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Leptin Secretion From the Epididymal Fat Pad Is Increased by the Sexual Maturation of the Male Rat

STANLEY J. NAZIAN

From the Department of Physiology & Biophysics, College of Medicine, University of South Florida, Tampa, Florida.

ABSTRACT: Although leptin has been implicated as an important factor in triggering the onset of puberty in females, much less is known about the role of this adipose tissue hormone in the sexual maturation of males. Previous work in the rat has suggested that the peripubertal rise in testosterone precedes an increase in leptin secretion, and it has been suggested that the testosterone rise induces the leptin increase. These studies examined some of the interactions between leptin secretion and the peripubertal testosterone rise in male rats. Serum leptin concentrations were significantly elevated in young adult male rats compared with immature rats. Cultured epididymal fat pads obtained from adult animals secreted significantly more leptin than did those obtained from immature rats. Castration of immature rats with or without testosterone replacement for 1 week did not result in a significant change in either the serum leptin concentrations or the ability of the epididymal fat pad to secrete leptin. Exposure of epididymal fat to 5 ng/mL of testosterone in vitro resulted in a significantly enhanced secretion of leptin into the media compared with plain media controls. These results confirmed that there is an increase in serum leptin concentrations with sexual maturation in the male rat. They also suggest that this increase is due to an enhanced ability of adipose tissue to secrete leptin. Within a normal physiologic range, testosterone may play a role in inducing this increased ability to secrete leptin.

Key words: Puberty, testosterone, adipose tissue.
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A relationship between body weight and the onset of puberty has been postulated from some time (Frisch and Revelle, 1974), and the basic idea that energy reserves have a significant influence on reproductive processes is widely held. The discovery of leptin (Zhang et al, 1994) provided the endocrine link between adipose tissue and the reproductive system.

Leptin is the protein product of the *Lep(ob)* gene. It is produced principally in white fat and is secreted in a hormonal fashion (Friedman, 1997; Sinha, 1997). Mice heterozygous for the *Lep*^{ob} allele lack leptin and are infertile. Leptin administration to such animals stimulates gonadotropin secretion and the growth of secondary sexual organs in both sexes (Barash et al, 1996). Exogenously administered leptin also appears to accelerate sexual maturation in normal female mice (Ahima et al, 1997; Chehab et al, 1997) and female rats (Cheung et al, 1997), whereas transgenetic mice that overexpress leptin experience early puberty (Yura et al, 2000).

Studies in rats, rhesus monkeys, and humans have not, for the most part, supported an obligatory role for leptin in the onset of puberty in males. Longitudinal studies in normally maturing boys have indicated that serum leptin is increased (Mantzoros et al, 1997) and decreased (Ahmed et al, 1999) during the early Tanner stages of puberty. Both longitudinal (Plant and Durrant, 1997; Mann et al, 2000) and cross-sectional (Urbanski and Pau, 1998) studies in rhesus monkeys have failed to support the notion that leptin serves as an acute trigger for the onset of sexual maturation. A detailed study in rats (Nazian and Cameron, 1999) has indicated that serum leptin concentrations rise during the course of sexual maturation. However, the onset of puberty, as judged by the onset of the peripubertal testosterone rise, secondary sexual organ growth, appearance of elongating spermatids and/or the peripubertal decline in serum follicle-stimulating hormone, preceded the increase in serum leptin. In contrast to these reports, an intriguing recent study in agonadal male monkeys (Suter et al, 2000) has suggested that the nocturnal levels of leptin are elevated just prior to the onset of the increase in luteinizing hormone pulse amplitude that is assumed to indicate hypothalamic puberty in these animals.

Although these studies failed to support the hypothesis that leptin induces puberty onset in male mammals, much of the data have left open the possibility that a rise in serum leptin concentrations may be a significant component of the pubertal process. Indeed, Urbanski and Pau (1998) have speculated that the peripubertal testosterone rise might have a significant stimulatory effect on leptin

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Correspondence to: Dr Stanley J. Nazian, Department of Physiology & Biophysics, College of Medicine, MDC Box 8, University of South Florida, Tampa, FL 33612 (e-mail: snazian@hsc.usf.edu).

