



Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce



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

Pilar J. Oviedo^{a,b}, Agua Sobrino^a, Andrés Laguna-Fernandez^a, Susana Novella^{a,c}, Juan J. Tarín^d, Miguel-Angel García-Pérez^a, Juan Sanchís^b, Antonio Cano^e, Carlos Hermenegildo^{c,*}

^a Research Foundation, Hospital Clínico Universitario, Valencia, Spain

^b Cardiology Department, Hospital Clínico Universitario, Valencia, Spain

^c Department of Physiology, University of Valencia, Valencia, Spain

^d Functional Biology and Physical Anthropology, University of Valencia, Valencia, Spain

^e Pediatrics, Obstetrics and Gynaecology, University of Valencia, Valencia, Spain

ARTICLE INFO

Article history:

Received 1 June 2010

Received in revised form 30 June 2010

Accepted 30 June 2010

Keywords:

Endothelial function

Estrogens

Migration

Cell cycle regulators

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.

© 2010 Elsevier Ireland Ltd. All rights reserved.

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

* Corresponding author at: Department of Physiology, Faculty of Medicine and Odontology, Av. Blasco Ibañez, 15, E 46010 Valencia, Spain. Tel.: +34 96 3864900; fax: +34 96 3864815.

E-mail address: carlos.hermenegildo@uv.es (C. Hermenegildo).

cyclin-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 de 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 (Sobrinho et al., 2010), and 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 μ M) (Tocris, Biogen, Madrid, Spain), cell permeable ROCK inhibitor Y-27632 (100 μ M) (Calbiochem, VWR International, Barcelona, Spain), and RhoA inhibitor C3 transferase cell permeable (1 μ g/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), as previously described (Oviedo et al., 2007), or by TaqMan Universal Mastermix reaction using Taqman probes: RhoA (Hs00236938.m1), Rho GEF-1 (Hs00180327.m1), cyclin D1 (Hs00277039.m1), cyclin A2 (Hs00153138.m1), cyclin B1 (Hs00259126.m1), CDK4 (Hs00364847.m1), CDK2 (Hs01548894.m1), CDK1 (Hs00364293.m1) and GAPDH (4326317E), all from Applied Biosystems (Foster City, CA, USA). Reactions were carried out in a 7900HT 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 μ g of protein were separated by 15% of SDS-polyacrylamide gel electrophoresis. Then, protein was transferred to PVDF sheets (Biorad, Madrid, Spain), incubated with anti RhoA 26C4 (sc-418), anti-cyclin D1 (sc-246), anti-CDK4 (sc-260), anti-cyclin A (sc-751), anti-CDK2 (sc-163), anti-cyclin B1 (sc-752), all from Santa Cruz Biotechnology, or anti-CDK1 (KAM-CC101) (Stressgen Biotechnologies, Tebu-bio, Barcelona, Spain), and developed with alkaline-labelled secondary antibodies: anti-mouse (for RhoA, cyclin D1, CDK1), anti-rabbit (for cyclins A and B1 and CDK4) or anti-goat (for CDK2), all from Sigma. Blots were digitalized 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 $\times$ 2 mm grid previously labelled with a rectangular shape (9 mm $\times$ 7 mm squares), allowed

to reach 100% confluence and starved for 24 h. Then, the monolayer was scraped with a disposable police rubber with a 2.5-mm-wide scraper to achieve a rectangular lesion, cells were rinsed and dishes were controlled 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 $\times$ 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-arabino furanoside 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 lesser 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 $\pm$ 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 ($117 \pm 6\%$ and $129 \pm 2\%$, 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 ($120 \pm 9\%$) 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 ($89 \pm 6\%$) or in combination with E2 ($90 \pm 7\%$) ($p < 0.05$ 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 ($141 \pm 6\%$) (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 Y-27632 was able to block the migratory effect created by E2 in a significant way ($p < 0.05$ vs. E2).

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).

