ISTANBUL UNIVERSITY PRESS

RESEARCH ARTICLE

Eur J Biol 2022; 81(1): 18-25

Leptin Enhances Nitric Oxide Production and Decreases Blood Flow in Rat Skeletal Muscle

Savas Ustunova[1], Aysegul Kapucu[2], Cihan Demirci Tansel[2]

[1]Bezmialem Vakif University, School of Medicine, Department of Physiology, Istanbul, Turkiye
[2]Istanbul University, Faculty of Science, Department of Biology, Istanbul, Turkiye

ORCID IDs of the authors: S.U. 0000-0003-1870-229X; A.K. 0000-0002-0946-1407; C.D.T. 0000-0001-7926-3089

Please cite this article as: Ustunova S, Kapucu A, Demirci Tansel C. Leptin Enhances Nitric Oxide Production and Decreases Blood Flow in Rat Skeletal Muscle. Eur J Biol 2022; 81(1): 18-25. DOI: 10.26650/EurJBiol.2022.1082814

ABSTRACT

Objective: There are a few studies related to nitric oxide (NO) and leptin interaction in the regulation of physiological events in skeletal muscle. Therefore, the aim of the present study is to investigate the interaction of leptin and NO in response to blood flow and nitric oxide synthase (NOS) distribution on rat skeletal muscle.

Materials and Methods: Twenty-four adult-male Wistar albino rats were divided into 4 groups: control (C), Leptin (LP) (50 µg/kg), L-NG-nitroarginine methyl ester (LN) (a non-specific nitric oxide synthase inhibitor, 10 mg/kg,) and L-NAME+Leptin (LN+LP) administrated groups. Drugs were administered via the right jugular vein. Hemodynamic parameters: mean arterial pressure, heart rate and blood flow were recorded during the experiment and at the end blood samples for biochemical analyses of leptin and nitrite/nitrate levels and gastrocnemius muscle tissues from the right hindlimb for NOS distribution were taken.

Results: Leptin infusion after L-NAME administration significantly decreased heart rate and blood flow ($p<0.05$, $p<0.001$). In addition, there was no change in the mean arterial pressure in the leptin group and leptin-administered L-NAME group. Leptin levels were the highest in the LP+LN group ($p<0.01$). Decreased nitrate/nitrate levels with L-NAME administration ($p<0.01$ vs C) reached control values by leptin infusion. Both endothelial NOS (eNOS) and neuronal NOS (nNOS) distributions were observed in the skeletal muscle cells of the leptin group.

Conclusion: Although NO synthesis is inhibited by L-NAME, it is concluded that leptin partially enhances NO production and leptin uses NO as a mediator in its physiological effects.

Keywords: Blood flow, leptin, nitric oxide, rat; skeletal muscle

INTRODUCTION

Leptin, a polypeptide hormone, which is coded by the obesity (*Ob*) gene, is mainly released from white adipose tissue and belongs to the cytokine family. The best-known function of leptin is the regulation of food intake and energy expenditure (1). In addition, leptin is functional in many metabolic events such as angiogenesis, hematopoiesis, and lipid/carbohydrate metabolism. It is also effective on functions of reproductive, cardiovascular, and immune systems (2). Leptin shows its effect through membrane-bound leptin receptors (OBR). The OBR gene expresses in the central nervous system (CNS) and in many peripheral tissues including skeletal muscle (3). When leptin binds to OBR, thermogenesis is stimulated through the PI3K and AMPK signaling pathways in the skeletal muscle, enhancing glucose and fatty acid metabolism. Therefore, leptin-mediated increase in the fatty acid oxidation reduces the formation of triacylglycerol and fat deposits to prevent lipotoxicity (4, 5).

Furthermore, when leptin in circulation crosses the blood-brain barrier, it acts on the hypothalamus to regulate energy balance acting and increases the sympathetic nerve activity in brown adipose tissue to provide

Corresponding Author: Savas Ustunova E-mail: sustunova@bezmialem.edu.tr
Submitted: 04.03.2022 • **Revision Requested:** 21.03.2022 • **Last Revision Received:** 23.03.2022 • **Accepted:** 04.04.2022 • **Published Online:** 29.04.2022

thermogenesis. In addition, because of sympathetic nervous system stimulation, leptin increases arterial blood pressure, which is correlated to high levels of leptin in continuous hypertension (6, 7).

The binding sites of leptin in the brain are also important in the control of the cardiovascular system. Therefore, it is suggested that leptin may affect cardiovascular system functions through its effects on the CNS. Furthermore, functionally active OBRs have been demonstrated to be present in endothelial cells, suggesting that endothelium, which plays an important role in the regulation of blood pressure, is one of the main targets of leptin (8-10).

Nitric oxide (NO), a gaseous transmitter and intracellular second messenger in biological systems, is one of the substances released by the endothelium to cause vasodilatation for regulating blood pressure. Previous studies have also shown that NO plays an important role in platelet aggregation, cytotoxicity, hypertension, diabetes, atherosclerosis, learning and memory function, and regulation of male sexual function (11). In addition, NO has many functions in skeletal muscle. These are stimulation and contraction of muscle, mitochondrial energy production, glucose metabolism and autoregulation of blood flow. Although expression of OBRs is present in endothelial cells, its effect on NO production is controversial (12-14).

There have also been some studies demonstrating that leptin increases NO release from endothelial cells (9, 10, 15). Although there have been studies to demonstrate leptin and NO interaction, the relationship between leptin and NO has not been fully explained in the regulation of physiological events in skeletal muscle. Therefore, this study was carried out to determine the relationship between leptin and NO in response to blood flow and NOS distribution on rat skeletal muscle.

MATERIALS AND METHODS

Animals and Study Groups

In the present study, 24 adult-male Wistar albino rats weighing 250-300 g were used. All animals were housed in a light- (12/12 hours light and dark period) and temperature-controlled room (22±2°C), fed with standard lab chow and tap water *ad libitum* until the day of the experiment. The study was reviewed and approved by the Experimental Animals Ethics Committee of Experimental Medicine Research Institute of Istanbul University (Date:18.10.2005, Number: 36).

