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# A Detailed Study on the Role of Sex Steroid Milieu in Determining Plasma Leptin Concentrations in Adult Male and Female Rats

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**We examined the effects of sex steroid milieu on plasma leptin levels in adult male and female rats. Since the body weight is known to influence leptin concentrations, the hormone was measured in rats with a very similar body weight (about 250 g) throughout this study. Plasma leptin levels were significantly higher in female than in male rats. Orchidectomy (ODX) caused a significant rise in leptin, and replacement of a physiological dose of testosterone (T) completely abolished the effect of ODX. Since the effect of tamoxifen (estrogen antagonist) coadministered with T on leptin levels in ODX rats was the same as that of T alone, it was suggested that the suppressive effect of T on leptin may be mediated by the androgenic potency of T, but not by its aromatized product, estradiol. In female rats, plasma leptin concentrations did not change significantly during the estrous cycle. Furthermore, leptin levels were not affected either by ovariectomy alone or by the administration after ovariectomy of physiological doses of estradiol, progesterone, or both. This is the first study to demonstrate in rats with a very similar body weight the existence of a clear sexual difference in plasma leptin levels, and also a suppressive action of T on the adipocyte hormone concentrations.** © 1999 Academic Press

Leptin, the *obese (ob)* gene product, is a hormone secreted by adipocytes (1, 2). Leptin is considered as a homeostatic signal that contributes to body weight regulation through modulating feeding behavior and energy expenditure (2, 3). Accumulating evidence indicates that in both humans (4-9) and rodents (10), females have higher levels of circulating leptin than their male counterparts at any level of adiposity. Recent studies in humans suggest that T, which has a suppressive effect on leptin production, may cause the lower level of leptin in men than in women (11-16). Although several studies reported that estrogen may also elevate circulating leptin levels in humans (17-20), conflicting reports also exist (15, 21, 22).

As for rodents, to the best of our knowledge there are only two previous studies which examined the effects of gonadal steroid milieu on circulating leptin concentrations. Shimizu *et al.* (17) reported a stimulatory effect of $E_2$ on serum leptin levels in female rats, but Amico *et al.* (23) reported that serum leptin concentrations did not change significantly during the estrous cycle of the rat. However, it has yet to be explored whether T suppresses leptin levels also in rats, and if it does, whether the androgenic potency of T itself or $E_2$ aromatized from T, may play a role. Moreover, it is largely unknown whether P, another ovarian steroid, is involved in regulating leptin levels in the general circulation.

Therefore, in this study we performed a detailed study on the role of sex steroid milieu in determining plasma leptin levels in adult male and female rats. In humans, strong relationships have been found between circulating leptin levels and BW, body mass index, and percentage of body fat (6, 24). Even though whether such relationships also exist in rats remains to be determined, we thought it important and thus elected to compare plasma leptin levels in rats with a very similar BW.

## MATERIALS AND METHODS

*Animals.* Adult male and female rats of the Wistar strain were used. They were housed in an air-conditioned room with controlled lighting (light on from 08:00 to 20:00 h), and were given free access to laboratory chow and tap water. All the following experiments were conducted in accordance with the Guidelines for Animal Experimentation, Hirosaki University.

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Abbreviations: T, testosterone; $E_2$, estradiol; P, progesterone; BW, body weight; ODX, orchidectomy; TAM, tamoxifen; sc, subcutaneous; OVX, ovariectomy.

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**TABLE 1**

Sex-Related Difference in Plasma Leptin Levels

| Group | Number of rats | Plasma leptin (ng/ml) | BW (g) |
|---|---|---|---|
| Intact males | 8 | 1.1 ± 0.3 | 252 ± 4 |
| Intact females (diestrus day 2) | 8 | 3.6 ± 0.5* | 249 ± 4 |

* Significantly different *vs.* intact males.

**TABLE 2**

Plasma Leptin Levels at Various Stages of the Estrous Cycle in Female Rats

| Group | Number of rats | Plasma leptin (ng/ml) | BW (g) |
|---|---|---|---|
| Proestrus | 8 | 3.5 ± 0.4 | 252 ± 4 |
| Estrus | 6 | 3.3 ± 0.3 | 250 ± 3 |
| Diestrus day 1 | 7 | 3.6 ± 0.4 | 248 ± 5 |
| Diestrus day 2 | 7 | 3.2 ± 0.4 | 251 ± 3 |

*Experiment 1: Sex-related difference in plasma leptin levels.* Between 12:00-13:00 h of day, 8 week-old intact male rats weighing about 250 g were rapidly decapitated within 30 seconds of removal from the cage. As for females, vaginal smears were taken daily from 11 week-old intact female rats. Rats displaying at least two consecutive cycles of 4-day duration and weighing about 250 g were sacrificed for blood collection between 12:00-13:00 h on diestrus day 2. Trunk blood was collected into plastic tubes containing EDTA-2Na (2.5 mg/ml blood) and centrifuged at 1,000 g for 20 min and 4°C. The plasma was kept frozen at −20°C until assayed for leptin.

