Abstract

The metabolic syndrome is a growing epidemic; it increases the risk for diabetes, cardiovascular disease, fatty liver, and several cancers. Several reports have indicated a link between hormonal imbalances and insulin resistance or obesity. Transgenic (TG) female mice overexpressing the human chorionic gonadotropin β-subunit (hCGβ+ mice) exhibit constitutively elevated levels of hCG, increased production of testosterone, progesterone and prolactin, and obesity. The objective of this study was to investigate the influence of hCG hypersecretion on possible alterations in the glucose and lipid metabolism of adult TG females. We evaluated fasting serum insulin, glucose, and triglyceride levels in adult hCGβ+ females and conducted intraperitoneal glucose and insulin tolerance tests at different ages. TG female mice showed hyperinsulinemia, hypertriglyceridemia, and dyslipidemia, as well as glucose intolerance and insulin resistance at 6 months of age. A 1-week treatment with the dopamine agonist cabergoline applied on 5-week-old hCGβ+ mice, which corrected hyperprolactinemia, hyperandrogenism, and hyperprogesteronemia, effectively prevented the metabolic alterations. These data indicate a key role of the hyperprolactinemia-induced gonadal dysfunction in the metabolic disturbances of hCGβ+ female mice. The findings prompt further studies on the involvement of gonadotropins and prolactin on metabolic disorders and might pave the way for the development of new therapeutic strategies.

Introduction

Metabolic syndrome is a growing epidemic worldwide that involves 1 out of 4 adult people, and its prevalence increases with age (Grundy 2008). The consensus statement provided by the International Diabetes Federation (IDF) defines the metabolic syndrome as a condition with abdominal obesity plus any two of the following: elevated plasma triglyceride levels, reduced high-density lipoproteins (HDLs), increased blood pressure, or increased fasting plasma glucose (Alberti et al. 2006). Obesity-associated insulin resistance is considered a cause-and-effect relationship since weight changes correlate with changes in insulin sensitivity (Kahn et al. 2006).
Qatanani & Lazar 2007). In this respect, hyperlipidemia is linked to insulin resistance, since insulin promotes fat cell differentiation, enhances adipocyte glucose uptake, and inhibits adipocyte lipolysis.

Although the role of prolactin in reproduction is well known, the participation of this hormone in weight gain and glucose homeostasis is still under debate. Patients with prolactinomas were reported to acquire weight gain and metabolic alterations (Greenman et al. 1998, Ben-Jonathan et al. 2008). However, it is still unclear whether these conditions are directly associated to hyperprolactinemia (Ciresi et al. 2013). Recent experimental evidence suggests that prolactin has a crucial role on the pancreas and the adipose tissue, most notably during development. Prolactin receptor-deficient mice (Prl r−/−) provided direct evidence that prolactin signaling is involved in adipogenesis by affecting energy balance and metabolic adaptation (Carré & Binart 2014). Furthermore, prolactin is shown to be essential for the development of pancreatic β-cell during the perinatal period (Auffret et al. 2013), and is therefore, involved in the manifestation of insulin resistance by stimulating insulin release and regulating adipokine release (Ben-Jonathan et al. 2008, Carré & Binart 2014). Prolactin was found to decrease glucose transporter 4 (GLUT4) mRNA expression that may cause a decreased glucose uptake in peripheral tissues (Nilsson et al. 2009). Moreover, prolactin induces pyruvate dehydrogenase kinase 4 (PDK4), whose activation is known to lead to decreased glucose oxidation (White et al. 2007). In addition, this hormone participates in perinatal brown adipocyte differentiation and function (Viengchareun et al. 2008), and also affects energy homeostasis through modulation of lipid metabolism (Carré & Binart 2014).

We have previously shown the implications of chronically elevated levels of hCG in the phenotype of transgenic (TG) mice. Particularly, female mice overexpressing the hCGβ− subunit (hCGβ+) exhibit precocious puberty, elevated serum levels of hCG, prolactin, testosterone, and progesterone, and present with infertility (Rulli et al. 2002, Ratner et al. 2012). Besides, hCGβ+ ovaries show hemorrhagic cysts and massive luteinization as a result of the active stimulation with hCG (Rulli et al. 2002, Ratner et al. 2012). Among the extragonadal phenotypes, these females develop obesity, pituitary macroprolactinomas, mammary gland tumors, and elevated bone density at older ages (Rulli et al. 2002, Yarram et al. 2003, Kuorelahti et al. 2007, Pakarainen et al. 2007, Ahtilainen et al. 2010, Ratner et al. 2012). In contrast to transgenic females, hCGβ+ males are fertile and exhibit normal levels of testosterone and prolactin (Rulli et al. 2003).