The rats were divided into 4 groups, each including 6 rats as follows: control (C), Leptin (LP) (50 µg/kg, #sc-471278, Santa Cruz Biotechnology, Texas, USA), L-NG-nitroarginine methyl ester (LN) (L-NAME, a non-specific nitric oxide synthase inhibitor, 10 mg/kg, Sigma-Aldrich Chemie Gmbh Munich, Germany) and L-NAME+Leptin (LN+LP) administrated groups. The control group had saline in the same volume at the same time points of drug administrations. All drugs and saline were administrated intravenously (IV) via an infusion pump (KDS 200/200P LEGACY, KD Scientific, Holliston, MA, USA) at a rate of 0.166 ml/min, so the total volume was 0.5 ml for each administration.

Experimental Design

The animals received intraperitoneal (ip) sodium pentothal (90 mg/kg) anesthesia. During the experiment, the rats were placed on a heating pad to control body temperature via a rectal probe (MLT1403, ADInstruments, Sydney, AUS) and body temperature was maintained at approximately 37°C. Tracheostomy was performed and spontaneous breathing was provided. Right carotid artery and right jugular vein were cannulated with a polyethylene (PE 50) cannula. Blood pressure was monitored via a pressure transducer (MLT0380/D, ADInstruments, Sydney, AUS) from the right carotid artery. All drugs were administered via a venous catheter which was attached to the right jugular vein. A small incision was made on the gastrocnemius muscle of the left hindlimb without causing any bleeding. To determine intramuscular blood flow, the needle probe of the laser Doppler flowmeter (Blood FlowMeter INL 191, ADIsntruments, Sydney, AUS) was carefully placed on the muscle surface. After a 30-min stabilization period the first drugs were applied and 20 min after the first drug administration, the second drugs were administered (Figure 1). All hemodynamic parameters were recorded during the experiment by a data acquisition unit (PowerLab 8/30, ADInstruments, Sydney, AUS) and then analyzed offline every 10 min.

At the end of the experiment, blood samples were collected directly from the heart for biochemical analyses and the tissue samples of the gastrocnemius muscle from the right hindlimb, which not had any intervention, were taken to determine the distribution of NOS enzymes, immunohistochemically.

Immunohistochemical Analysis

Gastrocnemius muscle tissues were fixed in 10% formalin and embedded in paraffin. In 4 µm sections taken from paraffin blocks, the distributions of eNOS and nNOS were examined immunohistochemically. For this purpose, the sections were kept overnight at 56°C, then passed through xylene and alcohol series and brought up to the water. The sections were incubated for antigen retrieval in citrate buffer (pH 6.0) (Thermo Scientific, AP-9003-500) for 10 minutes by microwaving slides and then allowed to cool to room temperature for 20 min and rinsed with distilled water. En-

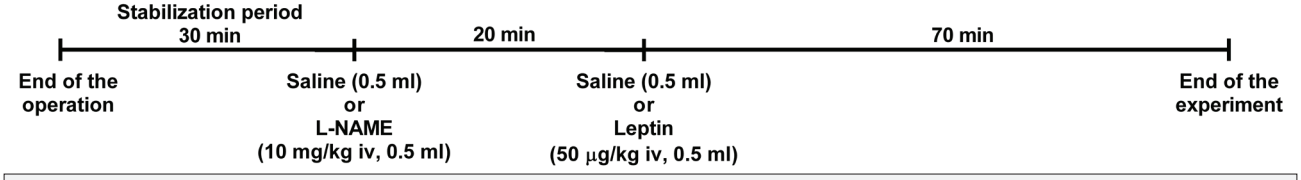

Figure 1. The timeline of experimental design.

dogenous peroxidase activity was eliminated with 0.3% H_2O_2 (Lab Vision, TA-060-HP) at room temperature for 10 min. The sections were rinsed with distilled water and washed for 10 min in phosphate buffered saline (PBS) three times in each step.

After washing, the sections were treated with blocking serum (Ultra V Block, Lab Vision, TA-60-UB, Värmdö, Sweden) for 5 min at room temperature. The muscle sections were then incubated with anti-eNOS (RB- 1711-P, NeoMarkers Fremont, CA) and nNOS (KAP-NO003, NeoMarkers Fremont, CA) antibodies at 4°C overnight. Antibodies were diluted in a ratio of 1:100 with an antibody diluent (UltrAb Diluent, Lab Vision, TA-125-UD, Värmdö, Sweden). Then, the sections were processed by the StrepABC procedure, following the manufacturer's instructions, using a goat anti-rabbit IgG (Labvision, TR- 125-BN and TR-125-HR, Värmdö, Sweden). The activity was demonstrated by the AEC (3-amino-9-ethyl carbazole) substrate kit (Labvision, TA-004-HAC, Värmdö, Sweden). The sections were counterstained with Mayer hematoxylin. The results of the immunohistochemistry were examined by two independent observers using a light microscope (Leica, Wetzlar, Germany) and photographed by Image Pro-Plus system.

Slides were blindly analyzed with ImageJ software (National Institutes of Health, Bethesda, Maryland, U.S.A.) by using a color deconvolution plug-in. For staining intensity, every section was assessed in 3 similar fields of view under 400X magnification. Images were analyzed to calculate the mean intensity of AEC, ranging from 0 (black) to 255 (total white). The final AEC intensity was calculated according to the formula $f = 255 - i$, (i = mean DAB intensity) with data acquired from the software; i ranges from 0 (zero = deep brown, highest expression), to 255 (total white) (16).

