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Central leptin replacement enhances chemorespiratory responses in leptin-deficient mice independent of changes in body weight

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Abstract

Previous studies showed that leptin-deficient (ob/ob) mice develop obesity and impaired ventilatory responses to CO₂ ($\dot{V}_E - \text{CO}_2$). In this study, we examined if leptin replacement improves chemorespiratory responses to hypercapnia (7 % CO₂) in ob/ob mice and if these effects were due to changes in body weight or to the direct effects of leptin in the central nervous system (CNS). $\dot{V}_E - \text{CO}_2$ was measured via plethysmography in obese leptin-deficient- (ob/ob) and wild-type-(WT) mice before and after leptin (10 µg/2 µl day) or vehicle (phosphate buffer solution) were microinjected into the fourth ventricle for four consecutive days. Although baseline $\dot{V}_E$ was similar between groups, obese ob/ob mice exhibited attenuated $\dot{V}_E - \text{CO}_2$ compared to WT mice (134±9 versus 196±10 ml min⁻¹). Fourth ventricle leptin treatment in obese ob/ob mice significantly improved $\dot{V}_E - \text{CO}_2$ (from 131 ± 15 to 197 ± 10 ml min⁻¹) by increasing tidal volume (from 0.38±0.03 to 0.55±0.02 ml, vehicle and leptin, respectively). Subcutaneous leptin administration at the same dose administered centrally did not change $\dot{V}_E - \text{CO}_2$ in ob/ob mice. Central leptin treatment in WT had no effect on $\dot{V}_E - \text{CO}_2$. Since the fourth ventricle leptin treatment decreased body weight in ob/ob mice, we also examined $\dot{V}_E - \text{CO}_2$ in lean pair-weighted ob/ob mice and found it to be impaired compared to WT mice. Thus, leptin deficiency, rather than obesity, is the main cause of impaired $\dot{V}_E - \text{CO}_2$ in ob/ob mice and leptin appears to play an important role in regulating chemorespiratory response by its direct actions on the CNS.

Keywords

Respiratory chemoreception; Obesity; Hypercapnia; Leptin; Ventilation

Introduction

Leptin, a satiety peptide secreted mainly by adipocytes in proportion to the degree of adiposity, crosses the blood–brain barrier via a saturable receptor-mediated transport system [1, 9] and acts through neural pathways to suppress appetite [27] and modulate sympathetic nervous system activity in various organs and tissues leading to increased thermogenesis, arterial pressure, and heart rate [13, 18, 26]. Previous studies suggest that leptin may also regulate respiratory function. For instance, leptin-deficient (ob/ob) mice are unable to increase ventilation during a CO₂ challenge, suggesting impaired ventilatory control [20, 29]. In addition, one previous study demonstrated that subcutaneous infusion of leptin in ob/ob mice improved respiratory responses to hypercapnia (high CO₂ concentration), suggesting a stimulatory effect of leptin on central chemorespiratory control [19]. However peripheral treatment with leptin can also affect peripheral systems that modulate ventilation. Recent findings that leptin receptors are present in carotid body [24] and lung tissue [14, 30] suggest that leptin may exert its action via peripheral mechanisms. Therefore, we investigated the direct central nervous system (CNS) effects of leptin on regulation of respiration.

It is also unclear whether the impaired ventilatory response to hypercapnia caused by leptin deficiency (e.g., in ob/ob mice) is due mainly to lack of direct actions of leptin or to the morbid obesity that develops secondary to hyperphagia and reduced energy expenditure.

Moreover, the impact of weight loss during leptin replacement therapy in contributing to the improvement of respiratory function has not, to our knowledge, been previously assessed. Therefore, the main goals of this study were to examine the CNS actions of leptin in modulating the chemorespiratory responses to hypercapnia in ob/ob mice and to determine if these effects were due to changes in body weight or are independent of leptin-induced weight loss.

Methods

The experimental procedures and protocols of this study conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center and by the Ethics Committee of the University of São Paulo, Ribeirão Preto, São Paulo, Brazil.

Animals

Male C57BL/6 J wild-type (WT) and C57BL/6 J-Lep^{ob} leptin-deficient mice (ob/ob) between 6 and 10 weeks of age purchased from the University of Campinas, SP, Brazil or from the Jackson Laboratory (Bar Harbor, ME, USA) were used in these studies.

