

Impact of Estradiol on Parametrial Adipose Tissue Function

Evidence for Establishment of a New Set Point of Leptin Sensitivity in Control of Energy Metabolism in Female Rat

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Estradiol has been implicated in the regulation of food intake; however, its effect seems to be exerted in a bimodal fashion. We examined whether a single injection of estradiol valerate (E₂V), lastingly effective, could induce changes in parametrial fat function that further induce a new set point of leptin sensitivity in the female rat. E₂V induced severe anorexia and loss of body weight between d 4 and 12 posttreatment. E₂V rats recovered normal food intake and departing body weights on wk 2 and 3 posttreatment, respectively; however, they did not reach body weights of control rats. On d 61 posttreatment, we found that unfasting E₂V, vs control, rats displayed increased E₂ and leptin circulating levels; reduced plasma tumor necrosis factor- α (TNF- α) concentrations; similar circulating levels of glucose, insulin, and triglyceride; and lower parametrial fat mass containing a higher number of adipocytes that, although normal in size, *in vitro* released more leptin. Metabolic responses to fasting indicated that unlike control animals, E₂V rats did not decrease triglyceride circulating levels, and that both groups decreased plasma glucose, leptin, and insulin, but not TNF- α , levels. High glucose load experiments indicated that E₂V animals displayed a better insulin sensitivity than control rats; did not significantly increase circulating leptin concentrations as control rats did; and, unlike control, significantly decreased plasma triglyceride levels. Our data strongly support a potent acute anorectic effect of E₂ and that, after several weeks, E₂ modified parametrial fat function and insulin sensitivity, protecting the organism against future unfavorable metabolic conditions.

Key Words: Food intake; anorexia; allostasis; cytokines; lipids.

Introduction

Fat distribution differs with gender, and this characteristic is dependent on a sex steroid hormone basis (1). Adipogenesis, a process involving adipocyte precursor cells—preadipocytes—mature adipocytes via transcriptional activation of adipose-specific genes (2,3), is positively modulated by several transcription factors (4,5) and influenced by the endocrine system (6–8). It has been proposed that while androgens block (9) adipogenesis estrogens stimulate (10) adipogenesis. This fact is strongly supported by the presence of specific sex steroid receptors in mammals' preadipocytes and adipocytes (11,12), thus indicating that these cells are targets for sex steroid hormones and, in turn, modulate growth of adiposity (1).

Consistent with the mentioned effects of sex steroids on adipogenesis, the adipocyte product of the *ob* gene, leptin, is also expressed and released on a sex steroid-dependent basis: while estrogens stimulate, androgens inhibit leptin expression and release (13,14). Reciprocally, leptin is also known to modulate sex steroid production in both sexes; by acting through its specific receptor, leptin inhibits estradiol production by ovarian granulosa cells (15) and testicular testosterone secretion (16). Thus, these data clearly indicate interplay between adipose tissue and gonadal steroids.

All actions of the pleiotropic protein finally converge in the control, by hypothalamic signaling, of food intake and energy balance (17). Estradiol has been directly implicated in the regulation of food intake (18), and the mechanism involved in this effect seems to be owing to its binding to specific receptors present in the arcuate nucleus, a main hypothalamic orexigenic site rich in neuropeptide Y (NPY) cell bodies (19). Although the exact role of estradiol on food intake remains controversial, it seems to be dependent on whether the effect of estradiol is analyzed after either acute (20) or chronic (21) administration, thus rendering orexigenic or anorexigenic activity, respectively; however, up until now, no studies have been conducted in order to clarify this discrepancy.

Because estradiol is implicated in the lipogenesis-lipolysis cycle (10,22), we hypothesized that acutely adminis-

