Neonatal hypothalamic androgenization in the female rat induces changes in peripheral insulin sensitivity and adiposity function at adulthood

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Abstract
It is recognized that there exists a link between hyperandrogenicity and insulin resistance. OBJECTIVE: By using the neonatally androgenized female rat we explored whether this treatment modifies peripheral insulin sensitivity and visceral fat function at adulthood.

EXPERIMENTAL DESIGNS: On day 5 of age, female Sprague-Dawley pups were injected, sub cutaneous, with either 50µl of sterile corn oil alone (CT) or containing 1.25 mg of testosterone propionate (TP) and further used for experimentation on day 100 of age. CT and TP rats were killed by decapitation in non-fasting condition and blood samples were kept frozen for measurement of different metabolites. Immediately after sacrifice, freshly dissected visceral fat pads were used for isolation of adipocytes, these cells were then incubated with medium alone or containing different concentrations of insulin in order to determine leptin secreted into the medium. Additionally, in vivo metabolic responses to intravenous high glucose load were performed in, 24 hour-fasting, CT and TP rats.

RESULTS: We found that neonatal androgenization induced adult animals displaying higher visceral adiposity mass, body weight and leptinemia than CT rats. No group differences were found in basal circulating levels of several hormones and metabolic parameters. The results of the high glucose load 90-min test indicated that TP and CT rats developed similar glycemia but this accounted because of an early significantly higher peak values of circulating insulin in TP than in CT rats, regardless of similar enhancement in circulating glucocorticoid concentrations in both groups. While high glucose load significantly increased, over the baseline, circulating leptin concentrations as early as 30 min post-glucose in CT rats, in TP animals, it significantly enhanced leptinemia only by the end of the test. Finally, results of in vitro incubations of isolated visceral adipocytes indicated that cells from androgenized rats spontaneously released more leptin than control cells, although they were less responsive than CT cells to insulin-induced leptin output. CONCLUSION: Our study strongly supports the hypothesis that development of insulin resistance seems to be dependent on early hyperandrogenicity.
Introduction

Clinical and experimental studies suggest that an interaction between insulin and sex hormones takes place. It has been reported that sex hormone therapy in healthy subjects [1], androgen treatment in females and estrogen administration in males, is able to induce insulin resistance. A very common cause of menstrual disturbances, chronic anovulation, and hyperandrogenism is, in premenopausal women, the polycystic ovary syndrome (PCOS) [2–4]. Frequently, hyperinsulinemia is a physiopathological finding associated to PCOS [4–6]; however, the overall mechanisms involved in the development of PCOS still remain unclear. While enhanced leptin levels could be a deleterious factor involved in different physiopathological conditions resulting in chronic anovulation [7], conversely, physiological levels of leptin are necessary for normal hypothalamo-pituitary-ovary axis function [8–10]. It is known that PCOS women frequently have associated obesity and insulin resistance [4–6, 11, 12]. Regarding the relationship between PCOS and insulin resistance, it has been reported the successfullness of the treatment of PCOS women with insulin-sensitizing agents [13, 14]. In fact, the therapy with these products normalizes insulin and leptin plasma levels, suggesting that hyperinsulinemia in PCOS patients may play an important role in abnormal adipose tissue function.

Hyperandrogenicity, in non-PCOS women, could be clinically manifested even in the absence of hyperandrogenemia [15, 16], and it can be overridden by androgen antagonist therapy [15, 16]. Clinical manifestation of hyperandrogenicity is frequently associated to insulin resistance, a powerful predictor of type 2 diabetes [17–19]. However, it must be stressed that they could be not accompanied by hirsutism, but, they could be recognized by centralized body fat, a male characteristic [20–22]. Furthermore, obese, postmenopausal, women treated with androgen enlarge their visceral fat mass [23].

