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Effects of Tibolone Metabolites on Human Endometrial Cell Lines in Co-Culture

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Abstract

In human endometrium, cell proliferation is regulated by ovarian steroids through heterotypic interactions between stromal and epithelial cells populating this tissue. We tested the proliferative effects of tibolone and its metabolites using endometrial co-cultures that mimic the normal proliferative response to hormones. We found that both the Δ^4-tibolone metabolite and the pure progestin ORG2058 counteract estradiol-driven epithelial cell proliferation. Surprisingly, the estrogen receptor binding 3-hydroxyl-metabolites of tibolone also counteracted estradiol-driven proliferation. Inhibition of proliferation by 3β-OH-tibolone was abrogated by low doses of the progesterone receptor antagonist mifepristone, This suggests that 3β-OH-tibolone is converted to a progestagenic metabolite. We found that the stromal cells used in the co-cultures express high levels of the ketosteroid dehydrogenase, AKR1C2, which is able to oxidize 3β-OH-tibolone back to tibolone. Thus the unexpected progestagenic effect of 3β-OH-tibolone in these co-cultures may be due to metabolic activity present in the stromal cells of the co-cultures.

Keywords

endometrial cell lines; tibolone; co-culture assay

Introduction

Regulation of cell proliferation in the endometrium results from complex interactions between the stromal and epithelial compartments, as demonstrated *in vivo* in the mouse and *in vitro* using tissue reconstruction. Co-cultures of normal human stromal and epithelial cells have shown that appropriate hormonal responses regulating epithelial cell proliferation depend on the presence of stromal cells or medium conditioned by them[15]. These latter studies point out the role of paracrine growth factors secreted by stromal cells as regulators of epithelial cell proliferation. The benefit of studies in this system, in principle, is that they may allow us to dissect the mechanisms involved in the endometrial response to hormones, including epithelial-stromal interactions that regulate tissue homeostasis.

The use of primary cell culture systems for physiological studies is difficult to implement systematically, due to patient to patient variations that impedes statistical analysis, and

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procuring endometrial tissue and generating primary cultures is a costly and time consuming enterprise. We have previously demonstrated that under our growth conditions, the Ishikawa cells can be substituted for the normal epithelial component. Recently, we described an immortalized endometrial stromal cell line, which can be substituted for the normal stromal component in the co-culture[18]. We believe that this co-culture system will be advantageous for the studies of hormonal effects in the endometrium, and are currently testing a variety of hormonal analogs.

In the present study we tested the effects of tibolone and its principal metabolites on endometrial proliferation in this co-culture system. Tibolone is a synthetic steroid that is used in many countries for peri- and post-menopausal hormone replacement therapy. A number of clinical studies report that tibolone causes relief of climacteric symptoms and provides benefits to bone health[1]. These effects resembling those of estradiol therapy are in contrast to other studies showing that in breast tissue, tibolone has no estrogenic/progestagenic effects[2], and that in endometrium, tibolone exerts progestagenic effects. Overall, the pattern of clinical effects induced by tibolone appears to be estrogenic in bone and brain, absent in the breast, and progestagenic in the endometrium.

This differential activity on various target tissues is attributed to the hormone receptors binding capacities of the metabolites of tibolone. The principal metabolites found in the blood include 3α- and 3β-hydroxy tibolone and to a lesser extent Δ^4-tibolone[5]. *In vitro*, tibolone itself binds poorly to hormone receptors, while the 3α- and 3β-hydroxy (OH) metabolites bind solely to the estradiol receptors with a preference for ERα over ERβ[6]. The Δ^4-tibolone metabolite binds the progesterone receptor. Thus, the proportions of estrogenic versus progestagenic metabolites formed within a tissue are thought to determine the hormonal effect of tibolone on different steroid responsive tissues. Tibolone has been proposed to belong to a new class of steroid analogs, called STEAR, which is an eponym for Selective Tissue Estradiol Activity Regulator[7].

