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Effect of TNF-α on Molecules Related to the Insulin Action in Endometrial Cells Exposed to Hyperandrogenic and Hyperinsulinic Conditions Characteristics of Polycystic Ovary Syndrome

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Abstract

Polycystic ovary syndrome (PCOS) affects not only ovarian functions but is also able to affect endometrium metabolism. Around 80% of women with PCOS are obese. High tumor necrosis factor (TNF)-α production and low adiponectin levels are characteristics of obesity. Interestingly, endometrium from obese women with PCOS presents an insulin-resistance condition, high TNF-α levels, and low adiponectin levels. However, TNF-α effect on molecules associated with insulin action in endometrial cells remains unclear. Therefore, the objective of this work was to evaluate TNF-α effect on expression of molecules associated with adiponectin (insulin sensitizing) and TNF-α signaling pathways and on Glucose Transporter type 4 (GLUT-4) levels in human endometrial cells under the characteristic conditions of hyperandrogenic/hyperinsulinic (HA/HI) PCOS. Two human endometrial stromal cell lines (T-HESC/St-T1b) under HA/HI conditions were used to assay the effect of high TNF-α concentration (100 ng/mL) on adiponectin, AdipoR1-AdipoR2 receptors, Adaptor protein phosphotyrosine interacting with PH domain and leucine zipper 1 (APPL1), Phospho-AMP-activated protein kinase T172 (p-AMPKT172), GLUT-4, Tumor necrosis factor receptor 1 (TNFR1)-Tumor necrosis factor receptor 2 (TNFR2) receptors protein levels, and nuclear factor κB (NFκB) nuclear content, by Western blot or immunocytochemistry. The NFκB participation in TNF-α effect on adiponectin expression was assayed using an NFκB inhibitor (pyrrolidine dithiocarbamate). The TNF-α increases the expression of molecules associated with its own signaling pathway ($P < .05$) and decreases the protein levels of adiponectin and its associated molecules ($P < .05$). Moreover, TNF-α increases NFκB nuclear content ($P < .001$), whereas with NFκB inhibition the decrease in adiponectin content induced by TNF-α was not observed. GLUT-4 levels were lower with TNF-α treatment ($P < .01$). Thus, in human endometrial stromal cells, high TNF-α levels negatively affect the insulin action through decreased adiponectin signaling and GLUT-4 protein. This could explain the failures observed in endometrial function of obese women with PCOS.

Keywords

endometrial cells, TNF-α, adiponectin, insulin, GLUT-4

Introduction

Polycystic ovarian syndrome (PCOS) is an endocrine-metabolic disorder characterized by the presence of hyperandrogenism accompanied by ovarian dysfunction, which is manifested by oligo-anovulation and/or polycystic ovaries detected by ultrasonography.[1]

Based on these diagnostic criteria, PCOS appears to be a very common pathology that affects between 5% and 20% of women of reproductive age.[2] These women experience three times more spontaneous abortions than women without PCOS.[3] In this regard, it is known that the endometrium from women with PCOS presents metabolic alterations associated with failures in

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insulin signaling, which may be part of the reason why these patients experience reproductive failures.[4] On this subject, our laboratory has demonstrated that lower levels of molecules related to insulin signaling, GLUT-4 translocation to the cell membrane, and to glucose uptake (including Phospho-Insulin Receptor Substrate 1 (p-IRS1), Phospho-Akt substrate of 160 kDa (p-AS160), and Glucose Transporter type 4 (GLUT-4) are found on endometria from women with PCOS with a high body mass index (BMI) compared to normal women.[5-7]

Another important consideration is that about 70% to 80% of patients with PCOS have some degree of obesity,[8,9] which is accompanied by functional defects on adipose tissue. These defects can be associated with the presence of a moderate but chronic inflammatory environment.[10] In addition, several studies indicate that PCOS induces a proinflammatory environment in a manner that is independent of the obesity condition.[10,11] Indeed, previous studies from our group have demonstrated that the expression of obesity marker molecules, such as tumor necrosis factor TNF-α and adiponectin, is altered in endometrial tissues from obese women with PCOS compared to obese women without the syndrome.[11,12] Significantly, the expression of TNF-α and of molecules related to its signaling pathway is elevated in the endometrial tissue from obese women with PCOS,[12] while the expression of adiponectin and its pathway molecules decreases in these tissues. This situation is also observed in an in vitro cell model of human endometrial stromal cell (HESC) cultures (T-HESC and St-T1b lines) subjected to hyperandrogenic and hyperinsulinic (HA/HI) conditions, emulating PCOS features.[11] Moreover, in this cell model HA/HI conditions increase TNF-α levels, which in turn reduce glucose uptake by these human endometrial cells.[12]

It is known that TNF-α is one of the main cytokines present in proinflammatory processes associated with obesity.[13] It is also a known negative regulator of insulin signaling, which has been verified in both animal models and in vitro assays, including insulin-resistance models created through the use of adipose cells.[14-17] On the other hand, adiponectin levels decrease in an obese condition, which is related to failures in insulin action; therefore, it can be stated that adiponectin has an important role as an insulin-sensitizing molecule.[18]

Interestingly, studies in adipose cells have shown that TNF-α decreases the expression and secretion of adiponectin,[19,20] while adiponectin may affect the proinflammatory and insulin-suppressive actions induced by TNF-α.[18,21] However, it is not known whether there is a relationship between a proinflammatory environment characterized by high levels of TNF-α and higher presence of macrophages,[10,12] with lower levels of adiponectin in the endometrium from obese women with PCOS.[11]

Therefore, in this work, through the use of an in vitro cell model consisting of HESCs under PCOS conditions, we evaluated whether high concentrations of TNF-α alter the expression of adiponectin- and insulin-related pathways. These alterations, which are associated with endometrial metabolism failures, could eventually lead to the reproductive failures observed in women with PCOS.

Materials and Methods

Cell Culture and Treatments

Telomerase-immortalized HESC lines T-HESC (ATCC, CRL-4003)[22] and St-T1b,[23] were cultured in DMEM/Ham F12 medium (Sigma Aldrich Co, St Louis, Missouri) with 10% fetal bovine serum treated with dextran carbon (Hyclone; Thermoscientific, New York), and 1× of antimycotic/antibiotic, at 37°C in a 5% CO_2 atmosphere until 80% confluence. Both cell lines were obtained from primary cultures of HESCs, which were immortalized with telomerase gene, as mentioned. The ESCs were obtained from endometrial biopsies of premenopausal women.[24,25] Cells (T-HESCs and St-T1b) were cultured directly on 4-well slides (Thermoscientific Nunc Lab-Tek II Chamber Slide System; ThermoFisher Scientific; 60 000 cells/well) or in 6-well plates (160 000 cells/well) in growth media for 24 hours at 37°C in 5% CO_2/atmosphere for immunocytochemistry (ICC) or Western blot (WB), respectively. Then, cultures were washed twice with sterile Dulbecco phosphate-buffered saline (PBS; GIBCO; Invitrogen Corporation, Camarillo, California). Cultures were further subjected to testosterone plus insulin treatment (100 nM each = HA/HI conditions) for 24 hours in serum-free medium. Testosterone and insulin concentrations were determined in previous studies from our laboratory.[5,6,26] Moreover, some cell cultures were also costimulated with insulin and testosterone (100 nM each) and TNF-α (100 ng/mL) or with testosterone, insulin, or TNF-α alone for 24 hours to determine TNF-α effect on protein expression. Basal condition corresponds to cultures with no hormonal stimulation. Since the behaviors are similar under all our experimental conditions, both lines of HESCs were used indistinctly.[11] Three independent experiments were performed for each treatment (n = 3; in duplicate). All experimental conditions and treatments were previously used in Oróstica et al.[12]