With regards to studies in the rat model, only changes in muscle insulin sensitivity have been reported after androgen treatment [24, 25]. In consistence with these findings, abnormalities in the glucose transporter 4, dysfunction of the glycogen synthase, decreased glycogen synthase protein, and diminished transcapillary insulin transport [24–26] have also been observed. In the present study we used the classical model of Barraclough [27], by which early hypothalamic androgenization in female rats induces some biochemical abnormalities at adulthood resembling those characterizing PCOS women (e.g. impaired pattern of LH secretion, anovulation and infertility). We presently add some evidence that early adrogenization in the female rat could be an important factor for the development of both peripheral insulin resistance and changes in visceral adipocyte function during adulthood.

Research design and methods

Animal model

The model employed in our research design has been previously reported [27], however, a brief description is followed. Adult male (300–330 g BW) and female (240–280 g BW) Sprague-Dawley rats were allowed to mate in colony cages in a light- (lights on from 07:00 to 19:00 h) and temperature (22°C)-controlled room. Rat chow and water were available ad libitum. Pregnant rats were transferred to individual cages. On day 5 after parturition, female pups were injected sc with either 50 µl of sterile corn oil alone (n = 20 rats; control group, CT) or containing 1.25 mg of testosterone propionate (Organon, Argentina) (n = 20 rats; TP group). Rats were weaned at 21 days of age and kept in individual cages in a light (lights on from 07:00 to 19:00 h)- and temperature (22°C)-controlled room, with rat chow and water available ad libitum; individual body weight (BW) was daily recorded (between 07:30 and 08:30 h) from the weaning day up to the experimental day. CT and TP rats were used for experimentation on day 100 of age [28].

Experimental designs

Experiment 1. Different groups of rats (8–10) were killed by decapitation (between 08:30 and 09:15 hours) in non-fasting condition. Immediately after decapitation, trunk blood was collected into 10 ml plastic tubes containing 0.5 ml of EDTA 10% solution. Blood samples were immediately centrifuged and plasma samples were kept frozen until measurement of different metabolites (see below). Immediately after sacrifice, freshly dissected visceral fat pads were weighted and used for isolation of adipocytes as previously described [29], although with minor modifications. Briefly, fat pads were slightly minced and placed in KREBS-MOPS medium (Sigma-Aldrich CO., St. Louis) containing 1% BSA and 0.1% collagenase (Sigma-Aldrich, Type 1), in a ratio of 3 ml of solution per gram of fat tissue. Tissues
were gently shaken at 37°C during 50 minutes in polypropylene flasks under 95% air-5% CO2 atmosphere, in a metabolic incubator. At the end of this period, cell suspensions were filtered through one layer of nylon cloth (300µm), transferred to conical polypropylene tubes and centrifuged at 100xg for 20 minutes at room temperature. Cells were then washed, 3 times, with medium alone to eliminate the stromavascular fraction and collagenase. Adipocytes were counted and diluted, with DMEM (Sigma-Aldrich)-1% BSA medium, pH 7.4, to the necessary volume to obtain approximately 4 x 10^5 adipocytes per 1 ml of medium. This volume was distributed into each 15 ml-conical polypropylene tubes and 0.1 ml of medium, alone or containing different concentrations of insulin (Sigma-Aldrich; final concentrations ranging between 0.1–5.0 nM), was added into the tubes. At least 6 tubes per condition were used in each experiment. Tubes were incubated, by shaking at 37°C, during 2 hours in 95% air-5% CO2 atmosphere. At the end of incubation tubes were centrifuged for 20 minutes at 100xg; and the infranatant was separated from adipocytes and kept frozen (–20°C) until measurement of medium leptin concentration.

Experiment 2. Metabolic responses to high glucose load were performed in, 24 hour-fasting, CT and TP rats (bearing an indwelling i.v. cannulae, implanted 48 hours before the experiment). Animals (n = 9 rats per group) were bled before (sample time zero) and at several times (5, 15, 30, 60 and 90 minutes) after glucose (2g per Kg BW) i.v. administration [30]. Plasma samples, split into aliquots, were kept frozen (–20°C) until determination of different parameters.

