Imprinting of Female Offspring with Testosterone Results in Insulin Resistance and Changes in Body Fat Distribution at Adult Age in Rats

Cecilia Nilsson,*§ Maria Niklasson,*§ Elias Eriksson,‡ Per Björntorp,*§ and Agneta Holmäng*§

**Department of Heart and Lung Diseases, ‡Department of Pharmacology, and the §Wallenberg Laboratory, Göteborg University, Göteborg, Sweden*

Abstract

In women, a relative hyperandrogenicity is statistically associated with insulin resistance and centralization of body fat, which are predictors for the development of non–insulin-dependent diabetes mellitus. The aim of this study was to evaluate the effect of androgenization of newborn female rats on insulin sensitivity at adult age. To mimic the neonatal androgen peak normally observed in male rats, female pups were administered one high dose of testosterone (T) subcutaneously within 3 h after birth. They were then given back to their mothers and followed to adult age. At the end of the week 9, tail samples were taken, showing no differences in fasting plasma concentrations of glucose, lactate, insulin, or free fatty acids between T-treated rats and controls. Plasma concentrations of T and progesterone were significantly lower in the T-treated rats, whereas no differences were found in the levels of corticosterone, estradiol, insulin-like growth factor I, or ACTH. After 10 wk, insulin sensitivity was studied with hyperglycemic and euglycemic hyperinsulinemic (5 mU insulin/kg/min) clamp techniques. The T-treated rats showed insulin resistance with both techniques, which was overcome with time and increasing insulin concentrations during the clamp measurements. The T-treated rats were also heavier and had increased relative weights of skeletal muscles and the spleen. Parametrial, retroperitoneal, and inguinal adipose tissues decreased in weight while mesenteric adipose tissue tended to increase, resulting in an ~ 30–50% larger mesenteric than other adipose tissues. It is concluded that neonatal T imprinting of female rats is followed by insulin resistance, changes in adipose tissue distribution, and an enlarged lean mass, without elevation of circulating T. Similar changes are seen in adult female rats or women receiving T. (*J. Clin. Invest.* 1998. 101:74–78.)

Key words: testosterone • neonatal exposure • insulin resistance • skeletal muscle • adipose tissue

Introduction

Diabetes in women with beard growth was described in 1921 (1). The polycystic ovary syndrome is characterized by hyperandrogenicity, centralization of body fat, severe insulin resistance, and impaired glucose tolerance, also in the absence of obesity (2, 3). In women selected at random from the population, a relative hyperandrogenicity without polycystic ovaries is statistically associated with insulin resistance, powerful predictors for the development of non–insulin-dependent diabetes mellitus (4–6). The risk of development of non–insulin-dependent diabetes mellitus is elevated ~ 10-fold in the lowest quintile of sex hormone binding globulin concentrations, an indicator of hyperandrogenicity (4), in comparisons with the four highest quintiles of sex hormone binding globulin concentrations. It should be noted that this relative hyperandrogenicity is not always apparent clinically as gross physical stigmatization with hirsutism or genital abnormalities. However, such women often have a centralization of body fat as indicated by an elevated waist/hip circumference ratio, a male characteristic (7, 8). Furthermore, at least when obesity is at hand, lean body mass is elevated (9).

Testosterone (T)[1] administered to female rats and to transsexual women is followed by severe insulin resistance (10–12). In rats this is due to a combination of abnormalities of insulin effects in muscle tissue. These consist of absence of translocation of glucose transporter 4 to the cell surface, deficient activation of glycogen synthase, a decrease of glycogen synthase protein, and a diminished transcapillary transport of insulin (10, 11, 13). Transsexual women receiving T have diminished subcutaneous fat, while visceral fat and lean body mass increase (14). Furthermore, in obese, postmenopausal women, androgen administration was followed by a diminution of abdominal and femoral subcutaneous fat, while visceral fat mass increased (15).

Notably, hyperandrogenicity is not always associated with elevated androgen levels in serum. Certain clinical manifestations of an increased androgenicity, such as hirsutism, male pattern baldness, and anovulation, may occur and may respond to treatment with androgen antagonists, also in women displaying normal serum levels of the androgens (16, 17). An enhanced responsiveness of the androgen receptor is one of several tentative explanations of these findings.

