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RESEARCH ARTICLE

The insulin-sensitizing mechanism of myo-inositol is associated with AMPK activation and GLUT-4 expression in human endometrial cells exposed to a PCOS environment

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Cabrera-Cruz H, Oróstica L, Plaza-Parrochia F, Torres-Pinto I, Romero C, Vega M. The insulin-sensitizing mechanism of myo-inositol is associated with AMPK activation and GLUT-4 expression in human endometrial cells exposed to a PCOS environment. *Am J Physiol Endocrinol Metab* 318: E237–E248, 2020. First published December 24, 2019; doi:10.1152/ajpendo.00162.2019.—Polycystic ovary syndrome (PCOS) is an endocrine-metabolic disorder characterized by hyperandrogenism and ovulatory dysfunction but also obesity and hyperinsulinemia. These characteristics induce an insulin-resistant state in tissues such as the endometrium, affecting its reproductive functions. Myo-inositol (MYO) is an insulin-sensitizing compound used in PCOS patients; however, its insulin-sensitizing mechanism is unclear. To understand the relationship of MYO with insulin action in endometrial cells, sodium/myo-inositol transporter 1 (SMIT-1) (MYO-transporter), and MYO effects on protein levels related to the insulin pathway were evaluated. SMIT-1 was assessed in endometrial tissue from women with normal weight, obesity, insulin resistance, and PCOS; additionally, using an in vitro model of human endometrial cells exposed to an environment resembling hyperinsulinemic-obese-PCOS, MYO effect was evaluated on p-AMPK and GLUT-4 levels and glucose uptake by Western blot, immunocytochemistry, and confocal microscopy, respectively. SMIT-1 was detected in endometrial tissue from all groups and decreased in PCOS and obesity ($P < 0.05$ vs. normal weight). In the in vitro model, PCOS conditions decreased p-AMPK levels, while they were restored with MYO ($P < 0.05$). The diminished GLUT-4 protein levels promoted by PCOS environment were restored by MYO through SMIT-1 and p-AMPK-dependent mechanism ($P < 0.05$). Also, MYO restored glucose uptake in cells under PCOS condition through a p-AMPK-dependent mechanism. Finally, these results were similar to those obtained with metformin treatment in the same in vitro conditions. Consequently, MYO could be a potential insulin sensitizer through its positive effects on insulin-resistant tissues as PCOS-endometrium, acting through SMIT-1, provoking AMPK activation and elevated GLUT-4 levels and, consequently, increase glucose uptake by human endometrial cells. Therefore, MYO may be used as an effective treatment option in insulin-resistant PCOS women.

AMPK; endometrium; GLUT-4; myo-inositol; polycystic ovary syndrome

INTRODUCTION

The polycystic ovary syndrome (PCOS) is an endocrine-metabolic disorder present in 5–20% of women in fertile age worldwide. PCOS is characterized by the presence of hyperandrogenism, ovulatory dysfunction, and/or ovarian cysts, causing a heterogeneous physio-pathological state that includes manifestations like acne, hirsutism, menstrual irregularities, chronic oligo/anovulation, and infertility (4, 36). The PCOS pathogenesis is still unclear (3); nevertheless, obesity and insulin resistance with women with PCOS present with overweight or obesity (24), and at least 70% of this subgroup have insulin resistance (25, 46). PCOS women have a high prevalence of central obesity accompanied by an increase of biochemical hyperandrogenism (1, 24). Central obesity promotes an oxidative stress state by increasing the circulating free fatty acids and proinflammatory cytokines as tumor necrosis factor-α (TNF-α), IL-6, and C-reactive protein. Additionally, these cytokines diminish circulating adipokines with insulin-sensitizing properties, such as adiponectin (25, 45). The hyperandrogenic, hyperinsulinemic, and proinflammatory environment present in women with PCOS affects biochemically the insulin-sensitive tissues as muscle, liver, adipose, and reproductive tissues, such as the endometrium. The endometrium is a tissue that responds to steroids and insulin and has crucial functions in recognition, adherence, and implantation of embryo (26). In PCOS, the metabolism and regulation of endometrial function are altered, and this state is exacerbated by hyperinsulinemia and obesity (46), increasing the risk of reproductive failures like infertility, pregnancy complications, and miscarriage. Additionally, the cellular responses to stimuli promoting the cellular proliferation and differentiation increase the risk in PCOS-hyperinsulinemic women to develop endometrial hyperplasia and adenocarcinoma (49, 50).

In previous reports from our laboratory, the expression of some molecules associated with insulin action in the endometrium of women with PCOS has been studied, and these reports offer an understanding, from the molecular point of view, of

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the endometrial characteristics of these patients, which generate the reproductive and endocrine-metabolic complications mentioned above. In fact, we have noted that in the endometrium of hyperinsulinemic PCOS women with elevated BMI, the activated state of IRS-1 Tyr-612, AKT Ser-473, and AS160 Thr-642 is altered, and the protein levels of GLUT-4 are diminished in comparison to control normoinsulinemic women (22). Additionally, when studying the endometrium of PCOS-obese women with insulin resistance, a chronic proinflammatory environment was found, characterized by increased endometrial levels of TNF-α (44). Moreover, in an in vitro model of human endometrial cells cultured under conditions that resembled the PCOS environment, a decrease was found in the activation of proteins associated to insulin signaling, such as AMPK, Thr-172, and lower levels of glucose transporter GLUT-4 (45) compared with the basal condition, indicating a decrease in the translocation of GLUT-4 to the membrane and, thereby, decreasing the uptake of glucose in this tissue.

Insulin sensitizers' well-established role in hyperinsulinemia and its relationship with obesity in the PCOS pathophysiology have advanced their inclusion as a therapeutic approach to reduce the metabolic dysfunction on these women (2, 3, 19). One of the main insulin sensitizers used is metformin; this biguanide exerts its effects in several insulin resistance tissues as skeletal muscle, adipose tissue, and reproductive tissues with metabolic activity as endometrium (12, 24, 40, 66). In fact, once inside the endometrial cell, metformin can stimulate several pathways, including the activation of AMPK by modifying AMP/ATP rates and LKB1 activation. The activation of AMPK is intimately related with GLUT-4 transcription and translation processes (12).

Despite the therapeutic success of metformin in PCOS-insulin resistance women, secondary effects such as reduced tolerance, gastrointestinal symptoms, and weight gain are present in at least 30% of patients taking it; additionally, because of its renal excretion mechanism, its prescription to patients with renal dysfunction must be evaluated (2, 21, 52, 66). In this context, interest in new insulin sensitizer compounds have been studied since the last decade. The inositol-derivate compounds, specifically myo-inositol (MYO), is one of the principal inositol derivates that has been studied lately and that presents important results in clinical studies when it is used as a pharmaceutical or supplemental compound (21, 41, 61). The MYO therapy in PCOS women has shown MYO to be a potential insulin-sensitizing compound, with better tolerance results when comparing with other insulin sensitizers, such as metformin (51, 52). Also, MYO has been observed to improve metabolism in women with PCOS. Important positive results in endocrine parameters have been reported, including improvements in testosterone, androstenedione, SHBG, and FSH levels (10).

MYO is a polyol cyclohexane derivate of inositol, an abundant natural compound found in plants and animals. It can be obtained intrinsically from glucose-6-phosphate or from a recovery pathway that involves the inositol-phosphate cycle, although it is principally obtained extrinsically through extracellular uptake using specific transport proteins (9, 18, 27). In cells, MYO enters the cells using coupled proton or sodium transport proteins. One of the main transporters for MYO described in humans is the sodium/myo-inositol transporter 1 (SMIT-1) (56). It is expressed in the brain, kidney, bone, pancreas, and heart (23, 37, 42, 62). After its uptake, MYO can be converted to its epimer analog, D-chiro-inositol, which is involved in glucose catabolism and insulin signaling; MYO and D-chiro are the structural bases for inositol glycans, which has been proposed as an insulin second messenger (33). Also, MYO can be methylated or phosphorylated by kinases and synthases, enzymes that convert MYO to second messengers (6, 20, 30).

