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OBESITY ALTERS THE UTERINE ENVIRONMENT BEFORE PREGNANCY

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Running title: Obesity alters the uterine environment before pregnancy

Abbreviations: BMI, body mass index; CAF, cafeteria diet; PCNA, proliferating cell nuclear antigen; GLUT, glucotransporter; IR, insulin receptor; HIF-1 α, hypoxia-inducible factor-1 α; PPAR, peroxisome proliferator-activated receptor; AR, adrenergic receptor.

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ABSTRACT

Obesity is a metabolic disorder that predisposes to numerous diseases and has become a major global public-health concern. 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 administration for 60 days induces obesity in female rats and theirs fetuses develop macrosomia. Given that in our model rats are not genetically modified and that obese mothers were fed standard chow during pregnancy, the aim of the current study was to test the hypothesis that obesity alters the intrauterine environment prior to pregnancy; and this may explain the exacerbated fetal weight gain. We found that uteri from obese rats during the oestrous phase developed insulin resistance through mechanisms that involve the induction of uterine hypoxia and the downregulation of the insulin receptor gene. Moreover, uterine cell proliferation was induced by obesity concomitantly with the reduction in the uterine contractile response to a β2 AR agonist, Salbutamol; and this may be consequence of the downregulation in the uterine β2 AR expression. We conclude that CAF-induced obesity alters the uterine environment in rats during the oestrous phase and may cause the fetal macrosomia previously described by us in obese animals. The lower sensitivity of the uterus to a relaxation stimulus (Salbutamol) is not a minor fact given that for implantation to occur the uterus must be relaxed for embryo nidation. Thus, the alteration in the uterine quiescence may impair implantation and, consequently, the foregoing pregnancy.

Keywords: Obesity; Uterus; Insulin; Glucose; adrenergic receptor; uterine contractile activity.

INTRODUCTION

The global epidemic of obesity includes an alarming rise in the number of reproductive aged women who are overweight (body mass index [BMI]≥25 kg/m^2) or obese (BMI≥30 kg/m^2). The National Health and Nutrition Examination Survey found that the 60% of women in the United States are overweight or obese upon entering pregnancy [1]. This trend has serious implications for the general health of women as well as their reproductive potential. Obese women are three times more likely to suffer from anovulatory infertility than patients with a normal BMI [2]. Even when they do ovulate, the time to conception is two-fold longer in overweight patients [3]. Moreover, obesity negatively impacts assisted reproduction outcomes by lowering implantation and clinical pregnancy rates, increasing miscarriage rates, and decreasing live birth rates when compared with normal-weight women [4-8]. However, it is still unclear whether these negative pregnancy outcomes are due to factors affecting the oocyte/embryo quality, the endometrium, or both. Regarding the latter, it has been described that insulin is implicated in the regulation of endometrial development, metabolism, and receptivity [9, 10]. Insulin resistance is commonly exhibited by obese women and it negatively influences implantation and subsequent pregnancy. It has been shown that obese women with normal glucose tolerance had a 40% decrease in the expression of GLUT4 in adipocyte membranes when compared with lean controls [11], suggesting tissue specific insulin resistance. Insulin sensitivity is also controlled by several transcription factors, among which hypoxia-inducible transcription factors (HIF) and peroxisome proliferator-activated receptors gamma (PPARγ) stand out regarding obesity. Hypoxic stress is associated with obesity due to the excessive adipose tissue deposition [12, 13] and it is known that hypoxia impairs insulin sensitivity [14, 15]. Hypoxia promotes the expression of HIFs both at gene and protein levels [16]. HIF acts as transcription factor and for functioning HIFα and HIFβ subunits should heterodimerize. Among HIFα isoforms (HIF1α, 2α and 3α), HIF1α stabilizes in hypoxia conditions and generally rapidly degrades in a normoxic environment, while HIF1β is

constitutively expressed [17]. On his behalf, PPARs are a group of ligand-activated nuclear hormone receptors that connect the environment represented by nutritional inputs to specific genetic programs controlling genes involved in inflammation, adipogenesis, lipid metabolism, and glucose homeostasis [18]. There are three different isoforms of PPARs in mammals: PPARα, PPARβ/δ, and PPARγ. Despite showing structural similarities, the three isoforms exhibit differences in ligand specificities, tissue distribution and functions. PPARγ becomes relevant in conditions of obesity since it contributes to the uptake of glucose and lipids, and when expressed ectopically, it promotes deposition of lipids in peripheral tissues [19]. In fact, PPARγ agonists, such as the thiazolidinediones pioglitazone and rosiglitazone, are currently prescribed as anti-diabetic drugs and act as insulin sensitizers [20]

Furthermore, obesity during pregnancy is associated with an abnormal intrauterine metabolic environment that has long-lasting effects on offspring, since the ability of the progeny to adapt to an adverse intrauterine environment is conferred prior to pregnancy [21]. In view of all the above, endometrial insulin resistance may potentially be one mechanism that negatively impacts fertility in obese patients, inducing long-lasting effects on the offspring as well.

In previous studies of our laboratory we found that cafeteria diet-induced obesity in female rats induced systemic and ovarian insulin resistance [21]. Obesity also impaired the reproductive outcome by reducing the ovarian reserve, altering ovulation [22], decreasing fertility rates and delaying conception [21]. Moreover, we showed that maternal pre-gestational obesity exacerbated fetal growth during gestation that resulted in fetal macrosomia despite all animals were fed standard chow during gestation, highlighting the importance of the maternal body weight at conception time [21]. In order to study the possible mechanisms responsible for these alterations, the purpose of the current study was to test the hypothesis that obesity alters the intrauterine environment prior to pregnancy.

In view of all the above, we first purpose to evaluate if obesity induce the development of uterine insulin resistance and whether HIF and PPARγ are involved in this disruption.

Moreover, it has been shown that insulin resistance alters responsiveness and contractile activity of uterine tissue [27], that higher uterine contractile activity is associated to lower pregnancy rate after *in-vitro* fertilization [28] and that obesity negatively impacts outcomes of assisted reproduction due to lower pregnancy rates [29, 30]. The uterine contractile activity is known to be controlled predominantly by adrenergic receptors (ARs) [31-33], whose levels are altered in several tissues as consequence of obesity [34-36], however it is still unknown if their uterine expression are modified as consequence of obesity. So, for all the above, we also propose to study whether the uterine ARs levels and contractile activity are altered as a consequence of obesity.

MATERIALS AND METHODS

Animal husbandry

Wistar rats (*Rattus Norvergicus*) were obtained from Bioterio Central, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires. 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. 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.).

Animal diets

Twenty-two days old female Wistar rats weighing 120-130 g were divided randomly into two groups distinguished by dietary composition: (1) *Control Group* was fed only standard rodent chow diet; (2) *Obese Group* was also offered with “cafeteria-style” (CAF) diet (a varying menu of highly palatable human foods comprising sausages, cheese, snacks, peanuts, vanilla and

chocolate biscuits). This animal model was described by other authors [23], has been previously successfully adapted and implemented by us, and showed that obesity is induced after 60 days of CAF diet administration [22]. Weight gain, abdominal circumference and body length were monitored twice a week.

Anesthesia and tissue collection

Animals were sacrificed when reach the first estrus stage after 60 days of diet protocols. For that purpose, anesthesia was performed using 50 mg/kg solution of ketamine (Brouwer, Buenos Aires, Argentina) associated with 10 mg/kg xylazine (Alfasan, Woerden, Holland) that were injected intraperitoneally into the inner side of one of the hind legs. Afterwards, euthanasia was performed by cardiac exsanguination and uteri were removed, divided in pieces and pieces were: (1) used fresh for myometrial contractile studies, (2) used fresh for uptake glucose analysis, (3) frozen for subsequent RNA extraction, (4) lysed for western blotting or (5) fixed in 4% (w/v) formaldehyde for 24 h, dehydrated, embedded in paraffin and cut into seven-micron sections. Sections were mounted on gelatin-coated glass slides and subsequent used for immunohistochemical studies.