Pyrrolidine Dithiocarbamate Treatment in St-T1b

To determine the participation of NFκB in TNF-α action on adiponectin expression, St-T1b cells were treated with an inhibitor of NFκB[27] activation denominated pyrrolidine dithiocarbamate (PDTC). A dose–response curve was created with 3 doses of the compound (10, 50, and 100 μM) for 1 hour (data not show), and 50 μM of PDTC was used in the following experiments. St-T1b cultures were stimulated with TNF-α (100 ng/mL) for 24 hours to determine the adiponectin levels or the cells were pretreated with 50 μM PDTC for 1 hour before the addition of the different stimuli for 24 hours: TNF-α (100 ng/mL), insulin (100 nM), and testosterone (100 nM), or costimuli with the three molecules. The adiponectin levels were determined by ICC.

Immunocytochemistry

Briefly, T-HESC and St-T1b cells were fixed with a solution containing 4% paraformaldehyde in PBS for 15 minutes at room temperature. The endogenous peroxidase activity was inhibited by incubation in 3% H_2O_2 (15 minutes) and

nonspecific binding was blocked with PBS-bovine serum albumin (BSA) 2% (10 minutes). Cells were incubated with the primary antibody overnight at 4°C: NFκB p65 subunit antibody (Rabbit Monoclonal; Cell Signaling #8242, Danvers, MA, USA; dilution 1/300), adiponectin antibody (Rabbit Monoclonal; Novus Biologicals NBP1-40607, Littleton, CO, USA; dilution 1/100), and GLUT-4 antibody (Rabbit Polyclonal; Sta. Cruz sc-7938 Biotechnology, Dallas, TX, USA; dilution 1/50). Then, cells were washed with PBS 1× solution 3 times and incubated with a horseradish peroxidase (HRP)-labeled secondary antibody to rabbit or mouse immunoglobulin G (IgG; KPL, Gaithersburg, MD, USA; dilution 1/300) for 2 hours at 37°C. Chromogenic mark was revealed using 3,3′-diaminobenzidine as substrate and counterstaining was performed with hematoxylin for 5 seconds. The analysis was performed by the measurement of positive pixel intensity with the use of the semiquantitative analysis tool-integrated optical density (IOD) of the Image-Pro Plus 6.2 software. The data are presented as IOD arbitrary units (AU). In the case of NFκB, the results were expressed as percentage of positive nuclei per well. The results were expressed as the mean (standard error of the mean [SEM]) per studied group.

Western Blot

Protein extraction was obtained from HESC or St-T1b cultures treated with the different stimuli for 24 hours using 100 μL radioimmunoprecipitation assay buffer per well (50 mM Tris-Base, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X100, and 0.1% sodium dodecyl sulfate). Also, 1× protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Rochester, New York) was added. Samples were centrifuged at 10 000*g* for 20 minutes at 4°C. The resulting supernatant was used to determine protein concentration with the bicinchoninic acid protein assay kit (Thermo Fisher Scientific). Total proteins (50 μg) were denatured and fractionated using 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels. The protein content was transferred to a nitrocellulose membrane and protein transference was confirmed by Ponceau S staining. The membranes were blocked for 1 hour in Tris-buffered saline with Tween 20 (TTBS) (20 mM Tris, pH 7.5; 137 mM NaCl; 0.1% Tween 20) containing 5% nonfat dry milk or 5% BSA for phosphorylated proteins. The membranes were washed twice for 5 minutes with TTBS and incubated overnight at 4°C with antibodies against TNFR1 (Rabbit Monoclonal; Cell Signaling #3736; dilution 1/500), TNFR2 (Rabbit Polyclonal; Cell Signaling #3727; dilution 1/500), NFκB p65 subunit (Rabbit Monoclonal; Cell Signaling #8242; dilution 1/500), adiponectin (Rabbit Monoclonal; Novus Biologicals NBP1-40607; dilution 1/300), adiponectin receptor 1, AdipoR1 (Rabbit Polyclonal; Alpha Diagnostic ADIPOR12-A; dilution 1/600), adiponectin receptor 2, AdipoR2 (Rabbit Polyclonal; Alpha Diagnostic ADIPOR22-A; dilution 1/600), APPL1 (Rabbit Monoclonal; Cell Signaling #3858; dilution 1/500), AMPKα (Rabbit Polyclonal; Cell Signaling #2532; dilution 1/500), and p-AMPKα T172 (Mouse Monoclonal; Cell Signaling #2535; dilution 1/500). β-Actin antibody (Mouse Monoclonal; Sigma-Aldrich A5441; 1/10 000) was incubated for 1 hour at room temperature. Then the blots were washed with PBS 1× solution 3 times and incubated for 1 hour at room temperature with an HRP-labeled secondary antibody to rabbit or mouse IgG (KPL, Maryland; dilution 1/5000). After antibody incubation, the membranes were washed 3 times for 5 minutes each with TTBS; the bound antibodies were detected with an enhanced chemiluminescence substrate, Western Lightning Plus-ECL (Perkin-Elmer, Waltham, MA, USA). Band intensities were quantified by scanning densitometry utilizing the UN-SCAN-IT software, version 5.1; Automated Digitizing System. The data correspond to AU. The results were expressed as the ratio of the housekeeping gene β-actin or the ratio between phosphorylated proteins and total protein levels.

Statistics Analysis

All data showed a nonparametric distribution and were analyzed by Kruskal-Wallis test. For multiple comparisons, Dunn posttest was used. P values <.05 were considered significant. Statistical tests were performed using Graph Pad Prism 5.0. All results correspond to mean of each condition (standard error).

Results

Effect of TNF-α on TNFR1 and TNFR2 Protein Levels in HESCs Under HA/HI Conditions

Previously, our group reported that endometria from obese women with PCOS show a significant increase in TNF-α levels and molecules related to its signaling pathway.[12] Considering this, it was assessed whether TNF-α treatment induces the expression of its own receptors in cultured HESCs after stimulation with high concentrations of testosterone and insulin. Testosterone and insulin were used in order to emulate the HA/HI endometrial environment characteristic of PCOS.

