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ORIGINAL ARTICLE

Proinflammatory environment and role of TNF-α in endometrial function of obese women having polycystic ovarian syndrome

L Oróstica[1], I Astorga[1], F Plaza-Parrochia[1], C Vera[1], V García[2], R Carvajal[3], F Gabler[4], C Romero[1,3] and M Vega[1,3]

BACKGROUND/OBJECTIVES: A high percentage of women having polycystic ovarian syndrome (PCOS) exhibit hyperinsulinemia and obesity. Transforming necrosis factor-α (TNF-α) is an adipokine that increases in obesity and negatively affects insulin action in several tissues, including the endometrium. In fact, it has been reported that insulin signaling is altered in the endometrium of PCOS women, affecting its reproductive function. The aim of this study was to determine the proinflammatory environment and TNF-α signaling in endometrium from obese women with PCOS, and also to evaluate the effect of TNF-α on endometrial cell energy homeostasis.

METHODS: Serum and endometrial tissues were obtained from four study groups: normal-weight, normal-weight-PCOS, obese and obese-PCOS (hyperandrogenemia/hyperinsulinemia) ($n = 7$ per group). Serum TNF-α level was assayed by enzyme-linked immunosorbent assay (ELISA); endometrial TNF-α level and its receptors (TNFR1/TNFR2) as well as nuclear factor (NF)-κB content were determined by immunohistochemistry. Finally, we evaluated TNF-α effect on glucose uptake in cultured human endometrial stromal cells (T-HESC) treated or not with testosterone/insulin resembling partially the PCOS condition.

RESULTS: TNF-α plasma levels were similar between groups, whereas cytokine levels and macrophage number increased in endometrium from obese-PCOS women ($P < 0.001$). Both receptor types were higher in obese vs normal-weight women, particularly TNFR2 content in the obese-PCOS group ($P < 0.001$). Furthermore, an increased NF-κB nuclear content in endometrium from obese-PCOS was observed ($P < 0.001$). Finally, TNF-α treatment of T-HESC cultures exhibited a decrease of glucose uptake ($P < 0.05$), although similar to cells treated with testosterone or testosterone/insulin/TNF-α.

CONCLUSIONS: These results suggest that the PCOS condition induces an inflammatory state exacerbated when obesity is present, where a higher TNF-α signaling is observed, all of which could affect glucose uptake in the tissue and may cause fertility failures in these women.

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INTRODUCTION

Polycystic ovarian syndrome (PCOS) is an endocrine disorder affecting 5–18% of women in reproductive age.[1,2] This pathology is characterized by clinical and/or biochemical hyperandrogenism, together with oligo- and/or amenorrhea or polycystic ovary assessed by ultrasonography.[2] Moreover, ~70% of women with PCOS have some degree of obesity that may or may not be accompanied by an insulin-resistant condition with compensatory hyperinsulinemia.[3] This condition could compromise the energy availability in insulin-responsive tissues, including the endometrium.[4,5]

It is known that endometrial tissue expresses the molecules involved in the insulin signaling pathway, with glucose metabolism being the main source of energy that fulfills endometrial normal function.[4,5] Otherwise, it is well documented that endometria from PCOS women exhibit alterations on the insulin pathway, indicating an endometrial insulin-resistant condition.[6–11] This situation could worsen if we add the alterations related to obesity.

In fact, obesity involves an altered expression pattern of adipokines secreted primarily by adipose tissue, including transforming necrosis factor-α (TNF-α), interleukin-6 and adiponectin, among others. These alterations could contribute to the proinflammatory environment that has been described as a mild but chronic proinflammatory condition in obese patients, affecting not only the adipose tissue, but also other target tissues of these adipokines.[12,13] It is known that adiponectin is an insulin sensitizer and anti-inflammatory molecule, whereas TNF-α is a proinflammatory cytokine and participates in obesity-related systemic insulin resistance by inhibiting tyrosine kinase of the insulin receptor in muscle and fat.[14–16] Studies from our laboratory indicate that the adiponectin pathway is decreased in the endometrium from obese women with PCOS compared with those tissues obtained from obese women without PCOS.[17] Furthermore, other investigators have observed that TNF-α and interleukin-6 can reduce the expression of both the transcript and protein of adiponectin in adipose tissue whereas TNF-α can increase interleukin-6 expression.[18] Interestingly, studies have shown that endometrial cells from normal fertile women express TNF-α and its levels vary throughout the menstrual cycle, giving it a role in the implantation process.[19] Moreover, TNF-α can induce

[1]Laboratory of Endocrinology and Reproductive Biology, Clinical Hospital, University of Chile, Santiago, Chile; [2]Faculty of Health Sciences, University of Antofagasta, Antofagasta, Chile; [3]Department of Obstetrics and Gynecology, Faculty of Medicine, Clinical Hospital, University of Chile, Santiago, Chile and [4]Department of Pathology, Faculty of Medicine, University of Chile, San Borja Arriarán Clinical Hospital, Santiago, Chile. Correspondence: Dr M Vega, Laboratory of Endocrinology and Reproductive Biology, Faculty of Medicine, Clinical Hospital, University of Chile, Av. Santos Dumont 999, Independencia, Santiago 8380456, Chile.
E-mail: mvega@hcuch.cl
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cell proliferation in endometrial cell cultures.[20] A major intracellular effector of the TNF-α canonical pathway is nuclear factor (NF)-κB.[21] This transcription factor has been related with insulin resistance in several animal models associated with an increase in free fatty acids in circulation or with other inherent factors to obesity.[21] Furthermore, studies show that NF-κB, principally the p65–p50 dimer, has been found more active in the liver of obese mice fed with a high-fat diet,[22] suggesting a role in insulin failures under this context.

As women with PCOS have increased incidence of implantation failure compared with the normal population,[23,24] it is of importance to study the effect of the obesity condition on endometrial function of obese women with PCOS and also throughout the menstrual cycle. However, it is unknown whether the signaling pathways of adipokines are present in this tissue, nor if these pathways are altered under both the conditions of obesity and PCOS. If this is the case, it could partially explain the failure of the glycoside metabolism observed in the endometrium from PCOS women.

Therefore, the present study aims to evaluate whether TNF-α signaling and the inflammatory environment are altered in endometrial tissue from obese women under hyperandroginemic and hyperinsulinemic conditions characteristics of an important subpopulation of patients with PCOS. In addition, it aims to assess whether TNF-α signaling, including NF-κB as its principal effector related to insulin resistance models, is able to affect glucose uptake in an *in vitro* model of human endometrial stromal cells exposed to hyperinsulinic and hyperandrogenic conditions in the presence of TNF-α, resembling in part the altered hormonal and obesity conditions found in PCOS women.