Uterine glucose uptake

Glucose uptake measurement was adapted from previous works [24]. Briefly, one uterine horn from each animal (n=8 rats/group) was isolated and divided into two halves that 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 with or without insulin (0.1 mU/ml) for 50 min at 37°C. Tisuues 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 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. Uteri were frozen in liquid nitrogen and processed as previously described by

us [21]. Aliquots of the lysate were used for protein measurement using Bradford and radioactivity in the solubilized tissue was measured in a liquid scintillation spectrometer.

Uterine homogenates and Western blotting

Uterine pieces (n=5 rats/group) were homogenized in Tris-buffer (100 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 0.5% NP40, 1% Triton, 1 mM PMSF) containing 1X protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The lysate was centrifuged at 4°C for 10 min at 10 000 × *g*, and the pellet was discarded. Protein concentrations in the supernatant were measured by Bradford assay (Bio-Rad, Hercules, CA, USA). After boiling for 5 min the uterine lysates, 90 µg of protein from each sample was applied to an SDS-polyacrylamide gel (10%) and electrophoresis was performed at 100 V for 1.5 h. The separated proteins were transferred onto PDVF membranes in transfer buffer (20% methanol, vol/vol; 0.19 M glycine; 0.025 M Tris-Base, pH = 8.3) for 1 h at 4°C. Blots were blocked for 1.5 h in TBS (4 mM Tris–HCl, pH = 7.5, 100 mM NaCl) containing bovine serum albumin (0.1%) at room temperature as previously described [25]. The primary antibodies used were: rabbit polyclonal anti-β2 AR (adrenergic receptor β2) (1:1000, overnight; Santa Cruz Biotechnology Inc., USA, sc: 9042), rabbit polyclonal anti-PPARγ (peroxisome proliferator-activated receptor γ) (1:500, overnight; Santa Cruz Biotechnology Inc., sc: 7196) and mouse monoclonal anti -Glyceraldehyde-3-PDH (GAPDH) (1:500, overnight; Millipore, USA, MAB374) that was used as an internal control. The identity of the bands was established by the use of molecular weight standards (14.3–200 kDa, Bio-Rad), which allows the identification of the bands of interest: β2 AR (56-85 kDa), PPARγ (54-57 kDa) and GADPH (38 kDa); which were absent in the negative control experiments performed in the absence of primary antibodies. Afterwards, blots were incubated for 1h with biotin-conjugated secondary antibodies: anti-rabbit IgG (1:2000; Millipore) or anti-mouse (1:500; DakoCytomation, USA, Eo-354) followed by streptavidin–peroxidase complex (1:2000; DakoCytomation). The specific signals were visualized using ECL detection solution

(Thermo Scientific, Illinois, USA) and acquired in an ImageQuant RT ECL (General Electric, Amersham Bioscience, Argentina) and software and quantified with Image J software (version 1.42q, National Institute of Health, USA). Densitometry analysis was performed with ImageJ software. Results are expressed as the relative intensity of β2 AR and PPARγ normalized against GAPDH.

Uterine RNA extraction and retrotranscription

Total RNA was extracted from uteri (n= 5 rats/group) 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 3U AMV Reverse transcriptase (Promega, Madison, WI, USA), 1uM oligo d(T)15 Primer (Dongsheng Biotech, Guangdong, China) and 1Mm Mix dNTPS (Dongsheng Biotech). The reaction mixture was incubated for 60 min at 42°C followed by 15 min at 70°C.

Polymerase Chain Reaction (PCR)

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. Primer sets used are detailed in table 1, where the specific annealing temperature and the number of cycles used for each pair of primers are also included. 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% agarose (Biodynamics, Buenos Aires, Argentina) gels. Gel images were taken with the ImageQuant RT ECL (General Electric) and software and quantified using Image J software (version 1.42q, National Institute of Health, USA). Density of the bands of interest was normalized to that of

GADPH in each sample. Negative controls were performed without reverse transcriptase or RNA.

Real time PCR (qPCR)

cDNA samples were diluted 1:2 before use. Quantitative real-time PCR (qPCR) was performed in a DNA Engine Opticon 2 Real-Time Cycler (Roche Applied Science) following the standard curve using FastStart Universal SYBR Green Master Mix (Roche) and primers at a final concentration of 0.9 μM. Primer sequences are shown in table 1. L30 gene was used to normalize for differences in concentrations of β2 AR cDNA samples.

Immunohistochemistry

Uteri cellular proliferation was assessed by immunohistochemical quantification of proliferating cell nuclear antigen (PCNA) [26]. For that purpose, uterine slides (n=5 rats/group) were incubated with 5% (w/v) non-fat milk at room temperature for 30 min for background blocking. Afterwards, endogenous peroxidase activity were blocked by incubation with 3% (v/v) hydrogen peroxide at room temperature for 15 min. Tissue sections were then incubated at 4°C overnight with mouse monoclonal anti-PCNA (1:200, overnight; Millipore, MAB424) as primary antibody. Controls were performed by omitting the primary antibody. Sections were, then, incubated with rabbit anti-mouse (1:300; DakoCytomation, Eo-354) at room temperature for 40 min; and, afterwards, incubation for 40 min with streptavidin–biotin horseradish peroxidase complex reagent (1:500; DakoCytomation) was used. Color development was performed with a solution containing 3,3′-diaminobenzidine (DakoCytomation) and sections were counterstained with hematoxylin. Finally, the sections were dehydrated, mounted and observed with an FV-300 Olympus light microscope and photographed. The number of cells with PCNA - positive (dun) nuclei present in the stroma and in the luminal and glandular epithelium was determined by counting 500 cells within 4 quadrant tissue sections from each uterine sample.

Immunofluorescence

Uterine localization of β2 AR were analyzed by immunofluorescence as previously described [27]. For that purpose, uterine slides (n=5 rats/group) were placed in a solution containing 0.01 M citrate buffer, pH 6.2 for 5 min in a microwave oven at 100 C at 600 W for antigenic recovery. Afterwards, background blocking that was achieved by incubating with 5% (w/v) non-fat milk at room temperature for 30 min. Tissue sections were then incubated at 4°C overnight with rabbit polyclonal anti-β2 AR (H-73) (1:200, overnight; Santa Cruz Biotechnology sc: 9042) as primary antibodies. Controls were performed by omitting the primary antibody. Sections were, then, incubated with goat anti rabbit (1:1000; Millipore) at room temperature for 40 min; and, afterwards, incubation for 40 min with streptavidin–biotin Alexa Fluo 488 conjugate (1:500; Molecular Probes) was used and nuclei were stained with propidium iodide (Invitrogen). Finally, sections were mounted in 50:50 PBS - glycerol and observed and photographed with an Olympus Bx-61 microscope for laser confocal microscopy attached to an Olympus FV-300 camera.

Myometrial contractile analysis

Small strips (~10 × 5 mm) of longitudinal myometrium were dissected from each animal (n=5 rats/group) and suspended in a separate 20 mL organ bath filled with KRB warmed at 37°C and gassed with 95% 0_2 - 5%CO_2 . Each strip was set to a resting tension of 9.8 mN (1gF); its contractile activity was recorded using isometric force transducers (Harvard Apparatus, South Natick, MA) connected to a bridge amplifier, which was in turn connected to a data acquisition system (Data Studio Pasco). According to Chaud et. al.[28], myometrial strips were left to stabilize for 30 min until regular phasic contractions were achieved. Afterwards, 20 min spontaneous baseline contractile function was determined before the accumulative addition of Salbutamol (0.05 ng/ml to 5000 ng/ml)[29], a selective β 2 –adrenoceptor agonist, applied at 7-min intervals. Chemicals were obtained from Sigma unless stated otherwise. The resultant

contractile activity measurements included the amplitude and the frequency of contractions as well as the activity integrals (area under the time-force curve).

