The anorectic effect of oestradiol does not involve changes in plasma and cerebrospinal fluid leptin concentrations in the rat

M Rocha, E Grueso and M Puerta

Department of Animal Biology II (Physiology), Faculty of Biological Sciences, Complutense University, 28040 Madrid, Spain
(Requests for offprints should be addressed to M Puerta; Email: mpuerta@eucmax.sim.ucm.es)

Abstract

Oestradiol is a potent anorectic agent that reduces both food intake and body weight. Since leptin is known to reduce food intake, we first analysed if the anorectic effect of oestradiol is driven by an increased leptin concentration in either cerebrospinal fluid or plasma. Oestradiol also reduces body weight and fat mass. Accordingly, a decrease in plasma leptin concentration can also be expected after an oestradiol-driven reduction in fat mass. To test this hypothesis was the second aim of this study. Female Wistar rats received oestradiol chronically during 14 days. During the first week of treatment there was a reduction in

food intake, body weight and fat mass that returned to initial values during the second week, but no changes in *ob* mRNA levels were found in white adipose tissue depots. There was no effect of treatment or time on plasma and cerebrospinal fluid leptin concentrations. Therefore, the anorectic effect of oestradiol is not driven by an increase in leptin concentration either in plasma or in cerebrospinal fluid, and the reduction in fat mass that oestradiol produces is not followed by a reduction leptin concentration.

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Introduction

It has been shown that white adipose tissue (WAT) produces the hormone leptin, the plasma concentration of which is directly related to the amount of body fat (Considine *et al.* 1996, Pratley *et al.* 1997, Greenberg & Boozer 1999) and is therefore considered to be the messenger signalling the size of the fat store to the central nervous system (CNS), in agreement with the lipostatic theory (Kennedy 1953, Hausberger 1959). On a short-term basis, plasma leptin concentrations also follow food intake patterns so that, despite an essentially unchanged amount of WAT during a 24 h cycle, nocturnal ingestive episodes in rats increase plasma leptin concentration, whereas no intake during the inactive phase of the cycle or fasting, decreases it (Ahima *et al.* 1996, Weigle *et al.* 1997, Pickavance *et al.* 1998). Therefore, leptin is considered both a lipostatic and a satiating signal.

The effects of oestradiol on energy balance have been known for a long time. Thus ovariectomized (ovx) rats receiving either daily injections or subcutaneous implants of oestradiol show both a reduced food intake and a reduced body weight (Wade 1975, Richard 1986) – effects that are also evident in intact rats (Puerta *et al.* 1990). These effects are probably driven by actions of oestradiol at a central level, as its administration to the hypothalamic nuclei of rats reduces food intake (Butera *et al.* 1990, Dagnault & Richard 1997, Lindblom *et al.*

2000). However, it can be argued that an enhancement of the leptin concentration either in plasma or in cerebrospinal fluid (CSF) could be involved. Certainly, oestradiol has been shown to increase *ob* mRNA expression in WAT of ovx rats (Shimizu *et al.* 1997, Brann *et al.* 1999) and in adipocytes isolated from ovx rats (Machinal *et al.* 1999), this increase being followed by an enhanced concentration of leptin in the culture medium. A single dose of oestradiol in ovx rats also increased plasma leptin concentration (Brann *et al.* 1999). The first aim of this study was therefore to determine if the anorectic effect of oestradiol was mediated by an increase in leptin concentration. It can also be reasoned that, as soon as food intake decreases, a decrease in plasma leptin concentration could be expected – a decrease that would be even greater once the concomitant body weight loss (and hence fat mass) began. In this way, the decreased leptin concentrations would counterbalance the oestradiol-induced reduction in food intake. The second aim of the study was to investigate this hypothesis.

