Gonadal suppression by a GnRH analogue does not alter somatic growth in rats with complete GH deficiency

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Abstract

Sexual dimorphism of somatic growth in rats appears to reflect differing actions of sex steroids. However, mechanisms of gonadal steroid effects on the somatotrophic axis are incompletely understood. To evaluate whether GH is involved in the effects of long-term gonadal suppression on somatic growth in rats, a GnRH agonistic analogue (GnRHa) was administered to normal Sprague-Dawley rats (controls) and to a strain of rats with complete growth hormone deficiency (GHD; $n=4-6$ in each group). Subcutaneous injection of GnRHa (2 mg/kg) or saline were given within 48 h after birth and repeated every 3 weeks. GnRHa treatment significantly reduced serum gonadal steroid levels in rats of both sexes with small testes in males and impaired development of internal genitalia in females. GnRHa-treated control females became significantly heavier ($P<0.01$ ANOVA for repeated measures) than

saline-treated rats beginning at 8 weeks. However, female GHD rats with GnRHa treatment did not differ in body weight from rats receiving saline. In male rats, GnRHa treatment did not change body weight in either control or GHD rats. Serum IGF-I concentrations did not differ between treatment groups in GHD and control rats of either sex. Hepatic GH binding was reduced significantly by GnRHa treatment in female control rats ($P<0.01$), but not in female GHD rats. These data suggest that sexual dimorphism in body size and its modulation by estrogens are independent of circulating IGF-I levels suggesting non-endocrine IGF-I-mediated mechanisms, and that GH-induced somatic growth is modulated by estrogens, but not androgens, in rats.

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Introduction

Interactions between the somatotrophic and the gonadotrophic axes play important roles in mammalian somatic growth. Manipulation of gonadal steroid secretory status clearly alters somatic growth in rodents (Jansson *et al.* 1985, Wehrenberg & Giustina 1992). However, the precise mechanisms by which gonadal steroids affect the somatotrophic axis are not fully understood.

In female rats elimination of estrogens by ovariectomy results in exaggerated growth, while administration of estradiol (E_2) inhibits somatic growth (Jansson *et al.* 1985). In addition, ovariectomy reduces circulating levels of growth hormone (GH), while E_2 administration increases mean GH levels (Carlsson *et al.* 1987, Borski *et al.* 1996). In contrast, circulating levels of insulin-like growth factor-I (IGF-I) increase after ovariectomy and decrease with E_2 administration (Handelsman *et al.* 1987, Borski *et al.* 1996). Furthermore, GH binding in liver is decreased by ovariectomy (Carmignac *et al.* 1993). Thus, elimination of estrogens in female rats is associated with enhanced growth, low GH, high IGF-I concentrations,

and decreased hepatic GH binding. These paradoxical links between growth, GH and IGF-I led to a hypothesis that estrogen action on the somatotrophic axis was mediated by inhibition of IGF-I production in the liver or elsewhere (Borski *et al.* 1996). However, ovariectomy in GH-insufficient dwarf rats did not significantly alter body weight gain (Gevers *et al.* 1995), suggesting that at least in female rats gonadal steroids may affect growth by altering GH actions.

In male rats, alterations of gonadal steroids have not produced consistent results. Body weight gain was attenuated in some studies (Siddiqui *et al.* 1989, Vanderschueren *et al.* 1994), but not in others (Jansson *et al.* 1985, Handelsman *et al.* 1987). Circulating IGF-I levels and hepatic GH binding either were increased (Handelsman *et al.* 1987) or unchanged (Siddiqui *et al.* 1989, Vanderschueren *et al.* 1994). Reasons for these inconsistencies are not clear, but aspects of growth related by gonadal steroids might be more sensitive to estrogens than to androgens.

In addition, the perinatal hormonal milieu might influence somatic growth and the somatotrophic axis during

later life. In male rats prepubertal castration did not reduce body weight gain as much as neonatal castration, and neonatal administration of testosterone clearly affected somatic growth (Jansson *et al.* 1985). Furthermore, a previous study has shown that prepubertal castration enhanced serum IGF-I concentrations, while neonatal administration of gonadotropin-releasing hormone (GnRH) antagonist abolished this pubertal rise of IGF-I (Handelsman *et al.* 1987). Thus, one suspects that gonadotropin elevation induced by gonadectomy might have contributed to the IGF-I rise during puberty. Accordingly, sex steroids may participate importantly in growth and in regulation of the somatotrophic axis both during puberty and during early postnatal life.

