Original research paper

Hypothalamic expression of anorexigenic and orexigenic hormone receptors in obese females *Neotomodon alstoni:* Effect of fasting

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Obesity is a world problem that requires a better understanding of its physiological and genetic basis, as well as the mechanisms by which the hypothalamus controls feeding behavior. The volcano mouse *Neotomodon alstoni* develops obesity in captivity when fed with regular chow diet, providing a novel model for the study of obesity. Females develop obesity more often than males; therefore, in this study, we analysed in females, in proestrous lean and obese, the differences in hypothalamus expression of receptors for leptin, ghrelin (growth hormone secretagogue receptor GHS-R), and VPAC, and correlates for plasma levels of total ghrelin. The main comparisons are between mice fed *ad libitum* and mice after 24 hours of fasting. Mice above 65 g body weight were considered obese, based on behavioral and physiological parameters such as food intake, plasma free fatty acids, and glucose tolerance. Hypothalamic tissue from obese and lean mice was analysed by western blot. Our results indicate that after *ad libitum* food access, obese mice show no significant differences in hypothalamic leptin receptors, but a significant increase of 60% in the GHS-R, and a nearly 62% decrease in VPAC2 was noted. After a 24-hour fast, plasma ghrelin increased nearly two fold in both lean and obese mice; increases of hypothalamic leptin receptors and GHS-R were also noted, while VPAC2 did not change significantly; levels of plasma free fatty acids were 50% less after fasting in obese than in lean animals. Our results indicate that in obese *N. alstoni* mice, the levels of orexigenic receptors in the hypothalamus correlate with overfeeding, and the fact that lean and obese females respond in different ways to a metabolic demand such as a 24-hour fast.

Keywords: Obesity, Hypothalamus, Ghrelin, Leptin, VPAC receptors, *Neotomodon alstoni*

Introduction

Obesity is currently a major health problem that is increasing worldwide. The prevalence of obesity is a consequence of interactions among multiple genes and lifestyle factors. For the past several years, various approaches have been used to identify the physiological and genetic bases of obesity; one of the strongest pieces of evidence about the development of obesity involves modifications of hormones and neurotransmitters that regulate appetite, energy expenditure, and the sensations of satiety in specific centers within the brain.[1,2] In order to understand the causes and consequences of this pathophysiological condition, several animal models of obesity have been used; most of them require nutritional or genetic manipulations such as high fat or hypercaloric diets.[3–5] Models in which animals develop obesity with standard rodent diets are particularly useful to understand whether the mechanisms altered in obesity occur regardless of the animal model used, or if they are a consequence of the specific method used to induce obesity.

When the Mexican volcano mouse *Neotomodon alstoni* is kept in captivity, *circa* 50% of individuals develop obesity.[6] Body weight (BW) gain, occurs earlier in females than in males. In this study, mice were considered obese based on BW above 65 g, or lean with BW below 45 g. The Lee index also differed between these BW groups.[6] Obese females have

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remarkably high levels of plasma leptin, as well as an increased concentration of triacylglycerides and insulin. Significant decreases in amplitude on the circadian rhythm of locomotor activity are more evident in obese females than in males, also a tendency to present overfeeding; these characteristics suggest the existence of a sex-related factor that may facilitate obesity in this species. The aforementioned alterations suggest that, in obese females of *N. alstoni*, a differential regulation of orexigenic and anorexigenic hormones may occur in the hypothalamus, where most of the areas that regulate food intake are located.

Food intake is modulated by multiple endocrine signals that are integrated in the hypothalamus. Ghrelin is an orexigenic hormone from the periphery (released mainly by the oxyntic cells within the stomach) that stimulates food intake.[7] Plasma ghrelin is elevated under conditions of physiological demand such as fasting.[8] Ghrelin up-regulates food intake and lowers energy expenditure, mainly through hypothalamic mediators, acting both centrally and peripherally. The growth hormone secretagogue receptor (GHS-R) is a component of the ghrelin signaling pathway and is involved in mediating its pleiotropic effects.[9] Ghrelin stimulates growth hormone secretion through activation of GHS-R, and it is also related to appetite, food intake, weight gain, and gastric emptying. In addition, ghrelin has been implicated in pathological weight gain, obesity, type 2 diabetes, and metabolic syndrome.[10]