Intracerebroventricular cannulation

Ob/ob and WT mice were anesthetized with 250 mg kg⁻¹ of tribromoethanol and immobilized in a stereotaxic apparatus (KOPF, California, USA). Under aseptic conditions, a steel cannula (14.0×0.6 mm) was implanted into the fourth cerebral ventricle using the mouse brain stereotaxic coordinates [11] from Bregma (AP -5.88 mm, DV -3.5 mm, and L 0.0 mm). The guide cannula was anchored into the fourth ventricle with three stainless steel screws and dental acrylic. A stylet was inserted to seal the cannula until use. Animals were allowed to recover for 7 days before intracerebroventricular (ICV) daily microinjections of leptin or vehicle, and ventilation measurements were performed. At the end of the experiment, animals were euthanized and the brains were removed to confirm the position of the cannula.

Ventilation measurements

Pulmonary ventilation ($\dot{V}_E$) was measured using whole-body plethysmography as previously described [17]. Briefly, mice were acclimated to a plethysmography chamber (700 ml) for 1 h, and the ports of exit or entrance for gas in the chamber were closed to produce an internal constant volume. Then, breathing frequency (f) and tidal volume (V_T) were measured by changes in the pressure inside the chamber caused by inspiratory and expiratory fluctuations. We used a spirometer model ML141 produced by AD Instruments (Colorado, USA) for measurements and analyzed the signals using a PowerLab system. The system was calibrated with injections of 0.2 ml of room air with the animal inside the plethysmography chamber. Minute ventilation was reported as the product of f and V_T .

Baseline $\dot{V}_E$ measurements were obtained with the animals' breathing room air before they were exposed to a hypercapnic gas mixture containing 21 % O₂+7 % CO₂+ 72 %N₂. The mice were exposed to hypercapnia for approximately 5 min before measurements were made during one additional minute at the end of hypercapnia.

Intracerebroventricular leptin treatment

Leptin (National Hormone & Peptide Program, CA, USA) was administered ICV once a day for four consecutive days at the dose of 10 µg dissolved in 2 µl phosphate buffer solution (PBS, pH=7.4) into the fourth ventricle of ob/ob and WT mice. This dose was chosen based on previous studies examining the acute CNS effects of leptin on cardiovascular function [25, 26]. The ICV injections were performed with the animals inside the plethysmography chamber. The microinjection was carried out using a 5-µl Hamilton syringe connected to the ICV guide cannula via a 10-cm polyethylene tubing (PE50). The volume (2 µl) was carefully injected during 1 min to prevent damage to the brain. This protocol was repeated for four consecutive days between 9:00 and 10:00 a.m. Another group of mice received only 2 µl of PBS ICV and was used as control.

Ventilatory measurements were made 1, 3, and 6 h after leptin or vehicle ICV injections. After each measurement, the mice remained in the plethysmography chamber with access to room air, and a new baseline period breathing room air was taken for at least 5–6 min before measurements under hypercapnic condition were performed at 1, 3, and 6 h post-leptin or vehicle injection.

Peripheral leptin treatment

To test whether the effect of ICV leptin replacement on pulmonary ventilation was due to peripheral actions of leptin caused by potential spillover into the systemic circulation, we included an additional group of ob/ob mice that received leptin peripherally (10 µg/day via subcutaneous injections) for four consecutive days. $\dot{V}_E$ was measured daily beginning 4 days before leptin treatment was started until the last day of leptin injection.

Food restriction (pair-weight) in ob/ob mice

To dissociate the direct effects of leptin from those caused by weight loss in ob/ob mice, ventilatory responses to CO₂ were also examined in lean pair-weighted ob/ob mice. After weaning, ob/ob mice were food restricted for 60 days to match the body weight of age-matched WT mice fed at libitum. After 60 days of food restriction, ventilatory parameters were measured with animals breathing room air (normoxia) and 7 % CO₂ (hypercapnia) and compared to those measured in WT and obese ob/ob mice.

Immunohistochemistry

Wild-type mice were anesthetized (250 mg kg⁻¹ of tribromoethanol) and perfused transcardially with 0.9 % saline followed by 100 ml of 4 % paraformaldehyde (Sigma). Brains were removed and submerged in 30 % sucrose for cryoprotection for two nights. Coronal sections (30 µm) were cut for single-label immunostaining. In this protocol each step was preceded by PBS rinses. Tissue sections were pretreated with 0.3 % hydrogen