Interestingly, we found that all the tibolone compounds tested inhibit E_2-driven proliferation of the epithelial component. We show that the stromal and epithelial cells express the ketosteroid dehydrogenase AKR1C2, which may allow for the conversion of 3βOH-tibolone back to tibolone[19]. From our observations, using the co-culture system, we conclude that tibolone counteracts E_2-driven proliferation in the uterus in a manner similar to progestins, and that the balance of metabolism of tibolone in the endometrium favors the production of the progestagenic metabolites.

Material and Methods

Reagents and Cell Culture

Tibolone, tibolone metabolites (3αOH-tibolone, 3βOH-tibolone, and Δ^4-tibolone), and progestin ORG2058 were generous gifts of Organon NV (Oss, Netherlands). Mifepristone and 17β-estradiol were obtained from Sigma (St. Louis, MO). The immortalized normal endometrial stromal cell line SHT290 (SHT) was developed in our laboratory18 and the Ishikawa (IK) epithelial endometrial adenocarcinoma cell line20 was obtained from Dr B. Lessey (Greenville Hospital System University Medical Center, Greenville South Carolina). Both cell lines were maintained in steroid-free medium (designated thereafter “JAC4”) consisting of a 1:1 mixture of Ham F12 (GIBCO, Invitrogen Corp, Carlsbad, CA) and M199 basic medium (Sigma)) supplemented with 4% charcoal-stripped fetal bovine serum (Hyclone, Logan UT), 0.25% ITS+ (insulin-transferrin-selenium plus lipoic acid, BD Biosciences, Becton Dickinson, Franklin Lakes, NJ), 0.1 mM phosphorylethanolamine (Sigma) and 2 mM glutamine (GIBCO) and antibiotic/antimycotic solution (hereafter referred to as ABAM; GIBCO) diluted to yield 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and

250 ng/ml amphotericin B. This medium and all other media were phenol red-free. All cultures described in this report were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Assessment of Cell Proliferation

Stromal cells grown in JAC4 medium were seeded in 12-well cluster culture plates (BD Bioscience) at 50,000 cells per well. The next day, Ishikawa cells grown in JAC4 were seeded onto cell culture inserts with 0.4 μm porosity (BD Bioscience) at a density of 4,000 cells per insert. The inserts were suspended above the adherent stromal cells so that they were covered by the culture medium. To assess proliferation, Ishikawa cells were detached from the inserts using a 1:1 mixture of versene (GIBCO) and trypsin-EDTA, thoroughly mixed to obtain single cell suspensions, and enumerated with a Coulter Counter (Beckman Coulter, Inc., Fullerton, CA).

To conduct the assay for measuring estrogenic activity assays, stromal cells were mixed with 0.4 ml of growth factor-reduced, phenol red-free Matrigel® (BD Biosciences) as previously described[17]. Co-cultures were equilibrated in steroid-free JAC4 for 48 hr prior to the addition of 10^{-8} M 17β-estradiol or tibolone metabolites. The cultures were subsequently treated with appropriate hormones in JAC4 for a period of 48 hr, switched to a serum-free medium (formulated as F12/M199 mixture, 0.25% ITS+, 0.25% BSA (Sigma), 20 ng/ml epidermal growth factor (EGF; BD Bioscience), ABAM, 2 mM glutamine) containing the same hormones for an additional 64 hr, after which the Ishikawa cells were counted.

To conduct assays for measuring progestagenic activity, co-cultures were equilibrated in JAC4 supplemented with 10^{-8} M 17β-estradiol for 48 hr, after which the medium was changed to JAC4 supplemented with a combination of estradiol 10^{-8} M and 5×10^{-7} M of one of the tibolone metabolites, or 10^{-8} M estradiol plus 10^{-7} M of the pure progestin ORG2058 for 48 hr. After 48 hr, the cultures were switched to the serum-free medium described above supplemented with estradiol and added compounds for an additional 64 hr, after which the Ishikawa cells were counted. Assays to evaluate tibolone did not include 10^{-8} M estradiol because tibolone is assumed to be converted to a mixture of estrogenic and progestagenic metabolites[21]. Control cultures used for the assay of progestagenic activity were treated with 10^{-8} M 17β-estradiol for the duration of the experiment.

