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Progesterone enhances vascular endothelial cell migration *via* activation of focal adhesion kinase

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Abstract

The mechanisms of progesterone on endothelial cell motility are poorly investigated. Previously we showed that progesterone stimulated endothelial cell migration *via* the activation of actin-binding protein moesin, leading to actin cytoskeleton remodelling and the formation of cell membrane structures required for cell movement. In this study, we investigated the effects of progesterone on the formation of focal adhesion complexes, which provide anchoring sites for cell movement. In cultured human umbilical endothelial cells, progesterone enhanced focal adhesion kinase (FAK) phosphorylation at Tyr^{397} in a dose- and time-dependent manner. Several signalling inhibitors interfered with progesterone-induced FAK activation, including progesterone receptor (PR) antagonist ORG 31710, specific c-Src kinase inhibitor PP2, phosphatidylinositol-3 kinase (PI3K) inhibitor wortmannin as well as ρ-associated kinase (ROCK-2) inhibitor Y27632. It suggested that PR, c-Src, PI3K and ROCK-2 are implicated in this action. In line with this, we found that progesterone rapidly promoted c-Src/PI3K/Akt activity, which activated the small GTPase RhoA/ρ-associated kinase (ROCK-2) complex, resulting in FAK phosphorylation. In the presence of progesterone, endothelial cells displayed enhanced horizontal migration, which was reversed by small interfering RNAs abrogating FAK expression. In conclusion, progesterone promotes endothelial cell movement *via* the rapid regulation of FAK. These findings provide new information on the biological actions of progesterone on human endothelial cells that are relevant for vascular function.

Keywords: progesterone • vascular endothelial cells • focal adhesion kinase • actin cytoskeleton • cell movement

Introduction

The endothelium plays a primary role in the regulation of vascular structure and functions. Endothelial dysfunction is a well-established response to cardiovascular risk factors and can be a promoter of atherosclerosis [1, 2]. Menopause is associated with endothelial dysfunction because of sex steroids reduction. In this regard, the impaired flow-mediated dilatation, reduced nitric oxide availability and abnormal endothelial morphology are found in post-menopausal women [3–5]. These functional abnormities may explain the increased incidence of cardiovascular disease in post-menopausal women.

Hormone replacement therapy (HRT) is believed to induce cardiovascular protection. However, clinical trials have challenged this assumption, reporting no cardiovascular benefit or even increased risk in post-menopausal women receiving HRT [6–10]. This apparent discrepancy is still under investigation. Recent years a 'healthy endothelium' concept has been proposed, suggesting that the cardiovascular effects of HRT may largely depend on intact endothelial function [11]. In this regard, the Cardiovascular Health Study demonstrated that favourable vascular effects of HRT are only limited to patients without extensive atherosclerosis [3]. In parallel, our previous work indicated that HRT improves the vascular function and preserves the morphological integrity of endothelial cells in relatively young post-menopausal women [12].

Endothelial cells are primary targets of sex steroids. It is well recognized that oestrogen improves endothelial function through genomic and non-genomic mechanisms [13]. By contrast, less is known on the progesterone's endothelial actions. We recently

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discovered that progesterone stimulates endothelial migration [14]. By promoting dynamic actin remodelling, progesterone activates the actin-regulatory protein moesin, which supports the formation of membrane filopodia and pseudopodia [14]. These structures are essential for cell migration by interaction with the extracellular matrix *via* anchorage proteins and focal adhesions (FAs), which provide the platform for cells to generate the locomotive force. Nevertheless, it remains largely unknown whether progesterone regulates endothelial cell adhesion to the extracellular matrix.

FAs are composed of various structural proteins and represent sites where a number of intra- and extracellular signalling events regulating cell migration take place. Focal adhesion kinase (FAK) is the pivotal molecule that controls FA formation. When the cells are at rest, FAK is auto-inhibited by an intra-molecular interaction of the FERM (Band 4.1, ezrin, radixin, moesin) domain with the kinase domain. Under the stimulation, FAK is phosphorylated at Tyr^{397}, leading to the subsequent phosphorylation of $Tyr^{576/577}$ in the catalytic loop, which is necessary for the full activation of the kinase domain. Activated FAK begins to partner with cell-membrane integrins with the assistance of other proteins such as paxillin and vinculin, resulting in FA formation and cell migration [15]. FAK activity is essential not only for tumour metastasis [16], but also for developmental processes controlling blood vessel formation [17].

Recently we found that progesterone modulates FAK activity in breast cancer cells [18]. In this study, we suggested that FAK is the target of progesterone in vascular endothelial cells and its activation plays important role in progesterone-stimulated endothelial cell migration. Therefore, we explored the regulatory actions of progesterone on FAK activity by using Western blot and immunofluorescence methods. The role of active FAK on endothelial migration was analysed using the wound healing assay. Moreover, by transfection with specific small interference RNA (siRNA) or overexpression plasmids, we characterized the signalling pathways initiated by progesterone receptor (PR) that lead to FAK activation.

