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Modulatory Role of Testosterone in Plasma Leptin Turnover in Rats

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We explored whether testosterone influences circulating leptin turnover in rats. Sham-operated male and female rats and 21-d gonadectomized rats treated or not treated with testosterone propionate were used. Anesthetized rats were implanted with an iv catheter, and then blood samples were drawn before and throughout a 60-min period following systemic leptin administration. Plasma testosterone, estradiol, and leptin concentrations were monitored. The results indicated that while gonadectomy blunted circulating concentrations of the homologous sex steroid, testosterone therapy, in gonadectomized rats, restored plasma testosterone concentrations to values found in normal male rats. Pharmacokinetic parameters evaluated during the test indicated the following: First, in the overall pharmacokinetic analyses, testosterone therapy in gonadectomized rats induced a more rapid disappearance of leptin from the circulation. Second, orchidectomy significantly enhanced the area under the curve (AUC) of circulating leptin, an effect fully reversed by testosterone treatment. Third, testosterone treatment in ovariectomized rats significantly decreased the AUC of leptin concentrations. Fourth, while gonadectomy alone did not modify circulating leptin half-life, conversely, testosterone therapy in gonadectomized rats decreased leptin half-life in the circulation. Finally, while orchidectomy reduced leptin body clearance, this parameter was increased by androgen therapy in gonadectomized rats. Our results strongly support that testosterone could play a main role in plasma leptin turnover by increasing leptin clearance rate and shortening plasma leptin half-life.

Key Words: Pharmacokinetic; sexual dimorphism; estradiol; gonadectomy.

Introduction

The hypothalamus is the main interface for the integration of systemic and brain stem afferent signals indicating the organism's energetic balance. After changes in energy metabolism, specific efferent responses are integrated in the hypothalamus to, in turn, maintain homeostasis. Leptin *(1)* is a 16-kDa protein that is mainly synthesized in adipocytes and secreted into the blood *(2)*. It is accepted that circulating leptin levels correlate well with body fat *(3)* and play a very important regulatory role in body fat stores. In fact, after entering into the hypothalamus *(4)*, leptin inhibits food intake *(5)* and increases energy expenditure by a hypothalamic feedback loop *(6)*.

Adipogenesis is a process positively modulated by several transcription factors *(7,8)* and influenced by the endocrine system *(9–11)*. Among the effects of sex steroid hormones, androgens have been proposed to block *(12)* and estrogens to stimulate *(13)* adipogenesis. This endocrine regulation is strongly supported by the presence of specific sex steroid receptors in fat tissue *(14,15)*, thus indicating that adipocytes are targets for sex steroid hormones and thus modulate the growth and function of adipose tissue *(16)*. Moreover, fat distribution differs with gender, and this characteristic seems to be dependent on a sex steroid hormone basis *(16)*. Consistent with the effects of sex steroids on adipogenesis, leptin seems to be expressed and released on a sex steroid–dependent basis: while estrogens stimulate, androgens inhibit leptin expression and release *(17,18)*. However, studies performed in humans *(19)* and rats *(20)* indicate that the differences in circulating leptin levels among sexes do not take place only because of sex differences in the relative amount of fat *(19,21)* or adipose tissue leptin mRNA expression *(20)*; moreover, both studies claim that circulating sex steroids may regulate plasma leptin concentrations by, e.g., modulating the leptin rate of degradation *(20)*. The aforementioned studies *(19–21)* strongly support that the rapid decrease in circulating leptin levels observed in boys, when they are compared before and after puberty, could be attributed to, at least in part, an increase in testicular testosterone production *(22)*. Reciprocally, leptin is also known to modulate sex steroid metabolism, in both genders, by acting through specific leptin receptor. Indeed, leptin inhibits estradiol (E_2) production by the ovary *(23)* and testosterone secretion by the testes *(24)*. All these data clearly indicate an important interplay between adipose tissue and gonadal functions.

It is known that gonadectomy decreases energy expenditure in both sexes *(25)*, and, therefore, changes in the half-

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lives of circulating hormones could be related to differences in energy expenditure, combined or not with an effect of sex steroids. Thus, the aim of the present study was to determine whether a testosterone molecular basis could be partially responsible for changes in the circulating leptin turnover process. For this purpose, intact and gonadectomized rats of both sexes, receiving or not receiving testosterone therapy, were intravenously injected with leptin and a pharmacokinetic analysis was performed, by using a two-pool model of plasma leptin distribution *(26)*, in order to determine the influence of testosterone on the removal of leptin from the circulation.