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secretion. Support for this hypothesis was provided by the results of experiments involving the transient ablation of prepubertal Leydig cells (Nazian and Cameron, 1999). Such treatment resulted in a delay in the rise in leptin secretion that corresponded to the delay in the peripubertal rise in testosterone. The experiments reported here were designed to further examine this hypothesis by examining leptin secretion by epididymal fat pads obtained from immature and young adult rats.

Materials and Methods

General

Animals—Male rats of a Sprague-Dawley-derived strain were obtained from Harlan Sprague-Dawley (Indianapolis, Ind). They were housed 2–4 per cage in shoebox-type cages with food and water available ad libitum under standard conditions of temperature (24°C) and lighting (12 hours light, 12 hours darkness). Animals were anesthetized to a surgical level with pentobarbital and then decapitated. Trunk blood was obtained and the serum frozen until assayed for leptin concentrations by radioimmunoassay. The experiments describe here were performed with the prior permission of the University of South Florida Institutional Animal Care and Use Committee.

Adipose Tissue Culture—Standard incubation media consisted of Dulbeccos modified Eagle medium (Sigma Chemical Company, St Louis, Mo) supplemented with 0.5% fetal calf serum, and antibiotics (gentamicin/penicillin/streptomycin). Individual epididymal fat pads were obtained and dissected free of connective tissue and blood vessels, and placed into 2–4 mL of ice-cold incubation media. They were weighed and minced using fine scissors into 2 mL of incubation media in a 6-well tissue culture plate. When all wells were ready, the plate was placed in a 37°C water-jacketed incubator (95% air, 5% carbon dioxide) for 1 hour. The media was then removed and replaced with fresh media containing 50 nM porcine insulin (I), 1 μM dexamethasone (DEX), or both 50 nM porcine insulin and 1 μM dexamethasone (DEX + I). After incubation for varying times, the media was removed, replaced with fresh media containing the appropriate additives, and frozen until assayed for leptin by radioimmunoassay. Preliminary experiments using pooled epididymal fat pads obtained from adult rats (Figure 1) indicated that under these conditions, basal leptin secretion into the media declined slightly after the first 24 hours and was thereafter essentially stable through 96 hours of culture. The addition of insulin alone had essentially no effect on leptin secretion, whereas DEX containing media resulted in an initial increase in leptin secretion that declined steadily through 96 hours of incubation. The DEX + I combination resulted in a significant increase in leptin secretion that was maintained through 96 hours. Based on these results, and our belief that the DEX + I combination reflects a more physiologically ''normal'' situation, the basic incubation protocol for most of the experiments reported here consisted of paired epididymal fat pads obtained from the same rat and exposed to either plain media (right) or media containing DEX + I (left) for 48 hours.

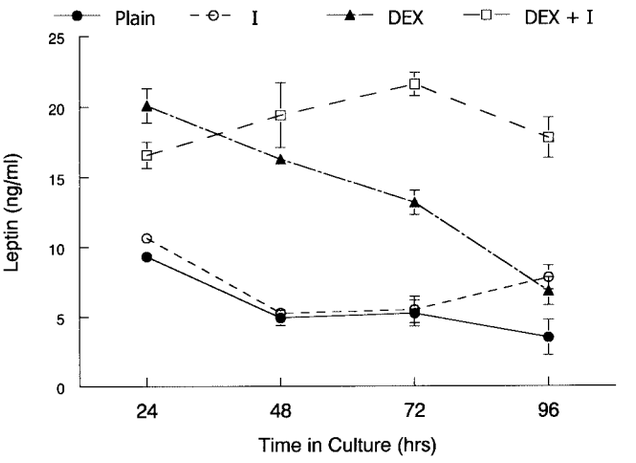

Figure 1. Time course of leptin secretion into the media from epididymal fat exposed to plain media, 50 nM porcine insulin (I), 1 μM DEX, or 1 μM DEX plus 50 nM insulin (DEX + I). Approximately 300 mg of minced tissue from an adult rat was used in each well (n = 4 wells each treatment).

