Progestogens regulate endothelial actin cytoskeleton and cell movement via the actin-binding protein moesin

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The endothelial effects of progestogens are poorly investigated. Actin remodeling and cell movement are fundamental for endothelial function and are controlled by the actin-binding protein moesin. In this work, we studied the effects of progesterone and medroxyprogesterone acetate (MPA) on actin remodeling, moesin activation and cell movement in human endothelial cells. Our findings show that progesterone and MPA trigger a rapid endothelial actin rearrangement, with the formation of cortical actin complexes, pseudopodia and membrane ruffles. Both progestogens trigger a rapid progesterone receptor (PR)-dependent moesin activation via a non-genomic signaling cascade involving G proteins, the small GTPase RhoA and the Rho-associated kinase (ROCK-2). In addition, MPA signaling also requires the recruitment of phosphatidylinositol-3 kinase (PI3K). Both progestogens enhance endothelial cell migration, which is prevented by moesin silencing or by blockade of PR, G proteins, PI3K, mitogen-activated protein kinases or ROCK-2. Progesterone and MPA potentiate 17β-estradiol (E2) induced-moesin activation. However, they partially reduce cell migration induced by E2. In conclusion, progesterone and MPA regulate endothelial cell movement by rapidly signaling to the actin-binding protein moesin and to the actin cytoskeleton. These findings provide new information on the biological actions of progestins on human endothelial cells that are relevant for vascular function.

Keywords: progesterone; medroxyprogesterone acetate; vascular endothelial cells; moesin; cell movement

Introduction

Vascular endothelial cells synthesize and release multiple active factors which regulate angiogenesis, inflammatory responses, vascular tone and permeability. The lack of correct endothelial function is associated with increased cardiovascular risk (Feletou and Vlahou, 2006). Endothelial dysfunction gradually ensues after the menopause (Taddei et al., 1996), associated with impaired flow-mediated dilatation (Herrington et al., 2001), declining of endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) bioavailability as well as abnormal endothelial morphology (Majmudar et al., 2000; Kubickiene et al., 2005). These functional alterations of endothelial cells likely contribute to the increased risks of cardiovascular diseases seen in post-menopausal women (Kannel et al., 1976; Guthrie et al., 2004).

Hormone replacement therapy (HRT) has been regarded as an effective tool to protect post-menopausal women from cardiovascular diseases (Barrett-Connor and Bush, 1991). However, major randomized clinical trials have failed to confirm the cardiovascular advantages of HRT (Manson et al., 2003). The Women’s Health Initiative (WHI) trial shows that combined HRT with conjugated equine estrogens (CEE) and medroxyprogesterone acetate (MPA) is associated with a non-significant increase of coronary heart disease (CHD) in post-menopausal women, whereas women receiving CEE alone in the sister cohort showed a non-significant decrease of coronary events, along with a significant reduction of a composite outcome of CHD events in younger women (Rossouw et al., 2002; Anderson et al., 2004). These findings raise the interest on the role of progestogens on the vascular system and in particular on endothelial cells.

Estrogen preserves endothelial function in vitro and in vivo (Mendelsohn and Karas, 1999; Mendelsohn and Karas, 2005). Exposure of endothelial cells to estrogen increases NO and prostaglandins synthesis and release which results in vasodilatation (Huang et al., 2001; Simoncini et al., 2000a,b). In addition, estrogen regulates endothelial proliferation (Fu et al., 2007), response to inflammation (Gerald et al., 2006), permeability (Groten et al., 2005) and morphology (Kubickiene et al., 2005). However, progesterone or other synthetic progestins have a variable influence on endothelial function.

For example, natural progesterone increases endothelial NO production whereas MPA is devoid of such action (Simoncini et al., 2004). In non-human primates, MPA has been shown to interfere with the athero-protective effects of estrogens, which does not happen with natural progesterone (Adams et al., 1990, 1997). In support of these observations, discrepant effects of progestins have also been described in other tissues (Nilsen and Brinton, 2003).

We recently characterized a novel set of actions of estrogen on the control of the actin cytoskeleton in human endothelial cells. By promoting dynamic actin remodeling, estrogen supports the formation of focal adhesion complexes and the development of specialized membrane structures such as ruffles and pseudopodia, thus inducing endothelial cell migration. These phenomena depend on the activation of the actin-regulatory protein moesin (Simoncini et al., 2006). These findings highlight new possible mechanisms through which estrogen...
might control a number of processes such as re-endothelialization of damaged vessels and angiogenesis. Nevertheless, the role of progestogens on endothelial cell migration and the possible mechanisms involved are yet unknown.