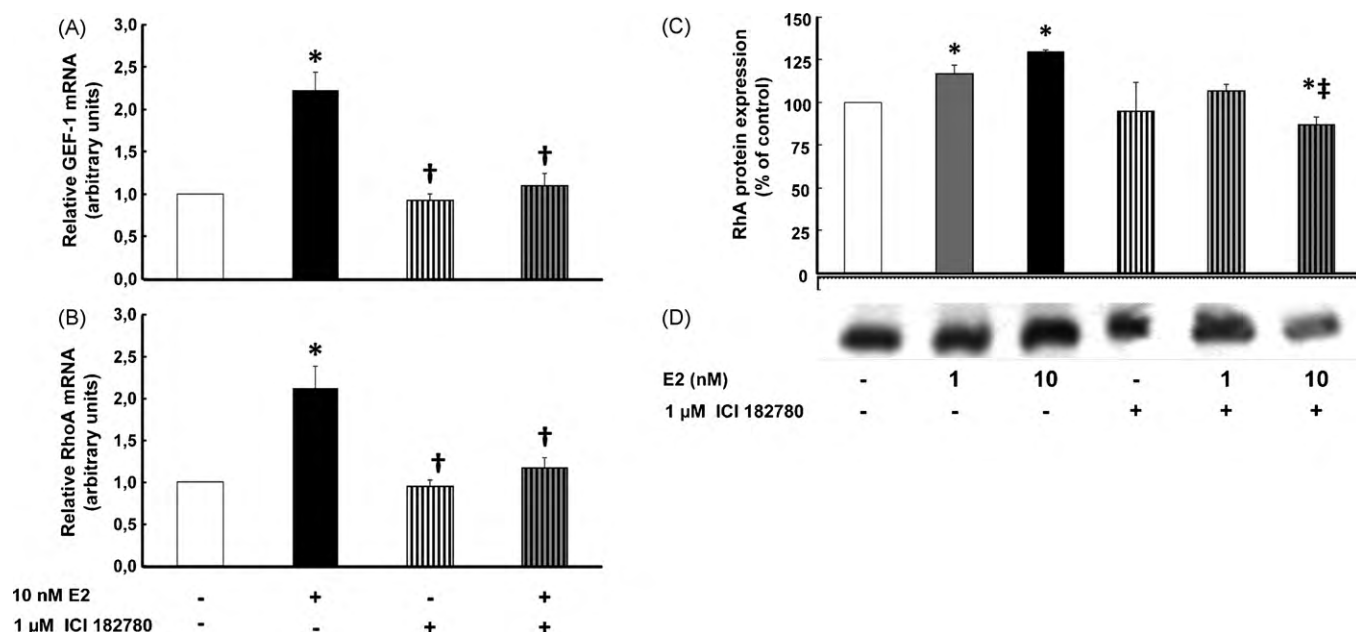


Fig. 1. Estradiol increased Rho GEF and RhoA in HUVEC in an ER-dependent manner. Quiescent HUVEC were exposed to 1–10 nM E2 with or without 1 μ M ICI 182780 for 24 h. Rho GEF-1 (A) and RhoA (B) mRNA levels were measured by qRT-PCR. RhoA protein levels were assessed by immunoblotting (C), and a typical image (D) is presented. Data are mean $\pm$ 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

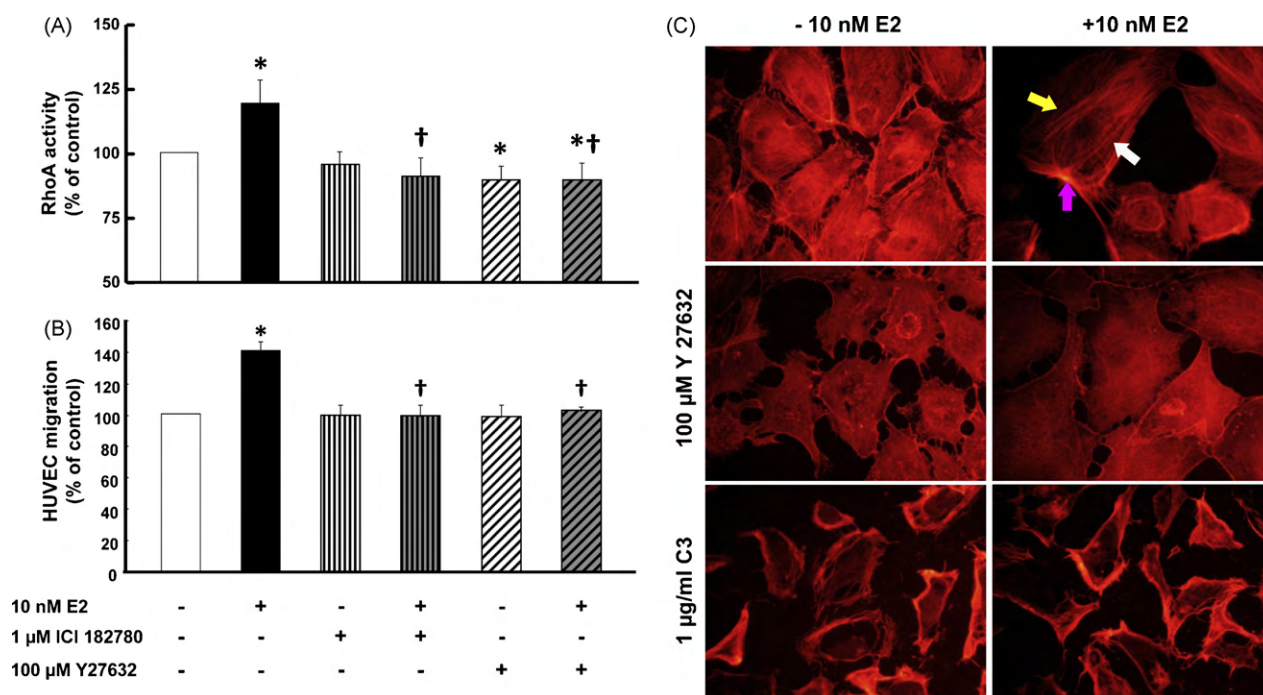


Fig. 2. Estradiol increased RhoA activity and cell migration in an ER and RhoA-dependent manner. HUVEC were serum starved for 24 h and exposed for 24 h to 10 nM E2 alone or combined either with 1 μ M ICI 182780 or with 100 μ M Y-27632. (A) RhoA activity was assessed as described in Section 2. Data are expressed as mean $\pm$ 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 $\pm$ 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 $\times$.