Statistical analysis

Experimental data are presented as the mean ± S.E.M unless otherwise is indicated and the number of animals used for each determination is indicated in the figure legends as n. Statistical analyses were carried out by using the Instat program (GraphPAD software, San Diego, CA, USA) and $P<0.05$ was considered statistically significant.

For the glucose uptake measurement and PCNA analysis, comparisons between groups were performed using two-way analysis of variance (ANOVA) followed by Bonferroni post- tests.
For contractile activity values (amplitude, frequency and activity integral), comparisons among all concentrations of Salbutamol and between control and obese rats were performed by two-way ANOVA with repeated measures followed by the Newman–Keuls test. The significance of the remaining results was determined using Student's t-test.

RESULTS

Obesity induces uterine insulin resistance

The results of the glucose uptake by uteri from control and obese rats are shown in Fig.1. Basal glucose uptake was similar in uteri from obese and control rats. On its behalf, insulin stimulated the uterine glucose uptake by nearly 1.6-fold in control rats ($P<0.05$), whereas insulin response was almost completely blunted in uteri from obese rats. These results show that uteri are insulin sensitive organs in normal conditions and that obesity induces uterine insulin resistance.

The induction of uterine insulin resistance by obesity involves uterine hypoxia and the downregulation of the insulin receptor gene

The fact of finding insulin resistance at uterine level led us to; first, analyze the local expression of the insulin receptor (InsR) gene. Decreased InsR mRNA levels was detected in uteri from obese animals when compared to controls ($P<0.05$, Fig. 2A). This result suggests that the uterine insulin resistance induced by obesity is, at least in part, due to a change in the local transcriptional regulation of the *InsR*. We next analyze the expression of the main molecules regulating the uterine glucose intake: glucotransporters *Glut-1* and *Glut-4*. The analysis of Glut-1 and Glut-4 mRNA levels revealed no difference in their uterine expressions between control and obese rats (Fig. 2B and C).

Hypoxic stress is commonly associated to obesity due to the excessive adipose tissue deposition [30]. It controls insulin sensivity as well as peroxisome proliferator-activated receptors (PPARs) does [31], among others. The action of hypoxic stress is mediated by hypoxia-inducible factor-1 a (HIF-1 α) [32]. So, we analyzed whether uterine *Hif-1*α and PPARγ expressions were modified by obesity. Higher *Hif-1*α gene expression was detected in uteri from obese animals compared to controls ($P<0.05$, Fig. 2D). Regarding PPARγ, its protein levels were similar in uteri from control and obese rats (Fig. 2E).

The uterine proliferative activity is increased by obesity

It has been described that insulin stimulates endometrial cellular proliferation [33]. Moreover, it is known that obesity is an independent risk factor for endometrial cancer [34] through mechanisms that involves increased inflammatory signaling and increased levels of insulin [35]. So, the uterine proliferative activity of control and obese rats was analyzed and its results are summarized in Fig. 3. PCNA immunostaining revealed that DNA synthesis varied between control and obese animals and it depends on the uterine compartment. The highest degree of cellular proliferation occurred in luminal epithelial cells followed by stromal cells (Fig. 3A) both in uteri from control (panels a and c) and obese rats (panels b and d). Both were higher in obese animals compared to controls ($P<0.01$ and $P<0.05$ respectively, Fig. 3B). The less mitotic

activity was seen in the glandular epithelial cells and no difference was detected in this compartment between control (panel e) and obese animals (panel f) (Fig. 3B).

Obesity reduces the uterine contractile response to a β2 AR agonist, Salbutamol, due to a local downregulation of β2 AR expression

It has been described that insulin resistance also alters responsiveness and contractile activity of uterine tissue [36] and the latter is known to be controlled predominantly by adrenergic receptors (ARs) [37-39], whose levels are altered in several tissues as consequence of obesity [40-42]. However the uterine expression of ARs under obesity conditions has not been studied so far. So, we first screened all uterine ARs isoforms in control and obese rats. α1A, α2C, α2A, α2B and β2 AR mRNAs isoforms were detected both in uteri from obese and control animals, being the β2 *AR* isoform the dominant in both groups (Fig. 4A). Moreover, β2 AR mRNA and protein levels were lower in uteri from obese animals compared to controls ($P<0.01$ and $P<0.001$ respectively, Fig. 4B and C, respectively). Regarding its localization, β2 AR showed an intense myometrial localization and relatively weak expression at luminal and glandular epithelium (Fig. 4D) both in uteri from obese and control animals. Having found lower uterine levels of β2 AR in obese rats, a key factor regulating relaxation of the myometrium, we next analyze whether obesity alters the uterine contractile activity. Examinations were only performed on myometrial strips which showed regular spontaneous contractile activity. Spontaneous uterine contractile activities are illustrated in Fig. 5A top and bottom traces. The analysis of the spontaneous contractile activities revealed that obesity did not alter the amplitude (Fig.5B), the frequency (Fig.5C) and, consequently, neither the activity integral of uterine contractions (Fig.5D).

To confirm that the lower levels of β2 AR found has a physiological role on uterine contractility, we next analyzed the response of the myometrium to Salbutamol, a selective agonist of β2 AR (Fig. 6A). Salbutamol decreased the amplitude of contractions at 0.5, 5, 50,

500 and 5000 ng/ml concentrations in the control group (P<0.001) and at 50, 500 and 5000 ng/ml in the obese group compared to the spontaneous contractile activity (P<0.01-0.001; Fig. 6B). The decrease was significantly (P<0.05-0.01) lower in the obese group after treatment at 5 and 50 ng/ml compared to controls.

Salbutamol administration significantly (P<0.001) decreased the frequency of contractions at 5, 50, 500 and 5000 ng/ml concentrations only in the control group compared to the spontaneous contractile activity (Fig. 6C). In the obese group, the contraction frequency was not modified by Salbutamol when compared to the spontaneous contractile activity. The frequency of contractions was significantly higher after treatment at the 50ng/ml, 500 ng/ml and 5000 ng/ml concentrations in the obese group compared to controls (P<0.001; P<0.05 and P<0.01, respectively).

When the activity integrals were calculated it was found that, in control group, Salbutamol induced a significant decrease at all the concentrations evaluated (0.05 to 5000 ng/ml) compared to the spontaneous contractile activity (P<0.001; Fig. 6D). In the obese group the decrease in the activity integral was detected at 5, 50, 500 and 5000 ng/ml compared to the spontaneous contractile activity (P<0.01-0.001). The decrease was significantly lower in the obese group after treatment at 0.05, 0.5, 5 and 50 ng/ml compared to controls (P<0.05, P<0.01, P<0.001 and P<0.001, respectively).

DISCUSION

It is becoming increasingly clear that the states of extreme positive energy balance and peripheral insulin resistance result in reproductive dysfunction [43]. In our group, we work with CAF induced-obesity in rats as animal model to investigate the response of reproductive tissues to obesity. CAF largely reflects the variety of highly palatable, energy dense foods that are prevalent in Western societies and that is associated with the current obesity pandemic. In previous works we demonstrated that CAF-induced obesity in rats is associated to peripheral

and ovarian insulin resistance; and that their reproductive performance is disrupted [21, 22]. Moreover, we showed that maternal pre-gestational obesity led to fetal macrosomia despite mothers fed standard chow during gestation.[21]. Therefore, the present study aimed to study if the uterine environment before pregnancy is also altered by CAF. Here, we show that obesity induces the development of uterine insulin resistance through mechanisms that involve the decrease in the gene expression of the *InsR*, without altering those of *Glut-1* and *Glut-4*. These results are in accordance with those found, by us, at the ovarian level [21] and given that insulin controls GLUT-4 trafficking, the fact that uterine *Glut-4* gene expression is not altered by obesity does not imply that GLUT-4 protein expression and/or trafficking is not altered in these animals. New experiments are being design in order to clarify that point. It has been postulated that differential insulin signaling of tissues leads to reproductive dysfunction [43] and here we describe for the first time that obesity induces uterine insulin resistance. This is not a minor fact, given that we have previously shown that fertility rates were lower and conception was delayed in the obese animals. Furthermore, we also showed that even in the face of a normal gestational environment, a pre-pregnancy exposure to a maternal CAF impairs fetal growth [21]. Thus, uterine insulin resistance may be involved in the reproductive alterations previously described by us in these animals. Moreover, it has been shown that the ability of the progeny to adapt to an adverse intrauterine environment is conferred prior to pregnancy and it is possible that the effects of maternal obesity may be transmitted to subsequent generations, having profound implications for human health [44]. All these evidences highlight the importance of studying how maternal obesity impairs the pre-conception environment to further be able to propose treatments for preventing/reverting that disruption. Thus, the metabolic intrauterine environment here described in cycling rats may be responsible not only for reproductive disruptions but also for alterations in the fetal growth that we previously described [21].

