*Original Article*

Reproductive Sciences
2017, Vol. 24(7) 1079-1087

DOI: 10.1177/1933719116678689
journals.sagepub.com/home/rsx
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# Molecular Mechanisms of Androstenediol in the Regulation of the Proliferative Process of Human Endometrial Cells

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## Abstract

Proliferation in endometria of women with polycystic ovarian syndrome (PCOS) is increased, similar to the biosynthesis of androstenediol (estrogenic metabolite). As previously shown, in human endometrial cells, androstenediol increases CYCLIN D1 levels and KI67 and decreases P27 content. The objective of the present investigation was to determine the mechanisms by which androstenediol promotes endometrial cell-cycle progression. Estrogen receptor α (ERα) activation and changes in CYCLIN D1 and P27 levels were evaluated by Western blot in T-HESC and St-T1b endometrial cell lines, using receptor antagonists; activation of PI3K-protein kinase B (AKT) and mitogen-activated protein kinases-extracellular signal–regulated kinases (MAPK-ERK)1/2 pathways was evaluated using PI3K, MAPK/ERK kinase (MEK)1/2, and RNA-polymerase II inhibitors. The data showed that androstenediol treatment significantly increases CYCLIN D1 and decreases P27 levels through ERα activation ($P < .05$). In addition, an increase in AKT/ERK1/2 phosphorylations was determined ($P < .05$). In the presence of RNA-polymerase II inhibitor, phosphorylation of AKT/ERK1/2 decreased ($P < .05$), meaning that endometrial cells need transcriptional activity to activate the kinases involved. It was also observed that PI3K action is required for P27 and CYCLIN D1 changes. Therefore, the action of androstenediol in endometria depends on PI3K-AKT and MAPK-ERK1/2 pathways activation, together with cell transcriptional machinery. This could be of clinical significance, as in pathologies such as PCOS, increased endometrial levels of androstenediol together with a high prevalence of endometrial hyperplasia and adenocarcinoma have been reported.

**Keywords**
endometria, PCOS, MAPK, PI3 K, androstenediol

## Introduction

Several investigations indicate that androstenediol is a steroid with estrogenic activity[1-5] that can be highly metabolized from the precursor dehydroepiandrosterone in several tissues including the endometrium.[6] A recent report described higher concentration of androstenediol in endometria from women with polycystic ovarian syndrome (PCOSE) compared to controls.[7] Furthermore, the same investigation showed that androstenediol exerts a positive action on the proliferation process of endometrial cells, through the increase in CYCLIN D1 and a decrease in P27 levels. On the other hand, high serum concentrations of androstenediol have been detected in women with endometrial carcinoma compared to control women, suggesting a potential role of this steroid in the pathophysiology of this carcinoma.[8]

Women with polycystic ovarian syndrome (PCOS) exhibit endometrial alterations, including an increase in hyperplasia and adenocarcinoma occurrence.[9-13] Several studies report an imbalance in proliferation-apoptosis processes in PCOSE, favoring progression of the cell cycle and mitosis, and decreasing apoptosis.[14-18] In fact, this pathological endometrial tissue presents high CYCLIN D1 (positive regulator of the cell cycle) and decreased P27 (negative regulator of the cell cycle) levels.[7,17] Additionally, in this tissue, an increased sensitivity to estrogens has been detected[19,20]; probably, the estrogen sensitivity is related to the augmented cell proliferation observed in PCOSE.

Two pathways highly related with the proliferation process are Mitogen-Activated Protein Kinases (MAPK) (Extracellular signal–regulated kinases [ERK] 1/2) and PI3K-Protein Kinase

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B (AKT). The MAPK pathway may be activated by estrogen and is capable of positively regulating CYCLIN D1 levels, by increasing its half-life.[21] Besides, evidences suggest that estrogens may induce transcription of CYCLIN D1 in a direct manner, as well as, indirectly through the activation of PI3K-AKT pathway.[22,23] Phosphorylated AKT at serine 473 (p-AKT) increases glycogen synthase kinase 3 beta (GSK3β) phosphorylation and thereby its inactivation; in turn, GSK3β may phosphorylate and promote the degradation of CYCLIN D1. Therefore, p-AKT favors in an indirect manner a longer half-life of CYCLIN D1.[24] Meanwhile, estrogens are able to reduce the amount of P27 that is associated with CYCLIN E/CDK2, through the PI3K-AKT pathway, this phosphorylation increases P27 degradation by proteasome and also it inhibits the entry of the molecule into the nucleus, which prevents the function of cell cycle negative regulator.[21,22,25] In this context, previous reports from our laboratory indicate that PCOSE has high levels of p-AKT (belonging to PI3K-AKT pathway) and elevated protein levels of RAS (belonging to MAPK pathway).[17]

Accordingly, in the present research, we evaluated a potential mechanism through which androstenediol could be acting, by generating changes in cell cycle regulatory molecules. This knowledge could increase our understanding of the disturbances in endometrial cell homeostasis present in the PCOS condition. Therefore, in the present investigation, using an in vitro model, we evaluated the action and possible mechanism of androstenediol on the generation of changes in cell cycle regulatory molecules.