SUBJECTS AND METHODS

Subjects

A total of 28 women were recruited and classified according to previous reports[17] in four groups: normal-weight, normal-weight-PCOS, obese and obese-PCOS. The PCOS was diagnosed according to the Androgen Excess and PCOS Society criteria, whose central criterion is hyperandrogenism,[2] determined in this case by the Free Androgen Index (biochemical hyperandrogenism) that considers total testosterone and sex hormone-binding globulin blood levels (normal value < 4.5). Therefore, the high ovarian androgen production and the decreased sex hormone-binding globulin blood level lead to a significantly higher Free Androgen Index in obese-PCOS and normal-weight-PCOS women (Table 1). Besides, obese-PCOS women who participated in this study presented hyperinsulinemia evaluated by an oral glucose tolerance test with 75 g load of glucose (normal values: fasting glucose < 110 mg dl^{-1}; glucose 120 min < 140 mg dl^{-1}). Thus, Insulin Sensitivity Index composite[25] and Homeostasis Model Assessment[26] values were abnormal in this group of patients and were diagnosed as women with hyperinsulinemia (normal values: insulin sensitivity index composite > 3 and homeostasis model assessment < 2.5) (Table 1). Therefore, obese-PCOS was the only group having hyperinsulinemia and hyperandrogenemia. Moreover, both groups of obese had a body mass index (BMI) of > 30 kg m^{-2}, whereas a BMI of < 25 kg m^{-2} was observed for both normal-weight groups, and the age of patients of the different studied groups was similar (Table 1). In addition, other pathologies that induce hyperinsulinemia or hyperandrogenemia were discarded as Cushing's syndrome and congenital adrenal hyperplasia. None of the women participants in the present investigation had received any treatment in the past 3 months before recruitment into the study. Endometrial and blood samples were obtained during the proliferative phase (PP) of the menstrual cycle; the endometrial dating was performed according to Noyes criteria[27] by an experienced pathologist. In addition, a reduced number of endometrial samples were obtained in the secretory phase (SP) of the menstrual cycle from obese women with or without PCOS to assess its proinflammatory status. Ethical Committees from the Faculty of Medicine and Clinical Hospital of the University of Chile approved this research where all subjects signed an informed written consent.

Determination of TNF-α blood concentration by ELISA

Serum samples from the four studied groups were used for detection of TNF-α levels by enzyme-linked immunosorbent assays (ELISAs). For the detection we used the commercial kit: TNF-α IMMULITE (Siemens, Berlin-Charlottenburg, Germany; Cat. No. #PILKNF-11). This cytokine was detected by a colorimetric reaction using an ELISA reader (Biotek EL800, Biotek Instruments Inc., Winooski, VT, USA) and absorbance measurements at 450 nm were performed using Gen5 1.06 program (Biotek Instruments Inc.).

Tissue preparation

Endometrial tissue samples from the four studied groups were obtained with a Pipelle suction curette from the corpus of the uteri and divided into two fragments. A fragment of each sample was fixed in 4% buffered formaldehyde for 24 h, embedded in paraffin and cut in 4 μm thick sections before histological and immunohistochemical studies. The other fragment of each sample was frozen in liquid nitrogen and stored at −80 °C to measure protein content. As previously reported,[17] the fragment of frozen samples was homogenized in RIPA buffer (50 mM Tris-Base, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100 and 0.1% SDS), and 1× protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Rochester, NY, USA) was added. The samples were centrifuged at 10 000 *g* for 20 min at 4 °C. The resulting supernatant was used to determine protein concentration with the BCA Protein Assay kit (Thermo Fisher Scientific).

Immunohistochemistry

Briefly, paraffin sections of human endometrial tissue were deparaffinized in xylene and gradually hydrated through graded alcohols.[17] The sections were incubated in 10 mM citrate buffer (pH 6.0) at 95 °C for 30 min, incubating the samples in 3% hydrogen peroxide for 15 min, preventing endogenous peroxidase activity. Nonspecific antibody binding was inhibited by incubating samples with the blocking solution (Histostain-SP, Invitrogen, Carlsbad, CA, USA) for 10 min. Then, samples were incubated with corresponding primary antibodies: TNF-α (rabbit polyclonal, Cell Signaling, Danvers, MA, USA; dilution 1:100), TNFR1 (rabbit

Table 1. Clinical and metabolic parameters of studied groups

Parameters	*Normal weight*	*Normal-weight-PCOS*	*Obese*	*Obese-PCOS*
Age (years)	26.6 ± 5.6	25.8 ± 2.7	27.4 ± 5.2	25.9 ± 2.4
BMI (kg m^{-2})	22.4 ± 2.1	25.6 ± 0.2	34.7 ± 4.8[a]	32.6 ± 3.4[b]
Testosterone (ng dl^{-1})	36.6 ± 10.7	40 ± 5.4[c]	22.6 ± 8.4	49.4 ± 9.2[b]
SHBG (nmol l^{-1})	68.9 ± 0.8	20.8 ± 2.7[c]	4.3 ± 2.4	25.7 ± 1.1[b]
FAI (reference < 4.5)	1.9 ± 0.6	7.3 ± 1.5[c]	2.3 ± 0.7	6.7 ± 1.9[b]
HOMA-IR (reference < 2.5)	1.49 ± 1.1	1.1 ± 0.2	2.3 ± 0.4	4.5 ± 0.9[d]
ISI composite (reference > 3)	8.1 ± 4.7	8.0 ± 1.8	4.6 ± 2.3	2.09 ± 0.3[d]

Abbreviations: BMI, body mass index; FAI, Free Androgen Index; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance; ISI, Insulin Sensitivity Index; PCOS, polycystic ovarian syndrome; SHBG, sex hormone-binding globulin. Studied groups were: normal-weight, normal-weight-PCOS, obese and hyperinsulinemic obese-PCOS. The values are mean ± s.e.m. [a]$P < 0.05$ between obese and normal-weight groups. [b]$P < 0.05$ between obese-PCOS and normal-weight groups. [c]$P < 0.05$ between normal-weight-PCOS and normal-weight groups. [d]$P < 0.05$ between obese-PCOS and the other groups.

monoclonal, Cell Signaling; dilution 1:100), TNFR2 (rabbit polyclonal, Cell Signaling; dilution 1:100), NF-κB p65 subunit (rabbit monoclonal, Cell Signaling; dilution 1:500) and CD68 (mouse monoclonal; Dako, Carpinteria, CA, USA; dilution 1:100) overnight at 4 °C. The internal control was carried out on adjacent sections incubated without the primary antibody. A biotinylated horseradish peroxidase secondary antibody was used for the detection of immune signal. Chromogenic revealed was developed by the streptavidin-peroxidase system and 3,3′-diaminobenzidine was used as substrate; counterstaining was performed with hematoxylin (Dako, Via Real). The slides were evaluated on an Olympus optical microscope (Olympus BX51TF, Tokyo, Japan) by measurement of positive pixel intensity with the use of the semiquantitative analysis tool IOD (Integrated Optical Density), using the Image Pro Plus 6.0 program (Media Cybernetics Inc., Rockville, MD, USA). Results were expressed as arbitrary units. To determine the translocation of NF-κB, we counted the number of positive p65 nuclei, the subunit that most frequently participates in the active NF-κB complex,[28] and the number of total nuclei in the tissue. Results were expressed as the percentage of positive nuclei in the tissue. To determine the proinflammatory environment, we evaluated the positive immunostaining of CD68 protein (macrophage marker) detected in endometria from the four studied groups. Results were expressed as the number of macrophages regarding the total tissue area.