With this background, it becomes important to elucidate the cause and effects of the relative hyperandrogenicity in a rather large part of the population of women. Androgen production may occur from the ovaries, adrenals, and adipose tissue in females (18, 19).

Of considerable importance in this context is the observation that adult female rats may display male-like behavior also

Address correspondence to Dr. Agneta Holmäng, Wallenberg Laboratory, Göteborg University, S-413 45 Göteborg, Sweden. Phone: 46-31-60-29-65; FAX: 46-31-82-37-62; E-mail: Agneta.Holmang@wlab.wall.gu.se

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1. *Abbreviation used in this paper:* T, testosterone.

in the absence of elevated serum levels of androgens, if treated with one dose of T in the neonatal phase. Immediately after birth, T production in male offspring results in imprinting of male secondary gender characteristics at adult age. Thus, in female rodents, a relative hyperandrogenicity may be induced either by neonatal imprinting with T or by administration of androgens to the adult animal (20, 21). Clinical studies suggest that early hormonal imprinting may influence androgenization not only in rodents but also in women (22–26).

In this report we describe the consequences at adult age of the administration of one dose of T to newborn, female rat offspring. The specific question asked was whether this is followed at adult age by insulin resistance as well as other characteristics of relative hyperandrogenicity in women, including centralization of body fat and elevated lean body mass. The results show that this is indeed the case.

Methods

Nulliparous time-mated Sprague-Dawley female rats (day 20 of pregnancy) were purchased from B&K Universal (Sollentuna, Sweden) and kept under controlled conditions (temperature 21–22°C, humidity 55–65%, light on from 5 a.m. to 7 p.m.) with one animal in each cage until parturition. The female pups were raised with a lactating mother until ~ 30 d of age; thereafter they lived in cages with five to six animals. All were fed with commercial rat chow, containing 18.5% protein, 4.0% fat, and 55.7% carbohydrates with sufficient supply of vitamins and minerals (Lactamin, Stockholm, Sweden) and were provided with tap water ad libitum. The study was approved by the Animal Ethics Committee of Göteborg University.

Study procedure. Immediately after birth, male pups were removed, and female pups were divided into two groups. One group (n = 22) was treated with T propionate (Apoteksbolaget, Stockholm, Sweden) (1 mg T dissolved in 0.05 ml sesame oil, subcutaneously) within 3 h after birth. As controls, 16 female pups received 0.05 ml sesame oil subcutaneously.

At 9 wk of age, tail blood samples were taken from 11 of 22 T-treated and 10 of 16 control rats for determination of glucose, lactate, insulin, T, progesterone, 17β-estradiol, corticosterone, FFA, IGF-I, and ACTH.

Euglycemic hyperinsulinemic clamp. At 10 wk of age, the rats (controls, n = 6; T, n = 11) were subjected to a euglycemic hyperinsulinemic clamp as described previously (10, 27, 28). Briefly, the animals were anesthetized with 170 mg/kg body wt Inactin (Research Biochemicals, Inc., Natick, MA). Catheters were then inserted into the left carotid artery for blood sampling, and into the right jugular vein for glucose and insulin infusions. The body temperature was maintained at 37°C with a heating blanket. Insulin was continuously infused at a rate of 5 mU/kg/min. To maintain plasma glucose at 7.0 mM, a 10% glucose solution in physiological saline was administered. The infusion rate was guided by glucose concentration measurements in 30-μl blood samples at regular intervals (every 5 min during the first 40 min, then every 10 min). Insulin concentrations were determined in 250-μl blood samples taken at 0, 40, 80, 120, 160, and 200 min of infusion. A total of < 2 ml blood was used for these determinations, compensated for by the infusion volumes. After 160 min, the clamp was terminated. At the completion of the clamp, the rats were killed with intravenous injections of KCl. One control rat died during the clamp.

Hyperglycemic hyperinsulinemic clamp. To determine the endogenous insulin secretion, the rats (controls, n = 10; T, n = 11) were subjected to a hyperglycemic clamp at 10 wk of age as described previously (28). The surgical procedure was performed as described above. Before starting the clamp, a 250-μl blood sample was taken, for determination of plasma glucose and insulin.