Statistical analysis

Each treatment was performed on a total of six co-cultures per experiment and the mean and standard deviation calculated. A Student T Test was applied to ascertain the significance ($p<0.05$) of differences in cell numbers between treatments. Each experiment was repeated at least twice.

Detection of AKR1C Message by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was prepared from cultured endometrial cells using an RNA purification kit from Promega Corp (Madison, WI). Reverse transcription was performed using AMV reverse transcriptase according to the manufacturer instructions (Roche, Indianapolis, IN) in the presence of random hexamers (GIBCO). PCR reaction products were loaded on a 2% agarose gel and electrophoresed in parallel to a DNA ladder standard. Subsequently the gel was stained with ethidium bromide. For negative controls, cDNA was replaced by water, and the absence of genomic DNA in the RNA samples was verified by showing that direct PCR amplification of the RNA samples without a reverse transcription step did not yield any product. All PCR products were normalized to the PCR product of hypoxanthine phosphoribosyl transferase 1 (Lesch-Nyhan syndrome) (*HPRT1*) which is unresponsive to variations of hormones. Primers

for the amplification of *HPRT1* were designed in house. The sequences of the primers for amplification of AKR1C2 and 3 were as described in Penning et al[22].

Results

Validation of the co-culture system with estradiol

We determined that, when grown alone, neither the stromal cells nor the Ishikawa cells used in the co-culture system responded to estradiol by an increase in proliferation (data not shown). Having previously demonstrated that in co-cultures, normal stromal cells regulate the estrogenic proliferative response of Ishikawa cells[15], we confirmed that co-culture of Ishikawa cells with the recently established immortalized normal stromal cell line SHT29018 also shows a significant effect of estradiol on the proliferation of Ishikawa cells (Figure 1). As described in material and methods, the assay for estrogenic activity is conducted over a period of 7 days (156 hr), during which the medium is changed every other day. If instead of renewing the medium every other day (Figure 1A), we renew the medium every day (Figure 1B), the proliferation of the Ishikawa cells in response to estradiol is greatly diminished, suggesting that preventing the accumulation of growth factors in the medium adversely affects the proliferative response of the Ishikawa cells induced by estradiol.

Proliferation of Ishikawa cells in the presence of tibolone and its metabolites

The tibolone metabolites were tested along with estradiol employing the co-culture assay used previously to assess E_2-induced cell proliferation. As shown in Figure 2A, while estradiol treatment gave a significant stimulation of Ishikawa cell growth, the 3αOH-tibolone and 3βOH-tibolone derivatives did not stimulate the proliferation of Ishikawa cells significantly. Notably, the 3-OH-tibolone derivatives were used at concentrations 50 times higher than estradiol; at these concentrations, one may expect a similar receptor activation[6]. Using the assay for estrogenic activity, we did not observe an effect on proliferation with Δ4-tibolone, the pure progestin ORG2058 (Figure 2B), nor tibolone (not shown). Thus none of the tibolone metabolites induced an estrogenic effect (Figure 2).

To determine whether these compounds exerted a progestagenic affect, the tibolone metabolites ability to inhibit estrogen-mediated proliferation was tested. In the progestagenic assay, the co-culture is primed with estradiol for two days to insure high expression of progesterone receptors, after which the medium is renewed with a combination of estradiol with the test progestagenic compound as described in material and methods. These studies showed that like the progestin ORG2058, Δ^4-tibolone counteracts the proliferative effect of estradiol (Figure 3). Curiously, the 3OH-tibolone metabolites also had an inhibitory effect but this effect was not as strong as either ORG2058 or Δ^4-tibolone.

