Differential actions of estrogen and SERMs in regulation of the actin cytoskeleton of endometrial cells


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ABSTRACT: Estrogen and selective estrogen receptor modulators (SERMs) differentially impact endometrial cell function, however, the biological basis of these differences is not established. Deregulated cell adhesion to the extracellular matrix, cell movement and invasion are related to endometrial disorders, such as endometriosis or endometrial cancer. Remodeling of the actin cytoskeleton is required to achieve cell adhesion and movement. Estrogen receptor (ER) regulates actin and cell membrane remodeling through extra-nuclear signaling cascades. In this article, we show that administration of 17β-estradiol (E2) and tamoxifen (TAM) to immortalized Ishikawa endometrial cells or to human endometrial stromal cells (ESC) results in remodeling of actin fibers and cell membrane. This is linked to rapid phosphorylation on Thr558 of the actin-binding protein moesin and enhanced migration and invasion of normal and Ishikawa cells. Raloxifene (RAL) does not result in moesin activation or actin remodeling. When endometrial cells are exposed to E2 in the presence of TAM or RAL, both SERMs interfere with the recruitment of moesin, with the remodeling of the cytoskeleton, and with cell movement and migration induced by E2. The differential actions of E2, TAM and RAL are linked to a distinct modulation of the extra-nuclear signaling of ER to G proteins and to the Rho-associated kinase. These findings increase our understanding of the actions of estrogen and SERMs in endometrial cells and highlight potential molecular targets to interfere with the estrogen-related altered cell adhesion encountered in endometrial disorders.

Key words: actin cytoskeleton / endometrial cells / estrogen / moesin / SERMs

Introduction

Endometrial morphology is dynamically regulated by the changing levels of sex steroid hormones during the menstrual cycle or pregnancy (Speroff et al., 1999). The cyclic architectural modifications observed in the endometrium are achieved through the interplay of a number of cellular events and one of the necessary steps is the re-structuring of the cytoskeleton. Remodeling of actin fibers is critical for the morphological organization of the cell membrane, for the generation of cell–cell interactions and for cellular adhesion to the extracellular matrix (Pollard and Borisy, 2003), and all these processes are required for the plasticity of tissues, including the endometrium. The control of cytoplasmic actin arrangement, notably the loss of stress fibers, is also a key factor for cell proliferation and for malignant transformation (Pawlak and Helfman, 2001).

Estrogens have been recently found to be effective regulators of several cytoskeletal components, particularly of actin fibers (Simoncini et al., 2006; Giretti et al., 2008). Through the control of actin organization estrogen regulates cell morphology and the interaction with the extracellular environment, and drives cell movement in a number of estrogen-sensitive tissues. An unbalanced exposure to estrogen and progesterone is a key factor for the development of endometrial carcinoma (Ito et al. 2007), and the modification by sex steroids of cell morphology and motility through the actin cytoskeleton might represent a step in endometrial transformation and may play a role for endometrial cancer cell spread, invasion and metastasis.

Estrogen controls different checkpoints involved in actin arrangement. For instance, estrogen activates selected members of the ezrin–radixin–moesin (ERM) family of actin-binding proteins (Simoncini et al., 2006; Giretti et al., 2008), that are key controllers of actin polymerization and interaction with the cell membrane (Pollard and Borisy, 2003). In endothelial and breast cancer cells this is achieved through the ERα-dependent recruitment of a rapid, extra-nuclear signaling cascade targeting the small GTPase RhoA and its downstream effector Rho-associated kinase (ROCK). This pathway leads to the sequential activation of moesin, of actin remodeling and of cell motility (Simoncini et al., 2006; Giretti et al., 2008).
The distinct control of estrogen receptors (ERs) by natural estrogens or by the available selective estrogen receptor modulators (SERMs) is the basis for their different effects in some tissues, such as the endometrium (Shang and Brown, 2002). The SERM tamoxifen (TAM), like 17β-estradiol (E2), increases endometrial cell proliferation (Pole et al., 2005), and its use in breast cancer patients is associated with an increased risk of endometrial hyperplasia and cancer (Rutqvist et al., 1995; Cohen, 2004). On the contrary, raloxifene (RAL) has no stimulatory effect in the endometrium (Cummings et al., 2002), and recent studies even suggest that the use of this SERM might result in reduced incidence of endometrial cancer (DeMichele et al., 2008). At present, there is no available information on whether these differential endometrial effects may be related to discrepant actions of estrogen and SERMs on endometrial cell cytoskeleton.