The glucose infusion consisted of two phases: (*a*) a 6-min priming dose, empirically calculated to raise plasma glucose levels to ~ 11 mmol/liter (60, 45, 30, 25, and 15 mg/kg, given at 0, 1, 2, 3, 4, and 5 min, respectively); and (*b*) a 60-min maintenance glucose infusion aimed at maintaining hyperglycemia (i.e., ~ 12 mmol/liter). Every 5 min, a 250-μl blood sample was taken, to measure the glucose concentration, and the glucose infusion rate was adjusted accordingly; insulin concentrations were also measured at the same time. At completion of the clamp (after 65 min), the adrenals, thymus, spleen, and heart were rapidly removed and weighed. The muscles of the hind limb (extensor digitorum longus, soleus, plantaris, and tibialis anterior) were also rapidly excised and weighed. Furthermore, the parametrial, retroperitoneal, inguinal, and mesenteric adipose tissues were dissected out and weighed. At the completion of the clamp, the rats were killed with intravenous injections of KCl. One T-treated rat died during the clamp.

Analytical methods. Blood was collected in heparinized microtubes and centrifuged immediately in a microcentrifuge (International Equipment Co., Needham, MA). Plasma concentrations of glucose and lactate were determined enzymatically by using 10-μl samples for simultaneous analysis on a YSI 2700 SELECT biochemical analyzer (Yellow Springs Instrument Co., Yellow Springs, OH). Plasma insulin was analyzed with the rat insulin RIA kit (Linco Research Inc., St. Charles, MO). T was measured with Coat-A-Count Total Testosterone (Diagnostic Products Corp., Los Angeles, CA), which is a solid phase radioimmunoassay.

Progesterone and 17β-estradiol were assayed with commercially available enzyme immunoassays (progesterone ELISA and oestradiol ELISA; Biomar Diagnostic Systems, Marburg, Germany).

For corticosterone determination, a radioimmunoassay, RSL ^{125}I Corticosterone RIA (ICN Biomedicals Inc., Irvine, CA), was used, and ACTH determinations were performed with an immunoradiometric assay (IDS, Boldon, UK). For analysis of IGF-1, an immunoenzymometric assay, OCTEIA IGF-1 kit (IDS), was used.

FFA were determined with an enzymatic colorimetric method (NEFA-C; Wako Chemicals, Neuss, Germany).

Statistical analysis. All results are presented as means±SE. Statistical methods used were Student's t test and, when several comparisons were performed, ANOVA (Stat-View program in the Macintosh system) followed by Fisher's least significant difference test for post-hoc analyses.

Results

Table I shows the weight of the total body and various tissues (adjusted) at the age of 10 wk. Body weight was significantly elevated in the T-treated rats. Both the soleus and tibialis muscles were also heavier in the T group compared with controls, whereas the extensor digitorum longus showed no difference. Among fat depots, the parametrial, retroperitoneal, and inguinal adipose tissue showed significantly lower weights in the T-treated group compared with controls, but the mesenterial fat depot weight tended to be elevated, although not significantly. No significant differences were found in the weights of the adrenals, thymus, or heart between the two groups. However, the spleen was significantly heavier in the T-treated rats.

Fasting plasma concentrations of glucose (6.1±0.3 and 5.8±0.2 mmol/liter), lactate (3.8±0.4 and 3.7±0.3 mmol/liter), insulin (9.2±1.2 and 8.3±0.8 mU/liter), and FFA (1.22±0.13 and 1.23±0.06 mmol/liter) did not differ between the control and T-treated rats, respectively, after nine experimental weeks. The plasma concentrations of T (0.20±0.01** and 0.29±0.02 nmol/liter, $^{**}P < 0.01$) and progesterone (80.7±6.8* and 161±30.5 nmol/liter, $^{*}P < 0.05$) were significantly lower in the T-treated group compared with controls. Estradiol (120±18 and 141±12 pmol/liter), corticosterone (750±33 and 774±25