Our Animal Care Committee approved experiments. Animals were killed by decapitation, accordingly to protocols for animal use, in agreement with NIH Guidelines for care and use of experimental animals.

Assays

Plasma glucose and triglyceride levels were determined, in fresh samples, by enzymatic assays from Wiener Argentina Laboratories.

Plasma and medium leptin concentrations were determined by a radioimmunoassay (RIA) previously developed in our laboratory [31]. The detection range of the standard curve was 0.4–15 ng/ml. The intra- and inter-assay coefficients of variation (CVs) were 5–8 and 10–13%, respectively.

Circulating concentrations of insulin were determined by a specific rat RIA developed in our laboratory. Briefly, rat insulin (used for both standard curves and radio-iodination) and guinea pig anti-porcine insulin serum (IS510) were purchased from Sigma-Aldrich. Insulin was labeled with 125I-Na (specific activity 15 Ci/mmol, from, Amershams Pharmacia Biotech, UK) by the chloramine-T method and purified by elution, after loading the radio-iodination mixture, from a Sephadex G-50 (Sigma Aldrich) (1.5x40 cm) column equilibrated with sodium phosphate (0.05 M)-BSA (5g/l)-sodium azide (10 mg/l) solution (pH 7.4). The detection range of the standard curve was 0.1–10 ng/ml. Unknowns or standards (200µl) were incubated overnight at 4°C in the presence of 50µl of anti-insulin guinea pig serum (final dilution 1:360,000) and 50µl (approximately 15,000 cpm) of tracer. Separation of bound and free fractions was achieved by addition of 500µl charcoal (2.5%)-dextran T 70 (0.25%), followed by 10 min incubation at 0°C (in an ice bath). Finally, tubes were centrifuged at 3,000 rpm for 15 min at 4°C and free radioactivity was counted. The intra- and inter-assay CVs were 3–7 and 8–11%, respectively.

Serum levels of LH and FSH were determined by double antibody RIAs as previously described [32]. Results are expressed in terms of the reference preparations (rat LH-RP3 and FSH-RP-2), supplied by the National Pituitary Hormone Program, Bethesda, Md., USA. The intra-assay and inter-assay CVs were less than 8% and 13%, respectively, for both assays.

Circulating concentrations of Testosterone (T) [33], estradiol (E2) [33] and corticosterone (B) [34] were determined by specific RIAs as earlier described in detail; CVs intra-assays ranged between 4–8%, and CVs inter-assays were within the range 9–12% for all RIAs.

Data analysis

Results are expressed as mean ± SEM. Mean values were compared by ANOVA or ANOVA with multiple determinations, followed by post-hoc comparisons with the Fisher’s test [35]. The area under the curve during the i.v. high glucose load test was calculated as previously reported [36]. P values lower than 0.05 were considered statistically significant.

Results

Effects of neonatal androgenization on changes in body weight over development and, adiposity and circulating concentrations of several metabolites at adult age.

Figure 1 (top) shows changes in BW values, in CT and TP rats, from weaning (age 21 days) up to the experimental day (age 100 days). As it can be observed, neonatal treatment with TP, although did not modify BWs up to age 37 days, was able to significantly (P < 0.05) increase BW values from day 38 of age, and the enhanced BW of TP rats persisted up to the experimental day (P < 0.05 or less vs., age-matched, CT values). For comparison purposes, BW values in different groups of 100 day-old rats are shown in Figure 1 (bottom A).

When fresh visceral fat was dissected and weighted, on day 100 of age, we found that rats treated with TP were characterized by enlarged (P < 0.01) visceral fat mass (Figure 1, bottom B).