Materials and Methods

Female Wistar rats, 200–220 g body weight, were acclimated to 28 °C (thermoneutrality). On day 0 of the experiment (221±1 g body weight), 50% of the animals received one 17β-oestradiol-filled Silastic capsule (Dow

Corning, MI, USA; 0.5 cm long; 3.2 mm o.d.; 1.5 mm i.d.). Control rats received empty implants. Capsules were inserted subcutaneously with the animal under ether anaesthesia. This method ensures a constant release of the hormone, increasing its plasma concentration for more than 1 month (Smith *et al.* 1977, Richard 1986, Nava *et al.* 1994). Rats were housed in individual cages with food and water available *ad libitum* and with a darkness:light cycle of 12 h:12 h, lights on at 0800 h. The food provided was a commercial diet (Panlab, Barcelona, Spain) containing: 66.7% carbohydrate, 19.3% protein, 3.4% fat, 4.9% cellulose and 5.7% mineral (w/w). Food intake and body weight were monitored daily. Food intake was measured by weighing what remained in the food cup. Animals were cared for and used in accordance with the principles of The Council of European Communities (86/609 EEC).

Both control and treated animals were killed (by decapitation) at different times (days 2, 8 and 14) during the experiment, between 1100 h and 1300 h. Just before the animal was killed, and with the animal under ether anaesthesia, CSF was obtained by the insertion of a 23-gauge butterfly needle into the cisterna magna and the gentle withdrawal of 80–100 µl CSF. Only samples without any blood contamination were processed. Vaginal smears were taken to corroborate the efficacy of treatment in oestradiol-treated rats. Blood was collected in a heparinized glass tube (10 IU/ml). Right-side WAT pads – inguinal, parametrial and retroperitoneal – were removed and immediately frozen in liquid N₂ and stored at –80 °C until required for analysis. Left-side pads – inguinal, parametrial, retroperitoneal, perirenal and periovaric – were weighed. Total depot weight was calculated as twice the left-side pad weight. The abundance of WAT was estimated as an adiposity index modified for that described previously (Taylor & Phillips 1996) and calculated as the sum of inguinal, parametrial, retroperitoneal, perirenal and periovaric depots divided by body weight and multiplied by 100.

Total cellular RNA was extracted using Ultraspec RNA reagent (Biotecx Laboratories, TX, USA), a modified procedure of the single-step method reported for total RNA isolation (Chomczynski & Sacchi 1987). RNA fractionation, blotting, hybridization and chemiluminescent detection were carried out according to previously published procedures (Trayhurn *et al.* 1994). In brief, RNAs were fractionated by agarose gel electrophoresis, transferred to a positively charged nylon membrane (Roche Molecular Biochemicals, Mannheim, Germany) by capillary blotting and crosslinked with UV light using a Foto/UV 21 (Fotodyne, WI, USA). The mRNA for *ob* gene was detected using a 33-mer antisense oligonucleotide probe 5'-labelled with a single digoxigenin ligand (5'-GGT CTG AGG CAG GGA GCA GCT CTT GGA GAA GGC-3'). The membranes were incubated sequentially with the oligonucleotide and with an anti-digoxigenin serum alkaline phosphatase-conjugated

antibody and processed essentially as in the procedures provided by Roche Molecular Biochemicals. CDP-star was used as the chemiluminescent substrate. Signals were visualized by exposing the membranes to a film (Hyperfilm ECL, Amersham Pharmacia Biotech, Amersham, Bucks, UK) and quantified by densitometry using NIH Image software. Membranes were stripped and reprobed for 18S rRNA to check the loading and transfer of RNA during blotting. 18S rRNA was detected using a 31-mer antisense oligonucleotide probe (5'-CGC CTG CTG CCT TCC TTG GAT GTG GTA GCC G-3').

Blood was centrifuged at 2000 g for 15 min at 4 °C. Plasma was withdrawn and stored at –80 °C until required for analysis. Plasma leptin concentrations were determined with a commercially available rat leptin RIA kit (Linco Research, St Charles, MO, USA). The limit of sensitivity for assay was 0.5 ng/ml and the limit of linearity 50 ng/ml. The intra- and interassay coefficients of variation were 4.6% and 5.7% respectively. CSF either from one rat or pooled from two rats was lyophilized and assayed by redissolution in a smaller volume than that indicated in the Linco kit for plasma samples. The other components of the kits were reduced accordingly. The standard curve obtained with reduced volumes was validated with the quality controls, as the values fitted their expected range. β-Hydroxybutyrate plasma concentration, measured as an index of starvation or severe fasting, was determined with a commercially available kit (Sigma Diagnostic, St Louis, MO, USA).