Radioimmunoassay and Statistics—Serum and media concentrations of leptin were determined using a radioimmunoassay kit obtained from Linco Research, Inc (St Louis, Mo) as used previously in this laboratory (Nazian and Cameron, 1999). For a given experiment, all samples were run in the same assay. Data were analyzed and significance determined by using SigmaStat for Windows (Jandel Scientific, Sausalito, Calif). For comparisons between 2 groups, the Student's *t*-test was used. For comparisons involving multiple measurements, a one-way or two-way analysis of variance (ANOVA), as appropriate, followed by the Student-Newman-Keuls test, was performed.

In Vivo Studies

Sexual Maturation—Ten immature (30–31 days old) and 10 young adult (70–71 days old) male rats were used. Individual minced epididymal fat pads from the immature rats were incubated for 48 hours with either plain media or media containing DEX + I. From each adult rat, 2 pieces of epididymal fat pad of approximately the same mass as a whole pad from an immature rat were obtained and treated similarly.

Castration and Testosterone Treatment—Immature (24-day-old) male rats were castrated via a single mid-scrotal incision while under methoxyflurane anesthesia. Immediately after the castration they received subcutaneous implants of Silastic capsules containing crystalline testosterone as described previously (Nazian, 1992). Castrated controls and sham-operated controls received empty capsules. Testosterone capsules were 0.5 and 2.0 cm long, and were intended to result in serum androgen levels just below and somewhat above the age normal values. Seven animals were used per group. One week after surgery and capsule implantation, the animals were anesthetized and individual epididymal fat pads were incubated in plain media or media containing DEX + I for 48 hours.

In Vitro Studies

Epididymal fat pads were obtained from immature (29-day-old) rats and incubated in plain media or media containing 5.0 or 50

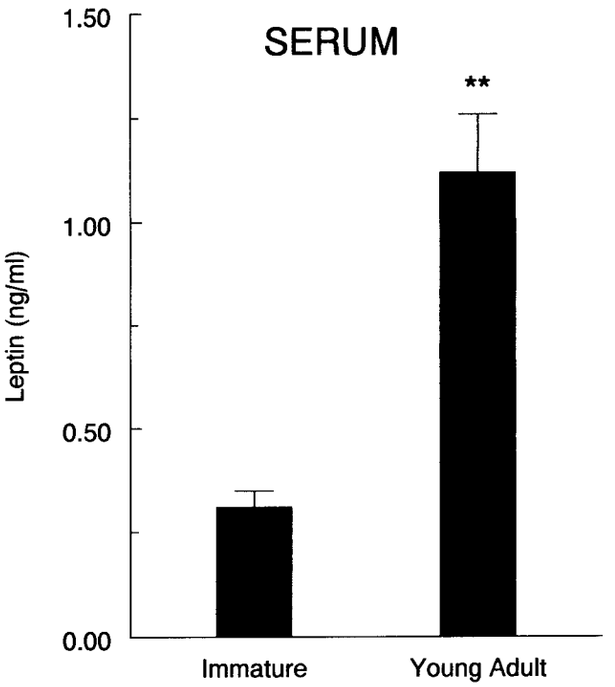

Figure 2. Serum concentrations (mean ± SEM) of leptin in immature and young adult male rats (n = 10). **$P < .01$ vs immature (Student's *t*-test).