peroxide (Sigma) for 30 min followed by a blocking step with 3 % BSA (Sigma) for 1 h, diluted in PBS and 0.25 % Triton X-100 (PDT). The tissue was incubated with primary antiserum for leptin receptor from Santa Cruz Biotechnology (OB-R 1:1,500 dilution) for 40 h at 4°C. Sections were then incubated with biotinylated donkey anti-rabbit immunoglobulin G (BA-1000, Vector; 1:600 in PBS) for 2 h at room temperature and with an avidin–biotin complex (Vector Elite kit; 1:100 in PBS) for an additional 1 h at room temperature. After rinsing, the sections were incubated in 0.04 % diaminobenzidine tetrahydrochloride (0.2 mg/ml; Sigma) and 0.01 % hydrogen peroxide dissolved in PBS. The reaction was terminated after 5–7 min with successive rinses in PBS. Tissue sections were mounted onto gelatin-coated slides, air dried overnight and dehydrated in alcohol, cleared in xylenes, and cover-slipped with Entellan (Merck).

Statistical analyses

The results are expressed as mean±SEM. The data were analyzed using GraphPad Prism 5 program (one-way- and two-way-) ANOVA with repeated measures followed by Bonferroni's post hoc test for comparison between control and experimental values within each group when appropriate. Statistical significance was accepted at level of $p < 0.05$.

Results

Ventilatory response to CO₂ in WT and obese ob/ob mice

Baseline $\dot{V}_E$ was similar between WT and obese ob/ob mice and there were no significant differences in tidal volume (V_T) or frequency (f) between the groups (Fig. 1). However, during hypercapnia (7 % CO₂) $\dot{V}_E - CO_2$ was significantly attenuated in obese ob/ob mice compared to WT mice (134 ± 9 vs. 196 ± 10 ml min⁻¹, Fig. 1). This reduced response to hypercapnia was mainly due to a smaller increase in V_T in ob/ob mice compared to WT mice (0.42 ± 0.02 vs. 0.62 ± 0.02 ml) since there was no difference in f during hypercapnia between the groups (Fig. 1).

Effects of ICV leptin administration on chemorespiratory response in ob/ob and WT mice

To determine whether leptin modulates pulmonary ventilation by direct activation of its receptors in the CNS, we examined the effects of ICV leptin replacement on basal minute ventilation as well as on $\dot{V}_E - CO_2$ in conscious obese ob/ob and WT mice. Our results demonstrate that central leptin injections for four consecutive days exerted a time-dependent stimulatory effect on $\dot{V}_E$ in leptin-deficient mice (Fig. 2) but not in WT mice (Table 1).

After 3 days of leptin treatment, baseline $\dot{V}_E$ increased in ob/ob mice mainly due to an increase in V_T when compared to vehicle-treated ob/ob mice values (Fig. 2). Leptin infusion into the fourth ventricle also enhanced the ventilatory response to CO₂ in ob/ob mice (from 86 ± 6 to 197 ± 10 ml min⁻¹) compared to vehicle treatment (from 54 ± 9 to 119 ± 8 ml min⁻¹, Fig. 3). The improvement of $\dot{V}_E - CO_2$ after leptin treatment was caused by an increase in V_T (~60 %), whereas no major alteration in f response to CO₂ was observed between leptin or vehicle treatment (Fig. 3 and Table 2).

Central leptin infusion into the fourth ventricle also gradually decreased body weight by ~18 % in obese ob/ob mice (from 38.8 ± 1 to 32.0 ± 1 g, before and after leptin, respectively) compared to ~5 % reduction in the vehicle group (from 40.7 ± 4 to 38.6 ± 4 g, before and after vehicle, respectively). Although the fourth ventricle leptin injections in WT mice did not alter $\dot{V}_E - CO_2$, it also promoted weight loss (24.1 ± 1 to 20.7 ± 1 g, $p < 0.05$).

Effects of peripheral leptin administration on chemorespiratory response in ob/ob mice

Subcutaneous leptin injection at the same dose-infused ICV did not significantly alter $\dot{V}_E - CO_2$ in ob/ob mice (Table 2). This finding suggests that the effect of leptin to improve ventilatory responses to hypercapnia in ob/ob mice when injected centrally was due to its direct action on the CNS and not by a peripheral effect of leptin resulting from potential spillover into the systemic circulation. Subcutaneous leptin injection at this dose also had no impact on the body weight of these mice (from 50.1 ± 5 to 49.7 ± 5 g).