## Experimental

### Materials

The used antibodies for Western blot were β-ACTIN (A 5441, Sigma, St. Louis, MO, USA); CYCLIN D1 (#2978 Cell Signaling Technology, Danvers, Massachusetts); P27 (#3686 Cell Signaling Technology); pERK1/2 (#sc-16982 Santa Cruz Biotechnology, California, USA); ERK1/2 (#9102 Cell Signaling Technology, Danvers, Massachusetts, USA); p-AKT (#9271 Cell Signaling); AKT (#9272 Cell Signaling Technology) and the anti-rabbit secondary antibody (#074-1806 KPL, Gaithersburg, Maryland). The inhibitors used were LY-294.002 hydrochloride (#L9908-1 mg, Sigma-Aldrich Co, St Louis, Missouri), U-0126 (#sc-222395A, Santa Cruz Biotechnology), 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy) phenol]-1H-pyrazole dihydrochloride (MPP) (#1991, Tocris Bioscience, London, United Kingdom) and α-amanitin (#4025, Tocris Bioscience).

### Cell Culture

The human endometrial stromal cell lines T-HESC (ATCC, CRL-4003)[26] and St-T1b[27] were used in this study. Both cell lines were obtained from primary cultures of endometrial stroma, T-HESC from the midsecretory phase and St-T1b from the proliferative phase. Both cell lines were immortalized with telomerase gene (hTERT) transfection using a retroviral system.[26,27] As previously reported,[7] the cells were propagated in DMEM HAM F12 medium without phenol red (Sigma-Aldrich Co), supplemented with 1.5 g/L sodium bicarbonate, 1× Insulin–Transferrin–Selenium (ITS) mixture, 500 ng/mL puromycin, 10% fetal bovine serum (FBS), and cultured at 37°C and 5% $CO_2$. In experiments, cells were cultured for 24 hours at a ratio of 800 000 cells/plate and then were treated for 20 minutes or 48 hours with 100 nM of androstenediol, 17β-estradiol or testosterone in DMEM HAM F12 without serum. Nontreated cells correspond to cell under basal conditions in each experiment. Androstenediol, estradiol, and testosterone stimuli were performed at 100 nM each, as previously determined.[7,28,29] According to the literature, LY-294.002 hydrochloride, a highly selective inhibitor of PI3 K, was used at a concentration of 10 μM.[28,30] While, U-0126 a highly selective noncompetitive inhibitor of MAPK/ERK Kinase (MEK)1/2, was used at 50 nM.[31] The antagonist of estrogen receptor α (MPP) and the inhibitor of RNA polymerase II (α-amanitin) were used at 1 μM and 0,1 μM, respectively.

### Western Blotting

The cells cultured in the presence of different stimuli were lysed on ice using RIPA buffer (50 mM Tris, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton, and 0.1% sodium dodecyl sulfate [SDS]) with a protease inhibitor cocktail (Roche, Basel, Switzerland). The samples were centrifuged at 10 000*g* for 20 minutes at 4°C. Protein concentrations were determined using the kit BCA protein assay (Pierce, Washington, USA). Forty micrograms of total protein for each sample was denatured, electrophoresed in 10% SDS-PAGE gel, and transferred to nitrocellulose membrane (Bio-Rad, California, USA). Then, membranes were blocked to unspecific binding using 10% skim milk in tween tris-buffered saline (TTBS) (20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20) for 1hour. Subsequently, the blots were washed in TTBS and incubated overnight at 4°C with antibodies against CYCLIN D1 (1:300), P27 (1:500), p-AKT (1:300), AKT (1:500), p-ERK1/2 (1:100), and ERK1/2 (1:500). Incubation with antibody to β-ACTIN (1:20 000) was performed for 1 hour at room temperature. Incubation with secondary antibody was performed for 1 hour at room temperature with anti-rabbit IgG (1:5000) or mouse (1:10 000) coupled to HRP. After washing, it was developed with a chemiluminescence system (Perkin Elmer, Waltham, Massachusetts). The intensities of the bands were quantified by densitometry using the software UN-SCAN-IT version 6.1. Results were expressed as a ratio of the densitometry of the protein of interest versus those of β-ACTIN. In experiments in cells, it was expressed as fold basal change.

### Statistical Evaluation

Each experiment was performed 3 times in duplicate. Comparisons between groups were performed by Mann-Whitney test. *P*-values < .05 were considered significant. Statistical tests were done using Stata 9 and Graph Pad Prism 4.0.