Even though LH/hCG receptors are detected in different nongonadal tissues, including the pancreas (Abdallah et al. 2004, Cole 2010), their physiological significance remains unclear. Our previous studies demonstrate that hyperprolactinemia is the main cause for the reproductive defects of adult hCGβ+ females, which can be prevented by a short-term treatment with the dopamine agonist cabergoline at the beginning of the reproductive age (Ratner et al. 2012). Conversely, the same treatment applied at 3 months of age failed to recover fertility. These findings demonstrate that the cabergoline treatment applied at a critical moment of the phenotype progression prevents hCG-induced abnormalities in these transgenic mice.

The aim of this study was to investigate the possible alterations of glucose and lipid metabolism in adult hCGβ+ females. The short-term treatment with cabergoline was followed in order to assess whether hyperprolactinemia influenced metabolism in the hypersecreting hCGβ+ females. Since hCGβ+ males do not exhibit changes in prolactin levels, this study was focused on females. Glucose and insulin tolerance tests were conducted at different ages, as well as determination of serum insulin concentration and pancreatic gene expression analysis. Since obesity was described as part of the extra-gonadal phenotype, serum triglycerides, cholesterol, and high-density lipoprotein cholesterol (HDL-C) were also measured in TG females.

Materials and methods

Animals

All the experiments were performed in TG female mice overexpressing the hCGβ− subunit under the control of the human ubiquitin C promoter (hCGβ+). Generation, housing, and genotyping of hCGβ+ with FVB/N genetic background have been previously described (Rulli et al. 2002). Wild-type (WT) littermates were used as controls. Mice were maintained under controlled conditions (12 h light:12 h darkness cycle, 21°C), and were given free access to laboratory chow and tap water. Food intake was monitored daily on females caged individually during 1 week. All experimental procedures were performed according to the NIH Guidelines for Care and Use of Experimental Animals, and approved by the Institutional Animal Care and Use Committee of the Instituto de Biología y Medicina Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas (IBYME-CONICET).
Cabergoline treatment

WT and hCGβ+ female mice of 5 weeks of age were injected i.p. with 500 μg/kg cabergoline (Laboratorios Beta S.A., Buenos Aires, Argentina) suspended in 0.25% (w/v) methylcellulose as vehicle (Tanaka & Ogawa 2005). The females received three injections of cabergoline, one every other day, during one week (hCGβ+cab) (Ratner et al. 2012). The females used as controls were injected with vehicle only.

Glucose homeostasis tests

Two, three, and six-month-old female mice were fasted for 6 or 3 h and blood was collected from the tail vein to perform glucose tolerance test (IGTT) or insulin tolerance test (ITT), respectively. Glucose (2 g/kg, dissolved in water) or insulin (0.75 IU/kg Humulin R, Eli Lilly Interamericana, Argentina) was administered by i.p. injection. Blood glucose was determined at time points 0, 30, 60, and 90 min according to manufacturer's recommendations using a glucometer Accu-Chek (Roche) (Andrikopoulos et al. 2008). The ITT was performed on the same group of animals one week after IGTT. In addition, glucose-stimulated insulin secretion was determined from serum samples of 6-h-fasted females of 3 and 6 months of age, at 0 and 30 min after glucose administration. Serum samples were obtained by centrifugation and stored at −20°C. Insulin levels were assessed by using the rat/mouse Insulin Elisa Kit (EZRMI-13K; Millipore).

Sample collection

Mice were weighed and killed by CO₂ asphyxiation at 6 months of age after 18 h fasting, and cardiac blood was obtained immediately thereafter. Serum samples were separated by centrifugation and stored at −20°C for biochemical analyses. Pancreata were perfused with RNAlater (Ambion) immediately after dissection, and then snap frozen and stored at −70°C for RNA isolation.