Leptin is mainly secreted by adipose tissue, and it is recognized as an endocrine signal related to the satiety process. It regulates energy balance through central circuits, including pro-opiomelanocortin neurons[11] that control food intake and energy expenditure. Leptin deficiency leads to chronic hyperphagia and massive obesity. However, in most obese humans, leptin levels are chronically elevated.[12] Persistent hyperleptinemia seems to be associated with leptin resistance, where signaling through the leptin receptor is reduced.[13] Circulating leptin concentrations are correlated with indices of body-fat content. Both leptin and ghrelin are endocrine signals out of balance in conditions of obesity.[14,15]

Pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) are also involved in food intake in various species.[16] Both ligands act through common receptors such as VPACR-1 and VPACR-2, also known as PACAP/VIP mutual receptors; VIP and PACAP are actively expressed in the hypothalamus and participate in the control of appetite and energy homeostasis.[17] Activated VPACR-1 and VPACR-2 increase intracellular calcium signaling in hypothalamic neurons containing neuropeptide Y.[18] Plasma VIP is notably reduced in obese women compared to lean women,[19] suggesting that the VIP signaling pathway through VPAC2 receptors might be playing an important role in the development of overweight and eventually obesity.[20]

This study focuses on comparing lean and obese *N. alstoni* females for the expression of the leptin, ghrelin, and VPAC2 receptors in the hypothalamus as well as their plasma ghrelin concentration under two conditions: feeding *ad libitum* and 24-hour fasting. Our results indicate that in obese females, overfeeding correlates with increased plasma ghrelin and hypothalamic GHS-R receptors and as well as with a decrease in VPAC2 receptors; no changes in leptin receptors were noted. In response to a 24-hour fast, obese and lean mice increase plasma ghrelin as well as the hypothalamic GHS-R and leptin receptor. These results provide initial evidence about the mechanisms that may be involved in the development of obesity in this species and may help to better understand obesity and its physio-pathology in a nontraditional animal model, the volcano mouse *N. alstoni*.

Methods

Animals and housing

N. alstoni were born and raised in the *vivarium* facilities of the Facultad de Ciencias (UNAM) as the F1 generation from wild-captured breeders. Commercial diet for laboratory rodents (Rodent Lab Chow 5001, Purina® Inc., MO, USA) and tap water were provided *ad libitum.* Room temperature was between 18 and 23°C, and light–dark cycles were set at 12:12 hours (photophase: 06:00–18:00, 200–250 lx). By the seventh month mice were separated by BW into two groups: lean (BW 43 ± 2 g) and obese (BW 70 ± 2 g). Mice with BW between 45 and 65 g were not included in this study. Obese mice above 65 g BW differ physiologically and behaviorally from lean mice, as previously mentioned.[6] Food intake was determined for 10 consecutive days by weighing chow pellets after lights-on and lights-off. The mice used in this study were in proestrous phase, determined by means of vaginal inspection, using cotton swabs and saline examined by microscopy to determine estrous phase. Tissue or blood samples were always collected between 12:00 and 15:00 hours during the photophase. All procedures described in this study were carried out in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and the Official Mexican Regulation for Experimentation in Animals (NOM-062-ZOO-1999). Lean and obese mice were compared in two feeding conditions: *ad libitum* and after a 24-hour fast.

Metabolite and hormone assay

Using heparinized capillary tubes, blood samples were taken from the retro orbital sinus of mice anesthetized

in a closed chamber with volatilized isofluorane (Dorin, Halocarbon Labs, NJ, USA) until unconscious then immediately removed for recovery; the next sample was collected 30 minutes later. After decapitation to collect brains, blood samples were also taken. Samples were centrifuged (3500 rpm/5 minutes), and then 400 µl of plasma was kept at −70°C until used. The analytical assays were performed according to the manufacturer's instructions (Mouse Ghrelin kit EZR GRT-91K Millipore, Billerica, MA, USA, Free Fatty Acids Kit, Bio Vision Research Products, San Francisco, CA, USA). All reactions were measured by means of a microplate reader (Spectramax 190, Molecular Devices, CA, USA). Glucose tolerance tests on five obese and five lean mice were performed as indicated elsewhere;[21] samples were taken from isofluorane-anesthetized animals that had been deprived of food for 4 hours. Blood samples were taken before (time 0) and 30, 60, 90, and 120 minutes after glucose intraperitoneal administration (solution of 50% glucose at a dose of 2.5 g glucose/kg/BW); glucose was quantified using reactive bands and a glucometer (Accutrend® GCT, Roche Diagnostics, IN, USA).