Materials and methods

Cell cultures and treatments

Human umbilical vein endothelial cells (HUVEC) were cultured as previously described [19]. Before treatments, HUVEC were kept 48 hrs in Dulbecco's modified Eagle medium (DMEM) containing steroid-deprived foetal bovine serum (FBS). Before experiments investigating non-transcriptional effects, HUVEC were kept in DMEM containing no FBS for 8 hrs. The inhibitors of RNA or protein synthesis, namely actinomycin D (Act D—10 μM) or cycloheximede (CHX—200 μM), were also used to eliminate the transcriptional effects. Whenever an inhibitor was used, the compound was added 30 min. before starting the treatments. Progesterone, pertussis toxin (PTX), PD98059, wortmannin (WM), Y-27632 were from Sigma-Aldrich (St. Louis, MO, USA) and 4-pregnen-3, 20-dione3-O-carboxymethyloxime: BSA (P-BSA) was from Steraloids (Steraloids Incorporation, Newport, RI, USA). 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo (3,4-d) pyrimidine (PP2) was from Calbiochem (EMD Biosciences, Germany). ORG 31710 was obtained from Organon Akzo Nobel (Organon BioSciences N.V., Molenstraat, Oss, The Netherlands).

Immunoblottings

Cell lysates were separated by SDS-PAGE. Antibodies used were: FAK (#3285; Cell Signaling Technology, Danvers, MA, USA), Tyr^{397}-phospho-FAK (#3283; Cell Signaling Technology), $Tyr^{576/577}$-phospho-FAK (#3281; Cell Signaling Technology), Thr^{34}-P-Akt (#07-789; Upstate, Lake Placid, NY, USA), Akt (#9272; Cell Signaling Technology), Src (#2108; Cell Signaling Technology) and Tyr^{416}-phospho-Src (#2101; Cell Signaling Technology). Primary and secondary antibodies were incubated with the membranes with standard protocol. Immunodetection was accomplished using enhanced chemiluminescence, which was acquired with a quantitative digital imaging system (Quantity One, BioRad, Hercules, CA, USA) to check for saturation. Overall emitted photons were quantified for each band, particularly for loading controls, which were homogeneously loaded.

Kinase assays

HUVEC were harvested in 20 mM Tris-HCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaCl, 0.5% octylphenoxy poly(ethyleneoxy)ethanol, branched (IGEPAL) and 0.1 mg/ml phenylmethanesulfonyl fluoride (PMSF). Equal amounts of cell lysates were immunoprecipitated with Rhotekin Rho binding domain (RBD) agarose (14-383; Upstate) *versus* GTP-RhoA or an Ab *versus* ρ-associated kinase (ROCK-2, C-20; Biotechnology, Inc., Santa Cruz, CA, USA). The immunoprecipitations (IPs) were washed three times with buffer containing 20 mM Tris-HCl, 10 mM EDTA, 150 mM NaCl, 0.1% IGEPAL and 0.1 mg/ml PMSF. For ROCK-2 activity assay, two additional washes were performed in kinase assay buffer (20 mM 3-(N-morpholino)propanesulfonic acid, 25 mM β-glycerophosphate, 5 mM ethylene glycol tetraacetic acid, 1 mM DL-dithiothreitol-DTT) and the samples were therefore resuspended in this buffer. Five micrograms of de-phosphorylated myelin basic protein (MBP; Upstate) together with 500 μM adenosine-5′-triphosphate and 75 mM $MgCl_2$ were added to each sample and the reaction was started 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 with antibodies recognizing RhoA (sc-418; Santa Cruz) or Thr^{98}-P-MBP (05-429; Upstate).

Cell immunofluorescence

HUVEC were grown on cover slips and exposed to treatments. Cells were fixed with 4% paraformaldehyde for 30 min. and permeabilized with 0.1% Triton X for 5 min. Blocking was performed with 3% normal

serum for 20 min. Cells were incubated with antibodies against Tyr^{397}-phospho-FAK. After washing the nuclei were counterstained with 4′-6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) and actin was stained with Texas Red-phalloidin (Sigma-Aldrich). The cover slips were 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 (Olympus, Shinjuku-ku, Tokyo, Japan). Pictures were photographed.

Transfection experiments

On-TARGETplus SMARTpool siRNA reagents against human c-Src (NM-198291), FAK (NM-005607) and control siRNA (D-001810-01-05) were purchased from Dharmacon (Thermo Fisher Scientific, Inc., Waltham, MA, USA). HUVEC were transfected with siRNA using Lipofectamine (Invitrogen, San Diego, CA, USA) according to the protocol. Cells (40% confluent) were serum-starved for 1 hr followed by incubation with 100 nM target siRNA or control siRNA for 6 hrs in serum-free media. The serum-containing media was then added (10% serum final concentration) for 42 hrs before experiments and/or functional assays were conducted. Target protein silencing was assessed through protein analysis up to 48 hrs after transfection.

Each plasmid (1.5 μg) was transfected into HUVEC using the Lipofectamine (Invitrogen) according to the manufacturer's instructions. The transfected plasmids were as follows: RhoA T19 and RhoA G14V, p85α or dominant-negative p85α (Δ p85α). These constructs were obtained from the Guthrie cDNA Resource Center (www.cdna.org). All the inserts were cloned in pcDNA3.1+. As control, parallel cells were transfected with empty pcDNA3.1+ plasmid. Cells (60–70% confluent) were treated 24 hrs after transfection, and cellular extracts were prepared according to the experiments to be performed.