The specific properties that confer to MYO its insulin-sensitizer effects and relationship with glucose metabolism through insulin signaling participation, are still unclear. MYO could exert its insulin-sensitizer effects by several mechanisms, including its metabolism and generation of IP3, PIP, PIP2, and PIP3 (20, 28, 30, 39, 47, 48). PIP3 acts as a second messenger in the insulin pathway through the activation of PI3K/AKT. On the other hand, the increase of phosphoinositides on the intracellular environment can exert important changes in energetic state of the cell that can be detected by energy sensor kinases as LKB1 and AMPK (8, 16). The scientific findings presented previously show that MYO could be a potential insulin-sensitizing agent to be used as an effective option in women with PCOS, given the evidence that its oral administration induces the recovery of the endocrine-metabolic state in these patients (21, 41, 48, 62). Nevertheless, at present, the mechanism through which MYO exerts its action on cells, is poorly understood. Therefore, it is relevant to gain knowledge on the molecules associated to the insulin pathway that could serve as targets for MYO action in human endometrial cells subjected to conditions that resemble PCOS and insulin resistance environments. Finally, the positive effect of MYO on insulin action could improve the reproductive failures associated with endometrial function observed in insulin-resistant women with PCOS.

MATERIALS AND METHODS

Subjects. A total of 24 women were recruited and classified in four study groups: normal weight (NW or control group), obese (OB), obese and insulin resistance (OB-IR), and obese-PCOS-insulin resistance (OB-PCOS-IR). The clinical and metabolic parameters of study groups are shown in Table 1. The PCOS was diagnosed, according to the Androgen Excess and PCOS Society criteria (4), whose main criterion is hyperandrogenism, determined biochemically by the Free Androgens Index (FAI normal values: <4.5), which is calculated from total testosterone and sex hormone binding globulin (SHBG) plasmatic levels; additionally, for PCOS diagnosis, hyperandrogenism was accompanied by an ovary dysfunction state, identified by oligo or amenorrhea and/or polycystic ovaries determined by ultrasonography. The hyperinsulinemic state in the two study groups was evaluated by an oral glucose tolerance test with a 75-g load of glucose (normal values: fasting glucose <110 mg/dL, glucose 120 min <140 mg/dL); Insulin Sensitive Index (ISI) composite (normal value: >3) and Homeostasis Model Assessment (HOMA) (normal value: <2.6) were also calculated (38). Women with obesity had a body mass index (BMI) >30 kg/m^2 and the NW group <25 kg/m^2. The age of the different groups was similar (Table 1). Pathologies that induce hyperinsulinemia or hyperandrogenemia were discarded as congenital adrenal hyperplasia and Cushing's syndrome. None of the participants had received any hormonal treatment during the past 3 mo before recruitment into the study and/or ovulation induction (e.g., gonadotropins and clomiphene citrate). All of these parameters have been used in previous studies from our laboratory (12, 25, 44). The blood and endometrial samples were obtained during the proliferative phase of menstrual cycle; the endometrial dating was performed according

Table 1. *Clinical and metabolic parameters of study groups*

Parameters/Study Groups	NW	OB	OB-IR	OB-PCOS-IR
n	6	6	6	6
Age, years	25.0 ± 1,6	27.5 ± 1.4	25,3 ± 1.8	29.3 ± 1.9
BMI, kg/m^2	23.1 ± 1.2	38.5 ± 2.9*	32.8 ± 1.5	36.4 ± 2.4*
Testosterone, ng/dL	38.9 ± 5.9	31.7 ± 4.7	31.5 ± 6.2	55.7 ± 5.9
SHBG, nmol/L	53.1 ± 3.1	38.7 ± 4.2	34.3 ± 4.4	22.3 ± 3.1*
FAI	2.60 ± 0.48	2.87 ± 0.31	3.38 ± 0.58	9.21 ± 1.12*†
Fasting glucose, mg/dL	92.3 ± 3.9	92.5 ± 2.6	92.8 ± 3.1	92.5 ± 4.8
Glucose 120 min, mg/dL	90.4 ± 6.5	104.8 ± 8.6	113.8 ± 7.7	124.3 ± 13.9
Fasting insulin, μUI/L	7.9 ± 0.9	7.6 ± 1.3	23.9 ± 5.6*†	27.5 ± 4.3*†
Insulin 120 min, μUI/L	42.5 ± 1.9	48.8 ± 7.1	127.9 ± 33.8	273.0 ± 82.6*†
HOMA-IR	1.79 ± 0.46	1.72 ± 0.27	5.57 ± 1.37*†	6.06 ± 0.82*†
ISI Composite	6.36 ± 0.45	6.28 ± 1.04	2.47 ± 0.66	1.42 ± 0.26*†

The values are expressed as means ± SE. BMI, body mass index; FAI, free androgens index; HOMA-IR, homeostasis model assessment-insulin resistance; ISI-Composite, insulin sensitivity index; NW, Control women with normal weight; OB, Control women with obesity; OB-IR, women with obesity and insulin-resistance; OB-PCOS-IR, women with obesity, PCOS and insulin-resistance; SHBG, sex hormones blinding globulin. Reference values: Obesity, BMI >30 kg/m^2; testosterone, 5.7–77 ng/dL; SHBG, 18–114 nmol/L; FAI <4.5 normal; Fasting glucose ≤100 mg/dL; glucose 120 min ≤ 140 mg/dL; fasting insulin ≤20 μUI/mL; insulin 120 min ≤60 μUI/mL; HOMA-IR, normal <2.6; ISI Composite, normal >3. *$P < 0.05$ compared with NW; †$P < 0.05$ compared with OB.

to Noyes criteria (43) by an experienced pathologist. 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.

Tissue preparation. Endometrial tissue samples from the four study groups were obtained with a Pipelle suction curette from de corpus of uteri; a fragment of each sample was fixed in 4% buffered paraformaldehyde for 24 h, embedded in paraffin and cut in 4-μm thick sections before histological and immunohistochemical studies, as previously reported (46, 49).

Cell culture and treatments. For the in vitro experimental model, telomerase-immortalized human endometrial stromal cell line St-T1b (55) was cultured in DMEM/Ham F12 medium (Sigma Aldrich, St. Louis, MO), with 10% FBS treated with antimycotic/antibiotic, at 37°C in a 5% CO_2 atmosphere until 80% of confluence (25, 50). Then, the cells were cultured in 6-well plates, 500,000 cells per well for Western blot (WB) and 24-well plates, 40,000 cells per well for immunocytochemistry (ICC) in growth media for 24 h at 37°C in 5% CO_2 atmosphere. Further, cultures were washed twice with sterile 1× Dulbecco's PBS (Gibco by Life Technologies, Carlsbad, CA), and subjected to treatments using serum-free medium as a vehicle. For treatments, the basal condition corresponds to vehicle. As it has been established in previous studies from our laboratory (25, 44, 45, 50), to represent the hyperandrogenic (HA), hyperinsulinemic (HI), and proinflammatory conditions present in endometria from insulin-resistant women with PCOS and/or obesity, stimuli with 100 nM testosterone (Sigma Aldrich), 100 nM insulin (HUMULINA), and 100 ng/mL TNF-α (Gibco by Life Technologies), were used, respectively. To evaluate its insulin-sensitizing effect under the above-mentioned conditions, 1 mM MYO was added as a costimulus. To evaluate the role of the transporter SMIT-1 on MYO in vitro effects, 1 mM phloridzin dihydrate was used as SMIT-1 inhibitor (Sigma Aldrich). Also, to evaluate the role of AMPK in MYO effect on GLUT-4 levels and glucose uptake in St-T1b cells, the AMP-kinase inhibitor dorsomorphin or Compound C (CC) was used (ref. ab120843; Abcam, Cambridge, UK). Compound C (6-[4-(2-Piperidin-1-ylethoxy) phenyl]-3-pyridin-4-ylpyrazolo [1,5-a]pyrimidine) is the only available agent that is used as a cell-permeable AMPK inhibitor (64). St-T1b cells were stimulated for 24 h (to evaluate GLUT-4 levels) or 48 h (to evaluate glucose uptake) with 100 mM testosterone, testosterone plus 1 mM MYO or testosterone plus 1 mM MYO and 20 μM CC; then, GLUT-4 levels were measured by Western blot analysis or glucose uptake by confocal microscopy. Lastly, to compare with the MYO insulin-sensitizing effects on protein expression, 20 μM metformin (Sigma Aldrich) was administered under the same conditions as used for MYO studies in this model. All treatments were done for 24 h, at 37°C, and in 5% CO_2 atmosphere. Four independent experiments were performed for each treatment.

