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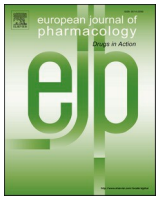

Regulation of human endothelial cell migration by oral contraceptive estrogen receptor ligands

Aida Dama [a,b], Chiara Baggio [a], Lucia Trevisi [c], Chiara Bolego [c], Andrea Cignarella [a,*]

[a] Department of Medicine, University of Padova, Padova, Italy
[b] Albanian University, Tirana, Albania
[c] Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Padova, Italy

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ABSTRACT

Ethinylestradiol (EE) and estetrol (E4) are the two main estrogenic agents used in combined oral contraceptives. These compounds have different binding affinity to and efficacy on estrogen receptors (ER) subtypes. We previously reported that treatment with estrogenic agents enhances angiogenesis via nongenomic, G protein-coupled estrogen receptor (GPER)-dependent mechanisms. However, the impact of EE and E4 on human endothelial function has been little investigated. EE and E4 (10^{-9}– 10^{-7} M) significantly enhanced migration of human umbilical vein endothelial cells (HUVECs) using scratch and Boyden chamber assays. Mechanistically, both agents increased accumulation of phosphorylated protein tyrosine kinase 2 on tyrosine 397 (FAK Y397), a key player in endothelial cell motility, after 30-min treatment. Treatment with increasing concentrations of EE, but not E4, enhanced accumulation of the glycolysis activator PFKFB3. Of note, effects of EE and E4 on endothelial migration and signalling proteins were abolished by addition of the GPER antagonist G36 (10^{-6} M). Thus, EE and E4 induced comparable endothelial responses *in vitro*, suggesting no apparent alterations of vascular remodelling and regeneration capacity by oral contraceptives containing these agents.

1. Introduction

17β-oestradiol (E2) induces protective cardiovascular and systemic metabolic effects by interacting with multiple cell types and receptors (Arnal et al., 2010; Cignarella et al., 2010). Oestrogen action is mediated by its interaction with oestrogen receptors (ERs), ERα, and ERβ, which operate as hormone-regulated transcription factors. Membrane ERs and the recently described G protein-coupled oestrogen receptor (GPER) may also mediate rapid, non-genomic effects. GPER mediates many of the acute as well as chronic effects of E2, and its roles in the cardiovascular system have been increasingly recognized (Barton et al., 2018). Endothelial cells (ECs) represent a principal target of E2 beneficial effects, including neovascularization, thus contributing to ameliorating ischemic damage. GPER and nuclear ERs are expressed in ECs, and may trigger overlapping or independent signalling pathways (Romano and Gorelick, 2018). The glycolytic enzyme activator 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) plays a pivotal role in endothelial function controlling pathophysiological angiogenesis (De Bock et al., 2013; Imbert-Fernandez et al., 2014; Xu et al., 2014). We previously reported that E2–induced angiogenesis is independent from genomic signalling and occurs via GPER-mediated up-regulation of PFKFB3 as a downstream effector (Trenti et al., 2017; Boscaro et al., 2020a).

While E2 effects on several aspects of endothelial function are well characterized (Arnal et al., 2010; Zahreddine et al., 2021), the action profile of the estrogenic ligands ethinylestradiol (EE) and estetrol (E4) has been little investigated. EE increases the release of bioactive NO by inhibition of superoxide anion production in bovine endothelial cells (Arnal et al., 1996). E4 binds ERα and ERβ, with higher affinity for ERα but with lower affinity compared with EE and E2 (Coelingh Bennink et al., 2008; Visser et al., 2008). E4 induces a relaxing response in arteries from a variety of vascular beds, at least in part via endothelium-dependent mechanisms, with lower potency than E2 (Hilgers et al., 2012). Of note, E4 activates only nuclear ERα but fails to elicit responses that are mediated by membrane ERα, characterising E4 as a novel natural selective oestrogen receptor modulator (SERM; Abot

Abbreviations: GPER, G protein-coupled estrogen receptor; HUVEC, human umbilical vein endothelial cells; EE, ethinylestradiol; E4, estetrol; FAK, protein tyrosine kinase 2; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3.

* Corresponding author. Department of Medicine, University of Padova, via Giustiniani 2, 35128, Padova, Italy.
E-mail address: andrea.cignarella@unipd.it (A. Cignarella).