We sought to clarify the mechanisms underlying the effects of prepubertal manipulation of gonadal steroids on the somatotrophic axis by studying completely GH-deficient rats. The dwarf rats used in previous investigations (Gevers *et al.* 1995) were only partially GH-deficient, with residual GH in the circulation representing approximately 10% of concentrations in normal rats (Carmignac & Robinson 1990). Animals used in the present study were completely deficient in GH, as confirmed by abnormal splicing of the GH gene and absence of GH products in the pituitary (Nogami *et al.* 1989, Takeuchi *et al.* 1990). The present study also characterizes prepubertal gonadal suppression with GnRH agonistic analogue (GnRHa) since early postnatal life. In this manner, potentially confounding effects from elevated gonadotropins and imprinting effects of neonatal androgens on the somatotrophic axis can be minimized.

Materials and Methods

Study protocol

The spontaneous dwarf rats were generously provided by Roussel Morishita Research Laboratories (Shiga, Japan). This animal has an autosomal recessive mutation in the GH gene resulting in complete absence of the GH protein while prolactin-, adrenocorticotropin-, thyroid-stimulating hormone- and luteinizing hormone-producing pituitary cells are unaffected (Nogami *et al.* 1989, Takeuchi *et al.* 1990). Thus, this unique model of dwarfism shows isolated, complete GH deficiency (GHD). Adult Sprague–Dawley rats were purchased from CLEA JAPAN INC. (Shizuoka, Japan) and used as control animals. GHD rats and Sprague–Dawley rats approximately 15 weeks old were crossed to obtain GHD and Sprague–Dawley newborn rats for the study. Pups were assigned randomly to either GnRHa or saline treatment groups.

Treatment groups for the first experiment were as follows: male GHD rats treated with GnRHa ($n=6$); male GHD rats treated with saline ($n=6$); female GHD rats treated with GnRHa ($n=5$); female GHD rats treated

with saline ($n=5$); male Sprague–Dawley rats treated with GnRHa ($n=5$); male Sprague–Dawley rats treated with saline ($n=6$); female Sprague–Dawley rats treated with GnRHa ($n=4$); female Sprague–Dawley rats treated with saline ($n=4$). The first dose of GnRHa or saline was given within 48 h after birth, after which GnRHa or saline injections were repeated every 3 weeks. The GnRHa, leuprolide acetate depot, was kindly provided by Takeda Chemical Industries Ltd (Tokyo, Japan). The dose of GnRHa (2 mg/kg, s.c.) was determined according to a previous study (Sudo *et al.* 1991), where this dosage produced marked gonadal suppression. Animals were weighed weekly to measure somatic growth. At the end of the study at age 15 weeks, rats were killed by cardiac exsanguination under ether anesthesia. Blood sampling and tissue sampling including liver and testis were performed. The wet weight of the testis was measured. Livers were frozen immediately on liquid nitrogen. Serum and livers were stored at -20°C until assayed.

In the second experiment, Sprague–Dawley rats were treated with GnRHa (male, $n=3$; female, $n=4$) or saline (male, $n=4$; female $n=5$) under the same treatment regimen as described for the first experiment, and serial tail vein blood sampling was performed every 3 weeks to evaluate serum IGF-I levels.

All rats were housed in animal facilities with 12 h light : 12 h darkness and an ambient temperature of 25°C . A block rodent diet (CA-1; CLEA JAPAN INC.) and tap water were provided and animals were allowed to feed and drink *ad libitum*. The protocol was approved by the Animal Studies Committee of the Tohoku University School of Medicine.

Hormonal assays

Serum levels of testosterone and E_2 were determined with commercially available RIA kits (DPC Japan, Tokyo). Respective minimum detectable values for testosterone and E_2 were 5 ng/dl and 5 pg/ml. Serum IGF-I concentrations were determined by a double antibody RIA using a rabbit antiserum to rh-met IGF-I (878/4) at a final titer of 1:250 000 following extraction of plasma binding proteins by an acid–ethanol cryo-precipitation technique (Breier *et al.* 1991). Excess insulin-like growth factor-II (IGF-II) was added to the assay buffer in order to remove the interfering effects of residual IGF binding proteins. This assay has been extensively validated for rat serum (Breier *et al.* 1994). Intra- and interassay coefficients of variation respectively were 5.9% and 8.5% for testosterone, 4.4% and 4.2% for E_2 , and 4.5% and 7.2% for IGF-I.