Some metabolic parameters in circulation, in basal unfasting condition, were not significantly affected by TP treatment, such as: glycemia, triglyceridemia and insulinemia, as well as plasma LH, FSH, testosterone and corticosterone levels (Table 1). Conversely, while basal circulating concentrations E2 were significantly (P < 0.05 vs. CT) lower in TP rats, those of leptin were significantly (P < 0.05 vs. CT) higher in TP rats.
significantly (P < 0.05 vs. CT) higher in this group (Table 1).

Impact of neonatal hypothalamic androgenization on metabolic responses to i.v. high glucose load.

Figure 2 shows the results of circulating levels of several parameters in basal condition (time zero) and on different times after i.v. administration of glucose (2 g/Kg BW) in, 24 hour-fasting, 100 day-old CT and TP rats. Figure 2A shows the results of circulating glucose levels before and several times after high glucose load. As depicted, circulating glucose concentrations were significantly (P < 0.05) enhanced over the respective baseline, in a similar fashion, in both groups at 5 and 15 minutes post-glucose administration, both groups of experimental animals displayed statistically similar peak values at 5 minutes post-glucose. Thereafter, circulating glucose values recovered the respective baseline values at 30 minutes post-glucose in both experimental groups, and remained the same up to the end of the experiment. No significant glucose tolerance differences between groups were found after statistical analysis calculating the area of the glucose concentration x time curves (6.44 ± 0.32 and 6.08 ± 0.35 g/l/90 min in CT and TP groups, respectively).

Table 1. Basal circulating levels of several metabolic parameters in, 100 day-old, control (CT) and neonatally androgenized (TP) female rats in non-fasting condition. Values are the mean ± SEM (n = 9 animals per group).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CT</th>
<th>TP</th>
</tr>
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<tbody>
<tr>
<td>Glucose (g/l)</td>
<td>1.22 ± 0.02</td>
<td>1.26 ± 0.04</td>
</tr>
<tr>
<td>Triglycerides (g/l)</td>
<td>1.41 ± 0.11</td>
<td>1.18 ± 0.09</td>
</tr>
<tr>
<td>LH (ng/ml)</td>
<td>1.55 ± 0.63</td>
<td>2.64 ± 0.56</td>
</tr>
<tr>
<td>FSH (ng/ml)</td>
<td>2.11 ± 0.61</td>
<td>3.14 ± 0.71</td>
</tr>
<tr>
<td>Estradiol (pg/ml)</td>
<td>65.39 ± 7.07</td>
<td>21.61 ± 2.43 +</td>
</tr>
<tr>
<td>Testosterone (ng/ml)</td>
<td>0.16 ± 0.01</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>Corticosterone (µg/dl)</td>
<td>10.11 ± 3.75</td>
<td>8.63 ± 2.74</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>2.89 ± 0.42</td>
<td>4.84 ± 0.61 +</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>1.33 ± 0.23</td>
<td>1.95 ± 0.36</td>
</tr>
</tbody>
</table>

Moreover, statistical analysis calculating the area of the insulin concentration x time curves indicated that significantly (P < 0.05) higher insulin concentrations were needed in TP (25.49 ± 1.69 ng/ml/90 min) than in CT (16.16 ± 4.05 ng/ml/90 min) rats to maintain similar glycemia throughout this test.

Figure 2C shows the results of plasma leptin levels, in both groups, before and after high glucose load. As it was found in unfasting rats, 24 hour-fasting TP rats displayed significantly (P < 0.05) higher plasma leptinemia than CT rats. While CT rats reached significantly (P < 0.05) higher plasma leptin concentrations than its baseline at 30 or more minutes post-carbohydrate load, conversely, TP rats attained significantly (P < 0.05) higher leptin circulating levels than their respective basal levels only 90 minutes post-glucose administration. It must be noted that at most of the times studied in this test, TP rats displayed significantly (P < 0.05) higher circulating leptin levels than their normal counterparts (see also Figure 2C). Significant (P < 0.01) differences between groups were found after statistical analysis calculating the area of the leptin concentration x time curves (3.74 ± 1.16 vs. 9.59 ± 1.31 ng/ml/90 min in CT and TP groups, respectively).

