Estradiol induces endothelial cell migration and proliferation through estrogen receptor-enhanced RhoA/ROCK pathway

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**ABSTRACT**

Migration and proliferation of endothelial cells are involved in re-endothelialization and angiogenesis, two important cardiovascular processes that are increased in response to estrogens. RhoA, a small GTPase which controls multiple cellular processes, is involved in the control of cell migration and proliferation. Our aim was to study the role of RhoA on estradiol-induced migration and proliferation and its dependence on estrogen receptors activity. Human umbilical vein endothelial cells were stimulated with estradiol, in the presence or absence of ICI 182780 (estrogen receptors antagonist) and Y-27632 (Rho kinase inhibitor). Estradiol increased Rho GEF-1 gene expression and RhoA (gene and protein expression and activity) in an estrogen receptor-dependent manner. Cell migration, stress fiber formation and cell proliferation were increased in response to estradiol and were also dependent on the estrogen receptors and RhoA activation. Estradiol decreased p27 levels, and significantly raised the expression of cyclins and CDK. These effects were counteracted by the use of either ICI 182780 or Y-27632. In conclusion, estradiol enhances the RhoA/ROCK pathway and increases cell cycle-related protein expression by acting through estrogen receptors. This results in an enhanced migration and proliferation of endothelial cells.

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1. Introduction

The integrity and functionality of the arterial endothelium play a crucial role in the physiology of circulation (Filipe et al., 2008), as well as in preventing the development of cardiovascular diseases (CVD), whose genesis is currently considered a consequence of the anatomical and functional disruption of the endothelium (Spyridopoulos et al., 1997). Both estrogen receptors (ER, α and β) are expressed on it (Venkov et al., 1996; Cid et al., 2002), and it is also a target for the action of sexual hormones, such as estrogens (O’Lone et al., 2007). CVD affect women and men in a different way (Regitz-Zagrosek, 2006), differences that may be a consequence of the influence exerted by sexual hormones.

RhoA is a small GTPase (20–40 kDa), member of the Rho GTPase family (RhoA, CDC42 and Rac), which is activated by guanine nucleotide exchange factors (GEFs) (Buchsbaum, 2007). Regulation of the actin cytoskeleton is the best understood and conserved function of these GTPases (Bar-Sagi and Hall, 2000). Moreover, Rho GTPases coordinate the intracellular responses to extracellular stimuli through the regulation of actin in vascular cells and regulate a series of functions involved in the pathogenesis of CVD (Shimokawa and Rashid, 2007).

The major RhoA downstream effector is Rho kinase (ROCK), which phosphorylates the myosin-binding subunit of myosin light chain (MLC) phosphatase, and thus inhibits myosin phosphatase activity, maintaining MLC in a contractile state (Horwitz and Parsons, 1999). The increased myosin contractility and resulting tension drives the formation of F-actin stress fibers and focal adhesions that play an essential role during migration (Rolfe et al., 2005; Sander et al., 1999).

Migration and proliferation of endothelial cells are closely involved in re-endothelialization and angiogenesis, two important cardiovascular processes increased in response to estrogens (Straub, 2007). Migration is the initiating and rate-limiting event, since it releases endothelial cells from contact inhibition and leads them to subsequent proliferation. Normally endothelial cells are quiescent, but after disruption they can re-enter the cell cycle (Spyridopoulos and Andres, 1998), whose progression is led by the...
cycdin-dependent kinases (CDK) associated with their respective cyclins (Andres, 2004).

We have previously shown that estrogens exert a proliferative effect on human umbilical vein endothelial cells (HUVEC) (Concina et al., 2000; Oviedo et al., 2007; Morales et al., 1995). Considering that RhoA would be one of the major regulatory pathways at the cardiovascular level (Loirand et al., 2006) and that the estradiol-stimulated RhoA pathway in endothelial cells has been poorly studied, our aims were the following: (1) to study whether estradiol modifies RhoA expression/activity; (2) to evaluate how estradiol-mediated migration or proliferation is affected by ER and RhoA inhibition; and (3) to investigate whether estradiol regulates the gene or protein expression of some key cell cycle-related proteins through ER or RhoA pathways.