Western blot

Mice in proestrous were sacrificed by decapitation at noon, either *ad libitum* or after 24 hours fasting (four lean and four obese); their brains were dissected, frozen in dry ice, and then kept at −70°C prior to analysis. The hypothalamus was identified in frozen brains under a stereoscopic microscope, using the anterior limit of the optic chiasm, dorsal third ventricle, and the caudal end of medial part of mammillary nucleus as anatomic references.[22] The brain tissue was removed and homogenized in four volumes of RIPA buffer (Cell Signaling, MA, USA) supplemented with complete protease inhibitors (Roche Diagnostics, IN, USA) using a Potter-Elvehjem teflon-glass homogenizer (70 rpm for 15–20 seconds). The hypothalamic homogenate was centrifuged at 12 000 rpm for 20 minutes at 4°C, and the supernatant was carefully recovered, aliquoted, and stored at −70°C until used. Total protein concentration was determined by the Bradford assay (Bio-Rad, CA, USA). The western blot was performed as indicated elsewhere.[23] Briefly, equal amounts of protein were mixed with 2X Laemmli sample buffer and incubated at 80°C for 5 minutes. The protein was separated on a 10% polyacrylamide gel, electroblotted onto a nitrocellulose membrane (Bio-Rad), blocked for 1 hour in 5% non-fat milk (Bio-Rad), and then incubated overnight at 4°C with anti-VPAC2 antibody (1:500 dilution; sc-30020R, Santa Cruz Biotech, CA, USA); anti-ghrelin receptor (GHS-R) antibody (1:500 dilution; ab85104, Abcam, Cambridge, MA, USA); anti-leptin receptor antibody (1:400 dilution; ab5593, Abcam), and anti-tubulin (1:1000 dilution; ab15246, Abcam). Membranes were washed and incubated for 2 hours with alkaline phosphatase-conjugated rabbit anti-goat secondary antibody (1:5000 dilution, Santa Cruz), and the bands were visualized using the alkaline phosphatase conjugate substrate kit (Bio-Rad) according to the manufacturer's instructions. Blots were digitalized and analysed with the Image J® software (version 1.38, NIH, MD, USA), normalized to the tubulin (~55 kDa) signal used as a loading control, and compared to the results of lean mice fed *ad libitum*. Molecular weights of proteins considered in the present comparisons were: the short form of leptin (~100 kDa), GHS-R (~43 kDa), and VPAC2 (~65 kDa).

Statistical analysis

The results are expressed as mean ± SEM unless otherwise indicated. Statistical analysis was done using the Statistica software (StatSoft Inc., OK, USA). Lean vs. obese groups were analysed by a Student's *t*-test, and (*) indicates a significant difference between groups or feeding conditions ($p < 0.05$).

Results

Body weight increase and food intake

Fig. 1A shows the average growth curves (±SD) of two groups of females ($n = 10$; followed for 4 months after weaning) in which the rate of BW gain diverged among groups. Obese animals (filled circles) can be distinguished from the tenth week. By the seventh month (week 24) BW differences were found ($p < 0.05$, $t = 15.71$). Daily food intake for lean and obese is shown in Fig. 1B ($n = 10$). Obese female mice ate about 30% more than lean ones at night (5.94 ± 0.33 g and 4.56 ± 0.22 g, respectively), but no differences were noted in daytime food consumption.

