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Obesity alters the ovarian glucidic homeostasis disrupting the reproductive outcome of female rats

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Abstract

Obesity constitutes a health problem of increasing worldwide prevalence related to many reproductive problems such as infertility, ovulation dysfunction, preterm delivery, fetal growth disorders, *etc.* The mechanisms linking obesity to these pathologies are not fully understood. Cafeteria diet (CAF) is the animal model used for the study of obesity that more closely reflects western diet habits. Previously we described that CAF induces obesity associated to hyperglycemia, reduced ovarian reserve, presence of follicular cysts and ovulatory impairments. The aim of the present study was to contribute in the understanding of the physiological mechanisms altered as consequence of obesity. For that purpose, female Wistar rats were fed *ad libitum* with a standard diet (*control group*) or CAF (*Obese group*). We found that CAF fed-rats developed obesity, glucose intolerance and insulin resistance. Ovaries from obese rats showed decreased glucose uptake and became insulin resistant, showing decreased ovarian expression of glucotransporter type 4 and insulin receptor gene expression respect to controls. These animals showed an increased follicular nitric oxyde synthase expression that may be responsible for the ovulatory disruptions and for inflammation, a common feature in obesity. Obese rats resulted subfertile and their pups were macrosomic. We conclude that obesity alters the systemic and the ovarian glucidic homeostasis impairing the reproductive outcome. Since macrosomia is a risk factor for metabolic and obstetric disorders in adult life, we suggest that obesity is impacting not only on health and reproduction but it is also impacting on health and reproduction of the offspring.
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Keywords: Obesity; Ovary; Insulin; Glucose; Glut-4; Fertility; Reproductive outcome

1. Introduction

The global obesity epidemic is currently one of the most serious health concerns in the developed world and is an emergent concern in the developing world [1]. The World Health Organization has recognized obesity as an epidemic of the 21st century. As this epidemic of metabolic disorders continues, the associated medical comorbidities, including those affecting reproduction, increase as well [2]. In particular, women with obesity or poorly controlled diabetes have an increased risk of infertility, miscarriage, obstetric complications, neonatal morbidity and mortality and birth defects in their offspring [3].

Nutrition is one of the key environmental factors that lead to subfertility or infertility not only in clinical medicine but also in animal production [4]. Under-nutrition, over-nutrition and obesity are associated with poor reproductive performance [5–9]. However, the physiological mechanisms that underlie these effects are poorly understood. It has been described that obesity produce insulin resistance in the classic target tissues of insulin action, such as the liver and muscle [10,11]. However, it is controversial whether the reproductive axis remains insulin sensitive in the setting of peripheral insulin resistance. Brothers et al. have described a direct role of insulin signaling in the gonadotroph during the genesis of obesity-induced infertility. There is little literature regarding the action of insulin in the reproductive axis downstream pituitary, however, it has been shown that the ovary is insulin sensitive [12,13]. In this regards, data from clinical and experimental studies supports that estrogens contribute to glucose homeostasis, besides their pivotal role in sexual development and reproduction [14]. It is well recognized that the menopause favors visceral fat deposition and insulin resistance, leading to a significant increase in type 2 diabetes risk [15]. In this regard it has been shown that in postmenopausal women hormonal replacement therapy reduces the incidence of type 2 diabetes [16,17]. In concordance with that, bilateral ovariectomy of monkeys and rodents

Abbreviations: GDM, gestational diabetes mellitus; GLUT, glucotransporter; IR, insulin receptor; IGF, insulin growth factor; IGF1R, insulin-like growth factor 1 receptor; GTT, glucose tolerance test; ITT, insulin tolerance test; AUC, area under the curve.

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was shown to impair insulin sensitivity and glucose metabolism, a deleterious effect that was reversed by the chronic administration of estrogens [18,19]. All these evidences show a pivotal role of estrogens in regulating glucose homeostasis. However, the mechanisms by which estrogens influence insulin sensitivity and glucose metabolism remain poorly understood.