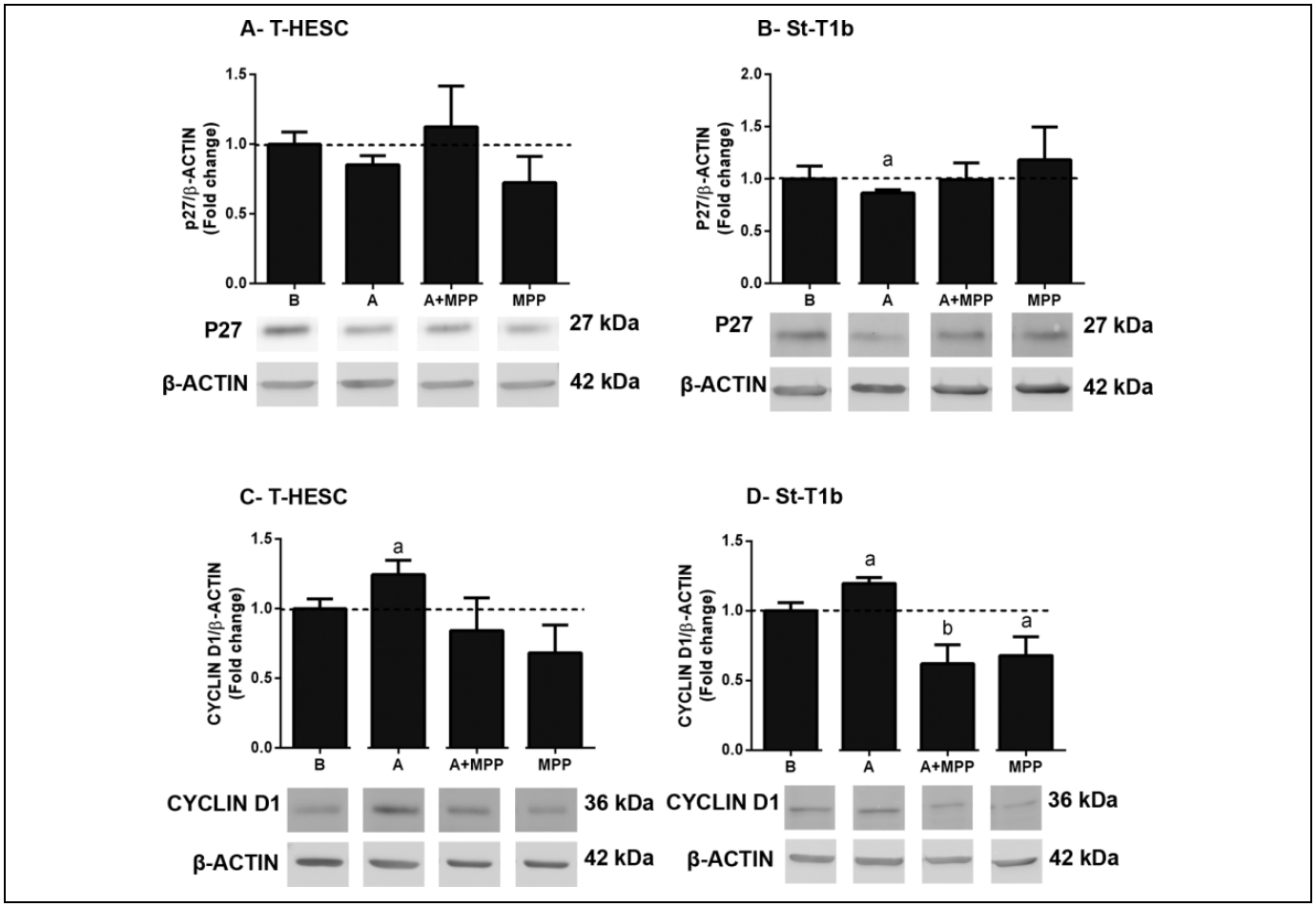


**Figure 1.** Semiquantification of protein levels of P27, CYCLIN D1, and β-ACTIN in human endometrial cell lines. Panel A, P27 in T-HESC. Panel B, P27 in St-T1b. Panel C, CYCLIN D1 in T-HESC. Panel D, CYCLIN D1 in St-T1b. Both cell lines were treated with estrogen receptor α antagonist MPP dihydrochloride for 30 minutes and then stimulated with androstenediol for 48 hours. A indicates androstenediol; B basal; A + MPP, androstenediol plus MPP. a: $P < .05$ versus B; b: $P < .05$ versus A. Results are expressed as mean ± standard error of the mean (SEM) fold change; n = 3 in duplicate.

## Results

### *Levels of P27 and CYCLIN D1 Under Stimulus of Androstenediol and Estrogen Receptor Antagonist in T-HESC and St-T1b Cells*

To assess whether androstenediol could affect the levels of regulatory molecules of the cell cycle through the estrogen receptor, T-HESC and St-T1b cells were treated with androstenediol and an estrogen receptor antagonist (MPP) for 48 hours (Figure 1). Results of P27 levels in T-HESC and St-T1b cells showed that androstenediol treatment decreased the protein content around 15% in both cell lines, being significant in St-T1b ($P = .03$), in agreement with our previous data.[7] No significant effect was obtained when cells were treated with androstenediol and the estrogen receptor antagonist, MPP (Figure 1A and B). On the other hand, protein levels of CYCLIN D1 increased with androstenediol stimulus, 25% in T-HESC ($P = .03$) and 20% in St-T1b ($P = .03$). Nevertheless, this increase disappears when cells were treated with the antagonist of estrogen receptor α, detecting significant differences in CYCLIN levels in cells stimulated with androstenediol, with and without MPP in St-T1b ($P = .002$; Figure 1C and D). Additionally, St-T1b cells stimulated with MPP alone showed decreased protein levels compared to basal condition ($P = .04$; Figure 1D).