Free fatty acid (FFA) concentrations are shown in Fig. 2A. Mice fed *ad libitum* did not show differences

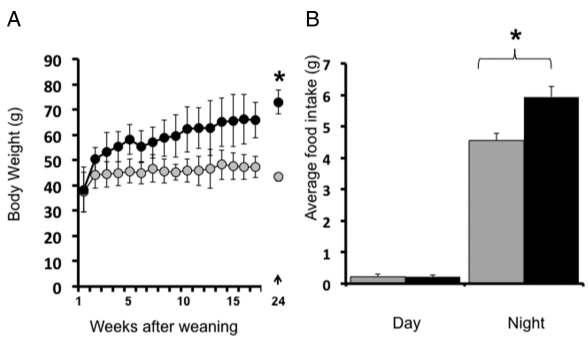

Figure 1 (A) Average (±SD) body weight after weaning over the course of 16 weeks. Obese (black circles) and lean (gray circles) mice can be distinguished. Animals used in this study were at week 24 after weaning (*$p < 0.05$). (B) The daily average meal intake (±SEM) in 7-month-old adults was evaluated for 10 days in obese (black bars) and lean mice (gray bars), indicating hyperphagia at night in obese mice.

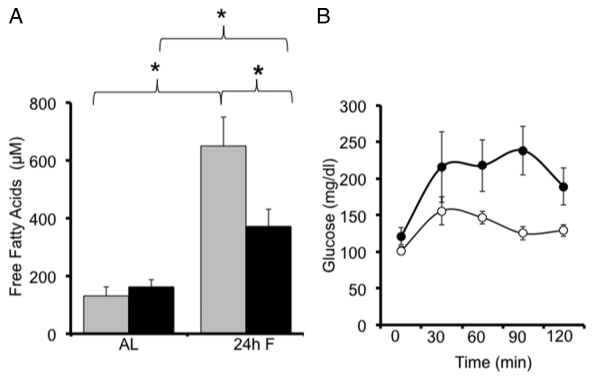

Figure 2 (A) Free fatty acids in plasma in *ad libitum* (AL) conditions and after 24 hours of fasting (24-hour F). A larger mobilization of FFA after 24-hour F is observed in lean (gray bars) than in obese mice (black bars). (B) A glucose tolerance test was performed in 4-hour fasted, obese (black circles) and lean mice (white circles). The area under the curve is larger in obese (*$p < 0.05$) than in lean mice.

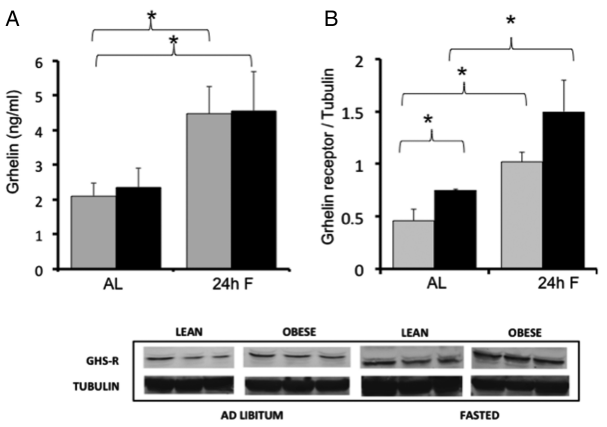

Figure 3 (A) Average plasma ghrelin (ng/ml) in obese (black bars) and lean mice (gray bars). A 24-hour fast increased circulating ghrelin by almost two-fold in both lean and obese mice. (B) The hypothalamic ghrelin receptor (GSH-R) content is higher in obese mice under both conditions tested. Representative immunoblots are shown in the box below.

between lean and obese animals (131 ± 13 μM vs. 162 ± 10 μM, respectively). Fasted lean mice, however, had an almost two-fold higher ($p < 0.05$, $t = 2.46$) concentration of FFA (650 ± 30 μM) than fasted obese ones (371 ± 25 μM). In lean mice the FFA concentration increased about four-fold between groups fed *ad libitum* and after a 24-hour fast ($p < 0.001$, $t = 5.57$), suggesting a greater mobilization of FFA during fasting than in the obese mice.

The glucose tolerance test indicated that glucose levels in obese mice did not return to the initial concentration within 2 hours after the glucose intraperitoneal injection in 4-hour fasted mice (Fig. 2B), indicating glucose intolerance. The area under the curve was about 170% larger in obese than in lean females (10270 ± 2038 vs. 3753 ± 768 relative units, respectively; $p < 0.05$, $t = 3.205$).