Two-way analysis of variance (ANOVA) was performed to compare WAT depot weight, plasma and CSF leptin concentrations, β-hydroxybutyrate plasma concentration and adiposity index, time and treatment being the variable factors. A Student–Newman–Keuls test was used for *post hoc* comparisons. Food intake, body weight and *ob* mRNA levels were evaluated by an unpaired Student's *t*-test between corresponding control and treated animals (Mann–Whitney U-test was used when data did not show Gaussian distribution). *P* < 0.05 was considered as statistically significant.

Results

All rats receiving oestradiol had oestrus-like vaginal smears. Food intake was severely decreased on oestradiol administration, but this effect was transitory and disappeared after 7 days of treatment. The greatest decrease took place on day 3 of treatment, when food intake in treated rats was only 80% that of the control group (*P* < 0.05; Fig. 1). Body weight reflected the reduction in food intake so that, after only 3 days of treatment, treated animals showed a reduced body weight, which was 95% of that in controls (*P* < 0.05). Again this effect was transient, differences disappearing after 8 days of treatment (Fig. 1).

Figure 1 Daily food intake and body weight in rats receiving oestradiol chronically. Each point represents the mean $\pm$ S.E.M. of results from 24 animals at days 0–2, 16 at days 3–8 and eight at days 9–14. * $P < 0.05$ compared with the respective control rats by Student's *t*-test (or a Mann–Whitney U-test on day 0 for food intake and on days 0 and 2 for body weight).

Figure 2 depicts the changes in individual WAT depots. Inguinal and parametrial depot weight were significantly lighter ($P < 0.05$) on day 2 of treatment (Fig. 2). The entire retroperitoneal, perirenal and periovaric mass was also reduced in treated animals on day 2, but the difference did not reach statistical significance (Fig. 2). No differences were found in WAT depot weight of control and treated rats on day 8 of treatment (Fig. 2). On day 14 of treatment, WAT depots were heavier in treated rats than in controls, although only the parametrial depot weight difference reached statistical significance ($P < 0.05$). The adiposity index changed in parallel with changes in individual depot weight, so that it decreased after 2 days of treatment ($P < 0.05$), was similar to control on day 8 and was greater than in controls on day 14 ($P < 0.05$; Table 1). Figure 3 depicts *ob* mRNA levels as arbitrary units in inguinal, parametrial and retroperitoneal WAT depots. Treatment with oestradiol did not change the *ob* mRNA level in any of the three depots at any time of treatment (Fig. 3).

There was no effect of treatment or time on plasma leptin concentration (Fig. 4). CSF leptin concentration

Figure 2 Weight of several WAT depots in rats receiving oestradiol chronically. Each bar represents the mean $\pm$ S.E.M. of eight animals for inguinal and parametrial depots and four animals for the retroperitoneal (RT), perirenal (PR) and periovaric (PO) depots. * $P < 0.05$ compared with respective controls.

was slightly decreased in treated animals on day 2 (Fig. 5) but the difference did not attain statistical significance. No differences in CSF leptin concentration were found on days 8 and 14 between treated and control rats (Fig. 5). There was no effect of treatment or time in plasma β -hydroxybutyrate concentration (Fig. 6).

Table 1 Adiposity index (sum of WAT depots (g) $\times$ 100)/body weight (g) in rats receiving oestradiol chronically. Values are means $\pm$ S.E.M. ($n = 4$ animals)

	Adiposity index		
	Day 2	Day 8	Day 14
Control	3.4 $\pm$ 0.2	3.8 $\pm$ 0.6	3.2 $\pm$ 0.2
Treated	2.6 $\pm$ 0.2*	3.0 $\pm$ 0.3	4.1 $\pm$ 0.3*

* $P < 0.05$ compared with respective control.

A. **B.**

Figure 3 (A) *ob* mRNA levels in different WAT depots in rats receiving oestradiol chronically. Each bar represents the mean $\pm$ S.E.M. of four to eight rats. (B) Northern blot of inguinal WAT at days 2, 8 and 14 of treatment. 18S rRNA was used to check loading and transfer.