Results

Basal Circulating Sex Steroid Levels in Different Groups of Experimental Animals

Basal testosterone plasma levels determined in anesthetized rats after 21 d of orchidectomy (ODX) were significantly ($p < 0.02$) decreased (ODX values vs male values), and testosterone propionate (TP) replacement therapy in ODX rats (ODX+TP) fully restored circulating testosterone levels to those measured in intact male rats (Fig. 1A). Basal plasma testosterone concentrations were very low in anesthetized intact female and ovariectomized (OVX) rats; however, TP administration in OVX rats (OVX+TP) enhanced ($p < 0.02$) their circulating testosterone concentrations (Fig. 1B) to values found in both intact male and ODX+TP rats.

Regarding circulating E_2 concentrations, no differences were noticed among the three experimental groups of anesthetized male rats, with the levels being at the detection limit of the assay (Fig. 1C). Conversely, in female rats, both OVX and OVX+TP animals displayed significantly lower ($p < 0.05$) E_2 levels than those found in intact rats (Fig. 1D).

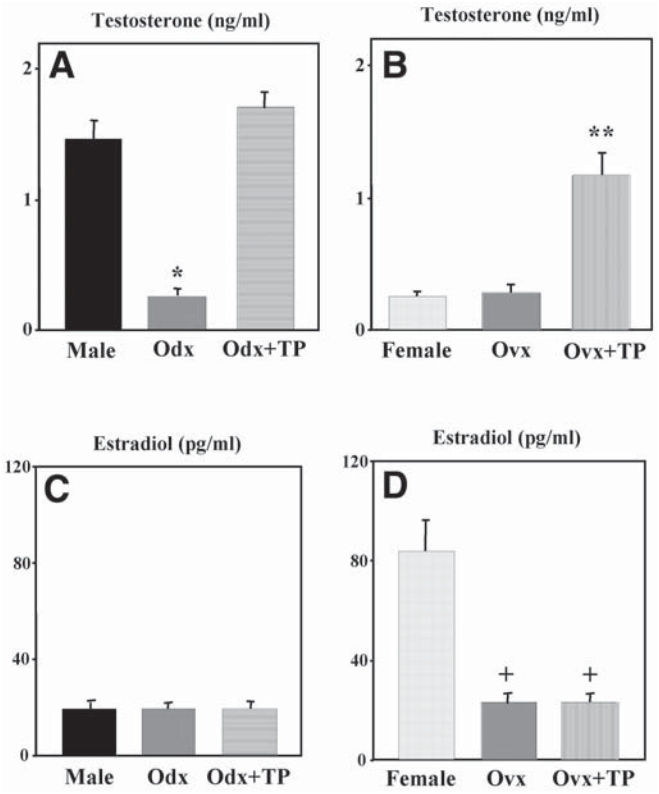

Fig. 1. Basal plasma levels of testosterone (**A,B**) and estradiol (**C,D**) in different groups of anesthetized rats of both sexes: sham-operated (male and female), 21-d gonadectomized (ODX and OVX), and ODX and OVX rats treated with TP (ODX+TP and OVX+TP). Values are the mean ± SEM ($n = 7$ rats/group). *$p <$ 0.02 vs male and ODX+TP values; **$p < 0.02$ vs female and OVX values; $^{+}p < 0.05$ vs female values.

Patterns of Circulating Leptin Concentrations in Intact, Gonadectomized, and Androgen-Treated Gonadectomized Rats of Both Sexes After IV Bolus Injection of Recombinant Murine Leptin

Figure 2 shows the patterns of plasma leptin levels, plotted after two-compartment pharmacokinetic analysis, in different groups of anesthetized rats of both sexes (male, ODX and ODX+TP: Fig. 2A; female, OVX, and OVX+TP: Fig. 2B) administered with leptin intraveneously (1.5 μg/100 g of body wt). As depicted, all groups examined displayed a biexponential time-dependent decrease in leptin circulating levels following iv bolus administration of leptin. Statistical analysis of the regression of the negative slopes of circulating leptin concentrations through time (when considered for the effects of sex, surgery, steroid treatment, time, and square of the time) indicated a significant ($p <$ 0.00001) time-dependent decrease in circulating leptin concentrations in all groups studied (b coefficient = –10.92 and $t = –9.53$ for time; b coefficient = 0.13 and $t = 7.06$ for the square of time). The theoretical regression model for the circulating leptin concentration ´ time curves was characterized by F (11, 204) = 75.47 ($p < 0.0001$) and adjusted $R^2 = 79.21\%$ (number of observations = 216). Important to denote is that gonadectomized rats treated with the androgen (ODX+TP and OVX+TP groups) displayed a significantly faster leptin disappearance from the circulation through time in TP-treated gonadectomized rats (b coefficient = –2.74; $t = –1.96$; $p < 0.05$) than the remaining groups of animals.