The WB analysis shows that TNFR1 and TNFR2 protein levels increase in the presence of TNF-α, either alone (1.6 ± 0.045 AU TNFR1; 1.43 ± 0.089 AU TNFR2) or with costimulation with insulin/testosterone/TNF-α (1.6 ± 0.051 AU TNFR1; 1.40 ± 0.077 AU TNFR2) versus basal condition (1.00 ± 0.002 AU; $P < .05$; Figure 1). A similar behavior is observed for NFκB protein levels under the same conditions (1.46 ± 0.043 AU in TNF-α alone or 1.35 ± 0.043 AU in insulin/testosterone/TNF-α; $P < .05$; Figure 1).

Effect of TNF-α on NFκB Translocation to the Nucleus in HESCs Under HA/HI Conditions

In order to determine whether TNF-α induces activation and subsequent translocation of NFκB to the nucleus, p65 nuclear content in endometrial cells was evaluated by ICC. Figure 2 shows that treatment with TNF-α alone (73.7% ± 11.1%) or TNF-α with insulin and testosterone (44% ± 11.2%)

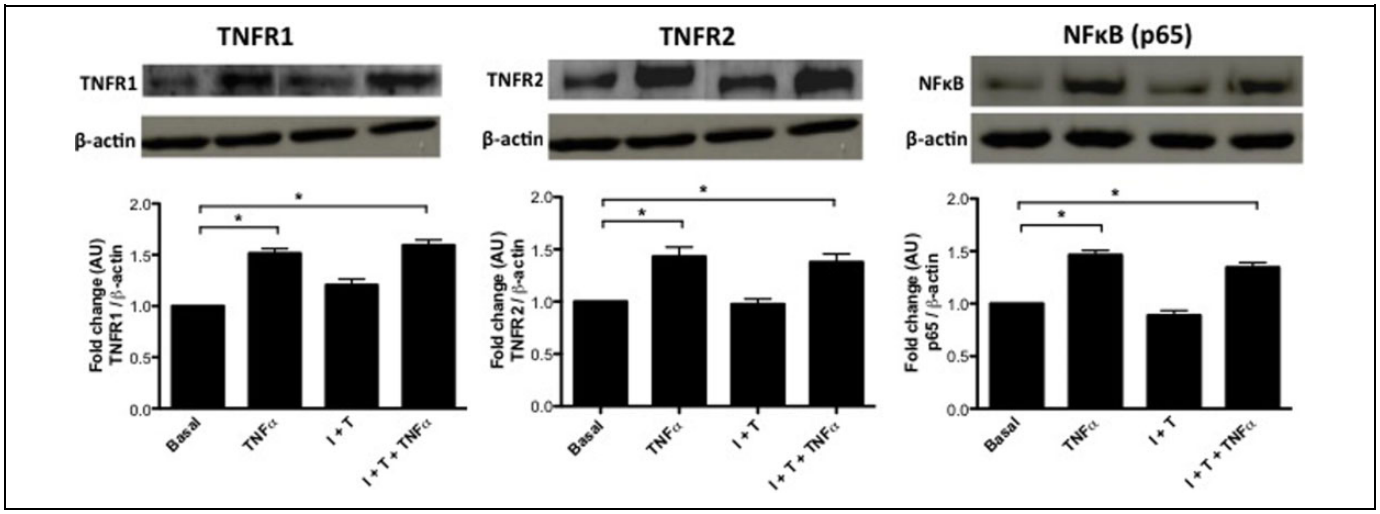

Figure 1. Protein levels of TNFR1, TNFR2, and NFκB in human endometrial stromal cells. The graphs show the levels of TNF-α 1 receptor (55 KDa band), TNF-α receptor 2 (75 KDa band), and subunit p65 of NFκB in T-HESC cultures treated with 100 ng/mL TNF-α, 100 nM insulin (I), 100 nM testosterone (T), or combined stimuli for 24 hours by WB. The data were normalized using the protein levels of β-actin (42 kDa) in each sample and analyzed as change in time with respect to baseline. The results are presented as arbitrary units (AU) and correspond to the mean (SE) of each group (n = 3 in duplicate, *P value < .05). TNF-α indicates tumor necrosis factor α; NFκB, nuclear factor κB; HESC, human endometrial stromal cell line; WB, Western blot; SE, standard error.

significantly increases the percentage of cells with positive nuclei compared to the basal condition (2.8% ± 0.1%) or to insulin/testosterone treatment (3.4% ± 0.4%; $P < .001$). Thus, these results are consistent with those observed in Figure 1, indicating that TNF-α is able to increase the expression/activation of molecules associated with its own signaling pathway in this cell model.

Effect of TNF-α on Adiponectin Levels and Molecules Associated With Its Signaling Pathway in HESCs Under HA/HI Conditions

Since a previous report from our group showed that HESCs have a reduced ability to uptake glucose in the presence of TNF-α or TNF-α/insulin/testosterone,[12] the effect of this cytokine on the expression of insulin-sensitizer molecules, such as adiponectin, was determined. With this aim, adiponectin, AdipoR1, AdipoR2, APPL1, and activated AMPK (p-AMPK T172) protein levels were determined by WB. The data show (Figure 3) that after 24 hours of cotreatment with insulin and testosterone, adiponectin (0.69 ± 0.1 vs 1.23 ± 0.2 AU), AdipoR1 (0.97 ± 0.2 vs 3.0 ± 1.0 AU), and AdipoR2 (0.67 ± 0.1 vs 1.28 ± 0.2 AU) protein content decreases compared to the untreated condition ($P < .05$). Regarding TNF-α effect on the expression of these molecules, a significant decrease in adiponectin (0.69 ± 0.07 vs 1.23 ± 0.2 AU), AdipoR1 (1.0 ± 0.2 vs 3.0 ± 1.0 AU), and AdipoR2 (0.62 ± 0.2 vs 1.28 ± 0.2 AU) protein levels was observed ($P < .05$). Interestingly, after costimulation with insulin, testosterone, and TNF-α, AdipoR1 protein levels were lower than after stimulation with TNF-α alone or insulin/testosterone (83% less vs 65% less; Figure 3). Thus, when cells are treated with the three stimuli, their inhibitory effects on the adiponectin pathway could be exacerbated.

Additionally, a similar result was obtained for APPL1 protein (Figure 4); in fact, when cells were stimulated with TNF-α (0.34 ± 0.07 vs 1.68 ± 0.18 AU) or insulin plus testosterone (0.61 ± 0.1 vs 1.68 ± 0.18 AU), a decrease in APPL1 content was observed, similar to the results obtained with the cotreatment of insulin/testosterone/TNF-α (0.32 ± 0.04 vs 1.68 ± 0.18 AU; $P < .05$). The APPL1 levels were 40% lower in the presence of TNF-α alone compared to insulin plus testosterone (Figure 4); however, this decrease was not statistically significant. Therefore, the different treatments used may partially explain why the adiponectin pathway is altered in patients with PCOS, which in turn negatively affects the action of insulin in endometrial cells. Importantly, only treatment with the three stimuli could induce a slight decrease in p-AMPK T172 (0.54 ± 0.08 vs 0.75 ± 0.05 AU; $P < .05$; Figure 4), indicating that TNF-α alone is not sufficient to alter AMPK activation.