Hepatic GH binding analysis

Hepatic GH binding assays were performed as described previously (Breier *et al.* 1988) and validated for rat liver (Singh *et al.* 1992, Gargosky *et al.* 1995, Ambler *et al.*

1996, Butler *et al.* 1996). In brief, microsomal membranes were prepared as follows. Liver tissue was thawed at 4 °C, cut into small pieces (~1 g), and washed in cold (4 °C) 0.3 M sucrose. The tissue was then weighed and homogenized (1:3 wt/vol) in 0.3 M sucrose containing aprotinin (106 kallikrein inhibitor units [KIU]/l; Trasylol, Bayer Pharmaceuticals, Botany, Australia). The homogenate was centrifuged at 1500 g for 20 min at 4 °C, and the resulting supernatants were centrifuged sequentially at 15 000 g for 20 min and 100 000 g for 90 min at 4 °C. The 100 000 g pellet was incubated with 4 M MgCl₂ (1:2 wt/vol ratio of initial liver weight) for 20 min at 4 °C to remove endogenously bound ligands (Breier *et al.* 1988). The preparation was then centrifuged at 48 500 g for 60 min at 4 °C, and the resulting pellet was suspended in 0.025 M HEPES buffer, pH 7.6, and centrifuged again at 48 500 g for 20 min at 4 °C. Aliquots of the final pellet were resuspended in cold 0.25 M HEPES buffer, pH 7.6, containing aprotinin 106 KIU/l at a ratio of 1 ml buffer/g wet wt initial liver tissue and frozen in aliquots at -20 °C until further analysis. Binding analysis was performed in triplicate using 50 µl of membrane preparation (the equivalent of 50 mg initial wet liver weight) per tube. The membrane preparations were incubated with approx. 30 000 c.p.m. of ¹²⁵I-bGH (batch #PR003, Dr W Baumbach, American Cyanamide Co, Princeton, NJ, USA) at a final incubation volume of 0.5 ml for 20 h. Non-specific binding was determined by addition of excess of unlabeled bGH (1000 ng/tube). Incubation was terminated by adding 3 ml of ice-cold 0.025 mol/l TRIS, 0.01 mol/l CaCl₂ buffer, pH 7.6. Bound and free hormone were separated by centrifugation at 3000 g for 45 min at 4 °C. Equilibrium was reached under these conditions and specific binding was completely reversible by the addition of excess of the appropriate ligand. The intra- and interassay coefficients of variation were 5.7% and 9.8% respectively. Protein concentrations of the membrane preparations for individual animals were determined by a modified Lowry method (Singh *et al.* 1992), and specific binding has been corrected for protein concentration.

Data analysis

Serial body weight data were assessed by ANOVA for two-factor repeated measurements, and other data were analyzed by ANOVA for factorial measurements, both followed by Fisher's protected least significant difference method with correction for multiple comparisons as appropriate. Statistical significance was accepted at $P < 0.05$. All values are expressed as the mean $\pm$ s.d.

Results

Body weight of male Sprague-Dawley rats was significantly greater than female ($P < 0.01$), and the males were

Figure 1 Mean body weight ($\pm$ s.d.) in Sprague-Dawley (control) rats (A) and GHD rats (B) given saline injections. Male Sprague-Dawley (control) rats (●), female Sprague-Dawley (control) rats (■), male GHD rats (○), and female GHD rats (□) are depicted. SD, Sprague-Dawley.

twice as heavy as females at 15 weeks (Fig. 1A). While male GHD rats also weighed significantly more than females ($P < 0.05$), sex difference in body weight was small (only 25% heavier at 15 weeks, Fig. 1B). Serum IGF-I concentrations were higher, and hepatic GH binding was lower, in male Sprague-Dawley rats than in females, while these sex differences were not observed in GHD rats (Fig. 2). Circulating testosterone and E₂ levels at 15 weeks were lower in GHD than in Sprague-Dawley rats, although the differences did not reach significance (GHD vs Sprague-Dawley for testosterone, 154.1 ± 83.5 vs 260.7 ± 152.4 ng/dl, $P = 0.07$; for E₂, 14.0 ± 5.9 vs 25.2 ± 12.9 pg/ml, $P = 0.06$).