### *Phosphorylation Levels of AKT at Serine 473 (p-AKT) in T-HESC and St-T1b Cells Stimulated With Steroids*

To further evaluate the mechanism by which androstenediol affects the cell cycle, the phosphorylated fraction of AKT was determined under steroid stimulation. In T-HESC, androstenediol and estradiol treatment for 48 hours showed an increase of p-AKT/AKT levels (460%; $P = .004$ and 300%; $P = .004$, respectively; Figure 2A). Similar data were obtained in St-T1b, where androstenediol and estradiol exerted an increment of p-AKT/AKT levels (80%; $P = .004$ and 48%; $P = .03$, respectively; Figure 2B). On the other hand, 48-hour testosterone treatment of T-HESC did not generate significant differences on the phosphorylation rate of AKT compared to basal

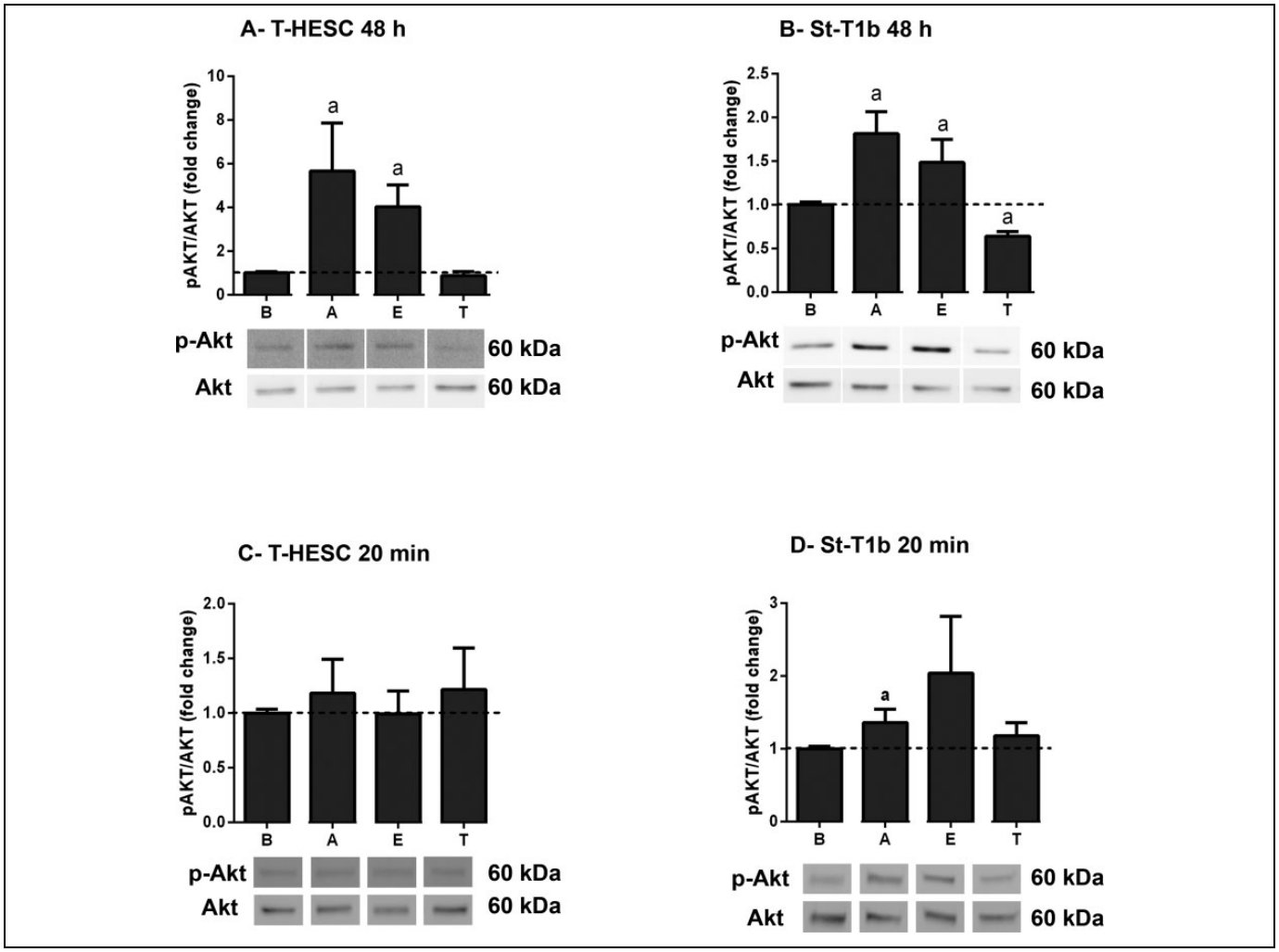


**Figure 2.** Semiquantification of the protein levels of AKT phosphorylated in serine 473 and of total AKT in human endometrial cell lines. Panel A, T-HESC treated for 48 hours. Panel B, St-T1b treated for 48 hours. Panel C, T-HESC treated for 20 minutes. Panel D, St-T1b treated for 20 minutes. A indicates androstenediol; B, basal; E, 17β-estradiol; T, testosterone. a: $P < .05$ versus B. Results are expressed as mean ± standard error of the mean (SEM) fold change; n = 3 in duplicate.

condition ($P = .34$). However, in St-T1b cells, a significant decrease of p-AKT protein levels was exerted under testosterone treatment ($P = .006$; Figure 2A and B).