Participation of NFκB in TNF-α Effect on Adiponectin Levels in HESCs Under HA/HI Conditions

To further evaluate NFκB participation in the decrease of adiponectin levels after TNF-α stimulation, an inhibitor of NFκB (PDTC) was used. For this purpose, HESCs were treated with insulin, testosterone, and/or TNF-α for 24 hours. Afterward, adiponectin levels were determined by ICC.

The results show that TNF-α (52 681 ± 8993 vs 10 266 ± 10 640 IOD AU) or insulin/testosterone/TNF-α (52 199 ± 4108 vs 136 905 ± 10 640 IOD AU) treatment decreases adiponectin protein levels in HESCs ($P < .05$; Figure 5). This

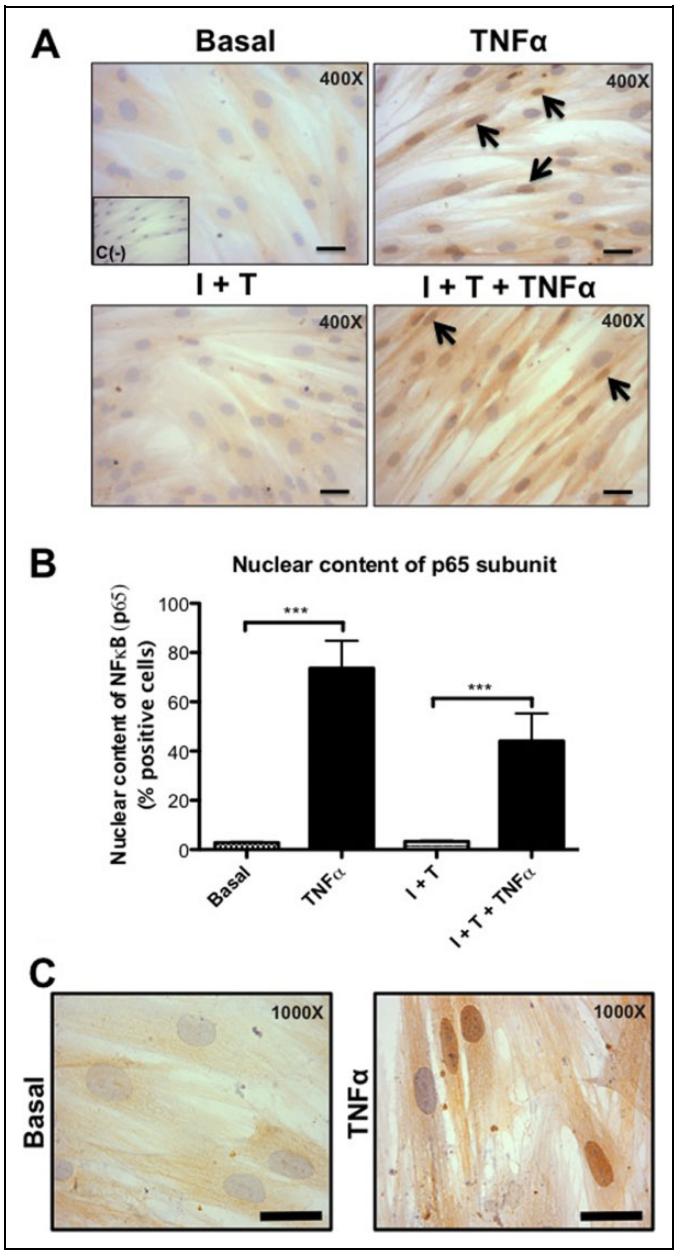

Figure 2. Subcellular localization of the p65 subunit of NFκB in human endometrial stromal cells. A, Micrographs of T-HESC cultures treated with 100 ng/mL of TNF-α, 100 nM insulin (I), 100 nM testosterone (T), or combined 24-hour stimulation for nuclear p65 detection by ICC. Arrows indicate positive nuclei for p65. B, Semiquantitative analysis of the immunopositive staining using the IOD tool. The values are presented as arbitrary units (AU) and correspond to the mean (SE) of each group (n = 3 in duplicate, ***P value < .001; inset: negative control; bar = 50 μm). C, Amplified microphotographs showing the nuclear localization of p65 in cells under a basal condition or treated with TNF-α (amplification: 1000×; bar = 50 μm). NFκB indicates nuclear factor κB; HESC, human endometrial stromal cell line; TNF-α, tumor necrosis factor α; ICC, immunocytochemistry; IOD, integrated optical density; SE, standard error.

result is similar to the one observed in Figure 3. Interestingly, pretreatment with PDTC completely reversed this decrease in adiponectin levels, whose levels remained similar to basal condition (156 056 ± 8993 vs 136 905 ± 10 640 IOD AU; Figure 5). Hence, it is highly possible that TNF-α inhibits the expression of adiponectin through NFκB activation, an effect that can also be observed under HA/HI conditions. These results could partly explain the mechanism by which TNF-α alters insulin action on its target cell.

Effect of TNF-α on GLUT-4 Levels in HESCs Under HA/HI Conditions

In previous studies from our laboratory, lower GLUT-4 and higher TNF-α levels were observed in endometria from hyperinsulinic and obese women with PCOS[7,12] compared to normal women; therefore, the effect of TNF-α on GLUT-4 levels in cells exposed to HA/HI conditions was determined (Figure 6). Results show that TNF-α decreases the expression of GLUT-4 in HESCs (41 617 ± 1334 vs 62 574 ± 3879 IOD AU; $P < .01$) independently of HA/HI conditions (45 513 ± 5183 vs 62 574 ± 3879 IOD AU; $P < .05$).

Discussion

The PCOS, which is characterized by high levels of androgens and ovarian dysfunction, among other signs, is a very prevalent disorder during the female reproductive age.[1-3] The alterations experienced by women with PCOS affect not only ovarian function but also the function of other tissues of reproductive importance, such as the endometrium. In fact, it has been shown that patients with PCOS have a higher rate of spontaneous abortions compared to healthy women.[26,27] Considering that a high percentage of women with PCOS are obese, adverse effects related to obesity could explain, at least in part, the alterations that our group has observed on insulin signaling in the endometrium from women with both PCOS and high BMI.[4]

In this regard, the results of the present study show that HESCs (under HA/HI conditions or not) respond to TNF-α action by increasing the expression of both TNF-α receptors and the protein content of NFκB, specifically the p65 subunit. Moreover, TNF-α induces a greater presence of p65 in the nucleus of these cells, indicating a greater cytokine-induced activation of NFκB. Considering the above, it can be suggested that in endometrial cells, independent of the HA/HI conditions, the presence of TNF-α favors the activation of its own signaling pathway. These results are in agreement with those found in the endometrium from obese women with PCOS, since higher TNF-α and TNFR2 levels are found on these women.[12] The above indicates that PCOS can induce a proinflammatory environment per se,[10] altering the expression of proinflammatory molecules, which generates a vicious circle that perpetuates chronic alterations in the endometrial tissue. Interestingly, TNFR2 is more related to proliferative and proinflammatory process than to apoptosis,[28] which is concordant to the fact that women with PCOS have a higher risk of developing endometrial hyperplasia and endometrial adenoma or carcinoma.[29,30]