Plasma ghrelin and hypothalamic ghrelin receptor

Total ghrelin (Fig. 3A) in plasma from mice fed *ad libitum* was no different in obese (2.36 ± 0.56 ng/ml) and in lean mice (2.11 ± 0.36 ng/ml); after a 24-hour fast, a nearly 100% increase was observed (lean 4.48 ± 0.79 ng/ml; obese 4.56 ± 1.14 ng/ml) relative to *ad libitum* conditions ($p < 0.05$, $t = 2.7$; Fig. 3A). Hypothalamic ghrelin receptor (GHS-R) evaluated by BW (Fig. 3B) was 63% higher in obese (0.75 ± 0.15 relative to tubulin) than in lean mice (0.46 ± 0.1) fed *ad libitum* ($p < 0.05$); however, in the 24-hour fasting condition, nearly 100% more GHS-R protein was detected in the hypothalamus of both lean and obese animals ($p < 0.05$; lean 1.02 ± 0.09; obese 1.5 ± 0.3 relative to tubulin). Representative immunoblots for GHS-R and tubulin are shown below the graph.

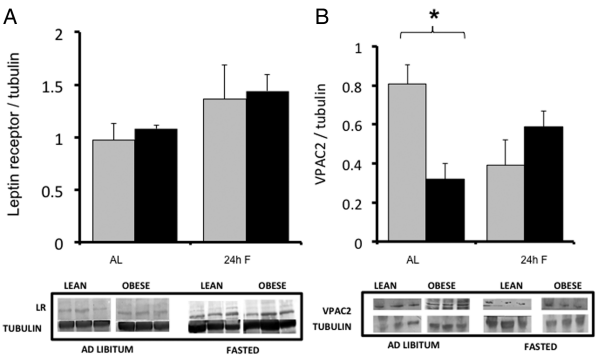

Figure 4 (A) There was no significant difference in the content of leptin receptor between lean and obese mice, but a tendency to increase was noted after 24-hour fasting. (B) Lower amounts of the VPAC2 receptor are present in the hypothalamus of obese than of lean mice. Lean (gray bars) and obese mice (black bars) (*$p < 0.05$). Representative immunoblots are shown.

Hypothalamic leptin and VPAC2 receptors

Figs. 4A and 4B show the average of relative concentration for the short form of the hypothalamic leptin receptor (~100 kDa) and VPAC2, respectively, as detected by western blot. Lean and obese mice had similar amount of leptin receptor in *ad libitum* conditions (0.97 ± 0.16; $n = 4$) and an increase was noted after 24-hour fasting, either in lean (1.36 ± 0.16) and obese mice (1.44 ± 0.15, respectively; $n = 4$ each); however, no statistical differences were noted.

Immunoblots (Fig. 4B) of mice fed *ad libitum* indicated a significantly lower amount of the VPAC2 receptor ($p < 0.05$) in obese than in lean mice (0.32 ± 0.08 vs. 0.81 ± 0.10, respectively; $n = 4$). In mice fasted for 24 hours, the difference between groups was reduced (0.59 ± 0.07 vs. 0.39 ± 0.13 for obese and lean mice, respectively; $n = 4$). A tendency

toward increased VPAC2 was observed in fasted obese mice, whereas there was a decreasing tendency in fasted lean mice, as compared with *ad libitum* conditions.

Discussion

The volcano mouse *N. alstoni* develops obesity after 4–7 months in *vivarium* conditions. Even when all animals were on the same feeding regimen, some remain lean while others become obese. This fact allows us to compare lean and spontaneous obese mice that were fed the regular rodent chow diet. Obese females are distinguished by gaining BW earlier than males. When the difference in BW become statistically significant, lean mice are more active than obese mice, and a tendency to hyperphagy was observed in obese females, leading to an even faster BW gain.[6] In this study, we studied 7-month-old mice; obese mice can be differentiated beginning in the third month after weaning, indicating that this phenomenon also exists in animals born and raised in the *vivarium*. Glucose tolerance tests (Fig. 1B) indicated that obese females showed a slower withdrawal of blood glucose than lean ones, suggesting possible insulin resistance. In previous studies, obese mice had shown similar glucose intolerance,[21] which is associated with hyperleptinemia. It is known that obesity and obesity-related changes in plasma leptin and adiponectin contribute to negatively modulate insulin resistance.[24] Likewise, earlier studies have found in obese mice, higher basal levels of plasma insulin than in lean mice, also indicating insulin resistance in both sexes.[6]