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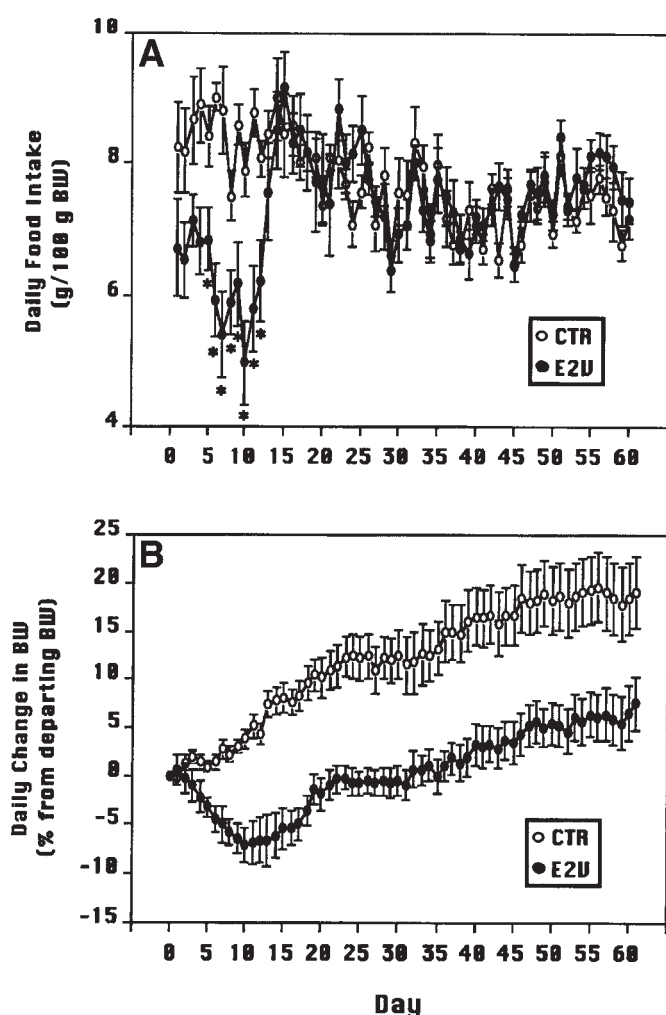


Fig. 1. Impact of im administration of vehicle alone (200 μ L of corn oil; control) or containing E₂V (2 mg/rat) on (A) daily food intake and (B) changes in body weight in adult female rats. Values are the mean \pm SEM ($n = 24$ rats/group). * $p < 0.05$ or less vs day-matched, control values. Change in body wt values (B), between d 4 and 61, in E₂V-treated rats are significantly different ($p < 0.05$ or less) vs day-matched control values.

tered estradiol, longtime effective (the valerate form; estradiol valerate [E₂V]) (23), could change parametrial adipose tissue function, rendering metabolic changes that could help researchers to better understand the effect of estradiol on feeding behavior.

Results

Acute and Lasting Effects of E₂V Treatment on Food Intake and Changes in Body Weight

E₂V injection (2 mg/rat, intramuscularly) was able to drastically reduce daily food intake during the first 2 wk posttreatment. However, only between d 4 and 12 following treatment was the amount of food eaten per rat significantly lower than in control rats (Fig. 1A). This effect was observed when high circulating estradiol levels were estab-

lished: ranging from 4120.21 ± 188.48 to 338.95 ± 31.56 pg/mL, between d 4 and 12 posttreatment, respectively (mean \pm SEM; $n = 6$ rats). Thereafter, no significant differences in food intake between control and E₂V rats were observed until the end of the experiment. Interestingly, body weight gain was (expressed as the mean \pm SEM of the percentage varied from the respective departing body weights) significantly arrested shortly after E₂V injection, and although these rats had recovered the departing body weights by 3 wk after treatment, they never achieved body weights equal to the range of control, day-matched rats (Fig. 1B). In fact, on the day before sacrifice, control rats (241.18 ± 6.52 g of body wt; $n = 18$) were significantly ($p < 0.05$) heavier than E₂V animals (217.23 ± 4.17 g of body wt; $n = 18$). More important, E₂ levels in 61-d-old animals were significantly ($p < 0.05$) higher in the E₂V than in the control group (155.75 ± 13.99 vs 65.89 ± 7.08 pg/mL, respectively).