Biochemical Analysis

Leptin and nitrite/nitrate levels were measured in blood samples which were drawn from the hearts of animals at the end of the experiment. Blood samples were centrifuged at 1500 *g* for 10 min at 4°C and the serum samples were used for analysis. Serum leptin levels were studied by an enzyme-linked immunosorbent assay (ELISA) using the Rat Leptin ELISA kit (DRG Instruments GmbH, Germany) according to the manufacturer's notes. Nitrite/nitrate levels in the serum were determined by the Griess method (17, 18). According to this reaction, nitrate is reduced in nitrite in the presence of nitrate reductase enzyme. Then, the mixture containing the sulfanilamide and N-(1-naphthyl) ethylene diamine dihydrochloride compounds, named the Griess reactive, is added into nitrite to obtain the colored diazo product. The resulting-colored product is measured spectrophotometrically at 540 nm.

Statistical Analysis

Data were analyzed using the GraphPad Prism 6.0 (GraphPad Prism, Version 6, Software Program, San Diego, CA) and were expressed as mean ± standard error of means (SEM). The time dependent results of hemodynamic parameters were compared by two-way ANOVA, while biochemical results of blood samples in different groups and area under the curves (AUC) for hemodynamic parameters were compared by one-way ANOVA followed with post-hoc Bonferroni's multiple comparison test. A value of $p \leq 0.05$ was considered as statistically significant.

RESULTS

Hemodynamic Results

There was no significant difference in the Mean Arterial Pressure (MAP) in the leptin administrated group when compared with the values of the C group at the same time intervals during the experiment. MAP was significantly increased within the first 10 minutes of L-NAME infusion in LN and LN+LP groups ($p<0.001$). Although these high levels tend to decrease, they were maintained until the end of the experiment which did not change much more with leptin administration in the LN+LP group. At the end of the experimental procedure (at the 90th min) MAP of the LN and LN+LP groups were still significantly higher than MAP levels of the C and LP groups ($p<0.001$, Figure 2A). Leptin and L-NAME also showed similar effects on systolic and diastolic pressure, both of which were compatible with

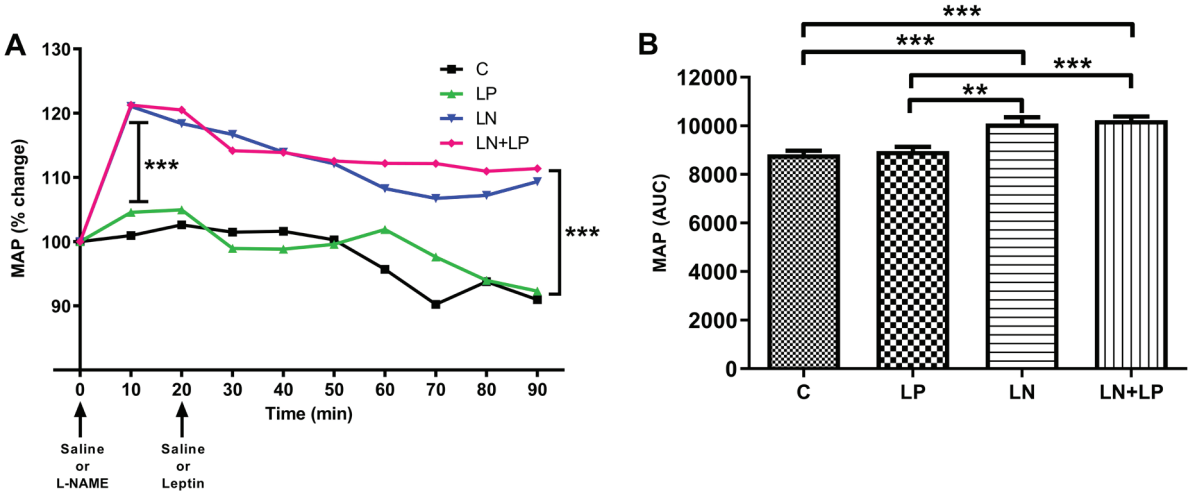

Figure 2. Comparison of % changes in mean arterial pressure during the experiment (A) and differences of area under curve (B) between the groups. MAP: mean arterial pressure, AUC: area under curve, C: Control, LP: Leptin, LN: L-NAME, LN+LP: L-NAME+Leptin groups (Data are expressed as mean±SEM, **p<0.01, ***p<0.001 statistically significance).

MAP (data not shown). To compare the cumulative difference between the groups during the experimental time, the AUC of hemodynamic parameters were calculated. AEC of both LN and LN+LP groups were very close to each other, as seen in the same way between the C and LP groups. However, the area under the curves of both LN and LN+LP was found to be significantly higher according to the control and leptin groups ($p<0.01$ and $p<0.001$ respectively, Figure 2B).

The heart rate (HR) was significantly decreased with L-NAME infusion in the LN and LN+LP groups ($p<0.05$). While these low levels slowly increased in the LN group and were close to that of the C levels at the end of the experiment, leptin administration slowed down this increase with respect to the LN group. Leptin and control groups had similar values during the experiment. At the end of the experimental procedure (at the 90th min) the LN+LP group had significantly lower HR according to all other groups ($p<0.05$, Figure 3A). When the AUC were analyzed, both C and LP groups had proximate values, which also were observed between LN and LN+LP groups. So, there was no significant difference in the HR during the experiment in the leptin administrated group when compared with the control group. On the other hand, the AUC of the LN+LP group was found to be significantly higher according to the C and LN groups ($p<0.05$, Figure 3B)

After administration of L-NAME, a statistically significant decrease in the blood flow, especially at the 20th minute of treatment, was observed in the LN and LN+LP groups, according to the C and LP groups ($p<0.001$). These low blood flow levels persisted over the duration of the experiment. Administration of leptin did not cause significant changes in blood flow compared to the C group until the 60th minute of the experiment. After that point, blood flow was significantly decreased ($p<0.001$). Leptin administration resulted in low blood flow in the LN+LP group until the end of the experiment and that was statistically