Furthermore, to evaluate whether changes in the phosphorylation of AKT occurs also in acute conditions, cells were evaluated under steroid stimuli for 20 minutes. The results showed that short stimuli did not generate changes in the levels of phosphorylation of AKT in T- HESC ($P > .05$; Figure 2C and D), whereas, in St-T1b cells, androstenediol generated an increase of p-AKT compared to basal condition ($P = .05$; Figure 2).

## *Evaluation of the Protein Levels of Phosphorylation of ERK1/2 at Threonine 202 and Tyrosine 204 in T-HESC Cells Stimulated With Steroids for 48 Hours*

The following results correspond to T-HESC cultures, as in general, the data obtained from both cell lines are quite similar. The data obtained from St-T1b cultures are shown in Supplemental Figures 1–3.

Based on the relevance of MAPK pathway in the proliferative process, T-HESC cultures were stimulated by steroids for 48 hours and the phosphorylated levels of ERK1/2 were evaluated. The evaluation of phosphorylated ERK fraction was separately determined as ERK1 and ERK2. Androstenediol increased the p-ERK1 content (38%; $P = .004$), whereas, the other stimuli did not generate changes compared to basal (Figure 3). Meanwhile, a significant increase of p-ERK2 was obtained under androstenediol treatment (58%; $P = .004$), similar to the result obtained with estradiol treatment (70%; $P = .02$) (Figure 3). Nevertheless, the treatment of T-HESC cells for 20 minutes showed that none of the stimuli generated significant changes in both ERK1 and ERK2 (data not shown).

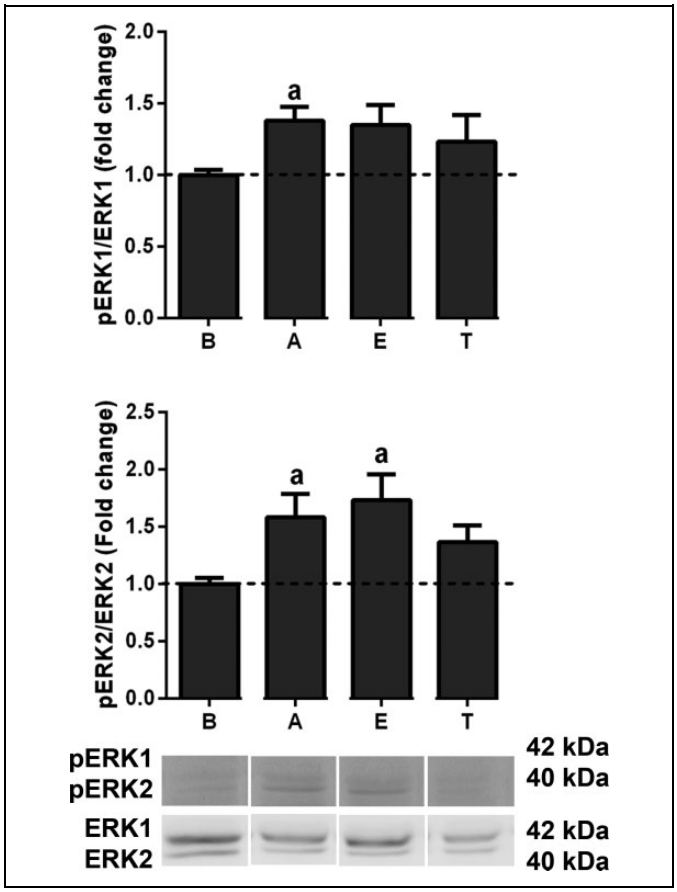


**Figure 3.** Representative Western blot and semiquantification of ERK1/2 phosphorylated in threonine 202 and tyrosine 204, and total ERK1/2 in human endometrial T-HESC cell line treated for 48 hours. A indicates androstenediol; B, basal; E, 17β-estradiol, T, testosterone. a: $P < .05$ versus B. Results are expressed as mean ± standard error of the mean (SEM) fold change; n = 3 in duplicate.

### *Effect of α-Amanitin and Androstenediol in p-AKT and p-ERK1/2 Levels in T-HESC Cells Stimulated for 48 Hours*

In order to determine whether the significant changes in p-AKT and p-ERK1/2 protein levels obtained with stimuli for 48 hours require the transcriptional machinery of the cell, an antagonist of RNA polymerase II (α-amanitin) was used. The increased levels of AKT phosphorylation under androstenediol treatment (Figure 2) are inhibited by the addition of α-amanitin to cell cultures ($P = .09$; Figure 4A). Similarly, the increased phosphorylation rate of ERK1 and ERK2 obtained when cells were treated with androstenediol (Figures 3), disappears when using RNA polymerase II inhibitor, α-amanitin (Figure 4B).

### *Effects of Androstenediol, PI3K Inhibitor (LY-294.002), and MEK1/2 Inhibitor (U-0126) in P27 Levels and CYCLIN D1 in T-HESC Cells*

Subsequently, the importance of PI3K-AKT or MAPK (ERK 1/2) pathways in the regulation of the cell cycle was determined using LY-294.002 and U-0126 inhibitors. For this purpose, P27 and CYCLIN D1 protein levels were evaluated. As to P27 levels, cell treatment with androstenediol plus LY-294.002 exerted an increase of 79% compared to cells stimulated only with the steroid ($P = .007$; Figure 5A), whereas, no significant differences in P27 levels compared to cells stimulated only with the steroid were generated under the treatment of U-0126 together with androstenediol (Figure 5B). On the other hand, the increased levels of CYCLIN D1 obtained with androstenediol stimulus, diminished significantly when cells were treated with LY-294.002 plus androstenediol ($P = .008$; Figure 5C). The use of U-0126 in addition to androstenediol generated similar results to basal conditions with respect to CYCLIN D1 protein levels. In contrast, androstenediol stimulus increased CYCLIN D1 levels ($P = .03$; Figure 5D).