Biochemical analyses

Serum cholesterol, triglycerides, and high-density lipoprotein cholesterol (HDL-C) concentrations were measured by colorimetric assays (BioSystems, Barcelona, Spain) according to the manufacturer's instructions. Serum lipid indices were calculated according to the following formulas: cholesterol/HDL-C (Castelli 1996); triglycerides/HDL-C (McLaughlin et al. 2003). The calculation of HOMA-IR (Homeostasis Model Assessment Insulin Resistance) was performed according to the formula of Matthews and coworkers (1985): (glucose mmol/dl × insulin mIU/mL)/22.5. The Quicki (Quantitative Insulin Sensitivity Check Index) was calculated consistent with Katz and coworkers (2000): 1/(log insulin mIU/mL + log glucose mg/dL).

hCG bioassay

The bioactive levels of circulating hCG were determined by the mouse testicular interstitial cell in vitro bioassay as described previously (Ding & Huhtaniemi 1989, Russi et al. 2002). Briefly, decapsulated testes from adult WT males were dispersed with collagenase type I (0.15 mg/mL) in M199 medium (Sigma-Aldrich) for 5 min at 34°C. The supernatant was filtered through nylon mesh (mesh size 100 μm) and the cell suspension was washed twice with M199 medium supplemented with 0.1% (w/v) bovine serum albumin (BSA; Sigma-Aldrich) and 20 mM HEPES (Sigma-Aldrich). Testicular interstitial cells obtained using this technique are predominantly Leydig cells, as described previously (Ding & Huhtaniemi 1989). Cells (50,000 cells/tube) were incubated with increasing concentrations of recombinant hCG as standard (AFP8456A, 20,000 IU/mg; NHPP, NIDDK), or with the serum samples, in a 95%O₂/5%CO₂ atmosphere at 34°C for 4 h. After incubation, supernatants were recovered by centrifugation and frozen at −20°C. The testosterone concentration in the supernatants was measured by radioimmunoassay, according to a method described previously (Ratner et al. 2012). The intra- and interassay coefficients of variation values were less than 12%.

In vivo peripheral tissue response to insulin

Six-month-old WT and hCGβ+ female mice were fasted for 4 h. Then, animals were anesthetized with 2% avertin (12 mL/kg i.p.). The abdominal cavity was opened and 2 IU/kg insulin was injected into the portal vein. At time points 0 and 5 min postinjection, portions of skeletal muscle were excised and flash frozen in liquid N₂ and stored at −70°C until used.

Western blot analysis

Skeletal muscle homogenates were prepared with lysis buffer (50 mM TRIS, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40-IGEPAL), 200 mM sodium orthovanadate (Na₃VO₄), 200 mM NaF,
and protease inhibitor cocktail (Roche). Concentration was determined by Lowry’s method (1951), using BSA as standard protein. Fifteen micrograms of protein from each sample was resolved by 10% SDS-PAGE under reducing conditions and transferred onto nitrocellulose membranes (Amersham Hybond-ECL, GE Healthcare). To reduce binding of nonspecific antibody, membranes were incubated for 1 h at room temperature in T-TBS blocking buffer. The membranes were then incubated overnight at 4°C with antibodies anti-AKT and anti-pAKT in T-TBS, 1% BSA (Cell Signaling; AKT, #9272S:1/500; pAKT, #4060S: 1/2000). Secondary goat anti-rabbit antibody conjugated with peroxidase HRP (Santa Cruz Biotechnology, # sc-2004: 1/5000) were used. For detection of actin, membranes were incubated overnight at 4°C with first antibody diluted in PBS-T, 1% BSA (Calbiochem, # cp01: 1:5000) followed by incubation with secondary goat anti-mouse IgM antibody conjugated with peroxidase HPR (Santa Cruz Biotechnology, # sc-2064:1/2000). Immunoreactive proteins were revealed by enhanced chemiluminescence (ECL-Plus, Amersham, GE Healthcare) using hyperfilm ECL (GE Healthcare) and band intensities were quantified using Scion Analyzer software.