Immunohistochemistry and immunocytochemistry. Briefly, for immunohistochemistry (IHC), paraffin sections of the endometrial samples were deparaffinized with HistoClear (National Diagnostics, Atlanta, GA) and gradually hydrated through graded alcohols. For antigenic recovery, the samples were incubated in 10 mM sodium citrate buffer (pH 6.0) at 95°C for 20 min. For immunocytochemistry (ICC) assays, St-T1b cells were fixed with 2% paraformaldehyde in 1× PBS for 2 min and then in 4% paraformaldehyde in 1× PBS for 15 min. Cells were permeabilized, incubating with 0.3% Triton X-100 (Winkler, RM Chile) for 15 min. For both, IHC and ICC, the endogenous peroxidase activity was prevented by incubating the samples in 3% hydrogen peroxide for 15 min. Inhibition of nonspecific binding was accomplished by incubating the samples with blocking solution 2–4% BSA/1× PBS for 20 min. Samples were incubated with primary antibody SMIT-1, 1:1,000 dilution (rabbit polyclonal; ref. ABS518, Sigma Aldrich), GLUT-4, 1:50 dilution (rabbit polyclonal; ref. SC-7938, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. An internal control was carried out on adjacent sections incubated without the primary antibody. After incubation, primary antibodies were washed, and samples were incubated with a biotinylated horseradish peroxidase secondary antibody anti-rabbit 1:300 dilution (ref. 115-035-003, Jackson Immunoresearch, West Grove, PA) for 2 h at 37°C. Chromogenic signal detection was revealed using the streptavidin-peroxidase system, 3,3′ diaminobenzidine was used as substrate, and counterstaining was performed with hematoxylin (Dako, Carpinteria, CA). The slides were evaluated on an Olympus optical microscope (Olympus BX51TF, Tokyo, Japan) and the Image Pro Plus 6.0 program (Media Cybernetics, Silver Spring, MD) was used for semiquantitative analysis of the positive pixel intensity, through the measurement of IOD (integrated optical density). The data are presented as IOD arbitrary units (AU).

Western blot analysis. Total protein content was obtained from St-T1b cultures treated with the different stimuli for 24 h using 150 μL radioimmunoprecipitation assay buffer per well (50 mM Tris-Base, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton, 0.1% sodium dodecyl sulfate; pH 8), and 1× antiprotease/phosphatase cocktail (Thermo Fisher Scientific, Waltham, MA). Lysate samples were collected in sterile tubes and then, 25% sonicated using 2–3 pulses of 5 s each to complete lysis. Samples were centrifuged at 20,000 *g* for 15 min at 4°C. The resulting supernatant was quantified for total protein concentration by bicinchoninic acid protein assay kit (Thermo Fisher Scientific). Fifty micrograms of total proteins were denatured and fractionated using 8% SDS-PAGE gels. The protein content was transferred to a nitrocellulose membrane, and the trans-

ference was confirmed by Ponceau S staining. The membranes were blocked for 1 h at room temperature using 5% nonfat dry milk on TBS with Tween-20 1× (TTBS; 20 mM Tris; 137 mM NaCl; 0.1% Tween-20; pH 7.5). The membranes were incubated at 4°C overnight with primary antibody SMIT-1 1:250 (rabbit polyclonal; ref. ABS518, Sigma Aldrich), p-AMPK Thr-172 and AMPK, both 1:400 (rabbit polyclonal; ref. CS 2535S and ref. CS 2532, Cell Signaling, Danvers, MA), β-actin 1:10,000 (mouse monoclonal; ref. A5441, Sigma Aldrich) diluted in blocking solution. To evaluate GLUT-4 levels, membranes were incubated with primary antibody GLUT-4 (rabbit polyclonal; ref. SC-7938, Santa Cruz Biotechnology) 1:500 dilution on 5% nonfat dry milk on TTBS 1× overnight at 4°C. After incubation, membranes were washed three times with TTBS and incubated for 1 h at room temperature with an HRP-labeled secondary antibody to rabbit (ref. 115-035-003) or mouse (ref. 223-005-024) IgG 1:5,000 dilution (Jackson Immunoresearch). Detection of bound antibodies was performed using an enhancer chemiluminescence substrate: luminol diluted in hydrogen peroxide (1:1) Westar Sun chemiluminescent substrate (Cyanagen, BO Italy). Chemiluminescent bands were visualized and photographically captured using Image Quant LAS 500 (General Electric, Boston, MA). Protein bands were semiquantified and analyzed by scanning densitometry with the Image J 1.52a program (National Institutes of Health, Bethesda, MD). The data correspond to AU, and the results were expressed as the ratio between molecule levels and housekeeping levels (β-actin) or the ratio between phosphorylated and total protein levels normalized to the basal conditions.

Quantitative real-time PCR assay. Human endometrial cells treated with the different stimuli were used to obtain total RNA samples using TRIzol (cat. no. 15596018; Ambion, Austin, TX) reagent, according to the manufacturer's instructions. The concentration of RNA was determined spectrophotometrically (A260:A280), while RNA integrity was determined by electrophoresis on a formaldehyde agarose gel under denaturing conditions. One microgram of total RNA was digested with DNase I and transcribed into complementary DNA (cDNA) by reverse transcription with M-MLV reverse transcriptase (cat. no. 28025-013; Invitrogen, Carlsbad, CA). PCR amplification of studied mRNA was assessed by using gene-specific primers: GLUT-4: sense 5′-ATCCTGATGACTGTGGCTCTGCT-3′ and antisense 5′-TCGTTCTCATCTGGCCCTAAATAC-3′; GAPDH: sense 5′-GAGTCAACGGATTTGGTCGT-3′ and antisense 5′-ATCCACAGTCTTCTGGGTG-3′, which was used as an internal control. Real-time PCR was performed on the Step One PCR System (Applied Biosystems, Foster City, CA) using SYBR Green QPCR master mix (no. 600828; Agilent Technologies, Santa Clara, CA). For the negative control reaction, no template was added to the reaction mixture. Values were expressed as relative copies of each transcript, and results are shown as a ratio of β-actin internal control. The mean (±SE) of these values was obtained for each studied group.

Glucose uptake assays. St-t1b cells were cultured with different stimuli for 48 h to evaluate the glucose uptake using 2-NBDG (2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) (cat. no. N13195; Thermo Fisher Scientific). Cells were maintained in 1 mL of glucose-free Krebs Buffer 1× (120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM $NaHCO_3$; at pH 7.2) with insulin 100 nM mM (Actrapid, B-1615, Novo Nordisk, Bagsværd, Denmark) for 20 min at 37°C to promote the insulin-dependent glucose uptake. Then, living cells were incubated with 500 μL of glucose-free Krebs Buffer 1X with 2,7 μL of 2-NBDG for 15 min at 37°C. Cells were washed with glucose-free Krebs Buffer 1×, and the fluorescence was visualized by confocal microscopy (C2+ Eclipse TI model, Nikon) using green channel (λ excitation laser: 488 nm) to obtain images. The fluorescence was measured using Image J Program (Fiji Version 2.0-rc-68/1.52g, Cambridge Astronomical Survey Unit). In addition, other plates with St-T1b cells were not treated with this acute insulin stimulus before incubation with 2-NBDG; the fluorescence from these cells was subtracted from fluorescence obtained in cells stimulated with insulin, to observe only the glucose uptake induced by insulin. The data correspond to AU obtained in each culture condition.

Statistics analysis. All data showed a nonparametric distribution and were analyzed by nonparametric (Kruskal-Wallis test) with Dunn's post hoc test for multiple comparisons and Mann Whitney *U*-test was used to compare two specific groups. A P value < 0.05 was considered significant. Statistical tests were performed using GraphPad Prism 6.0 program (San Diego, CA). All results correspond to the mean of each condition with ± SE (standard error of mean).

RESULTS

Protein levels of sodium/myo-inositol transporter 1 in human endometrial tissues. Scarce literature describing the presence of MYO transporter sodium/myo-inositol transporter 1 (SMIT-1) in endometrium has been reported; thus, it was relevant to assess the protein presence of SMIT-1 in this tissue and also evaluate whether hyperandrogenism, insulin-resistance, and obesity conditions present in PCOS, affect protein levels of SMIT-1. In this sense, semiquantitative analysis was performed in endometrial tissue of the four groups: NW, OB, OB-IR, and OB-PCOS-IR women. As shown in Fig. 1, the transporter was detected in both compartments of the endometrium (epithelium and stroma) in all studied groups. Additionally, a significant decrease of SMIT-1 levels was found in the OB-PCOS-IR group compared with the other groups ($P < 0.01$). Interestingly, the groups that presented obesity had decreased SMIT-1 levels compared with NW control group ($P < 0.05$); nevertheless, it is most likely that the PCOS condition determines the diminution of SMIT-1 levels in endometrium.