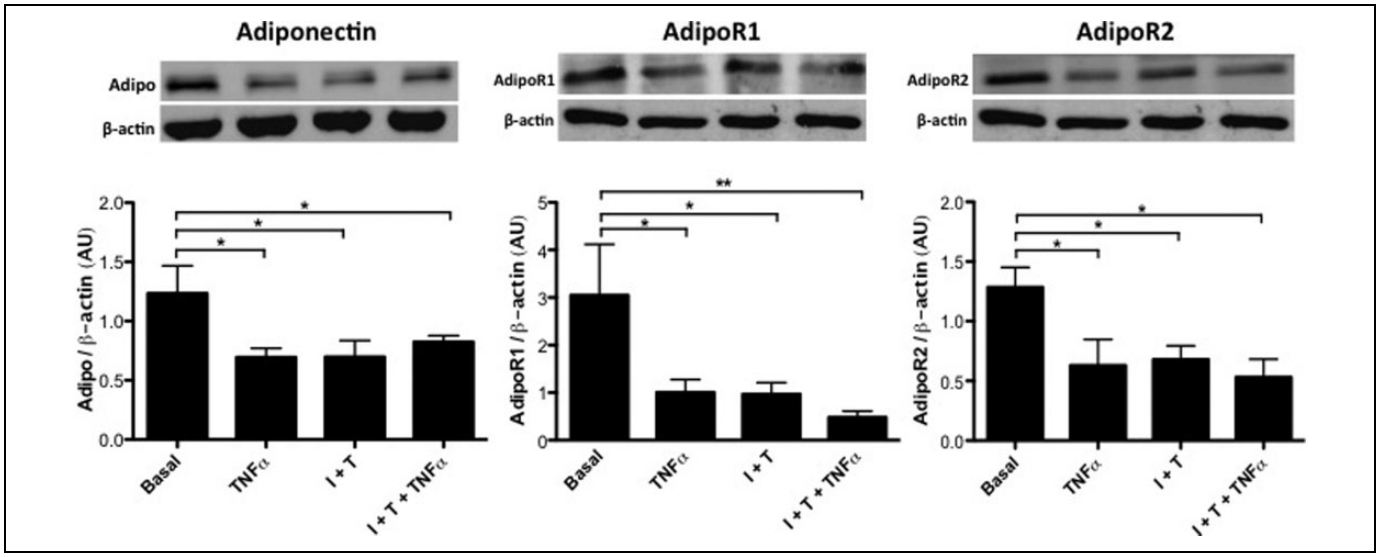

Figure 3. Protein levels of adiponectin, AdipoR1, and AdipoR2 in human endometrial stromal cells. The graphs show the levels of adiponectin (56 kDa band), AdipoR1 (42 kDa), and AdipoR2 (42 kDa band) in T-HESC cultures treated with 100 ng/mL TNF-α, 100 nM insulin (I), 100 nM testosterone (T), or combined stimuli for 24 hours by WB. The data were normalized using the protein levels of β-actin (42 kDa) and are expressed as the mean ± SEM of each group (n = 3 in duplicate, **P* value < .05; ***P* value < .01). HESC indicates human endometrial stromal cell line; TNF-α, tumor necrosis factor α; WB, Western blot; SEM, standard error of mean.

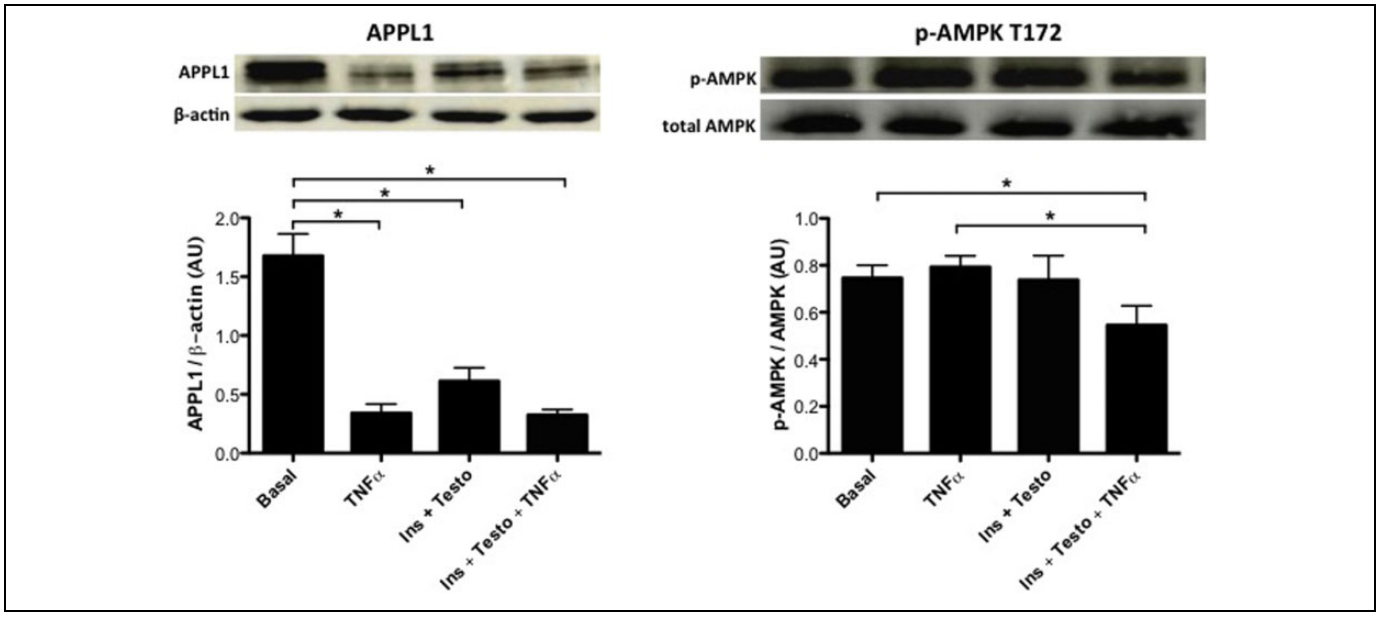

Figure 4. Protein levels of APPL1 and p-AMPK T172 in human endometrial stromal cells. The graphs show the levels of APPL1 (82 kDa band) and p-AMPK T172 (62 kDa band) in T-HESC cultures treated with 100 ng/mL TNF-α, 100 nM insulin (I), 100 nM testosterone (T), or combined stimuli for 24 hours by WB. The data were normalized using the protein levels of β-actin (42 kDa) in each sample for APPL1 or total AMPK levels (62 kDa) for p-AMPK. The results are presented as arbitrary units (AU) and correspond to the mean (SEM) of each group (n = 3 in duplicate, **P* value < .05). HESC indicates human endometrial stromal cell line; TNF-α, tumor necrosis factor α; WB, Western blot; SEM, standard error of mean.