## Discussion

The endometria from women having PCOS are characterized by a disruption in their hormonal environment and the regulatory mechanisms underlying the cell cycle, such as the proliferation process.[7,9-13,17] Therefore, to better understand the regulation of cell proliferation and to identify molecular mechanisms in endometrial cells, an in vitro model was used in the present study, where cells from 2 cell lines were treated with high concentration of androstenediol, partially resembling the PCOS condition as previously reported.[7] It is worth noting that the use of primary cell cultures of endometria from women with PCOS is unfeasible, mainly because of the small amount of endometrial tissue obtained from these patients by Pipelle Suction Curette.

As known, androstenediol is originated from dehydroepiandrosterone (DHEA), reaction catalyzed by the enzyme 17β-hydroxysteroid dehydrogenase (HSD).[32,33] Experimental evidences show that in the endometrium from women with PCOS, there is an increased metabolism of DHEA to androstenediol[6]; additionally, those tissues have higher androstenediol concentration compared to control endometria.[7] Therefore, the relevance to evaluate the action of androstenediol resides in that this steroid is eventually a good candidate to modulate the proliferative processes based on its estrogenic activity.[1,5,8]

As known, estrogens could bind to estrogen receptors and activate cell cycle progression favoring cell proliferation.[34] In this context, PCOSE has high levels of estrogen receptor α and coactivators,[20] which gives a high sensitivity to estrogen action that could potentiate the cell proliferation process. Reports described androstenediol to be an androgen given its structure of 19 carbons; however, from the point of view of its activity, it is capable to bind and activate estrogen receptors α and β.[3,5,35] Furthermore, increased levels of serum androstenediol have been found in women with endometrial carcinoma, attributing to this molecule a role in the development of endometrial pathology.[8] However, there are no studies to our knowledge linking this steroid with endometrial hyperplasia.

Previous studies from our group indicated that androstenediol added to endometrial stromal cell cultures increases the