Effect of MYO on SMIT-1 protein levels in human endometrial cells under PCOS conditions. In cultures of the endometrial cell line St-T1b, hyperinsulinemic (HI) and hyperandrogenic (HA) conditions, besides the proinflammatory environment that resemble PCOS, were evaluated to see whether SMIT-1 protein levels were modified. Also, the effect of MYO on the levels of the transporter was assessed in the same conditions. The data show that SMIT-1 is expressed in the cell line St-T1b and that its protein levels are not affected in the presence of HI/HA/TNF-α (Fig. 2), indicating that this transporter could be available for MYO transport in this cell model. Nevertheless, when MYO was added to the cell culture in HI/HA/TNFα condition, a significant decrease of SMIT-1 levels was found ($P = 0.0286$) compared with basal conditions.

Effect of MYO on p-AMPK Thr-172 levels in St-T1b cells under PCOS conditions. Considering the importance of glucose homeostasis for normal endometrial function and the insulin-sensitizing effect of MYO, the next step was to determine the potential molecular targets for MYO action. One of the studied molecules was AMPK, an energy sensor protein that interacts with proteins that participate on the insulin pathway, and in previous studies from our laboratory, lower p-AMPK Thr-172 levels were found in the endometrium of PCOS women (12). In the present study, these alterations were also found in the in vitro studies, where phosphorylation of AMPK significantly decreases in the presence of insulin + testosterone (HI/HA conditions; $P = 0.0013$ compared with basal condition) (Fig. 3*A*). Additionally, in the presence of a pro-inflammatory microenvironment (Fig. 3*B*), AMPK phosphorylation diminished significantly when TNF-α was present

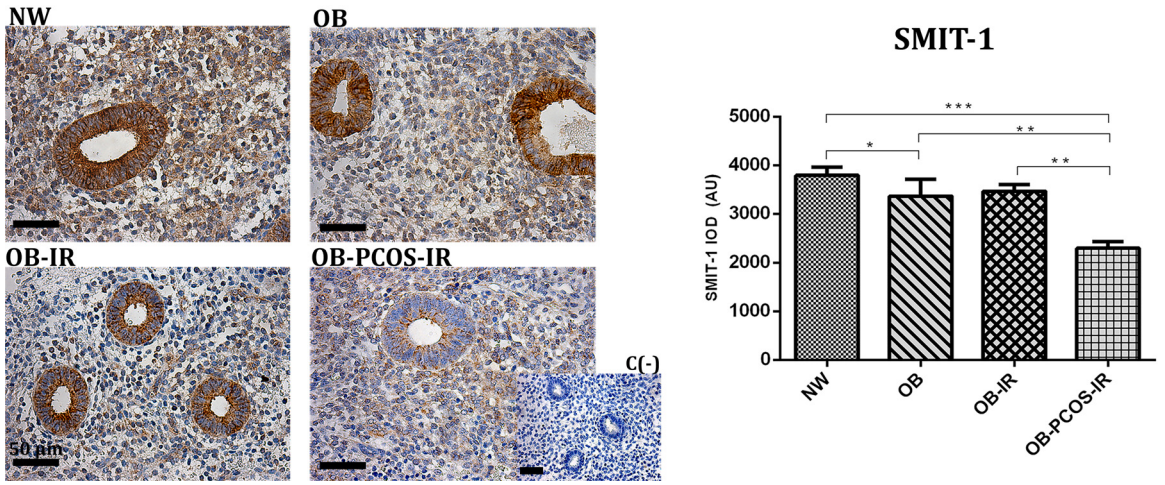

Fig. 1. Protein levels of sodium/myo-inositol transporter 1 (SMIT-1) in endometrial tissue from normal-weight (NW), obese (OB), obese-insulin resistance (OB-IR), and obese-PCOS-insulin resistance (OB-PCOS-IR) women. Levels were determined by immunohistochemistry. *Left*: microphotographs show the protein presence in epithelial and stromal compartments of endometrium identified by immunopositive brown color staining. *Right*: semiquantification of the protein levels in endometrial tissue was performed by Integrated Optical Density (IOD) tool of Image Pro Plus 6.0 program. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ analyzed by nonparametric test and Dunn's post hoc test. Data are shown as mean arbitrary units (AU) ± SE; $n = 6$ samples in each group. Bar: 50 μm; C(-): negative control.

individually or as a costimulus to HI/HA conditions ($P = 0.0079$). Interestingly, when MYO was added to the cell culture, it was capable of restoring phosphorylation to basal levels in all conditions ($P = 0.05$). These results suggest that AMPK could be one of the main targets for MYO action.

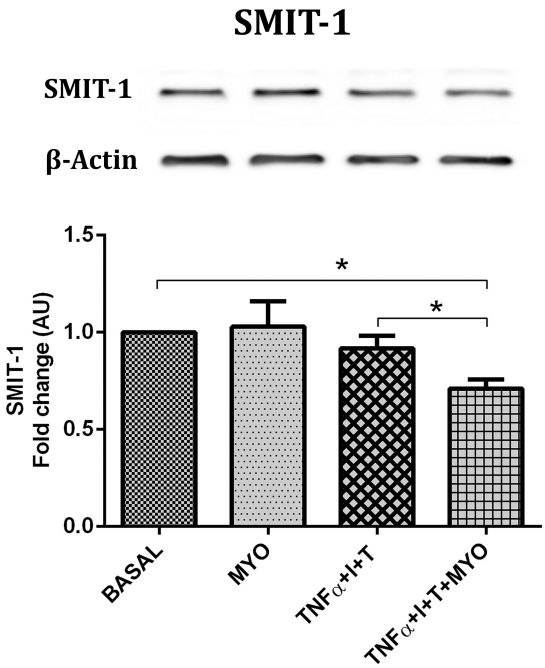

Fig. 2. Protein levels of sodium/myo-inositol transporter 1 (SMIT-1) in human endometrial cells under PCOS conditions. *Top*: graphs show the levels of SMIT-1 (79-kDa band) in St-T1b cultures treated with 1 mM Myo-inositol (MYO), 100 ng/mL TNF-α, 100 nM insulin (I), 100 nM testosterone (T), or combined stimuli for 24 h by Western blot. *Bottom*: data were normalized using the protein levels of β-actin (42 kDa) in each sample and analyzed as fold change with respect to baseline. The results are presented as arbitrary units (AU) and are the mean ± SE of each group; $n = 4$ in duplicate. $*P < 0.05$ analyzed by nonparametric test. TNF-α, tumor necrosis factor α.

Effect of MYO on GLUT-4 expression in St-T1b cells under PCOS conditions. Because of the activation of AMPK induces the transcription and translation of GLUT-4 in endometrial tissue, as we have previously reported (12), the next step was to evaluate GLUT-4 protein levels in HI/HA conditions and in the presence of MYO. As observed in Fig. 4*A*, when cells are cultured with insulin, testosterone, and MYO, the negative effects on GLUT-4 levels conferred by insulin plus testosterone are reverted to basal levels by the presence of MYO. In addition, quantitative PCR (qPCR) assays show a decrease of GLUT-4 mRNA levels with Insulin + Testosterone or TNF-α treatments compared with basal levels (Fig. 4*B*). Moreover, the presence of MYO under HI/HA or TNF-α conditions prevents the decrease of GLUT-4 mRNA levels. The latter finding is in agreement with a previous report (45) from our group that showed a negative effect of TNF-α on GLUT-4 protein levels. Thus, this positive effect of MYO on GLUT-4 levels could be related to the AMPK activation described previously, and MYO could exert its insulin-sensitizing effects through AMPK, as mentioned before.