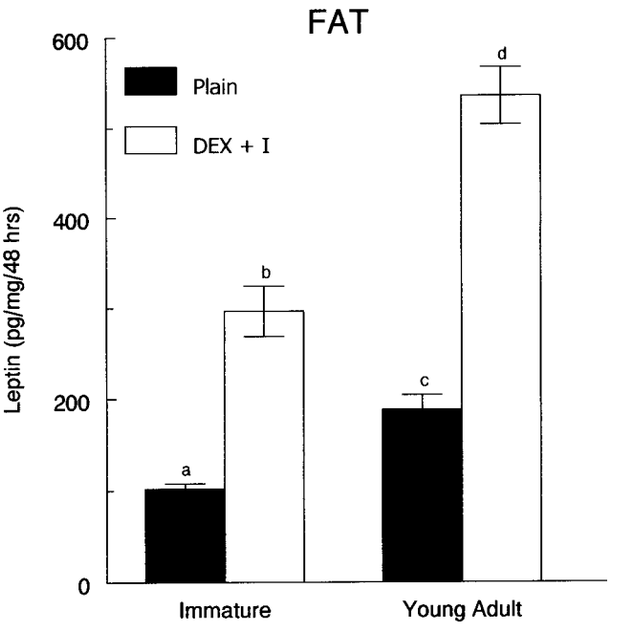

Figure 3. Secretion of leptin (mean ± SEM) into the media by epididymal fat pads obtained from the immature and young adult rats whose serum leptin values are shown in Figure 2. Paired fat pads were incubated for 48 hours in plain media (solid bars) or media containing 1 μM DEX plus 50 nM insulin (open bars). **(a–d)** Groups with different letters are significantly different from each other (Student-Newman-Keuls test).

ng/mL testosterone with or without added DEX + I. Media was replaced every 48 hours with identical media for 8 days.

Results

Sexual Maturation

Serum leptin concentrations (Figure 2) were significantly higher ($P < .01$) in young adult male rats (1.12 ± 0.14 ng/mL) compared with the concentration in immature animals (0.31 ± 0.04 ng/mL). Epididymal fat pads obtained from young adult rats secreted more leptin into the media than did fat pads obtained from immature rats (Figure 3) under basal conditions. Treatment with DEX + I increased leptin secretion by epididymal fat pads from both ages but appeared to be more effective on epididymal fat pads obtained from adult animals. Repeated measures two-way ANOVA indicated a significant effect of both age and treatment and a significant interaction between them ($P < .001$ for all 3 effects).

Testosterone In Vivo

As expected, castration of immature rats resulted in a significant reduction in prostate weight 1 week later (Figure 4, lower panel). Both sizes of testosterone capsule maintained prostate weight above that of sham controls. The 2.0-cm capsule resulted in larger prostate weights than the 0.5-cm capsule. Serum leptin concentrations were not altered by castration with or without testosterone replacement (Figure 4, upper panel). Leptin secretion from epididymal fat pads obtained from these rats (Figure 5) was similar regardless of in vivo treatment. Incubation with DEX + I resulted in a significant increase in leptin release into the media (repeated measures two-way ANOVA, $P < .001$) compared with plain media. There was not, however, a statistically significant effect of in vivo treatment nor any significant interaction.

Testosterone In Vitro

The effect of testosterone treatment in vitro for 8 days on the secretion of leptin from epididymal fat pads obtained from immature rats is shown in Figure 6. Incubation of fat pads with plain media led to a progressive decline in the amount of leptin appearing in the media regardless of the amount of testosterone in the media. Treatment of fat pads with DEX + I resulted in the expected increase in the amount of leptin secreted; however, this amount also appeared to decline with the length of time in culture for epididymal fat pads exposed to control media or media containing 50 ng/mL testosterone. The treatment with 5.0 ng/mL testosterone appeared to prevent this decline. Two-way ANOVA of this absolute data indicated a significant ($P < .01$) effect of both time and treatment, but no interaction. The lower panel of Figure 6 shows the results expressed as the increase in leptin secretion from the ep-