To prevent obesity the classical strategy is based on physical activity and reduced calorie intake. However, changing eating behavior and maintenance of ideal weight is difficult and hard to achieve not only *per se* but also because many of the infertile patients who attend infertility clinics at an age >30 years may not have much time to wait until they can lose weight because age itself is the major factor of declining fertility [3]. Thus, the identification of new molecular targets that can avoid, or at least to limit, the metabolic disturbances induced by obesity such as insulin resistance actually represents one of the most important public health challenge [20].

The development of animal models with metabolic dysfunction induced by diets with high caloric densities have been widely reported in the literature, as they can be used to reproduce the etiology, course and outcomes of human metabolic diseases [21–26]. In a previous study we have described that a western-style diet (cafeteria diet) induces obesity and hyperglycemia in rats concomitantly with multiple ovarian disruptions; *e.g.* ovulatory impairments, diminished estradiol levels, reduced ovarian reserve and follicular cysts development [27].

Based on the foregoing, the aim of the present work was to examine the influence of obesity on insulin sensitivity and glucose tolerance not only at systemic level but also locally in the ovary. Moreover, we wanted to evaluate the impact of obesity on the reproductive outcome.

2. Materials and methods

2.1. Animals and study protocol

Twenty two days old female Wistar rats (*Rattus Norvergicus*) weighing 120–130 g were obtained from Bioterio Central, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires. All research animals were treated in compliance with the guidelines for the care and use of animals approved by the Comité Institucional de Cuidado y Uso de Animales de Experimentación (CICUAL, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires) in accordance to principles of laboratory animal care (NIH Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, Washington, D.C.).

All rats had *ad libitum* access to water and standard rodent chow diet (ACA Nutrición Animal, Argentina) and were kept on a 12:12-h light–dark cycle at 22 °C. Rats were divided randomly into two 60-days intervention groups distinguished by dietary composition: (1) *Control Group* (n=20) was fed only standard rodent chow diet; (2) *Obese Group* (n=20) was also offered with a "cafeteria-style" diet (a varying menu of highly palatable human foods comprising sausages, cheese, snacks, peanuts, biscuits and chocolate biscuits). This animal model of obesity was adapted from previous studies [28] and has been previously successfully implemented by us [27]. Weight gain, abdominal circumference and body length were monitored twice a week.

The day of the first estrus phase after the 60thintervention day, animals were separated for 2 different studies: (1) Pre-gestational ovarian status studies: For that purpose, 10 animals (5 control and 5 obese) were subjected to a glucose tolerance test (GTT). Afterwards, these rats were sacrificed and ovaries were removed to perform PCRs, IHQs and to analyze the ovarian glucose intake. (2) Reproductive outcome studies: From a total of 30 animals (15 control and 15 obese), 10 were subjected to an insulin tolerance test (ITT). All rats were fed standard rodent chow from this moment and their reproductive outcome was evaluated.

2.2. Glucose tolerance test

For the glucose tolerance test (GTT), 6 h fasted control and obese rats were intraperitoneally injected with a bolus of glucose (2 g/kg) and blood glucose levels were determined at 0, 15, 30, 60, and 120 min after glucose challenge. Glycemia was measured in tail blood using glucose strips on an Accu-Chek Performa II instrument (Roche, Buenos Aires, Argentina). Data was collected for each individual animal and expressed as mean blood glucose concentration over time. The area under the curve (AUC) for glucose was calculated to evaluate glucose tolerance in control and obese animals [29].

2.3. Insulin tolerance test

The insulin tolerance test (ITT) was performed in 2 h fasted rats administrating a single intraperitoneal insulin injection (0.5 U/kg diluted in PBS) and blood glucose was sampled at times 0, 15, 30, 45, 60, 90, 120 and 150 min after insulin injection. Glycemia was measured in tail blood using glucose strips on an Accu-Chek Performa II instrument (Roche, Buenos Aires, Argentina). Insulin sensitivity for control and obese animals was estimated during ITT by the first-order rate constant of glucose disappearance (K_{ITT}) computed as the slope of the regression line of blood glucose against time during the first 60 min [30].