Cell culture and treatments

Telomerase-immortalized human endometrial stromal cell line T-HESC (ATCC, Manassas, VA, USA; CRL-4003)[29] was cultured in Dulbecco's modified Eagle's medium/Ham F12 medium (Sigma Aldrich, Co., St Louis, MO, USA) with 10% fetal bovine serum treated with dextran carbon (Hyclone Thermoscientific, Waltham, MA, USA) and 1× of antimycotic/antibiotics at 37 °C in a 5% CO_2 atmosphere until 80% confluence. Then, cells were cultured in 6-well plates, 700 000 cells per well in growth media for 24 h at 37 °C in 5% CO_2 atmosphere. The cells were washed twice with sterile Dulbecco's phosphate-buffered saline (GIBCO Invitrogen Corporation, Camarillo, CA, USA). The cultures were further subjected to testosterone plus insulin treatment (100 nM each) for 48 h in serum-free medium. Testosterone and insulin concentrations were determined in previous studies from our laboratory.[9,11] Moreover, some cell cultures were also costimulated with insulin and testosterone (100 nM each) and TNF-α (100 ng ml^{-1}) or with testosterone, insulin or TNF-α alone for 48 h to determine the effect on glucose uptake. Basal condition was cultures with no hormonal stimulation.

Glucose uptake assay

As previously reported,[11] cells were plated in 12-well plates at 150 000 cells per well. After hormone and cytokine stimuli, the cell cultures were incubated in transport media containing 15 mM Hepes buffer pH 7.6, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$ and 10 mM Deoxy-D-glucose. Uptake assays were performed in 500 µl of transport media containing 1 µCi ml^{-1} of 2-deoxy-D-[1,2(N)3H]glucose (PerkinElmer, Waltham, MA, USA). After 5 min of transport at room temperature, uptake was stopped with ice-cold stop solution (135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.2 mM $HgCl_2$). Cells were dissolved in 500 µl of lysis buffer (0.5 mM NaOH). To samples were added 3.5 ml liquid scintillation for radioactivity determination using a scintillation counter (Packard Tri-Carb 2100R Liquid Scintillation Counter (Perkin Elmer Packard, Waltham, MA, USA); 18% efficiency). Flavonoid quercetin (150 µm) was used to block GLUT1 transporters and to subtract the unspecific glucose uptake in each experiment.[11] Results of three independent experiments in duplicate were expressed as pmol glucose per µg total protein for each group.

Statistical analysis

All data showed a nonparametric distribution and were analyzed by Kruskal–Wallis test. For multiple comparisons, Dunn's post test was used. The *P*-values of < 0.05 were considered significant. Statistical tests were performed using Graph Pad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Determination of TNF-α serum levels

The present study assessed whether obesity and/or PCOS condition could alter blood levels of TNF-α by ELISA. The results show similar serum concentration of the adipokine in the four studied groups (normal-weight: 4.73 ± 0.25 ng ml^{-1}; normal-weight-PCOS: 4.52 ± 0.48 ng ml^{-1}; obese: 4.43 ± 0.37 ng ml^{-1}; obese-PCOS: 4.20 ± 0.08 ng ml^{-1}).

Determination of proinflammatory environment in endometrium: location and levels of TNF-α and CD68

Based on the failures in the insulin signaling and the lower adiponectin levels in endometrial tissue from PCOS women shown previously,[6–11,17] it was relevant to evaluate the local proinflammatory environment in endometria from the four study groups. In this context, the presence and location of TNF-α and macrophages were detected by immunohistochemistry in endometrial tissue. As shown in Figure 1a, the cytokine was detected in both compartments of the endometrium (stroma and epithelium) in all studied groups. Interestingly, we observed a significant increase of TNF-α in stroma and epithelium from obese-PCOS group compared with the other groups ($P < 0.001$). Coincidently, the presence of macrophages in the endometrium, detected through the levels of the protein marker CD68[ref. 30] (Figure 1b), revealed augmented number of macrophages in endometria from obese compared with normal-weight women (67%, $P < 0.01$). Even more, when the PCOS condition was present, the increased macrophage number was independent of whether the endometrium was obtained from normal-weight or obese woman. Nevertheless, a significant increase in macrophage number was observed in the obese-PCOS group, where hyperinsulinism and hyperandrogenism were present, compared with obese women (25%, $P < 0.05$).

Detection of TNF-α receptors type 1 and type 2 in endometrium

To assess the potential effect of TNF-α in endometrial tissue, the presence of both types of TNF-α receptors (type 1 or TNFR1 and type 2 or TNFR2) was detected in the four studied groups (Figure 2). We observed positive immune-reactivity for TNFR1 and TNFR2 in tissues from all groups of patients in both the stromal and epithelial compartment. When comparing normal-weight and normal-weight-PCOS or obese and obese-PCOS (Figure 2a), the PCOS condition increases TNFR1 levels with respect to their controls without the syndrome. The immune-detection of TNFR2 showed a similar pattern than TNFR1, with a significant increase in normal-weight-PCOS and obese-PCOS groups compared with normal-weight and obese groups without PCOS, respectively ($P < 0.001$) (Figure 2b). However, in endometria from obese-PCOS women we found a higher TNFR2 expression than in the other groups ($P < 0.001$), suggesting that when obesity, hyperinsulinism and PCOS conditions are present there is an increased TNFR2 level, together with TNF-α and the number of macrophages.

Based on the crucial role of endometria and of inflammatory molecules in the implantation process, we additionally determined the TNF-α system in a few number of endometrial tissue samples only from obese women with or without PCOS obtained in the mid SP of the cycle, as tissues from normal-weight women in the SP were not available. For this study, the expression of TNF-α, TNFR2 and CD68 was detected in their endometria by immunohistochemistry (IOD arbitrary units), where lower levels were found in obese-PCOS tissues compared with endometria from obese-PCOS women in PP of the menstrual cycle for the three studied proteins (TNF-α: 7936 ± 2500 (SP) and $247\,291 \pm 12\,000$ (PP), $P < 0.01$; TNFR2: 4371 ± 1200 (SP) and $167\,015 \pm 12\,500$ (PP), $P < 0.01$; CD68: 0.00057 ± 0.00021 (SP) and 124.6 ± 15.7 (PP) $P < 0.001$). According to these data, it is most likely that an alteration of the TNF-α system during the menstrual cycle is present in the endometrium from obese-PCOS women that could be associated with fertility problems in these women.