Impact of Estradiol Treatment on Parametrial Adiposity Function

Total parametrial fat mass was significantly ($p < 0.02$) reduced in E₂V vs control rats (2.81 ± 0.13 vs 4.21 ± 0.62 g, respectively; $n = 8$ rats/group); however, the number of adipocytes, obtained after enzymatic isolation of dissected fresh parametrial fat tissue, was significantly ($p < 0.05$) higher in the E₂V than in the control group ($n = 8$ rats/group; Fig. 2A). Figure 2B shows the results of leptin released into the medium by isolated adipocytes incubated in the absence or presence of several concentrations of insulin (0.1–2.0 nM). Spontaneous leptin release in vitro, by a similar number of adipocytes from both groups (400,000 cells/tube), was significantly ($p < 0.02$) higher in the E₂V than in the control cell group. Insulin-induced leptin release was found in a concentration-related fashion in control cells; conversely, no significant adipocyte responses to insulin were obtained in the E₂V cell group. Finally, histologic observations of parametrial fat tissue indicated that control (Fig. 3A) and E₂V (Fig. 3B) groups displayed cytoplasmic rim of mature unilocular adipocytes of similar size.

Longtime Effects of E₂V

Administration on Metabolic Responses to Fasting

No differences between groups were found in plasma glucose levels in nonfasting condition (1.21 ± 0.06 and 1.09 ± 0.04 g/L in control and E₂V groups, respectively) and in the response to fasting (0.79 ± 0.05 and 0.78 ± 0.04 g/L in control and E₂V groups, respectively; $p < 0.02$ vs respective nonfasting condition values). Similarly, no differences between groups were found in nonfasting plasma triglyceride levels (Fig. 4A), and whereas fasting significantly ($p < 0.05$ vs nonfasting condition) reduced plasma triglyceride levels in control rats, it failed in E₂V animals; thus, fasting in E₂V animals resulted in hypertriglyceridemia vs control rats (Fig. 4A). Unfasting plasma insulin concentra-

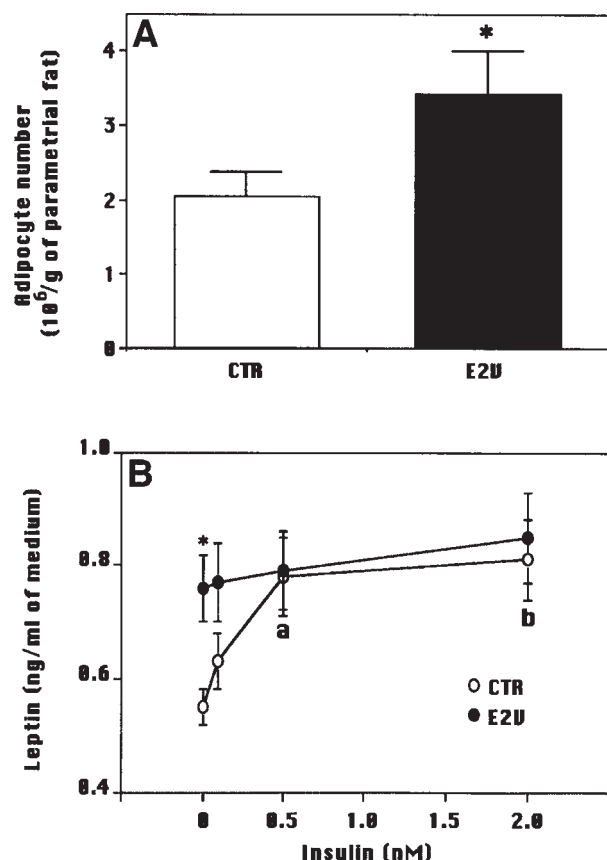


Fig. 2. (A) Isolated parametrial adipocyte number from unfasted control and E₂V rats on d 61 posttreatment. Bars represent the mean \pm SEM ($n = 8$ rats/group). (B) In vitro basal (insulin: 0 nM) and insulin-induced leptin release by isolated parametrial adipocytes from nonfasting control and E₂V rats on d 61 posttreatment. Values are the mean \pm SEM ($n = 3$ different experiments, with six tubes per point per experiment). a: $p < 0.05$ vs basal control values; b: $p < 0.01$ vs basal control values and $p < 0.05$ vs 0.1 nM insulin control values; * $p < 0.02$ vs control values in similar condition.

tions and the changes in circulating insulin levels after fasting were similar in both groups (Fig. 4B). E₂V rats displayed significantly ($p < 0.05$) higher nonfasting plasma leptin concentrations than control animals, and a significant ($p < 0.05$ vs the respective values in nonfasting condition) decrease in plasma leptin levels after fasting was found in both groups; nevertheless, fasting E₂V rats were hyperleptinemic vs control animals (Fig. 4C). Finally, while E₂V treatment significantly ($p < 0.02$ vs control nonfasting values) reduced plasma tumor necrosis factor- α (TNF- α) levels, fasting did not modify circulating TNF- α levels in any group (Fig. 4D).