To confirm that the inhibition of estradiol-driven proliferation was mediated by the progesterone receptor, we tested whether the effect of tibolone and its metabolites could be inhibited by inclusion of the progesterone receptor antagonist, mifepristone. In studies *in vitro*, it has been shown that in the endometrium, tibolone is mainly converted to the Δ^4- and the 3β-OH metabolites[21], therefore we only tested these two metabolites. Mifepristone at a concentration of 10^{-7} M largely antagonizes the effect of Δ^4-tibolone, but it did not influence the effect of 3β-OH-tibolone. Since mifepristone may express agonistic activity in our system we performed a dose-response study, titrating different concentrations of mifepristone versus a constant dose of 3β-OH-tibolone. Figure 4B shows that lower concentrations of mifepristone can indeed counteract the progestagenic effects of 3β-OH tibolone on estradiol-driven proliferation.

Expression of AKR1C enzymes in cultures

One explanation for the unexpected progestagenic effect of the 3βOH- metabolite could be the conversion of the 3β-OH- metabolite to tibolone and further to the Δ^{4-}-isomer in the co-culture system. Recently it has been shown that the ketosteroid dehydrogenase enzymes of the AKR1C family are capable of metabolizing the 3hydroxy-tibolone metabolites[19]. Although AKR1C1, AKR1C3, and AKR1C4 act as reductases, the AKR1C2 enzyme is capable of oxidizing the 3β-OH-tibolone metabolite back to tibolone. Using RT-PCR, we demonstrated that in the presence of estradiol both the AKR1C3 and AKR1C2 messenger RNAs are expressed by the Ishikawa cells and the stromal cells, raising the possibility that when expressed their activity influence the fate of steroids added to the co-culture (Figure 5). The presence of these enzymes and the observation that low concentration of mifepristone can block the effect of 3β-OH-tibolone together may explain the paradoxical progestagenic effects of 3OH-metabolite of tibolone in the co-culture.

Discussion

The Ishikawa endometrial adenocarcinoma cell line is widely used to evaluate the properties of human endometrial epithelium. We have shown previously that these cells recapitulate the epithelial responses to estradiols and progestins more accurately when they are co-cultured with human endometrial stromal cells. Previous findings indicated that the proliferative response induced by estradiol in the epithelial cells resulted from the secretion of paracrine factors by the estradiol stimulated stromal cells[15]. In the current study, experiment presented in Figure 1 show that secreted factors must accumulate over time to fully exert their regulatory effects on the proliferation of the epithelial component in the co-culture system. This effect demonstrates the determining role of the stromal cells in regulating the growth of epithelial cells.

The co-culture system allows testing of the progestagenic or estrogenic activities of compounds. It should be useful to dissect the complex effects of many hormones analogs on endometrial growth. Tibolone, an hormone replacement therapy drug that has been extensively studied in clinic, was an appropriate test compound, because it exerts differential effects on estrogen target tissues, depending on the local concentration of its various metabolites, as well as the ability of tssue enzymes to inactivate or store the metabolites through sulfation/desulfation.

In the endometrium, the overall long term effect of tibolone is progestagenic. Histological studies following treatment with tibolone for 3 months or longer revealed that the endometrium becomes atrophic, suggesting a progestagenic inhibition or lack of estrogenic stimulation of endometrial cell proliferation[3, 4]. In the laboratory, the progestagenic effects of tibolone on the endometrium have been demonstrated by measuring various progesterone-regulated factors. Early studies using cultures of fragments of endometrial tissue demonstrated that similar to progesterone, tibolone and Δ^4-tibolone counteracted the effect of estradiol on the production of prostaglandin $PGF_{2\alpha}$ [8]. Recent studies performed *in vitro* using normal endometrial stromal cells exposed to tibolone and its metabolites have demonstrated the expression of endpoints indicative of progesterone predominance[9, 10, 11]. Analysis of gene expression profiles by micro array analysis of cell lines reveals that tibolone has a profile similar but not identical to that of the progestagen MedroxyProgesterone Acetate (MPA)[12].