Role of MYO transporter SMIT-1 and AMPK in the MYO effect on GLUT-4 protein levels in human endometrial cells under hyperandrogenic condition. To determine the role of SMIT-1 as MYO transporter on changes of GLUT-4 protein levels, the sodium-transporter protein inhibitor phloridzin was used. On the basis of the negative effect of testosterone on GLUT-4 protein levels observed in the present study (data not shown), testosterone stimulus was selected to evaluate the mechanism by which MYO increases GLUT-4 levels. As observed in Fig. 5, phloridzin (FL) inhibited the MYO effect in testosterone plus MYO plus FL, reaching similar levels as the testosterone condition ($P < 0.01$). These results strongly suggest that the effect of MYO on GLUT4 levels is dependent of transporter SMIT-1. Furthermore, when we used an AMP-kinase inhibitor called dorsomorphin or compound C (CC) in cells treated with testosterone plus MYO, the effect of MYO was not observed (Fig. 6*A*), indicating that MYO increases

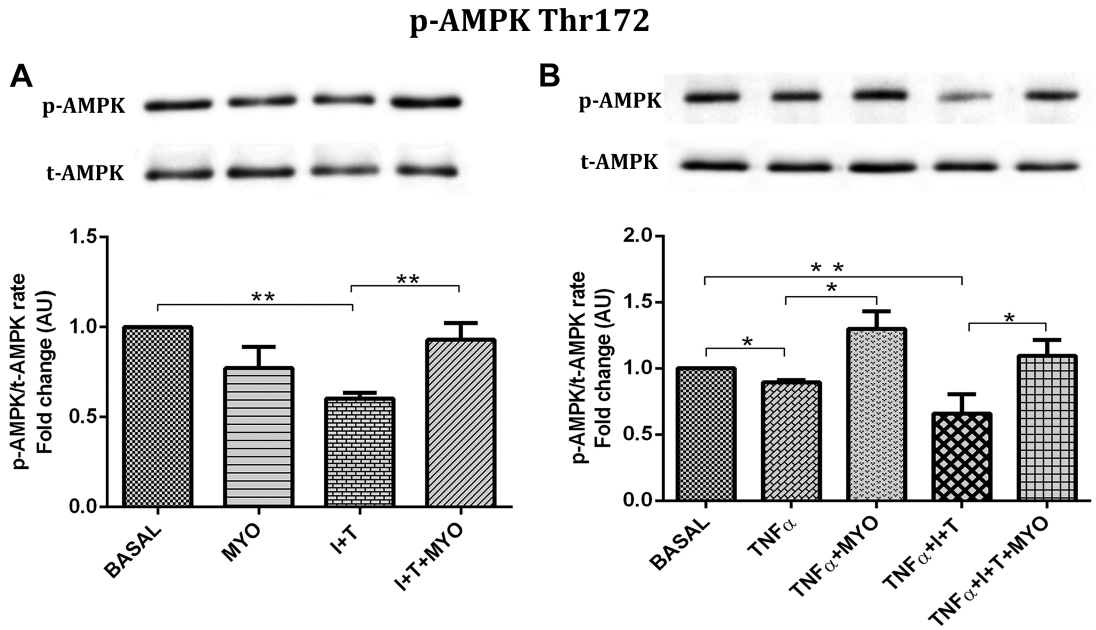

Fig. 3. Protein levels of p-AMPK Thr172 in human endometrial cells under PCOS conditions. The graphs show the levels of p-AMPK Thr172 (62 kDa band) in St-T1b cultures treated with 1 mM myo-inositol (MYO), 100 nM insulin (I), 100 nM testosterone (T), 100 ng/mL TNF-α, or combined stimuli for 24 h by Western blot. *A*: cells were evaluated under HI/HA (hyperinsulinemic and hyperandrogenic) conditions. *B*: cells were evaluated under HI/HA conditions plus a proinflammatory (TNF-α) stimulus. The data were normalized using the total AMPK protein levels (62 kDa) in each sample and analyzed as fold change with respect to baseline. The results are presented as arbitrary units (AU) and are the mean ± SE of each group. $n = 4$ in duplicate. *$P < 0.05$, **$P < 0.01$ analyzed by nonparametric test and Dunn's post hoc test. TNF-α, tumor necrosis factor α.

GLUT-4 expression by a mechanism dependent on AMPK phosphorylation in human endometrial cells. Also, to test the negative effect of CC on AMPK activation, we evaluated p-AMPK Thr-172 levels in St-T1b cells treated with CC. Fig. 6*B* shows a decrease of p-AMPK Thr-172 levels under CC stimulus. Interestingly, CC alone decreased GLUT-4 protein levels in these cells (Fig. 6*B*), indicating the importance of AMPK activation on GLUT-4 expression.

Effect of MYO on glucose uptake by St-T1b cells under hyperandrogenic condition. Since MYO induces an increase of GLUT-4 expression in St-T1b cells, it was first evaluated whether MYO was able to increase the glucose uptake in these cells. For this, we used 2-NBDG, a fluorescent tracer used for monitoring direct glucose uptake into living cells, which can be detected by confocal microscopy. The results (Fig. 7) show that testosterone treatment for 48 h decreased the glucose uptake, but when MYO (alone or plus testosterone) is present, this effect is prevented, indicating that MYO is capable of restoring the glucose uptake in human endometrial cells exposed to a hyperandrogenic environment characteristic of PCOS condition. Furthermore, to evaluate whether the effect of MYO on glucose uptake is dependent on the AMPK activation induced by MYO, cells were treated with CC for 48 h. Fig. 7 shows that MYO in the presence of testosterone and CC did not increase the glucose uptake in human endometrial cells. Even more, CC alone decreased the glucose uptake, and this effect is in agreement with the decrease of p-AMPK and GLUT-4 protein levels by CC treatment (Fig. 6*B*). Therefore, the results indicate that MYO increases the capacity of these cells to uptake glucose, and this effect could be dependent on AMPK activation.

Effect of metformin on AMPK activation and GLUT-4 protein levels in the human endometrial cells. Metformin is a widely described insulin sensitizer that restores the metabolic glucose state in insulin-resistant tissues such as PCOS-endometrium. Ex vivo reports from our laboratory (12) indicate that in obese-PCOS women treated with metformin, an increase of p-AMPK and GLUT-4 level was found in endometrial tissue compared with women with PCOS that are not taking metformin. On the basis of the obtained results for MYO on p-AMPK and GLUT-4 protein levels in HI/HA conditions described in this study (Figs. 3 and 4), we evaluated the effect of metformin under the same in vitro conditions as used for MYO. Fig. 8*A* shows that metformin augments phosphorylation levels of AMPK under HI/HA conditions ($P < 0.0188$ compared with Insulin + Testosterone), similar to the data obtained with MYO. In Fig. 8*B*, we observed that when cells were treated with metformin costimulated with Insulin plus Testosterone, the protein levels of GLUT-4 were normalized to control conditions ($P < 0.05$ compared with Insulin + Testosterone), similar to the results obtained with MYO stimuli. Therefore, MYO could have an insulin-sensitizing effect on endometrial cells by a similar mechanism as that of metformin.

DISCUSSION

There is strong scientific evidence that supports the use of MYO as an insulin-sensitizing compound in metabolic disorders, such as insulin resistance and hyperinsulinemic PCOS. In fact, the results of several clinical studies indicate that MYO improves the metabolic profile and restores insulin sensitivity in women with PCOS (11, 27, 41, 48, 58). Moreover, MYO may contribute toward correcting the dysfunction of hypotha-

