Leptin and Its Receptor Are Controlled by 17β-Estradiol in Peripheral Tissues of Ovariectomized Rats

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It has been widely shown that there is a complex interaction between sex steroids and leptin effects on body weight. In this sense, the absence of female sex steroids is linked to a significant increase in body weight, which seems to be related to an impairment of the central actions of leptin. The present study was designed to elucidate the effects of two different treatments with 17β-estradiol on leptin receptor and serum leptin levels in ovariectomized rats, a model of postmenopausal condition. Our results have shown that plasma leptin levels in ovariectomized rats were lower than in estradiol-treated animals, thereby supporting a positive effect of this steroid. Recent information has extended leptin actions to peripheral tissues, mainly to insulin-dependent tissues, this effect being related to metabolic actions. To better understand the peripheral effects of leptin and their possible regulation by estradiol treatment, we have analyzed leptin receptor expression in the skeletal muscle and the adipose tissue. Our results showed a tissue-specific regulation of this protein: Ob-Rb expression in the adipose tissue decreased when the time of treatment or the dose of estradiol administered increased, suggesting less sensitivity to leptin in this tissue, whereas in the skeletal muscle the changes in this protein followed the same profile as the plasma leptin levels. We think that this specific regulation could ensure a different response of each tissue toward the same serum leptin level. Further studies to clarify this situation are ongoing. Exp Biol Med 232:542–549, 2007

Key words: 17β-estradiol; leptin; leptin receptor; skeletal muscle; adipose tissue; rat

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Introduction

The loss of ovarian hormonal production associated with menopause is related to changes in body weight and is characterized by an increase in fat deposits, a decrease in insulin sensitivity, and a higher risk of cardiovascular disease (1). Hormone replacement therapy attenuates the postmenopausal increase of fat mass by about 60%, and this fact has a positive impact on cardiovascular risk factors (2). This action seems to be related to a dual effect of 17β-estradiol: a direct effect on tissues involved in lipid metabolism and an indirect effect on brain regions (including ventromedial hypothalamus and paraventricular nucleus) regulating satiety signals (3). However, the effects of 17β-estradiol seem to be dependent on the dose and the route of administration and also are related to deleterious effects, such an increase in the risk of cancer.

In the past few years, there has been a growing interest in leptin and its actions. Leptin is produced mainly in the white adipose tissue, and its discovery has confirmed the lipostatic theory of body weight maintenance developed from parabiosis data. When this protein crosses the blood–brain barrier, it provides the brain with information about the fat deposits of the body and thus acts as a part of the feedback mechanism that can function as a lipostat (for review, see Refs. 4 and 5). The regulation of plasma leptin levels is a very complex mechanism. Fasting is related to a decrease in plasma leptin levels, which are restored after refeeding in rats and humans (6, 7). Aging is linked to a decrease in leptin levels, and this decrease seems to be independent of body mass index (8). Moreover, leptin secretion also is regulated by different hormonal factors, including glucocorticoids (9), insulin (6), and sex steroids (10, 11). In this sense, it has been shown that plasma leptin levels are significantly higher in females than in males (12). Although until this moment the reason for this observation has not been completely elucidated, estrogen and testosterone seem to be involved (13, 14). The influence of estradiol on plasma leptin levels is supported by different studies that showed changes in leptin concentrations throughout the menstrual cycle, reaching the

peak in the luteal phase (15). Sivan *et al*. (16) have shown that leptin levels increased predominantly during the first half of pregnancy and fell dramatically in the immediate postpartum period. *In vitro* experiments showed positive effects of 17β-estradiol exposure on adipocyte leptin synthesis and secretion, supporting the stimulatory effect of 17β-estradiol on leptin secretion (11).