The aim of the present study was to establish whether estradiol, TAM or RAL might exert distinct regulatory actions on the actin cytoskeleton of human endometrial cells in primary culture or of a human immortalized endometrial cell line, leading to differences in cell motility. To this extent, we investigated if estradiol or the two SERMs differentially recruit the rapid signaling cascades that target the actin-binding protein moesin.

Materials and Methods

Cell cultures and treatments

The immortalized Ishikawa human endometrial adenocarcinoma cell line was obtained from the European Collection of Cell Culture (ECACC, Salisbury, UK). Ishikawa cells were grown in phenol-red free minimal essential medium (MEM) supplemented with l-glutamine (2 mM), 10% fetal bovine serum (FBS), 1% non-essential amino acids.

Normal human endometrial stromal cells (ESC) were obtained from 10 women undergoing surgical procedures requiring endometrial biopsy (diagnostic or operative hysteroscopy, diagnostic or operative laparoscopic procedures). These patients were included only when free of oral contraception, progestins, GnRH analogues or antagonists, or other hormonal medications for at least 3 months. Women with previous autoimmune, neoplastic, hepatic or thyroid disorders were excluded from the study. All women were younger than 40 and older than 17 years; the median age was 30 ± 7 years. All samples were collected during the follicular phase, based on the last menstrual period and histological examination of the samples. Each patient signed a written informed consent, and approval for this study was granted by the local institutional human investigations committee. ESC were obtained with the method described by Vigano et al. (2002). Endometrial cells were cultured to sub-confluence in phenol-red free Dulbecco’s minimal essential medium (DMEM) with 10% FBS and antibiotics.

Ishikawa and ESC were expanded in medium containing normal FBS until 24 h before treatment when all cells were shifted to medium containing charcoal-stripped FBS with no detectable amounts of sex steroids. Before experiments investigating non-transcriptional effects, Ishikawa or ESC were kept in culture medium containing no FBS for 8 h. All cells were incubated in a humidified tissue culture incubator at 37°C, 5% CO2 atmosphere.

Whenever an inhibitor was used the compound was added 30 min before the treatment. RAL was obtained from Eli Lilly Research Laboratories, E2, TAM, PTX (G proteins inhibitor), Y-27632 (ROCK-2 inhibitor), PD98059 (ERK1/2 MAPK cascade inhibitor) were from Sigma-Aldrich (Saint-Louis, MO, USA), ICI 182,780 (ER antagonist) was obtained by Tocris Cookson (Avonmouth, UK). RAL and PD98059 were dissolved in DMSO, E2, tamoxifen and ICI 182,780 were dissolved in 100% ethanol. PTX and Y-27632 were dissolved in PBS. The final concentration of the solvents was 1 μl of solvent per 1 ml of medium.

Immunoblottings

Cell lysates were separated by SDS-PAGE. Antibodies used were: mouse monoclonal anti-moesin (clone 38, Transduction Laboratories, Lexington, KY, USA), goat polyclonal anti-Thr588-P-moesin (sc-12895, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-ERα (clone TE111.SD11, NeoMarkers, Union City, CA, USA), goat polyclonal anti-ERβ (N-19; sc-6820, Santa Cruz Biotechnology), goat polyclonal anti-ROCK-2 (C-20; sc-1851; Santa Cruz Biotechnology), and rabbit polyclonal anti-Git3 (clone A-20; sc-410; Santa Cruz Biotechnology), mouse monoclonal anti-Thr98-P-myelin Basic Protein PMBP (clone P12, upstate), goat polyclonal anti-Actin (C-11):sc-1615 Santa Cruz Biotechnology). Primary and secondary Abs were incubated with the membranes using standard techniques. Immunodetection was accomplished using enhanced chemiluminescence. Chemiluminescence was acquired with a quantitative digital imaging system (Quantity One, Bio-Rad, Hercules, CA) allowing to check for saturation. Overall emitted photons were quantified for each band, particularly for loading controls, which homogeneously loaded.