Finally, Figure 2D shows the results of circulating B levels in this test. High glucose load increased basal B levels over the baseline (P < 0.05) on times 60 and 90 minutes in CT animals. On the other hand, TP rats developed a more rapid adrenal response to
Figure 2. Results of i.v. high glucose load (2 g/Kg BW) test in, 100 day-old, CT (open circles) and TP (closed circles) rats in 24 h-fasting condition. Circulating plasma levels of glucose (panel A), insulin (panel B), leptin (panel C), and corticosterone (panel D) before (sample time zero) and several times after high carbohydrate load in both experimental groups. Values are the mean ± SEM (n = 9 rats per group). * P<0.05 or less vs. the respective time zero values. +, P<0.05 or less vs. CT values in similar condition.

Figure 3. Concentration-related effect of insulin (0–5 nM) on leptin release in vitro by isolated visceral adipocytes from non-fasting, 100 day-old, CT and TP female rats. Values are the mean ± SEM (n = 3 different experiments, with 6 tubes per point per experiment). *, P<0.05 vs. respective basal values. +, P<0.05 vs. CT values in similar condition.
high carbohydrate load; in fact, plasma B levels were significantly (P < 0.05) enhanced over the baseline at 5 minutes post-glucose and remained the same up to the end of the experiment (Figure 2D). No significant differences between groups were found after statistical analysis calculating the area of the B concentration x time curves (32.88 ± 8.31 and 41.28 ± 6.71 µg/dl/90 min in CT and TP groups, respectively) during the test.

In vitro visceral adiposity function in control and neonatally-androgenized rats.

Figure 3 shows the results of leptin released into the medium by isolated adipocytes, obtained from visceral fat pads of CT and TP rats, incubated in the absence or presence of several concentrations of insulin (0.1–5.0 nM). Spontaneous leptin (Insulin 0 nM) release in vitro, by a similar number of adipocytes from both groups (400,000 cells per tube), was significantly (P < 0.02) higher in the TP than in the CT cell-group. Incubation of CT adipocytes with 0.1 nM insulin resulted in a significant (P < 0.05) output of leptin over the baseline, and the same accounted with all other insulin concentrations employed. Conversely, adipocytes obtained from TP rats required a much higher insulin concentration (0.5 nM or more) than CT adipocytes to significantly (P < 0.05) enhance leptin release over the respective baseline values. Important to denote is that TP adipocytes released significantly (P < 0.05) higher amounts of leptin than the CT cell-group, regardless of the incubation condition (Figure 3).

Discussion

Our study strongly suggests that hypothalamic androgenization in newborn female rats induced, when examined at adulthood, a significant increase in body weight, fat mass and circulating leptin levels; moreover, hyperinsulinemia in response to high glucose load and in vitro visceral fat insulin resistance distinguished these animals from their normal counterparts. These observations in TP rats, in basal non-fasting condition, were accompanied by lower estradiol levels than in CT animals and no significant differences in circulating levels of glucose, triglycerides, gonadotropins (LH and FSH) and insulin as well as in testosterone and glucocorticoid. The results of similar basal circulating levels of glucose, triglycerides, insulin, corticosterone, testosterone, gonadotropins as well as those of decreased plasma E2 concentrations in the adult, neonatally-androgenized, female rat are in agreement with data previously reported [28, 37, 38].

Although increased body weight and fat mass have also been previously reported in androgenized rats [37], to our knowledge, this is the first time showing that neonatally androgenized female rats displayed hyperleptinemia during adulthood. Moreover, our data on insulin resistance are in agreement with previously reported results [37] showing that insulin resistance at the muscle level is developed after neonatal androgen treatment in female rats, however, and of high relevance, we originally found that neonatally androgenized rats developed, during adulthood, peripheral insulin resistance and changes in adiposity function (leptin hypersecretion and insulin resistance).