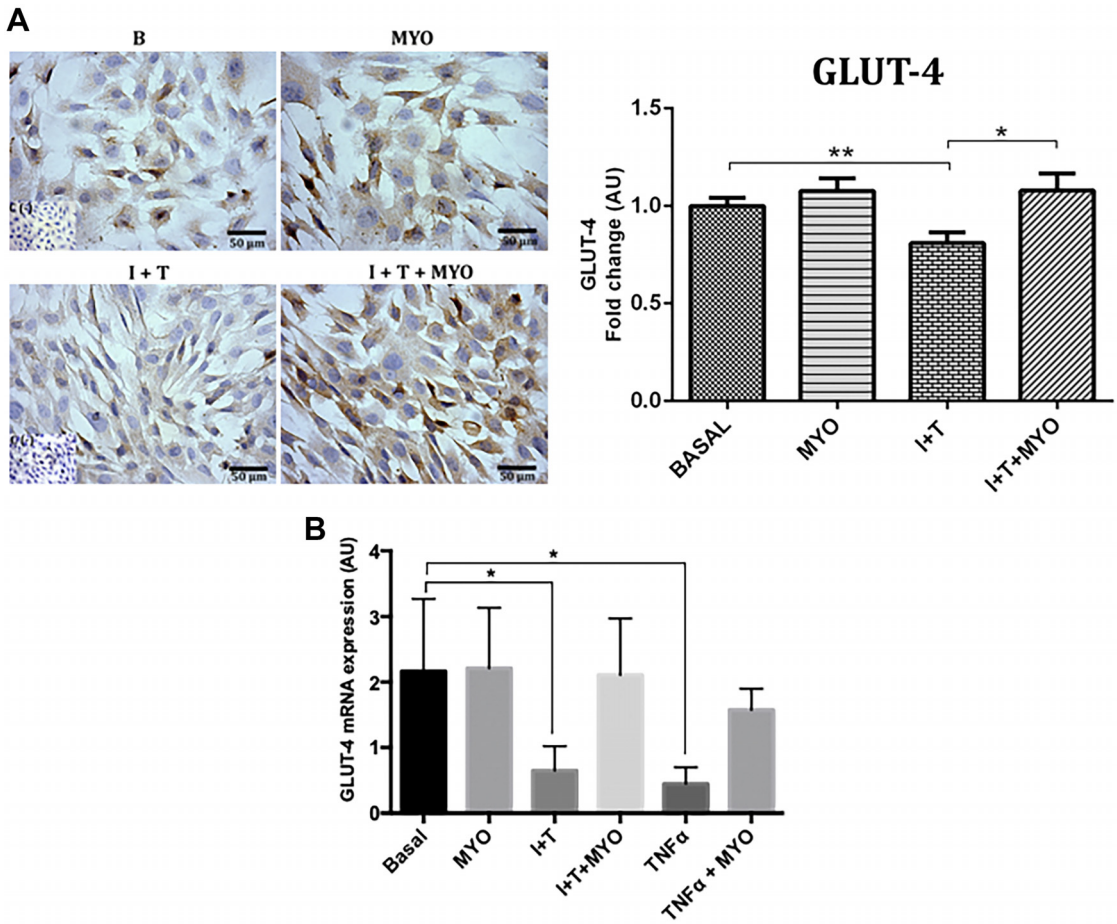

Fig. 4. GLUT-4 expression in human endometrial cells under PCOS conditions. *A*: microphotographs of St-T1b cells treated with 1 mM myo-inositol (MYO), 100 nM insulin (I), 100 nM testosterone (T), or combined stimuli for 24 h by immunocytochemistry. Semiquantitative analysis of the immunopositive staining using the Integrated Optical Density (IOD) tool of Image Pro Plus 6.0 program. The data were normalized using the basal levels in each experiment and analyzed as change in time with respect to baseline ($n = 4$ in duplicate, $^*P < 0.05$, $^{**}P < 0.01$; Bar: 50 μm; C(-): negative control). *B*: real-time PCR assay to evaluate GLUT-4 mRNA levels in St-T1b cells under the different stimuli ($n = 3$ in duplicate, $^*P < 0.05$). GLUT-4 mRNA levels were normalized by GAPDH levels. The results are presented as arbitrary units (AU) and are expressed as the mean ± SE of each group. The data were analyzed by nonparametric test and Dunn's post hoc test. GLUT-4, glucose transporter type 4.

lamic-pituitary-ovary axis in these women (51–53). Additionally, authors indicate that MYO oral therapy is safe in short- and long-term treatment approaches because of its good toleration (14, 21). When studying the molecular changes induced by MYO treatment to explain and understand the results described in the clinical studies, the literature is scarce; studies in hyperinsulinemic animal models mainly highlight that MYO oral therapy induces the translocation of GLUT-4 in insulin-sensitive tissues (14, 17); however, the main mechanisms by which MYO exerts this effect, are not yet elucidated. Considering this, we studied the effect of MYO on the levels of proteins related to insulin pathway in human endometrial cells subjected to conditions that resemble PCOS environment. It is important to note that the endometrium requires a high-energy supply to fulfill its reproductive functions. In this context, it is relevant to mention that PCOS women present reproductive failures associated with an abnormal endometrium function, and this could be explained, in part, by a deficient insulin action in this tissue.

MYO is known to be transported from the extracellular environment by specific transporters such as SMIT-1, whose presence in endometrial tissue is unclear. To settle this, in the present study, we found that SMIT-1 was expressed in all endometrial sample groups. Furthermore, obesity and PCOS conditions could promote a decrease in SMIT-1 levels. This would indicate an effect of hyperandrogenism in addition to proinflammatory cytokines, on levels of this transporter in the endometrium. In this sense, studies describe that proinflammatory cytokines related to obesity, like TNF-α, induce overexpression of the transcription factor TonEBP, which is associated with the increase of SMIT-1 expression (31, 32, 34). Also, it has been observed that changes in the expression of TonEBP are dependent on an osmotic equilibrium in the intra and extracellular environment (31). There is evidence that shows that patients with obesity can present plasma hypertonicity, represented by mild hypernatremia compared with normoweight individuals and alteration of the distribution of body fluids, which also contributes to insulin-resistant state, oxidative stress, and proinflammatory environment (59). These considerations indicate that there is a relationship of obesity with changes in the protein levels of SMIT-1. Since the PCOS endometrium presents several endocrine and metabolic altera-

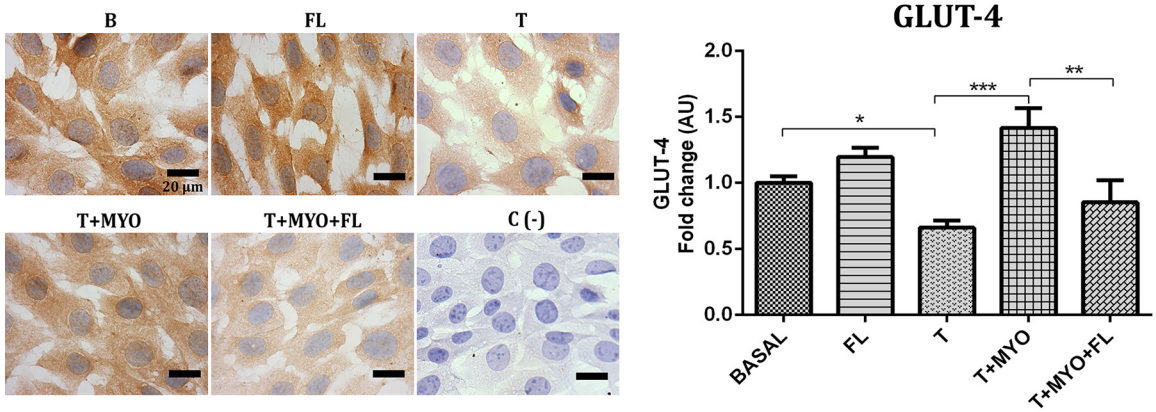

Fig. 5. Effect of phloridzin on protein levels of GLUT-4 in human endometrial cells under hyperandrogenic condition. Microphotographs show St-T1b cells treated with 0.1 mM phloridzin (FL), 1 mM myo-inositol (MYO), 100 nM testosterone (T), or combined stimuli for 24 h. Protein levels of GLUT-4 were evaluated by immunocytochemistry. Semiquantitative analysis of the immunopositive staining using the Integrated Optical Density (IOD) tool of Image Pro Plus 6.0 program. The results are presented as arbitrary units (AU) and are the mean ± SE of each group. $n = 4$ in duplicate, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, analyzed by nonparametric test and Dunn's post hoc test. Bar: 50 μm; C(-): negative control. GLUT-4, glucose transporter type 4.

tions, adjacent factors to hyperandrogenism, insulin resistance, and inflammation present in this pathology, can also affect SMIT-1 levels. To understand whether the three conditions besides the effect of MYO were directly related to SMIT-1 changes, in vitro experiments were performed in a model of human endometrial stromal cells. The results show a decrease in the levels of SMIT-1 in the presence of TNF-α and MYO (alone or with insulin and testosterone). This effect could be explained by the findings that indicate that TNF-α accelerates the uptake of MYO, promoting its intracellular accumulation in different cell types (32, 68), generating osmotic changes that would allow the regulation of SMIT-1 levels through an auto-regulation system (15, 68); however, more studies are needed to elucidate and understand this mechanism. Considering the presence of MYO transporter SMIT-1 in endometrium, and the scarce knowledge about its specific target proteins, we further study the MYO effects on proteins that play a crucial role in glucose metabolism of endometrium as AMPK and GLUT-4. These proteins have been previously studied and identified in our laboratory, as important targets of the negative effects of PCOS microenvironment on insulin action in the endometrium (12, 35, 45).

