Ulipristal Acetate Modulates the Expression and Functions of Activin A in Leiomyoma Cells

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Ulipristal Acetate Modulates the Expression and Functions of Activin A in Leiomyoma Cells

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Abstract

Uterine leiomyoma is the most common benign gynecological tumor in women of reproductive age and represents the single most common indication for hysterectomy. A development of new treatments is necessary for a medical management, and in this direction, several hormonal drugs are under investigation. Ulipristal acetate (UPA; a selective progesterone receptor modulator) is considered as one of the most promising because progesterone has a critical role in development and growth of uterine leiomyoma. The effect of steroids is partly mediated by growth factors like activin A which increases extracellular matrix expression contributing to the growth of leiomyoma. The present study aimed to test whether UPA acts on leiomyoma cells affecting expression and functions of activin A system. Cultured myometrial and leiomyoma cells were treated with UPA, and messenger RNA (mRNA) expression levels of activin A (inhibin βA [INHBA] subunits), its binding proteins (follistatin [FST] and FST-related gene), and its receptors (activin receptor-like kinase 4 [ALK4], activin receptor type [ActR] II, and ActRIIB) were evaluated. The effect of UPA on activin A modulation of fibronectin and vascular endothelial growth factor A (VEGF-A) mRNA expression in cultured myometrial and leiomyoma cells was also studied. Ulipristal acetate decreased INHBA, FST, ActRIIB, and Alk4 mRNA expressions in leiomyoma cultured cells. In addition, UPA was able to block the activin A-induced increase in fibronectin or VEGF-A mRNA expression in myometrial and in leiomyoma cultured cells. The present data show that UPA inhibits activin A expression and functions in leiomyoma cells, and this may represent a possible mechanism of action of the drug on uterine leiomyoma.

Keywords

ulipristal acetate, uterine fibroids, uterine leiomyoma, activin, follistatin, activin receptors, fibronectin, VEGF-A

Introduction

Uterine leiomyoma, or fibroid, is the most common benign gynecological neoplasia in women. It is a common cause of infertility and a leading indication for hysterectomy.[1] Leiomyoma is characterized by increased myometrial cell proliferation and by an excessive deposition of extracellular matrix (ECM) such as fibronectin[2,3]; its growth is dependent on ovarian sex steroid hormonal activity through local cytokines and growth factors.[3,4] Considering the central role of estrogens and progesterone in leiomyoma growth, gonadotropin-releasing hormone analog (leuprolide acetate) was approved by the United States Food and Drug Administration for uterine fibroids treatment. It is concomitantly used with iron therapy for the preoperative hematologic improvement in patients with anemia caused by uterine fibroids. Several randomized, placebo-controlled studies showed that leuprolide acetate significantly reduces uterine and myoma volume, with improved fibroid-related symptoms.[5-7]

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In addition, several potential therapies, like mifepristone (antiprogestin),[8] and selective progesterone receptor modulators (SPRMs) such as asoprisnil,[9] CDB-2914 (also known as ulipristal acetate [UPA]),[10-13] and CDB-4124 (also known as telapristone acetate),[14] have shown excellent therapeutic efficiency during the course of clinical trials (http://www.clinicaltrials.gov).

The CDB-2914 or UPA is considered as the closest therapeutic option. Ulipristal acetate (17a-acetoxy-11b-[4-N, N-dimethylaminophenyl]-19-norpregna-4,9-diene-3,20-dione) is a SPRM that binds to progesterone receptors A and B with high affinity.[15,16] The binding and antagonist potency of UPA with the glucocorticoid receptor is significantly reduced compared to mifepristone.[16,17] It reversibly blocks the progesterone receptor in its target tissues (uterus, cervix, ovaries, and hypothalamus) and acts as a potent, orally active antiprogestin.[18] In 2012, UPA (5 mg) was approved by European Medicines Agency for the treatment of moderate to severe symptoms limited to 3 months and presurgery. Some randomized clinical trials have shown that UPA significantly reduces leiomyoma and uterine volume, improving leiomyoma-related symptoms and quality of life without serious complications.[10-13] It is a considerable fact that an improved understanding of the molecular mechanisms of UPA action is of paramount importance for the development of an innovative therapeutic strategy, but the precise mechanisms by which UPA causes leiomyoma regression remain to be fully elucidated.