In this study, we investigated the effects of two commonly used progestogens, natural progesterone and MPA on endothelial cell movement. In particular, we studied the effects of these two compounds on moesin activation and actin cytoskeleton remodeling, with a focus on signal transduction. In addition, we also studied the effects of progesterone and MPA in the presence of estrogen.

Materials and Methods

Cell cultures and treatments

Human umbilical vein endothelial cells (HUVEC) were cultured as described (Simoncini et al., 2006). Before treatments, HUVEC were kept 48 h in Dulbecco’s minimal essential medium (DMEM) containing steroid-deprived fetal bovine serum (FBS). Before experiments investigating non-transcriptional effects, HUVEC were kept in DMEM containing no FBS for 8 h. 17β-estradiol (E2), progesterone, MPA, pertussis toxin, PD98059, wortmannin, Y-27632 were from Sigma-Aldrich (Saint-Louis, MO, USA), 4-pregnen-3,20-dione3-β-D-glucuronide from Sigma-Aldrich (Saint-Louis, MO, USA), bovine serum (FBS). Before experiments investigating non-transcriptional effects, HUVEC were kept in DMEM containing steroid-deprived fetal bovine serum (FBS). Before experiments investigating non-transcriptional effects, HUVEC were kept in DMEM containing no FBS for 8 h. 17β-estradiol (E2), progesterone, MPA, pertussis toxin, PD98059, wortmannin, Y-27632 were from Sigma-Aldrich (Saint-Louis, MO, USA), 4-pregnen-3, 20-dione3-β-D-glucuronide from Sigma-Aldrich (Saint-Louis, MO, USA), bovine serum (FBS). Before experiments investigating non-transcriptional effects, HUVEC were kept in DMEM containing steroid-deprived fetal bovine serum (FBS). Before experiments investigating non-transcriptional effects, HUVEC were kept in DMEM containing no FBS for 8 h. 17β-estradiol (E2), progesterone, MPA, pertussis toxin, PD98059, wortmannin, Y-27632 were from Sigma-Aldrich (Saint-Louis, MO, USA), 4-pregnen-3,20-dione3-β-D-glucuronide from Sigma-Aldrich (Saint-Louis, MO, USA), bovine serum (FBS). Before experiments investigating non-transcriptional effects, HUVEC were kept in DMEM containing steroid-deprived fetal bovine serum (FBS).

Results

Progesterone and MPA induce rapid cytoskeletal and cell membrane remodeling in human endothelial cells

We tested the effect of the administration of natural progesterone (P, 10 nM) or of the synthetic progestin MPA (10 nM) on actin arrangement in endothelial cells. At baseline, actin fibers were arranged longitudinally through the major axis of endothelial cells (Fig. 1). Treatment with progesterone or MPA induced a rapid localization of actin toward the edge of the cell membrane, where cortical actin complexes were formed, in parallel with the formation of specialized membrane structures, such as pseudopodia and membrane ruffles (Fig. 1). Visible changes of actin organization could be observed in ~75% of endothelial cells. These actions were rapid and time-dependent, being maximal at 15 min and after 30 min showed a reversal to the baseline arrangement (Fig. 1). The membrane-impermeable albumin–progesterone conjugate (PBSA, 10 nM) also induced actin reorganization after exposure to endothelial cells for 15 min (Fig. 1). All these actions were blocked by the addition of a selective progesterone receptor (PR) antagonist, ORG 31710 (10 μM), indicating that these effects are exerted through PR.