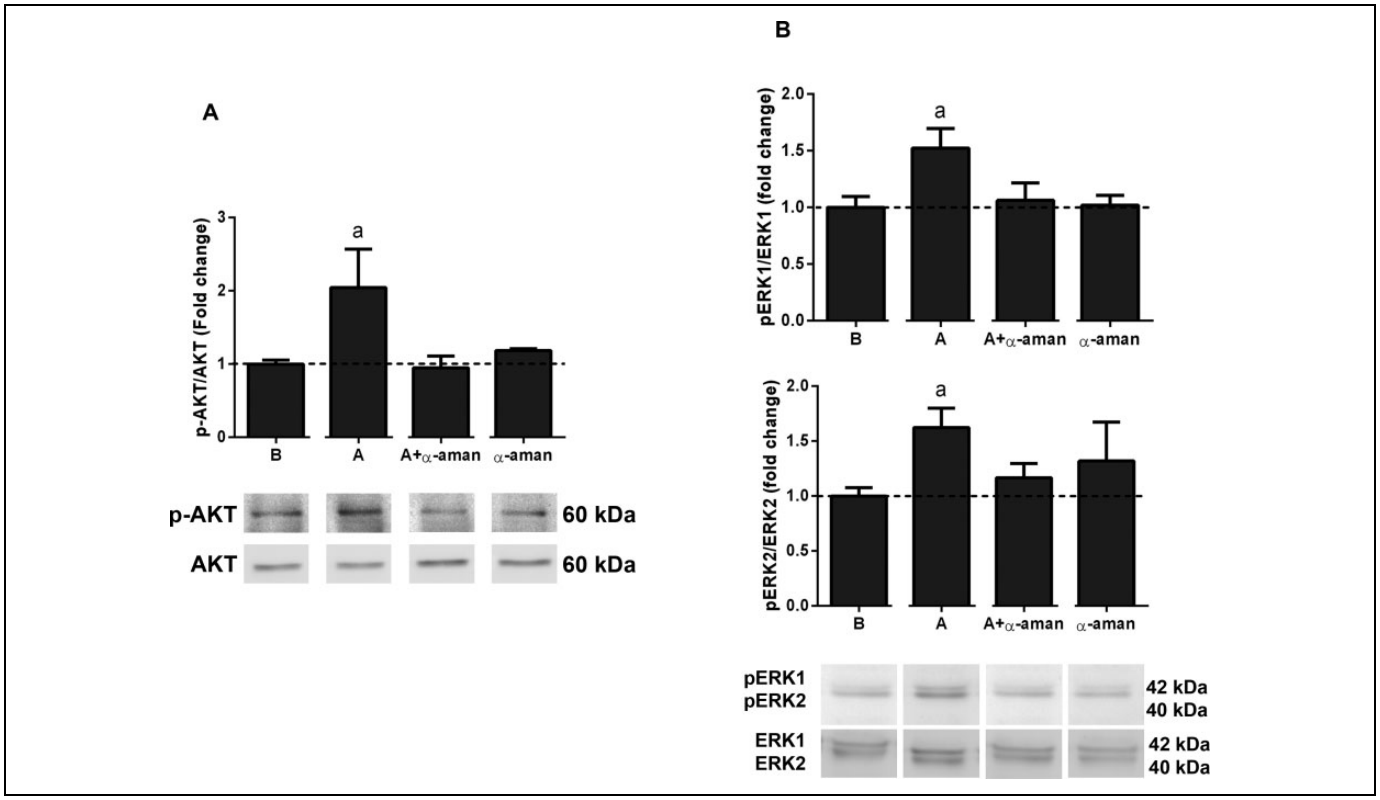


**Figure 4.** Semiquantification of protein levels of AKT phosphorylated in serine 473/total AKT (Panel A) and ERK1/2 phosphorylated in threonine 202 and tyrosine 204/total ERK1/2 (Panel B) in human endometrial cell line T-HESC. Cells were treated with RNA polymerase II inhibitor, α-amanitin (α-aman) for 30 minutes and then stimulated with androstenediol for 48 hours. A indicates androstenediol; B, basal; A + α-aman, androstenediol plus α-aman. a: $P < .05$ versus B. Results are expressed as mean ± standard error of the mean (SEM) fold change; n = 3 in duplicate.

percentage of cells in cycle,[7] similarly to the results obtained in endometrial tissues from women with PCOS, where an increased percentage of Ki67 positive cells was reported.[14] Moreover, it is remarkable that endometrial cells stimulated with androstenediol and estradiol behave similarly according to molecules related to cell proliferation (CYCLIN D1 and P27).[7] Therefore, androstenediol could act as a molecule with estrogenic activity in the conditions of the cell culture. To confirm this proposition, in the present investigation when we used estrogen receptor α antagonist (MPP dihydrochloride), the effects of androstenediol on P27 and CYCLIN D1 were inhibited (Figure 1). These findings could indicate that effects of androstenediol would be exerted through this subtype of estrogen receptor, which is highly expressed in PCOSE according to our previous report.[19] It has been described that estrogen receptor α has a role in increasing cell proliferation, whereas receptor-β would modulate receptor α, decreasing proliferation[36]; therefore, it was important to focus on the effect of androstenediol in estrogen receptor α.

Notably, in our study, we used androstenedione and testosterone stimuli together showing no change on proliferation compared to the basal condition.

In the present study, high concentrations of androstenediol for extended periods (48 hours) of time seem to be essential to induce a cellular response (Figure 2); similarly, a chronically elevated intratissular level of androstenediol in PCOSE was described.[7] In these conditions occurs the activation of pathways associated with proliferation, as PI3K-AKT or MAPK (ERK1/2; Figures 2 and 3) and the induced changes in protein levels of regulatory cell cycle molecules, such as CYCLIN D1 and P27, positive and negative regulator of the cell cycle, respectively.[7]

Apparently, the 48 hours of stimulus with androstenediol or estradiol allows synthesis or the activation of certain molecules, which in turn activate PI3K-AKT and MAPK (ERK1/2) pathway; therefore, it was found to augment phosphorylation of AKT and ERK1/2 in long stimuli but not in short ones (Figures 2 and 3). Even more, experiments performed with RNA polymerase II inhibitor (α-amanitin) showed a blockade of the effect of androstenediol in phosphorylation of AKT and ERKs (Figure 4). It is most likely that transcription of intermediary molecules stimulated by androstenediol is necessary for the activation of PI3K-AKT and MAPK (ERK1/2) pathways. One of these intermediary molecules could be the vascular endothelial growth factor (VEGF), whose levels are high in PCOSE,[37] together with the elevated concentration of androstenediol.[7]

Furthermore, the relevance of the transduction signal pathways in the modulation of cell cycle regulatory molecules was evidenced by the use of inhibitors of the activity of PI3K