Activin A, dimer of 2 inhibin βA (INHBA) subunits, is a growth factor belonging to the transforming growth factor β (TGF-β) family, and its expression and role have been documented in rat uterine tissue, human myometrial cell lines,[19] and human myometrial and leiomyoma tissue explants.[20] Activin A is highly expressed in leiomyoma tissue compared to the adjacent healthy myometrium[20] and has a cytostatic effect on primary myometrial cells but not in leiomyoma cells while increases fibronectin expression in leiomyoma cells demonstrating its potential fibrotic role in leiomyoma growth.[21]

Activin A system is quite elaborate and includes 2 binding proteins, follistatin (FST) and follistatin-related gene (FLRG),[22-24] and a complex receptor signaling. Two transmembrane serine/threonine receptor kinases are classified as type II (activin receptor type [ActR] II and ActRIIB) and type I (ActRIB; activin receptor-like kinase 4 [ALK4]). The ActRII binds activin with high affinity and facilitates activin binding to ALK4.[25] In the receptor complex, the constitutively active type II receptor kinase phosphorylates ALK4, and this phosphorylation leads to activation of the ALK4 kinase.[26] Once activated, ALK4 phosphorylates Smad2 and Smad3, which form part of the postreceptor signal transduction system.[27]

The present study aimed to evaluate the effects of UPA on the messenger RNA (mRNA) expression of activin A system (INHBA, FST, FLRG, ActRII, ActRIIB, and ALK4) in cultured myometrial and leiomyoma cells. Since activin A has a fibrotic role in leiomyoma[21] and in different biological systems modulates vascular endothelial growth factor (VEGF) expression,[28] a possible effect of UPA in modulating the activin A-induced expression of fibronectin and VEGF-A in cultured leiomyoma cells was also studied.

Materials and Methods

Materials

Activin A was purchased from R&D systems (Minneapolis, Minneapolis). Ulipristal acetate (CDB-2914) was supplied by PregLem SA (Geneva, Switzerland).

Sample Collection

Human uterine leiomyoma and matched myometrial tissues were obtained from caucasian women of reproductive age (age range: 41-49) indicated for hysterectomy due to fibroids. All patients gave their informed consent, and the permission of the Human Investigation Committee was granted. Tissue samples were taken only from women who had not received hormonal treatments during the 3 months prior to surgery. The size of the myomas varied between 3 and 10 cm, and their location was predominantly intramural.

Primary Myometrial and Leiomyoma Cell Culture

Myometrial and leiomyoma samples were placed into Hank balanced salt solution (Euroclone, Milan, Italy) after surgery and immediately transported to the laboratory for necessary actions. Samples were washed several times with Dulbecco phosphate-buffered saline (Invitrogen, Paisley, United Kingdom) to remove excess blood. After cutting myometrial and leiomyoma tissue into small pieces, samples were mixed in 0.1% type 2 (Invitrogen) or type 8 collagenase (Serva Electrophoresis GmbH, Heidelburg, Germany) in serum-free Dulbecco modified eagle medium (DMEM; Sigma, Milan, Italy) and incubated at 37°C for 5 to 6 hours with manual shaking. Digested cell suspensions were then centrifuged at 1200 rpm for 10 minutes and washed several times. Finally, cell pellets were dispersed in DMEM containing 10% fetal bovine serum (Sigma), 50 mg/L gentamicin (Lonza, Verviers, Belgium), 1% amphotericin B (Lonza), and 1% antibiotic–antimycotic solution (Sigma). Cells were plated in plastic dishes and maintained using the same media at 37°C in 95% air–5% CO_2. The growth medium was changed after 24 or 48 hours to remove unattached cells and thereafter twice a week. The purity of cells was assessed by staining with specific smooth muscle cell marker (α-sma). Cells used in these experiments were passaged less than 4 times. Myometrium and leiomyoma cells were treated with UPA at 10^{-6} mol/L, 10^{-7} mol/L, and 10^{-8} mol/L and negative control (only respective solvent, dimethyl sulfoxide in 0.1% as we used for UPA at higher dose) in serum-free DMEM for 24 hours. At the end of treatments, cells were lysed using TRIZOL reagent (Invitrogen) and stored at −80°C.