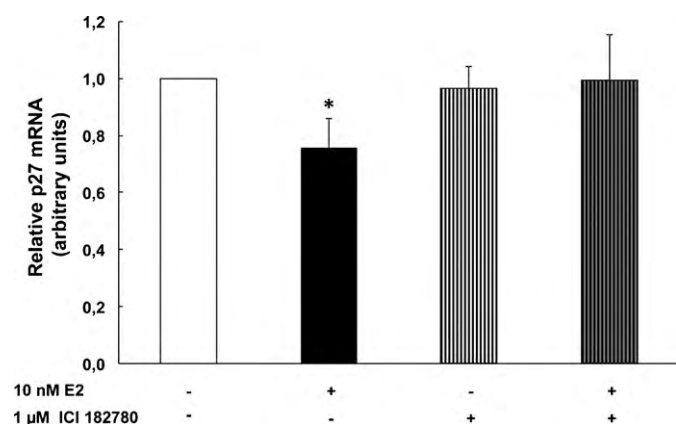


Fig. 3. Estradiol decreased p27 levels in an ER-dependent manner. Quiescent HUVEC were exposed to 10 nM E2 with or without 1 μM ICI 182780 for 24 h. Then, mRNA was isolated, reverse transcribed and amplified by qRT-PCR using specific primers. Data are expressed as the fold response over the control values $\pm$ SEM of duplicate determinations from seven to eight different experiments performed in cells from three different cultures. * $p < 0.05$ vs. control.

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 ($148 \pm 15\%$) and CDK4 expression ($140 \pm 17\%$). 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 $128 \pm 10\%$ for cyclin A2 and to $116 \pm 5\%$ 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 ($130 \pm 5\%$), as well as CDK1 ($117 \pm 2\%$), 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

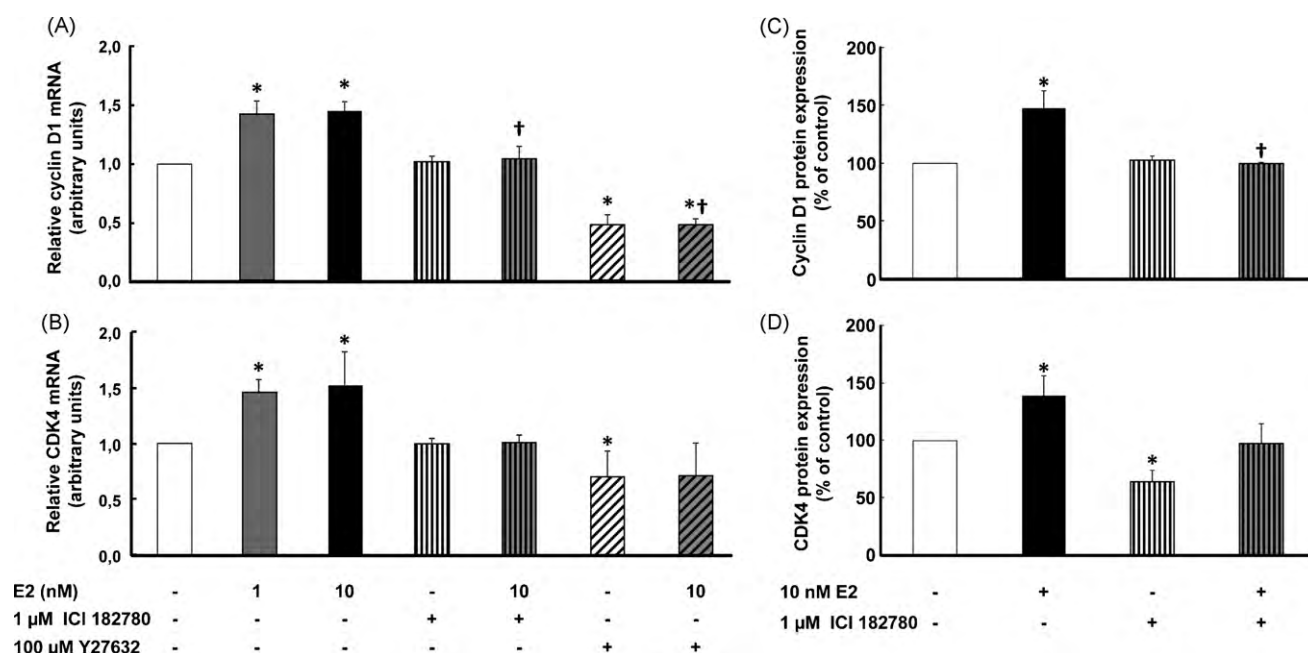


Fig. 4. Estradiol increased cyclin D1/CDK4 in an ER and RhoA-dependent manner. Quiescent HUVEC were exposed to 1–10 nM E2 with or without 1 μM ICI 182780 or Y-27632 for 24 h. Cyclin D1 (A) and CDK4 (B) mRNA levels were assessed by qRT-PCR, while cyclin D1 (C) and CDK4 (D) protein levels were measured by immunoblotting, as described in Section 2. Data are mean $\pm$ 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.