GnRHa treatment achieved gonadal suppression as evidenced by decreased serum concentrations of testosterone (control, 260.7 ± 152.4 vs 59.1 ± 23.3 ng/dl, $P < 0.01$; GHD, 154.1 ± 83.5 vs 48.9 ± 23.1 ng/dl, $P < 0.01$) or E₂ (control, 25.2 ± 12.9 vs 5.3 ± 2.0 pg/ml, $P < 0.05$; GHD, 14.0 ± 5.9 vs 5.2 ± 0.5 pg/ml, $P < 0.05$) and reduced testicular wet weight (control, 1.89 ± 0.13 vs 1.22 ± 0.06 g, $P < 0.01$; GHD, 0.70 ± 0.06 vs 0.46 ± 0.03 g, $P < 0.01$) or

Figure 2 Mean circulating IGF-I levels and hepatic GH binding ($\pm$ S.D.) at 15 weeks in Sprague-Dawley (control) rats (A) and GHD rats (B). Males, hatched columns; females, open columns; SD, Sprague-Dawley.

impaired development of the ovaries and uterus. Treatment with GnRHa did not significantly alter body weight, serum IGF-I levels or hepatic GH binding in either male Sprague-Dawley or male GHD rats (Fig. 3). However, body weight was significantly greater and hepatic GH binding was significantly lower in GnRHa-treated female Sprague-Dawley control rats ($P < 0.01$). However, in GHD female rats body weight and hepatic GH binding were not affected by GnRHa treatment. GnRHa treatment did not change circulating IGF-I levels in either female Sprague-Dawley or female GHD rats (Fig. 4). In addition, serial IGF-I measurement from the second experiment revealed that IGF-I levels were not affected by the GnRHa treatment at any time points before 15 weeks

Figure 3 Mean body weight (upper panels), circulating IGF-I levels (middle) and hepatic GH binding (lower) at 15 weeks in male Sprague-Dawley (control) rats (A) and GHD rats (B). Rats were treated either with saline (filled columns) or GnRHa (open columns). Values are $\pm$ S.D. SD, Sprague-Dawley.

of age in both male and female Sprague-Dawley control rats (Fig. 5).

Discussion

Three major mechanisms have been proposed for the effects of gonadal steroids on growth and the somatotrophic axes: mediation via GH secretory status (indirect effects); direct effects such as on local growth factors; and alteration of GH responsiveness by gonadal steroids. GH secretory status is known to be changed by modulation of gonadal steroids in rats as well as in humans (Jansson *et al.* 1985, Wehrenberg & Guistina 1992). In a study of dwarf rats, Gevers *et al.* reported that gonadectomy suppressed body weight gain in male rats but had no effect in female rats (Gevers *et al.* 1995). The authors suggested that androgens had such a direct action while estrogens altered GH secretion to affect growth. However, the same authors later reported a study where GnRHa had little effect on GH secretion, and postulated that growth enhancement

Figure 4 Mean body weight (upper panels), circulating IGF-I levels (middle), and hepatic GH binding (lower) at 15 weeks in female Sprague–Dawley (control) rats (A) and GHD rats (B). Rats were treated either with saline (filled columns) or GnRHa (open columns). Values are $\pm$ s.d. SD, Sprague–Dawley.

by GnRHa was due to a reduction of direct inhibition by estrogens (Gevers *et al.* 1998). In addition, an earlier study has shown that E_2 administration suppressed somatic growth in ovariectomized, hypophysectomized female rats, suggesting a direct effect of estrogens (Jansson *et al.* 1983). Whether the effects of gonadal steroids on growth are mediated through the somatotrophic axis therefore is not clear. Previous studies have used GH deficient rats with residual GH as high as 10% of normal. Other studies have used hypophysectomized rats, in which multiple pituitary hormones were insufficient. In the present study, we used the spontaneous dwarf rat, which has a splicing mutation in the GH gene (Takeuchi *et al.* 1990). Absence of GH protein in the pituitaries of these animals has been confirmed (Nogami *et al.* 1989). This animal model, then, showed isolated complete GH deficiency, and modulation of gonadal steroids could not alter GH secretory status as was possible in GH-insufficient dwarf rat models. Therefore, direct effects of gonadal steroid modulation on growth can be clarified in this model.

Figure 5 Mean circulating IGF-I levels ($\pm$ s.d.) in Sprague–Dawley (control) rats. Saline-treated male rats (■), GnRHa-treated male rats (□), saline-treated female rats (●), and GnRHa-treated female rats (○) are depicted. SD, Sprague–Dawley.