Immunohistochemistry

Pancreata from 6-month-old WT, hCGβ+ and hCGβ+cab female mice were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin wax. Endogenous peroxidase reactivity was quenched by a 20-min pretreatment with 10% methanol, 0.3% H2O2 in 0.01 M PBS (pH 7.4). For antigenic retrieval, sections were pretreated with citrate buffer (0.01 M, pH 6), and permeabilized by a 5-min incubation with 0.5% saponin in PBS and 5-min incubation with proteinase K (10ng/mL). Nonspecific proteins were blocked by subsequent incubation with protein blocking buffer (5% goat normal serum in PBS for PDX1, and 5% horse normal serum in PBS for NKX6.1) for 30 min. After several wash steps, sections were incubated with antibodies rabbit anti-PDX1 (Millipore, # 06-1379: 1/1000) and mouse anti-NKX6.1 (DSHB, # F55A10-S: 1/250) diluted in incubation buffer (2% goat normal serum in PBS for PDX1; 2% horse normal serum in PBS for NKX6.1) overnight in a humidified chamber at 4°C. On the second day, pancreata sections were washed and incubated with biotinylated secondary antiserum (goat anti-rabbit IgG; horse anti-mouse IgG, 1:500, Vector Lab, Burlingame, CA, USA) for 2h at room temperature. Finally, immunoreaction was visualized with 0.01% H2O2 and 0.05% 3,3-diaminobenzidine solution (in 0.05 M Tris–HCl, pH 7.6) and an avidin–biotin–peroxidase system (Vector Lab, Burlingame, CA, USA). Negative controls were performed in the absence of the primary antibodies.

RNA isolation and analysis of gene expression

Total RNA was isolated from pancreata as described previously (Gonzalez et al. 2011), using TRIZOL reagent (Invitrogen) according to the manufacturer’s protocol. Two micrograms of RNA were treated with DNase I (Invitrogen) and reverse-transcribed in a 20 µL reaction volume using M-MLV reverse transcriptase (Promega) and random hexameres (Biodynamics, Seattle, WA, USA). For quantitative real-time PCR (qPCR), primer sets were designed for the specific amplification of Ins1, Ins2, Gcg (Ins1 Fw: AAGCTGGTGGGCATCCAGTAACC, Table 1

<table>
<thead>
<tr>
<th>Body weight (g)</th>
<th>WT</th>
<th>hCGβ+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily food intake (g/mouse)</td>
<td>4.42 ± 0.20 (7)</td>
<td>4.43 ± 0.27 (7)</td>
</tr>
<tr>
<td>Abdominal white fat (g)</td>
<td>1.79 ± 0.35 (7)</td>
<td>1.79 ± 0.15 (4)</td>
</tr>
<tr>
<td>Bio hCG (IU/L)</td>
<td>1.79 ± 0.15 (7)</td>
<td>1.79 ± 0.15 (4)</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>152 ± 7 (5)</td>
<td>154 ± 10 (7)</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>0.21 ± 0.07 (5)</td>
<td>0.21 ± 0.07 (5)</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.78 ± 0.65 (5)</td>
<td>1.78 ± 0.65 (5)</td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.37 ± 0.02 (5)</td>
<td>0.37 ± 0.02 (5)</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>147 ± 14 (5)</td>
<td>147 ± 14 (5)</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>130 ± 14 (4)</td>
<td>130 ± 14 (4)</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>82 ± 4 (4)</td>
<td>82 ± 4 (4)</td>
</tr>
<tr>
<td>Cholesterol/HDL ratio</td>
<td>1.59 ± 0.18 (4)</td>
<td>1.59 ± 0.18 (4)</td>
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<tr>
<td>Triglycerides/HDL ratio</td>
<td>1.78 ± 0.11 (4)</td>
<td>1.78 ± 0.11 (4)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.E.M., the number of animals used in each determination is indicated in brackets. Student’s t-test: **P < 0.01; ***P < 0.001.
Ins1 Rev: GTTTGGGCTCCCATGGGCAAG; Ins2 Fw: CCGTGGCCTGCCTGCTCTT, Ins2 Rev: AGGTCTGAAGGTCACCTGCT; Gcg Fw: CTACACCTGTGCGAGCTCA, Gcg Rev: CTGGGGTCTCCCTGCTGTGCT), and cyclophilin A (Ppia) as an internal control (Ppia Fw: GCGTCTCCCTGAGCTGTT, Ppia Rev: AAGTCACCACCCTGGCAC). Each sample was assayed in duplicate using 4 pmol of each primer, SYBR Green Master Mix (Applied Biosystems), and 2–20 ng of cDNA in a total volume of 15 µL. Amplification was carried out in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). For the assessment of quantitative differences in the cDNA target between samples, the mathematical model of Pfaffl (2001) was applied. An expression ratio was determined for each sample by calculating 

\[
\frac{E_{\text{target}}}{E_{\text{Ppia}}} = \frac{(E_{\text{target}})^{\Delta Ct(\text{target})}}{(E_{\text{Ppia}})^{\Delta Ct(\text{Ppia})}},
\]

where E is the efficiency of the primer set and \(\Delta Ct = Ct (\text{reference cDNA}) – Ct (\text{experimental cDNA})\). The amplification efficiency of each primer set was calculated from the slope of a standard amplification curve of log (nanograms of cDNA) per reaction vs Ct value (\(E = 10^{-\text{slope}}\)). Efficiencies of 2 ± 0.1 were considered optimal. Results were expressed relative to a reference sample (WT chosen at random).