In our present study, tibolone and all its metabolites were found to be inactive when tested for estrogenic activity, measured by the stimulation of endometrial epithelial cell proliferation. In the protocol for progestagenic activity, tibolone metabolites were able to counteract the proliferation of Ishikawa cells that is induced by estradiol; this demonstrates their progestagenic activity. Thus, the co-culture system appears to closely mimic the effect of

tibolone *in vivo*. The progestagenic effects of tibolone and of the Δ^4-metabolite had been expected because tibolone is converted to its Δ^4-isomer in the endometrium, and Δ^4-tibolone has been shown to bind the progesterone receptor. In contrast, the activity of the 3OH-tibolone metabolites as progestin was surprising, as the 3-OH-metabolites are known to bind the estradiol receptors, not the progesterone receptor[6].

We next attempted to determine whether the progesterone receptor was involved in the abrogation of estradiol-driven proliferation. To do this, we conducted the progestagenic assay in the presence of the progesterone receptor antagonist mifepristone. The effect of the Δ^4-isomer on estradiol-driven proliferation could be counteracted by 10^{-7} M mifepristone. Lower concentrations of mifepristone suffice to abrogate the progestagenic activity detected in 3β-OH tibolone-treated culture (Figure 4B). Smaller amount of progestagenic compounds are generated in the 3β-OH-tibolone-treated cultures, and therefore lower concentrations of mifepristone are needed to counteract the effect of 3β-OH-tibolone. Higher concentrations of mifepristone are not efficacious perhaps because mifepristone has some intrinsic progestagenic agonist activity that may override its antiprogestagenic effects, as has been shown in other systems [23].

To explain how 3β-OH-tibolone could be metabolized in the co-culture system we examine the expression of the AKR1C enzymes. It had been shown previously that the ketosteroid dehydrogenase AKR1C2 can oxidize 3βOH-tibolone back to tibolone *in vitro*[19]. We attempted to confirm this hypothesis using the ketosteroid dehydrogenases inhibitors flufenamic acid[19] and cholanic acid. Unfortunately flufenamic acid reduced the estradiol driven increase in proliferation (perhaps because of its known effects on prostaglandin levels[24]. Studies with cholanic acid also were unsuccessful as the result of solubility problems. Cholanic acid could not be used at the concentration shown to inhibit AKR1C2 *in vitro*[25] without increasing the vehicle volume to an unacceptable percentage (data not shown). AKR1C-2 and -3 were expressed at higher levels in the stromal cells than in the Ishikawa epithelial cells. Since it is not clear whether expression of those metabolic enzymes are under the control of steroid hormones, this issue merits further investigation.

In summary, our results demonstrate the importance of the use of the co-culture model to study the regulation of cell proliferation in human endometrium. Applying this system to the study of tibolone we find that tibolone and its metabolites exert no estrogenic proliferative effects. In contrast, perhaps because of stromal metabolism, all tibolone compounds demonstrate progestagenic activity. These results are in agreement with clinical observations of an atrophic endometrium, indicative of an overall progestagenic effect.

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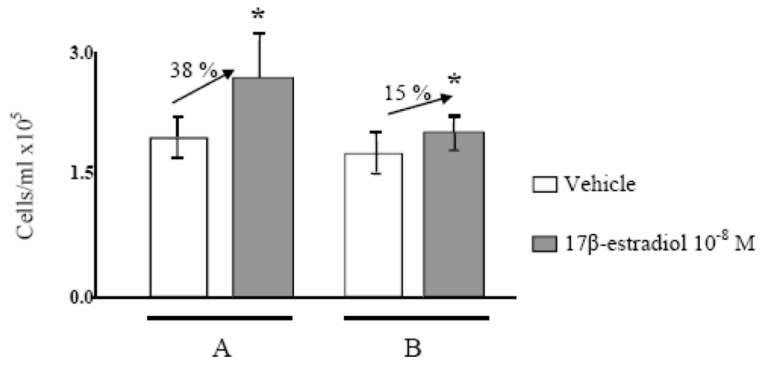

Figure 1.