It has been described that the excessive adipose tissue deposition produced by obesity leads to hypoxic stress [12, 13] that impairs insulin sensitivity [14, 15]. The fact of finding, here, that the uteri from obese rats shows higher *Hif-1*α expression controls shows for the first time that uterine hypoxia is induced by obesity. Moreover, we are showing that the uterine insulin resistance detected in obese animals may me consequence; not only due to decreased uterine *InsR* have levels, but it may involve the induction of uterine hypoxia. When the uterine levels the master regulator of glucose homeostasis, PPARγ, was evaluated; no differences were detected between uteri from control and obese rats. The fact that PPARγ levels are not altered does not imply that its function is not altered; thus, new experiments are being designed in order to evaluate this. However, if the uterine function of this factor were not modified by obesity, it could be concluded that PPARγ is not involved in the alteration in the uterine uptake of glucose induced by obesity.

Up to here, we show that obesity induces uterine insulin resistance due to a decrease in the uterine *InsR* levels through mechanisms that involves hypoxia/HIF1α signaling; without altering neither the uterine gene expression of *Glut-1* and *Glut-4* nor uterine PPARγ levels.

On the other hand, we cannot forget that insulin not only affects the uterine metabolism but it has also been described to stimulate endometrial cellular proliferation [33] and that obesity is an independent risk factor for endometrial cancer [34]. It has been shown that HIF1α is increased during carcinogenesis and progression of cervical cancer [45] and that the mechanisms involved in obesity-related endometrial carcinogenesis include increased levels of insulin [35]. Given that, here, obese rats showed higher uterine HIF1α levels as well as insulin resistance; the uterine proliferative activity was studied. In this regards, it is known that, as a mechanism to prevent cell division and immortalization, cells keep environment balance and self-regulation in the body by replicative senescence. Changes that occur in a link of this regulation process will make cells lose replicative senescence. Thus, the excessive proliferation of cells that carry this error induce tumors [46]. Our findings show that obesity, indeed,

induced higher endometrial proliferation rates, both in luminal epithelial cells and stromal cells than controls. These findings may be indicative of an increase in the vulnerability of the tissue to develop cancer. However, this increase in proliferation rates could be offset by an increase in the apoptosis rates; therefore additional experiments are being designed in order to evaluate this. The numerous uterine alterations produced by the obesity that we have described here led us to evaluate the impact of these on uterine function. In this regards, it has been shown that obesity negatively impacts outcomes of assisted reproduction due to lower implantation and pregnancy rates, higher miscarriage rates and decreased live birth rates as compared with normal-weight women [47, 48]. Here we show that the uterine contractile activity in response to a β2 AR agonist, Salbutamol, is reduced as a consequence of obesity. This indicates a lower sensitivity of the tissue to a relaxation stimulus; this is not a minor fact given that for implantation to occur the uterus must be relaxed, as this is a prerequisite for embryo nidation and decidual invasion [49]. So, the alteration in the uterine quiescence may impair a foregoing pregnancy. The lower sensitivity can be explained due to the downregulation in the β2 AR gene and protein uterine expression seen in these animals. This finding is consistent with those found by other authors who described an altered expression of ARs as a consequence of obesity in other tissues [40-42]. Estradiol is the primary utero-stimulant; it is an increase in estradiol levels triggers an in uterine contractility [49] and we have previously described that obese rats shows lower serum estradiol levels than controls [21]. So, we cannot fail to mention that the decrease in the uterine expression of ARsβ2 AR may be a consequence of the lower levels of estradiol. Weaknesses of our study include the inability to demonstrate a definitive cause-and-effect relationship of altered uterine environment, decreased fertility rates, delayed conception and/or fetal macrosomia. However, our data show for the first time that CAF-induced obesity impairs the uterine response to insulin, increases the uterine mitotic activity and alters the regulation of myometrial contractile activity; and these alterations may inevitably impair the reproductive outcome.

Moreover, we show that the mechanism by which obesity impairs the uterine response to insulin involves a decrease in the uterine levels of *InsR* as well as hypoxia/HIF1α signaling. Regarding the mechanisms involved in the alteration of the uterine contractile response to salbutamol by obesity, the downregulation in the uterine β2 AR expression is here described by us.

It has been shown that difficult embryo transfer stimulates uterine contractions and this has been postulated to lead to non-adherence of the embryo(s) to the endometrium, expulsion of the embryos from the uterine cavity shortly after embryo transfer, or both [50]. The same finding has been reported in a small study in natural cycles [51]. Thus, given that uterine contractile activity is a key process for ensuring uterine receptivity, obesity may have both negative short-term effects by impairing implantation through this mechanism, as well as long-term fetal effects due to inducing fetal macrosomia. If our hypothesis that the alteration in the uterine β2 AR levels is responsible for obesity induced implantation problems is borne out by further studies, the clinical implications could be important. For example, pharmacological agents that inhibit uterine contractions may be a potential therapeutic regimen for obese women with recurrent pregnancy loss or infertility, both common and increasing problems seen among the growing obese patient population. Using these agents may lead to an improvement in implantation and pregnancy rates in obese patients.

FIGURE CAPTIONS

Figure 1: ***Uterine insulin resistance is induced by obesity.*** Basal 2-deoxyglucose uptake in uteri from control (open bars) and obese (squared bars) animals and after insulin stimulation (filled bars). The data are given as the means ± S.E.M. (n=8). * P<0.05 respect to the basal condition.

Figure 2: ***The induction of uterine hipoxia and the downregulation of the insulin receptor levels are involved in the obesity induced- uterine insulin resistance.*** **(A)** Peroxisome proliferator-activated receptors gamma (PPARγ) protein levels and gene expression of: **(B)** Glucotransporter type 1 (GLUT-1)**, (C)** Glucotransporter type 4 (Glut-4)**, (D)** Insulin Receptor (InsR) and by **(E)** hypoxia-inducible factor-1 a (HIFα) in uteri from control (open bars) and obese (squared bars) animals. Representative gels are shown in the upper panel and the densitometric analysis are shown in the lower pannel. Values are expressed in arbitrary units (a.u.) as means ± S.E.M. of the specific optical density normalized against Glyceraldehyde-3-PDH (GAPDH), as housekeeping (n=5). * P<0.05 respect to the control group.

Figure 3: ***Uterine luminal epithelial and stromal cell proliferation is induced by obesity.*** **(A)** Cell proliferation visualized by PCNA immunostaining as dun nuclei in the uterus from control (a, c and e) and obese rats (b, d and f). Ge: glandular epithelium; Le: luminal epithelium. **(B)** Number of PCNA positive cells in different uterine compartments (luminal and glandular epithelial cells and stromal cells). Results are expressed as mean±SEM (*n*=5). * P<0.05 and ** P<0.01 respect to control group.