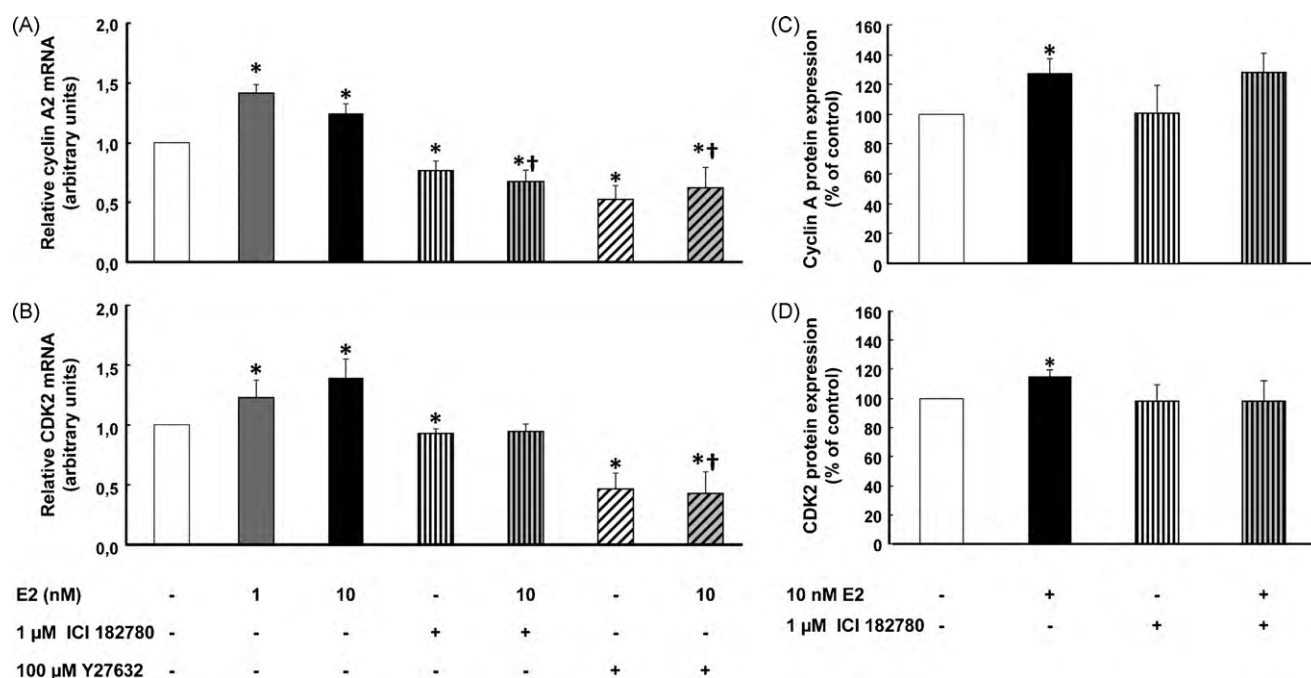


Fig. 5. Estradiol increased cyclin A2/CDK2 in an ER and RhoA-dependent manner. Quiescent HUVEC were exposed to 1–10 nM E2 with or without 1 μ M ICI 182780 or Y-27632 for 24 h. Cyclin A2 (A) and CDK2 (B) mRNA levels were assessed by qRT-PCR, while cyclin A2 (C) and CDK2 (D) protein levels were measured by immunoblotting, as described in Section 2. Data are mean $\pm$ 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.

