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CHRONIC VAGUS NERVE STIMULATION REDUCES BODY FAT, BLOOD CHOLESTEROL AND TRIGLYCERIDE LEVELS IN RATS FED A HIGH-FAT DIET

Abstract: *Chronic vagus nerve stimulation reduces body fat, blood cholesterol and triglyceride levels in rats fed a high-fat diet*

There is growing evidence that vagus nerve stimulation (VNS) exerts a suppressive effect on both short- and long-term feeding in animal models. We previously showed that VNS with high-frequency (10 Hz) electrical impulses decreased food intake and body weight in rats. In the present study, we investigated the effect of VNS with a low frequency (1 Hz) on the serum lipid concentrations, feeding behavior and appetite in rats fed a high-fat diet. The levels of appetite-regulating peptides were also assessed.

Adult male Wistar rats were subcutaneously implanted with a microstimulator (MS) and fed a high-fat diet throughout the entire study period (42 days). The left vagus nerve was stimulated subdiaphragmatically by rectangular electrical pulses (10 ms, 200 mV, 1 Hz, 12 h a day) generated by the MS. The daily food intake and body weight were measured each morning. At the end of the experiments, the serum glucose, cholesterol, triglycerides, low-density lipoproteins, high-density lipoproteins, ghrelin, leptin and nesfatin-1 concentrations were measured. The adipose tissue content was evaluated by the assessment of the weight of the epididymal fat pads.

Chronic VNS significantly decreased food intake, body weight gain and epididymal fat pad weight. VNS also lowered the total plasma cholesterol concentrations and triglyceride levels. Finally, the serum concentrations of nesfatin-1 were elevated, leptin levels were decreased, and ghrelin levels remained unchanged after VNS.

The study demonstrates that chronic electrical VNS exerts anorexigenic effects, lowering the blood concentration of lipids. Increased nesfatin-1 levels may contribute to these effects.

Key words: vagus nerve stimulation, high-fat diet, nesfatin-1, cholesterol, triglycerides

INTRODUCTION

Vagus nerve stimulation (VNS) is an alternative therapy to treat epilepsy and depression [1, 2]. It has also been under investigation for the management of various anxiety disorders [2], Alzheimer's disease, migraines [3], fibromyalgia [4], and tinnitus [5]. Moreover, the stimulation of the vagus nerve tends to increase the

production of anti-inflammatory cytokines and thus may lead to the increased rate of survival in experimental sepsis, hemorrhagic shock and ischemia–reperfusion injury models [6–9]. In such vagus nerve manipulations, VNS therapy has been surprisingly shown to induce, at least in some cases, alterations in food intake and weight decreases. These findings have encouraged many researchers to investigate the possible mechanisms of the weight loss after vagus nerve stimulation and to launch it as a possible new method for obesity treatment [10, 11].

Body weight, food intake and body fat content are regulated by multiple factors and controlled by short- and long-term regulation mechanisms. Food transported into the stomach and duodenum activates chemo- and mechanoreceptors, and these signals are transferred *via* the vagus nerve to the hind brain, where they are integrated and play a major role in the short-term regulation, reducing the size of meal consumed [12, 13]. Using extracellular recordings from the vagus nerve, Randich and Cox [14, 15] showed that the vagus conducts "satiety signals" from the jejunum when activated by fatty acid infusion. In the long–term control of food intake, the roles of various other mediators (ghrelin, leptin, nesfatin-1, orexins, neuropeptide Y) and structures (vagal afferent neurons — VAN, arcuate nucleus of vagus nerve — ARC, hypothalamus) must also be considered [13]; however, the vagal afferents play an essential role in such a regulatory system.

Thus, decreased food intake and weight gain in the animal models with vagus nerve stimulation must be considered as the result of the stimulation of brain centers, peripheral actions of vagal stimulation *via* short cholinergic reflexes and the combination of central and peripheral signals [13]. The VNS decreases food intake and body weight gain by signals transmitted from the gut to the brain, leading to the activation of the hypothalamic neurons and resulting in the state of satiety. Because vagal afferents transmit information to the brain not only from activated mechanoreceptors of the gastrointestinal tract but also from duodenal chemoreceptors, hepatic glucoreceptors and osmoreceptors [16], this hypothesis appears to have strong support.

We previously showed that short-term vagus stimulation affects food intake and decreases body weight in rats [17, 18]. Furthermore, Bugajski *et al.* [19] reported decreases in meal size, body weight and epididymal fat pad weight in obese rats. These data were confirmed by our recent work performed in rats fed a high-fat diet. The animals stimulated during a 6-week period with left VNS (10 Hz) revealed significant decreases in food consumption and body weight gain, lower fat accumulation and elevated levels of the anorexigenic peptide nesfatin-1 [20]. Moreover, VNS evoked changes in the nodose ganglia of the vagus nerve, affirming the afferent signal transmission of VNS signals [21]. Simultaneously, Ziomber *et al.* [22] showed that modulation of the left vagus by MS placed in rats with magnetic field exposure led to a decrease in body weight gain in growing animals and that this effect was correlated with a decrease in the leptin serum level. Disappointingly, in humans subjected to VNS, no changes in body weight were

observed [23, 24], or the data were controversial [25, 26]. Thus, the appropriate frequency, amplitude of impulses and character of the stimulation still need to be established in experimental models.