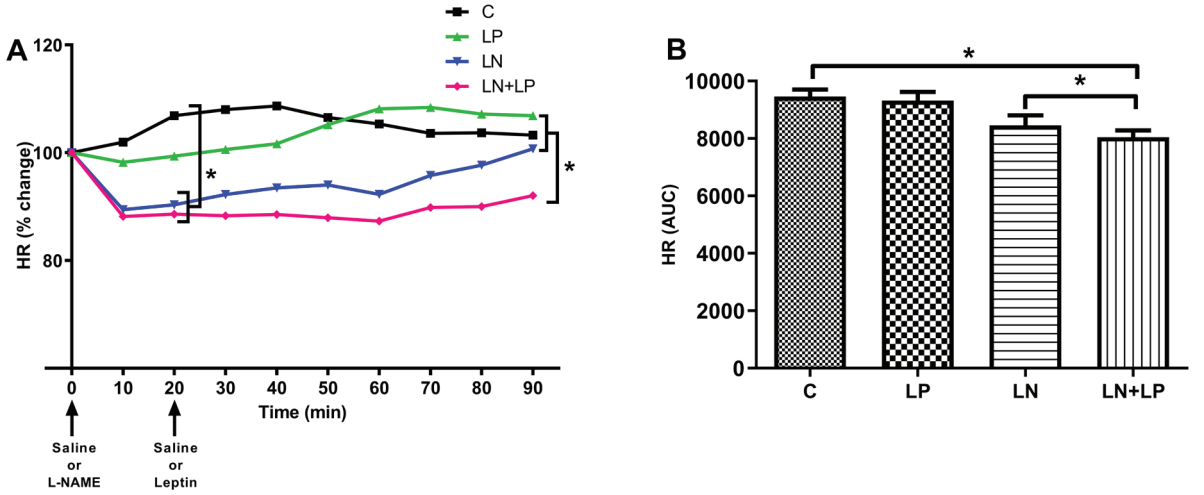

Figure 3. Comparison of % changes in heart rate during the experiment (A) and differences of area under curve (B) between the groups. HR: heart rate, AUC: area under curve, C: Control, LP: Leptin, LN: L-NAME, LN+LP: L-NAME+Leptin groups, C: Control, LP: Leptin, LN: L-NAME, LN+LP: L-NAME+Leptin groups (Data are expressed as mean±SEM, *p<0.05 statistically significance).

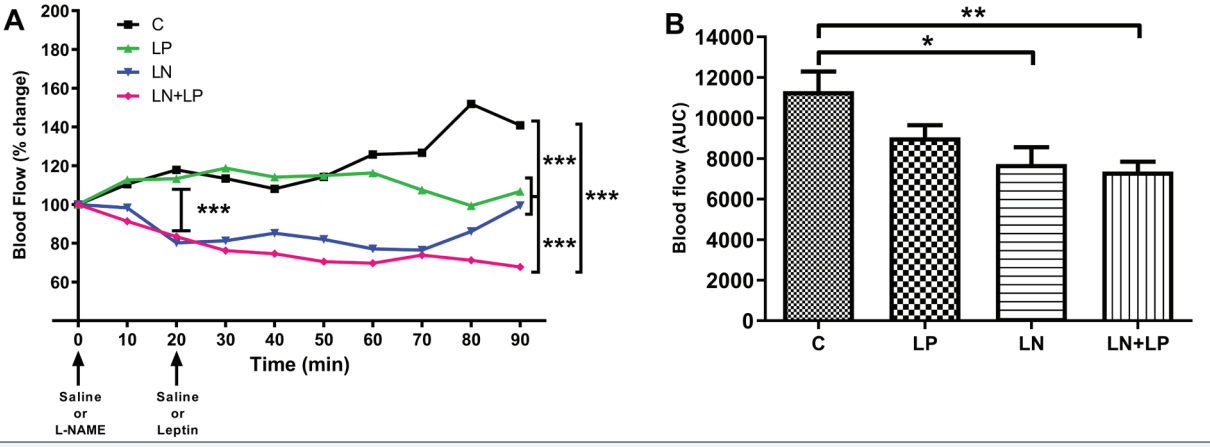

Figure 4. Comparison of % changes in blood flow during the experiment (A) and differences of area under curve (B) between the groups. AUC: area under curve, C: Control, LP: Leptin, LN: L-NAME, LN+LP: L-NAME+Leptin groups (Data are expressed as mean±SEM, *p<0.05, **p<0.01, ***p<0.001 statistically significance).

significant in accordance with other groups (p<0.001). However, in the LN group alone, blood flow returned to baseline levels at the end of the experiment (Figure 4A). When the area under the curves was examined, it was determined that the control group had a higher blood flow than that of all groups and this was statistically significant in both LN and LN + LP groups (p<0.001). L-NAME administration gave rise to a significant reduction in AUC of both LN and LN+LP groups when compared to LP group (p<0.001) (Figure 4B).

Biochemical Results

Serum leptin and nitrite/nitrate levels were measured in blood samples at the end of the experiment. It was determined that leptin levels of the LP, LN, and LN+LP groups were significantly increased according to that of the C group (p<0.05). The elevation in the LN+LP group was more significant (p<0.01) (Figure 5A). When the nitrite/nitrate levels were compared, the increase in the LP group was not significant, but there was a statistically significant decrease in the LN group compared to both C and LP groups (p<0.01, p<0.001, respectively). In the LN+LP group, the nitrite/nitrate levels were significantly higher than that of the LN group (p<0.01) but less than that of the LP group in a statistically insignificant manner and close to that of the C group values. In addition, nitrite/nitrate levels of the LP group were significantly elevated according to the LN group (p <0.001) (Figure 5B).