Progesterone and MPA rapidly induce moesin phosphorylation

Similar to E2 (Simoncini et al., 2006), both progesterone and MPA rapidly activated moesin in comparable time- and dose-dependent manners (Fig. 2A–D). Moesin activation was seen between 2 and 15 min after treatment with two progestogens (both 10 nM) and then declined after 30 min (Fig. 2A and B). The PR antagonist ORG 31710 (10 μM) prevented the action of both compounds (Fig. 2E). In the recent years, evidence has grown that a small subset of PR localized at the cell membrane after post-translational modifications (Ashley et al., 2006; Pedram et al., 2007). According to the rapid time-course of actin cytoskeleton rearrangement, moesin activation and de-activation, these actions are akin to non-genomic effects (Fu and Simoncini, 2007). Indeed, PBSA (10 nM) activated moesin similar to natural progesterone and this effect was sensitive to ORG 31710...
suggesting that PR localization at or near the cell membrane might be relevant for signaling to moesin. In support of this, immunofluorescence using anti-PR antibody linked to Texas Red showed that in endothelial cells with intact membrane (without the addition of triton X), a spotted pattern of PR staining was found on endothelial cell membrane (Fig. 2G). In contrast, in cells treated with Triton X, PR staining was concentrated in the nuclei, with sporadic staining in the periphery of the cells (Fig. 2G).

Figure 1: Actin cytoskeleton remodeling by progesterone or MPA. HUVEC were treated with progesterone (P, 10 nM), MPA (10 nM) or membrane-impermeable albumin-progesterone conjugate (PBSA, 10 nM) for the indicated time in minutes (0'–30'), in the presence or absence of ORG 31710 (1 μM). Actin fibers were stained with phalloidin linked to Texas red staining) and nuclei were counterstained with DAPI (blue staining). Immunofluorescence shows changes of actin fibers localization and the formation of specialized cell membrane structures (green arrows indicate longitudinal actin fibers, light blue arrows indicate pseudopodia, yellow arrows show ruffles and white arrows indicate focal adhesion complexes).

Figure 2: Progesterone and MPA activate moesin. Western blots show total cell amount of wild-type (Moesin) or Thr^558-phosphorylated moesin (P-Moesin). HUVEC were treated with progesterone (P) and MPA for different times (A and B) or in different concentrations (C and D). (E and F) HUVEC were exposed to progesterone (P, 10 nM), MPA (10 nM) or PBSA (10 nM) for 15 min, in the presence or absence of ORG 31710 (1 μM). (G) Endothelial cells were stained with an antibody versus PR (Texas red; red staining) in the absence or presence of 0.1% Triton X treatment. PR staining in the membrane or in the nuclei was shown.
Signaling cascades of progesterone and MPA

We recently characterized a novel mechanism of rapid signaling to the cytoskeleton activated by estrogen receptor alpha (ERα) in human endothelial cells that involves the recruitment of the G protein Go13 and leads to activation of the small GTPase RhoA and of its downstream effector Rho-associated kinase (ROCK-2) that activates moesin (Simoncini et al., 2006). To address the question of the relevance of these pathways for activation of moesin by PR in endothelial cells, we used pharmacological inhibitors of these intermediates in cells exposed to progesterone or MPA. Moesin activation by progesterone or MPA was prevented by the addition of the G protein inhibitor pertussis toxin (PTX, 100 ng/ml) or of the ROCK-2 inhibitor Y-27632 (10 μM) (Fig. 3A and B). On the contrary, PD98059 (5 μM), which inhibits the mitogen-activated protein kinase (MEK), was ineffective (Fig. 3A and B). However, differences in signaling were also observed, since wortmannin (30 nM), the inhibitor of phosphatidylinositol-3 kinase (PI3K), prevented the action of MPA but not that of progesterone (Fig. 3A and B). As control, ERK 1/2 and the PI3K downstream effector, Akt, were rapidly activated by progesterone and MPA (Fig. 3C and D) and PD98059 (5 μM) or wortmannin (30 nM) were effective in blocking these actions (Fig. 3C and D).

As previously reported (Menager et al., 1999; Vaiskunaite et al., 2000; Simoncini et al., 2006), RhoA is a crucial mediator conveying the upstream signaling evoked by various factors to its downstream target ROCK-2, which is a known activator of ezrin/radixin/moesin (ERM) proteins. RhoA was rapidly activated by both progestogens in endothelial cells and this was inhibited by ORG 31710 or PTX but not by PD98059 (Fig. 3E and F). Consistent with the previous findings, blockade of PI3K with wortmannin impaired RhoA activation by MPA but not by progesterone (Fig. 3E and F).