As the left and right vagal trunks feed different parts of the gastrointestinal tract and the contribution of both is important, the decision of which trunks should be stimulated remains controversial. Some previous works from our laboratory [17, 18] demonstrated that bilateral VNS seems to be more effective than unilateral stimulation, but others proved that unilateral stimulation could also be effective. However, to limit the possible side effects on the heart or lungs, we decided to apply chronic VNS by a microstimulator placed on the left vagus nerve. Electrodes were placed close to the gastro-esophageal junction to stimulate the small unmyelinated C fibers and avoid stimulating the fibers that join the trunk from the heart and lungs, as discussed by Val-Laillet *et al.* [27]. We used constant voltage microstimulators implanted for at least 6 weeks and used an intermediate frequency of stimulation at 1 Hz. Vagus nerve stimulation at a high frequency (20–30 Hz) was previously hypothesized to act mainly on the vagal afferents, influencing the brain centers, whereas the low-frequency (less than 5 Hz) VNS targets predominantly vagal efferents, evoking mainly anti-inflammatory effects [28]. Our previous experiments showed that VNS with 10 Hz exerts anorexigenic effects in rats fed a high-fat diet. Consequently, the aim of the current study was to evaluate the effects of chronic left VNS with a low (1 Hz) frequency on the long-term regulation of body weight and food intake in rats fed a high-fat diet. The serum glucose, cholesterol, triglyceride, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) concentrations were also measured. The development of obesity after a high-fat diet in this animal model has been already described and documented [19, 22, 29]. Furthermore, we evaluated the fat compartment of the experimental rats by measuring the epididymal fat pad, which reflects the total body fat mass [30]. We also investigated the blood levels of some appetite-regulating hormones, ghrelin, leptin and nesfatin-1, as they are known to play an important role in both the short- and long-term regulation of food intake.

MATERIAL AND METHODS

ANIMALS

Thirty-two male Wistar rats, housed in individual cages, were used in the experiment. Twenty-four animals were fed a high-fat diet (caloric distribution of the diet: protein 25.1%, fat 38.8%, carbohydrates 36.1%, metabolizable energy 4.34 kcal/g; Bento Kronen Products, Belgium) during the whole experiment, and eight rats were fed with the standard diet (caloric distribution of the standard diet: protein 26.7%, fat 7.9%, carbohydrates 65.4%, metabolizable energy 2.86 kcal/g; Labofeed, Poland).

The temperature was maintained at 23 ± 2°C, and the animals were placed on a 12 : 12 h dark/light cycle. Food and water were provided *ad libitum*. The Jagiellonian University Bioethical Committee approved the care and use of the animals (protocol number — 36/2008).

After 2 weeks of adaptation to the environmental conditions and the fat diet, the rats were starved for 12 hours and were operated under general anesthesia induced with sodium pentobarbital given intraperitoneally (Vetbutal, 0.25 mg/kg, Biowet, Pulawy, Poland). The rats were randomly divided into the following groups: (1) rats with an active microstimulator (MS) connected by electrodes to the left vagal nerve (MS group, n = 8), (2) animals with inactive MS without electrodes on the vagal nerve (sham group, n = 8), (3) intact rats without the MS and electrodes (control group, n = 8), and (4) rats fed the standard diet without the MS and electrodes (standard group, n = 8). The control group of animals (intact rats) was included in the study to eliminate any effects of the surgical procedures on the examined parameters. The group of animals fed the standard diet was included in the study to compare the effects of the high-fat diet on the rats. The sham-operated group served as the most important reference group for the assessment of food intake, body weight and epididymal fat pad weight.

EXPERIMENTAL PROTOCOL

The MS for vagus chronic stimulation (designed by the Institute of Electron Technology, Krakow, Poland) was sealed with silicone (Sylgard® 184 Silicone Elastomer, Dow Corning Co., Midland, MI, USA) placed during the surgery into the subcutaneous pocket. The electrodes of the MS were made from insulated silver wire (A-M Systems, Carlsborg, WA, USA). The unisolated ends of the electrodes were wrapped around the subdiaphragmatic left vagal nerve. The cathode and anode were positioned at a 0.5 cm distance. In the second group, a laparotomy was performed, and the inactive MS was implanted (sham group). In the control group (intact), no surgical manipulations were performed. Food was restored on the day after the operation. After a 1 week recovery period, the rats were placed into cages with electromagnetic field exposure, and the stimulation was started (day 1st).