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

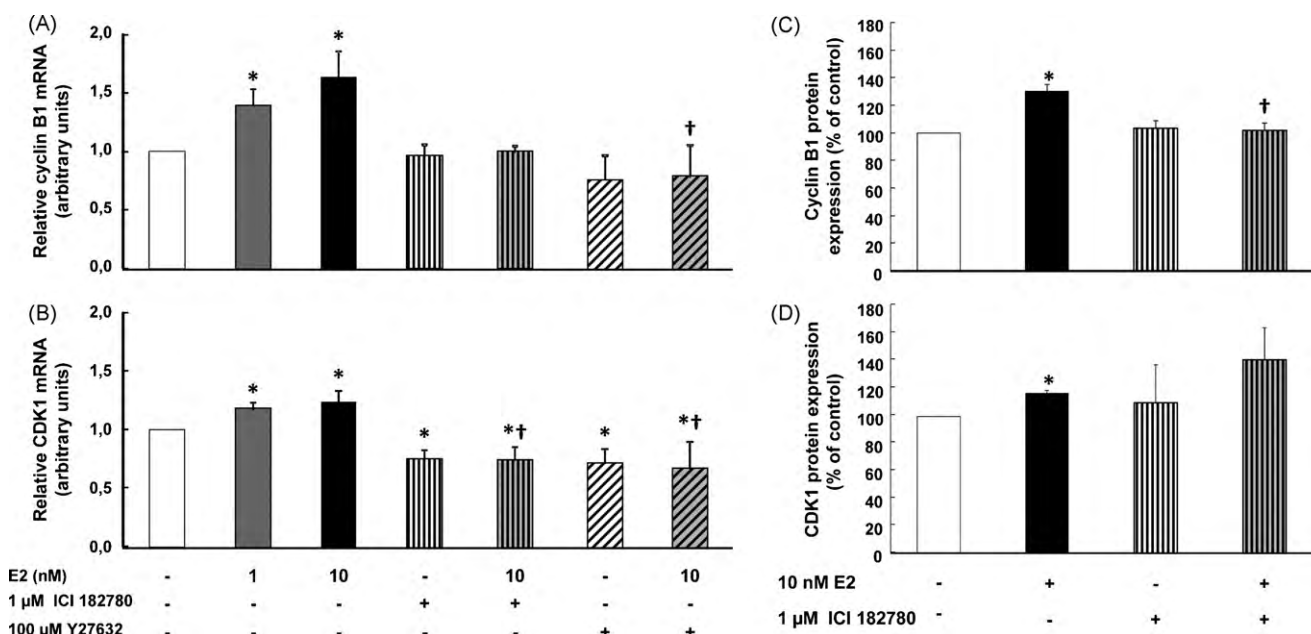


Fig. 6. Estradiol increased cyclin B1/CDK1 in an ER and RhoA-dependent manner. Quiescent HUVEC were exposed to 1–10 nM E2 with or without 1 μ M ICI 182780 or Y-27632 for 24 h. Cyclin B1 (A) and CDK1 (B) mRNA levels were assessed by qRT-PCR, while cyclin B1 (C) and CDK1 (D) protein levels were measured by immunoblotting as described in Section 2. Data are mean $\pm$ 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.

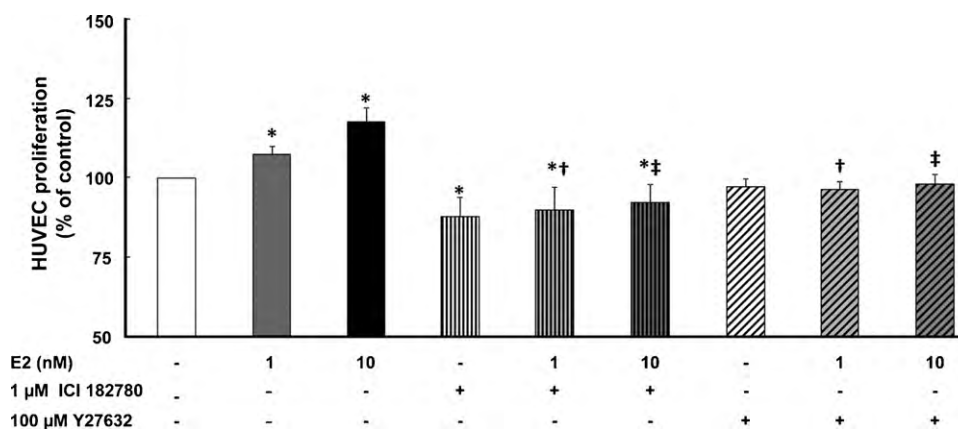


Fig. 7. Estradiol increased HUVEC proliferation in an ER and RhoA-dependent manner. Quiescent HUVEC were exposed to 1–10 nM of E2 with or without 1 μ M ICI 182780 or 100 μ M Y-27632 for 24 h. Cell proliferation was measured as described in Section 2. Data are expressed as mean $\pm$ SEM of sextuplicated determinations from 10 to 31 different experiments performed on cells from 8 different cultures. * p < 0.05 vs. control, † p < 0.05 vs. 1 nM E2, ‡ p < 0.05 vs. 10 nM E2.

al., 2008). Furthermore, E2-induced cyclin D1 expression (Schiff and Osborne, 2005) functions as a coactivator of the ER (Roberts, 1999). Therefore, and in agreement with our data, it is likely that the expression of these cell cycle-related proteins would be mediated by RhoA in response to E2 in an ER-dependent manner, as well as, cell proliferation.

Y27632 alone induces a greater effect than ICI 182780 on G1 and S-related cyclins and CDKs (Figs. 4 and 5). It could be due, by one side, to the used concentration (100 μ M), which affords the maximum effect in previous works (Rikitake et al., 2005). On the other side, Y27632 is also able to inhibit focal adhesion kinase (FAK) (Sinnott-Smith et al., 2001), another target of RhoA which regulates cell cycle progression (Oktay et al., 1999). Additionally, since ROCK is involved in proliferation, its inhibition may affect more deeply the expression of cyclins and CDKs involved in G1 and S phases (Croft and Olson, 2006). Consequently, the use of Y27632 may not only inhibit their gene expression, but also cell proliferation.

Since all the studied effects became much more evident after 24 h of treatment and involved changes both in mRNA and protein expression, we speculate that a genomic mechanism was involved. Nevertheless, we can not rule out a non-genomic mechanism, since it has been previously shown that E2 interacting with $G_{\alpha 13}$ triggers actin remodelling and migration of either endothelial or breast can-

cer cells mediated by RhoA/ROCK/Moesin pathway (Simoncini et al., 2006; Giretti et al., 2008).

To our knowledge, this is the first time that the Rho GEF-1/RhoA/ROCK pathway is evaluated in HUVEC under E2 treatment trying to unravel how this GTPase affects not only migration, but also cell cycle-related proteins and proliferation and their dependence on ER activation. This data are in accordance to previous reports of our group, in which endothelial cells exposed to estradiol showed an increased gene expression of those genes related to proliferation and to cellular actin cytoskeleton (Sobrinho et al., 2009).