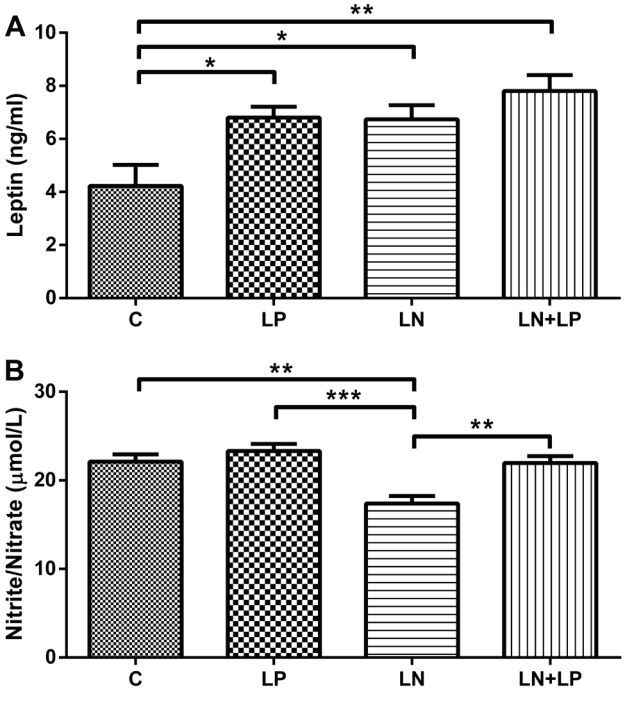

Figure 5. Serum leptin (A) and nitrite/nitrate (B) levels. C: Control, LP: Leptin, LN: L-NAME, LN+LP: L-NAME+Leptin groups (Data are expressed as mean±SEM, * p<0.05, **p<0.01, ***p<0.001).

Immunohistochemical Results

The distributions of eNOS and nNOS were investigated by immunohistochemical methods in the paraffin sections of control and drug administrated animals. In the control group, the reaction of eNOS and nNOS was determined in the perimysium, sarcolemma, and irregular connective tissue (Figure 6). In the leptin treated group, although the eNOS reaction was observed to be slightly dense in sarcolemma and more intense in the perimysium, the reaction was more pronounced around of the peripheral position of nuclei. The nNOS reaction was found to be similar in the same regions of the eNOS reaction, but at a lower density (Figure 6). The mean intensity of eNOS and nNOS reactions were significantly more than those of the C and LN groups (p<0.01, p<0.001, respectively) and were also denser from that of the LN+LP group which was significantly elevated in nNOS (Figures 7A-B). Both reactions were suppressed in the LN group. The eNOS reaction was denser in the sarcolemma whereas almost no nNOS reaction was observed in the control (Figure 6). After L-NAME administration, the eNOS reaction in the leptin-treated group was found to be mild in the sarcolemma and more in the vicinity of nuclei. The eNOS reaction was less than that of the leptin group and more than that of the control and L-NAME groups. The nNOS reaction was similar to that of the control values (Figures 6 and 7).

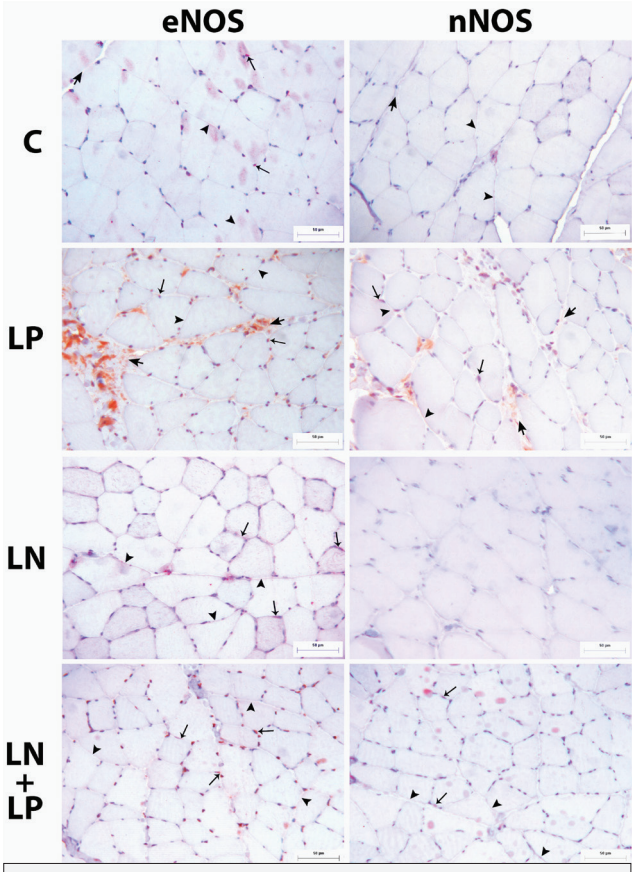

Figure 6. Immunohistochemically demonstrated eNOS and nNOS reactions in sarcolemma (➤), peripherally located nuclei (↑) and perimisium (↑) in gastrocnemius muscle. C: Control, LP: Leptin, LN: L-NAME, LN+LP: L-NAME+Leptin groups (Bar = 50 µm).

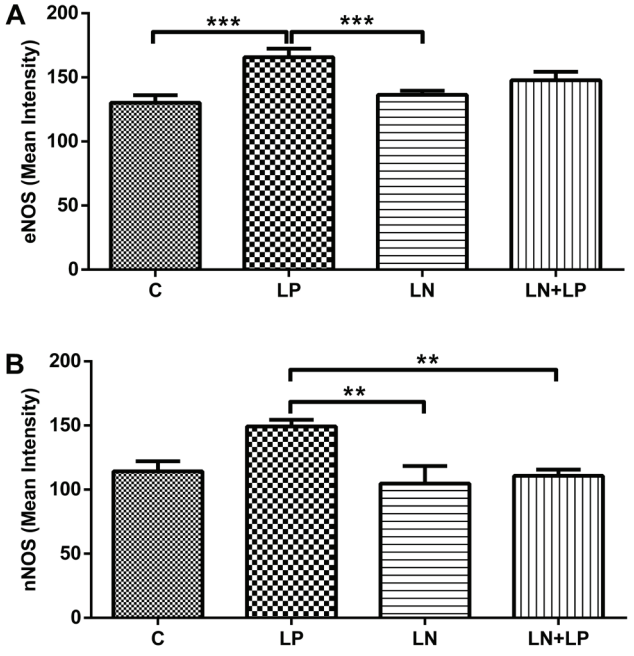

Figure 7. Mean intensity levels of eNOS (A) and nNOS (B) reactions in gastrocnemius muscle. C: Control, LP: Leptin, LN: L-NAME, LN+LP: L-NAME+Leptin groups (Data are expressed as mean±SEM, **p<0.01, ***p<0.001).