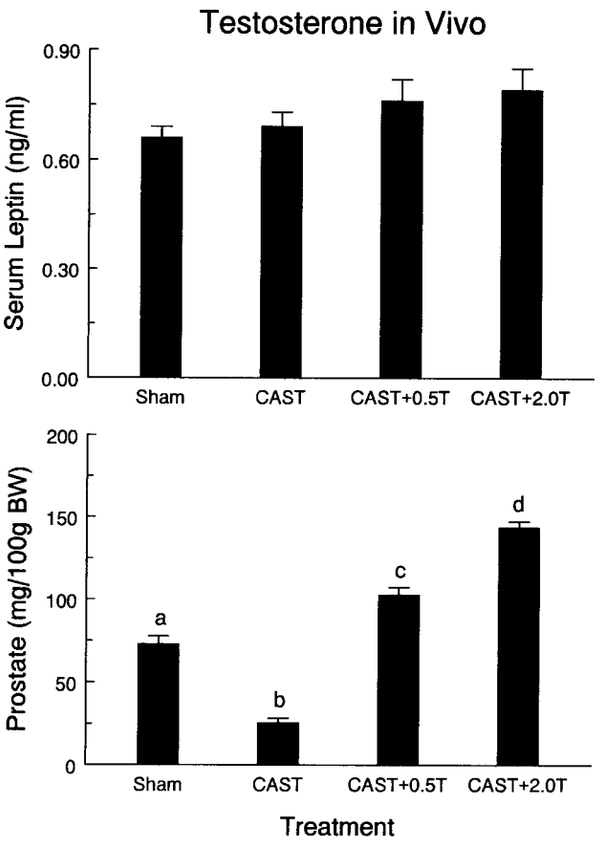

Figure 4. Relative prostate weight (mean ± SEM, lower panel) and serum leptin concentrations (mean ± SEM, upper panel) in immature rats sham operated (Sham) or castrated (CAST) and treated with empty or 0.5 cm (CAST + 0.5T) or 2.0 cm (CAST + 2.0T) testosterone-filled capsules. Animals were castrated on day 24 and killed 1 week later; n = 7 per group. **(a–d)** Groups with different letters are significantly different from each other (Student-Newman-Keuls test). There were no significant effects of treatment on serum leptin (ANOVA).

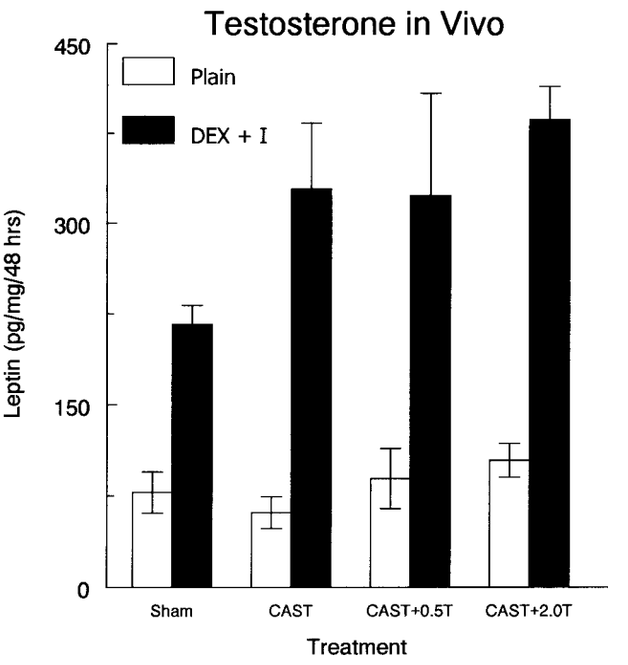

Figure 5. Secretion of leptin (mean ± SEM) into the media by epididymal fat pads obtained from the immature rats sham operated (Sham) or castrated (CAST) and treated with empty or 0.5 cm (CAST + 0.5T) or 2.0 cm (CAST + 2.0T) testosterone-filled capsules 1 week prior to being killed. Paired fat pads were incubated for 48 hours in plain media (open bars) or media containing 1 μM DEX plus 50 nM insulin (DEX + I; solid bars). Two-way ANOVA indicated a significant effect of incubation with DEX + I, but no effect of in vivo treatment and no interaction; n = 7 per group.