Cell migration assays

Cell migration was assayed with razor scrape assays as previously described [19]. Briefly, a razor blade was pressed through the confluent HUVEC monolayer into the plastic plate to mark the starting line. HUVEC were swept away on one side of that line. Cells were washed, and 2.0 ml of DMEM containing steroid-deprived FBS and gelatine (1 mg/ml) were added. Cytosine β-D-arabinofuranoside hydrochloride (Ara-C, Sigma) (10 μM), a selective inhibitor of DNA synthesis, which does not inhibit RNA synthesis was used 1 hr before the test substance was added. Ara-C is easy to enter into the cellular nucleus and it incorporates into DNA and inhibits DNA replication by the formation of cleavage complexes with topoisomerase I resulting in DNA fragmentation. Although the half-life of Ara-C was less than 1 hr in most of the cell lines, more than 80% of Ara-C DNA was retained at 24 hrs after drug removal [20]. Migration was monitored for 48 hrs. Fresh medium and treatment were replaced every 12 hrs. Cells were digitally imaged and migration distance was measured by using phase-contrast microscopy.

Statistical analysis

All values are expressed as mean ± S.D. Statistical differences between mean values were determined by Turkey–Kramer test for multiple comparisons. All differences were considered significant at $P < 0.05$.

Results

Progesterone activates FAK and increases the formation of focal adhesion complexes

First HUVEC was treated with progesterone in a range of concentrations (10^{-10}–10^{-7} mol/l). It is based on the fact that the serum level of progesterone in female is 1 to 90 ng/ml, which nearly equals to 0.3 to 300 nmol/l [21]. As shown in Figure 1A, progesterone rapidly induced FAK phosphorylation at Tyr^{397} (which corresponds to activation [15]) and $Tyr^{576/577}$ (which is necessary for the full activation of kinase domain [22]). Tyr^{397} of FAK was phosphorylated by progesterone at 10^{-8} mol/l with a visible effect from 5 min., a peak at 30 min. and a decline to basal levels after 1 hr (Fig. 1B).

Meanwhile, the sub-cellular localization of phosphorylated FAK in endothelial cells was examined by immunofluorescent analysis. In vehicle-treated control cells, the immunostaining of phosphorylated FAK was rather weak and actin fibres were longitudinally arranged in the cytoplasm (Fig. 1C). Short-term exposure to progesterone (10^{-8} mol/l) led to increased staining of phosphorylated FAK, which concentrated at the cell membrane where actin remodelling happened. These alterations could be observed as early as 5 min. and began to revert to baseline after 1 hr (Fig. 1C).

c-Src and phosphatidylinositol-3 kinase (PI3K)/Akt are required for FAK activation

The rapid time-lapse of FAK activation and de-activation suggests that this effect is likely to be extra-nuclear [23]. Indeed, progesterone still led to FAK activation even if RNA or protein synthesis was blocked in HUVEC with Act D (10 μM) or CHX (200 μM) (Fig. 2A). In addition, FAK activation was also induced by the membrane-impermeable albumin–progesterone conjugate (PBSA, 10 nM) (Fig. 2A).

To clarify the signalling molecules responsible for progesterone-induced FAK activation, we interfered with some of the cascades that mediate extra-nuclear actions of progesterone. Blockade of PR with the pure PR antagonist ORG 31170 (1 μM) completely abolished progesterone-dependent FAK activation, suggesting that PR is indispensable for progesterone's effect (Fig. 2B). Meanwhile, PI3K inhibitor WM (30 nM), non-receptor tyrosine kinase c-Src inhibitor PP2 (10 μM) and ROCK-2 Y27632 (10 μM) all largely impaired progesterone-induced FAK activation (Fig. 2B), indicating that c-Src, PI3K and ROCK-2 are required for this action.

On the contrary, progesterone-enhanced FAK phosphorylation was not altered by the G protein inhibitor PTX (100 ng/ml) or the mitogen-activated protein kinase kinase inhibitor PD98059 (5 μM) (Fig. 2B).

PR interacts with the tyrosine kinase c-Src [24] and this process is involved in the activation of PI3K [25]. In line with this, progesterone (10^{-8} mol/l) induced rapid phosphorylation of c-Src at the Tyr^{416} in the activation loop of the kinase domain, which