Consequently, the failures in insulin signaling observed in endometrium from obese women with PCOS may be the result of an increase in TNF-α activity, which may negatively affect insulin action through different mechanisms. One of these mechanisms is the increase in interleukin (IL)-6 expression via NFκB activation,[19,20,31] since this proinflammatory cytokine is closely related to insulin resistance and increased serum levels have been detected in obese women with PCOS.[32,33] Interestingly, IL-6 is able to decrease messenger RNA (mRNA) levels and adiponectin secretion in preadipocyte cultures, 3T3L1

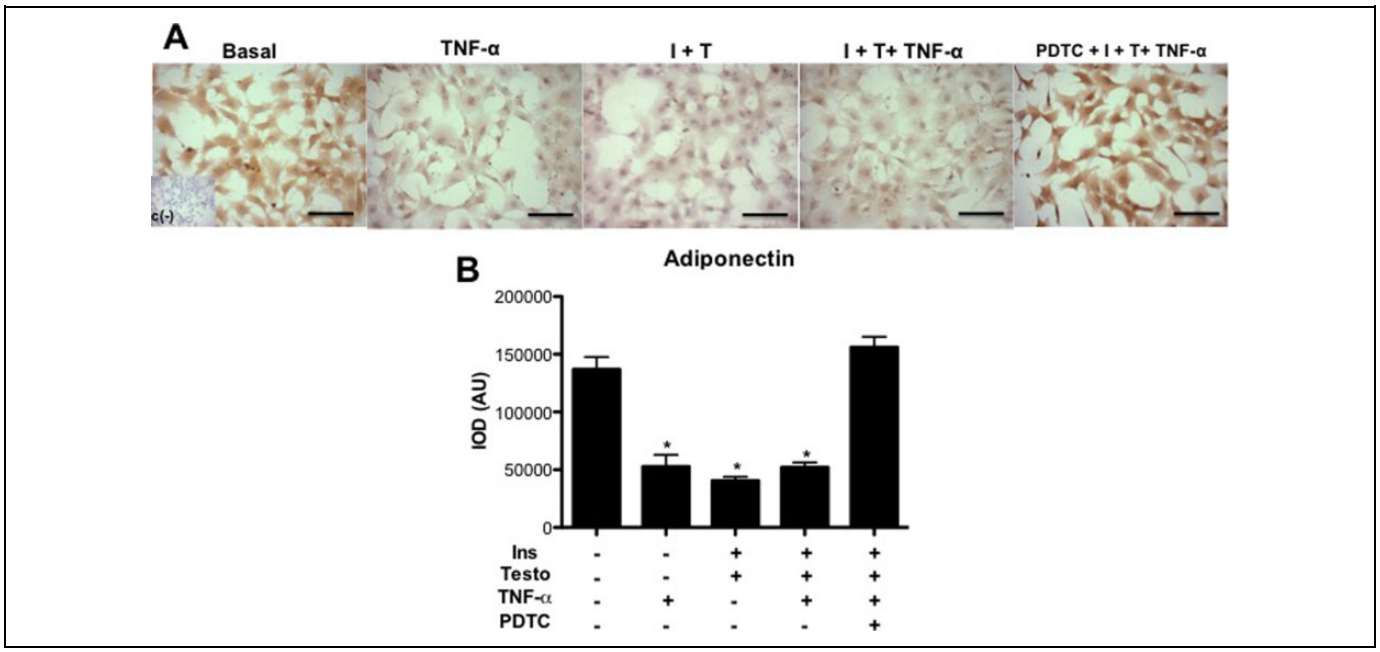

Figure 5. Effect of TNF-α and NFκB activation on adiponectin levels in human endometrial stromal cells pretreated with PDTC. A, Microphotographs of St-T1b cells previously treated with 50 μM of PDCT for 1 hour and then treated for 24 hours with insulin + testosterone (100 nM each) + TNF-α (100 ng/mL) for adiponectin detection by ICC. B, Semiquantitative analysis of the immunopositive staining using the IOD tool. The values are presented as arbitrary units (AU) and correspond to the mean (SEM) of each group (n = 3 in duplicate; **P* value <.05; inset: negative control; bar = 50 μm). TNF-α indicates tumor necrosis factor α; NFκB, nuclear factor κB; HESC, human endometrial stromal cell line; ICC, immunocytochemistry; IOD, integrated optical density; SEM, standard error of mean.