Leptin action is mediated through a specific receptor. To date, six different alternate isoforms of the leptin receptor have been described to be generated by alternative splicing of the leptin receptor gene (*db* gene), designated Ob-Ra to Ob-Rf with common extracellular and transmembrane domains. Only the long isoform of this receptor, Ob-Rb, presents a long intracellular domain with several motifs needed to interact with other proteins that are involved in different intracellular pathways; so Ob-Rb was initially considered to be the functional isoform of the receptor. However, the short isoforms seem to be involved in leptin transport and clearance and also seem to have a signaling capacity weaker than that of Ob-Rb, and its real physiologic role remains to be elucidated (17–19). The molecular structure of this receptor shows a high homology to the class I cytokine receptors, lacks intrinsic tyrosine kinase activity, and uses cytoplasmic-associated kinases of the JAK family. Ob-Rb is located mainly in the hypothalamic centers related to the regulation of food intake and energy balance; additionally, it also has been recently identified in insulin-sensitive tissues (for review, see Refs. 4, 19–22). The role of leptin in peripheral tissues remains partially unknown. Nevertheless, it has been demonstrated that direct effects of leptin included: in liver, improvement of insulin signaling and glucose metabolism (23); in skeletal muscle, favoring of lipid oxidation and thermogenesis (24, 25); and in adipose tissue, a selective reduction of leptin receptors led to a significant increase of adiposity and body weight (26). All these facts seem to suggest that the actions of leptin on body weight and food intake require not only hypothalamic actions, but also nonneural actions related to insulin-sensitive tissues. In this sense, the regulation of Ob-Rb could play a pivotal role in the regulation of leptin actions, and evidence showed that the plasma levels of 17β-estradiol are involved in the regulation of this receptor (27, 28). However, the relationship between Ob-Rb regulation by 17β-estradiol and leptin effects remains partially unknown.

Ovariectomized rats are considered a very useful model of postmenopausal condition. This experimental model is characterized by mild obesity, and it is useful to study the influence of hypoestrogenism on adiposity and the effect of hormonal replacement. To the best of our knowledge, the role of leptin and its receptor in this situation has not yet been fully elucidated. The chief aim of our study was to evaluate the influence of two different doses of 17β-estradiol (physiologic and supraphysiologic doses) on plasma leptin levels to better understand the regulation of leptin secretion. Moreover, we also have evaluated the changes in Ob-Rb expression in two main insulin-sensitive tissues; that is, skeletal muscle and adipose tissue.

Materials and Methods

Animals. Virgin female Wistar rats (from the Biotery of the Faculty of Medicine, University of Oviedo, Oviedo, Spain) weighing 250–280 g and kept under standard conditions of temperature (23°C ± 3°C), humidity (65% ± 1%), and a regular lighting schedule of 12:12-hr light:dark cycle (0800–2000 hrs) were used. The animals were fed with a standard diet (Panlab A04; Panlab, Barcelona, Spain). All animals had free access to water. Rats were weighed daily. All experimental manipulations were performed between 0930 and 1230 hrs. The University of Oviedo Ethics Committee, following the Guiding Principles for Research Involving Animals and Human Beings Recommendations from the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals, approved all of the animal experiments.

Estradiol Treatment. In the E groups different doses of 17β-estradiol were administered in order to simulate the plasma levels that we observed in normal pregnant rats (EA: 17β-estradiol 1.3 μg/kg body wt; EB: 17β-estradiol 0.65 μg/kg body wt). In the Ex100 group the doses of 17β-estradiol injected were 100 times higher than in the E groups (Ex100A: 17β-estradiol 130 μg/kg body wt; Ex100B: 17β-estradiol 65 μg/kg body wt), as previously described (29, 30).

Three days before initiating the hormonal treatment (Day −7), the rats were ovariectomized through a midline incision under sodium pentobarbital (50 mg/kg) anesthesia. Ovariectomized rats were separated randomly into three groups: control (V) and two groups treated with 17β-estradiol (E and Ex100), and were housed individually throughout the experiment. After surgery, ovariectomized rats were allowed 3 days to recover from surgery stress and decrease their hormonal levels. From Day −4 the rats were injected subcutaneously every 12 hrs (0900 and 2100 hrs) for 20 days with 0.1 ml of suspension in olive oil/ethanol (3:2 v/v) of 17β-estradiol (17β-estradiol; Sigma Chemical Co., St. Louis, MO). Control group (V) injected with vehicle (olive oil/ethanol, 3:2 v/v) was followed in parallel.

The hormonal treatment was applied according to Table 1. Groups (V, E, and Ex100) were divided randomly into three subgroups (six animals per subgroup): 6, 11, and 16 (according to the day of the experimental period on which the animals were killed). These days were selected because changes were found in the sensitivity to the insulin action similar to those described during pregnancy in the Wistar rat (29, 30).