2. Materials and methods

This investigation conforms to the principles outlined in the Declaration of Helsinki, was approved by the Ethical Committee of Clinical Research of the Hospital Clínico Universitario of Valencia, Spain, and written informed consent was obtained from all donors.

2.1. Cell culture and experimental design

Primary HUVEC were isolated, cultured, and identified as previously described (Sohlberg et al., 2010), and were used from passages 4 to 6. After cells reached no more than 75% of confluence, so that they were not contact-inhibited, they were starved and made quiescent for 24 h as previously reported (Ho et al., 2004).

Cells were exposed to the following treatment combinations: estradiol (E2) (1–10 nM) (Sigma, Alcobendas, Spain), non-selective ER antagonist ICI 182780 (1 μM) (Tocris, Biogen, Madrid, Spain), cell permeable ROCK inhibitor Y-27632 (100 μM) (Calbiochem, VWR International, Barcelona, Spain), and RhoA inhibitor C3 transferase cell permeable (1 μg/ml) (Cytoskeleton, Denver, USA). Control cells were exposed to vehicles.

2.2. Quantitative real time PCR (qRT-PCR)

Cells seeded into 25 cm² flasks were starved and treated for 24 h. After that, total cellular RNA was extracted, reverse transcribed (RT) and amplified, either by SYBRgreen reaction (cell cycle inhibitor p27), or by TaqMan Universal Mastermix reaction using Taqman probes: RhoA SYBRGreen reaction (cell cycle inhibitor p27), as previously described (Oviedo et al., 2007), or by TaqMan Universal Mastermix reaction using Taqman probes: RhoA (Hs00180327_m1), Rho GEF-1 (Hs001803272_m1), cyclin A (Hs00151318_m1), cyclin B1 (Hs00259126_m1), cyclin D1 (Hs00364847_m1), CDK2 (Hs01548949_m1), CDK1 (Hs00364293_m1) and GAPDH (4326317E), all from Applied Biosystems (Fosters City, CA, USA). Reactions were carried out in a 7000HT Fast Real-Time PCR System (Applied Biosystems). Each sample was amplified in triplicate for each gene. Data were analyzed with the SDS 2.2.2 software (Applied Biosystems), and the relative gene expression was calculated in relation to the housekeeping gene GAPDH.

2.3. Immunoblotting

Cells seeded into 25 cm² flasks were starved, treated for 24 h and lysed for protein extraction. From 50 to 75 μg of protein were separated by 15% SDS-polyacrylamide gel electrophoresis. Then, protein was transferred to PVDF sheets (Biorad, Madrid, Spain), incubated with anti RhoA 25C4 (sc-418), anti-cyclin D1 (sc-246), anti-cyclinD4 (sc-200), anti-cyclin A (sc-751), anti-cyclinD2 (sc-163), anti-cyclin B1 (sc-752), all from Santa Cruz Biotechnology, or anti-CDK1 (KAM-C101) (Stressgen Biotechnologies, Tebu-bio, Barcelona, Spain), and developed with alkaline-labelled secondary antibodies: mouse (for RhoA, cyclin D1, CDK1), rabbit (for cyclins A and B1 and CDK4) or goat (for CDK2), all from Sigma. Blots were digitized and the densities of spots analyzed. Equivalent protein loading and transfer efficiency were verified by staining for β-actin (from Sigma).

2.4. RhoA activity measurement

HUVEC were seeded onto six-well plates and allowed to reach no more than 60% of confluence. Following that, cells were serum starved for 24 h to ensure that RhoA was inactive before treatment. After 24 h of exposure to treatment, cells were lysed, protein concentration measured and the RhoA activity assessed by a commercial enzyme immunoassay (Cytoskeleton).

2.5. Migration measurement

Migration was assessed by a modification of the “scratch” wound healing assay (Favot et al., 2003). Briefly, HUVEC were seeded onto Petri dishes with 2 mm × 2 mm grid previously labelled with a rectangular shape (9 mm × 7 mm squares), allowed to reach 100% confluence and starved for 24 h. Then, the monolayer was scraped with a disposable potato rubber with a 2.5-mm-wide scraper to achieve a rectangular lesion, cells were rinsed and dishes were covered under microscope to assure that the four edges were free of cells. Cells were treated for 24 h and afterward, at least three selected non-adjacent fields for each treatment condition were acquired using a 4× objective microscope equipped with a CCD camera (Eclipse E-400, Nikon, Japan). Cell migration was quantified in each field by measuring the distance between the margin of the lesion and the most distant point on migrating cells. The 10 most mobile cells were analyzed with the UTHSCSA Image Tool Program.