Accumulation of stromal factors in the medium enhances estradiol-driven proliferative response of Ishikawa cells: Following the estrogenic assay protocol described in material and methods, set of samples A (6 co-cultures per treatment) was fed JAC4 medium for 48 hr, JAC4 medium supplemented with vehicle or estradiol 10^{-8} M for 48 hr and serum free medium with vehicle or estradiol 10^{-8} M for 64 hr. Set of samples B (6 co-cultures per treatment) received the same treatment except that the appropriate medium was renewed every 24 hr. Ishikawa cells were counted at the end of the treatment course as described in material and methods. % increase in proliferation is indicated on the figure. Bars indicate standard deviation. The asterisk indicates a p value of less than 0.05 (n=6, one experiment).

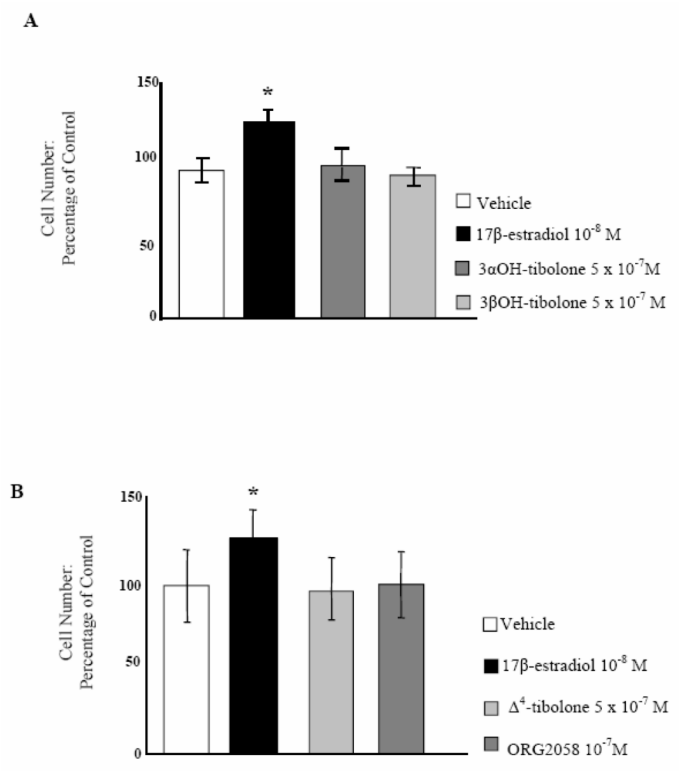

Figure 2.

Proliferative response of Ishikawa cells in the presence of estradiol or tibolone metabolites in co-cultures. Co-cultures were fed JAC4 medium for 48 hr, JAC4 medium supplemented with vehicle or test compound for 48 hr and serum free medium with vehicle or test compound for 64 hr. Ishikawa cells were enumerated in the Coulter counter. Cell numbers are presented as percentage of control. A) Comparison of the 3OH-tibolone metabolites and 17β-estradiol to vehicle treatment (n=6, 3 experiments). B) Comparison of Δ^4–tibolone, estradiol and progestin ORG2058 to vehicle treatment (n=6, 2 experiments). Cell numbers are presented as percentage of control. The asterisk indicates a p value of less than 0.05. Bars indicate standard deviations.

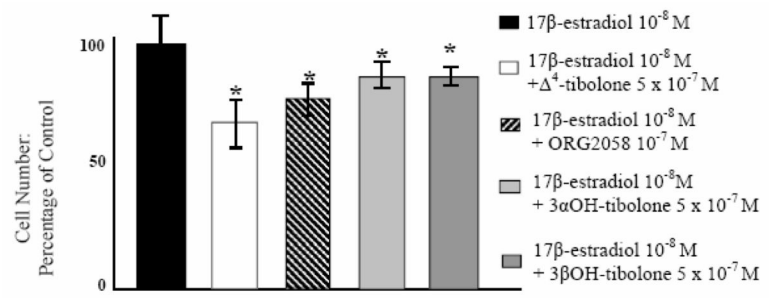

Figure 3.