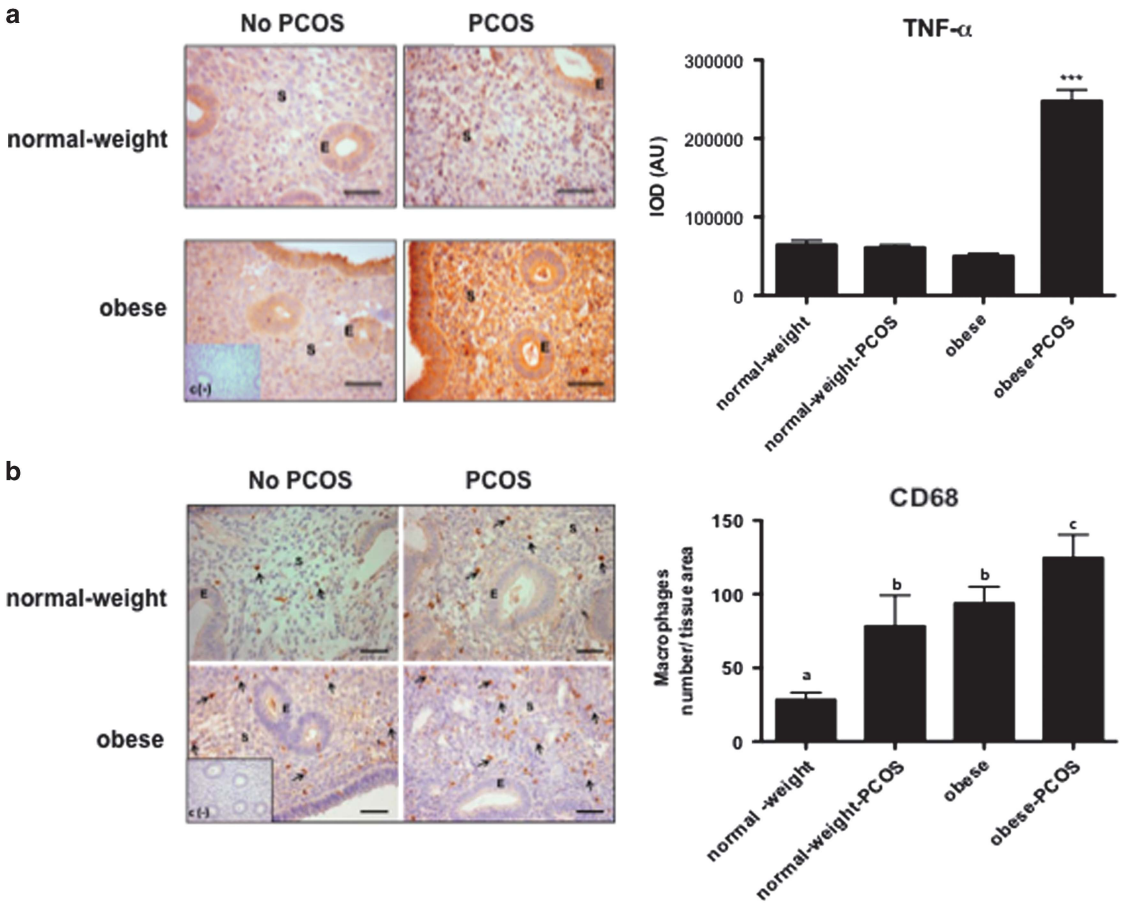

Figure 1. Detection of TNF-α protein and CD68-positive cells in endometrial tissue. TNF-α content (**a**) and percentage of CD68-positive cell (**b**) levels and location were assessed by immunohistochemistry. Photomicrographs (left hand) show the tissue location of TNF-α and CD68 by immunopositive brown color staining in endometrium from normal-weight, normal-weight-PCOS, obese and obese-PCOS women. Semiquantification (right hand) of each protein level in endometrial tissue was obtained by IOD tool. In (**a**), ***$P < 0.001$ obese-PCOS vs other groups. In (**b**), different letters indicate statistical significance (a≠b≠c with $P < 0.05$) and the arrows indicate the positive staining for CD68. C(−), negative control (inner box); E, epithelium; S, stroma. Bar = 50 μm; images at ×400. Nonparametric test and Dunn's post test. Data are shown as mean arbitrary units (AU) ± s.e.m., $n = 7$ samples in each group.

Detection of NF-κB (p65 subunit) in endometrial tissue

Regarding the above results, the TNF-α pathway could be more active in the endometrial tissue from obese-PCOS group. Therefore, to further analyze the effect of the cytokine in endometria, the presence of the p65 subunit of NF-κB in the nucleus was assessed in the endometrial tissue from the four studied groups (Figure 3). The data showed an increased number of positive nuclei in endometria from obese-PCOS (80%, $P < 0.001$) that clearly suggests a higher content of nuclear p65 in those endometria compared with the other groups, in accordance with an increased TNF-α activity in these tissues. This is consistent with the other results of the present study.

Effect of TNF-α on glucose uptake in human endometrial stromal cells (T-HESC) under hyperandrogenic and hyperinsulinic conditions

Given the high TNF-α level in endometrium from obese-PCOS group (Figure 1a) and the known negative effect of TNF-α on insulin signaling,[14–16] it was of relevance to investigate the effect of the cytokine in a functional cell process, such as glucose uptake. These experiments were performed in cultures of the human endometrial stromal cell line T-HESC in basal and under hyperandrogenic/hyperinsulinic conditions previously established in our laboratory[9] that resembles the PCOS microenvironment. These cultures were also treated with TNF-α concentration previously determined by a dose–response curve (data not shown). The results indicate that testosterone significantly decreases glucose uptake by these cells ($P < 0.05$) in agreement with our previous reports[9,11] (Figure 4). In addition, insulin partially reversed the negative effect of testosterone on glucose uptake. Interestingly, TNF-α also induces a negative effect on the ability of these cells to incorporate glucose ($P < 0.05$), and similar results were obtained with the co-administration of TNF-α/testosterone or TNF-α/testosterone/insulin.

DISCUSSION

PCOS is a condition that is mainly characterized by excessive production of androgens and in most cases is accompanied with insulin resistance and compensatory hyperinsulinemia. In this regard, our group has established that endometrium from women with PCOS have an altered expression of molecules involved in the insulin signaling pathway leading to failures in the insulin action.[5] This could explain in part the reproductive failures that occur in these women. These alterations could be exacerbated with the obesity condition present in a high percentage of PCOS patients.

Regarding obesity, it is known that the increase of adipose tissue involves changes in the molecular expression pattern of