2.6. Stress fiber formation

HUVEC were seeded onto fibronectin-pretreated sterile coverslips placed into 6-well plates, starved for 24 h and exposed to treatments for 24 h. Cells were then washed, fixed, permeabilized and stained with rhodamine-labelled phalloidin for actin filaments according to a commercial protocol (Cytoskeleton). Cells were observed under fluorescence microscopy equipped with a CCD camera (excitation 495 nm and emission 520 nm).

2.7. Cell proliferation measurement

Cell proliferation was measured as previously described (Oviedo et al., 2007) with a bromo-2-deoxyuridine (BrdU) colorimetric kit (Roche Diagnostics, Madrid, Spain). Cells treated with 1 mM citosine β-d-arabinofuranoside hydrochloride (Sigma) were used as the negative control (data not shown).

2.8. Measurement of cell viability

Cell viability was assessed as previously reported (Hermenegildo et al., 2005). None of the tested compounds showed a percentage of viability on HUVEC lower than 75% (data not shown).

2.9. Statistical analysis

ANOVA was applied for comparisons of means, and then Bonferroni’s test was performed. All p-values < 0.05 were considered significant. Data are mean ± SEM. Statistical analysis was carried out with Prism 4 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

To uncover the role of RhoA on E2-induced migration and proliferation of endothelial cells, we first measured the RhoA-regulatory protein, Rho GEF-1, by qRT-PCR in response to 10 nM E2, showing a significant increase (2.23 ± 0.22) (Fig. 1A). RhoA gene expression was also significantly increased in response to 10 nM E2 (2.13 ± 0.27) (Fig. 1B), resulting in an increased RhoA protein expression in response to 1–10 nM E2 (117 ± 6% and 129 ± 2%, respectively) (Fig. 1C and D).

These effects were mediated by ER since the use of ICI 182780 alone or combined with E2 significantly reverted RhoGEF-1 and not only RhoA gene expression, but also RhoA protein expression (p < 0.05 vs. E2).

Since Rho-GEF was expressed, it was likely that RhoA would become active in response to E2. Treatment with 10 nM E2 significantly increased the RhoA activity (120 ± 9%) which depended on the ER activation, since the use of ICI 182780 reversed RhoA activity to control levels. Moreover, the ROCK inhibitor Y-27632 significantly decreased the RhoA activity either alone (89 ± 6%) or in combination with E2 (90 ± 7%) (p < 0.05 vs. E2) (Fig. 2A).

To test whether RhoA-increased expression and activity resulted in a functional change, cell migration was assessed. HUVEC monolayer exposed to 10 nM E2 showed a significant increased cell migration (141 ± 6%) (Fig. 2B). On the one hand, cell migration depended on ER activation, since in the presence of the ER antagonist cell migration was maintained at control levels. On the other hand, this effect also depended on the RhoA pathway, since the use of ICI 182780 reversed RhoA activity to control levels. Moreover, E2 increased the formation of either dorsal stress fibers (which are connected to the substrate via focal adhesion) (Hotulainen and Lappalainen, 2006) or transverse arcs (Fig. 2C).
Fig. 1. Estradiol increased Rho GEF and RhoA in HUVEC in an ER-dependent manner. Quiescent HUVEC were exposed to 1–10 nM E2 with or without 1 μM ICI 182780 for 24 h. Rho GEF-1 (A) and RhoA (B) mRNA levels were measured by qRT-PCR. RhoA protein levels were assessed by immunoblotting (C), and a typical image (D) is presented. Data are mean ± SEM from four to eight different experiments performed on cells from four different cultures.* p < 0.05 vs. control, † p < 0.01 vs. 10 nM E2 and ‡ p < 0.0005 vs. 10 nM E2. HUVEC lost stress fibers and showed dendritic extensions when exposed either to Y-27632 alone or when this ROCK inhibitor was combined with E2, although cells remain well spread. In cells exposed to C3 transferase (RhoA inhibitor) alone or in combination with E2, stress fibers disappeared, there was a decrease in cell spreading, dendritic extensions were present and the cell body collapsed.