The day that animals were killed, and 12 hrs after the last estradiol injection, rats were anesthetized with sodium pentobarbital (50 mg/kg), and samples of skeletal muscle (flexor digitorum superficialis, extensor digitorum longus, soleus and extensor digitorum lateralis) and retroperitoneal

Table 1. Estradiol Treatment[a]

	Day												
Group	−7[b]	−6	−5	−4	−3	−2	−1	0	1	2	3	4	5
E	—	—	—	EA	EA	EA	EA	EA	EA	EA	EA	EA	EA
Ex100	—	—	—	Ex100A	Ex100A	Ex100A	Ex100A	Ex100A	Ex100A	Ex100A	Ex100A	Ex100A	Ex100A

[a] EA, 17β-estradiol 1.3 μg/kg body wt; EB, 17β-estradiol 0.65 μg/kg body wt; Ex100A, 17β-estradiol 130 μg/kg body wt; Ex100B, 17β-estradiol 65 μg/kg body wt.
[b] Rats were ovariectomized on this day.
[c] Day of killing.

adipose tissue were taken and immediately frozen in liquid nitrogen for future experiments. Finally, the animals were killed by bleeding.

Estradiol and Leptin Plasma Levels. The day of sacrifice, after pentobarbital anesthesia, blood samples (4 ml) were collected from the jugular vein into heparinized tubes and centrifuged at 3000 rpm for 20 mins at 4°C, and plasma was immediately drawn off and frozen at –20°C until assayed. Plasma 17β-estradiol was measured by radio immunoassay (RIA) using Immunchen kits of cover tubes (ICN Pharmaceuticals Inc., Costa Mesa, CA). The assay sensitivity was 10 pg/ml, and the intrassay coefficient of variation was 12.26%. Plasma leptin levels were measured by RIA using a kit from DGR Instruments (Marburg, Germany) for rat leptin. Cross-reactivity for human leptin was lower than 2%.

Preparation of Extracts from Adipose and Muscle Tissues and Western Blot Analysis. The samples of skeletal muscle and adipose tissue (100 mg wet weight) were prepared as previously described (31). Briefly, the samples were washed with ice-cold phosphate-buffered saline and homogenized immediately in 3 ml lysis buffer (50 m*M* Tris-HCl, pH 7.5; 150 m*M* NaCl; 1% Nonidet P40 [Roche Diagnostics, Barcelona, Spain]; 0.05% sodium deoxycholate; and 1 m*M* sodium orthovanadate) at 4°C. The extracts were centrifuged at 12,000 *g* at 4°C for 10 mins, and the protein content was determined by the Bradford dye-binding method (32). Similar-size aliquots (30 μg for skeletal muscle and 60 μg for adipose tissue) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 8% Tris-Acri-Bis), and proteins were electrotransfered from the gel to nitrocellulose membranes (Hybond-ECL; Amersham Pharmacia Biotech, Piscataway, NJ) as described by Towbin *et al.* (33). Nonspecific protein binding to the nitrocellulose membranes was reduced by preincubating the filter in blocking buffer (TNT, 7% bovine serum albumin [BSA]), and probed using a 1:5000 dilution of anti–Ob-R (antiserum sc-1834; Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were rinsed several times with blocking buffer without BSA, and protein bands were visualized by enzyme chemiluminescence (sc-2048; Santa Cruz Biotechnology) and quantified using a digital scanner (Nikon AX-110; Nikon, Tokyo, Japan) and the National Institutes of Health (Bethesda, MD) Image 1.57 software. Later, the membranes were incubated in stripping buffer (50 ml Tris-HCl 62.5 m*M,* pH 6.8; 1 g SDS; 0.34 ml β-mercaptoethanol) at 60°C. All membranes stripped were reprobed with a monoclonal anti–β-actin antibody (sc-1615, dilution 1.2500; Santa Cruz Biotechnology) to ensure equal protein loading. To help intergel comparisons during analysis, standards prepared from pooled skeletal heart also were run in each gel.

Statistics. Data are expressed as mean ± standard error of mean (SEM). Previously, we evaluated the Gaussian distribution of each variable. After this, the comparisons were made using an analysis of variance and the Student-Newman-Keuls test. $P \leq 0.05$ was considered significant. Statistical analysis was performed using SPSS v.6.01 for Windows (SPSS Inc., Chicago, IL).