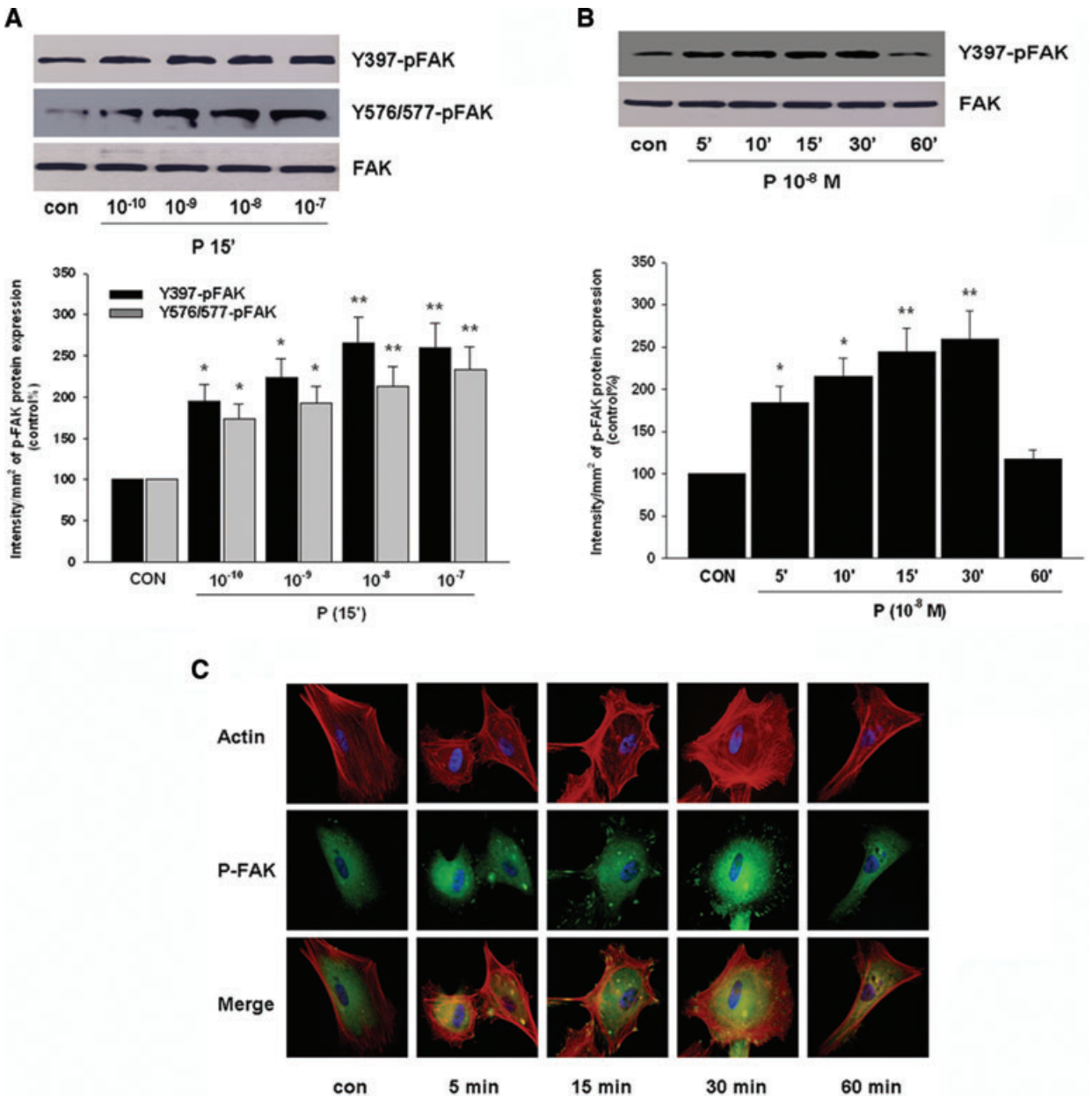

Fig. 1 Progesterone activates FAK and induces rapid actin cytoskeleton rearrangement in HUVECs. (**A**)–(**B**) show the dose- and time-dependent FAK activation in HUVECs after treatment with P (10 nM). Total cell amount of wild-type (FAK) or Tyr397- or Tyr576/577-phosphorylated FAK (p-FAK) are shown with Western blot. pFAK densitometry values were adjusted to FAK intensity, then normalized to expression from the control sample. $^{*}P < 0.05$ *versus* corresponding control; $^{*}P < 0.01$ *versus* corresponding control. (**C**) HUVECs were treated with P (10 nM) for the indicated time. Then the cells were stained with anti-phospho-Tyr397 FAK (p-FAK) linked to fluoresceinisothiocynate, actin was stained with phalloidin linked to Texas Red and nuclei were counterstained with DAPI. All the experiments were repeated three times with consistent results, and the representative images are shown.

corresponds to its activation [26] (Fig. 2C). Likewise, the PI3K downstream effector, protein kinase Akt, was also phosphorylated in response to progesterone (Fig. 2C). Moreover, the PI3K inhibitor WM had no effect on c-Src activation, whereas the c-Src inhibitor PP2 blocked Akt phosphorylation (Fig. 2C), indicating that c-Src was the upstream of Akt in this experimental setting.

The functional importance of c-Src/PI3K/Akt cascade in FAK activation was further elucidated by the transfection experiments.