To further characterize the role of PI3K and Go13 for the signaling recruited by PR in the presence of the different ligands, we transfected endothelial cells with a dominant negative form of the regulatory subunit of PI3K, p85α (Δp85α) or with a dominant negative Go13 construct (Go13 ΔQ226L/D294N). Transfection with Δp85α significantly impaired moesin phosphorylation induced by MPA but not by progesterone (Fig. 3G and H), confirming a key role of PI3K in MPA-dependent signaling. When cells were transfected with Go13 ΔQ226L/D294N, this fully blocked moesin activation induced by progesterone (Fig. 3G) whereas a lesser inhibitory effect was found on MPA-induced moesin activation (Fig. 3H). In addition, transfection of endothelial cells with both plasmids totally prevented moesin activation induced by both progesterone and MPA (Fig. 3G and H).

In the presence of PTX or Y-27632, there were no obvious changes in actin organization in endothelial cells exposed to progesterone or MPA (Fig. 3I). However, both progestogens still provoked rapid actin rearrangements of endothelial cells pre-treated with PD98059 (Fig. 3I). The PI3K inhibitor, wortmannin, blocked actin remodeling induced by MPA but not by progesterone (Fig. 3I).

Progestrone and MPA potentiated E2-induced moesin activation

As previously shown (Simoncini et al., 2006), E2 (1 nM) rapidly increased moesin phosphorylation in endothelial cells. This effect was substantially potentiated by the addition of progesterone (10 nM, mean increase versus E2 38.5%) or MPA (10 nM) (Fig. 4A, mean increase versus E2 24.6%). Intriguingly, the PR antagonist ORG 31710 inhibited the additive action of combining progesterone with E2 (Fig. 4A), and in the case of MPA, it also reduced the activation of moesin below that of E2 alone (Fig. 4A, mean decrease versus E2 85.7%). In parallel, actin remodeling and the formation of cortical actin complexes or pseudopodia were found when endothelial cells were exposed to progesterone and MPA in the presence of E2, but were largely reversed when the PR antagonist ORG 31710 was added to the combinations (Fig. 4B).

Effects of progesterone and MPA on endothelial cell movement

Finally, we studied the effects of progesterone and MPA on endothelial cell movement. In order to distinguish endothelial cell migration from proliferation, we pretreated endothelial cells with cytosome β-δ-arabinofuranoside hydrochloride (10 μM), a selective inhibitor of DNA synthesis which does not inhibit RNA synthesis. Both progesterone and MPA promoted endothelial cell horizontal mean migration (Fig. 5A–D). Endothelial cell migration induced by progesterone or MPA was largely blocked by interference with PR and G proteins (Fig. 5A–D). Lower inhibitory effects were found in the presence of the MAPK inhibitor, PD98059, of the PI3K inhibitor, wortmannin and of the ROCK-2 inhibitor, Y-27632 (Fig. 5A–D).

In order to check for the requirement of moesin for progesterone and MPA-promoted cell migration, we silenced moesin with antisense oligonucleotides (PONs) (Fig. 5E). Transfection with moesin antisense PONs greatly reduced the action of both progesterone and MPA on cell migration, whereas sense PONs had no impact (Fig. 5A–D).

E2 promoted endothelial cell migration as well. However, no additive effects were seen during progesterone or MPA co-administration. Actually, a significant reduction of cell migration was found during co-treatment with E2 and each progestogen (Fig. 5A–D). The PR antagonist ORG 31710 significantly reduced cell migration associated with both progestogens (Fig. 5A–D), but also slightly decreased the effect of the combination of E2 with each of the progestogens (Fig. 5A–D).

Discussion

A correct regulation of endothelial cell movement is critical to retain a healthy endothelium, allowing an effective regeneration of damaged or dysfunctional cells. We previously showed that the sex steroid estrogen promotes endothelial cell migration via an ER/Go13/RhoA/ROCK-2/moesin cascade, which is responsible for the estrogen-induced actin remodeling and cell migration (Simoncini et al., 2006). In the present work, we further found that progesterone and MPA enhance endothelial cell migration through moesin activation and actin rearrangement. These findings suggest that sex steroids are powerful cytoskeletal regulators in human endothelial cells and that this may turn into maintenance of cell function and integrity (Razandi et al., 2000; Simoncini et al., 2000a,b, 2003; McNeill et al., 2002).