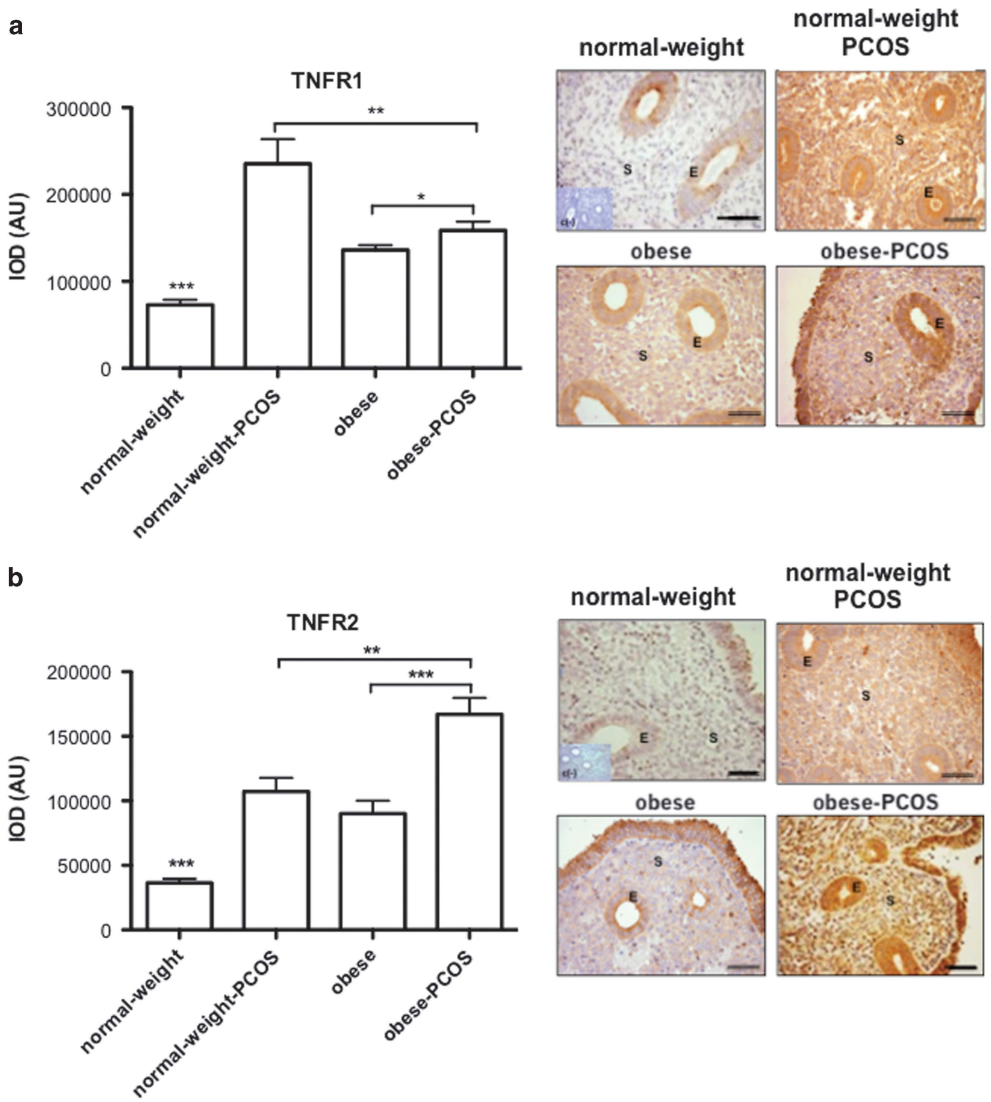

Figure 2. Protein levels of TNF-α receptors type 1 and 2 in endometrial tissue. TNFR1 (**a**) and TNFR2 (**b**) levels and location were determined by immunohistochemistry. Photomicrographs (left hand) show the tissue location of TNFR1 and TNFR2 by immunopositive brown color staining in endometrium from normal-weight, normal-weight-PCOS, obese and obese-PCOS women. Semiquantification (right hand) of the level of each protein in endometrial tissue was performed by IOD tool. In (**a**), $^{*}P < 0.05$ obese vs obese-PCOS; $^{**}P < 0.01$ normal-weight-PCOS vs obese-PCOS; $^{***}P < 0.001$ normal-weight vs other groups. In (**b**), $^{**}P < 0.01$ normal-weight-PCOS vs obese-PCOS; $^{***}P < 0.001$ normal-weight vs other groups and obese vs obese-PCOS. C(−), negative control (inner box); E, epithelium; S, stroma. Bar = 50 μm; images at ×400. Nonparametric test and Dunn's post test. Data are shown as mean arbitrary units (AU) ± s.e.m., $n = 7$ samples in each group.

these cells, altering the secretion of adipokines such as adiponectin (insulin sensitizing) or TNF-α (negative effect on insulin action), among others.[12,13,31,32] Recently, our group reported a diminished adiponectin expression in endometrium from obese women with PCOS, proving that these conditions do not only affect the ovary, but also other important reproductive tissues, such as the endometrium. Considering the antagonistic effect of TNF-α on insulin action and the low expression level of adiponectin, it was relevant to assess TNF-α signaling in the endometrium of obese-PCOS women and, importantly, the effect of this cytokine on the glucose uptake ability of endometrial cells exposed to the PCOS and obesity environment.

According to the results of the present investigation, no significant changes in serum TNF-α level were observed among the four study groups. However, it is unclear whether this plasma cytokine concentration is able to affect the normal function of tissues with a reproductive role, such as the endometrium. On the other hand, in this work the obesity was determined only by BMI of $>30\ kg\ m^{-2}$ without considering the waist circumference or the accumulation of visceral fat in these patients, with this fat giving the highest amount of TNF-α to circulation, eliciting a greater inflammatory environment under an obesity condition.[13]

In relation to the proinflammatory environment in the endometrial tissue, the increased cytokine level in obese women with PCOS could be associated in part to obesity, as it induces a mild but chronic inflammatory condition characterized by increased levels of cytokines like TNF-α. This cytokine is able to exert a negative action on the expression of adiponectin as a recognition site in its promoter is present;[33] therefore, it is likely that the increase in TNF-α content found in the present investigation in the endometrium of obese-PCOS women could be a potential cause of the adiponectin decrease found by García *et al.*[17] These data can explain some of the failures in the insulin signaling observed in endometria exposed to hyperinsulinemic