Figure 4: ***Uterine β2 AR expression is downregulated by obesity.*** **(A)** Screening of all adrenergic receptors (AR) mRNA isoforms showed dominant uterine β2 AR mRNA expression in both control and obese rats. **(B)** Gene β2 AR expression is downregulated in uteri from obese animals compared to controls (n=6; **P<0.01 control vs. obese). **(C)** Protein β2 AR levels are lower in uteri from obese animals compared to controls (n=5; ***P<0.001 control vs. obese). **(D)** β2 AR localization in the rat uterus (n=4) showed intense expression at myometrium and weak expression at epithelium both in control (a and c) and obese (b and d) animals. Alexa 488-labeled β2-AR antibody is in green, and propidium iodide-labeled nuclei are in red. Le: luminal epithelium; Ge: glandular epithelium; Lm: longitudinal muscular layer; Cm: circular muscular layer.

Figure 5: ***Obesity does not alter the spontaneous uterine activity of cycling rats during the oestrous phase.*** **(A)** Representative recordings of spontaneous contractile activity in uteri from control (top trace) and obese (bottom trace) rats. **(B)** Amplitude, **(C)** frequency and **(D)** activity integral of 20 min of uterine spontaneous contractile activity of control and obese rats (n=5).

Figure 6: ***The uterus becomes less sensitive to Salbutamol, a selective agonist of β2 adrenoceptors, as a consequence of obesity.*** **(A)** Representative recordings of uterine contractions in control (top trace) and obese (bottom trace) rats by increasing (0.05 ng/ml to 5000 ng/ml) concentrations of Salbutamol. Effect of increasing doses of Salbutamol on: **(B)** amplitude, **(C)** frequency and **(D)** activity integral of uterine contractions in control (open circles) and obese (filled circles) rats. The dose effects were analyzed for a 7 min period after treatments and normalized to the spontaneous activity measured during 20 min before the first agonist administration. Results are expressed as mean ± SEM (n=5). ϕ P<0.05, # P<0.01 and δ P<0.001 indicate significant differences compared to the spontaneous activity measured

of each group.* P<0.05, **P<0.01 and ***P<0.001 indicate significant differences between control and obese groups for the same agonist concentration.

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Gene	Primer secuence (5'-3')	Fragment size (pb)	Annealing temperature (°C)	Cycles (no.)	GenBank accession no.
Glut-4	F: ACTGGCGCTTTCACTGAACT	106	55	40	NM_012751
	R: CGAGGCAAGGCTAGATTTTG				
Glut-1	F: TGGCCAAGGAACACACGAATACTGA	105	56	40	NM_138827.1
	R: TGGAAGAGACAGGAATGGGCGAAT				
HIF-1a	F: CCTACTATGTCGCTTTCTTGG	185	52	35	NM_024359.1
	R: TGTATGGGAGCATTAACTTCAC				
InsR	F: ATCCGTCGCTCCTATGCTCTGGTGT	279	64,5	40	NM_017071
	R: GTTGGTCTTCAGGGCAATGTCGTTC				
GAPDH	F: CCATCAACGACCCCTTCATT	110	57	35	NM_017008.4
	R: GACCAGCTTCCCATTCTCAG				
L30	F: CCATCTTGGCGTCTGATCTT	200	58	35	NM_022699.3
	R: GGCGAGGATAACCAATTTC				
α1A AR	F:TCTTCCTAGTGATGCCCATTG	145	55,35	40	NM_017191
	R:GCTTTCTTGAACTCCTGGCTG				
α1B AR	F:CCTGTTCTCCACCCTAAAGC	140	55,05	40	NM_016991
	R:ACCCAAGGATACGCATGAAG				
α1D AR	F:AAAAGGCTGCCAAGACGT	133	55	40	NM_024483
	R:AAGATGACCTTGAAGACACCC				
α2A AR	F:GCGAGATCTACTTGGCCCTC	258	57	40	NM_012739.3
	R:CGTTAATCTTGCAGCTCGGC				
α2B AR	F:GTCTTCAACCAGGACTTCCG	147	55,6	40	NM_138505.2
	R:AGAGACTGTGGAGGTGGG				
α2C AR	F:TTCAAGCACATCCTCTTCCG	143	55,3	40	NM_138506.1
	R:GAACTCTGGAGAAGCCACAC				
β1 AR	F:CTGCTACAACGACCCCAAG	146	54,2	40	NM_012701.1
	R:TCTTCACCTGTTTCTGGGC				
β2 AR	F: GTACTGTGCCTAGCCTTAGC	118	58	40	NM_012492.2
	R: GGTTAGTGTCCTGTCAGGGAGG				
β3 AR	F:AGAACTCACCGCTCAACAG	137	54,65	40	NM_013108.2
	R:CATGGACGTTGCTTGTCTTTC				

Table 1: Details of primers used for PCR.

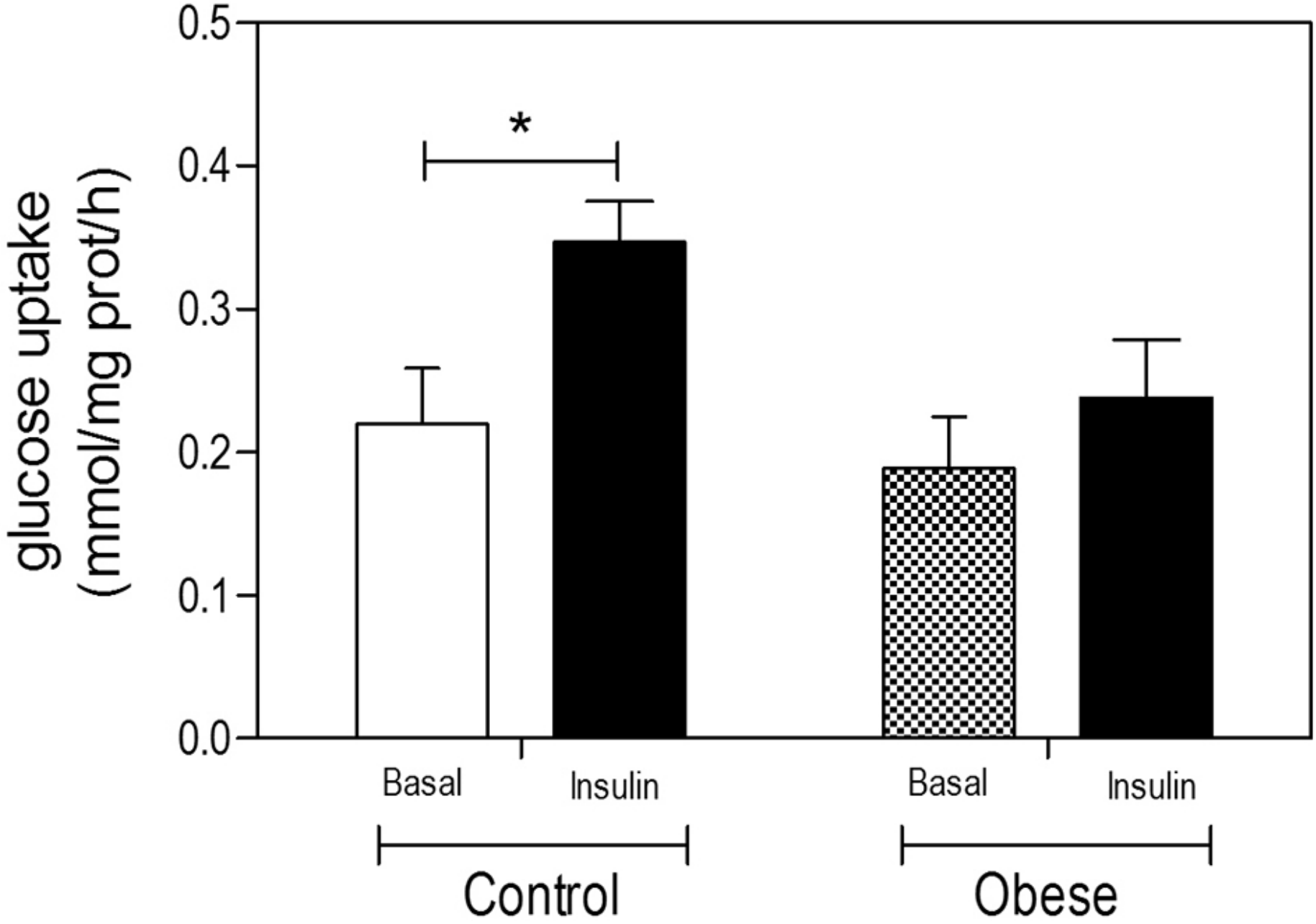

glucose uptake
(mmol/mg prot/h)
0.5
0.4
0.3
0.2
0.1
0.0
*
Basal
Insulin
Basal
Insulin
Control
Obese