Our data show that both progesterone and MPA transiently trigger actin rearrangements, in association with the formation of specialized membrane structures, such as pseudopodia and ruffles. These structures support the development of periodic waves of actin remodeling and the formation of integrin-dependent cell–extracellular matrix adhesion complexes (Giannone et al., 2004). Subsequently, the newly formed adhesion complexes are stabilized and locomotive forces are finally generated by the acto-myosin contractile machinery. By this manner, cells move forward in response to various stimuli (Yamazaki et al., 2005). The actions of progestogens on actin organization described in this work may provide new insight into a number of processes regulated by progestogens, such as progestosterone-promoted endometrial differentiation and vascularization or breast cancer metastasis (Kayisli et al., 2004; Carnevale et al., 2007).
Ezrin/Radixin/Moesin (ERM) proteins are key regulators controlling the dynamic process of actin cytoskeleton remodeling (Louvet-Vallee, 2000). Moesin represents the predominant ERM actin-binding protein in human endothelial cells (Berryman et al., 1993). When phosphorylated on Thr\(^{558}\), it serves as the bridge connecting actin fibers to plasma membrane, leading to the formation of cortical complexes. Our data show that moesin is phosphorylated by both progestogens between 2 and 15 min and that this declines after 30 min, time-consistent with the kinetics of actin rearrangement. According to its rapid activation and deactivation, these actions of the two

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**Figure 3:** Signaling cascades of progesterone and MPA.

(A and B) HUVEC were exposed to progesterone (P, 10 nM) or MPA (10 nM) for 15 min, in the presence or absence of the pure PR antagonist ORG 31710 (ORG 1 \(\mu\)M), the MEK inhibitor PD98059 (PD, 5 \(\mu\)M), of the PI3K inhibitor wortmannin (WM, 30 nM), of the G protein inhibitor, PTX (100 ng/ml), or of the ROCK-2 kinase inhibitor, Y-27632 (10 \(\mu\)M). Cell content of wild-type or phosphorylated moesin are shown. (C and D) Cells were exposed to progesterone (P, 10 nM) or MPA (10 nM) for 15 min, in the presence or absence of the MEK inhibitor PD98059 (PD, 5 \(\mu\)M), of the PI3K inhibitor wortmannin (WM, 30 nM). Cell content of wild-type or phosphorylated ERK1/2 or Akt are shown. (E and F) Active, GTP-bound RhoA was immunoprecipitated with Rhoteckin and subsequently assayed with western analysis with an anti-RhoA Ab (lower boxes). The upper boxes show the total RhoA content in the input. (G and H) Cells were exposed to 100 nM progestosterone or MPA for 15 min after transfection with dominant-negative p85\(^{\alpha}\) or \(G_{\alpha_{13}}^{\text{DN}}\) or the both for 48 h. Cell content of wild-type or phosphorylated moesin are shown. (I) Immunofluorescence shows changes of actin fibers localization and the formation of specialized cell membrane structures (green arrows indicate longitudinal actin fibers, light blue arrows indicate pseudopodia, yellow arrows show ruffles and white arrows indicate focal adhesion complexes).
progestogens are akin to other established non-genomic effects of sex steroids (Fu and Simoncini, 2007). In the present study, inhibition of G proteins blocked both progestogens-induced moesin activation and actin reorganization. In agreement, recent reports describe non-genomic PR signaling coupled to Gαi and Gβγ (Machelon et al., 1996; Lutz et al., 2000; Karteris et al., 2006), and our recent work shows that Gα13 interacts with ER to recruit moesin (Simoncini et al., 2006). In addition, the membrane-impermeable bovine serum albumin–progestosterone conjugate (PBSA) also induces moesin activation. Previous studies have shown that PBSA has no access through the cell membrane and that the progesterone–BSA conjugate is quite stable in the cell culture medium, without significant release of free progesterone that could enter the cell (Tischkau and Ramirez, 1993; Bandyopadhyay et al., 1998). This supports the hypothesis that a PR located at or near the cell membrane, where it could have a privileged access to G proteins (Zhu et al., 2003), might play a role in the signaling to moesin. Indeed, a spotted pattern of PR staining was found on the endothelial cell membrane.