Cell immunofluorescence

Ishikawa and ESC were grown on coverslips (2.5 × 10−4 cell/ml) they were incubated for 24 h and then exposed for 15 min to different treatments. Cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton for 5 min. Blocking was performed with PBS containing 1% bovine serum albumin for 30 min. Cells were incubated with Texas Red- phalloidin (Sigma) for 30 min which stains filamentous (F)-actin. After washing the nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Immunofluorescence was visualized using an Olympus BX41 microscope and recorded with a high-resolution DP70 Olympus digital camera. Cell membrane thickness and the gray level of extracellular area, cell membrane as well as cytoplasm were quantified using Leica QWin image analysis and image processing software (Leica Microsystems, Wetzlar, Germany). The remodeling of actin fibers and the morphological changes of the membrane were quantified by assessing the intensity of the actin fluorescence after conversion of the colored pixels to grayscale using the Leica QWin image analysis and processing software. This analysis was performed selecting random boxes including the extra- and intra-cellular space across the membrane, and the linear intensity of the signal was spatially recorded after visual identification by one examiner of the external and internal limits of the membrane. We sampled five areas per each cell, and we repeated this on 30 different cells per experimental condition. Each measurement of the extracellular, membrane or cytosol signal intensity in the 30 different cells per condition were used to calculate means ± SD.

Co-immunoprecipitation assays

Ishikawa cells were harvested in 100 mM Tris—HCl, pH 6.8, 4% SDS, 20% glycerol, 1 mM Na3VO4, 1 mM NaF and 1 mM phenylmethylsulfonyl-fluoride (PMSF). Equal amounts of cell lysates were incubated with 1 μg of precipitating Ab (Gαi3) overnight at 4°C under gentle agitation. Twenty-five microliter of a 1:1 protein A-agarose slurry were added, and the samples were rolled at 4°C for another hour. The samples were then pelleted, washed and resuspended in 50 μl of 2× Laemmli buffer for immunoblotting.
**Kinase assays**

Ishikawa cells were harvested in 20 mM Tris–HCl, 10 mM EDTA, 100 mM NaCl, 0.5% IGEPAL and 0.1 mg/ml PMSF. Equal amounts of cell lysates were immunoprecipitated with Rhotekin RBD agarose (14-383, upstate) versus ROCK-2 (C-20, Santa Cruz). The immunoprecipitates (IP) were washed three times with buffer containing 20 mM Tris–HCl, 10 mM EDTA, 150 mM NaCl, 0.1% IGEPAL (non-ionic, non-denaturing detergent) and 0.1 mg/ml PMSF. For ROCK-2 activity assay, two additional washes were performed in kinase assay buffer (20 mM MOPS, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM DTT) and the samples were therefore resuspended in this buffer. Five microgram of de-phosphorylated Myelin Basic Protein (Upstate) together with 500 μM ATP and 75 mM MgCl₂ were added to each sample and the reaction was started putting the samples at 30 °C for 20 min. The reaction was stopped on ice and by resuspending the samples in Laemmli Buffer. The samples were separated with SDS-PAGE and western analysis was performed using antibodies recognizing Thr98-P-myelin Basic Protein (05-429, upstate).

**Cell migration assays**

Cell migration was assayed with razor scrape assays. Briefly, a razor blade was pressed through confluent Ishikawa cell monolayers into the plastic plate coated with 10 μg/ml of type IV collagen to mark the starting line. Ishikawa cells were swept away on one side of that line. Cells were washed, and
2 ml of MEM containing steroid-deprived FBS, collagen type IV (10 mg/ml) and the test substance was added. Cytosine β-D-arabinofuranoside hydrochloride (Sigma) (10 μM), a selective inhibitor of DNA synthesis which does not inhibit RNA synthesis was used 1 h before the test substance was added. Migration was monitored for 48 h. Every 12 h fresh medium and treatment was replaced. Cells were digitally imaged and migration distance was measured by using phase-contrast microscopy using a digital ruler to measure migration distance in different sites of the dish.