Once MYO enters into cells, it can have different metabolic destinations that mainly depend on cellular function, within

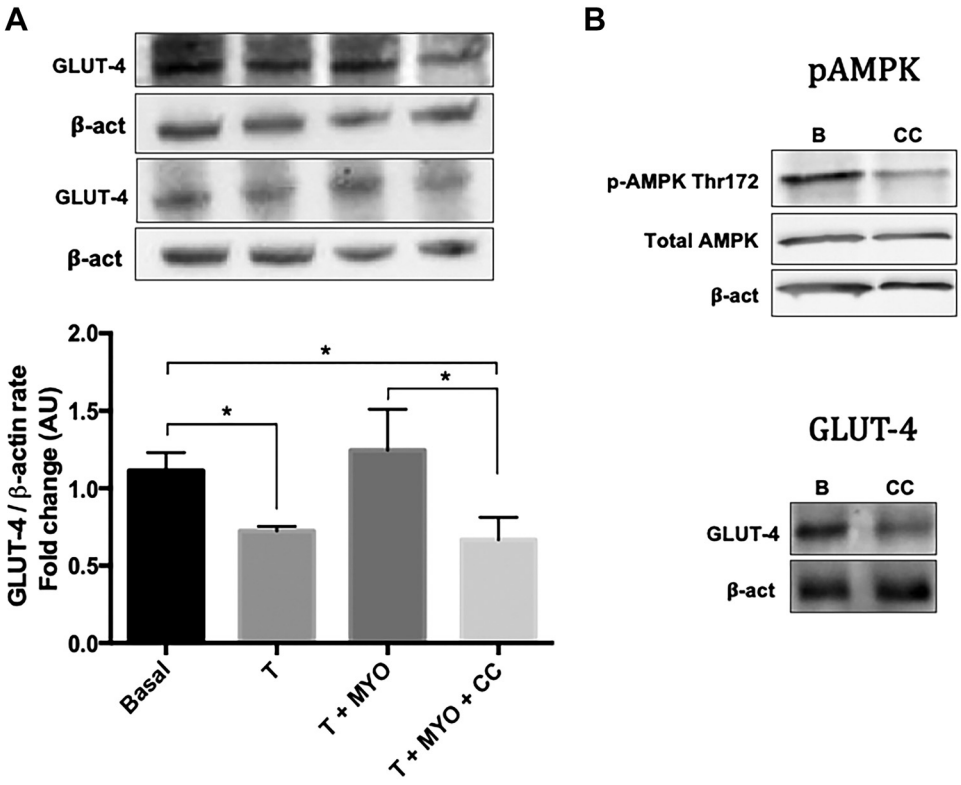

Fig. 6. Role of AMPK in the effect of MYO on GLUT-4 levels in human endometrial cells under hyperandrogenic condition. *A*: human endometrial cells (St-T1b cell line) were treated with 100 nM testosterone (T), 1 mM myo-inositol (MYO), 20 μM CC, or combined stimuli for 24 h. Protein levels of GLUT-4 were evaluated by Western blot analysis. The data were normalized using the protein levels of β-actin (43 kDa) in each sample and analyzed as fold change with respect to baseline. The results are presented as arbitrary units (AU) and correspond to the mean ± SE of each group. Three independent experiments were performed for each treatment ($n = 3$). $^{*}P < 0.05$ analyzed by nonparametric Mann-Whitney *U*-test. *B*: blots show the negative effect of CC (20 μM) on protein levels of p-AMPK Thr-172 (*top*) and GLUT-4 (*bottom*) in St-T1b cells. GLUT-4, glucose transporter type 4; CC: Compound C or Dorsomorphin.

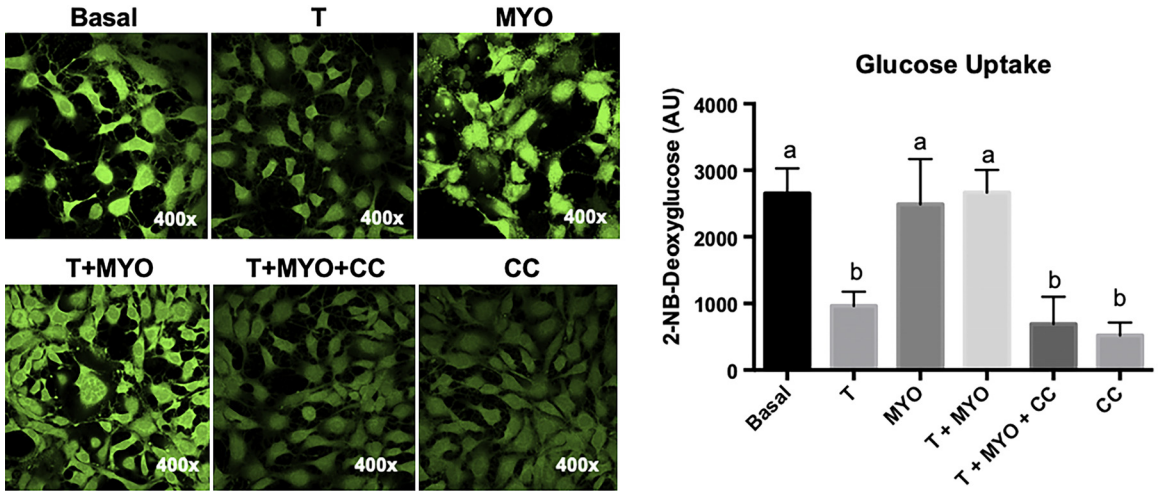

Fig. 7. Effect of myo-inositol (MYO) and role of AMPK on glucose uptake by endometrial cells under hyperandrogenic condition. Human endometrial cells (St-T1b cell line) were treated with 100 nM testosterone (T), 1 mM myo-inositol (MYO), 20 μM Compound C (CC), or combined stimuli for 48 h. Then, cells were incubated with 2-NB-deoxyglucose (2-NBDG) before of an acute insulin stimuli. Fluorescence was detected by Confocal Microscopy. *Left*: microphotographs show the fluorescence (green) from cells under the different conditions. The florescence signal was measured using ImageJ software. The results are presented as arbitrary units (AU) and correspond to the mean ± SE of each group. Three independent experiments were performed for each treatment ($n = 3$). a ≠ b with $P < 0.001$ analyzed by nonparametric Mann-Whitney *U*-test. Image amplification: ×400. CC: Compound C or dorsomorphin; 2-NBDG: 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-deoxyglucose.

which we can mention its conversion to second messengers, such as phosphatidylinositol (4,6) phosphate (PIP2), which is directly related to the insulin signaling pathway (28). PIP3 is generated from PIP2 phosphorylation by phosphatidylinositol kinases in response to insulin action. The increase and availability of the phosphatidylinositol pool would stimulate the activation of the insulin pathway in the steps after PI3K activation. On the other hand, the mechanism of action of MYO could be related to the state and energy balance of the cells in response to the changes generated in the pool of phosphatidylinositol, these changes could be detected by kinases, such as AMPK; in this sense, the levels of p-AMPK Thr-172 were analyzed in the in vitro model under PCOS-obesity conditions in the presence of MYO. As a result, the activation of AMPK significantly decreased in these conditions, but when MYO was added, AMPK phosphorylation was restored, increasing to basal levels in all three conditions. This interesting finding strongly suggests that AMPK could be one of the MYO target proteins, which would explain the insulin-sensitizing effect of this compound reported in clinical studies mentioned before. AMPK is activated with the cytosolic enhancement of AMP/ATP by allosteric activation by AMP and is also activated by phosphorylation of the serine-threonine kinase, LKB1 (29). A probable mechanism by which MYO stimulates the activation of AMPK could be through the enzyme inositol phosphate multikinase (IPMK). Recently, it has been described that this enzyme is capable of activating both AMPK by phosphorylation in its α-subunit and LKB1, constituting a key element for the regulation of AMPK (6, 8). The mechanism by which MYO activates IPMK is through its insertion into the phosphoinositide cycle, which is responsible for regulating the free circulation of phosphatidylinositol and inositol phosphate in the cell. This process promotes phosphorylation and dephosphorylation reactions by different inositol phosphate kinases and phosphatases, including IPMK, one of the key enzymes in the regulation of this cycle (28, 48). It is relevant to mention that the mechanism of action of metformin described by various authors also involves the participation of these proteins (7, 60).

In previous studies, our laboratory has reported that in endometrium, AMPK plays an important role on the regulation of protein expression of GLUT-4, through the activation of its downstream substrate, the myocyte enhancer factor 2A, a transcription factor that is implicated in the regulation of GLUT-4 gene expression (12). In addition, GLUT-4 is one of the main proteins described in the literature that promotes the MYO proposal as an insulin-sensitizing compound; however, this effect has only been previously studied in animal models (14, 64, 65). On the basis of this consideration, expression levels of GLUT-4 were evaluated to understand the mechanism of action and insulin-sensitizing effect of MYO in human endometrial cells under PCOS conditions. In this model, the increase of protein levels of GLUT-4 in cells treated with MYO strongly indicates that the increase in GLUT-4 levels could be in response to increased phosphorylation (activation) of AMPK, caused by the effect of MYO in the same studied conditions. To confirm the participation of MYO on changes in molecules of the insulin pathway, experiments were carried out in the presence of phloridzin, a specific inhibitor for sodium transporters as SMIT-1 (13, 67). The results of the present investigation indicate that the MYO uptake mechanism in endometrial cells is through its SMIT-1 transporter, allowing MYO to exert its metabolic effects on this in vitro model. This finding is relevant, considering that in the present study, we observed that endometrium from insulin-resistant women with obesity and PCOS show a decrease of SMIT-1 levels.