ER modulation of different target genes implicates more mechanisms than estrogen receptor response element (ERE). Thus, ER α regulates the transcription of target genes through direct binding to EREs, or by modulating the activity of other DNA bound transcription factors at alternative DNA sequences (Shu et al., 2010). As far as we know, a direct interaction between ER and RhoA gene has not been described. Regarding cell cycle-related proteins, estradiol has shown to induce significantly cyclin D1, CDK activities and down-regulate the CDK inhibitors p21 and p27 (Ru et al., 2006). In cyclin D1 gene, ERE sequences have been identified in the proximal promoters (Caldon et al., 2009), which activity would be also mediated by recruitment of a c-Jun/c-Fos/ER α complex to the tetradecanoyl phorbol acetate-responsive element of the gene (Cicatiello et al., 2004), or FoxA1 and NFIC (Eeckhoutte et al., 2006). Moreover, protein kinase C1 (PKC1)-interacting protein RBCK1 would support transcription of ER α and cyclin B1 (Gustafsson et al., 2010).

Our data may provide new insights over the effects that RhoA displays in response to E2 stimulus on endothelial cells and considering that the genesis of CVD is currently understood as a disturbance of the anatomic and functional integrity of the endothelial monolayer (Spyridopoulos et al., 1997) and that postmenopausal women have a higher risk to develop CVD (Mendelsohn and Karas, 1999). Then, any effort to enlighten how estrogens may improve migration and proliferation, two processes deeply involved in re-endothelialization, will result in a better management of these patients, even more considering that women who initiate hormone therapy closer to menopause tend to have reduced CVD risk compared with those who start it later (Rossouw et al., 2007; Miller and Mulvagh, 2007).

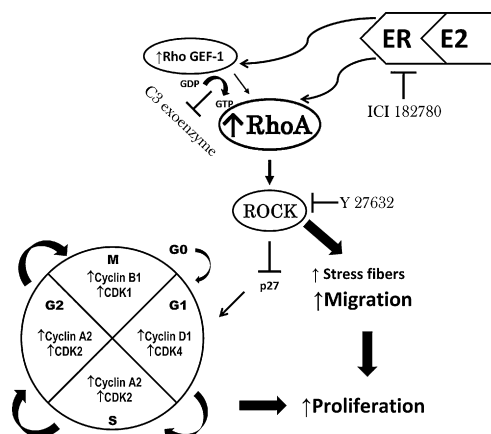


Fig. 8. Proposed signalling pathway. E2 acting through ER is able to activate in HUVEC Rho GEF-1 and RhoA which in turn, acting on its downstream effector ROCK, increases by one side stress fibers formation and migration and, on the other side, cell cycle-related proteins such as cyclin D1/CDK4, cyclin 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-

liferation 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.

Acknowledgments

We are indebted to Mrs. Rosa Aliaga and Mrs. Elvira Calap for their excellent technical assistance. We are also thankful to Dr. Rosario Gil, Mr. Jose Benavente and Dr. Amparo Ruiz of the Pathology Department, University of Valencia for their valuable help with migration experiments. *Grants:* Supported by the Spanish Ministerio de Ciencia e Innovación, Instituto de Salud Carlos III (Grants FIS 06/0589 and Red HERACLES RD06/0009/0005), Consellería de Sanidad (Grants AP121/2008, AP117/10 and GE-021/10) and Consellería de Educación, Generalitat Valenciana (Grant GVPRE/2008/276). P.J. Oviedo holds a Sara Borrell postdoctoral position from Instituto de Salud Carlos III and A Sobrino is a fellowship recipient from the FPI program (BFPI 06/145) from Conselleria de Educación, Generalitat Valenciana, Spain.