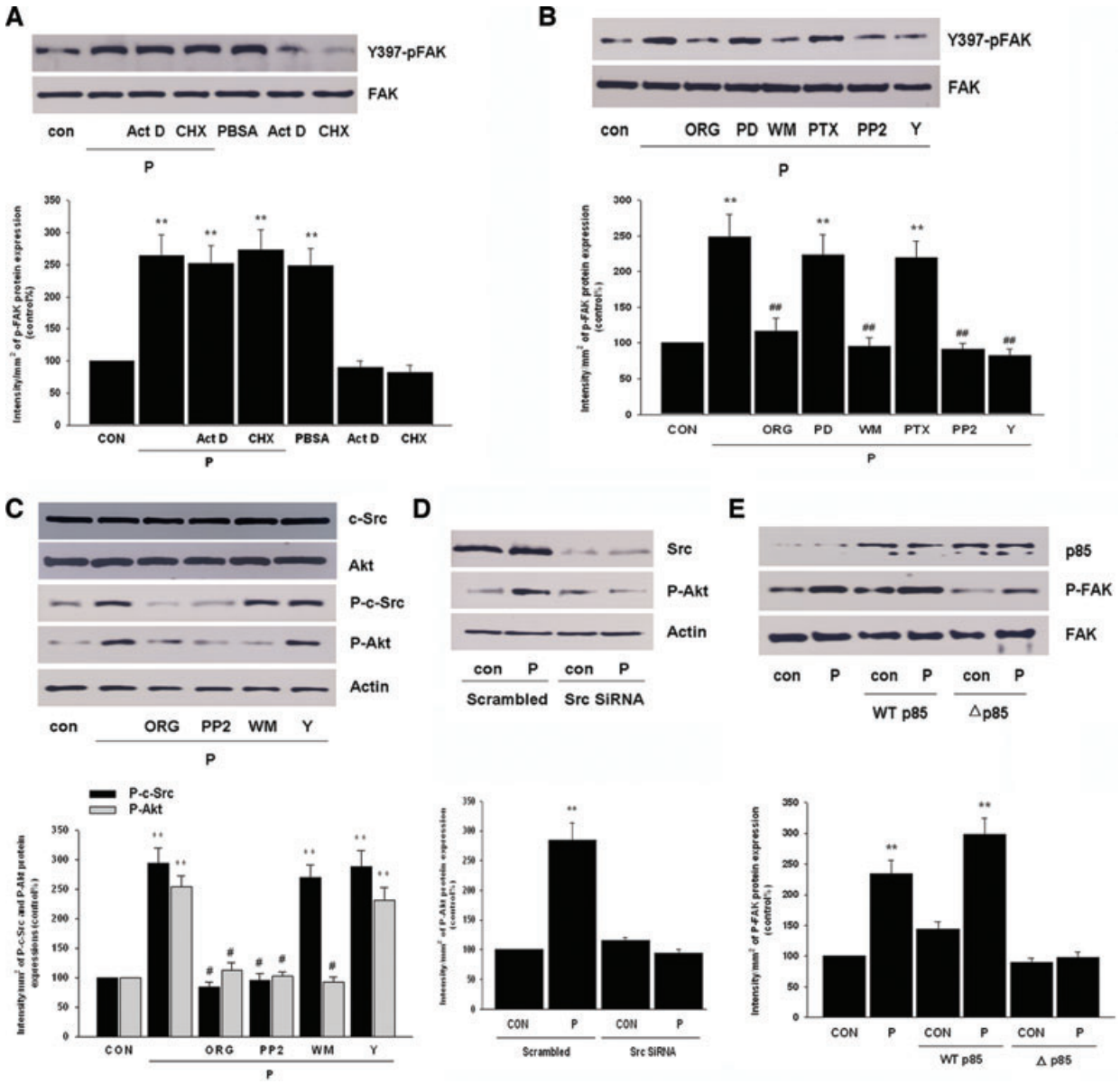

Fig. 2 c-Src and PI3K/Akt are implicated in progesterone-induced endothelial FAK phosphorylation. (**A**) HUVECs were treated with P (10 nM) or PBSA (membrane impermeable, 10 nM) for 15 min., in the presence or absence of Act D (10 μM) or CHX (200 μM). Cell content of wild-type or phosphorylated FAK are shown. Y397-pFAK densitometry values were adjusted to FAK intensity, then normalized to expression from the control sample. $^{**}P < 0.01$ *versus* control. (**B**) HUVECs were exposed to P (10 nM) for 15 min., in the presence or absence of the pure PR antagonist ORG 31710 (ORG—1 μM), of the mitogen-activated protein kinase kinase inhibitor PD98059 (PD—5 μM), of the PI3K inhibitor WM (30 nM), of the G protein inhibitor, PTX (100 ng/ml), of the Src kinase inhibitor, PP2 (10 μM) and of the ROCK-2 inhibitor, Y-27632 (Y—10 μM). Y397-pFAK densitometry values were analysed as above mentioned. $^{**}P < 0.01$ *versus* control. $^{\#\#}P < 0.01$ *versus* P. (**C**) HUVECs were exposed to P (10 nM) for 15 min., in the presence or absence of the ORG 31710 (ORG—1 μM), of the PP2 (10 μM), of the WM (30 nM), and of the Y-27632 (Y—10 μM). P-c-Src and P-Akt densitometry values were adjusted to actin intensity, then normalized to expression from the corresponding control sample. $^{**}P < 0.01$ *versus* corresponding control. $^{\#\#}P < 0.01$ *versus* corresponding progesterone. (**D**) HUVECs were exposed to 10 nM P for 15 min. after transfection with 100 nM c-Src siRNAs or control scrambled siRNAs for 48 hrs. Total c-Src, P-Akt and actin amounts and statistics for P-Akt densitometry are shown. $^{**}P < 0.01$ *versus* corresponding scrambled control. (**E**) HUVECs were exposed to P (10 nM) for 15 min. after transfection with wild-type p85α (WT p85α, 1.5 μg) or dominant-negative p85α (Δp85α, 1.5 μg) for 48 hrs. Cell contents of p85α, wild-type p85, phosphorylated FAK and statistics for densitometry are shown. $^{**}P < 0.01$ *versus* corresponding control without transfection. All these experiments were performed in triplicates and representative images are shown.