The animals from the MS and sham groups were placed individually into plastic cages and exposed to the magnetic field. The cages were connected to the generator of sinusoidal waves with an amplifier (Neurostimulator NSE 002, Electron Technology Institute, Krakow, Poland), and a 30 kHz pulsating magnetic field was generated. The magnetic field served as an external source of current in the MS wires connected to the left vagus nerve. The theoretical background for this method has been described by Zaraska *et al.* [31]. The parameters of the magnetic field were set experimentally to match the amplitude, duration and frequency of impulses used in our experiment. The third (control) and fourth (standard) groups of rats were placed into cages outside the magnetic field. The parameters of the impulses generated by MS were based on our previous studies and set as follows:

unipolar rectangular pulses, duration 10 ms, amplitude 200 mV, and frequency 1 Hz. The stimulation of the animals started every day at 6 p.m. and lasted 12 h until 6 a.m. the following morning (dark phase stimulation) during the whole experiment because food intake is predominantly nocturnal in rats and the amount of food consumed during the light phase does not exceed 10% [32].

The daily food intake and body weight were measured each morning during the entire study. The amount of the daily food intake was determined by subtracting the amount of food remaining from the amount given 24 h before. At the end of the experiment (day 42nd), immediately after the stimulation stopped, all the rats (non-fasted) were killed by decapitation and weighed. Both epididymal fat pads, located between the cauda epididymis and the distal extremity of the testis, were dissected from the animal and weighed. The epididymal fat pad/body weight ratio was calculated by dividing the fat pad weight by the total body weight.

BIOCHEMICAL ANALYSIS

Blood samples collected at the end of the experiment were taken into tubes containing aprotinin (0.6 TIU per 1 ml of blood; Sigma-Aldrich, USA), which were left for 30 minutes for clot formation. After centrifugation at 1500 ×*g* for 20 min at 4°C (Megafuge 1.0R, Heraeus Instruments), the serum samples were collected and frozen at –80°C until further analysis. Serum aliquots were prepared from each sample, and the glucose, hepatic enzymes (aspartate and alanine transaminases), triglyceride, total cholesterol, LDL and HDL levels were measured with the chemistry immune-analyzer, Olympus AU 600. Three serum aliquots were prepared, and the ghrelin, leptin, and nesfatin-1 levels were measured by the radioimmunoassay method (RIA), according to the protocols provided by the manufacturer (Phoenix Pharmaceuticals Inc., USA). All measurements were performed in duplicate.

STATISTICAL ANALYSIS

The data are expressed as the means and standard deviation (SD). The results were analyzed by one-way analysis of variance (ANOVA), followed by the post-hoc LSD test, with the STATISTICA 8.0 software package (StatSoft, Tulsa). The statistical significance was set at $P < 0.05$.

RESULTS

FOOD INTAKE AND BODY WEIGHT

In rats fed the high-fat diet, the food intake, body weight and epididymal fat pad weight were significantly increased compared with the animals fed the standard diet (Table 1).

Electrical stimulation of the left vagal nerve reduced the total food intake in the MS group compared with the sham and control groups. The differences were significant (P = 0.02 MS vs. sham; P = 0.04 MS vs. control). No differences between the sham and control groups were observed (P > 0.05; Table 1).

Although the final body weight was not significantly different in the MS group compared with the unstimulated and control animals, the VNS significantly reduced the body weight gain. The mean body weight gain in the MS group was 25.6% of the initial body weight, but it was 30.7% in the sham group and 28.7% in the intact group. Neither the high-fat diet nor VNS influenced the feed conversion efficiency. The amount of the total calorie intake during the experiment was significantly increased in the animals fed the diet rich in fat compared with the rats fed the standard diet.

Table 1

Mean food intake, body weight and epididymal fat pad weight in MS left vagus nerve stimulation (1 Hz), sham operated, control (intact)and standard rats

	Sham	MS-1 Hz	Control	Standard
Food intake during experiment (g)	1025 ± 65	891 ± 89*	1042 ± 78	1210 ± 89
Food intake during experiment (kcal)	4448 ± 282	3867 ± 386*	4522 ± 338	3436 ± 254
Feed conversion efficiency # (kcal/g)	28.1	28.6	30.1	30.9
Initial body weight (g) — day 1	516.5 ± 43,0	526.9 ± 24.4	524.8 ± 64.8	526.5 ± 25.2
	Sham	MS-1 Hz	Control	Standard
Final body weight (g) — day 42	675.0 ± 69.4	662.7 ± 30,0	700.3 ± 67.8	629 ± 22.7
Body weight gain (g)	158.5 ± 27,0	135.9 ± 8.9*	150.5 ± 50.4	111,3 ± 19.5
Body weight gain increment over initial body weight (%)	30.7	25.6*	28.7	20.8
Epididymal fat pad weight — EFP (g)	14.5 ± 3.52	11.6 ± 1.5*	15.0 ± 2.6	11.3 ± 2.6
EFP/body weight ratio (‰)	21.3 ± 3.3	17.6 ± 2.8*	21.3 ± 2.2	17.4 ± 4.4

Asterisks (*) indicate significant differences between the MS-1 Hz group and the sham and control groups.
the feed conversion efficiency was measured as the total food intake expressed in kcal divided by the weight gain for each animal during the experiment [33].