**Cell invasion assays**

Cell invasion was assayed using the BD BioCoatTM Growth Factor Reduced (GFR) MatrigelTM Invasion Chamber (BD Bioscience, USA). In brief, after rehydrating the GFR Matrigel inserts, the test substance was added to the wells. An equal number of Control Inserts (no GFR Matrigel coating) were prepared as control. A 0.5 ml of Ishikawa cell suspension (2.5 × 10⁴ cells/ml) was added to the inside of the inserts. The chambers were incubated for 24 h at 37°C, 5% CO₂ atmosphere. After incubation, the non-invading cells were removed from the upper surface of the membrane using cotton tipped swabs. Then the cells on the lower surface of the membrane were stained with Diff-Quick stain. The invading cells were observed and photographed under the microscope at 100× magnification. Cells were counted in the central field of triplicate membranes. The invasion was calculated as the % invasion test cell/% invasion control cells.

**Statistical analysis**

All values are expressed as (mean ± SD). Statistical analysis of the data was performed using one-way analysis of variance (ANOVA) followed by Tukey–Kramer Multiple-Comparisons Test. P < 0.05 was considered as statistically significant.
Results

Effects of estradiol, TAM and RAL on endometrial cell membrane and actin cytoskeleton remodeling

In order to test the hypothesis that estrogen and SERMs might have different effects on endometrial cancer cell morphology we assayed the effects of estradiol, TAM or RAL on actin fibers arrangement in ER+ Ishikawa endometrial adenocarcinoma cells. E2, TAM and RAL were provided to cells at different concentrations (0.1–100 nM) for 15 min. At baseline, actin fibers were arranged longitudinally in Ishikawa cells, that displayed regular cell borders (Fig. 1A–C). In cells exposed to E2 or TAM, a rapid loss of stress fibers, indicated by the disappearance of the regular, longitudinally-oriented cytoplasmic actin fibers (Chhabra and Higgs, 2007), and a shift of the fibers toward the edge of the membrane were seen (Fig. 1A, B). This cytoskeletal remodeling was accompanied by the development of membrane ruffles, visible as sheet-like membrane protrusions, and of pseudopodia (identified as weakly adherent longitudinal protrusions) (Chhabra and Higgs, 2007) at sites enriched in actin (Fig. 1A, B). In contrast, cells treated with RAL showed no modification of actin organization at any concentration tested (Fig. 1C). These changes corresponded to parallel modifications in the thickness of the cell membrane and in the actin fluorescence intensity ratio between membrane and cytoplasm (Fig. 1A–C).

The pattern of remodeling of actin fibers and cell membrane observed in Ishikawa cells with estrogen, TAM or RAL was also found in primary human ESC (Fig. 1D).

Estradiol and TAM, but not RAL, recruit the actin-binding protein moesin

Based on our previous finding that the actin-regulatory protein moesin mediates the cytoskeletal effects of estrogen in endothelial and breast cancer cells (Simoncini et al., 2006; Giretti et al., 2008), we investigated whether this protein might play a role in the endometrial actin rearrangement induced by E2 and TAM. The phosphorylation on Thr558 (corresponding to activation) of moesin was enhanced by short (15 min) incubations with E2 and with TAM (starting from 1 nM), but not by RAL in Ishikawa cells (Fig. 2A–C). Similar effects were found in ESC (Fig. 2D). The total cell content of moesin did not change during this period (Fig. 2A–D).
Estradiol and TAM, but not RAL, trigger moesin activation and cytoskeletal remodeling via an ER/G protein/ROCK-dependent signaling pathway

The recruitment of moesin by E2 and TAM in Ishikawa cells was not altered by inhibition of the ERK1/2 cascade with PD98059 (Fig. 3A–C). On the other hand, blockade of ER with ICI 182,780 or of G proteins with pertussis toxin (PTX) completely abolished moesin activation in Ishikawa cells (Fig. 3A–C) and ESC (Fig. 3D–F). In addition, interference with the kinase responsible for Thr558-phosphorylation of moesin, Rho-associated kinase (ROCK-2), using the inhibitor Y-27632 (ROCKi) also resulted in blockade of moesin phosphorylation in the presence of E2 or TAM (Fig. 3A–F). RAL was not effective in modulating moesin phosphorylation in Ishikawa or ESC (Fig. 3A–F), and the addition to RAL of ICI 182,780, PD 98059, or Y-27632 reduced moesin phosphorylation to a level below baseline.