RNA Extraction and Real-Time PCR

Total RNA was extracted using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. Samples were digested with a ribonuclease-free deoxyribonuclease (PromegaCorp, Madison, Wisconsin), and the RNA was cleaned up and concentrated using an RNeasy microkit (QIAGEN, Milan, Italy). We performed the reverse transcriptase (RT) using the high-capacity complementary DNA (cDNA) RT kit (Applied Biosystems, Foster City, California) with 1 μg RNA, and we performed the TaqMan real-time PCR for all the genes analyzed. The following TaqMan gene expression assays (Applied Biosystems) were used: Hs00170103_m1 (INHBA), Hs00610505_m1 (FLRG), Hs00246260_m1 (FST), Hs00923299_m1 (Alk4), Hs01012007_m1 (ActRII), Hs00609603_m1 (ActRIIB), Hs00365052_m1 (fibronectin), Hs00900055_m1 (VEGF-A), and Hs99999909_m1 (HPRT, used as housekeeping gene).[29]

The thermal cycle protocol was performed with initial denaturation at 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds, using 100 ng cDNA in a final reaction volume of 20 μL. The blank for each reaction, consisting of amplifications performed in the absences of RT enzyme, was performed.

Data Analysis

PRISM software (GraphPad Software, Inc, San Diego, California) was used to perform Kruskal-Wallis test, followed by post hoc Dunn multiple comparison test. Data are presented as the mean $\pm$ standard deviation of 3 separate experiments performed on different cultures and were considered significantly different when $P < .05$. All experiments were done in triplicates.

Results

Effect of UPA on Activin A, Its Binding Proteins, and Receptors in Cultured Leiomyoma Cells

Cultured human myometrial or leiomyoma cells were treated with UPA at varied concentrations (10^{-8} mol/L, 10^{-7} mol/L, and 10^{-6} mol/L) for 24 hours and mRNA expression of INHBA, FST, FLRG, ALK4, type II (ActRII), and type IIB (ActRIIB) was evaluated. We found that, in leiomyoma cells, UPA significantly decreased the expression of INHBA, FST, ActRIIB, and ALK4 mRNA compared to the nontreated controls (NT), while the same effect was not observed in myometrial cells (Figure 1).