That moesin is relevant for cell movement induced by sex steroids is supported by the demonstration that moesin silencing leads to a significant reduction of progesterin-induced cell migration. Of note, although the activation of moesin is enacted through rapid extra-nuclear actions of PR, the progestin-dependent enhancement of endothelial cell migration is a process that requires a longer time to be observed. As it is not possible to block gene expression in endothelial cells for more than a few hours (as this results in loss of viability), it is difficult to dissect the contribution of the non-genomic or of the genomic pathways of PR to cell migration. Previous publications on ER show that some actions of estrogen on gene expression are started by the recruitment in the cytoplasm of extra-nuclear signaling pathways, such as PI3K (Pedram et al., 2002). Thus, it is likely that the recruitment of G proteins, PI3K and moesin by PR ultimately results in the regulation of genes that play a role in cell migration. In addition, our previous results indicate that moesin expression is enhanced by long-term treatment with estrogen in endothelial cells (Simoncini et al., 2006), thus the enhanced endothelial cell movement observed in the presence of progestins likely depends on a complex interplay of extra-nuclear and nuclear effects of PR.

The evidence of the recruitment of the RhoA/ROCK-2 cascade in moesin activation is consistent with previous findings indicating that RhoA and ROCK-2 play a crucial role in activating ERM proteins (Menager et al., 1999; Vaikunnaite et al., 2000; Simoncini et al., 2006). Our results show that inhibition of G proteins results in a reduced activity of RhoA, implying that RhoA/ROCK-2 may represent the link between activation of G proteins by PR and moesin. Indeed, G proteins are able to control RhoA activity by modulation of the Rho guanine-nucleotide exchange factor (RhoGEF) (Ueda et al., 2008).

Interestingly, slight differences in signaling were observed between progestosterone and MPA, since PI3K was exclusively involved in MPA-induced moesin activation. This is consistent with other unpublished observations in breast cancer cells where we find that PI3K is recruited by interaction of PR with c-Src in the presence of MPA but not of progesterone (Simoncini, unpublished data). In endothelial cells, we find that activation of moesin in the presence of progesterone is prevented by a dominant negative Gα13 construct but not by a non-functional PI3K, indicating that progesterone drives PR to recruit Gα13 but not PI3K to signal to RhoA and moesin. On the contrary, MPA simultaneously recruits PI3K and Gα13 to activate RhoA and moesin. To explain this finding, it could be speculated that the conformation of PR bound by MPA allows a better interaction and activation of PI3K versus the conformation of PR bound by progesterone. This would allow MPA-bound PR to recruit two separate and parallel pathways to signal to RhoA, differently from progesterone-bound PR.

The spectrum of binding to PR and other steroid receptors varies significantly among progestins, leading to variable cellular effects, including in endothelial cells (Simoncini et al., 2004, 2007). Indeed, unlike progesterone, MPA is able to bind and activate other steroid receptors including the androgen and glucocorticoid receptors, which might also potentially explain the difference in signaling of progesterone versus MPA. Indeed, binding of MPA to glucocorticoid receptors results in interference with the anti-inflammatory actions of hydrocortisone in endothelial cells which are not exerted by natural progesterone (Simoncini et al., 2004).

Notwithstanding the differential requirement of PI3K for moesin activation by progesterone or MPA, this enzyme is still relevant for progesterone- and MPA-induced cell migration. Similarly, MAPK are involved in progestogens-promoted cell migration regardless of the absence of a role for moesin activation. These discrepancies are obviously due to the complexity of the cell movement process that is regulated by multiple internal and external signals which go well beyond the control of a single protein, like moesin (Chien et al., 2005).

When progesterone or MPA are provided together with E2, an increase of moesin phosphorylation is seen, indicating additive