Figure 1

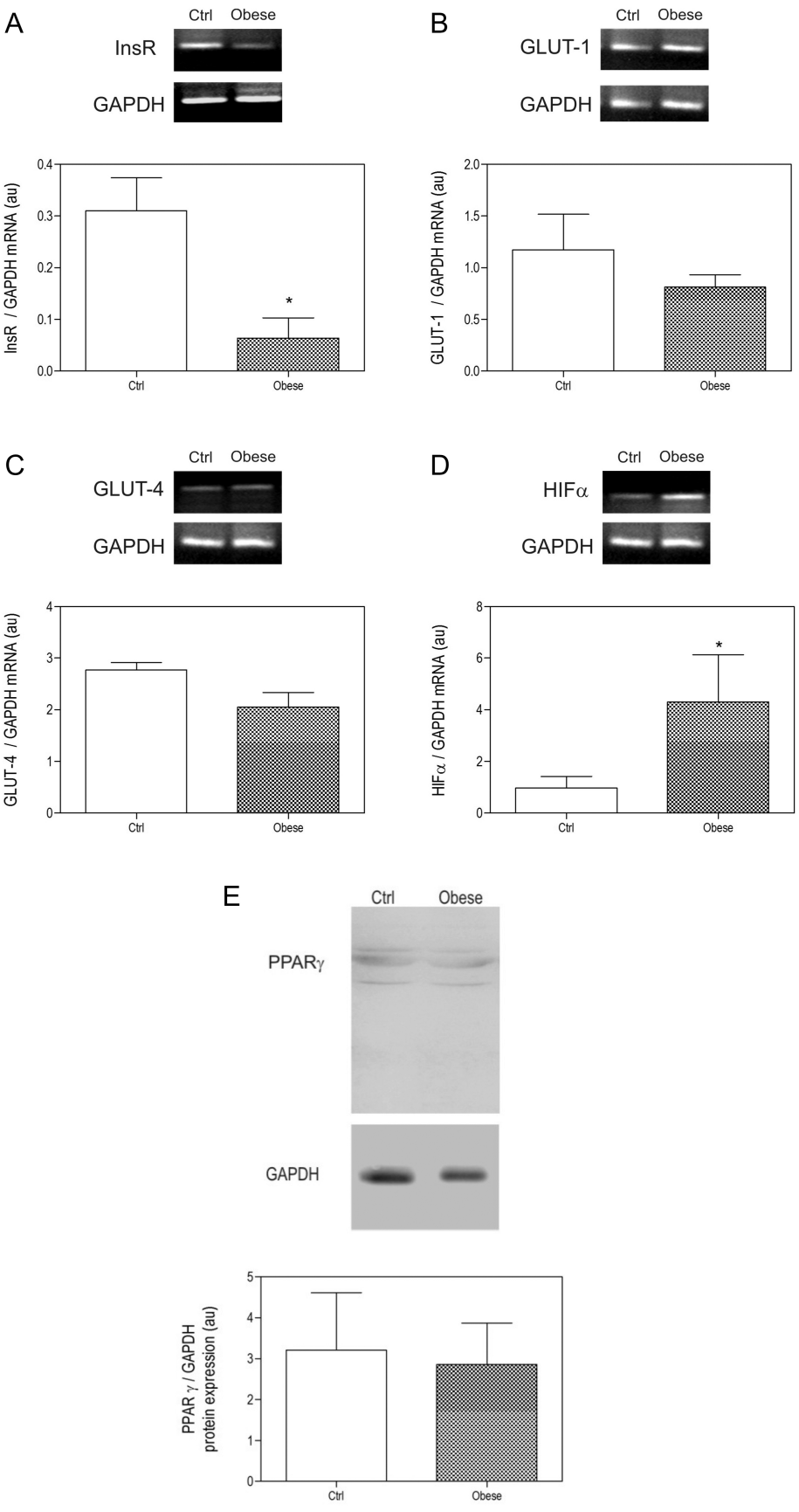
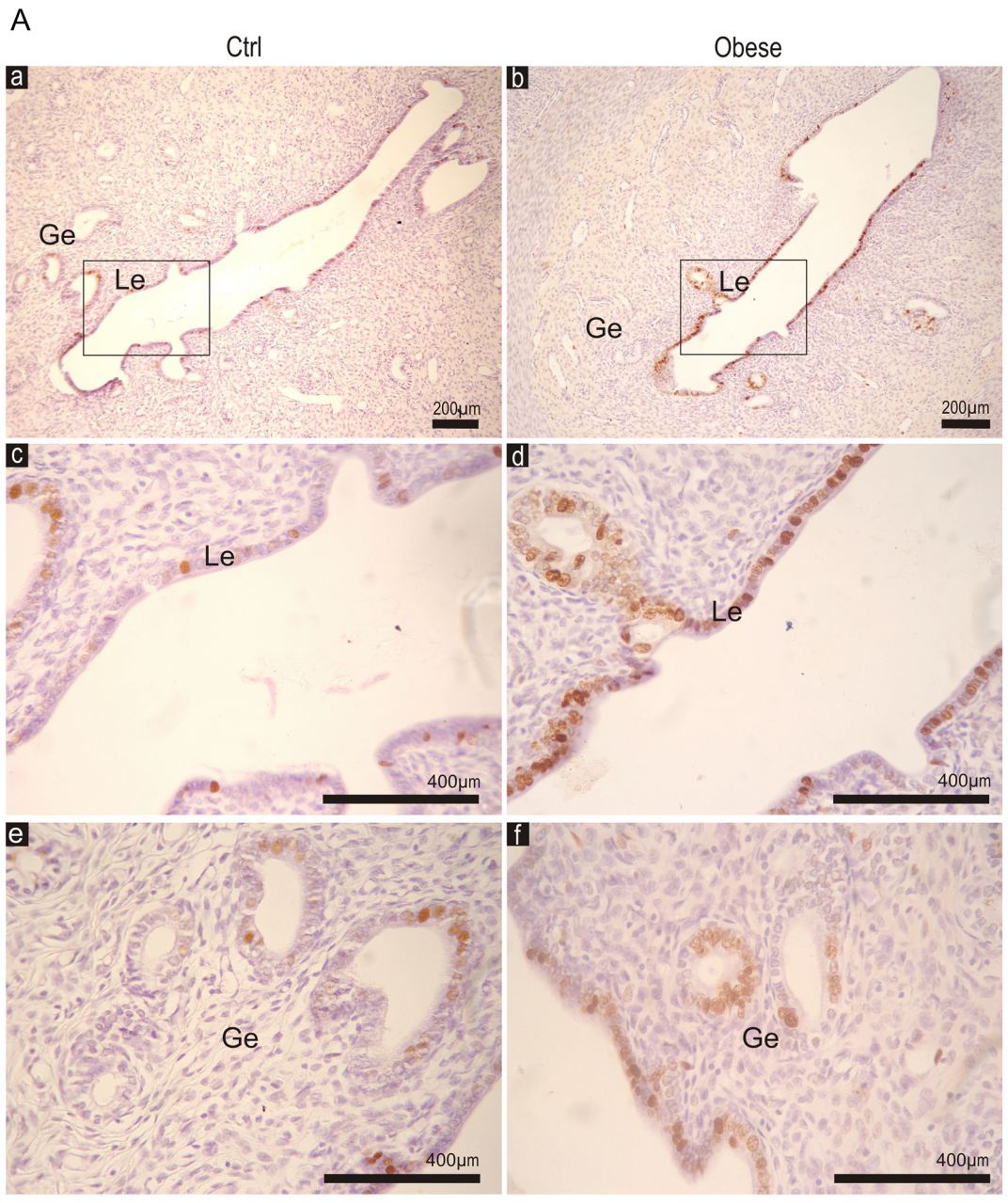