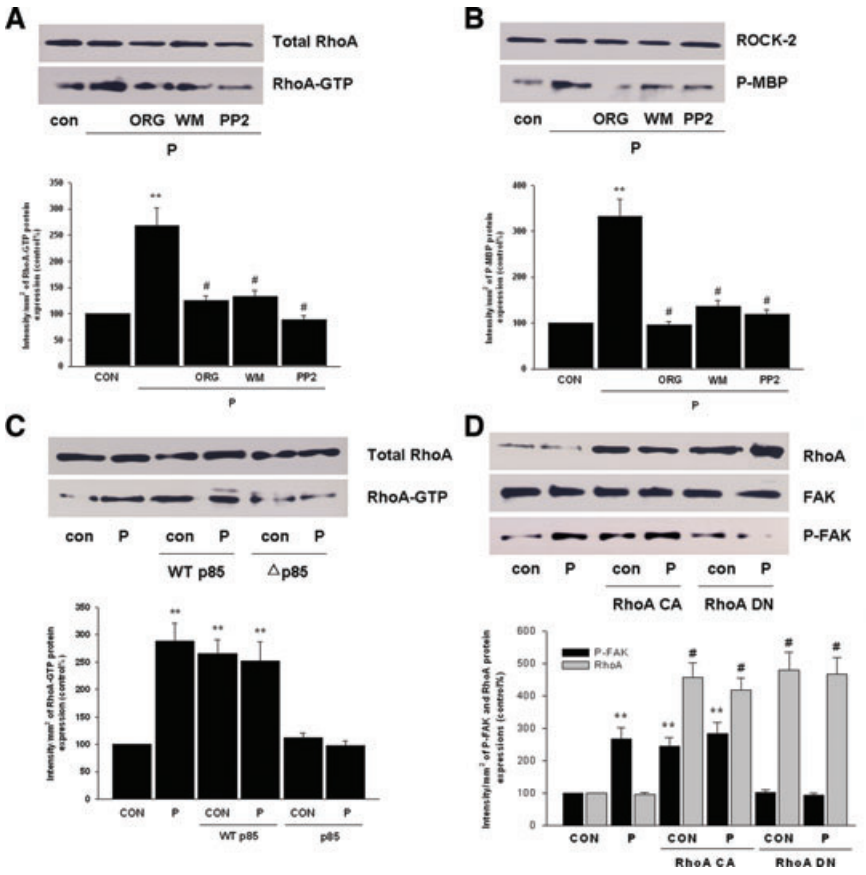

Fig. 3 RhoA and ROCK-2 are activated during PR signalling to FAK in HUVECs. (**A**) RhoA activity was assayed in HUVECs treated with P (10 nM) for 15 min. in the presence or absence of the pure PR antagonist ORG 31710 (ORG—1 μM), of the PI3K inhibitor WM (30 nM), or of the Src kinase inhibitor, PP2 (10 μM). Active, GTP-bound RhoA was immunoprecipitated with Rhotekin and subsequently assayed with Western analysis with an anti-RhoA Ab (lower boxes). In order to confirm that the protein amounts used in immunoprecipitation in each treatment sample are really equivalent, we used the same amount of total protein as 'input' to detect the total RhoA protein level, which was shown in the upper blot. RhoA-GTP densitometry values were adjusted to total RhoA intensity, then normalized to expression from the control sample. $^{**}P < 0.01$ *versus* control. $^{\#}P < 0.01$ *versus* P. (**B**) HUVECs were treated with 10 nM P for 15 min. in the presence or absence of ORG 31710 (ORG—1 μM), of WM (30 nM), of PP2 (10 μM). ROCK-2 was immunoprecipitated with a specific Ab and the IPs were used to phosphorylate the bait protein, MBP. ROCK-2 kinase activity is shown as the amount of phosphorylated MBP (P-MBP). P-MBP densitometry values were adjusted to ROCK-2 intensity, then normalized to expression from the control sample. $^{**}P < 0.01$ *versus* control. $^{\#}P < 0.01$ *versus* P. (**C**) HUVECs were exposed to P (10 nM) for 15 min. after transfection with wild-type p85α (WT p85α) or dominant-negative p85α (Δp85α) for 48 hrs. Active, GTP-bound RhoA was assayed and total RhoA content was analysed in the input. RhoA-GTP densitometry values were adjusted to total RhoA intensity, then normalized to expression from the control sample. $^{**}P < 0.01$ *versus* control without transfection. (**D**) HUVECs were either mock-transfected or exposed to constitutively active or dominant-negative RhoA (RhoA CA or RhoA DN). Cells were then treated with P (10 nM) for 15 min. and wild-type or phosphorylated FAK were analysed. pFAK and RhoA densitometry values were adjusted to FAK intensity, then normalized to expression from the corresponding control sample. $^{**}P < 0.01$ *versus* corresponding control without transfection. $^{\#}P < 0.01$ *versus P*. All these experiments were performed in triplicates and representative images are shown.

Progesterone was unable to activate Akt when c-Src expression was silenced with specific siRNA (Fig. 2D). In addition, transfection with a dominant-negative form of the regulatory subunit of PI3K, p85α (Δp85α), resulted in the impairment of FAK activation, whereas the transfection of a wild-type p85α construct (WT p85α) showed an additive effect with progesterone (Fig. 2E). The p85 subunit were overexpressed in both WT p85α and Δp85α plasmid transfection group (Fig. 2E), implying an efficient transfection in our experimental settings. Taken together, these results suggest that the c-Src/PI3K/Akt cascade is implicated in the PR-dependent FAK activation.