The presence of ERα and ERβ in Ishikawa cells is not constant in culture, therefore, we checked the expression of these receptors with western analysis, that revealed both ERα and ERβ in Ishikawa cells and ESC, as well as in ER+ T47D breast cancer cells that were used as control (Fig. 3G, H).

Estradiol and TAM, but not RAL, induce the interaction of ERα with Gα13 and the later signaling to ROCK-2 and to the actin cytoskeleton

We previously identified that ERα targets the actin-binding protein moesin via the interaction with the G protein, Gα13, which starts a cascade leading to recruitment of the Rho-associated kinase,
ROCK-2 (Simoncini et al., 2006; Giretti et al., 2008). In order to test if the differential behavior of E2 and TAM versus RAL might be due to a distinct activation of this process, we did co-immunoprecipitation studies in Ishikawa cells to test the interaction of ERα and Gα13 in the presence of the compounds under study. These experiments showed that the interaction between ERα and Gα13 is enhanced in the presence of E2 and TAM, although it is not affected by RAL (Fig. 4A).

In addition, in the presence of E2, ROCK-2 is functionally activated, as shown by enhanced Thr-phosphorylation of the bait protein MBP by ROCK-2 IP obtained from Ishikawa cells (Fig. 4B). ROCK-2 activation by E2 was prevented by blocking G proteins with PTX (Fig. 4B). In comparison, TAM resulted in a weak but visible recruitment of ROCK-2, although no ROCK-2 activation was seen in the presence of RAL (Fig. 4B).

In agreement with the previous results, the remodeling of the actin cytoskeleton induced by E2 or TAM in ESC was dependent on ER, G proteins and ROCK-2, as shown by actin staining in the presence or absence of the specific inhibitors (Fig. 4C–E).

TAM, but not RAL, inhibits moesin activation by E2
The SERMs TAM and RAL compete with estrogens for binding to ERs and may block estrogen action in some instances. Co-treatment of Ishikawa cells with E2 (1 nM) in association with increasing concentrations of TAM or RAL revealed that the E2-induced moesin activation is counteracted by TAM, even at low concentrations (Fig. 5A), although it is not affected by RAL (Fig. 5B).

Effects of estradiol, TAM and RAL on endometrial cancer cell motility and invasion
To delineate whether the biochemical changes observed with E2 and TAM in normal endometrial cells and in Ishikawa cells are associated with effects on cell motility, we performed *in vitro* migration assays. After partially scraping Ishikawa cells (growth-inhibited with Cytosine β-D-arabinofuranoside hydrochloride, an inhibitor of DNA synthesis which does not inhibit RNA synthesis) out of the cell culture dish, we monitored the movement of the remaining cells for the following 48 h. Exposure to E2, and to a lesser extent to TAM, resulted in an enhanced migration toward the scraped area (Fig. 6A, B). On the opposite, RAL had no visible effect on Ishikawa cell motility (Fig. 6A, B). The co-administration of TAM or RAL with E2 (all at 1 nM) both resulted in a blockade of Ishikawa cell motility induced by E2 (Fig. 6C, D).

In addition to assaying horizontal motility, we performed three-dimensional invasion assays to test the effect of estrogen or SERMs on Ishikawa cell invasive properties. Administration of E2 to growth-inhibited Ishikawa cells resulted in enhanced invasion of the matrix (Fig. 7A–C). The effect of E2 was blocked by the ER-antagonist ICI 182,720 and by the G protein inhibitor PTX (Fig. 7A–C). In addition, co-treatment with E2 plus TAM or E2 plus RAL significantly reduced the effect of estradiol (Fig. 7A–C). The administration of either TAM or RAL alone did not increase the invasive behavior of Ishikawa cells (Fig. 7A–C).

Discussion
Deregulated endometrial cell remodeling, proliferation, adhesion and interaction with the extracellular matrix are important to a variety
of endometrial disorders, including dysfunctional uterine bleeding, infertility and endometriosis (Fujiwara et al., 2003; Klemmt et al., 2007; Dimitriadis et al., 2009). In addition, they play a relevant role in endometrial hyperplasia and cancer (Lessey et al., 1995; Bamberger et al., 1998; Dai et al., 2002; Stefansson et al., 2004).