Lasting Effects of E₂V Treatment on Metabolic Responses to High Glucose Load

Figure 5 shows the results of several parameters evaluated in 24-h fasting rats before (sample time zero) and several times after ip high glucose load. Whereas plasma glucose

levels were similar in both groups 30 min postcarbohydrate load, at 60 min postinjection, circulating glucose levels were significantly ($p < 0.05$) lower in E₂V than in control rats. Finally, at 120 minutes postglucose, E₂V but not control rats had recovered the respective basal plasma glucose values (Fig. 5A). Circulating insulin concentrations (Fig. 5B) were similar in both groups 30 and 60 min postglucose load, whereas at 120 min postglucose E₂V rats recovered basal insulin values, but control rats showed higher ($p < 0.05$) plasma insulin levels than basal values. Regarding adipocyte responses to high glucose, whereas plasma triglyceride levels did not vary at any time examined in control rats (Fig. 5C), plasma lipid concentrations significantly ($p < 0.05$ vs respective basal values) decreased 60 and 120 min postglucose in E₂V rats (Fig. 5C). Interestingly, whereas plasma leptin levels were significantly ($p < 0.05$ vs respective basal values) enhanced 120 min postglucose in control rats, high carbohydrate load did not modify plasma leptin levels in E₂V animals (Fig. 5D). As expected, carbohydrate load did not change plasma TNF- α levels in any group (not shown).

Discussion

Our study clearly indicates that acute E₂V administration resulted in a potent anorexigenic signal and induced loss in body weight. The anorectic activity of E₂ took place when high circulating levels were attained. Thereafter, 2 wk posttreatment, the profound anorectic effect of E₂ disappeared and rats ate normally, thus recovering their initial body weights by 3 wk posttreatment. However, by the end of the period examined, estrogenized rats did not reach the same body weight range of age-matched control animals and had significantly lower parametrial fat mass than their counterparts. Important is that 61-d-old E₂V rats were hyperleptinemic and displayed E₂ plasma levels (see Results) similar to those attained by the end of pregnancy (162.87 ± 36.21 pg of E₂/mL; mean \pm SEM; $n = 6$ rats), a state characterized by relative leptin resistance (24). This finding strongly supports that, similar to that accounted in the pregnant rat, a new set point in the control of energy homeostasis might be established in the E₂V rat model.

Regarding the role of E₂ in the regulation of food intake, it has been reported that ovariectomy enhances eating and that E₂ replacement therapy reverses this effect (25); these results have been attributed to a direct effect of E₂ on hypothalamic NPY neurons (19). However, paraventricular nucleus (PVN) NPY production in ovariectomized (OVX) rats could increase (20) or decrease (21), depending on whether estradiol is administered on a short-term basis or in a sustained fashion, respectively. NPY neurons are known to communicate directly with other orexigenic signals, such as galanin-producing neurons (26), and the pattern of expression of the hypothalamic galanineric system seems to be also regulated by estradiol (27). Similarly, the expression of some anorexigenic pathways (e.g., PVN corticotropin-