Table I. Weights of Total Body, Extensor Digitorum Longus (EDL), Soleus (SOL) and Tibialis (TIB) Muscles, Parametrial, Retroperitoneal, Mesenterial, and Inguinal Adipose Tissue, Adrenals, Thymus, Heart, and Spleen (grams/kg of Body Wt.) in 10-wk-old T-treated and Control Rats

	Control	T
	n = 10	*n = 11*
Body wt (grams)	234±3	278±9*
EDL	0.39±0.01	0.41±0.02
SOL	0.34±0.01	0.40±0.02‡
TIB	1.69±0.02	1.76±0.03‡
Parametrial	14.1±0.8	8.1±0.5§
Retroperitoneal	12.1±1.2	9.4±0.3‡
Mesenteric	14.0±0.6	15.3±0.7
Inguinal	12.6±0.7	10.4±0.6‡
Adrenals	0.166±0.015	0.168±0.012
Thymus	2.18±0.09	2.28±0.14
Heart	2.98±0.09	3.04±0.09
Spleen	2.26±0.08	2.67±0.11*

Data are means±SE. ‡$P < 0.05$, §$P < 0.01$, *$P < 0.001$.

nmol/liter), IGF-I (169±5 and 190±11 μg/liter), and ACTH (2.2± 0.1 and 2.8±0.5 pg/ml) did not differ between the two groups.

Euglycemic hyperinsulinemic clamp. Fig. 1 depicts the glucose disposal rate during the euglycemic hyperinsulinemic clamp. The T-treated rats had a significantly lower glucose uptake at 40 min. Plasma insulin concentrations were 96±10 and 110±4 mU/liter (NS), in controls ($n = 5$) and in T-treated rats ($n = 11$), respectively. When plasma insulin levels had reached steady state levels (80 min 112±8 and 127±4 mU/liter, NS, and 160 min 108±12 and 130±6 mU/liter, NS, in controls and in T-treated rats) there were no significant differences in glucose disposal rates between the two groups.

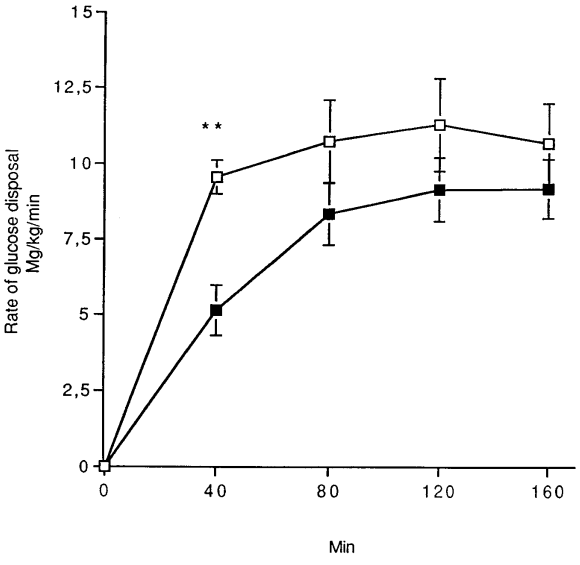

Figure 1. Total body glucose uptake during hyperinsulinemic euglycemic clamp (5 mU/kg/min) measurements in T-treated rats (*filled squares*) and control rats (*open squares*). Plasma glucose concentrations were ~ 7 mmol/liter. For other experimental conditions see Methods. Data are means±SE. Glucose uptake was significantly (**$P < 0.01$) lower in T-treated rats ($n = 11$) compared with control rats at 40 min ($n = 5$).

Plasma glucose was the same in both groups of animals throughout the insulin clamp (7.0±0.1 and 6.9±0.1 mmol/liter, NS, in controls and in T-treated rats, respectively).