Statistical analysis

Data are presented as the mean ± s.e.m. Statistical analysis for comparing two sets of data was performed with Student’s t-test for two independent groups. In those experiments where the effects of two factors (genotype and treatment) were studied, the two-way ANOVA was performed. The two-way ANOVA with repeated measures was used for the glucose and insulin tolerance tests. Bonferroni’s post hoc test was used to establish the level of significance between group pairs. The trapezoidal rule was used to determine the area under the curve (AUC). Data were transformed when required. A P value less than 0.05 was considered to be statistically significant.

Results

Hormonal and metabolic status of hCGβ+ female mice

We have previously demonstrated that at 6 months of age, hCGβ+ female mice showed pronounced disturbances in their gonadal and nongonadal phenotype (Rulli et al. 2002, Ratner et al. 2012). As was confirmed in
Table 1, hCGβ+ females exhibited a significant increase in body weight, abdominal white fat depot, and serum levels of bioactive hCG as compared with WT (P < 0.01) (Rulli et al. 2002, Ratner et al. 2012). These changes, however, were not accompanied by an increase in the daily food intake. At this age, serum levels of insulin and triglycerides were elevated in TG females (P < 0.001). However, serum fasting glucose, cholesterol, and HDL-C levels did not show differences between WT and hCGβ+ females (Table 1). In addition, the atherogenic (or Castelli) index, represented by the ratio of cholesterol/HDL-C, did not show significant differences between the groups, whereas the triglycerides/HDL-C ratio showed a statistically significant increase in hCGβ+ females as compared with WT females (P < 0.001). From fasting insulin and glucose data, we calculated the surrogate indexes of insulin sensitivity and resistance HOMA-IR and QUICKI. The values of HOMA-IR were significantly higher in the hCGβ+ group compared with WT (P < 0.05; Table 1), as observed for groups of mice with decreased insulin sensitivity. Conversely, the value of QUICKI was significantly lower in the hCGβ+ group compared with WT (P < 0.05), also indicative of diminished insulin sensitivity.

Age-dependent changes in the glucose homeostasis of hCGβ+ females

In order to study a possible correlation with age, IGTT and ITT were performed in 2-, 3-, and 6-month-old WT and hCGβ+ females. No differences were found in IGTT at 2 months of age (Fig. 1A). At 3 and 6 months of age, TG females showed glucose intolerance, represented by a delay in glucose clearance and an increase in glucose
levels through the different time points analyzed after glucose administration (Fig. 1B and C). Accordingly, the total glucose levels accumulated during the 90 min of the assay, represented as the AUC, were significantly increased in hCGβ+ females as compared with WT (P < 0.01).

The ITT performed in 2- and 3-month-old TG females showed a quick decline in glucose levels at 30 min after insulin administration, and remained low thereafter, as it was observed in WT females at the same ages (Fig. 2A and B). However, 6-month-old hCGβ+ females showed elevated glucose levels after insulin administration, which remained high through the different time points analyzed (Fig. 2C). Accordingly, the AUC resulted elevated in hCGβ+ females (P < 0.01).

The insulin secretion capacity in response to glucose administration was performed in 3- and 6-month-old
WT and hCGβ+ females (Fig. 3A). The glucose response was first measured at 30 min, meaning that only the second phase of insulin secretion was detected in this study (Caumo & Luzi 2004). TG mice from both ages exhibited elevated basal insulin levels as compared with WT females. The insulin secretion of WT females from both ages showed a correct response to glucose stimulation with at least a 2.5-fold increase at 30 min after glucose administration (P < 0.05). Conversely, 3- and 6-month-old hCGβ+ mice exhibited an impaired glucose-stimulated response, being only a 0.8- and 1-fold increase, respectively (Fig. 3A).