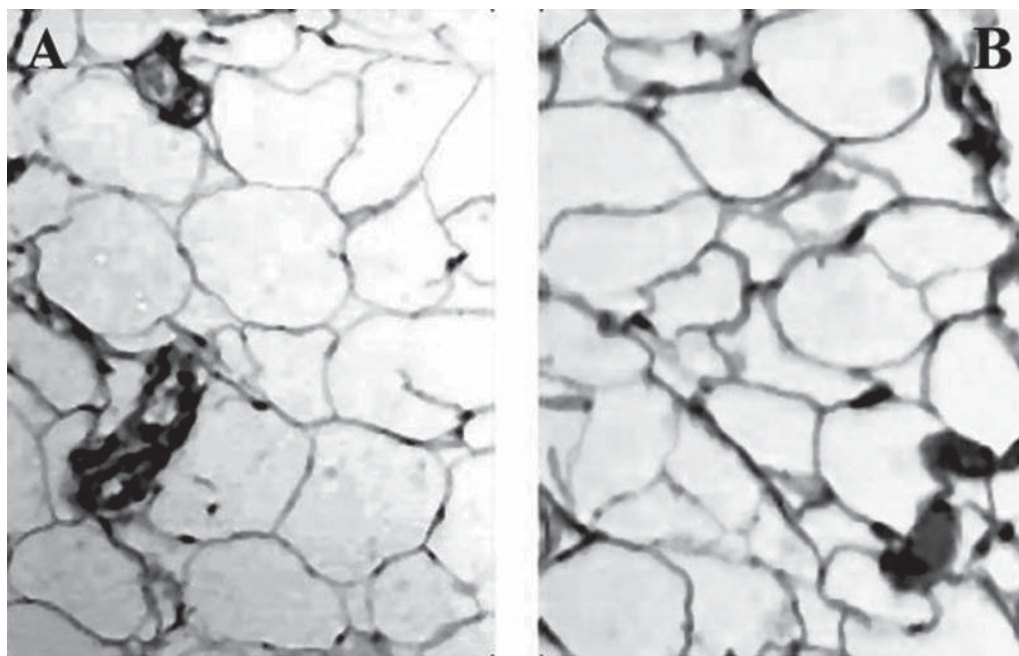


Fig. 3. Hematoxylin and eosin-stained parametrial adipose tissue from (A) control and (B) E₂V rats, 61 d posttreatment, showing cytoplasmic rim of mature unilocular adipocytes of similar size in both groups; magnification: $\times 20$.

releasing hormone and arcuate nucleus proopiomelanocortin) (18) seems to be under the regulatory control of E₂ (28). Because leptin administration in intact and OVX mice induced similar weight loss, a lack of E₂ modulation on the leptin effect has been claimed (29). However, it has been shown that E₂ is able to modify the expression of hypothalamic leptin receptor isoforms (30). Therefore, changes in the hypothalamic sensitivity to leptin could be an additional factor contributing to the establishment of a new set point of leptin action in the estrogenized rat model.

A sex steroid dependency of adipogenesis has been accepted, but, contrary to the proadipogenic effects attributed to E₂ (10), this steroid has also been implicated in parametrial (22) fat lipolysis. Positive and negative effects of E₂ on lipolysis and lipogenesis, respectively, have been claimed (31). However, the antilipogenic effect of E₂ is strongly supported by both, with results showing spontaneous increases in adipocyte hyperplasia and hypertrophy in the E₂ receptor α knockout mouse model (32), and studies indicating that E₂ inhibits adipogenesis in cultured mouse cell lines (33,34) by reducing peroxisome proliferator-activated receptor- γ mRNA expression (33). We found that the lasting effect of E₂ in the intact female rat induced an enhancement in both plasma leptin levels and parametrial adipocyte leptin output in vitro. Our data find support in the already recognized stimulatory effect of estrogen on parametrial fat leptin secretion in culture (13). Interestingly, we found in the estrogenized rat model decreased parametrial fat mass, regardless of increased parametrial fat adipocyte number and in vitro leptin output, without changes in adipocyte size,

data clearly indicating enhanced lipolysis (12). It is possible that increased leptin output could play a cooperative role for enhanced lipolysis by acting through an autocrine mechanism (35).

It is important that E₂V rats showed increased plasma triglyceride levels (vs control rats) in fasting condition; the increase in triglycerides seems to be a phenomenon dependent on enhanced estradiol production, as it was described during pregnancy (36). The data indicate that another characteristic metabolic state is shared during both E₂ therapy and pregnancy, thus supporting that changes in the set point of energy control in E₂V rats has occurred. However, after high glucose load, and contrary to that accounted in control rats, while fasting plasma leptin concentrations did not change, circulating triglycerides levels decreased in E₂V animals. The lack of in vivo changes in circulating leptin levels post-glucose load, a test normally characterized by increased insulin secretion, fully agrees with our in vitro data. In fact, and in an opposite way to that occurring in control parametrial fat, incubation of isolated adipocytes from E₂V rats not only released a high amount of leptin, but also were unresponsive to insulin stimulation. Regarding the modification in triglyceride levels post-glucose load, our data could indicate that, in hyperleptinemic rats, circulating triglycerides could result in the immediate fuel to be cleared after high carbohydrate load-stimulated metabolism. Additionally, our results fully agree with previous experiments showing that adenovirus gene transfer-induced hyperleptinemia, in normal rats, resulted in the depletion of triglyceride contents in liver, pancreas, and skeletal muscle (37).