Effect of tibolone metabolites or progestin on the estradiol-driven proliferation of Ishikawa cells in co-culture. Co-cultures were fed JAC4 medium supplemented with 10^{-8} M E_2 for 48 hr, JAC4 medium supplemented with E2 10^{-8} M combined with vehicle or indicated test compound for 48 hr and serum free medium with E2 10^{-8} M combined with vehicle or indicated test compound for 64 hr. Ishikawa cells were enumerated in the Coulter counter. Cell numbers are presented as percentage of control (n=6, 2 experiments). The asterisk indicates a p value of less than 0.05. Bars indicate standard deviations.

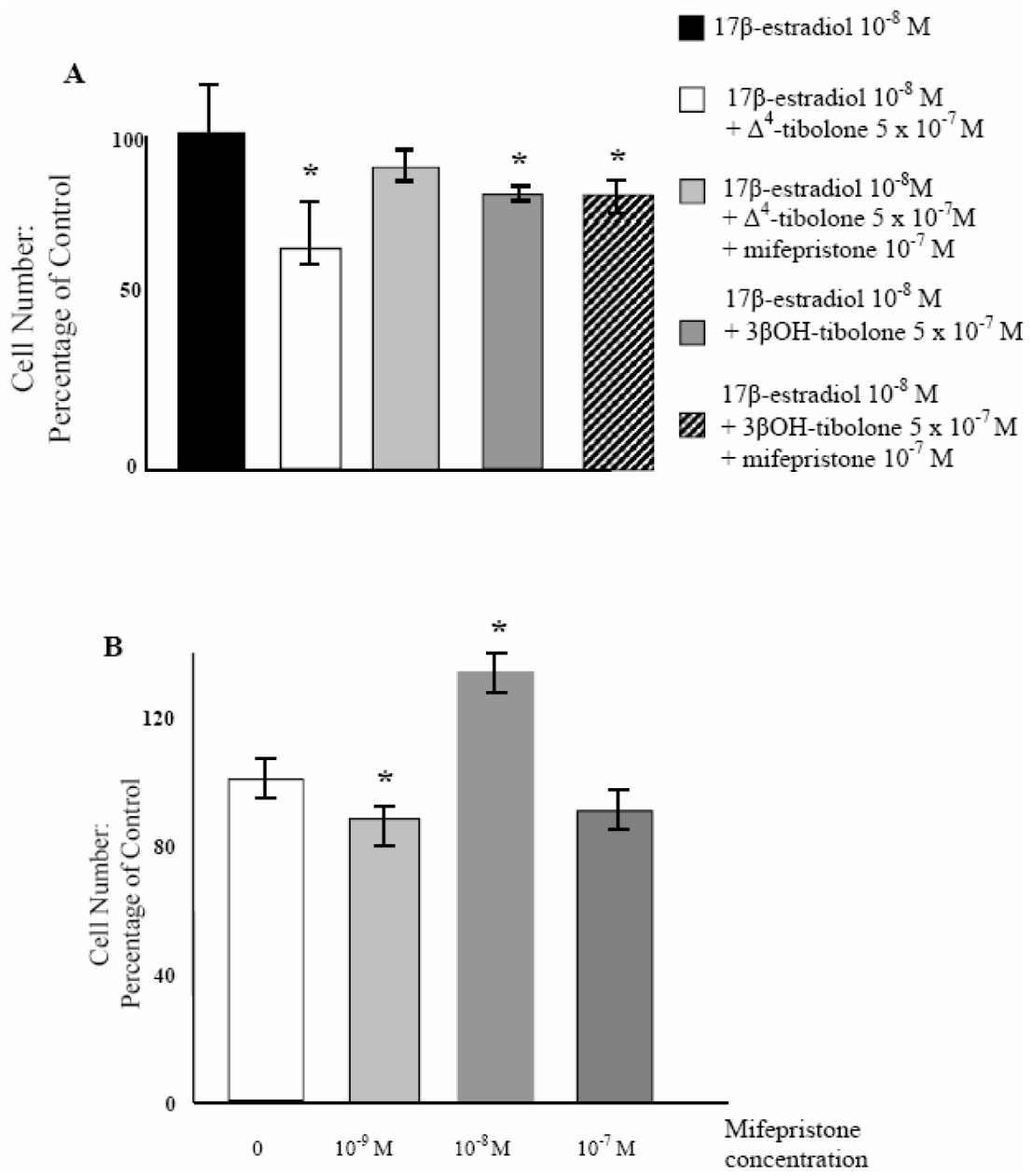

Figure 4.

Effect of the progesterone antagonist mifepristone on the regulation of Ishikawa cells estradiol-driven proliferation by Δ^4-tibolone and 3βOH-tibolone. A) Abrogation of Δ^4-tibolone effect on estradiol-driven proliferation by 5×10^{-7} M Mifepristone. Co-cultures were fed JAC4 medium supplemented with 10^{-8} M E_2 for 48 hr, JAC4 medium supplemented with E_2 10^{-8} M combined with vehicle or indicated test compound (with or without added mifepristone) for 48 hr and serum free medium with E_2 10^{-8} M combined with vehicle or indicated test compound (with or without added mifepristone) for 64 hr. Cell numbers are presented as percentage of control. (n=6, 2 experiments). The asterisk indicates a p value of less than 0.05. Bars indicate standard deviations.