Cellular interaction with the extracellular environment is achieved through the development of dynamic remodeling of the actin cytoskeleton, leading to waves of formation and disassembly of focal adhesion sites and changes in cell membrane morphology (Pollard and Borisy, 2003). Eventually, these events lead to the generation of membrane protrusions (such as lamellipodia and filopodia) and traction forces that allow the cells to move (Zamir and Geiger, 2001; Mitra et al., 2005).

Moesin, a member of the ERM family, is an actin-binding protein that plays an important role in cell motility by linking the actin cytoskeleton to a variety of membrane-anchoring proteins (Tsukita and Yonemura, 1999; Louvet-Vallee, 2000). In quiescent conditions moesin exists in an auto-inhibited conformation and phosphorylation of Thr558 within the C-terminal actin binding domain by the Rho-associated kinase (ROCK), results in a conformational change and in the association with the scaffold protein, ERM-binding protein 50 on moesin’s NH2-terminal end and with F-actin on moesin’s COOH-terminal end to mediate the linkage of microfilaments to membranes in cell surface microvilli (Oshiro et al., 1998). Recent evidence indicates that proteins of the ERM family have important regulatory roles in cancer biology. For instance, ezrin is over-expressed in highly aggressive sarcomas (Khanna et al., 2004; Yu et al., 2004), as well as in breast cancer, being associated with higher metastasis rate (Elliott et al., 2005). Likewise, moesin expression and sub-cellular localization have been found to be deranged in metastatic breast cancers (Giretti et al., 2008).

Related to the present article, ezrin is over-expressed in invasive endometrial cancers (Chen et al., 2001) and is required for in vivo invasion and metastatic potential of endometrial cancer cells (Ohtani et al., 2002). Endometriosis is also characterized by an increased expression of ezrin (Ornek et al., 2008), possibly suggesting that this protein may have a role in the attachment of ectopic endometrial cells to the peritoneum.

In the present article, we report that E2 and TAM rapidly activate a ER/Gα13/RhoA/ROCK/moesin signaling pathway in human endometrial Ishikawa cells and in primary cultured stromal cells. This cascade of events is related to the activation of actin remodeling and to the development of cell membrane morphological changes, as well as with the enhancement of cell horizontal movement and
invasion of a three-dimensional matrix in normal and immortalized endometrial cells. These findings are consistent with recent reports in other endometrial cell lines that show estrogen activation of endometrial cancer movement through the recruitment of extranuclear signaling of ERs to mitogen-activated protein kinases and phosphatidylinositol-3-OH kinase (Acconcia et al., 2006; Gentilini et al., 2007). In addition, the present results are in line with our recent reports, that identified the recruitment of the Gα13/RhoA/ROCK/moesin signaling cascade by estrogen and progesterone receptors in endothelial and breast cancer cells linked to cytoskeletal remodeling and cell movement and invasion (Simoncini et al., 2006; Fu et al., 2008a, b; Giretti et al., 2008). In these settings, ligand-bound ERα or PRs act as a G protein-coupled receptors recruiting the G protein Gα13, which signals to the small GTPase RhoA leading to the activation of the Rho-associated kinase ROCK-2. This kinase is responsible for the phosphorylation and activation of moesin and for the ensuing estrogen- or progesterone-induced endothelial and breast cancer cell enhanced interaction with the extracellular matrix and targeted migration (Simoncini et al., 2006; Fu et al. 2008a, b; Giretti et al., 2008).

A significant finding of this study is that RAL does not induce moesin phosphorylation, cytoskeletal changes or endometrial cell movement as opposed to E2 or TAM. The lack of agonistic activity of RAL on endometrial cells is well established both in vitro (Somjen et al., 1996) and in animals (Sato et al., 1996), although the molecular basis of the different action versus TAM or estradiol at this level is not fully established. In contrast with these results, our previous work shows that RAL is able to trigger actin remodeling and moesin

Figure 7 Effects of estradiol, TAM or RAL on Ishikawa cell invasion.