Furthermore, this study shows that MYO's effect on GLUT-4 levels and glucose uptake occurs via a p-AMPK-dependent mechanism in human endometrial cells. Other studies from our group, indicate that the hyerandrogenic condition (characteristic of PCOS) decreases glucose uptake in endometrial cells (44, 54) at 48 h of treatment. The present work

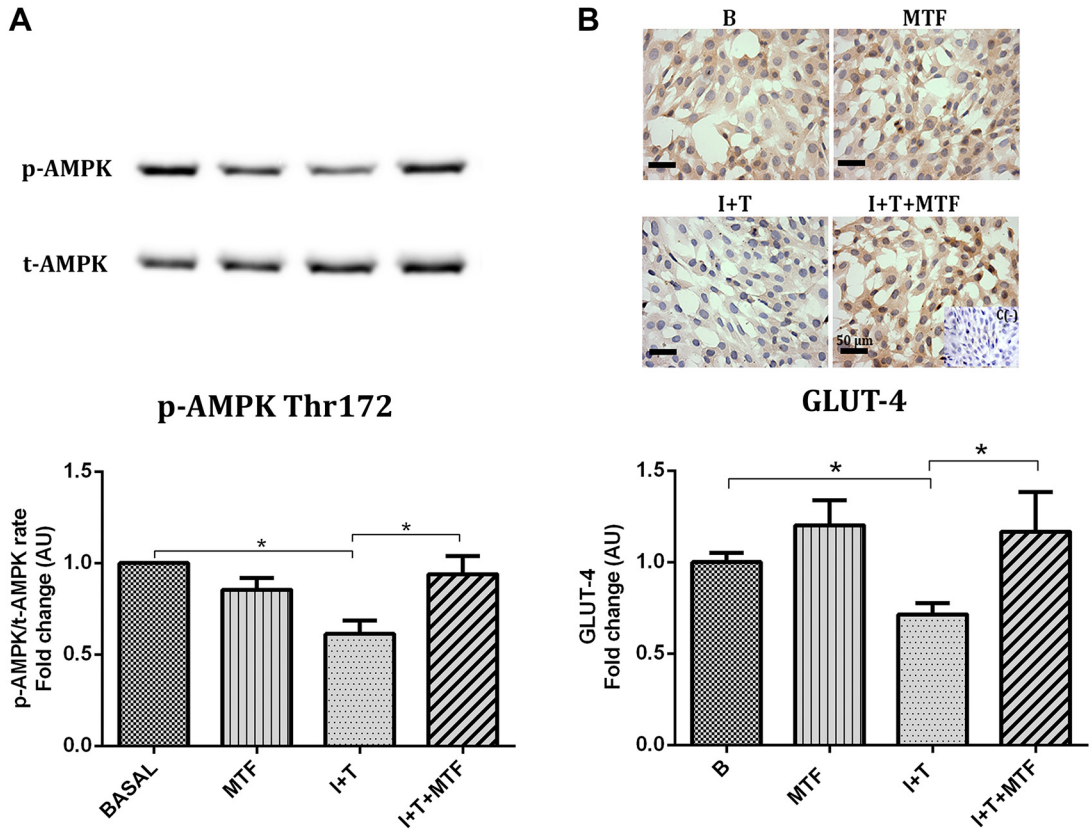

Fig. 8. Effect of metformin on protein levels of p-AMPK Thr-172 and GLUT-4 in human endometrial cells. Human endometrial cells (St-T1b cell line) were treated with 20 μM metformin (MTF), 100 nM insulin (I), 100 nM testosterone (T), or combined stimuli for 24 h. *A*: Western blot analysis for protein p-AMPK Thr-172 levels in St-T1b cells. The data were normalized using the total AMPK protein levels (62 kDa) in each sample and analyzed as fold change with respect to baseline. *B*: microphotographs of St-T1b cells for GLUT-4 protein levels analysis by immunocytochemistry and semiquantitative analysis of the immunopositive staining using the Integrated Optical Density tool of Image Pro Plus 6.0 program. The data were normalized using the basal levels in each experiment and analyzed as fold change with respect to baseline. The results are presented as arbitrary units (AU) and are the mean ± SE of each group. $n = 4$ in duplicate, $^*P < 0.05$ analyzed by nonparametric test and Dunn's post hoc test. Bar: 50 μm. C(-): negative control. GLUT-4, glucose transporter type 4.

confirms that testosterone reduces the capacity of endometrial cells to uptake glucose and that MYO can prevent that reduction. This is noteworthy, considering the failures in insulin signaling that have been observed in women with PCOS without obesity or insulin resistance. Also, MYO alone shows a positive effect on glucose uptake in these cells, but not in GLUT-4 or p-AMPK levels at 24 h (Figs. 3 and 4), suggesting other possible mechanisms induced by MYO to promote glucose uptake that need longer periods of stimulation. Nevertheless, our data indicate that MYO is able to reverse the effect of different stimuli [testosterone (with or without insulin) or TNF-α] on insulin's action in a pathologic endometrium. In addition, the results described so far permit us to propose that the insulin-sensitizing mechanism of MYO in human endometrial cells exposed to PCOS conditions could be through the increase of AMPK activation, which has positive effects on protein levels of GLUT-4, allowing its translocation to the membrane and, thus, increase the glucose uptake in those cells. However, the nonspecific effect of CC to inhibit AMPK activation should be considered (5). In fact, it is known that CC can inhibit other kinases, such as ERK8, MNK1, PHK, MELK, and Src. Interestingly, in an unpublished study from our group, we have shown that kinases like ERK can have a role in the pathophysiology of the endometrium in PCOS women. In this regard, we cannot discard the participation of other proteins (such as ERKs) in the effect of MYO on GLUT-4 expression. Nevertheless, our results show the inhibition of AMPK by CC in endometrial cells, and it is most likely that this effect may induce a decrease in GLUT-4 levels in the presence of MYO. This is in agreement with another study that shows an effective inhibition of AMPK by CC (63).

Finally, to compare the effects of MYO previously described, experiments were performed with metformin, a widely described insulin-sensitizing compound that also has its effect in the endometrial tissue (12, 57, 69). The obtained results clearly show that the response of endometrial cells to the action of metformin is similar and comparable to that obtained with MYO, even when observed the effect of MYO and metformin alone, indicating a positive effect of these drugs on insulin pathway in a pathologic tissue. This is important because, as mentioned, in a study by our laboratory, we show that metformin is capable of increasing AMPK activation and GLUT-4 levels in the endometrium from hyperinsulinemic-PCOS women treated with this drug (12). In addition, results obtained from the present study are in agreement with results observed in clinical studies that compared the effects of MYO and metformin on women with PCOS. These studies have shown an improvement in metabolic features, such as an insulin sensitivity profile, glucose levels, and BMI in the both treated groups. However, an important consideration is the better

tolerance by women to the MYO compared with metformin treatment (21). Consequently, the results obtained in this study allow us to conclude that MYO exhibits insulin-sensitizing effects in women with insulin-resistance and a proinflammatory PCOS endometrium, through SMIT-1. Once in the intracellular compartment, MYO could cause metabolic changes in the cells, stimulating the activation of AMPK and increasing protein levels of GLUT-4 and the glucose uptake by these cells.

These findings are relevant because of the endometrium's need for a high-energy supply in order to fulfill its reproductive functions. More importantly, MYO is a potential insulin-sensitizing agent that could be used as an effective treatment option in women with PCOS by improving their metabolic parameters as well as their reproductive status.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.O., F.P.-P., and M.V. conceived and designed research; H.C.-C., L.O., and I.T.-P. performed experiments; H.C.-C., L.O., and F.P.-P. analyzed data; H.C.-C., L.O., F.P.-P., and M.V. interpreted results of experiments; H.C.-C. and L.O. prepared figures; H.C.-C. and L.O. drafted manuscript; H.C.-C., L.O., and M.V. edited and revised manuscript; L.O., F.P.-P., I.T.-P., C.R., and M.V. approved final version of manuscript.

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