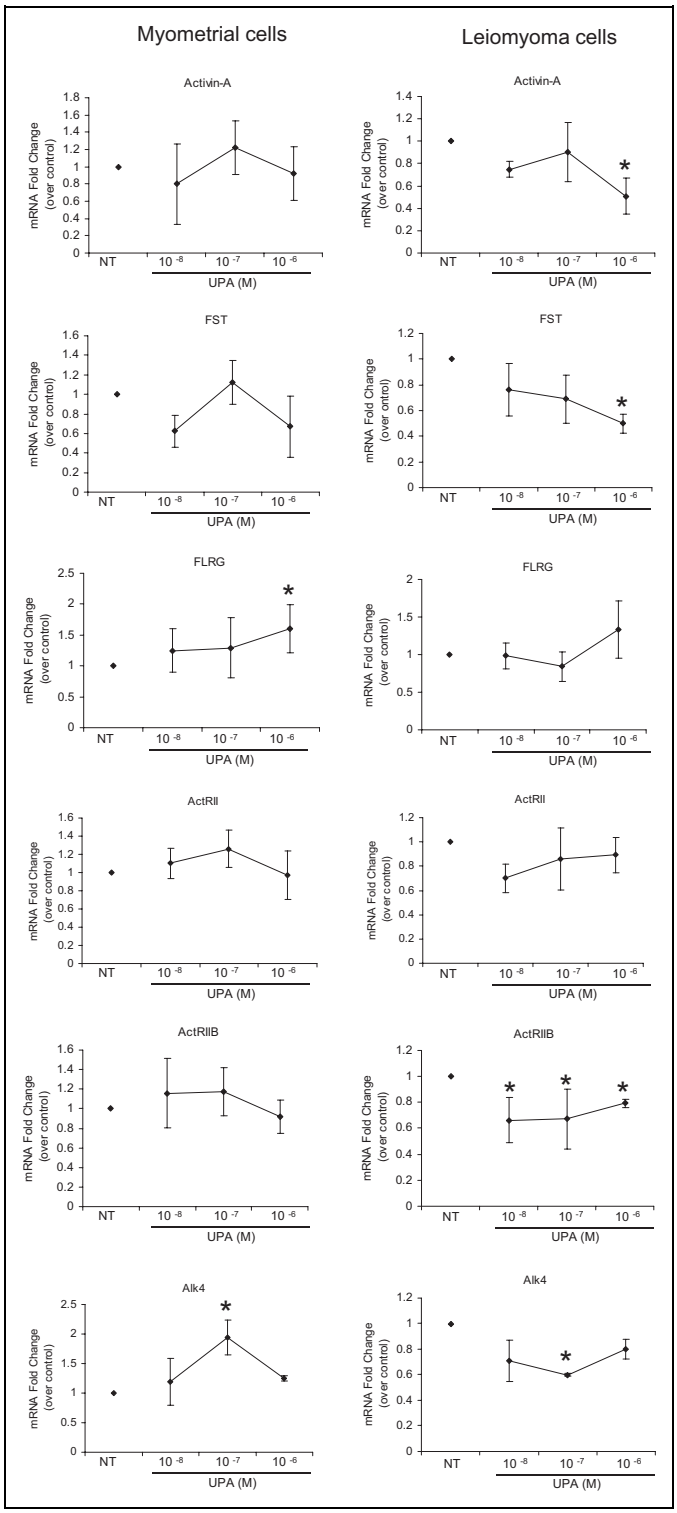

Figure 1. Expression of activin A, follistatin, follistatin-related gene (FLRG), and activin receptors (ActRII, ActRIIB, and activin receptor-like kinase 4 [ALK4]) in cultured myometrial and leiomyoma cells in response to ulipristal acetate treatment (from 10^{-8} to 10^{-6} mol/L; n = 3). *, $P < .05$. ActR indicates activin receptor type.

Effect of UPA on Activin A-Mediated Actions in Cultured Leiomyoma Cells

The possible influence of UPA on activin A modulation of fibronectin or VEGF-A mRNA expression was studied in cultured myometrial or leiomyoma cells. As shown in Figure 2, activin A treatment (4 nmol/L) for 24 hours significantly increased the mRNA level of fibronectin in both cell types, while in the presence of UPA, this effect was significantly reduced. Similarly, the administration of UPA at 10^{-6} mol/L