Results

Plasma 17β-estradiol concentrations in V, E, and Ex100 groups are shown in Figure 1. As expected, ovariectomy led to a significant decrease in plasma estradiol levels compared with estradiol-treated groups. The results obtained in the E group were similar to normal pregnancy at 5, 10, and 15 days of pregnancy (29, 30). In this group, we found similar values at 6 and 11 days of treatment and a significant increase between 6 and 16 and 11 and 16 days of treatment. The results obtained in the Ex100 group were significantly higher than those of the E and V groups, increasing progressively through the experiment.

Figure 2 shows the body weight, plasma leptin levels, and the relationship between both parameters (Fig. 2A, B, and C, respectively) registered in ovariectomized and estradiol-treated groups. The body weight was always significantly lower in estradiol-treated groups than in the control group (Fig. 2A) and, obviously, the body weight increased significantly until the end of the experiment. Plasma leptin levels were always significantly higher in estradiol-treated groups than in the V group, although at Day 11 we only registered differences with statistical significance between the Ex100 and V groups. The length of treatment promoted a significant increase in plasma leptin levels at the end of the experiment (Day 16) in all the experimental groups (Fig. 2B). Finally, the relationship between leptin plasma level and body weight (Fig. 2C) was always significantly higher in estradiol-treated groups than

Table 1. (Extended)

	Day										
Group	6[c]	7	8	9	10	11[c]	12	13	14	15	16[c]
E	EB	EB	EB	EB	EB	EA	EA	EA	EA	EA	
Ex100	Ex100B	Ex100B	Ex100B	Ex100B	Ex100B	Ex100A	Ex100A	Ex100A	Ex100A	Ex100A	

in the control group. Moreover, this relationship increased significantly until the end of the experiment in the three groups that were studied.

Ob-Rb is the main isoform involved in peripheral leptin signaling, so we focused our study on this isoform of the leptin receptor. Figure 3 shows the amount of Ob-Rb in the skeletal muscle of experimental groups using Western blot experiments. We observed a progressive increase of this protein through the treatment in control and estradiol-treated groups. The comparison between experimental groups at Day 6 showed that there are no differences between control and estradiol-treated animals, whereas on Days 11 and 16 the amount of Ob-Rb in skeletal muscle was significantly higher in estradiol-treated animals than in the control group.

Figure 4 shows the variations of Ob-Rb levels in the adipose tissue of V, E, and Ex100 animals. The absence of female sex steroids in the V group is related to a progressive decrease in this protein through the duration of the experiment. In the E group, the amount of Ob-Rb increased significantly between Days 6 and 11, whereas it decreased between Days 11 and 16 of treatment. Finally, in the Ex100 group we detected a significant decrease in this protein at the end of the experiment (Day 16). Comparison of Ob-Rb expression between groups shows that at Day 6 the control group presented the highest levels of adipose Ob-Rb protein. At Day 11, it was found that Ob-Rb levels were significantly higher in the E group than in groups V and Ex100; however, the highest levels of Ob-Rb were found in the Ex100 group at Day 16 of treatment.

Discussion

The present study confirms not only the implication of 17β-estradiol in the regulation of plasma leptin level, but also, to the best of our knowledge, this is the first study that confirms that this steroid is directly linked to a tissue-specific regulation of Ob-Rb expression in the skeletal muscle and the adipose tissue.

Estradiol-treated animals showed higher plasma leptin levels than the control group as shown in Figure 2B, which demonstrates that 17β-estradiol is responsible for the increase in plasma leptin levels in ovariectomized rats. Moreover, when we corrected the plasma leptin levels for the body weight, we observed that there were no differences between the estradiol-treated animals (Fig. 2C). Therefore, we believe that the effect of 17β-estradiol on plasma leptin levels seems to be independent of the dose administered and the estradiol plasma levels. Our results are in agreement with those reported by several authors (10, 11, 34, 35) who have demonstrated that ovariectomized rats showed a significantly lower expression of leptin mRNA in the adipose tissue and decreased plasma leptin levels compared with estradiol-treated rats. The mechanism responsible for