Progesterone-induced FAK activation: role of RhoA/ROCK-2

The small GTPase RhoA and its downstream effector ROCK-2 function as the modulators of actin cytoskeleton by linking upstream signalling events to actin-binding proteins such as moesin or FAK [27, 28]. Recently we have shown that progesterone enhanced RhoA/ROCK-2 activities in breast cancer cells [18]. Similarly, treatment of HUVEC with progesterone increased the amount of active, GTP-bound RhoA (Fig. 3A) and of functionally activated ROCK-2, shown by enhanced Thr-phosphorylation of the bait protein MBP by ROCK-2 immunoprecipitates (IPs) (Fig. 3B).

The activation of RhoA/ROCK-2 is mediated by c-Src and PI3K, because inhibitors of both kinases blocked their activation induced by progesterone (Fig. 3A, B). In line with this, RhoA activation was impaired by transfection with Δp85α, whereas transfection with WT p85α increased RhoA activation in the presence or absence of progesterone (Fig. 3C).

Y27632, the inhibitor of ROCK-2, was shown to largely impair progesterone-induced FAK activation (Fig. 2B), suggesting that RhoA/ROCK-2 is implicated for this action. In support, when HUVEC

Table 1 Silencing of FAK with siRNA prevents progesterone-enhanced cell migration

Treatment group		Cell migration distance (mean ± S.D., μm)
Scrambled siRNA	Control	24.6 ± 4.8
	Progesterone	45.7 ± 7.2*
FAK siRNA	Control	22.5 ± 3.2
	Progesterone	21.4 ± 5.3

Cells were transfected with 100 nM target siRNA for FAK or scrambled siRNA for 48 hrs and then treated with progesterone (10 nM) for 48 hrs. Cell migration distances were measured and values are presented within brackets as mean migration distance (μm) ± S.D.
*$P < 0.01$ *versus* scrambled siRNA control.

Table 2 Progesterone-enhanced cell migration was inhibited by relevant signalling inhibitors

Treatment group	Cell migration distance (mean ± S.D., μm)
Control	23.4 ± 5.1
Progesterone	48.2 ± 9.4*
Progesterone + ORG	24.7 ± 5.5†
Progesterone + WM	20.6 ± 3.6†
Progesterone + PP2	17.4 ± 3.8†
Progesterone + Y	22.6 ± 4.2†

Cells were treated with progesterone (10 nM) for 48 hrs, in the presence or absence of ORG 31710 (ORG—1 μM), of WM (30 nM), of PP2 (10 μM) or of Y-27632 (Y—10 μM). Cell migration distances were measured and values are presented within brackets as mean migration distance (μm) ± S.D.
*$P < 0.01$ *versus* control; †$P < 0.01$ *versus* progesterone.

were transfected with a RhoA constitutively-active construct (RhoA G14V), FAK phosphorylation was ligand-independently enhanced (Fig. 3D). On the contrary, transfection of a dominant-negative RhoA construct (RhoA T19N) resulted in a significant reduction of progesterone-induced FAK phosphorylation (Fig. 3D).

FAK activation is critical for HUVEC migration

Finally the role of FAK activation in endothelial cell migration was interrogated. Consistent with our previous work [14], progesterone markedly enhanced horizontal migration (Table 1). This effect was completely blocked by silencing FAK with siRNAs. The enhancement of endothelial migration induced by progesterone was also prevented by blocking PR with ORG 31710, by blocking c-Src with PP2, PI3K with WM, ROCK-2 with Y-27632 (Table 2).

Discussion

Female sex hormones have vascular benefits and this is largely attributed to their direct actions on vascular endothelial cells. It is well documented that oestrogen is the potent agent to maintain endothelial morphology and functions [13]. However, little is known about the progesterone's effects on endothelial cells. The major finding of this proposal is the identification of endothelial FAK as the target of progesterone that promotes endothelial cell migration.

Endothelial migration is critical for the physiological or pathophysiological processes such as vessel repair, angiogenesis and wound repair. Cell migration is a highly integrated process implemented by actin reorganization. Our previous studies showed that progesterone induces dynamic actin remodelling and the formation of membrane filopodia and pseudopodia, which are primary steps for endothelial cell migration [14]. This event is linked to the rapid activation by PRs of actin-binding protein moesin, which belongs to the ezrin/radixin/moesin family [29]. Protruded membranes then contact the substrate and form novel integrin-dependent FAs, which provides the platform for cells to generate the locomotive force [30]. Besides its ability to provoke actin cytoskeleton remodelling, progesterone is also revealed to increase the formation of FAs, indicating that progesterone may impact on endothelial migration through the modulation of multiple migratory steps.

We here find that progesterone rapidly activates Tyr^{397} phosphorylation of endothelial FAK, leading to the formation of FA complexes. Endothelial FAK is essential for vascular morphogenesis and vascular repair due to its central role on endothelial cell migration [31, 32]. For example, in the transgenic mice which overexpressed FAK in vascular endothelial cells, the number of vessels in the granulation tissue of healing wound is significantly increased [33]. Likewise, the inhibition of FAK phosphorylation results in reduced endothelial repair in cell wounding model [34]. These findings highlight the relevance of the active endothelial FAK in the maintenance of vascular functions and integrity. The identification of endothelial FAK regulation by progesterone may thus offer important mechanistic insights to better understand the beneficial cardiovascular effects of this natural hormone [35–37].