Ventilatory responses to CO₂ in lean ob/ob mice compared to WT and obese ob/ob mice

In order to dissociate the effects of obesity from the effects of leptin deficiency in causing impaired ventilatory responses to hypercapnia, we investigated ventilatory responses to CO₂ in additional groups of lean WT, obese ob/ob, and lean ob/ob mice that were pair-weighted from weaning to match the body weight of WT control mice. At 12 weeks of age, lean pair-weighted ob/ob mice had similar body weight compared to WT mice fed ad libitum (26 ± 1 and 27 ± 1 g). However, despite being lean, baseline minute ventilation was slightly lower in lean ob/ob mice (44 ± 4 ml min⁻¹) compared to the other groups (53 ± 3 and 59 ± 1 ml min⁻¹, for WT and obese ob/ob mice, respectively) (Fig. 4). Pair-weighted lean ob/ob mice also exhibited attenuated $\dot{V}_E$ to hypercapnia compared to WT control mice but similar to the impaired $\dot{V}_E$ observed in obese ob/ob mice (Fig. 4). Both lean and obese ob/ob mice showed smaller increases in V_T during CO₂ stimulation (0.37 ± 0.01 and 0.42 ± 0.02 ml) compared to WT mice (0.62 ± 0.02 ml) (Fig. 4). The respiratory frequency did not differ among groups.

Expression of leptin receptor in brainstem areas of breathing control

We used OB-R antibody polyclonal IgG recommended for detection of both short and long forms of the leptin receptor. The images were analyzed by a qualitative method according to the presence or absence of leptin receptors in areas associated with breathing control in the pons and medulla (Fig. 5). Positive immunostaining was observed in parabrachial nucleus (PBN) and locus coeruleus (LC) in pontine region (A–D panels), in raphe pallidus nucleus (E–G panels) in the rostral portion of the medulla, in ventral facial area 7 N (H–J panels), Böttinger complex (BötC), nucleus ambiguus, and rostral ventrolateral group of neurons (K–N panels) as well as in the caudal region of the nucleus of tractus solitarius (NTSc) (O–Q panels).

Discussion

This study demonstrated that ICV leptin replacement ameliorates impaired ventilatory responses to hypercapnia in leptin-deficient mice. We also showed that this brain-mediated effect of leptin to improve pulmonary ventilation in ob/ob mice cannot be explained by

potential spillover of leptin into the systemic circulation. In addition, we demonstrated in an unprecedented manner that pair-weight lean ob/ob mice do not exhibit any improvement in ventilatory response to CO₂, suggesting that leptin deficiency and not obesity is the main cause of impaired ventilatory responses in leptin-deficient ob/ob mice. Therefore, our data suggest that the improvement in chemorespiratory responses in obese ob/ob mice during central leptin replacement was independent of reduced body weight since lean ob/ob mice also exhibited a similar degree of impaired hypercapnic ventilatory responses as observed in obese ob/ob mice.

Leptin-deficient mice are extremely obese and have depressed ventilatory responses to CO₂. We hypothesized that central leptin replacement in these mice would have a rapid effect on ventilatory responses to hypercapnia before any significant changes in body weight could be observed. However, our results demonstrated that the effects of leptin on the control of respiratory function are not immediate and require 3 to 4 days before a significant improvement in ventilation is observed. As a consequence of leptin replacement, the obese ob/ob mice lost approximately 10 to 20 % of their body weight which could have contributed to the improvement in ventilatory function. To account for the effect of obesity in causing the respiratory deficit in obese ob/ob mice and to determine the role of weight loss in contributing to improved ventilatory responses to hypercapnia during leptin treatment, we investigated the responses to hypercapnia in lean, pair-weighted, ob/ob mice. The results from these experiments showed that the major cause of the impaired ventilatory response in ob/ob mice is leptin deficiency rather than obesity, and that weight loss in obese ob/ob during leptin treatment does not play a major role in mediating the effects of leptin to improve ventilatory responses to hypercapnia.

Although the precise mechanisms by which leptin modulates respiratory function are not well understood, our data are consistent with the idea that they may involve changes in gene expression and protein synthesis, thus requiring a few days for full effects. Our results also suggest the possibility that leptin may act in chemorespiratory nuclei that control ventilatory response to CO₂ since we observed that leptin receptors are expressed in neurons within PBN, LC, NTS, and BötC among other nuclei in the hindbrain responsible for the control of pulmonary ventilation. However, additional studies are needed to directly test the role of leptin receptors in these specific groups of neurons in mediating the effects of leptin on pulmonary ventilation.