EPIDIDYMAL FAT PAD WEIGHT

The fat pad weight, reflecting the total body fat content, was significantly higher in the sham group than in the MS group (P = 0.036). For the control group, the fat pad weight was also significantly higher than that in the MS group (P = 0.04). The mean epididymal fat pad weight relative to the body weight (fat pad/body weight ratio) was significantly lower (17.6‰) for rats with active MS compared with the sham animals (21.3‰) and the control animals (21.3‰) (Table 1). No differences between the sham and control groups were observed.

BLOOD BIOCHEMICAL ANALYSES

There were no significant differences between the VNS-treated rats and the sham, control, and standard rats with respect to the level of glycemia and the levels of aspartate and alanine transaminases. The high-fat diet significantly influenced the blood lipid composition by elevating the levels of triglycerides, total cholesterol, high-density lipoproteins and low-density lipoproteins (Table 2). VNS considerably reduced the blood triglyceride levels compared with the sham (P = 0.025) and control (P = 0.040) groups (Figures 1 and 2). The total serum cholesterol levels were significantly lower in the rats with active MS than in the sham animals (P = 0.045) and control animals (P = 0.032). The HDL concentrations remained unchanged, whereas VNS slightly, but not significantly, lowered the LDL cholesterol levels.

Table 2

The blood serum levels of glucose, triglycerides, total cholesterol (TC), high-density lipoproteins (HDL), low-density lipoproteins (LDL), and aspartate (AspAT) and alanine (AlAT) transaminases in MS left vagus nerve stimulation (1 Hz), sham operated, control (intact) and standard rats

	Sham	MS-1 Hz	Control	Standard
Glucose (mmol/L)	7.71 ± 0.49	7.72 ± 0.42	8.11 ± 0.69	7.37 ± 1.11
Triglycerides (mmol/L)	2.32 ± 1.30	1.86 ± 0.58 *	2.0 ± 0.34	1.41 ± 0.75
TC (mmol/L)	2.26 ± 0.21	2.04 ± 0.48 *	2.39 ± 0.17	1.89 ± 0.27
HDL (mmol/L)	0.95 ± 0.08	0.97 ± 0.11	1.12 ± 0.10	0.89 ± 0.12
	Sham	MS-1 Hz	Control	Standard
LDL (mmol/L)	0.57 ± 0.21	0.49 ± 0.09	0.57 ± 0.10	0.35 ± 0.23
AspAT (UI/L)	153 ± 19	186 ± 17	160 ± 28	170 ± 53
AlAT (UI/L)	68 ± 10	60 ± 7	65 ± 11	67 ± 17

Asterisks (*) indicate significant differences between the MS-1 Hz group and the sham and control groups.

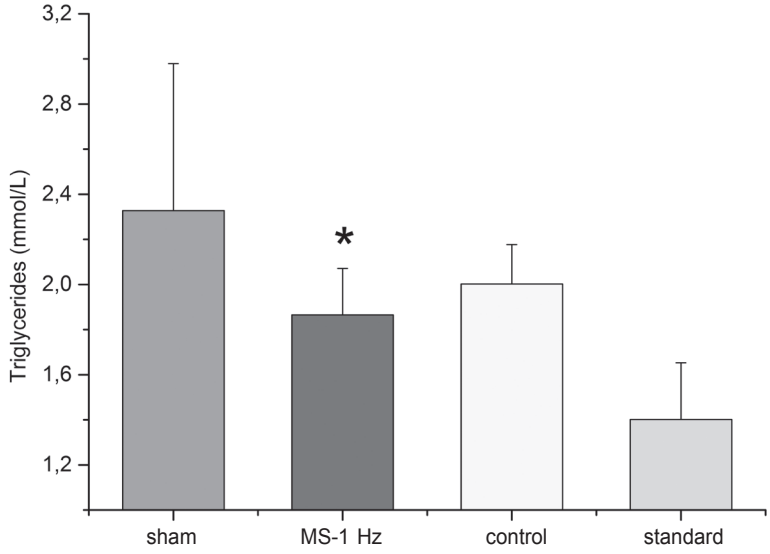

Fig. 1. Triglyceride blood serum concentration after left vagus nerve stimulation (MS-1 Hz) with the frequency of 1 Hz in the sham, control and standard groups (n = 8 for each group). The triglyceride level decreased in the MS-1 Hz group. Data are presented as the mean and standard deviation. *P < 0.05 compared with the sham and control groups