Effects of Gonadectomy Alone and Gonadectomy Combined with Testosterone Therapy on Plasma Leptin Turnover in Rats

Pharmacokinetic analysis of circulating leptin concentrations indicated that orchidectomy significantly ($p < 0.02$) increased the area under the curve (AUC) of plasma leptin levels in rats of male gender (ODX vs male values; Fig. 3A); conversely, ovariectomy did not modify this parameter, throughout the period of time studied (Fig. 3B). Inter-

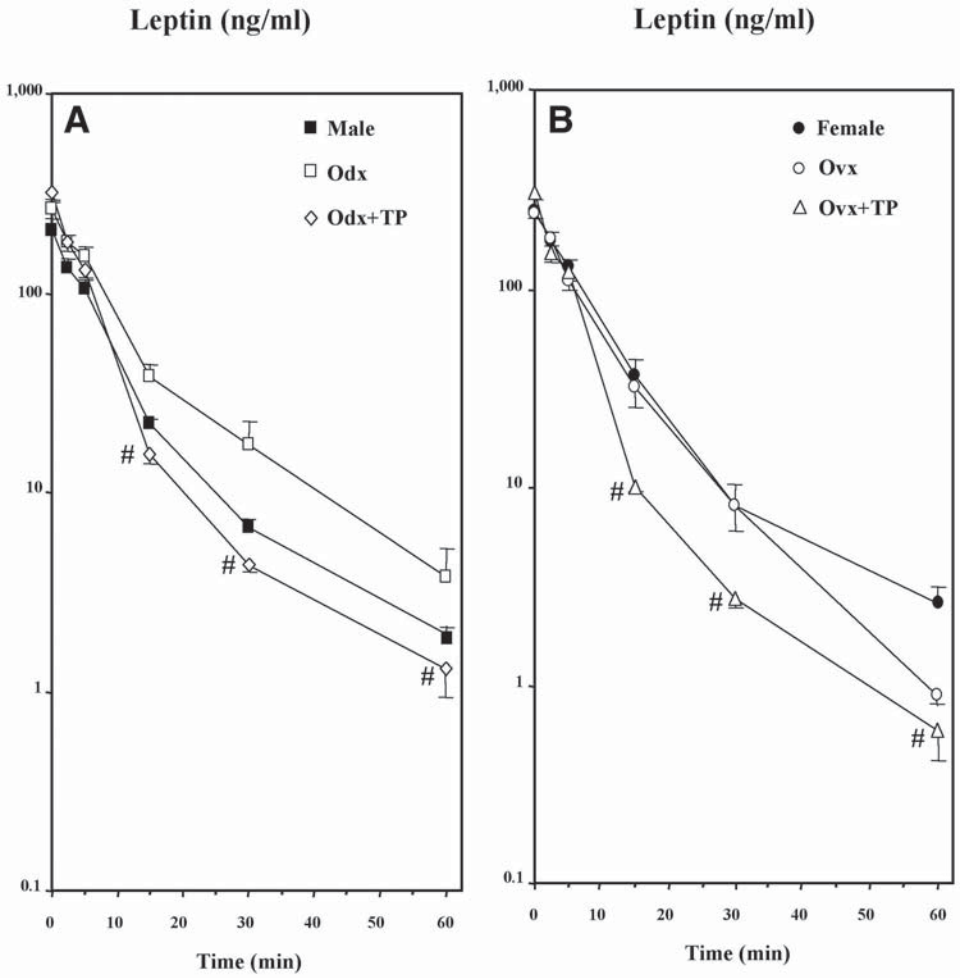

Fig. 2. Concentration-time curves of circulating leptin levels throughout a 1-h period in iv bolus leptin-administered sham-operated (male and female), 21-d gonadectomized (ODX and OVX), and ODX and OVX treated with TP anesthetized male (**A**) and female (**B**) rats. Values are the mean ± SEM ($n = 7$ rats/group). #Negative slopes of curves plotted with TP-treated rat values are significantly ($p < 0.05$) different from slopes of all remaining group curves.

estingly, TP replacement therapy fully reversed ($p < 0.05$) the effect of ODX alone on the AUC of leptin in circulation (ODX+TP vs ODX values; Fig. 3A), and, as pharmacologic therapy in OVX rats, TP significantly ($p < 0.05$) decreased the AUC of circulating leptin concentrations compared with values found in female rats (Fig. 3B).