References

- Andres, V., 2004. Control of vascular cell proliferation and migration by cyclin-dependent kinase signalling: new perspectives and therapeutic potential. *Cardiovasc. Res.* 63, 11–21.
- Bar-Sagi, D., Hall, A., 2000. Ras and Rho GTPases: a family reunion. *Cell* 103, 227–238.
- Buchsbaum, R.J., 2007. Rho activation at a glance. *J. Cell Sci.* 120, 1149–1152.
- Caldon, C.E., Sergio, C.M., Schutte, J., Boersma, M.N., Sutherland, R.L., Carroll, J.S., Musgrove, E.A., 2009. Estrogen regulation of cyclin E2 requires cyclin D1 but not c-Myc. *Mol. Cell. Biol.* 29, 4623–4639.
- Chen, J., Guerriero, E., Lathrop, K., SundarRaj, N., 2008. Rho/ROCK signaling in regulation of corneal epithelial cell cycle progression. *Invest. Ophthalmol. Vis. Sci.* 49, 175–183.
- Cicatiello, L., Addeo, R., Sasso, A., Altucci, L., Petrizzi, V.B., Borgo, R., Cancemi, M., Caporali, S., Caristi, S., Scafoglio, C., Teti, D., Bresciani, F., Perillo, B., Weisz, A., 2004. Estrogens and progesterone promote persistent CCND1 gene activation during G1 by inducing transcriptional derepression via c-Jun/c-Fos/Estrogen receptor (progesterone receptor) complex assembly to a distal regulatory element and recruitment of cyclin D1 to its own gene promoter. *Mol. Cell. Biol.* 24, 7260–7274.
- Cid, M.C., Schnaper, H.W., Kleinman, H.K., 2002. Estrogens and the vascular endothelium. *Ann. N. Y. Acad. Sci.* 966, 143–157.
- Coleman, M.L., Marshall, C.J., Olson, M.F., 2004. RAS and RHO GTPases in G1-phase cell-cycle regulation. *Nat. Rev. Mol. Cell Biol.* 5, 355–366.
- Concina, P., Sordello, S., Barbacanne, M.A., Elhage, R., Pieraggi, M.T., Fournial, G., Plouet, J., Bayard, F., Arnal, J.F., 2000. The mitogenic effect of 17beta-estradiol on in vitro endothelial cell proliferation and on in vivo reendothelialization are both dependent on vascular endothelial growth factor. *J. Vasc. Res.* 37, 202–208.
- Croft, D.R., Olson, M.F., 2006. The Rho GTPase effector ROCK regulates cyclin A, cyclin D1, and p27Kip1 levels by distinct mechanisms. *Mol. Cell. Biol.* 26, 4612–4627.
- Eeckhoutte, J., Carroll, J.S., Geistlinger, T.R., Torres-Arzuay, M.I., Brown, M., 2006. A cell-type-specific transcriptional network required for estrogen regulation of cyclin D1 and cell cycle progression in breast cancer. *Genes Dev.* 20, 2513–2526.
- Favot, L., Martin, S., Keravis, T., Andriantsitohaina, R., Lugnier, C., 2003. Involvement of cyclin-dependent pathway in the inhibitory effect of delphinidin on angiogenesis. *Cardiovasc. Res.* 59, 479–487.
- Filipe, C., Lam Shang Leen, L., Brouchet, L., Billon, A., Benouaich, V., Fontaine, V., Gourd, P., Lenfant, F., Arnal, J.F., Gadeau, A.P., Laurell, H., 2008. Estradiol accelerates endothelial healing through the retrograde commitment of uninjured endothelium. *Am. J. Physiol. Heart Circ. Physiol.* 294, H2822–H2830.
- Giretti, M.S., Fu, X.D., De Rosa, G., Sarotto, I., Baldacci, C., Garibaldi, S., Mannella, P., Biglia, N., Sisoni, P., Genazzani, A.R., Simoncini, T., 2008. Extra-nuclear signalling of estrogen receptor to breast cancer cytoskeletal remodelling, migration and invasion. *PLoS ONE* 3, e2238.
- Gustafsson, N., Zhao, C., Gustafsson, J.A., hlman-Wright, K., 2010. RBCK1 drives breast cancer cell proliferation by promoting transcription of estrogen receptor α and cyclin B1. *Cancer Res.* 70, 1265–1274.
- Hengst, L., Reed, S.I., 1996. Translational control of p27Kip1 accumulation during the cell cycle. *Science* 271, 1861–1864.
- Hermenegildo, C., Oviedo, P.J., Garcia-Perez, M.A., Tarin, J.J., Cano, A., 2005. Effects of phytoestrogens genistein and daidzein on prostacyclin production by human endothelial cells. *J. Pharmacol. Exp. Ther.* 315, 722–728.
- Ho, P.Y., Liang, Y.C., Ho, Y.S., Chen, C.T., Lee, W.S., 2004. Inhibition of human vascular endothelial cells proliferation by terbinafine. *Int. J. Cancer* 111, 51–59.
- Horwitz, A.R., Parsons, J.T., 1999. Cell migration—Movin' on. *Science* 286, 1102–1103.
- Hotulainen, P., Lappalainen, P., 2006. Stress fibers are generated by two distinct actin assembly mechanisms in motile cells. *J. Cell Biol.* 173, 383–394.
- Loirand, G., Guilluy, C., Pacaud, P., 2006. Regulation of Rho proteins by phosphorylation in the cardiovascular system. *Trends Cardiovasc. Med.* 16, 199–204.
- Mendelsohn, M.E., Karas, R.H., 1999. The protective effects of estrogen on the cardiovascular system. *N. Engl. J. Med.* 340, 1801–1811.
- Miller, V.M., Mulvagh, S.L., 2007. Sex steroids and endothelial function: translating basic science to clinical practice. *Trends Pharmacol. Sci.* 28, 263–270.
- Morales, D.E., McGowan, K.A., Grant, D.S., Maheshwari, S., Bhartiya, D., Cid, M.C., Kleinman, H.K., Schnaper, H.W., 1995. Estrogen promotes angiogenic activity in human umbilical vein endothelial cells in vitro and in a murine model. *Circulation* 91, 755–763.