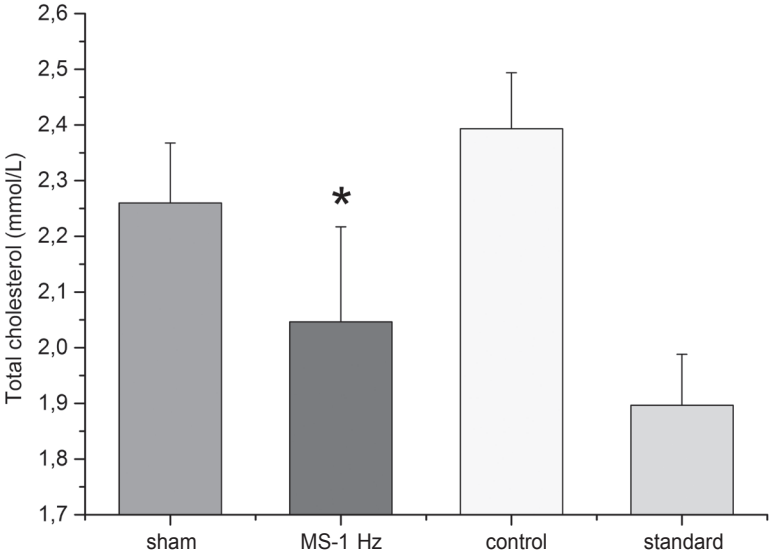

Fig. 2. Total cholesterol blood serum concentration after left vagus nerve stimulation (MS-1 Hz) with the frequency of 1 Hz in the sham, control and standard groups (n = 8 for each group). The cholesterol level significantly decreased in the MS-1 Hz group. Data are presented as the mean and standard deviation. *P < 0.05 compared with the sham and control groups

GHRELIN

There were no significant differences in the ghrelin serum levels between the VNS-treated rats and the sham, control, and standard rats (352.9 ± 72.9 pg/mL in the MS group, 411.7 ± 68.8 pg/mL in the sham group, 357.2 ± 73.1 pg/mL in the control and 352.2 ± 42.2 pg/mL in the standard group). Neither the high-fat diet nor VNS influenced the serum ghrelin level in the experimental animals.

LEPTIN

The high-fat diet elevated the leptin serum levels in the examined groups, especially the control and sham groups. The leptin serum level was significantly decreased in the VNS group compared with the sham (P = 0.007) and control (P = 0.013) groups (9.274 ± 2.39 ng/mL in the stimulated group, 11.5 ± 1.82 ng/mL in the sham group, 13.3 ± 3.66 ng/mL in the control and 8.73 ± 1.3 ng/mL in the standard group; Figure 3). No differences between the sham and control groups were found.

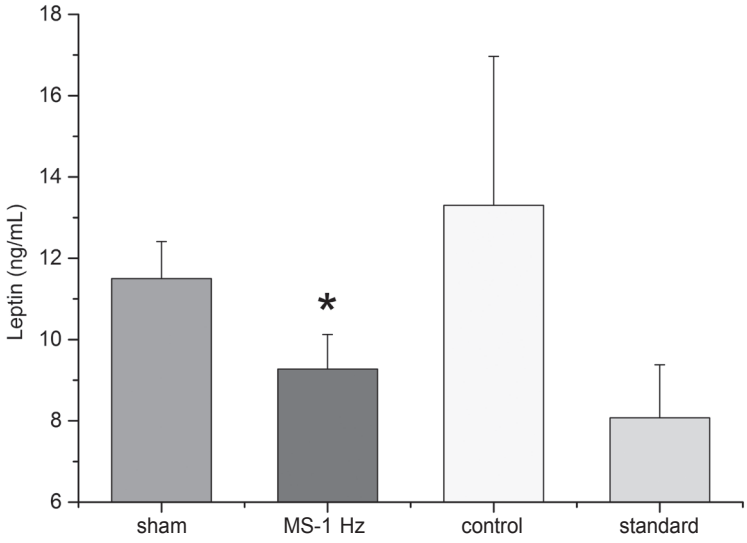

Fig. 3. Leptin serum concentration after left vagus nerve stimulation (MS-1 Hz) with the frequency of 1 Hz in the sham, control and standard groups (n = 8 for each group). The leptin level significantly decreased in the MS-1 Hz group. Data are presented as the mean and standard deviation.
*P < 0.05 compared with the sham and control groups

The high-fat diet lowered the nesfatin-1 serum levels in the examined animals. Vagus nerve stimulation significantly increased the nesfatin-1 serum level compared with the sham (P = 0.009) and control (P = 0.01) groups (990.1 ± 221 pg/mL in the stimulated group, 784.5 ± 273.4 pg/mL in the sham group, 766.25 ± 146.8 pg/mL in the control and 1026.3 ± 219.1 pg/mL in the standard group; Figure 4). No differences were found between the sham and control groups.