Analysis of circulating leptin initial half-life values ($t_{½}i$) in our model indicated that no gender-dependent (male vs female) differences were found (Fig. 3C,D). Interestingly, while gonadectomy alone did not change leptin $t_{½}i$ when compared to values in sham rats of both sexes, TP therapy in both ODX and OVX rats was able to significantly ($p < 0.05$ vs ODX and OVX values, respectively) decrease this parameter (Fig. 3C,D). Finally, no effects of gender and gonadectomy, either alone or combined with TP treatment, on final leptin half-life ($t_{½}f$; estimated approximately in 13 min) were observed (data not shown).

The results of total body leptin clearance (CL) indicated that ODX, but not OVX, induced a significant ($p < 0.03$ vs male values) decrease in leptin CL, and TP therapy in orchidectomized animals (ODX+TP) fully reversed the effect of ODX alone ($p < 0.01$) on leptin CL. In addition, TP-treated OVX rats displayed a significantly ($p < 0.05$ vs female values) enhanced leptin CL rate (Fig. 4A,B).

Finally, the volume of leptin distribution at steady state (*Vss*) was not modified by gonadectomy alone or combined with TP treatment regardless of gender (see Fig. 4C,D).

Discussion

The present study demonstrates a clear effect of testosterone on plasma leptin turnover, an effect observed in gonadectomized rats of both sexes. Although a sexual dimorphism *(21)* in circulating leptin levels in normal rats has been