Since TG female mice showed profound alterations in glucose metabolism at 6 months of age, we further analyzed the peripheral insulin sensitivity at this age. To this aim, we determined the status of insulin-induced AKT phosphorylation in skeletal muscle obtained from fasted WT and hCGβ+ mice (Fig. 3B). As expected, WT females showed a three-fold increase in insulin-stimulated AKT phosphorylation (P < 0.05), whereas TG females exhibited a severely impaired AKT activation, with levels comparable to basal level of both groups (P < 0.05). TG females showed an increase in total AKT as compared with WT (P < 0.05).

Taken together, these results showed that 6-month-old hCGβ+ females exhibited peripheral insulin resistance and impaired glucose tolerance, being the most important disruptions in glucose homeostasis.

Effect of cabergoline on the glucose and lipid homeostasis of hCGβ+ females

Treatment with the dopaminergic agonist cabergoline was carried out to analyze the influence of hyperprolactinemia on serum insulin, glucose, and triglycerides. It was confirmed that a short-term treatment with cabergoline administered at 5 weeks of age to hCGβ+ females (hCGβ+ cab) was effective in the normalization of prolactin levels (P < 0.001; Fig. 4B) and body weight (P < 0.05; Fig. 4A) at 6 months of age (Ratner et al. 2012). In addition, cabergoline treatment restored serum triglycerides (P < 0.001; Fig. 4D) and insulin (P < 0.05; Fig. 4C) to normal levels in comparison to hCGβ+ control females. Cabergoline treatment administered to 5-week-old WT females (WTcab) did not produce any effect on the parameters studied (Fig. 4A, B, C and D).

Effect of cabergoline on IGTT and ITT of hCGβ+ females

IGTT and ITT were performed to further analyze the influence of prolactin on the glucose homeostasis of the TG females (Fig. 5). The IGTT showed a similar clearance in WT and WTcab females, with significantly increased glucose levels of hCGβ+ females at 30, 60 (P < 0.001), and 90 (P < 0.01) min after glucose administration (Fig. 5A). hCGβ+ females treated with cabergoline showed a significant reduction in
glucose levels at 30 min, as compared with the results obtained for hCGβ+ females. In line with this, the AUC exhibited similar results (Fig. 5B).

The ITT demonstrated that cabergoline treatment fully prevented the appearance of insulin resistance in TG females (Fig. 5C). This was confirmed by analyzing the AUC: complete normalization occurred in cabergoline-treated TG females with respect to control hCGβ+ females (P < 0.05; Fig. 5D).

Effect of cabergoline on pancreatic Ins1, Ins2, Gcg, PDX1, and NKX6.1 in hCGβ+ females

Due to the effectiveness of the cabergoline treatment in normalizing the glucose homeostasis of TG females, we assessed the gene expression for preproinsulin (Ins1 and Ins2) and glucagon (Gcg) in pancreatic tissue of 6-month-old hCGβ+ and hCGβ+ cab females. In agreement with the increased serum levels of insulin, hCGβ+ females exhibited significantly increased gene expression of both Ins1 and Ins2, as compared with WT females (P < 0.05, Fig. 6A). The cabergoline treatment restored the expression levels of the genes for insulin to the level obtained in WT mice (Fig. 6A). By contrast, the expression of Gcg did not show significant differences among the groups studied (Fig. 6A). The cabergoline treatment applied to WT females did not affect the expression levels of the genes analyzed.

In addition, we performed immunohistochemistry for two well-known markers of β-cell maturity and identity, PDX1 and NKX6.1. The presence of both markers was detected in the pancreatic islets of WT, hCGβ+, and hCGβ+ cab, and the expected nuclear localization was observed (Fig. 6B).

Discussion

The influence of hormones on glucose and lipid metabolism may be evidenced, among others, in various clinical conditions such as hormone replacement therapy, pregnancy, menopause, and hyperandrogenic states. Several models have been useful for understanding the pathophysiology of the metabolic syndrome (Kennedy et al. 2010, Guo 2014). We report here a TG mouse model that shows a clear link between alterations of the gonadotropin axis and metabolic dysfunctions.