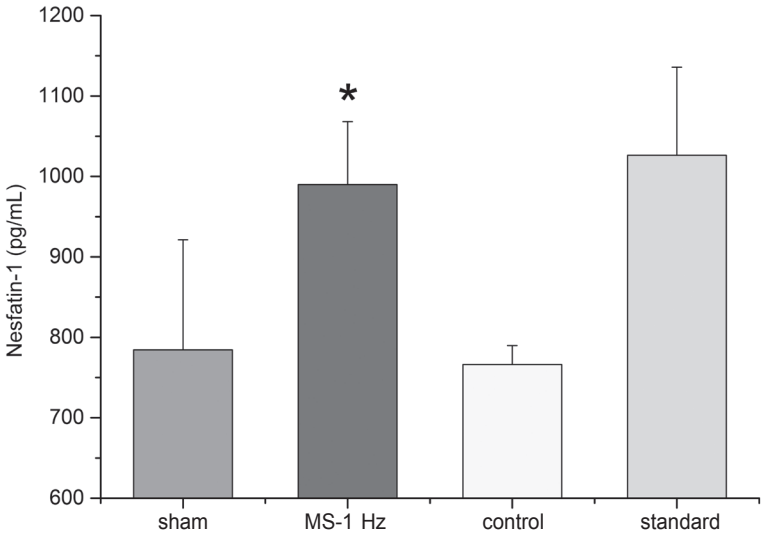

Fig. 4. Nesfatin-1 serum concentration after left vagus nerve stimulation (MS-1 Hz) with the frequency of 1 Hz in the sham, control and standard groups (n = 8 for each group). The nesfatin-1 level significantly increased in the MS-1 Hz group. Data are presented as the mean and standard deviation. *P < 0.05 compared with the sham and control groups

DISCUSSION

Vagus nerve stimulation is a relatively new method adopted for obesity treatment. So far, the exact mechanism of the reduction in body weight has remained unclear. This animal study was performed using a high-fat diet because obesity induced by high-fat diet mimics obesity in humans and is widely considered an appropriate model for studying dietary obesity [34–36). However, the use of such a diet evokes significant changes in the appetite regulating-peptide levels. A high-fat diet increases, but fasting decreases, the nesfatin-1 protein levels and secretion by adipose tissue, and a positive correlation between the body mass index and circulating nesfatin-1 levels in humans has been reported [37, 38]. As for leptin, a short-term, high-fat diet lowers the circulating leptin concentration

[39], whereas in animals fed a high-fat diet for a long time, a significant increase in the leptin levels is present [40, 41]. The ghrelin concentration decreases after feeding with diets rich in fat [40, 42]. Thus, the experimental results may differ as they depend on the animal model applied, and the results should be considered with caution.

We showed previously that low frequency VNS affects the short-term volume regulation of food intake and decreases the body weight in rats [17, 18], but these experiments were performed in rats fed the standard diet. Furthermore, we reported a significant decrease in body weight and food consumption after left VNS with a 10 Hz frequency in rats fed a high-fat diet [20]. Those data encouraged us to examine whether such effects could be evoked by VNS with a lower frequency (1 Hz). Our hypothesis appeared to be correct, although the 1-Hz stimulation seems to be less effective than VNS with 10 Hz. There were significant decreases in the food intake, body weight gain and epididymal fat pad weight in animals treated with chronic vagus nerve stimulation with the microstimulator set at 1 Hz compared with the sham and control animals. We also examined the effects of VNS on the feed conversion efficiency. The VNS-treated rats required an intake of approximately 30 kcal to gain 1 g of weight. No differences were found among the groups of animals used in our experiment. Conversely, Banni *et al.* [33] observed a significantly decreased feed conversion efficiency by chronic VNS, suggesting that peripheral mechanisms may be involved in weight loss after VNS. Our data did not support their conclusions.

Most of the VNS studies performed on rats showed either a decrease in body weight gain or weight loss [19, 22, 29]. Similar observations were reported in growing pigs by Matyja *et al.* [43], Val-Laillet *et al.* [27], and Sobocki *et al.* [44] and in rabbits by Sobocki *et al.* [45]. Some studies revealed other effects of VNS: activation of different brain structures [46], decrease in plasma insulin growth factor — 1 (IGF-1) concentration [44], altered myoelectric activity [43] or increase in gastric emptying and increase in the amplitude of gastric contraction [19]. The effects of vagus nerve stimulation on body weight and food consumption observed in several animal models indicate that this vagal manipulation may evoke satiety signals that are normally transmitted toward the brain centers controlling appetite, body weight and adipose tissue accumulation. The use of different models of stimulation (time, voltage, and species of animals) limits the benefits of these results. However, this growing knowledge of VNS from basic studies allowed some pilot clinical studies to be started [10, 11].