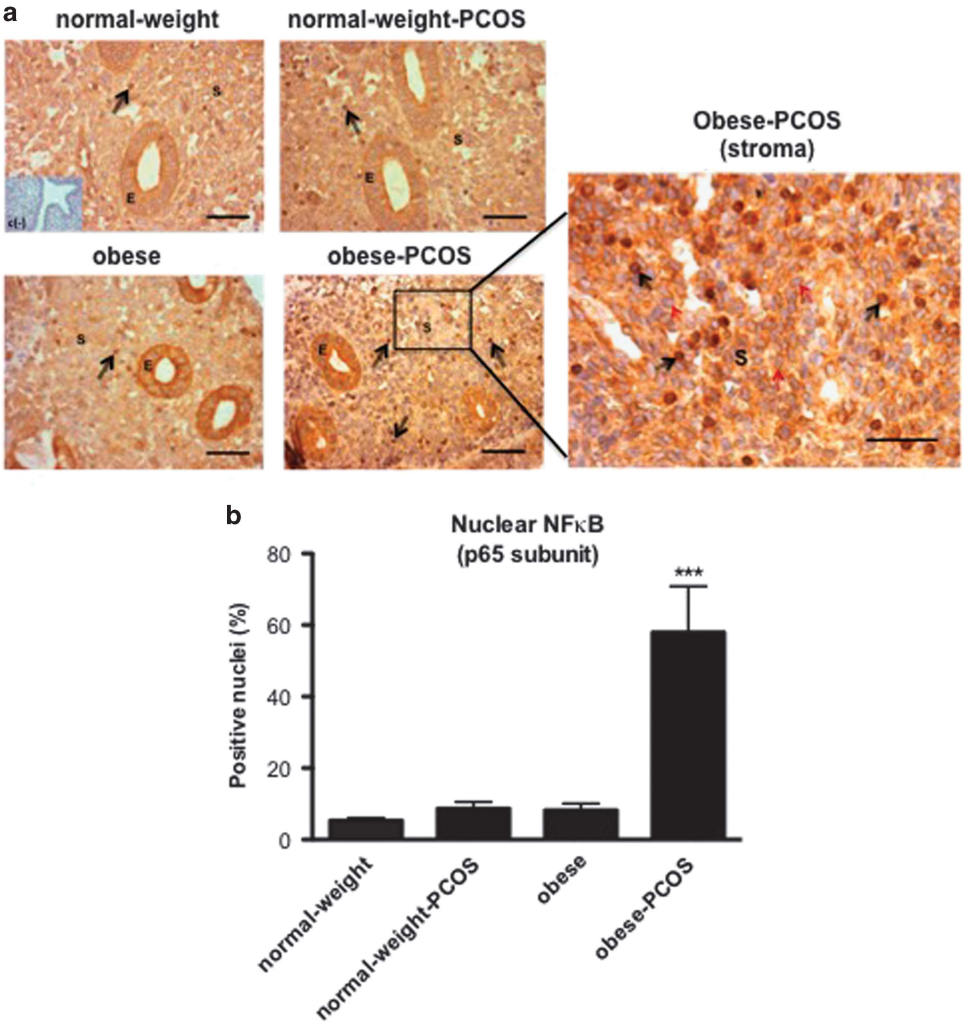

Figure 3. Detection of NF-κB nuclear p65 subunit in endometrial tissues. In (**a**), arrows in the photomicrographs indicate positive nuclei for p65 subunit of NF-κB and location in endometrial tissue from normal-weight, normal-weight-PCOS, obese and obese-PCOS women assessed by immunohistochemistry. The photograph with zoom on the right shows positive nuclei (black arrows) and negative nuclei (red arrows) for p65 subunit in endometrial stroma from obese-PCOS woman. In (**b**), semiquantification of the immunopositive staining shows the percentage of positive nuclei per total area of endometrial tissue in each group. ***$P < 0.001$ obese-PCOS vs other groups. C(−), negative control (inner box); E, epithelium; S, stroma. Bar = 50 μm; Images at ×400. Nonparametric test and Dunn's post test. Data are shown as mean ± s.e.m., $n = 7$ samples in each group.

and hyperandrogenic environment, present only in the group of obese women with PCOS. Accordingly, the increased number of macrophages in obese-PCOS endometrium could be the cause of the greater amount of TNF-α generated in these tissues. Consequently, this inflammatory condition could trigger negative effects on insulin signaling and metabolism of the endometrial tissue.

In the PCOS condition without obesity, a proinflammatory environment can also be found. In fact, in the case of the normal-weight-PCOS group, the macrophage number could be positively regulated by the hyperandrogenic condition present in the tissue. It is known that androgen induces increased production of TNF-α by macrophages, as reported in ovaries of rats with hyperandrogenism.[34] In this regard, there is evidence that PCOS involves an inflammatory process *per se*,[35] as shown by the higher levels of C-reactive protein in PCOS women compared with controls who match in BMI and age.[36] In addition, one study showed that visceral adipocytes obtained from non-obese women with PCOS promote the release of free fatty acids into the blood that could induce a mild proinflammatory environment in these women.[37] Hence, all these studies might indicate that obesity and/or PCOS can elicit a proinflammatory environment. Besides, when both conditions are present, an exacerbation of this environment at the endometrial level could be generated.

Some investigations have shown that TNF-α expression and its receptors change throughout the menstrual cycle, being higher in the SP where it could participate in the remodeling/proliferation process, as part of the normal function of the endometrium (bleeding or implantation process if the oocyte is fertilized).[19,38,39] Therefore, the results obtained in endometrium from obese-PCOS women during the mid SP showed an altered TNF-α system; this alteration could affect the implantation process and successful pregnancy in PCOS women with spontaneous ovulation, as the importance of immune cell activity during this process is known. Regarding this, the alteration of TNF-α system in the endometrium from obese-PCOS women could be strongly related to the hyperinsulinemic condition present in this study group. In fact, a work by Nestler and colleagues[40] indicate that a hyperinsulinemic condition in women with PCOS where luteal phase was induced by clomiphene may alter the proinflammatory environment and

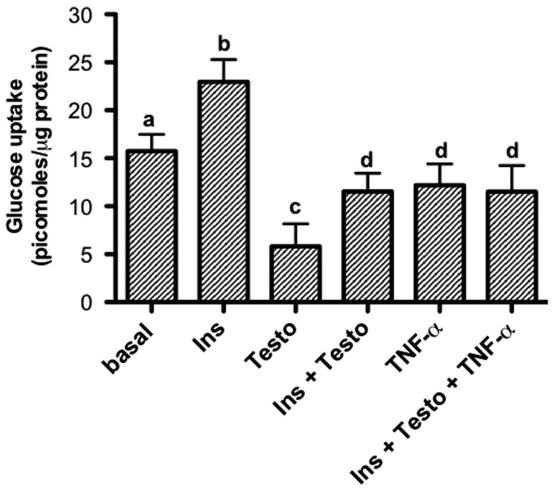

Figure 4. Effect of TNF-α on glucose uptake by the human endometrial stromal cell line T-HESC. Detection of tritiated-glucose in human endometrial stromal cells (T-HESC line) treated with different stimuli for 48 h using a scintillation counter. Basal, serum-free medium; Insulin (Ins), 100 nM; testosterone (Testo), 100 nM; TNF-α, 100 ng ml^{-1}. Different letters indicate statistical significance (a≠b≠c≠d with $P < 0.05$). Nonparametric test and Dunn's post test. Data are shown as mean ± s.e.m., $n = 3$ independent experiments in duplicate.

endometrium receptivity by a decrease in serum levels of regulatory molecules of immune response, such as glycodelin (secreted primarily by the endometrium) and IGFBP-1. These molecules modulate the accession process in the maternal–fetal interface, whereas treatment with metformin increases levels of both molecules in these women.[40]

Regarding the proliferative endometrium from obese women with PCOS, the increased levels of TNF-α and TNFR2 suggest a higher TNF-α activity.[38] Interestingly, genetic alterations of TNF-α and TNFR2, specifically some polymorphisms, have been associated with the presence of PCOS and hyperandrogenism in women.[41,42] The latter has also been linked to an increased amount of soluble TNFR2 in blood and with insulin resistance and a high BMI in women, contributing to an inflammatory state and the insulin-resistant condition.[42–44] On the other hand, because of the presence of a death domain, TNFR1 is mainly associated with apoptotic signals induced by TNF-α, whereas TNFR2 relates primarily to proliferative effects of immune cells and cytotoxicity through activation of molecules such as NF-κB or activator protein-1.[38,45,46] Moreover, obesity and insulin resistance studies have identified TNFR2 and NF-κB as the main effectors of TNF-α to induce inflammatory effects on various systems.[21,22,43,44] Considering that in our study the only group that has hyperinsulinemia and hyperandrogenemia was the obese-PCOS group, it is possible that when both conditions are present, the expression of TNF-α/TNFR2 increase significantly, suggesting an effect on proinflammatory condition in these women. This condition could induce adverse effects on insulin action through the activation of p65-NF-κB affecting the endometrial function, as evidenced by the higher content of nuclear NF-κB in these tissues. Thereby, active NF-κB can promote gene expression of proinflammatory cytokines, such as TNF-α.[47] The latter could exacerbate and perpetuate a state of chronic local inflammation in endometrium from obese-PCOS women.