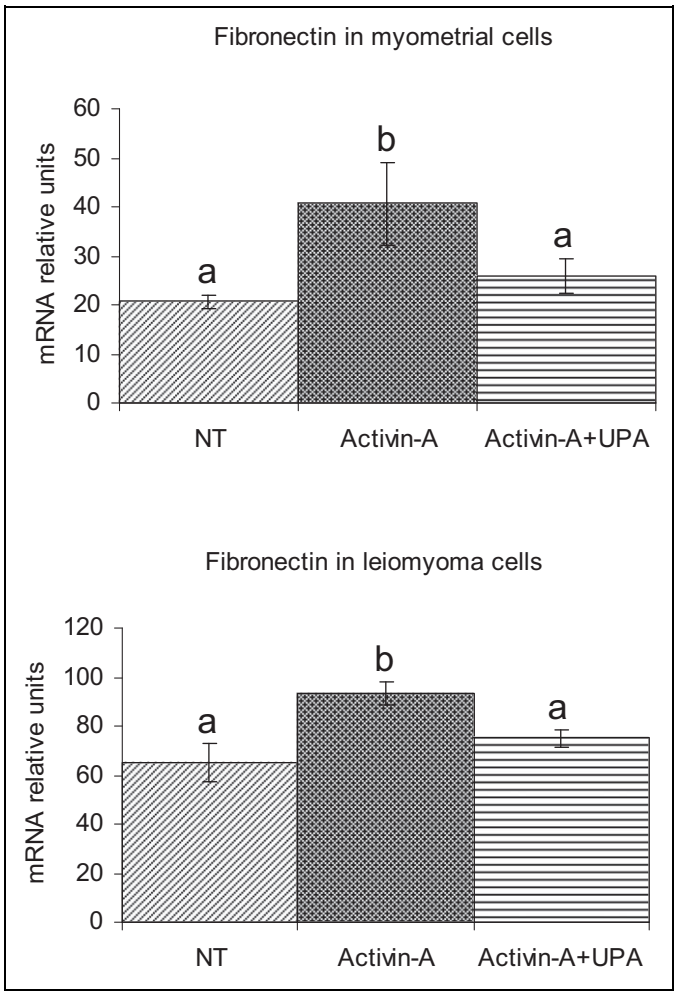

Figure 2. Effect of ulipristal acetate (10^{-6} mol/L) on activin A-induced fibronectin messenger RNA (mRNA) expression in cultured myometrial and leiomyoma cells. Values (mean ± standard deviation) that are significantly different from one another ($P < .05$) are indicated by different letters (n = 3).

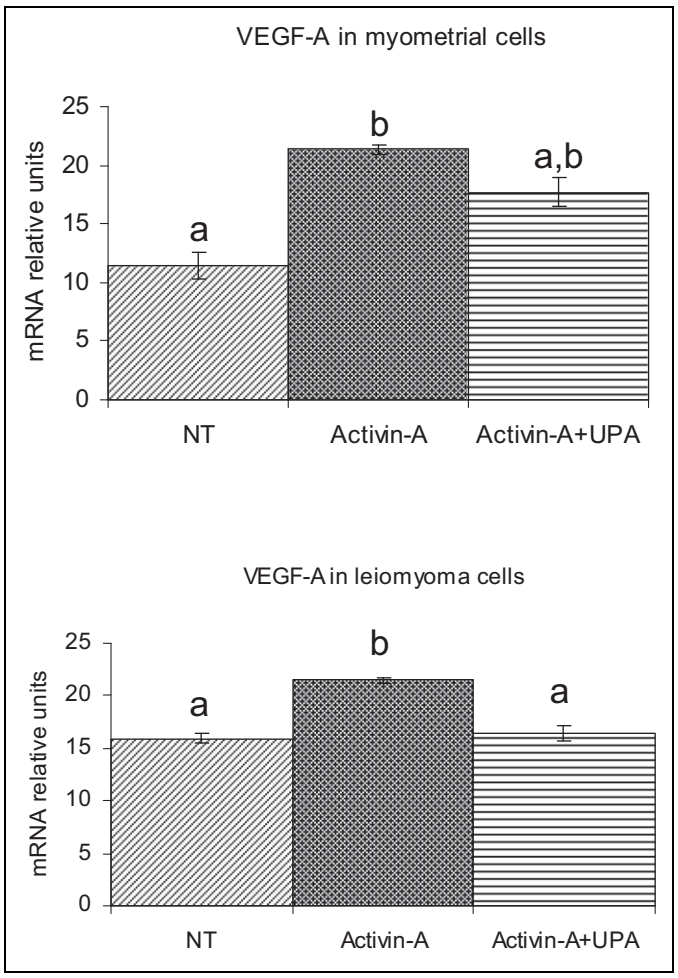

Figure 3. Effect of ulipristal acetate (10^{-6} mol/L) on activin A-induced vascular endothelial growth factor A (VEGF-A) messenger RNA (mRNA) expression in cultured myometrial and leiomyoma cells (n = 3). Values (mean ± standard deviation) that are significantly different from one another ($P < .05$) are indicated by different letters (n = 3).

significantly reduced the effect of activin A on VEGF-A mRNA expression induction in both myometrial and leiomyoma cells (Figure 3).