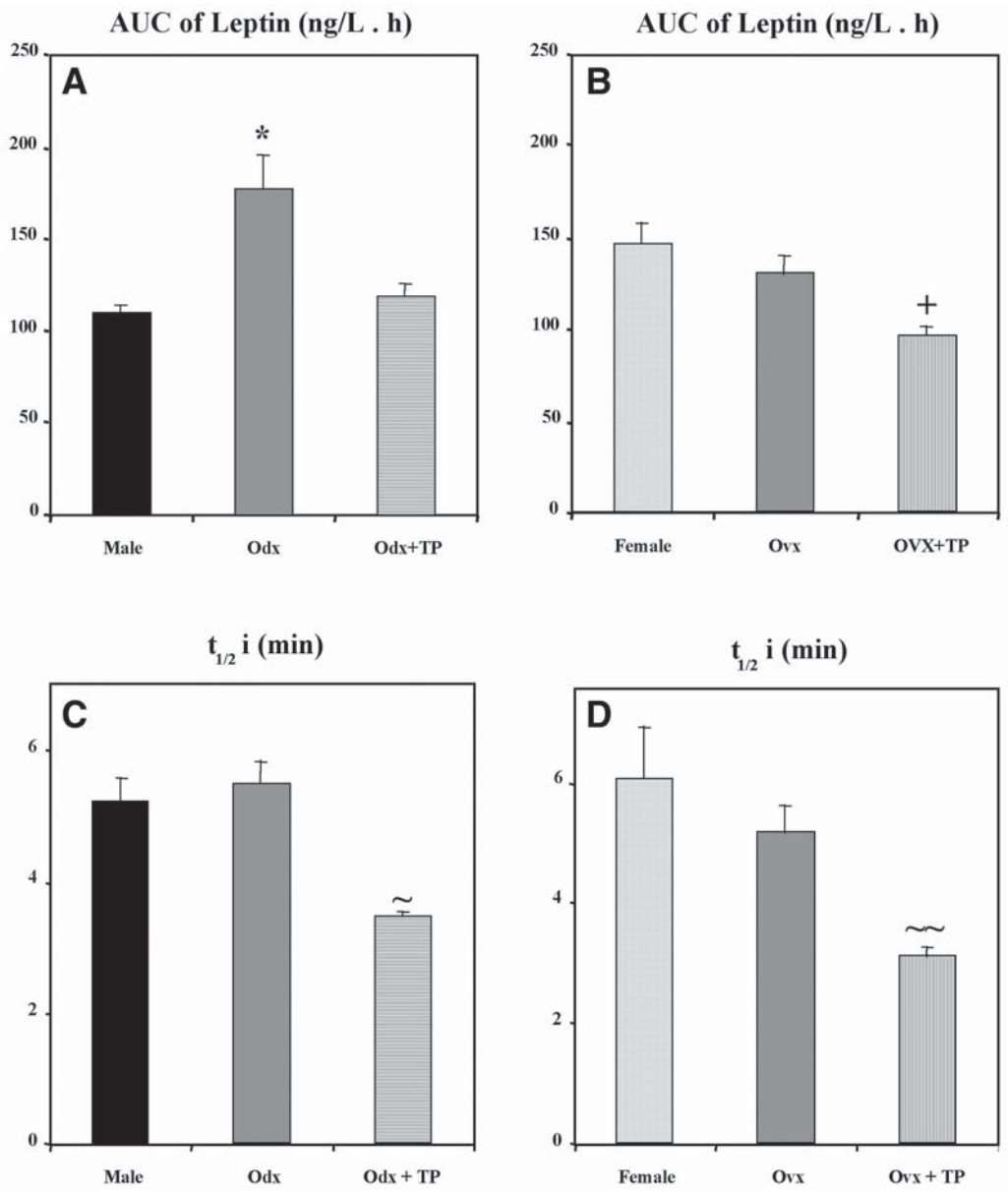

Fig. 3. AUC of plasma leptin levels and initial ($t_{½}i$) leptin half-life values obtained after fitting a two-compartment model to individual concentration-time curves in male (**A** and **C**, respectively), and female (**B** and **D**, respectively) rats. Values are the mean ± SEM ($n = 7$ rats/group). *$p < 0.05$ or less vs male and ODX+TP values; $^{+}p < 0.05$ vs female values; ~$p < 0.05$ vs ODX values; ~~$p < 0.05$ vs OVX values.

claimed, up to now, it has only been speculated that endogenous sex steroids could be responsible for that sexual dimorphism *(19,21,22,27)*. We demonstrated that differences in circulating leptin levels between male and female rats could be owing to, at least in part, an enhancing effect of testosterone on plasma leptin turnover.

To our knowledge, this is the first evidence sustaining a sex steroid basis for the differences in circulating leptin turnover. It is accepted that the pharmacokinetics of circulating leptin is influenced by its binding to plasma carrier molecules, the rate of leptin synthesis, and leptin binding to different tissues, creating an additional pool of endogenous leptin *(26)*, thus indicating that all these factors could influence circulating leptin levels. We demonstrated that within the context of a two-pool model of leptin in circulation *(26)*, the concentration of sex steroid in plasma represents an important factor influencing plasma leptin concentrations. The surgical removal of endogenous testosterone (ODX) decreased plasma leptin turnover owing to a significant decrease in leptin CL rate, while leptin half-life remained unchanged. This occurred in association with an enhancement in the AUC of circulating leptin after injection of the adipokine. Conversely, the removal of endogenous estrogens by ovariectomy did not affect plasma leptin turnover. Interestingly, testosterone therapy in ODX rats completely reversed the decreased plasma leptin turnover induced by