Figure 4: Progesterone and MPA potentiate E2-induced moesin activation. HUVEC were exposed to E2 (1 nM) or E2 plus progesterone (P, 10 nM) or MPA (10 nM) for 15 min, in the presence or absence of the pure PR antagonist ORG 31710 (ORG, 1 μM). (A) Cell content of wild-type or phosphorylated moesin are shown. (B) Changes of actin fibers localization and the formation of specialized cell membrane structures are demonstrated (green arrows indicate longitudinal actin fibers, light blue arrows indicate pseudopodia, yellow arrows show ruffles and white arrows indicate focal adhesion complexes).
Figure 5: Progesterone and MPA enhance endothelial cell migration.
HUVEC were scraped out of the cell culture dish and the extent of migration of the remaining cells was assayed in the presence of Ara-C (see text). Cells were exposed to progesterone (P, 10 nM) or MPA (10 nM) for 48 h, in the presence or absence of E2 (1 nM), or of the pure PR antagonist ORG 31710 (ORG, 1 μM), the MEK inhibitor PD98059 (PD, 5 μM), of the PI3K inhibitor wortmannin (WM, 30 nM), of the G protein inhibitor, PTX (100 ng/ml), of the ROCK-2 kinase inhibitor, Y-27632 (10 μM), of the antisense oligonucleotides (PONs) (AS, 4 μM) or of the control sense PONs (S 4 μM). (A and C) The experiments were performed in triplicates and representative images are shown. (B and D) Cell migration distances were measured and values are presented as % of control. Data representing the migration distance of cells from the starting line are expressed as mean ± SD. Statistical significance between groups are indicated in the graphs. The arrows indicate the direction of migration. The upper black lines indicate the starting line and the lower black lines indicate the mean migration distance. (E) Moesin expression was detected by western blot in HUVECs transfected with moesin antisense PON for the indicated time.
effects between E2 and progestogens. However, compared with E2 alone, there is a significant reduction of cell migration when endothelial cells are exposed also to progesterone or MPA. These phenomena suggest that estrogen and progestins may act co-operatively in the rapid signaling to moesin, whereas this may not extend to longer-term actions, such as the modification of cell phenotype linked to cell movement. Indeed, the recruitment of ER and PR each turns into the regulation of a complex subset of target genes, whose function on cell movement is not yet investigated. Moreover, recent findings indicate that PR may act as an ER antagonist in certain circumstances, altering the ability of ER to interact with estrogen response elements and to trigger gene expression (Zheng et al., 2007). In addition, the blockade of PR with ORG 31710 inhibits moesin activation and cell migration induced by the combination of E2 with each progestin. A similar observation has been reported in breast cancer cells, where the up-regulation of breast cancer resistance protein expression induced by the combination of E2 plus progesterone was abolished by the PR antagonist RU-486 (Wang et al., 2006). One possible explanation of these observations could be that PR cross-talks with ER to accomplish these functions, and the presence of progesterone or MPA might facilitate this cross-talk. On the other hand, the presence of the PR antagonist may interfere with the ability of ER to serve as a scaffold to ER, thus antagonizing the function of both ER and PR. This would be consistent with the established ability of the ER antagonist ICI 182 780 to block PR signaling in breast cancer cells (Migliaccio et al., 1998) but additional studies will be necessary to provide a definitive explanation.

In theory, the interference of progestins with the estrogen-induced endothelial cell migration may reduce estrogen’s potential to support the continuous repair of injured endothelial areas, thus offsetting some protective actions of estrogen on the vessels. However, whether this is of any relevance in vivo cannot be predicted by this study. Likewise, although it might be tempting to hypothesize that the interference of the progestins with estrogen-supported endothelial migration might have played a role in determining the negative results of the clinical trials where women received HRT with both estrogen and progestins, such as the Heart and Estrogen/Progestin Replacement Study HERS or the WHI studies (Hulley et al., 1998; Rosouw et al., 2002) this is just a matter of speculation. Thus, the exploration of the relevance of the promotion of endothelial movement by sex steroids for vascular function or dysfunction should be the matter of future studies.

In conclusion, our findings indicate that both progesterone and MPA enhance endothelial cell movement. These actions are mediated by activation of the actin-binding protein moesin, resulting in actin remodeling via partially different signal transduction pathways. In addition, progesterone and MPA interfere with estrogen induced-moesin activation and endothelial cell movement. These findings provide new mechanisms of actions of PR in human endothelial cells, establishing the signaling to RhoA/ROCK-2 and moesin as a prominent function of this receptor implicated in the control of endothelial cytoskeleton and cell movement. Moreover, these results add new clues for better understanding the pharmacological actions of progestins, which might be of use in the clinical setting.

Author’s Role
All authors meet the qualification of MHR. X.-D.F and M.F. designed and carried out the experiments, analyzed the data, drafted and revised the manuscript; A.M.S., L.G. and M.S.G. carried out some experiments; A.R.G. raised funds for the project, discussed the project and the results, reviewed the manuscript; T.S. raised funds for the project, designed the experiments, analyzed the data, drafted and revised the manuscript.

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