Ishikawa cells were treated for 24 h with E2 (10^{-9} M), in the presence or absence of the pure ER antagonist ICI 182,780 (ICI—100 nM), of the G protein inhibitor, PTX (100 ng/ml) or RAL (10^{-9} M) or TAM (10^{-9} M). Other cells received a combination of E2 (10^{-9} M)+TAM (10^{-9} M) or E2 (10^{-9} M)+RAL (10^{-9} M). The invading cells were photographed at 100 x magnification and counted in the central field of triplicate membranes. (A) Indicates the mean number of invading cells ± SD from three separate experiments. *P<0.05 versus Con. **P<0.05 versus E2. (B) Shows the differential invasion expressed as percentage of invading cells in treated cells/percentage of invading cells in control. *P<0.05 versus Con. **P<0.05 versus E2. (C) Shows sample images of invasion in the different conditions. TAM, tamoxifen; RAL, raloxifene.
phosphorylation in ER+ breast cancer cells, leading to enhanced cell migration and invasion (Flamini et al., 2008). This discrepancy may indicate that the rapid extra-nuclear signaling of ER triggered by SERMs may be to some extent cell-specific, similar to what is well established for gene regulation.

The potential relevance of the anti-estrogenic action of RAL in the endometrium have been recently highlighted by a large-scale clinical trial indicating that women receiving RAL are significantly protected from endometrial cancer as compared with normal controls and even more in comparison to women receiving TAM, where an increased risk is found (DeMichele et al., 2008). This is consistent with studies that show that RAL blocks the stimulatory effect of estradiol on the endometrium (Sato et al., 1996), as well as the progression of transplanted endometrial carcinoma cells in mice (Gottardis et al., 1990). Although the molecular basis for the different action of RAL versus E2 or TAM in endometrial cells has not been completely elucidated, there is evidence that these ER ligands induce a different three-dimensional conformation of the ligand-binding domain of the receptor, which is related to a distinct pattern of interaction with co-activators or co-repressors in cells and thus with different gene regulation (Dutertre and Smith, 2000). Our work expands on this area, highlighting how different SERMs also turn into separate cellular actions because of differences in the recruitment of rapid, extra-nuclear pathways linked to the control of the actin cytoskeleton.

On the other hand, in the presence of E2, which potently increases endometrial cell movement, both SERMs, TAM and RAL, act as anti-estrogens, resulting into a marked blockade of both horizontal cell migration and invasion of three-dimensional matrices, highlighting that both SERMs are able to interfere with ER activation by E2 and with its later extra-nuclear signaling.

Our data also demonstrates that interference with the recruitment of the Goq11/RhoA/ROCK/moesin cascade in endometrial cells disrupts the E2- or TAM-induced enhanced attachment and migration. This might offer a potential new way to control the endometrial actions of E2 or TAM, with potential usefulness in a range of benign or malignant disorders where endometrial attachment to the extra-cellular matrix is deranged, such as dysfunctional uterine bleeding, endometriosis or endometrial cancer.

Although we identify a series of signaling steps that are differentially regulated by E2, RAL or TAM in endometrial cells, there are a number of unanswered questions. For instance, how does TAM promote phosphorylation of moesin and membrane modifications and yet it inhibits migration and to some extent, invasion when added to estradiol? And why is it that RAL does not inhibit the phosphorylation of moesin induced by E2 and still it interferes with its effects on migration and invasion? One likely explanation is that the functional regulation of cell migration and invasion is regulated through multiple, redundant signaling intermediates, and moesin is only one of the players in the game. Further elucidation of the role of this protein for endometrial cell movement or differentiation is required to have a better understanding of the fundamental biological differences in the actions of estrogen or SERMs on these cells.

In conclusion, this article describes the differential regulation of cell membrane morphology, actin cytoskeleton remodeling and migration and invasion of endometrial cells by estradiol, TAM and RAL. Through a different regulation of ER extra-nuclear signaling, estradiol and TAM enhance endometrial cell cytoskeletal remodeling and movement, whereas RAL does not. These findings help to understand the molecular basis of the actions of estrogen and SERMs in endometrial cells, and could provide new rationale for the clinical findings on the endometrial actions of SERMs.

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References


Ezrin and moesin are membrane-cytoskeletal linker proteins that play crucial roles in cell adhesion, migration, and invasion. Ezrin is necessary for osteosarcoma metastasis, and moesin is involved in cell development and tissue-specific expression. The phosphorylation of moesin by rho-associated kinase (Rho-kinase) plays a crucial role in the formation of microvilli-like structures. Ezrin interacts with Galpha13 to drive actin remodeling and endothelial cell migration via the RhoA/Rho kinase/moesin pathway.