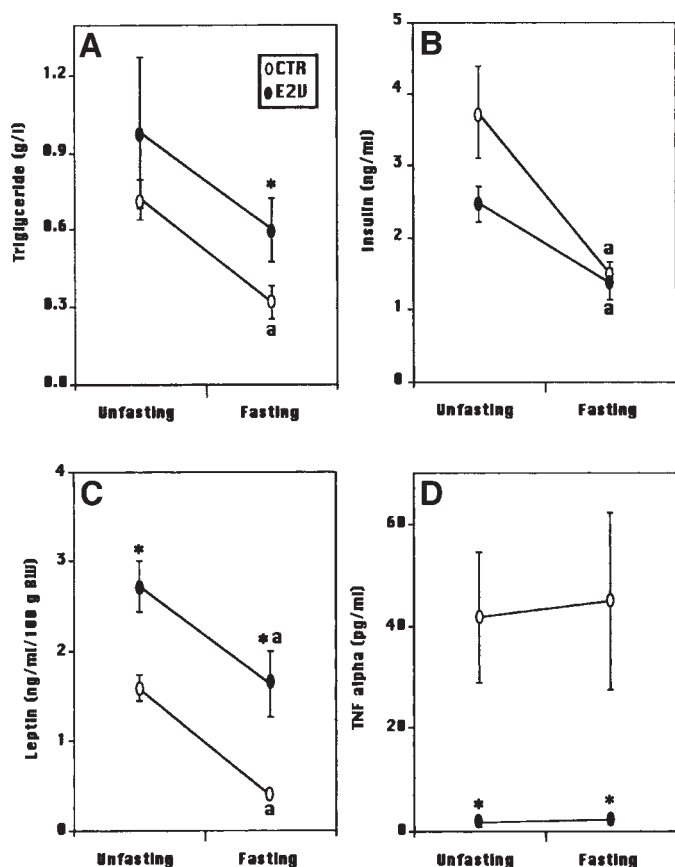


Fig. 4. Plasma concentrations of (A) triglyceride (TG), (B) insulin, (C) leptin, and (D) TNF- α in control and E₂V rats examined, on d 61 posttreatment, in nonfasting and after 24-h fasting conditions. Values are the mean \pm SEM ($n = 8$ rats/group condition). a: $p < 0.05$ or less vs respective group values in nonfasting condition; * $p < 0.05$ or less vs control values in similar condition.

We demonstrated that estrogenized rats have developed, as occurred in other animal models (38,39), tissue-specific changes in insulin sensitivity, such as improved in vivo peripheral insulin sensitivity, after high glucose load, and unresponsive parametrial adipocytes to insulin stimulation; however, it must be stressed that these adipocytes spontaneously secreted high leptin, a protein known to inhibit insulin binding in adipocytes (40). These facts probably indicate that changes in parametrial adipocyte function could be contributing to the development of a new set point in the control of energy balance in estradiol-treated rats. Although we cannot be precise as to the exact mechanisms modified that could explain the enhanced insulin sensitivity in hyperleptinemic E₂V rats, leptin overexpression (41) and estradiol treatment (42) have been proposed to improve insulin sensitivity. Additionally, decreased circulating levels of TNF-

