenous nesfatin-1 in respiratory control. Moreover, considering the expression of the peptide in centers closely associated with respiratory regulation, and also the effect of the peptide on the regulation of the respiratory-closely related cardiovascular system and energy balance, suggests that endogenous nesfatin-1 may play a physiological role in respiratory regulation. Again obtaining more potent hyperventilation effect with nesfatin-1 in satiated rats show that endogen nesfatin-1 has a physiological role in respiratory control. Because satiety enhances peripheral or central nesfatin-1 expression and also increases oxygen demand related with energy metabolism. Despite the study focuses on the effect of the nesfatin-1 in the respiratory control, further studies are needed to explain the mechanism of the nesfatin-1-induced respiratory responses.

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