To test the effect of E2 and RhoA on cell cycle proteins, p27 gene expression was assessed in order to assure that cells were able to re-enter the cell cycle and then progress through the different phases

Fig. 2. Estradiol increased RhoA activity and cell migration in an ER and RhoA-dependent manner. HUVEC were serum starved for 24 h and exposed for 24 h to 10 nM E2 alone or combined either with 1 μM ICI 182780 or with 100 μM Y-27632. (A) RhoA activity was assessed as described in Section 2. Data are expressed as mean ± SEM of duplicate determinations from five to ten different experiments performed on cells from four different cultures. (B) Cell migration was measured as described in Section 2. Quantification is expressed as mean ± SEM of the measurements of at least 3 fields from seven to nine different experiments performed on cells from 6 different cultures. * p < 0.05 vs. control, † p < 0.05 vs. 10 nM E2. For visualization of stress fibers (C), quiescent HUVEC were plated onto coverslips and treated for 24 h. Cells were then fixed, stained with rhodamine-labelled phalloidin and visualized by fluorescence microscope. E2 stimulated the formation of dorsal stress fibers (yellow arrow), transverse arcs (white arrow) and focal adhesion (pink arrow) formation, which disappeared in the presence of Y-27632 or C3. Images were taken at a magnification of 40×.
in response to E2 treatment. Indeed, E2 significantly decreased p27 levels to 0.76 ± 0.10, an effect reversed in the presence of ICI 182780 (Fig. 3).

After 24 h of HUVEC exposure to 1–10 nM E2, the gene expression of G1 phase cell cycle-related proteins cyclin D1 and CDK4 was significantly increased (Fig. 4A and B). These effects depend on the ER activation, since the treatment with ICI 182780 decreased it to control levels. Furthermore, this effect was also dependent on the RhoA activation, since the exposition to Y-27632 showed the same inhibitory profile either for cyclin D1 (p < 0.05 vs. E2) as well as for CDK4 (Fig. 4A and B). Protein expression revealed a similar pattern; 10 nM E2 significantly increased cyclin D1 (148 ± 15%) and CDK4 expression (140 ± 17%). ICI 182780 decreased cyclin D1 in comparison to E2 (p < 0.05), whereas the effect on CDK4 was not significant (p = 0.16) (Fig. 4C and D).

The 1–10 nM E2 significantly increased S phase cell cycle-related proteins, cyclin A2 and CDK2 gene expression. These effects were mediated by ER since ICI 182780 decreased cyclin A2 (p < 0.05 vs. E2), and also were mediated by RhoA activation, since the use of Y-27632 significantly decreased not only cyclin A2 expression, but also CDK2 (p < 0.05 vs. E2) (Fig. 5A and B). According to data for mRNA, protein expression was increased in response to 10 nM E2 to 128 ± 10% for cyclin A2 and to 116 ± 5% for CDK2. When cells were exposed to ICI 182780, there were no changes in cyclin A2 or in CDK2 protein expression (Fig. 5C and D).

M phase cell cycle-related proteins were also evaluated. Thus, 1–10 nM E2 significantly increased cyclin B1 gene expression (1.40 ± 0.16 and 1.65 ± 0.22, respectively) and CDK1 (1.19 ± 0.05 and 1.26 ± 0.10, respectively) (Fig. 6A and B). ICI 182780 significantly decreased CDK1 expression (p < 0.05 vs. E2). Y-27632 significantly decreased cyclin B1 and CDK1 expression (p < 0.05 vs. E2) (Fig. 6A and B). In relation to protein expression, 10 nM E2 significantly increased both cyclin B1 (130 ± 5%), as well as CDK1 (117 ± 2%), whereas ICI 182780 decreased cyclin B1 protein expression to control levels (p < 0.05 vs. E2). CDK1 levels remained unaltered after ICI 182780 exposure (Fig. 6C and D).

All these changes resulted in a dose-dependent, E2-increased HUVEC proliferation after 24 h of treatment. This effect was dependent on ER and RhoA activity, since it was significantly counteracted by the ER antagonist ICI-182780 and by the ROCK inhibitor Y-27632 (Fig. 7).