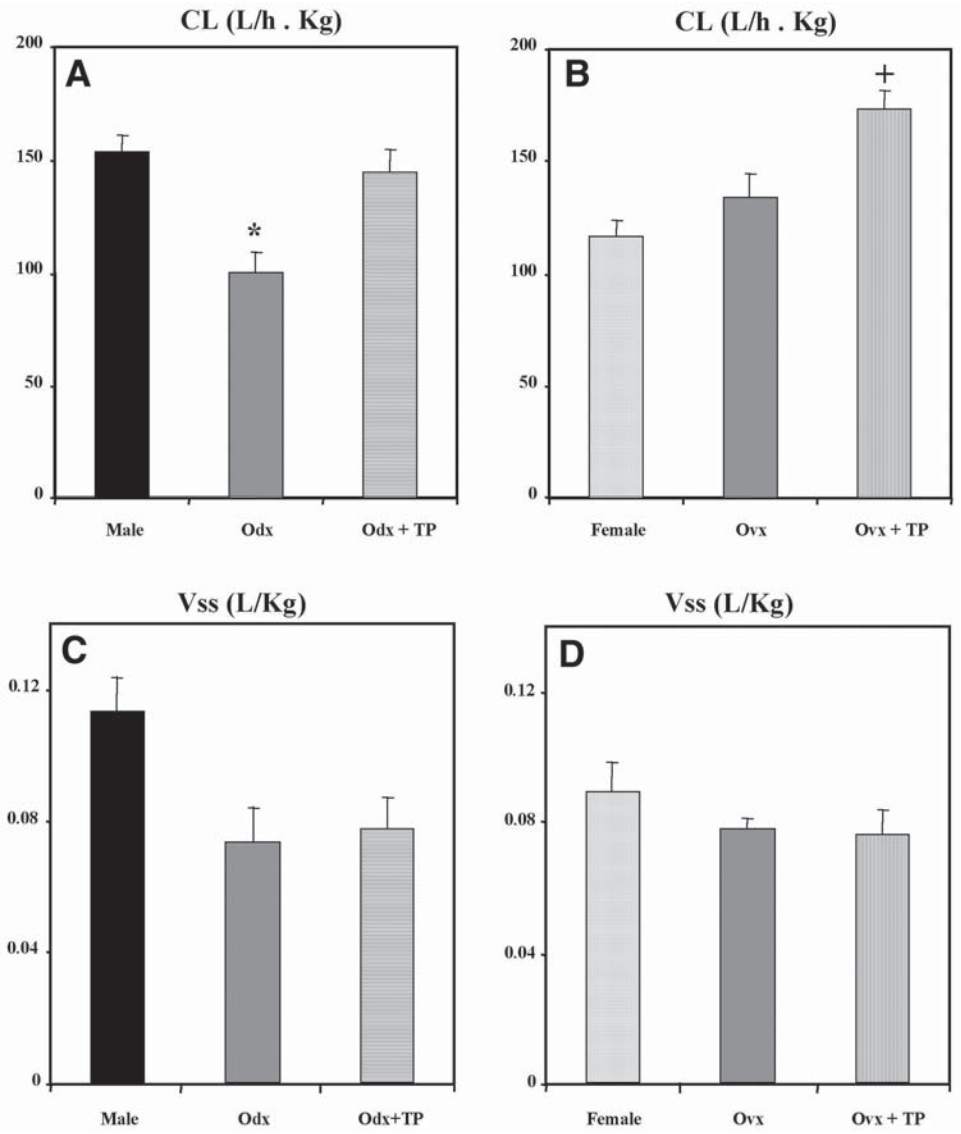

Fig. 4. CL and volume of leptin distribution at steady state (*Vss*) obtained after fitting a two-compartment model to individual concentration-time curves in different groups of male (**A** and **C**, respectively) and female (**B** and **D**, respectively) rats. Values are the mean ± SEM (n = 7 rats/group). *$p < 0.03$ or less vs male and ODX+TP values; $^{+}p < 0.05$ vs female values.

orchidectomy alone by significantly increasing leptin CL rate and decreasing initial leptin half-life. Important to denote is that the leptin half-life found in our normal rats, of both sexes, is in agreement with the one previously reported in normal rats, although estimated by a different method *(25)*. Our data indicate that circulating estrogens, as suggested earlier *(28)*, did not influence plasma leptin turnover.

It must be strongly stressed that the effect of testosterone was also observed in ovariectomized rats, and, again, the mechanisms involved seem to be similar to those found in orchidectomized rats. In fact, TP therapy in OVX rats enhanced leptin CL rate, in association with a reduction in both leptin AUC and initial leptin half-life. Thus, our results strongly support a sex steroid basis, rather than a gender dependency, of the effect of testosterone on plasma leptin turnover. Our results also indicate that testosterone was effective for increasing circulating leptin turnover, in contrast to physiologic levels of estrogen, thus suggesting a predominant enhancing role of androgens in plasma leptin turnover. Important to remark is that the surgical ablation of gonadal tissues, as an additional potential factor for modification of leptin CL, only marginally reduced *Vss* in rats of male gender, regardless of whether TP therapy was applied or not. Thus, our data strongly support a testosterone molecular basis for the differences in plasma leptin levels and turnover between sexes, regardless of body weight. In fact,

the effect of circulating testosterone concentrations takes place similarly in rats of different gender and body weight.