As already mentioned, glucose availability is crucial for human endometrium to fulfill its normal function. The results of the present study show that testosterone is able to decrease glucose uptake by endometrial cells, whereas cotreatment with insulin partly reversed this effect in agreement with previous findings from our laboratory.[9,11] Furthermore, the present work and other studies from our laboratory have shown that these cells respond to the insulin treatment by significantly increasing glucose uptake.[9,11] Nevertheless, in the cotreatment conditions, insulin was not able to overcome the negative effect of testosterone or TNF-α on glucose uptake. Therefore, it is most likely that other factors present in the environment of pathological endometrium alter the insulin action. On the other hand, TNF-α is also capable of decreasing glucose uptake by these cells, indicating a negative effect of the cytokine on insulin signaling, as described by other studies.[48,49] In addition, the cotreatment of T-HESC with testosterone–insulin–TNF-α also decreases glucose uptake by these cells. However, testosterone and TNF-α can induce negative effects on insulin action probably through different pathways. In this context, the higher inflammatory environment and elevated TNF-α level in endometrium from obese women with PCOS do not rule out the participation of other cytokines such as interleukin-6 that is also elevated in the serum of obese patients and has a negative effect on insulin action.[50–52] Finally, all these evidences suggest that a condition of insulin resistance associated with obesity can adversely affect the energetic metabolism of endometrium from PCOS women through the increase in proinflammatory condition characterized by a higher activity of TNF-α/TNFR2/NF-κB system in the tissue that could directly affect the insulin signaling or affect insulin sensitizer molecules such as adiponectin, also decreased in endometrium from obese women with PCOS.[17]

In conclusion, these and previous results clearly indicate that the PCOS condition can affect the insulin action in the endometrial tissue. Furthermore, obesity may exacerbate this effect by providing an additional inflammatory environment in the tissue, causing disruptions in the action of insulin and glucose homeostasis, the main source of energy for the normal function of endometria. Altogether, these findings may explain in part the fertility failures found in these women.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1 Diamanti-Kandarakis E. Polycystic ovarian syndrome pathophysiology, molecular aspects and clinical implications. *Expert Rev Mol Med* 2008; **10**: e3.
2 Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W *et al.* Positions statement: criteria for defining polycystic ovary syndrome as a predominantly hyperandrogenic syndrome: an Androgen Excess Society guideline. *J Clin Endocrinol Metab* 2006; **91**: 4237–4245.
3 The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. *Fertil Steril* 2004; **81**: 19–25.
4 Frolova AI, Moley KH. Glucose transporters in the uterus: an analysis of tissue distribution and proposed physiological roles. *Reproduction* 2011; **142**: 211–220.
5 Oróstica L, Rosas C, Plaza-Parrochia F, Astorga I, Gabler F, García V *et al.* Review: altered steroid metabolism and insulin signaling in PCOS endometria: impact in tissue function. *Curr Pharm Des* 2016; **22**: 1–11.
6 Fornes R, Ormazabal P, Rosas C, Gabler F, Vantman D, Romero C *et al.* Changes in the expresión of insulin signaling pathway molecules in endometria from PCOS women with or without insulin resistance. *Mol Med* 2010; **16**: 129–136.
7 Rosas C, Gabler F, Vantman D, Romero C, Vega M. Levels of Rabs and WAVE family proteins associated to translocation of GLUT4 to the cell surface in endometria