Hyperglycemic hyperinsulinemic clamp. Basal plasma glucose concentration before the experiments was 6.9±0.3 mmol/liter for controls ($n = 10$) and 7.1±0.3 mmol/liter ($n = 10$) (NS) for T-treated rats. The aim was to maintain the glucose level at ~ 12 mmol/liter (5 mmol/liter above basal). The mean glucose level observed during the clamps was 11.7±0.2 mmol/liter for controls and 11.7±0.3 mmol/liter for T-treated rats. The stability of the plateau level (at 4–65 min) of glucose concentrations obtained was reflected by the coefficient of variation, which averaged 0.9±0.02% for controls and 0.8±0.03% for T-treated rats (Fig. 2 *a*). The endogenous insulin response provoked by imposed hyperglycemia (Fig. 2 *b*) was biphasic and sustained in both groups. There was no significant difference in basal insulin secretion between the two groups. However, the glucose-induced insulin secretion was significantly higher in the T-treated rats during the first 25 min, but this difference disappeared during the final 35 min of the clamp.

Discussion

The main results of this study were a pronounced insulin resistance, increased body and muscle weights, and a regional change in the distribution of depot fat in adult female rats, which had received one injection of T immediately after birth corresponding to the peak T concentration in newborn male pups (20). These changes occurred without elevation of circulating T; in fact T was lower in these rats than controls. These results contrast to an earlier study, where no differences in T levels were found between the two groups (29). This may be due to different methodological approaches, tail sampling versus decapitation.

Neonatal androgenization of female rats is known to induce morphological changes in the central nervous system and to irreversibly influence behavior (30). The hypothalamic regulation of the pituitary is profoundly influenced; thus, as shown in an early study by Barraclough (31), female rats receiving one dose of T in the neonatal period become anovulatory. In line with this observation, progesterone levels were found to be significantly reduced in the T-treated animals in this study.

The possibility that the insulin resistance, induced by neonatal administration of T to female rats, is partly secondary to this androgenization of the hypothalamus should not be excluded. Indeed, both estrogen and progesterone have been shown to regulate insulin sensitivity in rat (32); disrupted sex steroid cyclicity may thus contribute to the insulin resistance observed. Moreover, the possibility that an influence of neonatal androgenization on the secretory pattern of growth hormone (33) may lead to an altered insulin sensitivity (34) should be taken into consideration.

However, neonatal androgenization appears to influence peripheral tissue also, independent of the hypothalamus (35). The T injection might have changed the regulatory mechanism(s) involved in insulin sensitivity irreversibly. If this is the