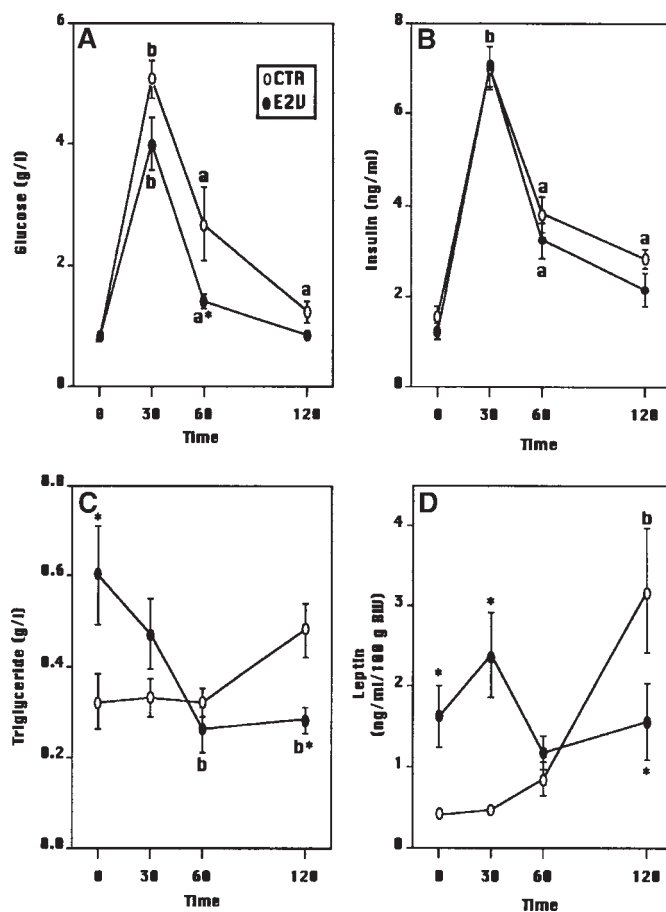


Fig. 5. Circulating plasma levels of (A) glucose, (B) insulin, (C) triglyceride (TG), and (D) leptin before (sample time zero) and several times after high glucose load (3 g/kg of body wt; intraperitoneally) in 24-h fasting, control, and E₂V rats on d 61 posttreatment. Values are the mean \pm SEM ($n = 8$ rats/group). a: $p < 0.02$ or less vs sample time zero values of the same group; b: $p < 0.01$ or less vs all other time values of the same group; * $p < 0.05$ or less vs control values in similar condition.

α , a cytokine known to reduce insulin sensitivity (43) and leptin secretion (44), could be another factor supporting our data. However, because of the enhanced parametrial adipose tissue function in the estrogenized rat, the involvement of other adipocyte product(s) in the development of some of the changes found in our study should not be discarded. For instance, adiponectin (45) is able to decrease glucose levels (45) by increasing insulin sensitivity (46) and triglyceride oxidation (47); moreover, adiponectin (48) and estradiol (49) suppress TNF- α production.

In summary, we found that high circulating levels of estradiol act acutely as a very strong anorectic signal. Thereafter, once declined in circulation, estradiol was able to modify parametrial adipose tissue function and to enhance peripheral insulin sensitivity. Our results provide strong evidence for an adaptation of the appetite-regulating circuitry in the

estrogenized rat model rendering a new homeostatic control that will probably protect the organism against unfavorable conditions such as starvation.

Materials and Methods

Animals and Experimental Design

The estrous cycle of adult Sprague-Dawley rats was followed for 1 wk. Individually housed rats were weighed and then injected intramuscularly with 200 μ L of corn oil alone (control; 204.67 ± 6.76 g of body wt; $n = 18$) or containing 2 mg of E₂V (201.17 ± 3.95 g of body wt; $n = 18$) as previously reported (23). Rats were kept in a temperature (20–22°C)-controlled room (12-h light/dark cycle, lights on at 7:00 AM) with water available ad libitum; food was provided daily in a controlled manner (50 g of Purina diet/d) at 8:00 AM. The following day, rat body weight and the remaining food per cage were recorded. The amount of food eaten was calculated by the difference between 50 g and the grams of food found in the cage; then, the amount of food eaten over the last 24 h was rectified with respect to body weight at the end of that period of time and expressed in grams of food eaten by 100 g of body wt. This parameter was recorded until the experimental day (d 61 after treatment). In addition, the estrous cycles of rats were followed every day, from d 1 to d 60 posttreatment, at noon.