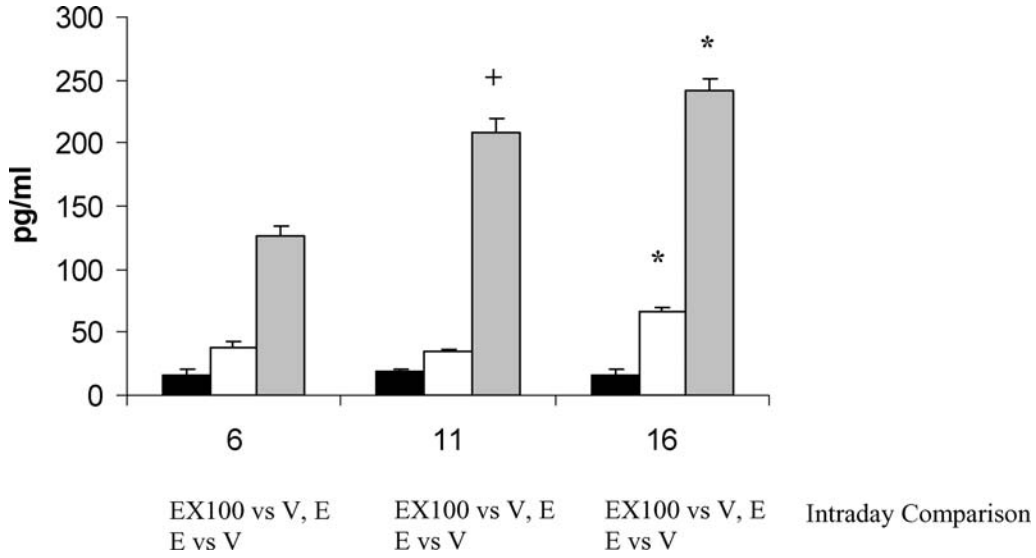

Figure 1. Plasma 17β-estradiol levels in ovariectomized rats (V [black bars]) and estradiol-treated (E [white bars] and Ex100 [gray bars]) rats at 6, 11, and 16 days of the experiment. Results are expressed as means ± SEM for six animals. Only significant differences are shown. (*, 16 vs. 6 and 11; $^{+}$, 11 vs. 16.)