Impaired ventilatory responses to hypercapnia have also been observed in agouti mice which have increased expression of agouti protein, an endogenous antagonist of melanocortin receptors [23]. This finding is consistent with the possibility that leptin-induced activation of the melanocortin system may be an important pathway by which leptin may mediate its effects on ventilation. This possibility is also supported by the fact that the melanocortin system has been shown to participate in other important actions of leptin including appetite and body weight regulation [6, 8], sympathetic activation [13, 26], glucose homeostasis [7], and blood pressure regulation [8, 13]. Melanocortin 4 receptors also are present in brain stem nuclei, including the NTS [16]. The role of the melanocortin system in controlling the response to hypercapnia is therefore an important area for further investigation.

Another important observation from our study was that the fourth ventricle leptin infusion for four consecutive days markedly reduced body weight in ob/ob and in WT mice. This observation suggests that in addition to the widely investigated effect of leptin to regulate body weight homeostasis via activation of leptin receptors in the hypothalamus, the brainstem may also play an important role in the regulation of body weight. Although previous acute studies have shown that leptin injection in brain stem nuclei reduces food intake [12, 16], few studies have examined whether this effect is maintained when leptin is administered over several days. We cannot rule out the possibility, however, that there may have been some recirculation of the leptin injected into the fourth ventricle back to the third and lateral ventricles, and that the resulting weight loss in our study was caused, in part, by activation of leptin receptors located in the hypothalamus. Indeed leptin effects on hypothalamic nuclei can also modulate breathing responses via projections to the phrenic nerves and to respiratory nuclei in the medulla [15, 32].

Obesity is a major cause of sleep apnea syndrome [2, 3, 22] and a positive correlation between plasma leptin levels and the degree of sleep apnea has been demonstrated [31]. Patients with severe apnea have higher circulating leptin compared to patients who have mild degree of apnea [21, 22]. Continuous positive airway pressure treatment in patients with sleep apnea syndrome can normalize ventilation and reduce plasma leptin levels without significantly changing body weight [4, 21, 31]. These observations, at first glance, appear to contradict our data and previous studies demonstrating impaired respiratory function in animals with leptin deficiency [19, 20] or leptin receptor mutations [10]. One potential explanation for these apparent contradictory findings is that obesity may cause resistance to the effects of leptin on ventilatory function, as has been previously demonstrated to occur for the appetite suppressant action of leptin [5, 25, 28].

In summary, we demonstrated that leptin deficiency, and not obesity per se, is the major cause of impaired ventilatory responses to hypercapnia in ob/ob mice. Our data also suggest that the fourth ventricle leptin replacement markedly improves baseline ventilation and ventilatory responses to CO₂ in ob/ob mice but not in WT mice despite promoting weight loss in both groups. These results, together with the observation that peripheral leptin administration at the dose given centrally and that preventing obesity by food restriction failed to improve ventilation in ob/ob mice, indicate that leptin modulates ventilation and ventilatory responses to hypercapnia via its direct actions on the CNS independent of weight loss. These observations provide new insights into potential mechanisms leading to breathing disorders in obesity and remain an important area for further investigation.

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Fig. 1.

Ventilation ($\dot{V}_E$), tidal volume (V_T), and frequency of ventilation (f) during breathing of room air or 7% CO₂ in obese ob/ob (gray box) and WT mice (black box) ($n=5/\text{group}$). Data expressed as mean $\pm$ SEM

Fig. 2.

Daily effects of ICV leptin (*black box*) or vehicle (*white box*) treatment in ob/ob mice

breathing 7 % CO₂ ($n=7$ /group). The measurements of pulmonary ventilation ($\dot{V}_E$), tidal volume (V_T), and frequency (f) were performed daily before and 1, 3, and 6 h after leptin ICV microinjections. Data expressed as mean±SEM

Fig. 3.

Ventilatory response ($\dot{V}_E$) and changes in tidal volume (V_T) and frequency of ventilation (f) from room air to 7 % CO₂ in obese ob/ob mice after 3 days of ICV leptin (*black box*) or vehicle (*white box*) treatment ($n=7$ /group). Data represent the measurements obtained 6 h after the last injection of leptin or vehicle. Data expressed as mean±SEM

Fig. 4.

Ventilatory responses to CO₂ (7 % CO₂), tidal volume (V_T), and frequency of ventilation (f) in obese ob/ob mice, $n=5$, 62 ± 1 g (gray box), lean ob/ob mice, $n=7$, 26 ± 1 g (white box), and wild-type mice, $n=6$, 27 ± 1 g (black box). Data expressed as mean $\pm$ SEM

Fig. 5.