DISCUSSION

The understanding of the effects of leptin on the CNS and peripheral systems was led to thinking that there may be links between leptin and obesity, hypertension, and cardiovascular diseases (2). Experiments in ob/ob mice have shown that the absence of leptin causes abnormalities in the vascular endothelium and in the contraction-relaxation functions of vessels (19). Expression of leptin in fibroblasts and the presence of OBRs in vascular endothelium and cardiac muscle cells suggest that leptin has cardiovascular effects which might be mediated by NO (20). Therefore, in this study, we aimed to find out whether leptin exerts its cardiovascular effects through NO.

Leptin increases sympathetic nerve activity in rodents and humans; however, leptin only consistently increases BP chronically in rodents. The ability of leptin to increase BP in rodents is via both hypothalamic and extrahypothalamic regions.

It has been reported that administration of high dose intracerebroventricular leptin increases arterial blood pressure and HR by central neural mechanisms (21). In another study, no significant change in arterial pressure, HR, renal blood flow or renal cortical blood flow was observed by administration of leptin or saline in rats with similar initial values. It has also been suggested that leptin has no effect on systemic blood pressure and arterial blood flow (22). In our study, after a single dose of IV 50 µg/kg leptin administration, MAP and HR were found to be similar to that of the saline-administered control group, and there was no significant difference between them during the entire experiment. Similarly, Frühbeck (15) reported that after 90 minutes of administration of a single dose of IV saline and 10, 100, 1000 µg/kg of leptin, there was no change in blood pressure and an insignificant increase in HR. Mitchell et al. (23) reported that there was no significant change in MAP and HR between leptin-administered and non-administered animals when they infused a total of 1 mg/kg leptin to animals for 3 hours, while blood pressure decreased regularly in both groups during the experiment. Frühbeck (15) also reported an increase in MAP and a decrease in HR after the first 10 minutes of NOS inhibition, which was performed with L-NAME (30 mg/kg). In our study, when we used 10 mg/kg L-NAME, we found that MAP in this group was significantly higher, and HR was significantly lower than the control values at the same time intervals throughout the experiment. However, the HR was close to the control at the end of the experiment. In the leptin-administered L-NAME group, the increase in the MAP values was more dramatic and the changes in blood pressure were similar to the L-NAME group. The low values of HR after L-NAME administration were maintained until the end of the experiment in animals treated with leptin after L-NAME. HR levels in the LN+LP group was the lowest at the end of the experiment according to others. In addition, Dunbar et al. (24) reported that leptin administration decreased the blood flow in the iliac and mesenteric arteries without a change in the renal artery, and increased the MAP by decreasing the blood flow in the splanchnic vascular beds and skeletal muscle. It has also been reported in other studies that leptin did not cause a significant change in mesenteric, hindlimb and renal blood flow (9, 23). However, Mitchell et al. (23) also reported that L-NAME significantly increased regional blood flow when they applied it for 3 hours. In addition, it was noted that there was no significant change in renal and hindlimb blood flow when leptin was administered (23). In our study, no change in blood flow was observed in the one-hour period after leptin administration. However, after this period, there was a significant decrease in blood flow of the leptin-administered group until the end of the experiment. In addition, in our study, a regular decrease in blood flow was observed after L-NAME administration. In leptin and L-NAME administered animals, while the hindlimb blood flow decreased over time, this decrease reached a significant level only at the end of the experiment.

Bełtowski et al. (22) compared plasma leptin concentrations in the leptin (single dose, IV, 1000 µg/kg) administered groups at the different time intervals. The highest plasma leptin level was obtained at the 15th minute; after this minute the values began to decrease, tending to approach the control, and reached the control value at the 195th minute. Our samples were taken at the 90th minute, and in parallel with Beltowski's data, the serum leptin levels of the animals in the leptin and L-NAME groups were significantly higher. However, the increase in serum leptin levels was more dramatic in the leptin administered L-NAME group. Bełtowski et al. (25) suggested that NO products increased because of leptin administration, but the leptin values of the groups were close to each other. It was stated that there was no change in plasma leptin level in the group that was administered only L-NAME. In our study, on the

contrary, serum leptin levels were observed to be significantly higher in the L-NAME and leptin-administered L-NAME groups compared to the control group. It has been suggested in different studies that leptin exerts its effects not only through NO, but also through the sympathetic nervous system and endothelial-derived hyperpolarizing factor (25-27). In rodents, there is strong evidence for the role of leptin in an increase in SNA (28). Therefore, we think that leptin levels were also increased in the L-NAME group in order to compensate for the suppression of NO levels and to activate other possible pathways. Indeed, it was observed that inhibition of NO synthesis caused an increase in leptin level as expected.