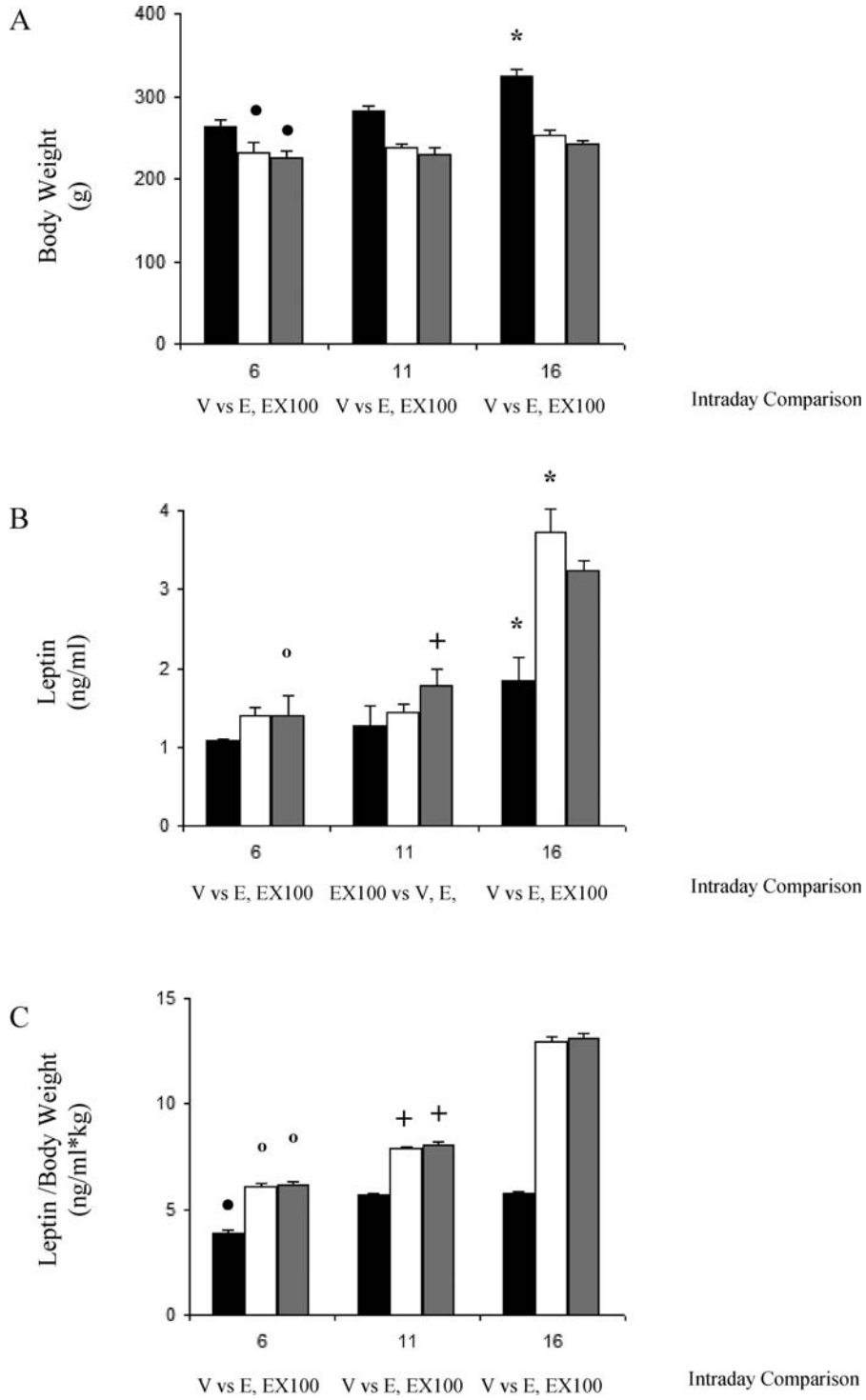

Figure 2. Body weight (A), plasma leptin level (B), and relative plasma leptin levels (C) in ovariectomized rats (V [black bars]) and estradiol-treated (E [white bars] and Ex100 [gray bars]) rats at 6, 11, and 16 days of the experiment. Results are expressed as means ± SEM for six animals. Only significant differences are shown. (^{o}, 6 vs. 11 and 16; $^{+}$, 11 vs. 16; *, 16 vs. 6 and 11; $^{\bullet}$, 6 vs. 16.)

estradiol regulation of leptin plasma level remains unknown, but a direct action of estrogen on the adipocyte leptin gene has been proposed, supported by the identification of a half-palindrome of the estrogen-responsive element in the leptin gene promoter. In this sense, *in vitro* studies have demonstrated that the expression of leptin mRNA is overexpressed, and its secretion also increases when adipocytes from ovariectomized rats are incubated with 17β-estradiol (11, 36).

Most of the studies considered to date have shown that plasma leptin levels are correlated to body fat content; the adipose tissue, being the main tissue involved in leptin