It has been earlier found [28] that TP rats, when compared to CT animals, although did not display differences in hypothalamic LHRH and anterior pituitary gonadotropins content, conversely, they showed an altered pulsatile rhythm of gonadotropin release in plasma due to decreased trough ad average mean values of LH-FSHH and diminished FSH peak values; increased LH:FSH ratio; and impaired FSH response to LHRH stimulus in vivo. Thus the permanent sterility of these rats seems to be mainly due to altered pituitary function after priming their hypothalamus with testosterone; thus, changes in hypothalamic LHRH secretion into the portal blood could also be an additional factor for impaired gonadotrope function.

We presently observed that in TP rats, although no differences in circulating LH and FSH levels, vs. CT rats, were found, conversely, the ratio of LH:FSH concentrations in circulation augmented from 0.73, in CT rats, to 0.84 (P < 0.05; n = 9 rats per group); a characteristic similar manifest in PCOS women [2–4]. It has been found that plasma gonadotropins are lower in TP treated than in normal female rats only when determined before puberty, thereafter, and probably due to an enhancement in hypothalamic LHRH synthesis to in turn induce puberty [28], circulating gonadotropins levels became indistinguishable from those of controls when compared on day 60 or more of age [28].

The possibility that the development of insulin resistance could be secondary due to hypothalamic androgenization, at the neonatal age, should not be discarded. Similarly, an effect of TP treatment on the pattern of GH secretion [39] could be leading to an alteration in insulin sensitivity [40]. Additionally, a recent study in aromatase-knockout male mice [41] demonstrates that, at adult age, these animals develop hyperandrogenemia, high body weight, glucose intolerance and insulin resistance when compared with data obtained in wild-type litter-mates; interestingly, some of these effects can be reversed by estrogen therapy [41] in this strain of mice. Thus, our data, in combination with those above mentioned clearly indicate that high testosterone levels at the neonatal age could result in a very strong perturbation in the mechanisms involved in cell glucose uptake during adult life. High androgen levels in women have been associated with insulin resistance [1, 42], however, in normal men, pharmacological doses of testosterone for a long period of time did not impair glucose tolerance [43]. Thus, hyperandrogenemia seems to influence insulin sensitivity in a sex-dependent fashion. This sex/gender difference in the development of insulin resistance could be due to individual changes in the ratio of circulating steroids [41], in fact, we found that the T:E2 ratio significantly (P < 0.005) increases from 0.0027 ± 0.0003 (in CT rats) to 0.0075 ± 0.0009 as a consequence of the neonatal androgenization.

It is known that androgens affect adipocyte metabolism via androgen receptor-mediated mechanisms.
Moreover, we demonstrated that neonatally-androgenized female rats have developed, as occurred in other animal models, tissue specific changes in insulin sensitivity, such as impaired in vivo and in vitro insulin sensitivity after high glucose load and adipocytes exposed to insulin stimulation, respectively. However, it must be stressed that these rats are hyperleptinemic, due to both higher visceral adiposity and spontaneously adipocyte leptin output, thus high leptin levels could play an important role in inhibiting insulin binding in adipocytes [47]. Additionally, in our high glucose load test, we found that the early significant increase, over the baseline, in glucocorticoid secretion, a well known anti-insulinergic compound, could be another factor contributing to insulin resistance in TP-treated rats [48]. Finally, although not examined in our study, because neonatal androgenization in rat renders in changes in adipose tissue function, production of other adipokines could also be altered, e.g. TNFα; it is well known that this cytokine is able to reduce insulin sensitivity [49].

In summary, our data strongly support that neonatal androgenization resulted in increased visceral fat and leptin production associated with impaired peripheral insulin sensitivity during adulthood. Also, this study add, although indirectly, new evidence to address whether or not leptin could be involved in the pathogenesis of the development of PCOS in premenopausal women [50]. Furthermore, our results provide strong evidence for the hypothesis of a development of insulin resistance due to early hyperandrogenicity in the female gender.

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