In vitro studies have demonstrated that leptin stimulates NO production from the endothelium and causes NO-mediated vascular relaxation (10). However, the results of *in vivo* studies are not so conclusive. While some investigators reported the presence of NO in leptin-induced vascular relaxation (15), some investigators found the opposite (23, 29). Frühbeck and Gómez-Ambrosi (30) and Mastronardi et al. (31) observed an increase in plasma concentrations of nitrite/nitrate, the NO metabolites, after leptin administration. However, the source of NO has not been determined exactly. In our study, it was determined that serum nitrite/nitrate levels were significantly decreased in the L-NAME group compared to the control animals, as expected. When leptin was given to L-NAME treated animals, it was observed that there was a significant increase in serum nitrite/nitrate levels compared to that of the L-NAME group, and this value approached the control values. In the study by Bełtowski et al. (22) the highest nitrite/nitrate levels were obtained at the 45th and 75th minutes, respectively, after the administration of leptin (1000 µg/kg). At the 105th minute, this rate dropped to about one third. In our study, the failure to observe the expected increase in the leptin-administered group may be due to the time the blood samples were taken (after the 90th minute) and the dose of leptin administered.

NO is a mediator produced in endothelial cells that regulates vascular tone in a paracrine manner. There are studies showing NO production and NOS expression via leptin and its effects. It has been demonstrated that IV administration of leptin to Wistar albino rats increases serum NO level by 90% (15). In addition, it has been shown that administration of a single dose of leptin increases the level of nitrite/nitrate and cGMP, which is the secondary messenger of NO, in plasma and urine (22). In many different studies using L-NAME, it has been suggested that leptin increases NO production in ventricular cells, which in turn regulates cardiac contraction (32). It has also been indicated that expression of NOS isoforms is made in skeletal muscles of all mammals, including a muscle-specific type, nNOS. Immunohistochemical methods have shown that nNOS is found in the sarcolemma of muscle fibers, eNOS in the microvascular endothelium, and iNOS in leukocytes near muscle fibers and some endothelial cells (12, 33-37). In our study, nNOS and eNOS were also observed in the sarcolemmas of the fibrils, but the distribution ratios vary between groups. The increase in eNOS reactions in animals treated with leptin alone and L-NAME+Leptin may be due to the stimulatory effects of exogenous leptin administration on NO production.

In our study, when eNOS and nNOS distributions in the tissues were evaluated immunohistochemically, the strongest reaction was observed around the nuclei of the cells in the leptin group compared to all other groups. The fact that the reactions are intense around the nuclei suggests that NO plays a role in cellular transcription processes. Carbó et al. (38) suggested that protein synthesis was stimulated in the skeletal muscle, which they isolated 3.5 hours after 100 µg/kg IV leptin administration, and this was accomplished via an unknown mediator by leptin. In our study, the presence of eNOS and nNOS reactions around the nucleus after the leptin-administered groups strengthens the possibility that eNOS and nNOS may be the possible mediators that were not clearly known in the above-mentioned study. It was also determined that the distribution of eNOS in the tissue increased with the application of leptin after L-NAME while nNOS also showed a distribution like the control. The results here indicate that L-NAME may exert its inhibitory effect predominantly through nNOS. The eNOS reaction around the nuclei in the leptin-administrated L-NAME group, similar to the leptin group, suggests that leptin maintains its effect in various cellular events by stimulating eNOS.

In summary, with this study we demonstrated that a single dose IV leptin infusion did not cause any difference in mean arterial pressure with or without NO inhibition. Leptin administration significantly decreased heart rate and blood flow with the inhibition of NO. Both eNOS and nNOS distributions in skeletal muscle and nitrite/nitrate levels in serum samples were increased with leptin treatment which indicates that leptin induces NO production.

CONCLUSION

As a conclusion, the effect of leptin on blood flow, blood pressure and heart rate were investigated in the presence and absence of NO. Although NO synthesis is inhibited by L-NAME, we conclude that leptin partially enhances NO production and leptin uses NO as a mediator in its physiological effects.

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- S.U., C.D.T.; Data Acquisition- S.U., A.K.; Data Analysis/Interpretation- S.U., A.K., C.D.T.; Drafting Manuscript- S.U., C.D.T.; Critical Revision of Manuscript- S.U., C.D.T.; Final Approval and Accountability- S.U., A.K., C.D.T.

Conflict of Interest: Authors declared no conflict of interest.

Financial Disclosure: This work was supported by the Research Fund of Istanbul University [grant number T-744/13092005].