As previously demonstrated, hCGβ+ females exhibit elevated levels of hCG; progesterone, testosterone, and prolactin; precocious puberty associated with a transient increase of serum estradiol; and infertility at adulthood (Rulli et al. 2002, Ratner et al. 2012). Besides, hCGβ+ females show obesity, mainly with abdominal fat accumulation, macroprolactinomas, mammary adenocarcinoma, and increased bone mineral density at older ages (Rulli et al. 2002, Yarram et al. 2003, Kuorelahti et al. 2007, Ahtiainen et al. 2010, Bachelot et al. 2013). These extra-gonadal phenotypes of the hCGβ+ females are abolished by gonadectomy, indicating that ovarian hCG hyperstimulation with abnormal gonadal hormone production is directly or indirectly responsible for the extra-gonadal phenotype observed in this model (Rulli et al. 2002). Furthermore, we have shown that a short-term treatment with the dopamine agonist cabergoline to hCGβ+ females abolishes hyperprolactinemia, normalizes steroid hormone levels, and prevents the development of mammary tumors and pituitary adenomas in adulthood, thus demonstrating the pivotal role of prolactin on certain phenotypic alterations of hCGβ+ females (Ratner et al. 2012). In the present study, we showed that the endocrinological alterations induced by chronic hCG overproduction lead to significant metabolic dysfunctions associated with hyperinsulinemia, glucose intolerance, and impaired glucose-stimulated insulin secretion that precedes/ accompanies the development of insulin resistance. The failure of β-cell function in this model is evident, since despite hyperinsulinemia, basal glucose ranged within the normal values, but not after an i.p. glucose load. Besides, hypertriglyceridemia and high triglyceride/HDL-C index were found in adult female mice.

It is well known that AKT activation is involved in insulin sensitivity in peripheral organs. Specifically, in the skeletal muscle, insulin activates, via IRS1, the signaling pathways that involve phosphatidylinositol (PI) 3-kinase and its downstream effector AKT, which mediates glucose uptake by leading to membrane translocation of GLUT4 (Bjornholm et al. 1997). Given the evidence of insulin resistance and hyperinsulinemia with normoglycemia in TG females, we analyzed the activation of AKT in skeletal muscle. It seems that two phenomena coexist in this model. First, high basal AKT expression with the consequent increase in basal AKT phosphorylation, and secondly, the impaired AKT activation after insulin stimulation. A similar situation was found in a mouse model under high-fat diet (Liu et al. 2009). These animals also showed insulin resistance and hyperinsulinemia with normoglycemia. Moreover, basal AKT phosphorylation was increased, thus showing an adaptation of the system to the high insulin levels, and allowing the maintenance of glucose levels within the normal range (Liu et al. 2009). In the same way, we found an increase in basal AKT

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phosphorylation that would explain the normoglycemia observed. These findings, together with the inability to respond to exogenous insulin stimulation, suggest that the high AKT protein levels would lead to a saturation of the AKT kinase activity, thus producing a decrease in the rate of insulin-stimulated phosphorylation and explaining the insulin resistance observed in our model. Similar results were observed in humans, with altered AKT activation in skeletal muscle under hyperinsulinemic conditions (Karlsson et al. 2005). In addition, the db/db mice model, as well as obese diabetic human patients, also exhibit decreased AKT phosphorylation but unaltered basal total AKT in skeletal muscle (Shao et al. 2000, Gosmanov et al. 2004).

The role of prolactin on reproduction has been extensively studied, but implications of this hormone on metabolism, body weight, and energy regulations are an open issue. Pregnant and lactating women exhibit hypoadiponectinemia due to prolactin influence on secretion and expression of adiponectin (Asai-Sato et al. 2006). In hamsters, the inhibition of prolactin secretion by bromocriptine has led to a reduction in fat deposits, without reducing food intake or body weight (Freeman et al. 2000). It has been reported that prolactin is involved in adipose tissue differentiation as well as regulation of energy expenditure (Auffret et al. 2012). The absence of prolactin receptor in Prl KO mice prevents high-fat diet-induced weight gain, despite increased food intake through an increase in energy expenditure and metabolic rate. In an opposite way, in our model, hyperprolactinemia would be one of the main effectors on the weight gain by inducing accumulation of white abdominal fat depot and decreasing energy expenditure, with no change in food intake.

The lactogenic hormones during pregnancy enhance insulin production in response to the growing metabolic demands on the mother and affect pancreatic islet development in the fetus (Ben-Jonathan et al. 2008). Interestingly, the hyperprolactinemic state due to a selective disruption of the dopamine D2 receptor in the lactotropes of female mice (lacD2KO) leads to increased body weight, triglycerides, and glucose intolerance, but the response to insulin was preserved (Perez Millan et al. 2014). The short-term treatment with cabergoline provoked a recovery of glucose tolerance and a complete reversal of insulin resistance, as well as a significant reduction in insulin and triglyceride levels. In this regard, cabergoline effectively prevented the hyperprolactinemia-associated metabolic dysfunctions in TG mice. These findings provide strong evidence that elevated prolactin has a key role for the metabolic alterations in hCG overproducing females by acting directly on the target organs, and indirectly via alteration of the steroid hormone production. This could be explained by the persistent stimulus of prolactin together with hCG. This induces a significant increase in ovarian Lhgr accompanied by a massive ovarian luteinization, which results in elevated levels of progesterone and testosterone (Ratner et al. 2012).