4. Discussion

From our outcomes, E2 acting through an ER-dependent mechanism acts as an activator of RhoA, which in turn stimulates cell migration and proliferation.

Furthermore, stress fibers, the best characterized and most accessible multiprotein cellular contractile mechanism and whose formation is promoted by RhoA, were enhanced by E2, which guarantees the contractile force necessary in migrating cells. In this regard, it was evaluated how stress fibers formation were affected under ROCK inhibition by Y-27632 (which competes with ATP for...
binding to the catalytic site) and under RhoA inhibition by C3 exoenzyme (which acts through ADP ribosylation) (Rolfe et al., 2005) finding that in both cases the stress fibers and the focal adhesions disappeared, disrupting the cytoskeleton organization. Nevertheless, after the use of Y-27632 cells remain well spread, whereas after the use of C3, cell spread decreased. One explanation may be that C3 inhibits the active form of the RhoA and Y-27632 acts on its downstream effector, but the protein is not affected directly.

E2 has shown to exert not only a migratory effect on HUVEC (Morales et al., 1995; Simoncini et al., 2006), but also to enhance cyclins A and B1 gene expression and proliferation (Oviedo et al., 2007). Subsequently, we studied whether the RhoA pathway, through ER activation, affected the expression of the main cell cycle-related proteins, finding that the most important of them were up-regulated.

In that sense, it has been previously reported that RhoA induces post-translational destabilization and decreases the activity of p27 allowing cells to re-enter cell cycle (Hengst and Reed, 1996), induces cyclin D1 expression (Coleman et al., 2004) and the nuclear translocation of CDK2 promoting cell cycle progression (Chen et

![Fig. 5. Estradiol increased cyclin A2/CDK2 in an ER and RhoA-dependent manner. Quiescent HUVEC were exposed to 1–10 nM E2 with or without 1 μM ICI 182780 or Y-27632 for 24 h. Cyclin A2 (A) and CDK2 (B) mRNA levels were assessed by qRT-PCR, while cyclin A2 (C) and CDK2 (D) protein levels were measured by immunoblotting, as described in Section 2. Data are mean ± SEM of three to five different experiments performed on cells from four different cultures.* p<0.05 vs. control, † p<0.05 vs. 10 nM E2.](image)

![Fig. 6. Estradiol increased cyclin B1/CDK1 in an ER and RhoA-dependent manner. Quiescent HUVEC were exposed to 1–10 nM E2 with or without 1 μM ICI 182780 or Y-27632 for 24 h. Cyclin B1 (A) and CDK1 (B) mRNA levels were assessed by qRT-PCR, while cyclin B1 (C) and CDK1 (D) protein levels were measured by immunoblotting as described in Section 2. Data are mean ± SEM of three to five different experiments performed on cells from four different cultures.* p<0.05 vs. control, † p<0.05 vs. 10 nM E2.](image)
it has been previously shown that E2 interacting with G...Nevertheless, we can not rule out a non-genomic mechanism, since...

24 h of treatment and involved changes both in mRNA and protein...only inhibit their gene expression, but also cell proliferation. (Croft and Olson, 2006). Consequently, the use of Y27632 may not...

is involved in proliferation, its inhibition may affect more deeply...Y27632 is also able to inhibit focal adhesion kinase (FAK) (Sinnett-Smith et al., 2001), another target of RhoA which regulates...Fig. 8. Proposed signalling pathway. E2 acting through ER is able to activate in HUVEC Rho GEF-1 and RhoA which in turn, acting on its downstream effector ROCK, increases by one side stress fibers formation and migration and, on the other side, cell cycle-related proteins such as cyclin D1/CDK4, cyclin A2/CDK2 and cyclin B1/CDK1, promoting cell proliferation. ER: estrogen receptor; E2: estradiol; ROCK: Rho kinase; CDK: cyclin dependent kinase.

4.1. Conclusions

Taken together, the present findings suggest that E2 enhances HUVEC migration, cell cycle-related protein expression and pro-
literation through the RhoA/ROCK pathway in an ER-dependent manner, as showed in Fig. 8.

Disclosure statement

The authors have nothing to disclose.

Conflict of interest statement

The authors have declared no conflict of interest.

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