In long-term VNS (0.05 Hz, 102 days) in obese rats fed a high-fat diet performed by Bugajski *et al.* [19], a decrease in weight loss resulting from diminished food intake and the reduction of the body fat compartment, as measured by the fat pad mass, were reported. Similar results published by Ziomber *et al.* [22] in growing rats, using a different frequency of stimulation (0.1–1.0 Hz) and an amplitude ranging from 50 to 200 mV, resulting in a decreased body weight gain of the rats

examined. An interesting study concerning the relationship between vagus stimulation and appetite was published by Val-Laillet *et al.* [27]. The authors performed bilateral VNS with current stimulators (2 mA, 30 Hz, 500 μs pulse, ON 30 s, OFF 5 min) on obese mini-pigs. The VNS animals did not significantly gain weight compared with the sham animals, food consumption was decreased by 18% in the VNS mini-pigs, and sweet-food consumption was also diminished compared with the sham animals; these effects lasted for more than 14 weeks. It should be mentioned here that Val-Laillet used current stimulation instead of constant voltage stimulation to avoid possible, additional voltage drops, decreasing the current at the nerve level and potential insufficiency for stimulation [47]. However, our data showed that VNS increases the c-Fos positive neurons in the nodose ganglion of the left vagus nerve (stimulated) [21], in the nucleus of the solitary tract [20] and in the arcuate nucleus of the hypothalamus (yet unpublished results). Moreover, the study by Helmers *et al.* [48] proved that the range of output current settings between 0.75 and 1.75 mA with pulse width settings of 250 or 500 μs may result in optimal stimulation. Thus, we may conclude that constant voltage stimulation with the parameters used in our model is sufficient and effective.

Visceral fat or abdominal fat is located inside the abdominal cavity, packed between the organs. Visceral fat is composed of several adipose depots, including mesenteric, epididymal white adipose tissue and perirenal depots. Visceral fat is considered to be adipose tissue, whereas subcutaneous fat is not considered as such. An excess of visceral fat is known to be related to the grade of obesity. In rodents, one of the particular regions where visceral fat accumulates is the epididymal fat pad. The epididymal fat pads represent only a small part of the total body weight, but previous studies showed that the epididymal fat pad weight calculated as a proportion of the total body weight is highly correlated with the total body fat in mice and rats [30]. In our experiment, the body composition was altered by vagus nerve stimulation: the epididymal fat pad mass relative to body weight was significantly lower in rats with VNS compared with the control and sham animals. This observation correlated with data from previous studies [19, 20, 29]. Such a decrease in fat depots corresponds to the lowered food consumption and diminished weight gain caused by VNS. Furthermore, the blood cholesterol and triglyceride levels were significantly reduced. These effects were caused, in our opinion, by a reduced calorie intake. Other peripheral effects of VNS, such as increased lipid metabolism or excessive energy spending, are not excluded but are unlikely because VNS did not influence the feed conversion efficiency. However, VNS opens new possibilities for the treatment of excessive blood lipid contents connected to obesity.

The significant decrease in the fat content in the VNS-treated rats is related to the changes in appetite and regulating peptide concentrations, especially leptin. Most of the published reports are consistent with the correlation of the leptin expression and its release with the amount of body fat and number of adipocytes,

and its blood concentration mainly depends upon the adipose tissue content as this peptide is generated by fat cells [49, 50]. Indeed, in our experiment, we observed a significant leptin decrease after VNS, correlating with a decreased weight gain and reduction in food consumption. It is well documented that leptin is involved in the regulation of feeding behavior and energy expenditure [50, 51]. Leptin decreases food intake under the physiological condition. However, obesity in humans is generally associated with leptin resistance, which is progressively established during a hypercaloric diet and accompanied by a progressive increase in the serum leptin levels [49], and the leptin level in this group remains high. It should also be mentioned here that vagus nerve circuits play a role in leptin secretion. In 2003, Cigaina *et al.* reported that a decrease in the leptin level significantly correlated with weight loss in patients with gastric pacing [52]. Wang *et al.* [53] did find any changes in the leptin level either after vagus nerve dissection or after gastric bypass in rats. However, the leptin concentration was significantly diminished and correlated with weight loss in both studies. Recently, De Lartigue *et al.* [54] demonstrated that diet-induced obesity led to the development of leptin resistance in vagal afferent neurons (VAN) and that leptin signaling in VAN is required for appropriate cholecystokinin signaling and satiation, limiting meal size and duration [55]. In previous studies [20, 22], we reported that the rats with vagus nerve stimulation have decreased plasma leptin concentrations. If VNS reduces food intake by leptin release, we would expect an increase in the leptin concentration, but we have obtained the opposite results. We conclude that the observed decrease in the leptin concentration after VNS is associated with the diminished body weight gain and loss of the adipose tissue content rather than with the vagus nerve manipulations.