2.4. Anesthesia and pre-gestational tissues collection

The first estrus phase after the 60thintervention day, 10 animals (5 from control and 5 from obese group) were subjected to euthanasia after performing anesthesia with a 50 mg/kg solution of ketamine (Brouwer, Buenos Aires, Argentina) associated with 10 mg/kg xylazine (Alfasan, Woerden, Holland) that were injected intramuscularly into the inner side of one of the hind legs. Afterwards, ovaries were removed. One ovary from each animal was used fresh for uptake glucose analysis. Half of the remaining ovary was frozen for subsequent RNA extraction and the other half was fixed in 4% (w/v) formaldehyde for 24 h, dehydrated, embedded in paraffin and cut into seven-micron sections. Ovarian sections were mounted on gelatin-coated glass slides and subsequent used for immunohistochemical studies.

2.5. Glucose uptake by ovaries

The ovarian glucose uptake measurement was adapted from previous works [31]. Briefly, one ovary from each animal was isolated and divided into two halves. Both halves were incubated in Krebs-Ringer bicarbonate (KRB) buffer (117 mMNaCl, 4.7 mMKCl, 2.5 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 24.6 mM NaHCO3, pH 7.4) containing 2 mM pyruvate for 50 min at 37 °C. Ovaries were transferred to KRB containing 1 mM 2-deoxy-D-[1,2–3 H(N)]glucose (3 mCi) and incubated for an additional 10 min with or without insulin (0.1 mU/ml) at 30 °C. Incubation and transport buffers were continuously gassed with 95% O_2–5% CO_2. Transport was terminated by immersion in ice-cold KRB containing 80 mM cytochalasin B. Ovaries were frozen in liquid nitrogen and processed as previously described [32]. Aliquots of the lysate were used for protein measurement using Bradford and radioactivity in the solubilized tissue was measured in a liquid scintillation spectrometer.

2.6. Ovarian RNA extraction and polymerase chain reaction (PCR)

Total RNA was extracted from the ovaries using Trizol reagent (Invitrogen, *CA*, USA) according to the manufacturer's protocol. cDNA was synthesized by incubating 2 μg of extracted RNA in a buffer containing, 3 U AMV Reverse transcriptase (Promega, Madison, WI, USA), 1 μM oligo d(T)15 Primer (Dongsheng Biotech, Guangdong, China) and 1 mM Mix dNTPS (Dongsheng Biotech). The reaction mixture was incubated for 60 min at 42 °C followed by 15 min at 70 °C. cDNA (2 μl, selected to work within the linear range) was amplified by PCR in a buffer containing 0.5 U Taq-DNA polymerase (Invitrogen), 0.2 mM of each primer (Invitrogen), 0.2 mM of each dNTP, 1.5 mM MgCl2 and each specific primer. The primer sets used are detailed in Table 1 where the specific annealing temperature used for each pair of primers is included as well as the number of cycles used. The optimum cycle number was determined for each primer pair, so that signals were always in the exponential portion of the amplification curve. Each cycle consisted of: denaturation at 95 °C for 15 s, primer annealing at the specific temperature for 30 s and extension at 72 °C for 15 s. PCR products were electrophoresed on 2%

Table 1
Details of primers used for PCR

Gene	Primer sequence (5″-3″)	Fragment size (bp)	Annealing temperature (°c)	Cycles no.	GenBank accession no.
Glut-4	F: ACTGGCGCTTTCACTGAACT R: CGAGGCAAGGCTAGATTTTG	106	55	40	NM_012751
InsR	F: ATCCGTCGCTCCTATGCTCTGGTGT R: GTT GGT CTT CAG GGC AAT GTC GTT C	279	64,5	40	NM_017071
GAPDH	F: CCATCAACGACCCCTTCATT R: GACCAGCTTCCCATTCTCAG	110	57	35	NM_017008