Androgens affect lipid metabolism by increasing the activity of lipoprotein lipase and hepatic lipase, by causing an increase in triglycerides, LDL-C, and decrease the levels of HDL-C (LaRosa 1995). Estrogens, on the contrary, increase HDL-C and decrease LDL-C levels (Gillmer et al. 2006). In hamsters, the inhibition of prolactin secretion by bromocriptine has led to a reduction in fat depots, without reducing food intake or body weight (Freeman et al. 2000, Tikkanen 1996). The influence of androgens on lipid metabolism was also demonstrated in female rats under prenatal androgen treatment, which developed dyslipidemia and hepatic steatosis in adulthood. These changes would be the consequence of increased adipose tissue and insulin resistance induced by prenatal androgenization (Demissie et al. 2008). A similar metabolic alteration has been described in adult rats following early postnatal administration of testosterone (Alexander et al. 2007). Exposure to high levels of gonadal steroids, especially testosterone and progesterone throughout life (Rulli et al. 2002), could be one of the predisposing factors for dyslipidemia in this TG model, which is reinforced by the presence of obesity and insulin resistance.

The characteristic hyperprolactinemia in hCGβ+ females is a possible player in the adaptation of the pancreas to an increased insulin demand. Some lines of evidence serve as support for this purpose, as activation of prolactin receptor in the pancreas may be responsible for the increase in islet β-cells during pregnancy (Ben-Jonathan 2008, Huang et al. 2009, Huang 2013). In vitro exposure of islets to prolactin increases insulin secretion and β-cell proliferation and decreases the threshold of insulin response to glucose (Huang et al. 2009). However, Prl KO mice showed islet and β-cell hypoplasia, reduced pancreatic insulin mRNA levels, a blunted insulin secretory response to glucose, and mild glucose intolerance (Freemark et al. 2001). During pregnancy of heterozygous Prl+/− mice, pancreatic islet adaptation to blood glucose and the functioning mass of β-cell are affected (Huang et al. 2009).

Sex hormones collectively have the ability to reduce the sensitivity to insulin. It is known that estrogen and progesterone increase the pancreatic secretion of insulin and induce insulin resistance (Garcia et al. 1983,
González et al. 2000, Livingstone & Collison 2002). These behave as counterregulatory hormones of glucose homeostasis during the early stages of pregnancy, and as a result, β-cell hyperplasia and increased pancreatic insulin secretion is observed (Macotela et al. 2009).

Rats and mice have two structurally similar insulin genes, Ins1 and Ins2. Both genes are functional, but there is no consensus about their relative expression in rodent β-cells (Roderigo-Milne et al. 2002). Changes in glucose metabolism of hCGβ+ females were accompanied by a significant increase in the expression of both genes. This confirmed that hyperinsulinemia resulted from the overproduction of insulin in pancreatic β-cells of the hCGβ+ females. The identity of insulin-producing pancreatic β-cells was confirmed by the visualization of the specific markers PDX1 and NKX6.1 in hCGβ+ mice. Besides, cabergoline treatment was able to significantly reduce the Ins1 and Ins2 mRNA levels, in concordance with the normalization of serum insulin in transgenic females.

In light of metabolic syndrome as a growing epidemic, animal models are good tools to determine the pathophysiological basis of this disease and to know how the risk for other diseases increases. This transgenic model overexpressing hCG gives us the possibility to study the consequences of hormone alterations in metabolic dysfunctions. Hyperprolactinemia associated with an altered gonadal function would explain the altered occurrence of high levels of gonadal steroids and prolactin, considering that all these changes were manifested after the occurrence of high progesterone levels.

Consequently, the potential direct participation of hCG in the metabolic process deserves future studies.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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mice producing high levels of human chorionic gonadotropin. 


Elevated steroidogenesis, defective reproductive organs, and infertility in transgenic male mice overexpressing human chorionic gonadotropin. 


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