Although we did not find any sex-related difference in absolute circulating leptin concentrations in freely moving intact rats (data not shown), it should be taken into account that, in these age-matched rats, males were heavier than females (see Results), and, as a consequence, although not screened, males could be bearing more adiposity than females. However, in a previous study in rats *(20)*, it was reported that animals of both sexes, displaying a similar adiposity index and in conditions in which sex-related differences in adipose tissue ob mRNA expression disappear, the sexual dimorphism in circulating leptin levels remained. Moreover, it has been reported that gender/sex-dependent differences in circulating leptin levels are independent of leptin mRNA expression in rat adipose tissue *(20)*. Those studies *(19–21)* further support that some other mechanism(s) could also modulate leptin turnover; our results therefore add new information about the role of sex steroids in circulating leptin turnover.

It is known that kidney function displays a sexual dimorphism *(29)* and that an androgen basis for that effect has been claimed *(30)*. However, although the kidney is the key element involved in the clearance of circulating leptin, a predominant renal clearance mechanism remains controversial *(31,32)*. Glomerular filtration appears to be the most important mechanism for acute leptin clearance *(32,33)* since after kidney ablation the liver needs several hours before it can play a predominant role in leptin inactivation *(34)*. Additionally, because the kidneys express leptin receptors *(35)*, which could participate as transport molecules of leptin from the blood to the renal tubular fluid, an effect of sex steroids on kidney ob receptor expression should be not excluded. An effect of sex steroids on blood leptin-binding protein levels *(36)*, as it occurs with several other circulating hormone-binding proteins *(37)*, could participate in the testosterone-dependent mechanisms influencing plasma leptin turnover. Nevertheless, androgens could be playing an inhibitory effect on leptin-binding proteins and, thus, as a compensatory mechanism, contributing to the faster removal of leptin from the circulation to maintain homeostasis. This could involve the increase in the CL rate of the excess free leptin, which in both lean humans *(38)* and rats (unpublished results) represents 50% (approximately) of the total circulating pool of basal leptin levels.

Although it has been published that ovariectomy in women could slightly increase serum soluble leptin receptor levels *(39)*, our study demonstrates that ovariectomy did not influence plasma leptin turnover, thus suggesting a more important androgen modulation of leptin CL rate; conversely, estrogens could positively influence circulating leptin levels owing to their enhancing effect on adipocyte leptin production *(40)*. It is accepted that hormone levels in circulation are directly related to the process of production, release, changes in response to particular effectors, and degradation, thus helping to maintain homeostasis. The pharmacokinetic analysis of circulating hormones provides valuable information on, although theoretically, the speed of response to changes, and on the synthesis-degradation process of an organism to maintain homeostasis. High leptin turnover could well represent high energy expenditure; however, it could allow the organism faster responses and rapid adaptive mechanisms to recover homeostasis *(25)*. Conversely, low circulating hormone turnover represents a correlation with long-term regulation of different functions *(25)*.

Regarding some additional aspects of the biologic significance of our findings, it could be possible that the low-amplitude pulse of leptin secretion in blood, which characterizes male rats *(41)*, could result from both the enhancing and decreasing effects of testosterone on leptin CL and half-life, respectively. Thus, a higher-frequency pulse of leptin output in circulation in male than in female rats *(41)* is necessary for rapid integration of anorexigenic and orexigenic signals, at the hypothalamic level, for maintaining homeostasis.

In summary, our data suggest that androgens could contribute to the faster adaptation of males than females to acute changes in energy metabolism. Thus, gender-dependent differences in the adipostat *(25)*, the mechanism by which energy stores are held relatively constant, could be under a sex steroid–related control of plasma leptin turnover. Finally, whereas testosterone enhances leptin turnover, endogenous estrogens could also contribute to sex differences in circulating leptin levels by increasing adipocyte leptin production *(40)*, tissue sensitivity to leptin *(42)*, and leptin pulse amplitude of secretion in plasma *(41)*.