A
Ctrl
Obese
InsR
GAPDH
InsR / GAPDH mRNA (au)
0.4
0.3
0.2
0.1
0.0
*
Ctrl
Obese
B
Ctrl
Obese
GLUT-1
GAPDH
GLUT-1 / GAPDH mRNA (au)
2.0
1.5
1.0
0.5
0.0
Ctrl
Obese
C
Ctrl
Obese
GLUT-4
GAPDH
GLUT-4 / GAPDH mRNA (au)
4
3
2
1
0
Ctrl
Obese
D
Ctrl
Obese
HIFα
GAPDH
HIFα / GAPDH mRNA (au)
8
6
4
2
0
*
Ctrl
Obese
E
Ctrl
Obese
PPARγ
GAPDH
PPAR γ / GAPDH
protein expression (au)
5
4
3
2
1
0
Ctrl
Obese

Figure 2


A
Ctrl
Obese
a
b
Ge
Le
Le
Ge
200µm
200µm
c
d
Le
Le
400µm
400µm
e
f
Ge
Ge
400µm
400µm

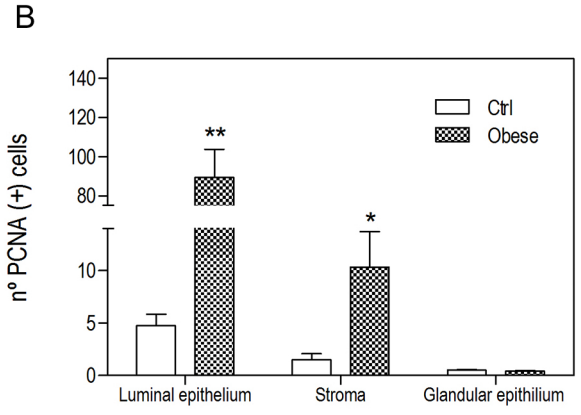

B
nº PCNA (+) cells
140
120
100
80
10
5
0
**
*
Ctrl
Obese
Luminal epithelium
Stroma
Glandular epithilium

Figure 3

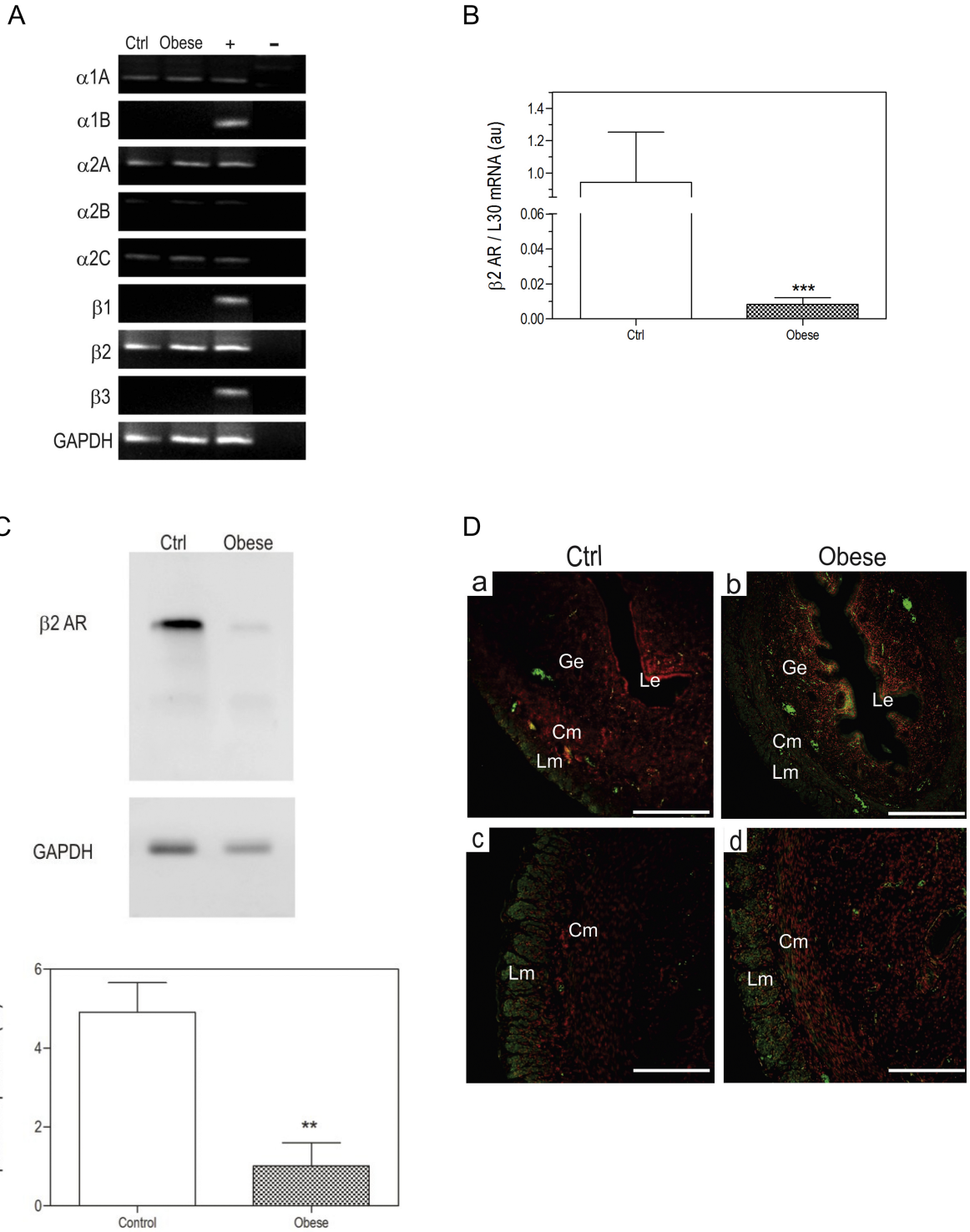

A
Ctrl
Obese
+
-
α1A
α1B
α2A
α2B
α2C
β1
β2
β3
GAPDH
B
β2 AR / L30 mRNA (au)
1.4
1.2
1.0
0.06
0.04
0.02
0.00
***
Ctrl
Obese
C
Ctrl
Obese
β2 AR
GAPDH
β2 AR / GADPH protein expression (au)
6
4
2
0
**
Control
Obese
D
Ctrl
Obese
a
b
c
d
Ge
Le
Cm
Lm