Photomicrographs ($\times 2.5$, $\times 20$, and $\times 100$ in detail) of coronal sections showing positive immunostaining for leptin receptor (OB-R) in brainstem nuclei of WT mice. (A–D) Parabrachial nucleus (PBN) and locus coeruleus nucleus (LC); (E–G) Rafe pallidus nucleus (RPa); (H–J) ventral facial area (7 N); (K–N) Bötzing complex (BötC), nucleus ambiguus (NA), and rostral ventrolateral group of neurons (RVL); (O–Q) commissural nucleus tracts solitarius (NTSc). Bar scale=500 μ m

Table 1

Effects of 3 days ICV leptin (10 μ l/2 μ l) or phosphate buffer (PBS, 2 μ l) microinjections on V_E -CO₂ in wild-type mice

		PBS		LEPTIN	
		Room air	7 % CO ₂	Room air	7 % CO ₂
Before	V_E	41 $\pm$ 2	171 $\pm$ 12	53 $\pm$ 5	179 $\pm$ 3
	V_T	0.21 $\pm$ 0.02	0.52 $\pm$ 0.03	0.27 $\pm$ 0.02	0.56 $\pm$ 0.01
	f	202 $\pm$ 14	330 $\pm$ 8	192 $\pm$ 9	327 $\pm$ 5
After 1d	V_E	47 $\pm$ 3	169 $\pm$ 15	53 $\pm$ 5	165 $\pm$ 15
	V_T	0.26 $\pm$ 0.03	0.52 $\pm$ 0.02	0.27 $\pm$ 0.02	0.52 $\pm$ 0.04
	f	186 $\pm$ 12	323 $\pm$ 17	192 $\pm$ 9	313 $\pm$ 10
After 2d	V_E	43 $\pm$ 3	159 $\pm$ 9	47 $\pm$ 3	164 $\pm$ 11
	V_T	0.24 $\pm$ 0.01	0.49 $\pm$ 0.01	0.24 $\pm$ 0.01	0.51 $\pm$ 0.03
	f	176 $\pm$ 15	322 $\pm$ 12	194 $\pm$ 9	315 $\pm$ 8
After 3d	V_E	47 $\pm$ 3	170 $\pm$ 13	43 $\pm$ 3	151 $\pm$ 7
	V_T	0.25 $\pm$ 0.01	0.53 $\pm$ 0.02	0.21 $\pm$ 0.01	0.48 $\pm$ 0.02
	f	190 $\pm$ 4	317 $\pm$ 19	202 $\pm$ 4	311 $\pm$ 6

Values are means $\pm$ SE. V_E , pulmonary ventilation ($\text{ml} \cdot \text{min}^{-1}$); V_T , tidal volume (ml); f respiratory frequency ($\text{breaths} \cdot \text{min}^{-1}$). PBS group (n=6), body weight variation from 22.7 $\pm$ 1 g to 22.9 $\pm$ 1 g. Leptin group (n=9), body weight from 24.1 $\pm$ 1 g to 20.7 $\pm$ 1 g (P<0.05)

Table 2Effects of 3 days ICV or subcutaneous leptin microinjections (10 µg/day) on V_E-CO₂ in ob/ob mice

		LEPTIN		LEPTIN	
		Intracerebroventricular		Subcutaneous	
		Room air	7%CO ₂	Room air	7%CO ₂
Before	V _E	86±6	131±15	56±5	111±8
	V _T	0.26±0.07	0.38±0.04	0.24±0.02	0.35±0.02
	f	216±59	330±9	239±23	316±12
After 1d	V _E	71±5	125±13	52±2	108±7
	V _T	0.28±0.05	0.38±0.04	0.23±0.02	0.35±0.02
	f	249±48	331±5	232±22	309±12
After 2d	V _E	95±2	129±18	49±3	119±10
	V _T	0.34±0.04	0.39±0.03	0.22±0.02	0.36±0.02
	f	277±40	332±17	223±17	328±13
After 3d	V _E	94±5	170±14 [‡]	45±2	112±6
	V _T	0.33±0.04	0.49±0.03 [‡]	0.23±0.02	0.34±0.02
	f	261±14	345±10	203±22	325±14

Values are means ± SE. V_E, pulmonary ventilation (ml.min⁻¹); V_T, tidal volume (ml); f respiratory frequency (breaths.min⁻¹). ICV group (n=7), body weight variation from 38.8±1 g to 32.1±1 g (P<0.05). Subcutaneous group (n=9), body weight from 50.1±5 g to 49.7±5 g.

[‡]p<0.05 vs. before treatment was started