Discussion

As expected, oestradiol treatment reduced food intake, in agreement with previous findings (Wade 1975, Richard 1986, Puerta *et al.* 1990). Thus, after 3 days receiving oestradiol, treated rats ate only 80% of the amount consumed by the controls. Nonetheless, no increase in plasma or CSF leptin concentration was found at that time, which excludes an increase in leptin signalling as the origin of the reduction in food intake. The oestradiol-driven reduction in food intake reduced the body weight of the treated animals to 95% the body weight of controls. A substantial part of this weight loss was due to fat loss, as judged by both the smaller weight of the WAT depots and the smaller adiposity index on day 2. Both reduced food intake (Ahima *et al.* 1996, Weigle *et al.* 1997, Pickavance *et al.*

Figure 4 Plasma leptin concentration in rats receiving oestradiol chronically. Each point represents the mean $\pm$ S.E.M. of four to six animals.

1998) and reduced fat mass (Greenberg & Boozer 1999), are known to be followed by decreased plasma leptin concentration. However, despite the presence of both

Figure 5 CSF leptin concentration in rats receiving oestradiol chronically. Each bar represents the mean $\pm$ S.E.M. of four to six samples.

Figure 6 β -Hydroxybutyrate plasma concentration in rats receiving oestradiol chronically. Each point represents the mean $\pm$ S.E.M. of seven or eight animals.

these negative influences – reduced food intake and fat loss – in our rats on day 2, no decrease in either *ob* mRNA levels in WAT or in plasma leptin concentration were found, which meant that the compensatory increase in food intake predicted by the adipostatic theory was not taking place. Therefore, it appears that, to allow the sustained reduction in food intake we have observed, other compensatory factors promoting leptin synthesis were also taking place. Indeed, oestradiol has been shown to increase *ob* mRNA expression in WAT of ovx rats (Shimizu *et al.* 1997, Brann *et al.* 1999) and in adipocytes isolated from ovx rats (Machinal *et al.* 1999), this increase being followed by an enhanced leptin concentration in the culture medium. A single dose of oestradiol also increased plasma leptin concentration in ovx rats (Brann *et al.* 1999). This peripheral stimulatory action of oestradiol should be taken into consideration in our treated rats, in which it was most probably a contributory factor determining the actual plasma leptin concentration. Summing up, our results indicate that, when the plasma oestradiol concentration increases, a decrease in food intake supervenes, with a concomitant reduction in fat mass and body weight. However, in contrast to what might be expected from the adipostatic and satiety roles of leptin, neither an increase in plasma or CSF leptin concentrations as the origin of reduced food intake was found, nor did any reduction in plasma leptin or CSF concentrations take place after food intake and fat mass reduction. Indirectly, our results suggest that oestradiol brings about its effect on food intake by acting at a central level, probably acting upon nervous nuclei controlling food intake and satiation, as suggested by others (Butera *et al.* 1990, Dagnault & Richard 1997, Lindblom *et al.* 2000). The similar leptin concentrations in both CSF and plasma of control and treated rats also suggest that oestradiol does not change leptin entrance into the CNS, although this was not demonstrated in our study. Nonetheless, results presented in this paper do not exclude oestradiol modulating hypothalamic leptin sensitivity as another mechanism to reduce food intake. Leptin CSF values observed in our study are in agreement with those previously published for rats (Ishizuka *et al.* 1998, Grueso *et al.* 2001).

Seven days after the implantation of the Silastic capsules, food intake had returned to that of untreated controls. After 14 days of treatment, body weight of treated rats was almost similar to that of controls. However, the plasma oestradiol concentration was still increased at that time (Nava *et al.* 1994). In other words, the effects of oestradiol were not permanent, which is in agreement with previous findings (Wade 1975). Certainly, if present for a long period of time, the potent negative effect of oestradiol on energy balance could lead to potentially fatal body weight losses. Therefore, the existence of a control mechanism is not surprising. Our results showed that, in intact rats, the anorectic effects of oestradiol were evident for 3 days, but from this time onwards they become less potent until they

disappeared, which suggest a desensitization process. The 3-day period of time before the initiation of the suggested desensitization would allow oestradiol to exert its anorectic effect to the full during the physiological increases in the hormone – that is, during the 10–12 h of pro-oestrus in every oestrus cycle (Ter Haar 1972, Butcher *et al.* 1974, Wade 1976) and during the last day of pregnancy (Lu *et al.* 1998). It seems reasonable to argue that those anorectic episodes could be beneficial either for mating or for labour respectively, as they release the female from the constraints of searching for food during those periods of time.

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