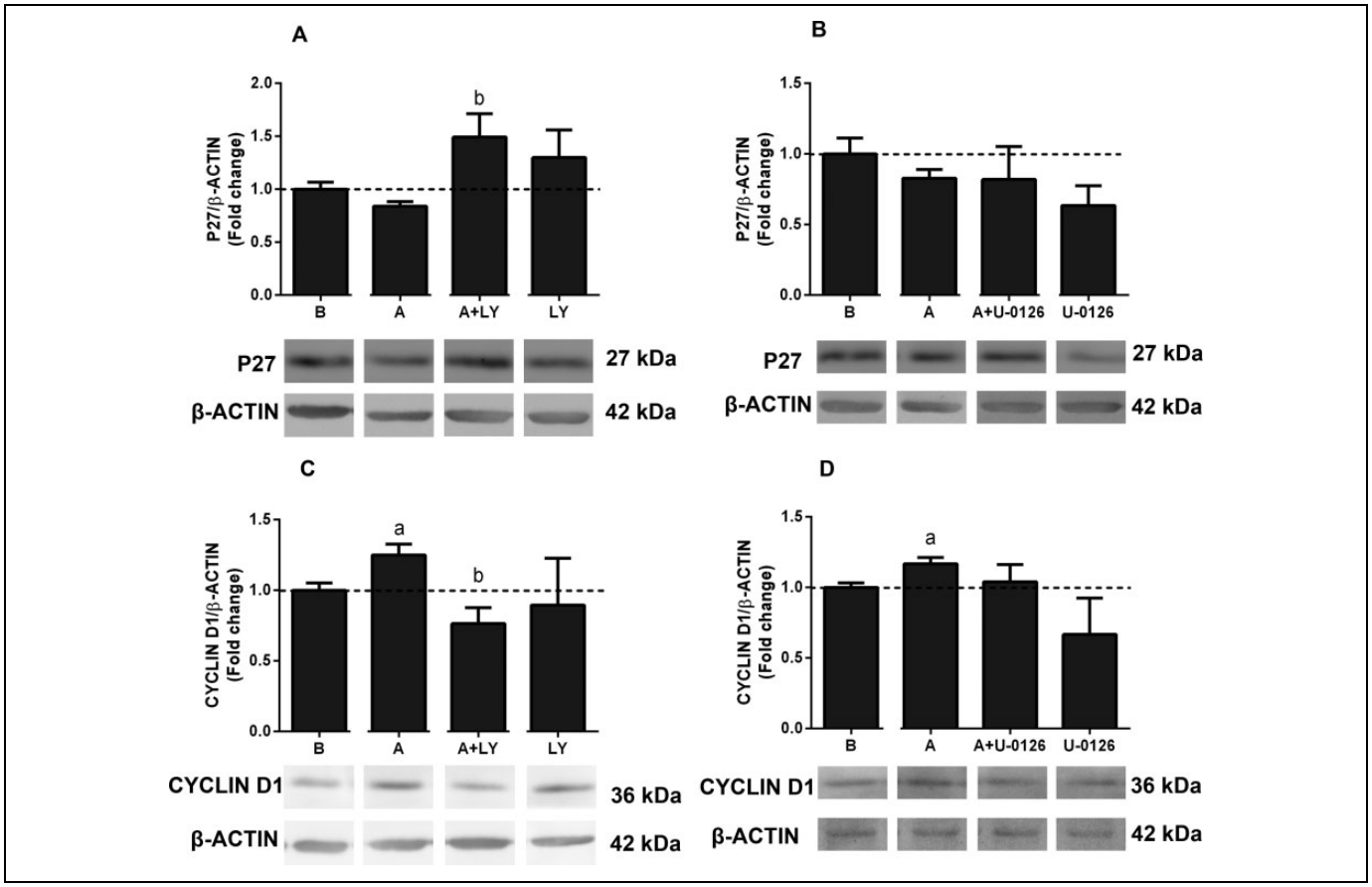


**Figure 5.** Semiquantification of protein levels of P27, CYCLIN D1, and b-ACTIN in human endometrial cell line T-HESC. Cells were treated with LY-294.002 hydrochloride (inhibitor of PI3K; LY) or U-0126 (inhibitor of MEK1/2) for 30 minutes and then stimulated with androstenediol for 48 hours. Panel A: P27 in cells treated with LY, Panel B: P27 in cells treated with U-0126, Panel C: CYCLIN D1 in cells treated with LY, and Panel D: CYCLIN D1 in cells treated with U-0126. A indicates androstenediol; B, basal; A + LY, androstenediol plus LY-294.002; A + U-0126, androstenediol plus U-0126. a: $P < .05$ versus B; b: $P < .05$ versus A. Results are expressed as mean ± standard error of the mean (SEM) fold change; n = 3 in duplicate.

(LY-294.002 hydrochloride) and MEK 1/2 (U-0126), where it was determined that androstenediol is particularly important in regulating CYCLIN D1 content and to a lesser extent P27 (Figure 5). Therefore, PI3K-AKT and MAPK (ERK1/2) pathways would be required for the regulation of cell cycle proteins, P27 and CYCLIN D1 (Figure 5), in agreement to the data obtained previously in PCOSE.[7]

In our experimental conditions, testosterone had an antiproliferative role contrasting with androstenediol or estradiol (Figures 2 and 3). This point is of importance in our model in which women with PCOS are hyperandrogenic.[38] Therefore, endometria of women with PCOS present high testosterone levels, with antiproliferative action, and high androstenediol levels, with pro-proliferative action. The increase of the proliferative response of this tissue would be caused, in part, by the imbalance of these 2 opposite forces, dominating the effect of androstenediol and/or estrogens.[7]

In summary, the data of the present investigation propose that androstenediol increases the activation of PI3K/AKT and MAPK (ERK1/2) pathways, through estrogen receptor α. Both signal transduction pathways could modulate the levels of the cell cycle regulators P27 and CYCLIN D1. This mechanism may be involved in the pathophysiology of the development of endometrial hyperplasia and/or adenocarcinoma detected in a subpopulation of women with PCOS.

## Acknowledgments

The authors thank the laboratory team for its collaboration. The authors are grateful to the women who donated tissue.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by FONDECYT (grant numbers 1100299, 1130053); Support Grant CONICYT Doctoral Thesis (grant number 24121153). CONICYT Doctoral National Fellowship (grant number 21100275).

## Supplementary Material

Supplementary material is available for this article online.

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