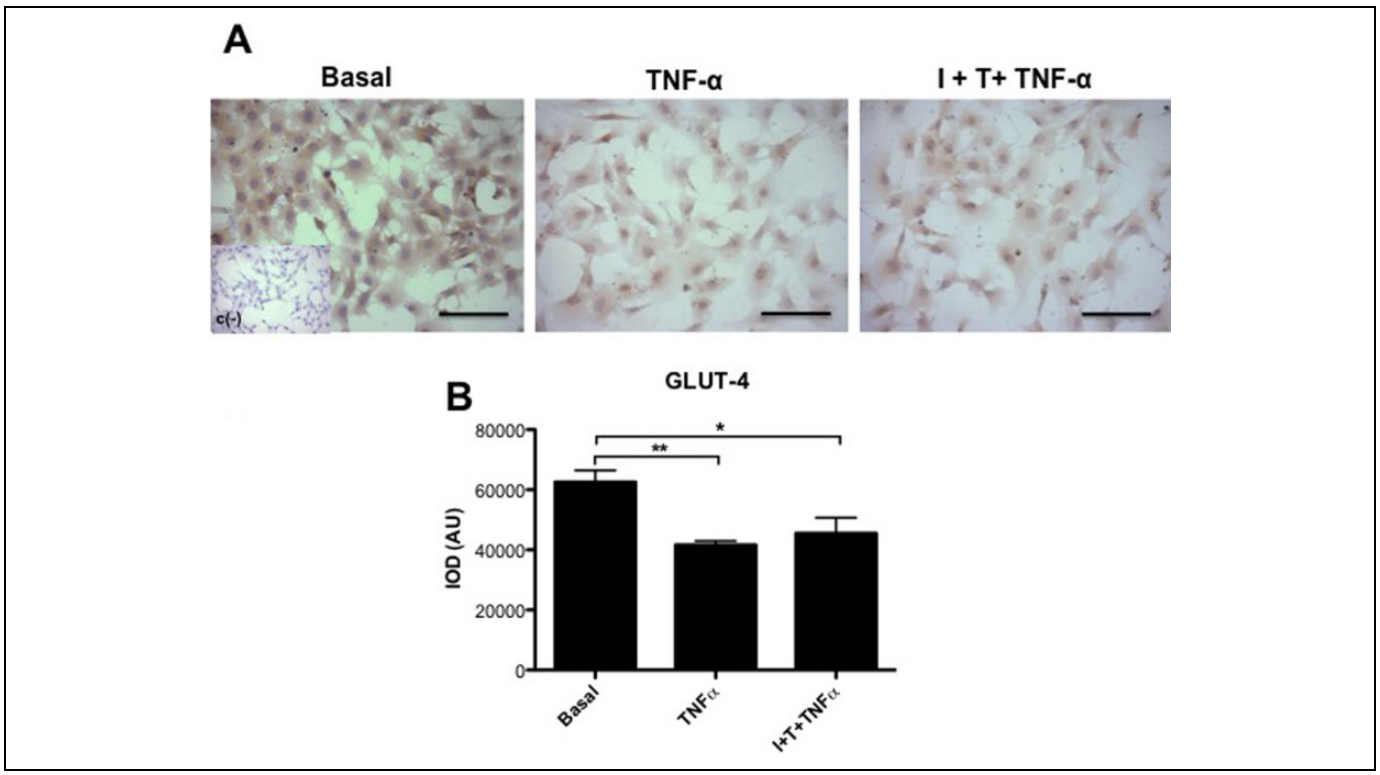

Figure 6. Effect of TNF-α on GLUT-4 levels in human endometrial stromal cells. A, Microphotographs of St-T1b cells treated with insulin + testosterone (I + T, 100 nM each) or insulin + testosterone + TNF-α (100 ng / mL) for 24 hours for the GLUT-4 detection by ICC. B, Semiquantitative analysis of the immunopositive staining using the IOD tool. The values are presented as arbitrary units (AU) and correspond to the mean (SEM) of each group (n = 3 in duplicate; **P* value <.05; inset: negative control; bar = 50 μm). TNF-α indicates tumor necrosis factor α; ICC, immunocytochemistry; IOD, integrated optical density; SEM, standard error of mean; GLUT-4, glucose transporter type 4.

cells.[34,35] This strongly supports the hypothesis that IL-6 is involved in insulin failures in these cells; however, this has not yet been evaluated in the endometrial cell model.

The present work shows for the first time that in human endometrial cells exposed to HA/HI conditions, TNF-α not only decreases adiponectin expression but also decreases protein levels of molecules related to the adiponectin signaling pathway, including the 2 types of adiponectin receptors (AdipoR1 and AdipoR2) and APPL1 (the main adapter molecule of adiponectin signaling pathway).[36] Several studies indicate that the increase or decrease in the total protein content of APPL1 in adiponectin target cells is sufficient to generate alterations in adiponectin effects.[18,36,37] Accordingly, TNF-α could decrease APPL1 expression or alter APPL1 activity by regulating the expression of molecules with the ability to inhibit APPL1 effect, such as APPL2. As reported elsewhere, the APPL2 isoform has opposite effects to APPL1 regarding the insulin-sensitizing effects of adiponectin.[38,39]

Furthermore, in the presence of the three stimuli, a significant decrease in p-AMPK, a key molecule in the regulation of cell energy homeostasis, was observed. The decrease in AMPK activation by TNF-α could be related to this cytokine capacity, activating the mTOR/S6K system, which in turn inhibits IRS1 activation,[17] among other effects. In contrast, AMPK activation favors insulin action and cellular metabolism by inhibiting mTOR/S6K activation and, therefore, it decreases the risk of developing insulin resistance or diabetes.[40] This result is consistent with the findings of Carvajal et al, in which they found lower levels of p-AMPK T172 in endometrium from hyperinsulinemic/obese women with PCOS compared to normal women.[7] Thus, in endometrial tissues and ESC cultures, the increase in TNF-α could result in a significant decrease in insulin action.

Another aim of this work was to evaluate the participation of NFκB on TNF-α effect on adiponectin expression in endometrial cells under HA/HI conditions. It is known that TNF-α induces kinase activation, including JNK activation, which can not only affect IRS1 activation[18,41] but also inhibit the positive action of peroxisome proliferator–activated receptor-γ (PPARγ) on the increase of adiponectin expression in adipose cells.[34] Involvement of NFκB in this TNF-α action remains unclear; however, it has been suggested that NFκB activation could affect the action of kinases related to the activity of the adiponectin gene transcriptional corepressors.

Lastly, another mechanism by which TNF-α could alter insulin action is through a decrease in GLUT-4 expression. This effect could partially explain results previously found on human samples and the in vitro model: lower GLUT-4 levels in the endometrium from women with PCOS with high BMI[7] and diminished glucose uptake on endometrial cells exposed to high TNF-α concentrations.[12] Also, since AMPK activation participates in a metformin-induced pathway to increase GLUT-4 expression, this result could be related to the lower p-AMPK levels observed in cells treated with TNF-α and the lower p-AMPK levels found in endometrium from hyperinsulinemic/obese women with PCOS.[7] In fact, the decrease in GLUT-4 levels in these tissues can be significantly restored with metformin treatment.[7] Interestingly, this result is concordant with those found in the endometrium from obese women with PCOS, where alterations in the FOXO1/PPARγ system (regulator system of GLUT-4 expression) and lower protein and mRNA levels of GLUT-4 were observed.[42]

An important aspect of this work is that the cell model used, despite being an in vitro model, allows for the discrimination between several signals present in local environment in endometrium from obese woman with PCOS that can affect insulin action in these tissues.[12] The results suggest that independent of obesity or HA/HI conditions, high levels of proinflammatory cytokines, such as TNF-α, may be affecting insulin action in the endometrium. This is an important aspect that could be considered in the clinical practice. The secondary outcome of this work was understood partially as the mechanism by which TNF-α induces negative effects on insulin action in endometrial cells.

In conclusion, the results shown in the present work indicate that the higher TNF-α levels found in endometrial cells could potentially induce several alterations: it alters the expression of molecules associated with the adiponectin pathway, it increases the expression of molecules related to its own signaling pathway, and it lowers GLUT-4 levels in these cells. All of the above are in agreement with the greater proinflammatory environment found in endometrial tissue from obese women with PCOS and the concomitant increase in local TNF-α in these tissues.[12] These disturbances could result in a decrease in the insulin action, altering the normal function of this tissue and possibly being a major cause of reproductive failure in these women.

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References

1. Azziz R, Carmina E, Dewailly D, 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(11):4237-4245.
2. Azziz R, Carmina E, Chen Z, et al. Polycystic ovary syndrome. *Nat Rev Dis Primers*. 2016;2:16057.