B) Abrogation of 3βOH-tibolone effect on estradiol driven proliferation by a lower dose of mifepristone. Co-cultures were fed JAC4 with 10^{-8} M estradiol for 48hrs, followed by a

combination of 10^{-8} M estradiol, 5 10^{-7} M 3β-OH Tibolone, with increasing concentrations of mifepristone. Control was treated with estradiol, 3βOH-tibolone and vehicle. Cell numbers are presented as percentage of control (n=6, 1 experiment). The asterisk indicates a p value of less than 0.05. Bars indicate standard deviations.

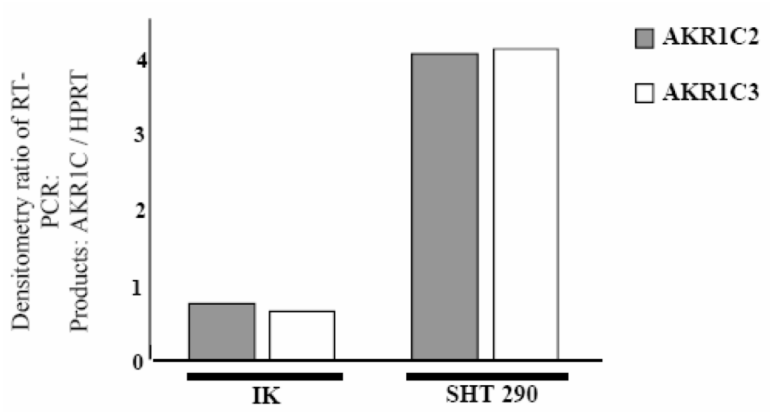

Figure 5.

Expression of steroid metabolizing enzymes in stromal cells and Ishikawa cells. RT-PCR amplification of AKR1C3 and AKR1C2 mRNA in co-cultured endometrial cells. At the end of 5 days of co-cultures in the presence of 10^{-8} M 17β-estradiol, the stromal cells and Ishikawa cells were harvested separately for RNA preparation and RT-PCR was performed as described in Materials and Methods. In one experiment, the PCR reaction products were quantitated by densitometry and normalized to the value of HPRT expression in the same sample. Results are presented here in arbitrary densitometric units.