- O'Loone, R., Knorr, K., Jaffe, I.Z., Schaffer, M.E., Martini, P.G.V., Karas, R.H., Bienkowska, J., Mendelsohn, M.E., Hansen, U., 2007. Estrogen receptors {alpha} and {beta} mediate distinct pathways of vascular gene expression including genes involved in mitochondrial electron transport and generation of reactive oxygen species. *Mol. Endocrinol.* 21, 1281–1296.
- Okta, M., Wary, K.K., Dans, M., Birge, R.B., Giancotti, F.G., 1999. Integrin-mediated activation of focal adhesion kinase is required for signaling to Jun NH2-terminal kinase and progression through the G1 phase of the cell cycle. *J. Cell Biol.* 145, 1461–1470.
- Oviedo, P.J., Hermenegildo, C., Tarin, J.J., Cano, A., 2007. Raloxifene increases proliferation of human endothelial cells in association with increased gene expression of cyclins A and B1. *Fertil. Steril.* 88, 326–332.
- Regitz-Zagrosek, V., 2006. Therapeutic implications of the gender-specific aspects of cardiovascular disease. *Nat. Rev. Drug Discov.* 5, 425–439.
- Rikitake, Y., Kim, H.H., Huang, Z., Seto, M., Yano, K., Asano, T., Moskowitz, M.A., Liao, J.K., 2005. Inhibition of Rho Kinase (ROCK) leads to increased cerebral blood flow and stroke protection. *Stroke* 36, 2251–2257.
- Roberts, J.M., 1999. Evolving ideas about cyclins. *Cell* 98, 129–132.
- Rolfe, B.E., Worth, N.F., World, C.J., Campbell, J.H., Campbell, G.R., 2005. Rho and vascular disease. *Atherosclerosis* 183, 1–16.
- Rossouw, J.E., Prentice, R.L., Manson, J.E., Wu, L., Barad, D., Barnabei, V.M., Ko, M., LaCroix, A.Z., Margolis, K.L., Stefanick, M.L., 2007. Postmenopausal hormone therapy and risk of cardiovascular disease by age and years since menopause. *JAMA* 297, 1465–1477.
- Ru, L.W., Chen, C., Liu, S., Safe, S., 2006. 17beta-estradiol (E2) induces cdc25A gene expression in breast cancer cells by genomic and non-genomic pathways. *J. Cell Biochem.* 99, 209–220.
- Sander, E.E., ten Klooster, J.P., van Delft, S., van der Kammen, R.A., Collard, J.G., 1999. Rac downregulates Rho activity: reciprocal balance between both Gtpases determines cellular morphology and migratory behavior. *J. Cell Biol.* 147, 1009–1022.
- Schiff, R., Osborne, C.K., 2005. Endocrinology and hormone therapy in breast cancer: new insight into estrogen receptor-alpha function and its implication for endocrine therapy resistance in breast cancer. *Breast Cancer Res.* 7, 205–211.
- Shimokawa, H., Rashid, M., 2007. Development of Rho-kinase inhibitors for cardiovascular medicine. *Trends Pharmacol. Sci.* 28, 296–302.
- Shu, F.J., Sidell, N., Yang, D., Kallen, C.B., 2010. The tri-nucleotide spacer sequence between estrogen response element half-sites is conserved and modulates ER[alpha]-mediated transcriptional responses. *J. Steroid Biochem. Mol. Biol.* 120, 172–179.
- Simoncini, T., Scorticati, C., Mannella, P., Fadiel, A., Giretti, M.S., Fu, X.D., Baldacci, C., Garibaldi, S., Caruso, A., Fornari, L., Naftolin, F., Genazzani, A.R., 2006. Estrogen receptor {alpha} interacts with G{alpha}13 to drive actin remodeling and endothelial cell migration via the RhoA/Rho Kinase/Moesin pathway. *Mol. Endocrinol.* 20, 1756–1771.
- Sinnett-Smith, J., Lunn, J.A., Leopoldt, D., Rozengurt, E., 2001. Y-27632, an inhibitor of Rho-associated kinases, prevents tyrosine phosphorylation of focal adhesion kinase and paxillin induced by bombesin: dissociation from tyrosine phosphorylation of p130cas. *Exp. Cell Res.* 266, 292–302.
- Sobrino, A., Mata, M., Laguna-Fernandez, A., Novella, S., Oviedo, P.J., Garcia-Perez, M.A., Tarin, J.J., Cano, A., Hermenegildo, C., 2009. Estradiol stimulates vasodilatory and metabolic pathways in cultured human endothelial cells. *PLoS ONE* 4, e8242.
- Sobrino, A., Oviedo, P.J., Novella, S., Laguna-Fernandez, A., Bueno, C., Garcia-Perez, M.A., Tarin, J.J., Cano, A., Hermenegildo, C., 2010. Estradiol selectively stimulates endothelial prostacyclin production through estrogen receptor α . *J. Mol. Endocrinol.* 44, 237–246.

- Spyridopoulos, I., Andres, V., 1998. Control of vascular smooth muscle and endothelial cell proliferation and its implication in cardiovascular disease. *Front. Biosci.* 3, d269–d287.
- Spyridopoulos, I., Sullivan, A.B., Kearney, M., Isner, J.M., Losordo, D.W., 1997. Estrogen-receptor-mediated inhibition of human endothelial cell apoptosis. Estradiol as a survival factor. *Circulation* 95, 1505–1514.

- Straub, R.H., 2007. The complex role of estrogens in inflammation. *Endocr. Rev.* 28, 521–574.
- Venkov, C.D., Rankin, A.B., Vaughan, D.E., 1996. Identification of authentic estrogen receptor in cultured endothelial cells. A potential mechanism for steroid hormone regulation of endothelial function. *Circulation* 94, 727–733.