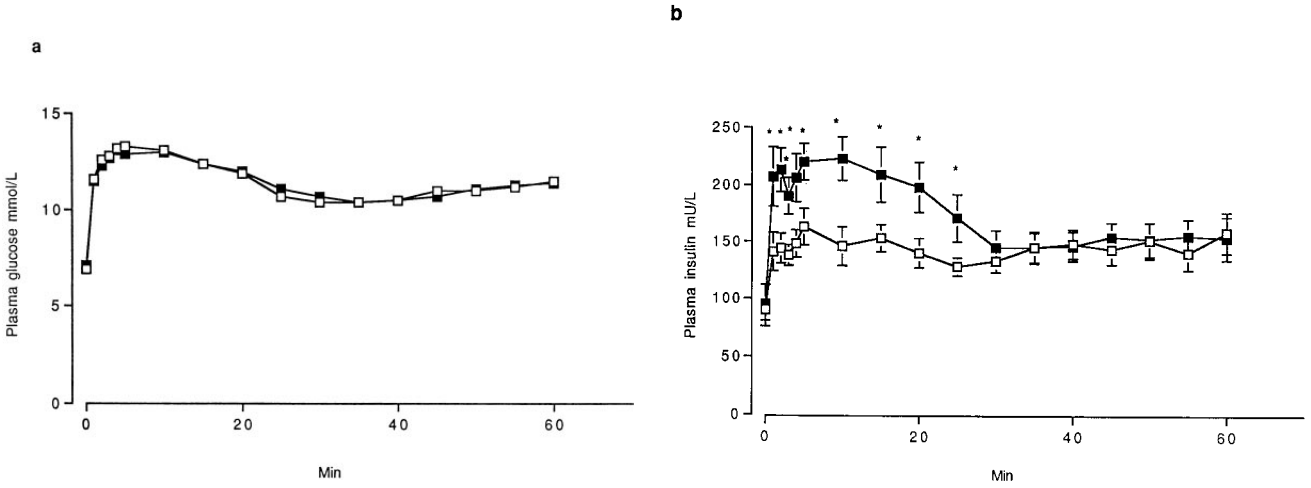

Figure 2. (*a*) Hyperglycemic clamp (i.e., imposed hyperglycemia via continuous, monitored glucose infusion; see Methods) in T-treated rats (*filled squares*) and control rats (*open squares*). Plasma glucose concentrations were ~ 12 mmol/liter. Data are means±SE. T-treated rats (n = 10), control rats (n = 10). No significant differences. (*b*) Endogenous insulin secretory response to a hyperglycemic clamp. Experimental conditions and symbols as in *a*. Plasma insulin concentrations were significantly (*$P < 0.05$) higher in T-treated rats during the first 25 min of the clamp. Data are means±SE.

case, then muscle tissue seems to be the probable location, because the clamp techniques measure mainly insulin sensitivity of muscle (36). Given the similarity between the effect on insulin sensitivity of neonatal and adult androgenization, respectively, and given the fact that T has been shown to diminish capillary density and transcapillary transport of insulin in muscle and also to decrease the insulin response of the translocation of glucose transporter 4 and of glycogen synthase, the possibility that neonatal androgenization may cause insulin resistance by a direct action on muscle cells does not seem unlikely (10, 11, 13).

Any of these abnormalities might be responsible via an "imprinting" effect of T at birth. Furthermore, the density of the androgen receptor might have changed, because T is known to upregulate the density of its own receptor in several tissues (37, 38). This would be expected to be followed by increased sensitivity to androgens and induction of insulin resistance (10). None of these possibilities was examined here. However, T administration to adult female rats is followed by an early decrease of the glucose disposal rate, where the transcapillary insulin transport rate seems to be the limiting factor (our unpublished observations). Since the same phenomenon was observed in this study, a slow transcapillary transport rate of insulin might be involved. Body weight was elevated in the T animals. This might be explained mainly by the well-known anabolic effects of T on muscle tissue, and the weights of the soleus and tibialis were indeed elevated.

Adipose tissue was diminished in all regions measured except mesenteric fat, where a tendency to higher weight was seen, although not significant. This meant that after T treatment the mesenteric fat depot became the largest of the adipose tissues measured (~ 30–50%), whereas this was not the case in controls. T exerts profound effects on adipose tissue metabolism via an androgen receptor (39). Lipid mobilization is enhanced at several regulatory levels, including increased density of the lipolytic adrenergic receptors (40), elevated activity of the adenylyl cyclase (41, 42), and probably of the protein kinase A and/or hormone-sensitive lipase (41, 43). Furthermore, the activity of lipoprotein lipase, the main regulator of lipid uptake in adipocytes, is inhibited by T (44). The net effect of these events would be expected to be a diminution of fat mass, and this was indeed found in all adipose tissues, except in the mesenteric region. This is an unexpected finding because in normal rats the density of the androgen receptor is highest in the mesenteric region in comparison with the other adipose tissues (39). Whether this means that the androgen receptor density is changed with regional preference by T imprinting cannot be decided.

Plasma concentrations of FFA did not differ between the two groups, therefore it seems unlikely that this factor was involved in the mechanism inducing insulin resistance.

The elevated spleen weight in the T-treated animals was not part of a generalized tissue enlargement, because other internal organs showed no differences. Whether this was a sign of involvement of the immune system (45, 46) is not clear.

In general, the results of T imprinting in female rats are remarkably similar to those seen in women with a spontaneous relative hyperandrogenicity, as well as in adult female rats and transsexual and obese, postmenopausal women receiving androgens. In these studies a marked insulin resistance (2–6, 10–12), centralization of adipose tissue mass (3, 4, 7, 14, 15), and an enlarged lean body mass (9, 14) were found. In women and adult rats this occurs with elevated T levels in circulation, whereas this was not the case in the imprinted rats studied here. This seems to leave the possibility for explanation of the observed perturbations in apparently normal women with a relative hyperandrogenicity in the population of both perinatal imprinting by T and an ongoing interaction between overproduced circulating T and tissues. The results of this study have suggested that imprinting by T of newborn female pups can occur with remaining stigmata in the regulation of insulin sensitivity and body composition in adult life. To elucidate the

mechanisms involved in different tissues requires additional studies.

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