*Experiment 2: Plasma leptin levels in cycling female rats at various stages of their estrous cycle.* As in Experiment 1, also in this study were used female rats which displayed at least two consecutive estrous cycles of 4-day duration. When they weighed about 250 g, vaginal smears were taken between 09:00-10:00 h on the day of the experiment. The stage of the estrous cycle was determined for individual animals, *i.e.* the proestrus, estrus, diestrus day 1, and diestrus day 2. After the rats had been kept undisturbed for about 3 h, they were rapidly decapitated and trunk blood was collected between 12:00-13:00 h. The subsequent treatment of samples was done in the same manner as in Experiment 1.

*Experiment 3: Effects of gonadectomy and sex steroid treatment after gonadectomy on plasma leptin levels in male and female rats.* For male rats, 5 groups were prepared, *i.e.* sham ODX, ODX, ODX + T, ODX + T + TAM, and ODX + $E_2$ groups. Sham ODX and ODX were performed under light ether anesthesia when the animals were 6 weeks of age and weighed about 200 g. ODX + T, ODX + T + TAM, and ODX + $E_2$ groups received until sacrifice a daily sc injection of T (5 mg/kg BW, Nakarai Chemicals, Ltd., Kyoto, Japan), a combination of T (5 mg/kg BW) and TAM (1 mg/kg BW, Sigma Chemical Company, St. Louis, MO), or $E_2$ benzoate (2.5 $\mu$g/kg BW, Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) dissolved in sesame oil, respectively. Both sham ODX and ODX groups were treated daily with sesame oil only. All the treatments were started on the day of ODX or sham ODX. When the animals weighed about 250 g, all the 5 groups were decapitated for blood collection between 12:00-13:00 h of day. Since the BW gain was smaller in ODX and ODX + $E_2$ groups than in the remaining three groups, the former two groups were sacrificed 24-28 days after ODX. The remaining three groups were all sacrificed 12-16 days after ODX or sham ODX. The subsequent treatment of blood samples was done in the same manner as in Experiment 1.

Also for female rats, 5 groups were prepared, *i.e.* sham OVX, OVX, OVX + $E_2$, OVX + P, and OVX + $E_2$ + P groups. Sham OVX and OVX were performed under light ether anesthesia when the animals were 9 weeks of age and weighed about 230 g. OVX + $E_2$, OVX + P, and OVX + $E_2$ + P groups received until sacrifice a daily sc injection of $E_2$ benzoate (2.5 $\mu$g/kg BW), P (5 mg/kg BW, Mochida Pharmaceutical Co., Ltd.), or a combination of these, respectively. Both steroids were dissolved in sesame oil for injection. Sham OVX and OVX groups were treated daily with sesame oil only. All the treatments were started on the day of OVX or sham OVX. When the animals weighed about 250 g, all the 5 groups were decapitated for blood collection between 12:00-13:00 h of day. Since sham OVX rats were normally cycling, they were decapitated on diestrus day 2. Since the BW gain was larger in both OVX and OVX + P groups than in the remaining three groups, the former two groups were sacrificed 12-14 days after OVX, and the remaining three groups were decapitated 24-28 days after OVX or sham OVX. The subsequent treatment of blood samples was done in the same manner as in Experiment 1.

*Leptin assay.* Plasma leptin levels were determined by a rat leptin RIA kit produced by Linco Research (St. Louis, MO). The sensitivity of the assay was 0.5 ng/ml. Both the intra- and interassay coefficients of variation were less than 10% in the assay.

*Statistical analyses.* Results were expressed as the mean ± S.E.M. One-way ANOVA followed by Scheffe's post-hoc test was used to analyze the data. Differences were considered significant if $P$ was smaller than 0.05.

## RESULTS

At almost the same BW, intact female rats had 3.3 times higher levels of plasma leptin than intact males (Table 1). In cycling female rats, plasma leptin concentrations did not vary significantly across the estrous cycle (Table 2). With respect to the effect of sex steroid milieu on leptin levels in male rats, it is worth noting that ODX group had 2.9 times higher levels of leptin than sham ODX group. This effect of ODX was completely abolished by the T treatment, and the effect of combined administration of T and TAM was similar to that of T alone. The administration of $E_2$ to ODX rats did not significantly change the elevated leptin levels induced by ODX (Table 3). By contrast, in female rats gonadectomy was without effect on plasma leptin concentrations. The administrations of $E_2$, P, and a com-

**TABLE 3**

Effects of Sex Steroid Milieu on Plasma Leptin Levels in Male Rats

| Group | Number of rats | Plasma leptin (ng/ml) | BW (g) |
|---|---|---|---|
| Sham ODX | 8 | 1.1 ± 0.3 | 253 ± 5 |
| ODX | 9 | 3.2 ± 0.5* | 250 ± 3 |
| ODX + T | 7 | 1.5 ± 0.4 | 251 ± 4 |
| ODX + T + TAM | 7 | 1.6 ± 0.3 | 248 ± 4 |
| ODX + $E_2$ | 8 | 3.0 ± 0.4* | 249 ± 3 |

* Significantly different *vs.* sham ODX, ODX + T, and ODX + T + TAM groups.