Materials and Methods

Animals, Surgery, and Treatment

Adult male (300–380 g of body wt) and randomly cycling female (180–220 g BW) littermate Wistar rats were used in all experiments (n = 7 rats/group). Rats were kept in a light (lights on from 7:00 AM to 7:00 PM)- and temperature (22°C)-controlled room with rat chow and water available ad libitum. Several groups of rats were submitted (8:00 AM 9:00 AM) to bilateral gonadectomy (ODX and OVX groups) or sham operation (male and female groups), under light ether anesthesia. Starting on d 1 after surgery, rats received TP as previously reported *(43)*, but with minor modifications. Briefly, 100 μL of sterile corn oil either alone (male, female, ODX, and OVX groups) or containing 0.5 mg/kg of body wt of TP (ODX+TP and OVX+TP groups) was injected (subcutaneously, between 3:00 PM and 3:30 PM) once every 2 d until the experimental day. Seven rats per group were used in our design and mean body weight values (in grams ± SEM) of these animals were as follows: male = 370.91 ± 10.18; ODX = 349.19 ± 12.74; ODX+TP = 358.91 ± 11.98; female = 215.01 ± 6.21; OVX = 221.54 ± 6.71;

OVX+TP = 224.66 ± 5.39. TP treatment allowed us (determined in preliminary experiments; data not shown) to transiently maintain circulating concentrations of testosterone at the level that characterizes normal male rats.

Experimental Designs

Twenty-one days after surgery, rats of different groups were anesthetized (starting between 8:00 AM and 9:00 AM; 15 mg of ketamine–10 µL of Rompun/100 g of body wt) and an indwelling Silastic catheter was intravenously implanted. 60 min later, blood samples were drawn (300 µL) before (sample time zero) and several times (2.5, 5, 15, 30 and 60 min) after iv administration of recombinant murine leptin (Sigma; 1.5 µg/100 g of body wt/100 µL of heparinized NaCl solution). Basal plasma testosterone and estradiol concentrations were measured by specific radioimmunoassays (RIAs). Circulating leptin concentrations, in all groups of rats and throughout the period of time examined, were submitted to a two-compartment pharmacokinetic model analysis based (with elimination from the central compartment) on the following biexponential equation: $C = C_i \cdot e^{-\lambda i \cdot t} + C_f \cdot e^{-\lambda f \cdot t}$ *(44)*, in which C is the leptin plasma concentration at any time, t, C_i is the initial and C_f is the terminal preexponential coefficient; and λ_i and λ_f are the initial and final rate constants *(45)*, respectively. Different pharmacokinetic parameters were derived from the parameter estimates obtained by the fitting procedure according to standard methods *(46)*. Briefly, the area under the concentration-time curve, from zero to infinity (AUC), of plasma leptin concentrations was defined as $C_i/\lambda_i + C_f/\lambda_f$. The maximum concentration of circulating leptin was calculated from the concentration-time-plotted natural logarithmic values by extrapolation to time zero (C_{max}). CL was determined as administered leptin dose/AUC. The volume of leptin distribution at steady state (Vss) was calculated as administered leptin dose $\times [(C_i/\lambda_i^2)+(C_f/\lambda_f^2)]/[(C_i/\lambda_i)+(C_f/\lambda_f)]^2$. The initial (*i*) and final (*f*) half-life values were obtained as $\ln(2)/\lambda_i$ and $\ln(2)/\lambda_f$, respectively.

Experiments were approved by our animal care committees. Animals were killed by decapitation according to protocols for animal use in agreement with National Institutes of Health guidelines for the care and use of experimental animals.

Assays

Testosterone levels in plasma were measured by a specific RIA as previously described and validated *(47)*; intra- and interassay coefficients of variation (CVs) ranged between 4–7 and 9–13%, respectively. Circulating concentrations of estradiol were determined by specific RIA as previously detailed *(47)*; intra- and interassay CVs were 4–6 and 10–12%, respectively. Leptin circulating levels were determined by a specific RIA (range of the standard curve: 0.5–20 ng/mL) from our laboratory *(48)*, with intra- and interassay CVs of 4–7 and 9–11%, respectively. This method displays full crossreactivity with rat and murine leptin.

Data Analysis

Pharmacokinetic data (leptin concentration × time curves) from all six groups studied were submitted to the least squares regression analysis for time, sex, surgery, and testosterone treatment conditions *(49)*.

The results of the different variables examined (AUC of leptin levels, leptin CL, leptin half-lives, and *Vss*) after pharmacokinetic studies and basal circulating sex steroid concentrations were analyzed by multiple variable multivariate analysis of variance *(49)*. Data are expressed as the mean ± SEM. Mean values were considered statistically different when p values were at least <0.05 after Bonferroni's correction owing to multiple comparisons.

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