Our study suggests that sexual dimorphism in circulating IGF-I levels and hepatic GH receptor concentration appears to be GH-dependent. In Sprague–Dawley control rats marked sexual dimorphism was seen in circulating IGF-I levels and hepatic GH binding, while they did not differ between sexes in GHD rats. In addition, although our male GHD rats were significantly heavier than females as originally reported (Okuma & Kawashima 1980), the difference was relatively small, only 25% at 15 weeks. In contrast, male Sprague–Dawley rats weighed twice as much as female Sprague–Dawley rats. These results suggest that the sexual dimorphism seen in control rats may be mediated mainly by sexually dimorphic GH secretion and action. Other factors independent of GH, such as direct actions of sex steroids, would have only minor effects on sexually dimorphic postnatal somatic growth in rats.

As shown in the present study, suppression of gonadal function by GnRHa did not alter body weight gain in male or female GHD rats. In Sprague–Dawley males, GnRHa treatment did not alter body weight. Orchiectomy generally inhibits body weight gain, but it was also reported that prepubertal orchiectomy had little effect on growth (Jansson *et al.* 1985). The effect of orchiectomy on somatic growth appears to be smaller than that of ovariectomy; reduction of body weight gain in orchiectomized rats was less than 10%, while in female ovariectomized rats, body weight increased by 30% (Gevers *et al.* 1998). Moreover, gonadal suppression induced by GnRHa was not complete in our study. Testicular weight was reduced by only 50% in Sprague–Dawley rats treated with GnRHa despite testosterone levels being markedly reduced. Thus, another reason for a lack of GnRHa effect on body weight in male rats may be incomplete gonadal suppression. On the other hand, in female Sprague–Dawley rats, GnRHa-induced ovarian hypofunction augmented body weight gain as repeatedly reported in ovariectomized rats (Jansson *et al.* 1985,

Wehrenberg & Guistina 1992). However, GnRHa did not have any effect in female GHD rats. This suggests that the effects of gonadal steroids in female rats are mediated primarily by GH. Our study also has demonstrated that hepatic GH binding was not altered in tissues from GHD rats despite reduced estrogens, which indicates that modulation of hepatic GH binding by estrogens also requires the presence of GH.

Circulating IGF-I levels were not altered by GnRHa treatment in either sex or in either strain of rats. To examine whether GnRHa treatment can change IGF-I levels before 15 weeks of age, serum concentrations of IGF-I were measured every 3 weeks in Sprague–Dawley rats in the second experiment. The results indicated that, in both male and female rats, IGF-I levels were not different between the treatment groups. Since GnRHa did not affect growth in male rats, lack of effects on hepatic GH binding and unchanged serum IGF-I concentrations were not surprising. However, reduction of estrogens by GnRHa resulted in enhanced growth in control Sprague–Dawley rats as outlined above; this augmentation was accompanied by reduced hepatic GH binding and unchanged serum IGF-I concentrations. Studies of ovariectomized rats also showed a reduction in hepatic GH binding but circulating IGF-I levels were increased (Handelsman *et al.* 1987, Gevers *et al.* 1995, Borski *et al.* 1996). Augmented IGF-I concentrations resulting from elimination of estrogens have been linked to enhanced growth, but no analogous relationship has been observed in male rats. Unaltered levels of serum IGF-I in our study might be related to inhibited gonadotropin levels, since elimination of estrogens was accomplished by ovariectomy in other studies. Alternatively, suppression of gonadal function during early post-natal life might have abolished the later rise in IGF-I. An earlier study has demonstrated that neonatal administration of a GnRH antagonist abolished the pubertal rise of IGF-I (Handelsman *et al.* 1987). Thus, early postnatal inhibition of gonadotropins can affect IGF-I levels at puberty.

In summary, an increase in body weight seen in female control rats with GnRHa treatment was not observed in GHD rats. This suggests that the GH-induced somatic growth in rats is modulated by estrogens but not androgens. Although the present study did not determine whether androgens have any direct effects on growth, possible small direct effects of androgens on body weight as previously suggested (Gevers *et al.* 1995) may explain a slightly greater body weight in male GHD rats than females. Reasons for lack of change in IGF-I levels with suppression of gonadal function by GnRHa are unclear. The sexual difference in body size and its modulation by estrogens seem to be independent of circulating IGF-I levels suggesting non-endocrine IGF-I-mediated mechanisms. The complex interrelationship between the gonadotropic and the somatotrophic axes requires further study to clarify details of mechanisms and interactions among the various influences.

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