**TABLE 4**

Effects of Sex Steroid Milieu on Plasma Leptin Levels in Female Rats

| Group | Number of rats | Plasma leptin (ng/ml) | BW (g) |
|---|---|---|---|
| Sham OVX (diestrus day 2) | 7 | 3.2 ± 0.4 | 248 ± 4 |
| OVX | 8 | 3.7 ± 0.5 | 251 ± 3 |
| OVX + $E_2$ | 8 | 3.6 ± 0.3 | 250 ± 3 |
| OVX + P | 7 | 3.4 ± 0.5 | 249 ± 4 |
| OVX + $E_2$ + P | 8 | 3.7 ± 0.4 | 252 ± 4 |

bination of the two steroids to OVX rats did not significantly change leptin levels (Table 4).

## DISCUSSION

Throughout this study, we determined plasma leptin levels in rats with a very similar BW (about 250 g). This is because it is known that BW is an important parameter in comparing circulating leptin levels between individuals (6, 24). We found that plasma leptin levels were significantly higher in female than in male rats. Previous studies by other investigators have already reported a sex-related difference in circulating leptin levels with female preponderance in both humans (4-9) and mice (10) at any level of adiposity. Even so, the present study is the first to demonstrate the distinct sex difference in leptin concentrations in rats with a very similar BW. It has recently been shown that the presence of T in men, at least in part, mediates the lower leptin levels in men than in women (11-16). However, whether this is also the case with rodents was not examined in previous studies. In the present study, we found for the first time that also in the rat T exerts a suppressive effect on circulating leptin levels. ODX rats had a 2.9-fold higher level of leptin than sham ODX rats, and this rise induced by ODX was completely abrogated by the daily treatment of T at such a physiological dose as employed in our previous study (25). This finding alone, however, does not necessarily indicate that androgenic property of T suppresses leptin production, because part of T is aromatized to $E_2$. In order to differentiate these two possible mechanisms, in this study we also tested the effect of TAM (estrogen antagonist) coadministered with T on leptin levels in ODX rats. The dose of TAM (1 mg/kg BW) we used was appropriate as reported in our previous study (26). The result was that the effects of T alone and T + TAM on leptin levels were almost the same, which suggests that T may inhibit leptin production through its androgenic potency. This conclusion is supported by another observation in this study that $E_2$ was unable to decrease plasma leptin levels in ODX rats. The suggested role of the androgenic property of T in regulating leptin production in the rat agrees with a previous human study that T and its active metabolite dihydrotestosterone were able to suppress leptin secretion and leptin messenger ribonucleic acid in primary culture of adipocytes (11).

With respect to the role of sex steroid milieu in regulating plasma leptin levels in female rats, the results we obtained were largely negative. Plasma leptin concentrations did not change significantly across the estrous cycle of normally cycling female rats. Moreover, leptin levels were not affected either by OVX alone or by the administration after OVX of $E_2$, P, or a combination of these. The dosages of $E_2$ and P we gave to OVX rats were physiological as reported in our previous study (25). The lack of significant changes in leptin during the rat estrous cycle is in keeping with the recent report by Amico *et al.* (23). The same conclusion was reached in one human study by examining circulating leptin levels across the menstrual cycle (22), but several conflicting reports also exist (17, 19, 20). The reason for this discrepancy remains uncertain. Prior to the present study, only Shimizu *et al.* (17) examined the effects of OVX and OVX + $E_2$ on circulating leptin levels in the rat. At odds with our data, they reported that $E_2$ treatment for OVX rats significantly elevated leptin concentrations. However, both Shimizu *et al.* (17) and we found that leptin levels were statistically the same between sham OVX and OVX rats. These results suggest that physiological concentrations of ovarian steroids may not exert a significant effect on leptin production.

Previous human studies reported inconsistent results concerning the effects of hormonal therapy on circulating leptin levels. Kohrt *et al.* (21) and Palmert *et al.* (15) concluded that estrogen has no discernible effect on leptin concentrations in females, whereas Elbers *et al.* (18) reported that estrogen treatment in combination with antiandrogen significantly increased serum leptin levels in males. A recent *in vitro* study of Casabiell *et al.* (27) reported that leptin secretion from human omental adipose tissue was significatnly stimulated by $E_2$ in women but not in men, and P and estrone were without effect in both sexes. However, to what extent this effect of $E_2$ on leptin release *in vitro* is reflected on circulating levels of the adipocyte hormone remains unknown.

Taken together, the heretofore reported effects of female sex steroids on circulating leptin levels are inconsistent in both humans and rats. Although this may indicate complicated actions of $E_2$ on leptin production depending on the gender or other factor(s), it is also possible that the action of $E_2$, even if it exists, is weak as compared to that of T. Thus, it may be that the only convincing action of sex steroids on circulating leptin concentrations is that of T, which is inhibitory in both humans and rats.

In summary, in this study we examined the effects of sex steroid milieu on plasma leptin concentrations in male and female rats excluding the interference of BW on the adipocyte hormone levels. Leptin level was significantly higher in female than in male rats. This sexual difference appears to ensue, at least in part, through the suppressive effect of T on the adipocyte hormone. In female rats, physiological levels of ovarian steroids were without effect on plasma leptin concentrations.

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