Experiment 1

Experiment 1 was performed in order to determine some metabolic parameters in basal condition and the response to 24 h of fasting. Individually caged nonfasting rats, from control and E₂V groups (with indwelling iv cannulae implanted 48 h before the experiment), were bled (8:00 AM) and left undisturbed for 24 h without access to food until bleeding on the next morning in fasting condition (8:00 AM). Plasma samples were split into aliquots and kept frozen (–20°C) until determination of glucose, triglyceride, estradiol, TNF- α , insulin, and leptin concentrations.

Experiment 2

Metabolic responses to high glucose load were determined in 24-h fasting control and E₂V rats (with indwelling iv cannulae implanted 48 h before the experiment). Animals were bled before (sample time zero) and at several times (30, 60, and 120 min) after ip administration of glucose (3 g/kg of body wt) (50). Plasma samples, split into aliquots, were kept frozen (–20°C) until determination of different parameters.

Experiment 3

Intact animals from both groups were killed by decapitation, in nonfasting condition, and parametrial fat tissue was dissected, weighted, and used for isolation of adipocytes, as described next, or processed for light microscopy. All experimentation received official approval from our institutional animal care committee.

Histologic and Functional Studies of Adipose Tissue

Parametrial adipose tissue of five animals from each group was fixed in Bouin's fluid and embedded in paraffin. Sections of 4 μ m were obtained at different levels of the blocks and stained with hematoxylin and eosin.

Isolation of adipocytes was performed as previously described (51) with minor modifications. Briefly, dissected parametrial fat (from both experimental groups) was slightly minced and placed in KREBS-MOPS medium (Sigma-Aldrich, St. Louis, MO) containing 1% bovine serum albumin (BSA) and 0.1% collagenase (Type 1; Sigma-Aldrich), in a ratio of 3 mL of solution/g of fat tissue. Tissues were gently shaken at 37°C for 50 min in polypropylene flasks under a 95% air–5% CO₂ atmosphere, in a metabolic incubator. At the end of this period, cell suspensions were filtered through one layer of nylon cloth (300 μ m), transferred to conical polypropylene tubes, and centrifuged at 100g for 20 min at room temperature. Cells were then washed three times with medium alone to eliminate the stromal vascular fraction and collagenase. Adipocytes were counted and diluted with Dulbecco's modified Eagle's medium (Sigma-Aldrich)–1% BSA medium, pH 7.4, to the necessary volume to obtain approx 4×10^5 adipocytes/1.4 mL of medium. This volume was distributed into each of 15-mL conical polypropylene tubes and 0.1 mL of medium, alone or containing different concentrations of insulin (Sigma-Aldrich; final concentrations ranging from 0.1 to 2.0 nM), was added to the tubes. At least six tubes per condition were used in each experiment. The tubes were incubated, by shaking at 37°C, for 2 h under a 95% air–5% CO₂ atmosphere. At the end of incubation, the tubes were centrifuged for 20 min at 100g, and the infranatant was separated from adipocytes for leptin measurement.

Determination of Hormones and Metabolites

Plasma glucose and triglyceride levels were determined by enzymatic assays from Wiener Argentina. Circulating concentrations of estradiol were determined by specific radioimmunoassay (RIA) as detailed previously (52); coefficients of variation (CVs) for intra- and interassays were 4–6 and 10–12%, respectively. Insulin plasma concentrations were determined by a specific rat insulin enzyme immunoassay kit from SPI-BIO (France); the standard curve was between 0.08 and 10 ng/mL, and intra- and interassay CVs were 2–4 and 6–8%, respectively. TNF- α circulating levels were determined by enzyme-linked immunosorbent assay (Bio-trak Assay; Amersham, UK); the standard curve was between 30 and 2500 pg/mL, and intra- and interassay CVs were 3–5 and 7–9%, respectively. Finally, plasma and medium leptin concentrations were assayed by a specific RIA described previously (53). The intra- and interassay CVs were 5–8 and 10–13%, respectively.

Data Analysis

Data, expressed as the mean \pm SEM, were analyzed by analysis of variance (ANOVA), followed by Fisher test for

comparison of different mean values (54). ANOVA with repeated measures, followed by the student-Newman-Keuls test for comparison of different mean values, was used when appropriate (54).

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