Figure 4

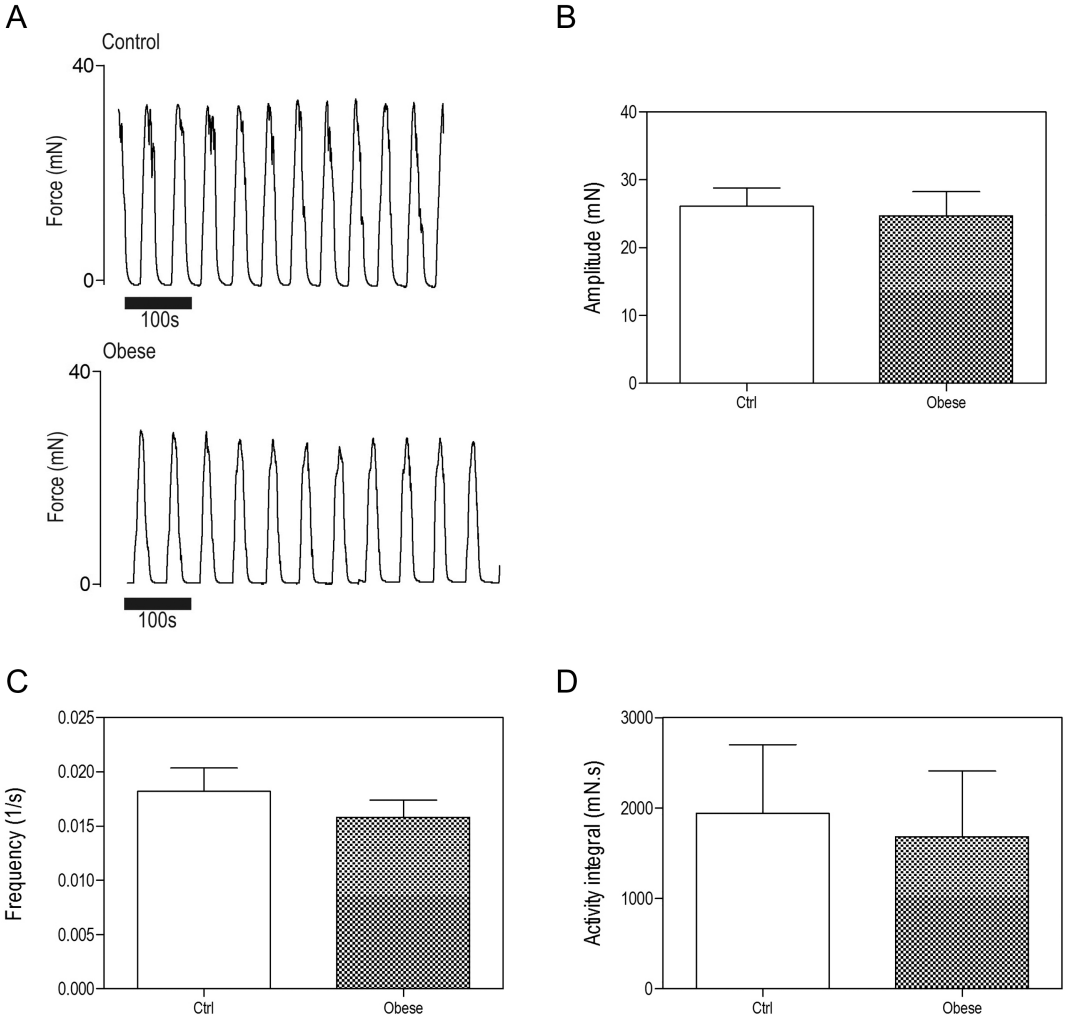

A
Control
40
Force (mN)
0
100s
Obese
40
Force (mN)
0
100s
B
40
30
20
10
0
Amplitude (mN)
Ctrl
Obese
C
0.025
0.020
0.015
0.010
0.005
0.000
Frequency (1/s)
Ctrl
Obese
D
3000
2000
1000
0
Activity integral (mN.s)
Ctrl
Obese

Figure 5

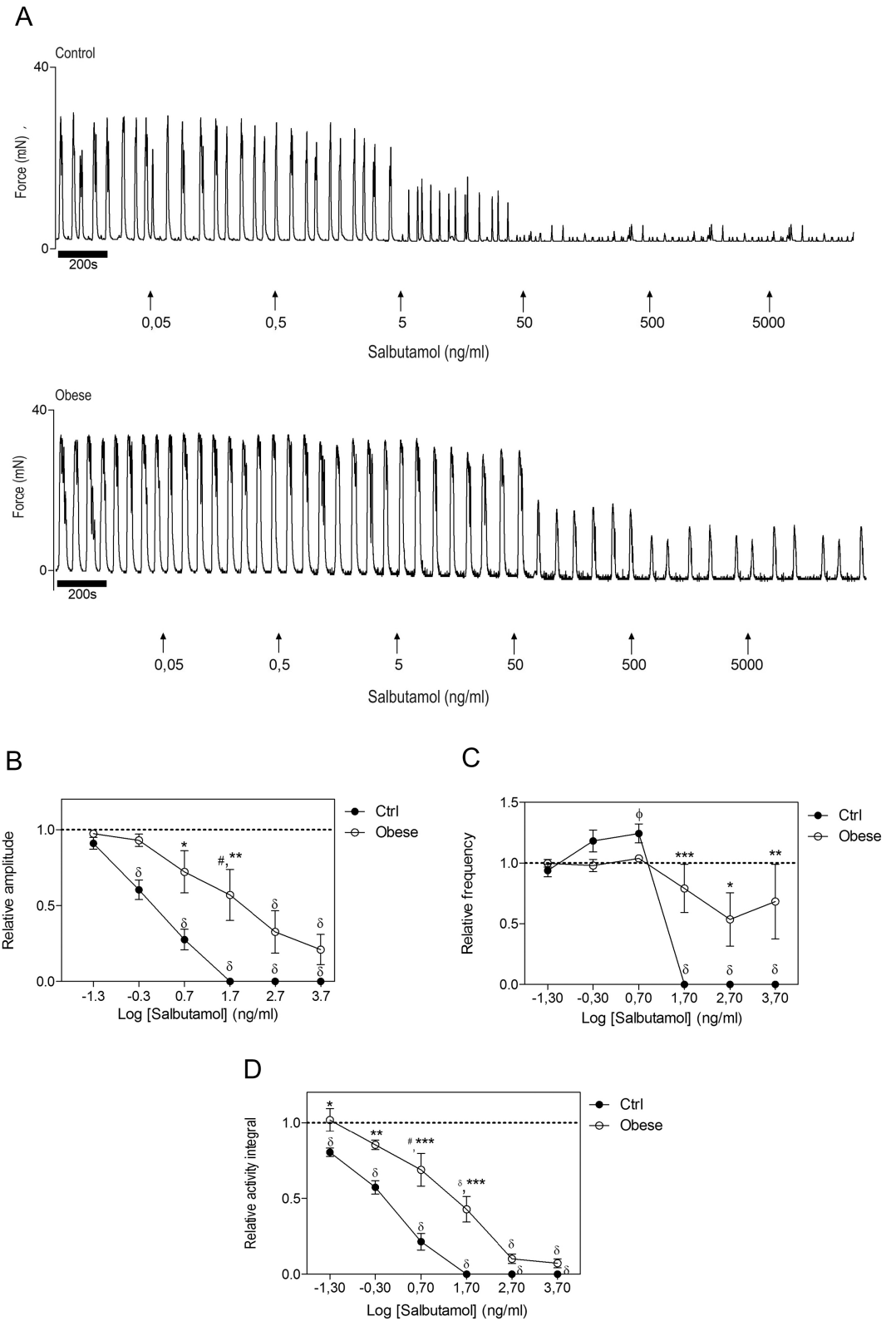

A
Control
40
0
Force (mN)
200s
0,05
0,5
5
50
500
5000
Salbutamol (ng/ml)
Obese
40
0
Force (mN)
200s
0,05
0,5
5
50
500
5000
Salbutamol (ng/ml)
B
Relative amplitude
1.0
0.5
0.0
-1.3
-0.3
0.7
1.7
2.7
3.7
Log [Salbutamol] (ng/ml)
Ctrl
Obese
C
Relative frequency
1.5
1.0
0.5
0.0
-1,30
-0,30
0,70
1,70
2,70
3,70
Log [Salbutamol] (ng/ml)
Ctrl
Obese
D
Relative activity integral
1.0
0.5
0.0
-1,30
-0,30
0,70
1,70
2,70
3,70
Log [Salbutamol] (ng/ml)
Ctrl
Obese

Figure 6