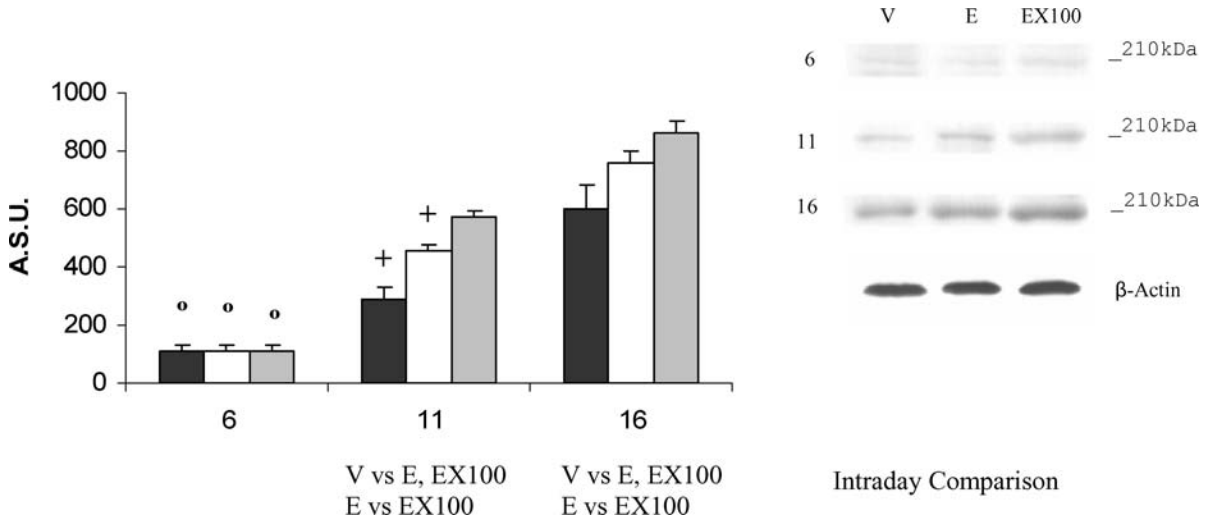

Figure 3. Ob-Rb protein levels in skeletal muscle of ovariectomized (V [black bars]) and estradiol-treated (E [white bars] and Ex100 [gray bars]) rats. Proteins were isolated with lysis buffer, and lysates were separated on an 8% polyacrylamide gel and immunoblotted with anti–Ob-R antibody. Scanning densitometric analysis was performed on five experiments. Data are expressed as mean ± SEM. Only significant differences are shown. A.S.U., arbitrary scanning units. (^{o}, 6 vs. 11 and 16; $^{+}$, 11 vs. 16.)

production, increases in ovariectomized rats (Fig. 2; Ref. 35). Our results show that leptin plasma levels are significantly lower in ovariectomized than in estradiol-treated rats, although it has been widely reported that ovariectomy leads to a significant increase in body fat content. This observation also has been reported by other authors (34, 35, 37), and it has been suggested that the decrease in the production of leptin associated with ovariectomy related to estradiol-treated animals could be the reason leading to the increase in body weight, because of the reduced control of appetite and the lower amount of energy spent. However, the leptin receptor also seems to be involved in the imbalance between ovariectomy, leptin, and body weight. In this sense, Kimura *et al*. (27) have shown that ovariectomy is directly related to a decrease in hypothalamic Ob-Rb that could impair the signaling that leads to a suppression of food intake, thereby resulting in an increase in body weight. Our results regarding the Ob-Rb expression in the adipose tissue of ovariectomized rats (Fig. 4) are in agreement with those of Kimura *et al*. (27), which showed a "downregulation" of this protein through the experiment, whereas plasma leptin levels increased, which also is suggestive of a desensitization of the adipose tissue to the effects of autocrine leptin. In this sense, it has been reported that leptin can affect fat mass regulation in a direct way through a direct inhibition of lipogenesis without the involvement of the central nervous system (38). Taking into consideration all of the data, we hypothesize that the weight

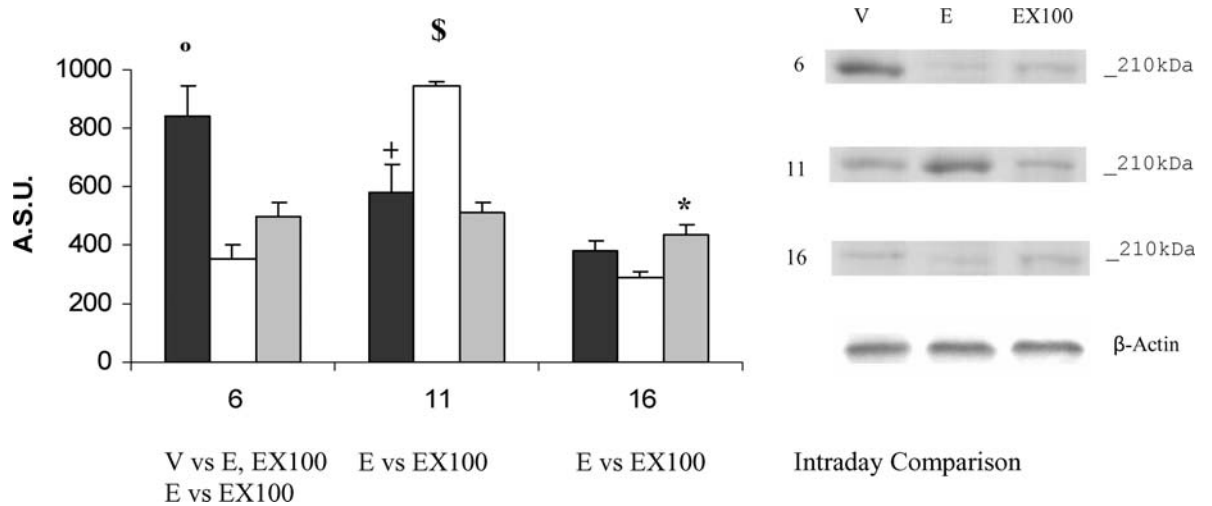

Figure 4. Ob-Rb protein levels in adipose tissue of ovariectomized (V [black bars]) and estradiol-treated (E [white bars] and Ex100 [gray bars]) rats. Proteins were isolated with lysis buffer, and lysates were separated on an 8% polyacrylamide gel and immunoblotted with anti–Ob-R antibody. Scanning densitometric analysis was performed on five experiments. Data are expressed as mean ± SEM. Only significant differences are shown. A.S.U., arbitrary scanning units. (^{o}, 6 vs. 11 and 16; $^{+}$, 11 vs. 16; *, 16 vs. 6 and 11; $^{\$}$, 11 vs. 6 and 16.)