The phosphorylation of FAK is characterized by the rapid time lapse and is insensitive to RNA and protein synthesis inhibitors, indicating that this action induced by progesterone is achieved *via* extra-nuclear signalling cascades. Although the definition of the exact membrane-binding site for progesterone is beyond the scope of this article, there is a possibility that signalling responsible for FAK activation are initiated from liganded-membrane PRs. Indeed, the existence of membrane-localized PRs in different tissues (including vascular endothelial cells) is well established [38–40]. Whatever the sub-cellular localization of PRs that are required to start signalling to endothelial FAK, the present findings concur with our previous reports in suggesting that membrane

steroids receptors may be relevant in the regulation of motility of different type of cells, such as vascular endothelial cells, breast cancer cells and neuron cells [19, 28, 41].

Although the present study displays a tight relationship between FAK activation and endothelial cell motility, the overall contribution of this rapid action to the long-term migration remains to be clarified. Indeed, progesterone may also alter FAK activity through conventional nuclear actions [42]. For instance, long-term treatment with progesterone or synthetic progestin medroxyprogesterone acetate (MPA) increases the expression and phosphorylation of FAK and the formation of FA complexes, resulting in enhanced migration of vascular endothelial cells or of breast cancer cells [12, 43].

A critical step for full enzymatic activity of FAK is the Tyr^{397} phosphorylation in the kinase domain, followed by the phosphorylation of $Tyr^{576/577}$ in the catalytic loop [44, 45]. A variety of stimuli, such as mechanical stress or the activation of growth factor receptors, catalyse Tyr^{397} phosphorylation and the active FAK functions as a molecular switch for multiple signalling outputs [46, 47]. In this cascade of events, c-Src acts as a central hub relaying upstream signals to FAK as well as conveying signals from FAK to downstream effectors [15]. It has been reported that PR contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src [24]. In line with this, the present study finds that progesterone activates c-Src that is required for FAK activation and endothelial migration. As alternative mechanisms, other studies also indicate the central role of c-Src in endothelial migration by recruiting and activating downstream effectors like protein kinase C or matrix metalloproteinase [48, 49].

This study indentifies that PI3K, a well-known downstream of c-Src [25], is activated by c-Src and is required for FAK activation. This is consistent with previous reports showing that the c-Src/PI3K/Akt pathway is implicated in Tyr^{397} FAK phosphorylation in cancer cells [18, 50]. The activation of PI3K/Akt results in the recruitment of the RhoA/ROCK-2, which is implicated in the regulation of endothelial functions such as cytoskeleton organization, motility, proliferation, adhesion and apoptosis [51]. Our findings do show that the activation of RhoA/ROCK-2 results in endothelial FAK phosphorylation and migration. In line with this, RhoA/ROCK cascade is shown to activate FAK in cardiac myocytes [27]. However, the mechanism through which RhoA/ROCK-2 activates FAK is still not completely understood. In this regard, it has been suggested that phosphorylated caveolin-1 relays signals from RhoA/ROCK-2 to FAK [52]. Caveolin-1 is regarded as a molecular switch for some of the extra-nuclear actions of sex steroids in vascular endothelial cells [53]. It would thus be interesting to interrogate the role of caveolin-1 in progesterone-induced FAK phosphorylation.

Of note, although it is shown that RhoA/ROCK cascade is the intermediate between PI3K to FAK, it is still possible that PI3K directly interacts with FAK and results in FAK activation, because the interaction of the p85 regulatory subunit of PI3K with FAK has been shown to trigger FAK phosphorylation [50, 54]. Intriguingly, active FAK is able to interact with p85 to increase its activity, thus creating a positive feedback loop [55]. Therefore, further exploration on the relevance of endothelial PI3K and FAK is needed.

In this work, we observed the endothelial actions of natural progesterone, but not of synthetic progesterone such as MPA, the compound widely used in combined HRT. It has been documented that natural progesterone or synthetic progestins have a variable influence on endothelial function. For example, natural progesterone increases endothelial nitric oxide production whereas MPA is devoid of such action [56]. In non-human primates MPA has been shown to interfere with the atheroprotective effects of oestrogens, which does not happen with natural progesterone [57, 58]. The intracellular signalling pathways recruited by progesterone and MPA are also divergent, even though they exert the similar biological action. In this regard, we have demonstrated that progesterone induces rapid activation of actin-binding protein moesin and endothelial or breast cancer cell migration through G protein, whereas MPA requires PI3K/Akt activation to complete the same effects [14, 28]. Therefore, the effect on FAK activation and the signalling recruited by progesterone cannot be extended to other synthetic progestins. Future research will be necessary to address the regulatory effect of MPA on endothelial FAK activation.

In conclusion, it is found that progesterone promotes endothelial cell movement by facilitating the formation of FA complexes *via* the rapid activation of FAK, which is mediated by c-Src/PI3K/Akt and RhoA/ROCK-2 cascade. These findings provide new information on the biological actions of progesterone on human endothelial cells that are relevant for vascular function.

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Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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