3. Goodarzi MO, Dumesic DA, Chazenbalk G, Azziz R. Polycystic ovary syndrome: etiology, pathogenesis and diagnosis. *Nat Rev Endocrinol*. 2011;7(4):219-231.
4. Orostica L, Rosas C, Plaza-Parrochia F, et al. Altered steroid metabolism and insulin signaling in PCOS endometria: impact in tissue function. *Curr Pharm Des*. 2016;22(36):5614-5624.
5. Rosas C, Gabler F, Vantman D, Romero C, Vega M. Levels of rabs and WAVE family proteins associated with translocation of GLUT4 to the cell surface in endometria from hyperinsulinemic PCOS women. *Hum Reprod*. 2010;25(11):2870-2877.
6. Fornes R, Ormazabal P, Rosas C, et al. Changes in the expression of insulin signaling pathway molecules in endometria from polycystic ovary syndrome women with or without hyperinsulinemia. *Mol Med*. 2010;16(3-4):129-136.
7. Carvajal R, Rosas C, Kohan K, 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(8): 2235-2244.
8. Barber TM, McCarthy MI, Wass JA, Franks S. Obesity and polycystic ovary syndrome. *Clin Endocrinol (Oxf)*. 2006;65(2): 137-145.
9. Barber TM, Dimitriadis GK, Andreou A, Franks S. Polycystic ovary syndrome: insight into pathogenesis and a common association with insulin resistance. *Clin Med (Lond)*. 2016;16(3): 262-266.
10. Duleba AJ, Dokras A. Is PCOS an inflammatory process? *Fertil Steril*. 2012;97(1):7-12.
11. Garcia V, Orostica L, Poblete C, Rosas C, Astorga I, Romero C, et al. Endometria from obese PCOS women with hyperinsulinemia exhibit altered adiponectin signaling. *Hor Metab Res*. 2015; 47(12):901-909.
12. Oróstica L, Astorga I, Plaza-Parrochia F, et al. Proinflammatory environment and role of TNF-alpha in endometrial function of obese women having polycystic ovarian syndrome. *Int J Obes (Lond)*. 2016;40(11):1715-1722.
13. Vilcek J, Lee TH. Tumor necrosis factor. New insights into the molecular mechanisms of its multiple actions. *J Biol Chem*. 1991; 266(12):7313-7316.
14. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science*. 1993;259(5091):87-91.
15. Hotamisligil GS, Murray DL, Choy LN, Spiegelman BM. Tumor necrosis factor alpha inhibits signaling from the insulin receptor. *Proc Natl Acad Sci U S A*. 1994;91(11): 4854-4858.
16. Hauner H, Petruschke T, Russ M, Rohrig K, Eckel J. Effects of tumour necrosis factor alpha (TNF alpha) on glucose transport and lipid metabolism of newly-differentiated human fat cells in cell culture. *Diabetologia*. 1995;38(7):764-771.
17. Zhang J, Gao Z, Yin J, Quon MJ, Ye J. S6K directly phosphorylates IRS-1 on Ser-270 to promote insulin resistance in response to TNF-(alpha) signaling through IKK2. *J Biol Chem*. 2008; 283(51):35375-35382.
18. Guerre-Millo M. Adiponectin: an update. *Diabetes Metab*. 2008; 34(1):12-18.
19. Wang B, Jenkins JR, Trayhurn P. Expression and secretion of inflammation-related adipokines by human adipocytes differentiated in culture: integrated response to TNF-alpha. *Am J Physiol. Endocrinol Metab*. 2005;288(4):E731-E740.
20. Wang B, Trayhurn P. Acute and prolonged effects of TNF-alpha on the expression and secretion of inflammation-related adipokines by human adipocytes differentiated in culture. *Pflugers Arch*. 2006;452(4):418-427.
21. Mirza SS, Shafique K, Shaikh AR, Khan NA, Anwar Qureshi M. Association between circulating adiponectin levels and polycystic ovarian syndrome. *J Ovarian Res*. 2014;7:18.
22. Krikun G, Mor G, Alvero A, et al. A novel immortalized human endometrial stromal cell line with normal progestational response. *Endocrinology*. 2004;145(5):2291-2296.
23. Zhang JJ, Xu ZM, Zhang CM, et al. Pyrrolidine dithiocarbamate inhibits nuclear factor-kappaB pathway activation, and regulates adhesion, migration, invasion and apoptosis of endometriotic stromal cells. *Mol Hum Reprod*. 2011;17(3):175-181.
24. Giudice LC.Endometrium in PCOS: Implantation and predisposition to endocrine CA. *Best Pract Res Clin Endocrinol Metab*. 2006;20(2):235-244.
25. Samalecos A, Reimann K, Wittmann S, et al. Characterization of a novel telomerase-immortalized human endometrial stromal cell line, St-T1b. *Reprod Biol Endocrinol*. 2009;7:76.
26. Ormazabal P, Romero C, Quest AF, Vega M. Testosterone modulates the expression of molecules linked to insulin action and glucose uptake in endometrial cells. *Horm Metab Res*. 2013; 45(9):640-645.
27. 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(4):1017-1026.
28. Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. *Cell Death Differ*. 2003;10(1):45-65.
29. Villavicencio A, Goyeneche A, Telleria C, et al. Involvement of Akt, Ras and cell cycle regulators in the potential development of endometrial hyperplasia in women with polycystic ovarian syndrome. *Gynecol Oncol*. 2009;115(1):102-107.
30. Plaza-Parrochia F, Orostica L, Garcia P, et al. Molecular mechanisms of androstenediol in the regulation of the proliferative process of human endometrial cells. *Reprod Sci*. 2017;24(7): 1079-1087.
31. Bouwmeester T, Bauch A, Ruffner H, et al. A physical and functional map of the human TNF-alpha/NF-kappa B signal transduction pathway. *Nat Cell Biol*. 2004;6(2):97-105.
32. 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;2011:389317.
33. Vgontzas AN, Trakada G, Bixler EO, et al. Plasma interleukin 6 levels are elevated in polycystic ovary syndrome independently of obesity or sleep apnea. *Metabolism*. 2006;55(8): 1076-1082.
34. Liu M, Liu F. Transcriptional and post-translational regulation of adiponectin. *Biochem J*. 2009;425(1):41-52.

35. Fasshauer M, Kralisch S, Klier M, et al. Adiponectin gene expression and secretion is inhibited by interleukin-6 in 3T3-L1 adipocytes. *Biochem Biophys Res Commun*. 2003;301(4):1045-1050.
36. Deepa SS, Dong LQ. APPL1: role in adiponectin signaling and beyond. *Am J Physiol Endocrinol Metab*. 2009;296(1): E22-E36.
37. Mao X, Kikani CK, Riojas RA, et al. APPL1 binds to adiponectin receptors and mediates adiponectin signalling and function. *Nat Cell Biol*. 2006;8(5):516-523.
38. Wang C, Xin X, Xiang R, et al. Yin-Yang regulation of adiponectin signaling by APPL isoforms in muscle cells. *J Biol Chem*. 2009;284(46):31608-31615.
39. Cheng KK, Zhu W, Chen B, et al. The adaptor protein APPL2 inhibits insulin-stimulated glucose uptake by interacting with TBC1D1 in skeletal muscle. *Diabetes*. 2014;63(11):3748-3758.
40. Patti ME, Kahn BB. Nutrient sensor links obesity with diabetes risk. *Nat Med*. 2004;10(10):1049-1050.
41. Rutledge AC, Adeli K. Fructose and the metabolic syndrome: pathophysiology and molecular mechanisms. *Nutr Rev*. 2007; 65(6 pt 2):S13-S23.
42. 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(1):123-131.