gain in ovariectomized rats (Fig. 2A; Refs. 27, 29, 35) might also be related not only to a decrease in hypothalamic Ob-Rb expression (27), but also to a decrease in the Ob-Rb expression in the adipose tissue. Moreover, leptin is able to modulate its own synthesis through an ultra-short negative feedback loop in the adipose tissue (39), so we believe that the increase in plasma leptin levels observed at the end of the experiment in ovariectomized rats could be related to a decrease in Ob-Rb expression in the adipose tissue.

On the other hand, it has been shown that estradiol treatment was able to increase hypothalamic Ob-Rb content (28), suggesting that the lower food intake and body weight in ovariectomized rats treated with estradiol are not only related to an increase in leptin synthesis (Fig. 2), but also to an "upregulation" of leptin signaling in the central nervous system. Our results show that estradiol treatment also can modulate leptin receptor expression in the adipose tissue in a time-dependent way, suggesting that this effect can be influenced by the dose of 17β-estradiol employed. In this sense, Meli *et al*. (14), using an experimental setup longer than ours (7 and 21 weeks), have shown that the time of treatment plays a pivotal role in the regulation of Ob-Rb. At 7 weeks of treatment, the expression of Ob-Rb was significantly lower in estradiol-treated animals than in ovariectomized ones; just the opposite of what happened at 21 weeks. Our results seem to indicate that an increase in the time of treatment or dose of estradiol employed can be related to a decrease in Ob-Rb expression in the adipose tissue, suggesting less sensitivity to effects of the leptin in this tissue. It has been published that leptin clearly impairs insulin actions in the adipose tissue (40), and our group has previously reported that estradiol is able to modulate insulin sensitivity in a dose-dependent way (30, 31). Moreover, a cross-talk between insulin and leptin signaling at a molecular level also has been reported (41). We have recently shown that estradiol is able to interact with insulin signaling (30, 31). More research is needed to better understand the complex interaction between insulin, leptin, and estradiol signaling.

A careful revision of the bibliography showed considerably fewer data on leptin actions in the skeletal muscle, and this issue remains controversial. However, skeletal muscle leptin actions can be classified into two sections: (i) actions related to insulin-dependent glucose uptake—most of the studies indicate that these effects are mediated centrally (42)—and (ii) actions regarding lipid partitioning (for review, see Ref. 40). In this sense, leptin actions in the skeletal muscle are directly linked to both an inhibition of lipogenic effects of insulin and to a stimulation of free fatty acid (FFA) oxidation in the skeletal muscle. Contrary to the results found in the adipose tissue, the Ob-Rb regulation in the skeletal muscle (Fig. 3) follows the same profile as the plasma leptin levels (Fig. 2B), showing that 17β-estradiol treatment is leading to an "upregulation" of Ob-Rb expression in a dose- and time-dependent way. We believe that this situation could be favoring that the skeletal muscle of estradiol-treated rats was more sensitive to leptin actions, promoting an increase of FFA oxidation and an inhibition of insulin lipogenic effects. In this sense, we propose that this tends to increase lipid metabolism and could be also related to the lower amount of adipose tissue present in ovariectomized rats treated with estradiol, and we cannot discard a possible relation to changes in insulin sensitivity.

In summary, to the best of our knowledge this is the first study that demonstrates that 17β-estradiol is able to modulate the leptin receptor in peripheral tissues in a dose-, time-, and tissue-dependent way. Considering all of the data, we suspect that the effects of this steroid on the leptin system could ensure a different response from the skeletal muscle and the adipose tissue toward the same plasma leptin level. However, more studies on the peripheral effects of leptin in whole-body animals are needed to clarify the role of 17β-estradiol regulation and to understand the complex relationship between leptin and insulin signaling.

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