Discussion

The present study shows for the first time that treatment with UPA reduces the expression of INHBA, FST, ActRIIB, and Alk4 mRNA in cultured leiomyoma cells. Since activin A and FST are highly expressed in leiomyoma specimens compared to the adjacent healthy myometrium,[14] the finding that UPA decreases their expression highly suggests the biological relevance of those molecules in leiomyoma cells. In addition, the reduced expression of activin receptors by UPA in leiomyoma cells further supports that activin pathway is a potential target of this drug in leiomyoma cells. It is interesting to note the selective action in leiomyoma cells, while the same effect was not shown in myometrial cells. This observation underlines the different behavior of the tumoral smooth muscle cells (leiomyoma cells) compared to the myometrial cells. Indeed, this differential effect is in agreement with recent data showing that activin A has a fibrotic effect in cultured leiomyoma cells more than in myometrial cells.[21]

Ulipristal acetate is clinically used at 5 mg and 10 mg/d; therefore, the data obtained present some limitation considering that it is unlikely that 10^{-6} mol/L concentration will be achieved at the tissue level. On the other hand, the in vitro experimental model may have different behavior compared to the in vivo situation (ie, the cells could be less responsive). Therefore, the reduced activin A and activin receptors in leiomyoma cells suggest that the activin system may be targeted by UPA and that this may represent one of the possible mechanism of UPA in the treatment of uterine fibroids.

In line with this expectation, we found that UPA was able to interfere with the actions of activin A on fibronectin and VEGF, in leiomyoma cells. Indeed, activin A modulates ECM turnover in different biological systems,[30-34] and a fibrotic role

of activin A in leiomyoma growth is supported by the evidence of increasing fibronectin mRNA expression in both myometrial and leiomyoma cells.[21] The same effect is induced by TGF-β, which stimulates fibronectin mRNA expression in both myometrial and leiomyoma cells.[35] Interestingly, in the present study, the increased fibronectin mRNA expression induced by activin A was prevented by UPA, suggesting that it may also inhibit leiomyoma growth by suppressing activin A fibrotic role in leiomyoma. Uterine leiomyoma is considered as a fibrotic disorder that contains 50% more ECM proteins than the corresponding myometrium.[36] Fibronectin is one of the most important ECM molecules, which plays a role in cell adhesion, cell morphology, surface architecture, tissue repair, and wound healing as well as regulation of cell growth and differentiation.[37-39]

Expression of VEGF mRNA also resulted in a target for UPA, and angiogenesis plays an important role in the regulation of leiomyoma growth,[4,40-42] VEGF being a potent vascular endothelial cell mitogen.[43] The VEGF mRNA and VEGF-A protein are detected in myometrium and leiomyoma,[44-47] and uterine leiomyoma exhibits an expression of VEGF-A higher than that in adjacent normal myometrium, supporting a role of local angiogenesis for the development and growth of these tumors.[46] For the first time, the present data also showed that activin A increases the expression levels of VEGF-A mRNA in myometrial and leiomyoma cells, while UPA was able to prevent this effect.

In conclusion, the present study shows that UPA downregulates the mRNA level of activin A, FST, ActRIIB, and Alk4 in leiomyoma cells and abolishes the action of activin A on the induction of fibronectin and mRNA level of VEGF-A in both myometrial and leiomyoma cells, thus indicating that the activin A signaling system may be part of the inhibitory effect of UPA in leiomyoma growth.

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Authors' Note

Mario Castellucci and Felice Petraglia contributed equally to this work.

Declaration of Conflicting Interests

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