ididymal fat pad exposed to DEX + I compared with that from the pad exposed to media without DEX + I. Two-way ANOVA of the data in this form also results in a significant ($P < .01$) effect of testosterone treatment, but no effect of time and no interaction.

Discussion

The data reported here clearly indicate that the serum concentrations of leptin are higher in adult male rats than they are in male rats prior to the onset of puberty. They also strongly suggest that the reason for this developmental increase is an increased ability of the adipose tissue to secrete leptin.

Although there is solid support (Barash et al, 1996; Ahima et al, 1997; Chehab et al, 1997; Cheung et al, 1997) for the proposal that leptin plays a significant role in the onset of female puberty, the evidence for a similar role in male mammals is weak. Indeed, investigators studying humans (Ahmed et al, 1999), rhesus monkeys (Plant and Durrant, 1997; Urbanski and Pau, 1998; Mann et al, 2000), and rats (Nazian and Cameron, 1999) have concluded that leptin is not a trigger that induces sexual maturation in the male. Rather, they have proposed that appropriately elevated levels of leptin have a permissive effect (Urbanski and Pau, 1998), or that an increase in the serum concentrations of leptin is a consequence of other maturational events, or both. Separate groups working in primates (Urbanski and Pau, 1998) and rodents (Nazian and Cameron, 1999) have proposed that the maturational increase in leptin concentrations is induced by the peripubertal testosterone rise. The data presented here suggest that, at least prior to puberty onset, testosterone has little or no effect on leptin secretion. Castration of immature male rats did not alter either the serum levels of leptin or the ability of the epididymal fat pads to secrete leptin, regardless of testosterone replacement, at least at the levels used in these experiments. It was expected that the larger testosterone capsule would result in early pubertal levels of serum testosterone, thereby mimicking an early peripubertal rise. Based on the response of the prostate, both capsules achieved this result, provid-

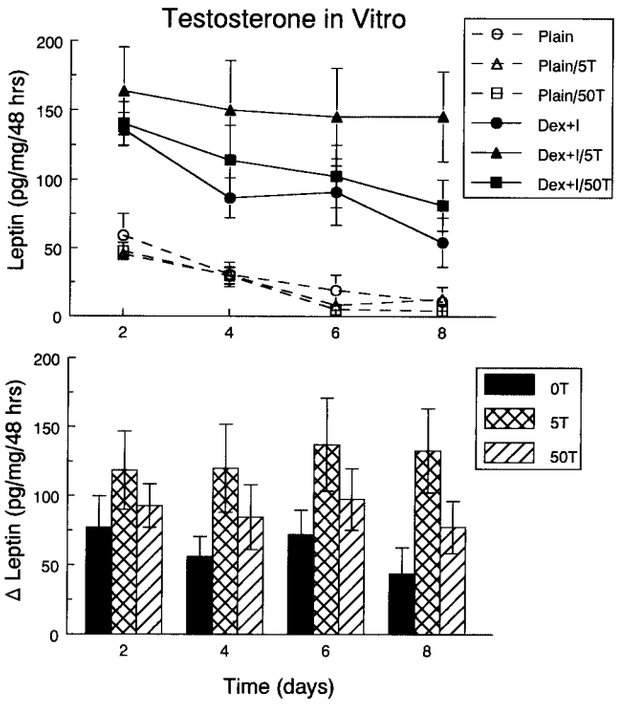

Figure 6. Upper panel: Leptin secretion (mean ± SEM) by paired epididymal fat pads obtained from immature rats and incubated in plain media (open symbols) or media containing 1 μM DEX plus 50 nM insulin (solid symbols). Media also contained 0 (circles), 5.0 (triangles), or 50.0 (squares) ng/mL testosterone. Two-way ANOVA of this absolute data indicated a significant ($P < .01$) effect of both time and treatment, but no interaction. Lower panel: The increment in leptin secretion induced by the DEX + I treatment. Two-way ANOVA of the data in this form also results in a significant ($P < .01$) effect of testosterone treatment, but no effect of time and no interaction; n = 6 per group.