In this study we found that the concentration of FFA, measured in *ad libitum* conditions (Fig. 2A), was slightly higher in obese than in lean females, which agrees with a previous analysis of triacylglycerides determined at the same time of day.[6,21] Fasting induces the mobilization of stored fat and the elevation of FFA levels.[25,26] This response was clearly present in lean mice, but it was notably attenuated in the obese group. At present we have not determined the mechanism of this effect, but it is highly suggestive that obese mice might show a reduction in the lypolytic activity, activated either by endocrine signaling or by sympathetic innervation of white adipose tissue.[27]

The data for total plasma ghrelin and its hypothalamic receptor (GHS-R) indicated a potential enhancement in the signaling by this hormone in obese female mice. An elevated level of the GHS-R in obese mice is consistent with the hypothesis that ghrelin has an orexigenic effect that is enhanced under conditions of physiological demand such as fasting.[7] This observation could be associated with the hyperphagy showed by the obese animals (Fig. 3B). In this study we observed that circulating levels of ghrelin as well as the amount of its receptor in the hypothalamus are clearly higher in obese than in lean mice. Preliminary observations by our group indicate that active ghrelin is also higher in obese mice (unpublished results), which suggest that all the mechanisms regulated by this hormone could be increased; nevertheless, the effect of fasting has yet to be addressed for this hormone. It has been suggested that ghrelin exerts its inhibitory effects on insulin secretion by regulating *glucemia*; it has been noted before that obese mice of *Neotomodon* present a higher serum concentration of insulin than lean,[6,21] consistent with insulin resistance detected in the former by the glucose tolerance test (Fig. 2B). This result may be of interest for future studies about the way in which obesity may attenuate or interfere with some of the metabolic feedback regulation in the hypothalamus, in a model where obesity seems to be related to increased ghrelin signaling.

The studies on the leptin receptor and its downstream peptidergic pathways have reconfirmed the crucial role of the hypothalamus in the regulation of food intake and energy balance.[13,28] Hyperleptinemy is more severe in obese female *N. alstoni* mice, and this trait is indicative of leptin resistance. In this study, we did not note significant changes in the amount of hypothalamic leptin receptor between obese and lean female mice fed *ad libitum* (Fig. 4A), and both groups show an increase in leptin receptor to the 24-hour fasting condition, consistent with other studies.[28] It is possible that leptin effect in obese may be insufficient because of reduced leptin transport into the brain, possibly due to the effect of high triacylglycerides in blood, as observed in this species[6] and elsewere.[29,30] It is expected that leptin concentrations in blood decrease after fasting, but this remains to be studied.

It was reported that leptin signaling is required for normal PACAP expression in the hypothalamic ventro-medial nucleus in mice.[31] VPAC receptors are involved in the regulation of hypothalamic neurosecretion for the homeostatic control of the circadian clock, among other processes.[32] The differences in the amount of VPAC2 receptor observed in *ad libitum* and fasting conditions indicate that in *N. alstoni*, VIP–PCAP signaling is also part of a multicomponent regulatory mechanism that is altered during the condition of obesity. In mice and rats, ghrelin and leptin are endocrine signals estradiol-sensitive;[33] the differences observed in females of *N. alstoni* may change regarding the estral cycle, therefore it is possible that some differences in leptin receptor may be observed if tested in different phases of the cycle.

Obesity blunts important mechanisms in the hypothalamus, in which signals seem to be inadequately transduced, as in the case of leptin.[13,15] In this study we did not observe significant changes in leptin

receptors; further research on the STAT-3 transduction pathway, one of the regulatory pathways of leptin in the hypothalamus, may give us a more integrated mechanism of obesity and leptin resistance in this animal model, with its susceptibility for developing obesity mainly through hyperphagy when provided with a standard diet. This study is a first approach to exploring the mechanisms that are associated with the spontaneous obese condition in female volcano mice and possibly related to modifications in food intake and hunger-satiety signals. This novel model improves our understanding of the orexigenic and anorexigenic hypothalamic signals that are altered during obesity, causing different responses to a stress condition such as fasting.

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