REFERENCES

1. Paz-Filho G, Mastronardi CA, Licinio J. Leptin treatment: facts and expectations. Metabolism 2015; 64(1): 146-56.

2. Zhang Y, Chua S, Jr. Leptin Function and Regulation. Compr Physiol 2017; 8(1): 351-69.
3. Kokta TA, Dodson MV, Gertler A, Hill RA. Intercellular signaling between adipose tissue and muscle tissue. Domest Anim Endocrinol 2004; 27(4): 303-31.
4. Sletten AC, Peterson LR, Schaffer JE. Manifestations and mechanisms of myocardial lipotoxicity in obesity. J Intern Med 2018; 284(5): 478-91.
5. Minokoshi Y, Toda C, Okamoto S. Regulatory role of leptin in glucose and lipid metabolism in skeletal muscle. Indian J Endocrinol Metab 2012; 16(Suppl 3): S562.
6. Ciriello J. Leptin in nucleus of the solitary tract alters the cardiovascular responses to aortic baroreceptor activation. Peptides 2013; 44: 1-7.
7. Smith PM, Ferguson AV. Cardiovascular actions of leptin in the subfornical organ are abolished by diet-induced obesity. J Neuroendocrinol 2012; 24(3): 504-10.
8. Hassan MJM, Bakar NS, Aziz MA, Basah NK, Singh HJ. Leptin-induced increase in blood pressure and markers of endothelial activation during pregnancy in Sprague Dawley rats is prevented by resibufogenin, a marinobufagenin antagonist. Reprod Biol 2020; 20(2): 184-90.
9. Rahmouni K, Jalali A, Morgan DA, Haynes WG. Lack of dilator effect of leptin in the hindlimb vascular bed of conscious rats. Eur J Pharmacol 2005; 518(2-3): 175-81.
10. Winters B, Mo Z, Brooks-Asplund E, Kim S, Shoukas A, Li D, et al. Reduction of obesity, as induced by leptin, reverses endothelial dysfunction in obese (Lepob) mice. J Appl Physiol 2000; 89(6): 2382-90.
11. Robbins RA, Grisham MB. Nitric oxide. Int J Biochem Cell Biol 1997; 29(6): 857-60.
12. Buchwalow IB, Minin EA, Samoilova VE, Boecker W, Wellner M, Schmitz W, et al. Compartmentalization of NO signaling cascade in skeletal muscles. Biochem Biophys Res Commun 2005; 330(2): 615-21.
13. Grozdanovic Z. NO message from muscle. Microsc Res Tech 2001; 55(3): 148-53.
14. Stamler JS, Meissner G. Physiology of nitric oxide in skeletal muscle. Physiol Rev 2001; 81(1): 209-37.
15. Frühbeck G. Pivotal role of nitric oxide in the control of blood pressure after leptin administration. Diabetes 1999; 48(4): 903-8.
16. Fuhrich DG, Lessey BA, Savaris RF. Comparison of HSCORE assessment of endometrial beta3 integrin subunit expression with digital HSCORE using computerized image analysis (ImageJ). Anal Quant Cytopathol Histpathol 2013; 35(4): 210-6.
17. Arneth W, Herold B. Nitrate nitrite determination in sausages after enzymatic reduction. Fleischwirtschaft 1988; 68(6): 761-4.
18. Goshi E, Zhou G, He Q. Nitric oxide detection methods in vitro and in vivo. Med Gas Res 2019; 9(4): 192.
19. Ren J. Leptin and hyperleptinemia-from friend to foe for cardiovascular function. J Endocrinol 2004; 181(1): 1-10.
20. Castracane VD, Henson MC. Leptin. Endocrine Updates: Springer; 2006.
21. Correia MLG, Morgan DA, Sivitz WI, Mark AL, Haynes WG. Leptin acts in the central nervous system to produce dose-dependent changes in arterial pressure. Hypertension 2001; 37(3): 936-42.
22. Bełtowski J, Jochem J, Wójcicka G, Żwirska-Korczala K. Influence of intravenously administered leptin on nitric oxide production, renal hemodynamics and renal function in the rat. Regul Pept 2004; 120(1-3): 59-67.
23. Mitchell JL, Morgan DA, Correia MLG, Mark AL, Sivitz WI, Haynes WG. Does leptin stimulate nitric oxide to oppose the effects of sympathetic activation? Hypertension 2001; 38(5): 1081-6.
24. Dunbar JC, Hu Y, Lu H. Intracerebroventricular leptin increases lumbar and renal sympathetic nerve activity and blood pressure in normal rats. Diabetes 1997; 46(12): 2040-3.
25. Bełtowski J, Wójcicka G, Jamroz-Wiśniewska A. Role of nitric oxide and endothelium-derived hyperpolarizing factor (EDHF) in the regulation of blood pressure by leptin in lean and obese rats. Life Sci 2006; 79(1): 63-71.
26. Kang K-W, Ok M, Lee S-K. Leptin as a key between obesity and cardiovascular disease. Journal of Obesity & Metabolic Syndrome 2020; 29(4): 248.
27. Jamroz-Wiśniewska A, Gertler A, Solomon G, Wood ME, Whiteman M, Bełtowski J. Leptin-induced endothelium-dependent vasorelaxation of peripheral arteries in lean and obese rats: role of nitric oxide and hydrogen sulfide. PLoS One 2014; 9(1): e86744.
28. Simonds SE, Pryor JT, Cowley MA. Does leptin cause an increase in blood pressure in animals and humans? Current opinion in nephrology and hypertension 2017; 26(1): 20-5.
29. Lembo G, Vecchione C, Fratta L, Marino G, Trimarco V, d'Amati G, et al. Leptin induces direct vasodilation through distinct endothelial mechanisms. Diabetes 2000; 49(2): 293-7.
30. Frühbeck G, Gómez-Ambrosi J. Modulation of the leptin-induced white adipose tissue lipolysis by nitric oxide. Cell Signal 2001; 13(11): 827-33.
31. Mastronardi CA, Yu WH, McCann SM. Resting and circadian release of nitric oxide is controlled by leptin in male rats. Proc Natl Acad Sci 2002; 99(8): 5721-6.
32. Sweeney G. Leptin signalling. Cell Signal 2002; 14(8): 655-63.
33. Brevetti LS, Chang DS, Tang GL, Sarkar R, Messina LM. Overexpression of endothelial nitric oxide synthase increases skeletal muscle blood flow and oxygenation in severe rat hind limb ischemia. J Vasc Surg 2003; 38(4): 820-6.
34. Punkt K, Naupert A, Wellner M, Asmussen G, Schmidt C, Buchwalow IB. Nitric oxide synthase II in rat skeletal muscles. Histochem Cell Biol 2002; 118(5): 371-9.
35. Punkt K, Zaitsev S, Park JK, Wellner M, Buchwalow IB. Nitric oxide synthase isoforms I, III and protein kinase-Cθ in skeletal muscle fibres of normal and streptozotocin-induced diabetic rats with and without Ginkgo biloba extract treatment. Histochem J 2001; 33(4): 213-9.
36. Vranić TS, Bobinac D, Jurisić-Erzen D, Muhvić D, Sandri M, Jerković R. Expression of neuronal nitric oxide synthase in fast rat skeletal muscle. Coll Antropol 2002; 26: 183-8.
37. Yan Z, Chen Z. Modulation of nitric oxide synthase isoenzymes in reperfused skeletal muscle. Chin J Traumatol 2000; 3(2): 76-80.
38. Carbó N, Ribas V, Busquets S, Alvarez B, López-Soriano FJ, Argilés JM. Short-term effects of leptin on skeletal muscle protein metabolism in the rat. J Nutr Biochem 2000; 11(9): 431-5.