ing testosterone levels that were biologically equivalent to those found in much older animals. It is possible that 1 week of exposure to these elevated levels of testosterone was a temporally inadequate stimulus. However, examination of the temporal relationship between the serum concentrations of testosterone and leptin during the sexual maturation of the male rat (Nazian and Cameron, 1999) suggests that a peripubertal rise in leptin secretion follows the peripubertal rise in testosterone by less than 1 week. The possibility remains that higher levels of testosterone, dihydrotestosterone, or both, would have resulted in an increase in leptin secretion.

The data from in vitro treatment of epididymal fat pads provides some support for this latter suggestion. A dose of testosterone equivalent to a moderately high adult normal value was capable of maintaining the responsiveness of the epididymal fat pad to the DEX + I treatment, whereas much higher doses or a lack of testosterone resulted in a decline over time. Perhaps the role of testosterone on leptin secretion is more subtle than anticipated, with adult levels resulting in a maintenance of responsiveness to other factors.

The observation that an effect of testosterone is only evident in the presence of DEX + I reinforces our belief that the presence of these additives results in more physiologically ''normal'' incubation conditions, at least with respect to leptin metabolism. The absence of either one of these substances results in either a slow (in the case of insulin) or immediate (in the case of DEX) decline in the ability of epididymal fat to secrete leptin (see Figure 1).

Several recent studies have examined the direct effect of gonadal steroid hormones on leptin. Although evidence seems to be accumulating that estrogens have a positive effect on leptin in females (Shimizu et al, 1997; Brann et al, 1999; Kristensen et al, 1999), the results are somewhat less clear when males are considered. It has been reported that in the male rat, castration reduces leptin messenger RNA (mRNA) expression in adipocytes obtained from subcutaneous fat but increases its expression in adipocytes obtained from perirenal fat (Machinal et al, 1999). Other studies have concluded that androgens decreased expression of leptin mRNA in epididymal and retroperitoneal fat (Wu-Peng et al, 1999).

There also does not appear to be a consensus on the effects of androgens on the serum levels of leptin in vivo or its secretion from adipose tissue fragments cultured in vitro. Testosterone has been reported to decrease serum leptin concentrations in hypogonadal men (Jockenhovel et al, 1997; Sih et al, 1997), and to reverse a castration-induced rise in leptin levels in male rats (Watanobe and Suda, 1999). This latter effect has been claimed to be the result of a reduction in the mass of the adipose tissue induced by testosterone (Pinilla et al, 1999). Other workers have found no effect of androgens on the secretion of leptin from adipose tissue cultured in vitro (Kristensen et al, 1999).

The major difference between these studies and the work reported here is the age of the animals used. The work summarized in the preceding paragraph was performed exclusively in adult animals, whereas the testosterone studies reported here were performed either in immature rats or with adipose tissue obtained from immature males. Complex changes take place during puberty that include both profound alterations in function as well as in the hormonal milieu. In fact, it was the working hypothesis of these studies that a difference in adipose tissue function was included in these changes.

Clearly, the epididymal fat pads obtained from adult male rats were capable of secreting more leptin than those obtained from immature rats. Although there are reports (Shimizu et al, 1997; Machinal et al, 1999) that fat from different regions varies in its ability to secrete leptin, this observation can probably account for the increase in serum leptin concentrations seen with the sexual maturation of the male rat.

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