Ghrelin, a peptide produced predominantly in the stomach and duodenum, evokes an orexigenic effect, increasing food intake in both animals and humans [56]. The mechanism of action involves the vagus nerve and activation of neurons in the hypothalamus [57, 58]. The ghrelin level increases before a meal and sharply falls after the start of the meal, suggesting a possible role in the induction of a meal [59]. Moreover, the systemic ghrelin level is negatively correlated with body weight and adiposity and increases with weight loss, whereas weight gain leads to decreased systemic ghrelin levels [60]. Ghrelin administration leads to weight gain in experimental animals by stimulating food intake, decreasing energy expenditures and spontaneous activity and promoting adipogenesis [61]. Obese individuals have decreased ghrelin levels [62], which can be normalized after diet-induced weight loss [63].

We previously reported an increase in the ghrelin serum concentration after electrical vagus nerve stimulation with the frequency at 10 Hz [20]. Data from the current study, however, did not confirm such an observation. Work by Cigaina *et al.* [64] and Gallas *et al.* [65] described an increase in the ghrelin plasma concentration after gastric stimulation. Although gastric pacing does not directly

stimulate the vagus nerve, the authors postulated the involvement of the vagus nerve in ghrelin release after gastric pacing. In contrast, Li *et al.* [66] revealed that gastric electrical stimulation in diet-induced obese rats reduced food intake, body weight and gastric emptying but that the peripheral modulation of plasma ghrelin level was not related to the stimulation effects. Although such results are not entirely conclusive, we may suggest that the observed reduction in the body weight and inhibition of food intake after VNS are due to other mechanisms rather than ghrelin mediators or metabolic pathways.

Nesfatin-1 is an 82 amino acid anorexigenic peptide derived from its precursor protein, NEFA/nucleobindin2 (NUCB2), which was identified by Oh [67]. Nesfatin-1 administered into the third cerebral ventricle significantly reduces food intake in rats. Twenty-four-hour fasting effectively reduces the NUCB2 expression in the paraventricular nucleus (PVN) [68], and nesfatin-1 immunopositive neurons in the PVN are activated by refeeding [69], indicating that nesfatin-1 plays an important role as a regulator of food intake. Nesfatin/NUCB2-immunopositive neurons are also located in the arcuate nucleus (ARC) and co-localize with proopiomelanocortin (POMC) and CART (Cocaine-and-Amphetamine Responsive Transcript) neurons [67, 68]. Nesfatin-1 crosses the blood–brain barrier in both the blood to brain and brain to blood directions [70, 71]. As reported by Shimizu *et al.* [72], nesfatin-1 given peripherally (by intraperitoneal injection) diminished the food intake. The study by Stengel *et al.* showed that nesfatin-1-immunopositive cells are present in the rat gastric mucosa and that most of the nesfatin-1-immunopositive cells also co-expressed ghrelin [73]. Moreover, the down-regulation of NUCB2 mRNA in gastric endocrine cells after 24-h fasting proved that nesfatin/NUCB2 gene expression might be regulated by the nutritional status. That mechanism was elucidated by Li *et al.*, who examined nesfatin-1 level fluctuations in diabetes mellitus patients after oral glucose ingestion [74]. He observed the reduction in the fasting nesfatin-1 concentration, which in his opinion may be one of the appetite-related hormones involved in diabetic hyperphagia. However, glucose ingestion did not affect the plasma nesfatin-1 level, suggesting that gastric chemosensation is not sufficient for the nesfatin-1 response. Tan at al., in a study on obese patients, found that the increase in the nesfatin-1 level correlated positively with body mass index (BMI) and fat mass in obesity and also observed that plasma nesfatin-1 was negatively correlated with nesfatin-1 in the cerebrospinal fluid, suggesting nesfatin-1 resistance in obese subjects [75].

In our experiment, the nesfatin-1 level was significantly elevated after vagus nerve stimulation. Thus, we suggest that vagus nerve stimulation increases the nesfatin-1 serum concentration, causing the anorexigenic effects observed in our study. The data from studies by Shimizu *et al.* partially support our hypothesis, as intraperitoneally administered M30, the active mid-segment of nesfatin-1, induced anorexia *via* the vagus nerve in mice whereas this effect was abolished in capsaicin-pretreated animals [76].

The present study demonstrates that food intake and body weight gain are decreased by long-term VNS at 1 Hz in rats fed a high-fat diet, leading to decreased levels of cholesterol and triglycerides. These data support our hypothesis that the electrical signals generated by a microstimulator and conducted by vagal afferents as satiety signals can modify the central regulation of body weight, food intake and body fat content. VNS causes changes in the nesfatin-1 levels, which may contribute to observed reductions in body weight and fat. Vagus nerve neuromodulation opens new possibilities for obesity management.

CONFLICT OF INTEREST STATEMENT

None declared.

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