from control and hyperinsulinemic PCOS women. *Hum Reprod* 2010; **25**: 2870–2877.
8 Kohan K, Carvajal R, Gabler F, Vantman D, Romero C, Vega M. Role of the transcriptional factors FOXO1 and PPARG on gene expression of SLC2A4 in endometrial tissue from women with polycystic ovary syndrome. *Reproduction* 2010; **140**: 123–131.
9 Ormazabal P, Romero C, Quest AFG, Vega M. Testosterone modulates the expression of molecules linked to insulin action and glucose uptake in endometrial cells. *Horm Metab Res* 2013; **45**: 1–6.
10 Carvajal R, Rosas C, Kohan K, Gabler F, Vantman D, Romero C *et al.* Metformin augments the levels of molecules that regulate the expression of the insulin-dependent glucose transporter GLUT4 in the endometria of hyperinsulinemic PCOS patients. *Hum Reprod* 2013; **28**: 2235–2244.
11 Rosas C, Oróstica L, Poblete C, Carvajal R, Gabler F, Romero C *et al.* Hyperandrogenism decreases GRP78 protein level and glucose uptake in human endometrial stromal cells. *Reprod Sci* 2015; **23**: 761–770.
12 Mitchell M, Armstrong DT, Robker RL, Norman RJ. Adipokines: implications for female fertility and obesity. *Reproduction* 2005; **130**: 583–597.
13 Cao H. Adipocytokines in obesity and metabolic disease. *J Endocrinol* 2014; **220**: T47–T59.
14 Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 1993; **259**: 87–91.
15 Hotamisligil GS, Budavari A, Murray D, Spiegelman BM. Reduced tyrosine kinase activity of the insulin receptor in obesity-diabetes. Central role of tumor necrosis factor-α. *J Clin Invest* 1994; **94**: 1543–1549.
16 Peraldi P, Hotamisligil GS, Buurman WA, White MF, Spiegelman BM. Tumor necrosis factor (TNF)-α inhibits insulin signaling through stimulation of the p55 TNF. receptor and activation of sphingomyelinase. *J Biol Chem* 1996; **271**: 13018–13022.
17 García V, Oróstica L, Poblete C, Rosas C, Astorga I, Romero C *et al.* Endometria from obese PCOS women with hyperinsulinemia exhibit altered adiponectin signaling. *Horm Metab Res* 2015; **47**: 901–909.
18 Hajri T, Tao H, Wattacheril J, Marks-Shulman P, Abumrad NN. Regulation of adiponectin production by insulin: interactions with tumor necrosis factor-α and interleukin-6. *Am J Physiol Endocrinol Metab* 2011; **300**: E350–E360.
19 Salama SA, Kamel MW, Diaz-Arrastia CR, Xu X, Veenstra TD, Salih S *et al.* Effect of tumor necrosis factor-ð¼ on estrogen metabolism and endometrial cells: potential physiological and pathological relevance. *J Clin Endocrinol Metab* 2009; **94**: 285–293.
20 Nair S, Nguyen H, Salama S, Al-Hendy A. Obesity and the endometrium: adipocyte-secreted proinflammatory TNF α cytokine enhances the proliferation of human endometrial glandular cells. *Obstet Gynecol Int* 2013; **2013**: 368543.
21 Rutledge AC, Adeli K. Fructose and the metabolic syndrome: pathophysiology and molecular mechanisms. *Nutr Rev* 2007; **65**: S13–S23.
22 Cai D, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J *et al.* Local and systemic insulin resistance resulting from hepatic activation of IKK-β and NF-κB. *Nat Med* 2005; **11**: 183–190.
23 Giudice LC. Endometrium in PCOS: implantation and predisposition to endocrine CA. *Best Pract Res Clin Endocrinol Metab* 2006; **20**: 235–244.
24 Quezada S, Avellaira C, Johnson MC, Gabler F, Fuentes A, Vega M. Evaluation of steroid receptors, coregulators, and molecules associated with uterine receptivity in secretory endometria from untreated women with polycystic ovary syndrome. *Fertil Steril* 2006; **85**: 1017–1026.
25 Matsuda M, DeFronzo RA. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. *Diabetes Care* 1999; **22**: 1462–1470.
26 McAuley K, Mann J, Chase J, Lotz T, Shaw G. Point: HOMA--satisfactory for the time being: HOMA: the best bet for the simple determination of insulin sensitivity, until something better comes along. *Diabetes Care* 2007; **30**: 2411–2413.
27 Noyes RN, Hertig AT, Rock J. Dating the endometrial biopsy. *Fertil Steril* 1950; **1**: 3–25.
28 Chen LF, Green WC. Shaping the nuclear action of NF-kB. *Nat Rev Mol Cell Biol* 2004; **5**: 392–401.
29 Krikun G, Mor G, Alvero A, Guller S, Schatz F, Sapi E *et al.* A novel immortalized human endometrial stromal cell line with normal progestational response. *Endocrinology* 2004; **145**: 2291–2296.
30 Falini B, Flenghi L, Pileri S, Gambacorta M, Bigerna B, Durkop H *et al.* PG-M1: A new monoclonal antibody directed against a fixative-resistant epitope on the macrophage-restricted form of the CD68 molecule. *Am J Pathol* 1993; **142**: 1359–1372.
31 Wang B, Jenkins JR, Trayhurn P. Expression and secretion of inflammation-related adipokines by human adipocytes differentiated in culture: integrated response to TNF-α. *Am J Physiol Endocrinol Metab* 2005; **288**: E731–E740.
32 Guerre-Millo M. Adiponectin: an update. *Diabetes Metab* 2008; **34**: 12–18.
33 Liu M, Liu F. Transcriptional and post-translational regulation of adiponectin. *Biochem J* 2009; **425**: 41–52.
34 Figueroa F, Motta A, Acosta M, Mohamed F, Oliveros L, Forneris M. Role of macrophage secretions on rat polycystic ovary: its effect on apoptosis. *Reproduction* 2015; **150**: 437–448.
35 wDuleba AJ, Dokras A. Is PCOS an inflammatory process? *Fertil Steril* 2012; **97**: 7–12.
36 Kelly CC, Lyall H, Petrie JR, Gould GW, Connell JM, Sattar N. Low grade chronic inflammation in women with polycystic ovarian syndrome. *J Clin Endocrinol Metab* 2001; **86**: 2453–2455.
37 Faulds G, Rydén M, Ek I, Wahrenberg H, Arner P. Mechanisms behind lipolytic catecholamine resistance of subcutaneous fat cells in the polycystic ovarian syndrome. *J Clin Endocrinol Metab* 2003; **88**: 2269–2273.
38 Haider S, Knöfler M. Human tumour necrosis factor: physiological and pathological roles in placenta and endometrium. *Placenta* 2010; **30**: 111–123.
39 Tabibzadeh S, Satyaswaroop PG, von Wolff M, Strowitzki T. Regulation of TNF-α mRNA expression in endometrial cells by TNF-α and by oestrogen withdrawal. *Mol Hum Reprod* 1999; **5**: 1141–1149.
40 Jakubowicz DJ, Seppälä M, Jakubowicz S, Rodriguez-Armas O, Rivas-Santiago A, Koistinen H *et al.* Insulin reduction with metformin increases luteal phase serum glycodelin and insulin-like growth factor-binding protein 1 concentrations and enhances uterine vascularity and blood flow in the polycystic ovary syndrome. *J Clin Endocrinol Metab* 2001; **86**: 1126–1133.
41 Yun JH, Choi JW, Lee KJ, Shin JS, Baek KH. The promoter -1031(T/C) polymorphism in tumor necrosis factor-alpha associated with polycystic ovary síndrome. *Reprod Biol Endocrinol* 2011; **9**: 131.
42 Peral B, San Millán JL, Castello R, Moghetti P, Escobar-Morreale HF. The methionine 196 arginine polymorphism in exon 6 of the TNF receptor 2 gene (TNFRSF1B) is associated with the polycystic ovary syndrome and hyperandrogenism. *J Clin Endocrinol Metab* 2002; **87**: 3977–3983.
43 Fernandez-Real JM, Broch M, Ricart W, Casamitjana R, Gutierrez C, Vendrell J *et al.* Plasma levels of the soluble fraction of tumor necrosis factor receptor 2 and insulin resistance. *Diabetes* 1998; **47**: 1757–1762.
44 Johansson A, Ahrén B, Forsberg H, Olsson T. Testosterone and diurnal rhythmicity of leptin, TNF-alpha and TNF-II receptor in insulin-resistant myotonic dystrophy patients. *Int J Obes Relat Metab Disord* 2002; **26**: 1386–1392.
45 Markel TA, Crisostomo PR, Wang M, Herring CM, Meldrum DR. Activation of individual tumor necrosis factor receptors differentially affects stem cell growth factor and cytokine production. *Am J Physiol Gastrointest Liver Physiol* 2007; **293**: G657–G662.
46 Micheau O, Tschopp J. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell* 2003; **114**: 181–190.
47 Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-κB activity. *Annu Rev Immunol* 2000; **18**: 621–663.
48 del Aguila LF, Claffey KP, Kirwan JP. TNF-alpha impairs insulin signaling and insulin stimulation of glucose uptake in C2C12 muscle cells. *Am J Physiol* 1999; **276**: E849–E855.
49 Massaro M, Scoditti E, Pellegrino M, Carluccio MA, Calabriso N, Wabitsch M *et al.* Therapeutic potential of the dual peroxisome proliferator activated receptor (PPAR)α/γ agonist aleglitazar in attenuating TNF-α-mediated inflammation and insulin resistance in human adipocytes. *Pharmacol Res* 2016; **107**: 125–136.
50 Hajri T, Tao H, Wattacheril J, Marks-Shulman P, Abumrad NN. Regulation of adiponectin production by insulin: interactions with tumor necrosis factor-α and interleukin-6. *Am J Physiol Endocrinol Metab* 2011; **300**: E350–E360.
51 Fulghesu AM, Sanna F, Uda S, Magnini R, Portoghese E, Batetta B. IL-6 serum levels and production is related to an altered immune response in polycystic ovary syndrome girls with insulin resistance. *Mediators Inflamm* 2011; ID 389317.
52 Escobar-Morreale HF, Luque-Ramírez M, González F. Circulating inflammatory markers in polycystic ovary syndrome: a systematic review and metaanalysis. *Fertil Steril* 2011; **95**: 1048–1058.