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et al., 2014). Whether EE and E4 affect endothelial function via GPER in human cells is at present unclear.

EE is contained in a large number of combined oral contraceptive formulations and menopausal hormone therapy medications, whereas E4 is a natural oestrogen that is produced exclusively during pregnancy by the foetal liver. E4 (formulated as the monohydrate) is a component of oral contraceptives that also contain drospirenone, which were approved by the FDA and the EMA in 2021. The estrogenic agent may impact on the cardiovascular safety of combined oral contraceptives (Dinger et al., 2016). Unlike E2 and EE, E4 apparently does not affect circulating hepatic factors, suggesting a reduced risk of thromboembolic events when being used as a contraceptive agent (Creinin et al., 2021).

In the present study, we compared the modulation of human endothelial cell migration and the underlying signalling pathways by EE and E4, both of which are active ingredients of several oral contraceptive medications.

2. Materials and methods

2.1. Drugs and chemicals

Cell culture reagents and foetal bovine serum (FBS) were purchased from Fisher Scientific Italia (Rodano, Milan Italy). Endothelial cell growth supplement (ECGS), 17α-ethynylestradiol (EE), and estetrol (E4) were from Merck Life Sciences (Milan, Italy). Collagen (rat-tail) was from Roche (Basel, Switzerland). The GPER antagonist (±) -(3aR*,4S*,9bS*)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-8-(1-methylethyl)-3H cyclopenta[c]quinoline (G36) and the PFKFB3 inhibitor 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) were purchased from Tocris Bioscience (Bristol, United Kingdom).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated as previously described (Bolego et al., 2006) from human umbilical cords collected after delivery from full-term normal pregnancies at the Obstetrics and Gynaecological Unit of Padua University Hospital. The procedure was approved by Padua University Hospital Ethics Committee. All experiments were performed in accordance with relevant guidelines and regulations. HUVECs were grown at 37°C and 5% CO_2 in complete medium (M199 supplemented with 15% FBS, 100 μg/ml ECGS, 100 U/mL heparin, 2 mM glutamine and 40 μg/mL gentamicin). Experiments with E2, EE and E4 were performed using phenol-free M199 medium supplemented with 5% FBS, 40 μg/ml gentamicin, 100 μg/ml ECGS and 100 UI/mL heparin. Cells were switched to complete phenol red-free M199 72 h before each assay and to culture medium with 5% serum overnight before experiments using estrogenic agents.

2.3. Western blot

HUVECs (3×10^5 cells) were seeded in 35-mm dishes in complete culture medium. Cells were treated as indicated and lysed with lysis buffer (PBS supplemented with 1.2% Triton X-100, Roche cOmpleteTM inhibitor cocktail 1X, 2.5 mM NaF, 2 mM sodium pyrophosphate, 4 mM Na orthovanadate and 1 mM PMSF). After centrifugation at 10,000 *g* for 15 min, supernatants were harvested for SDS-PAGE and Western blotting essentially as described (Trenti et al., 2017). Protein quantification was performed using the BCA assay (Merck). Proteins (45 μg) were separated on SDS-PAGE and transferred onto PVDF membranes (Amersham Hybond-P, Cytiva, Buccinasco, Milan, Italy). Membranes were then blocked and probed using the following primary antibodies: mouse anti-FAK (1:1000), rabbit anti-phospho Y397 FAK (1:1000), rabbit anti-PFKFB3 (1:5000) and rabbit anti-GAPDH (1:10,000), all from Abcam (Cambridge, United Kingdom). After washing, membranes were incubated with appropriate secondary HRP-conjugated antibodies (Vector Laboratories, Newark, CA, USA) at 1:10,000 dilution. Bands were detected by chemiluminescence using the LiteAblot Turbo (Euroclone, Pero, Milan, Italy). Images were acquired with Azure Imaging System (Azure Biosystems, Dublin, CA, USA). Densitometric analysis of bands was performed with ImageJ 1,47v (NIH, Bethesda, MD, USA). Data are expressed as relative protein levels with respect to the loading control GAPDH.

2.4. Chemotaxis assay

Chemotaxis experiments were performed in a 48-well modified micro-chemotaxis chamber (Neuro Probe, Gaithersburg, MD, USA) using 8-μm nucleopore polyvinylpyrrolidine-free polycarbonate filters coated with 10 μg/ml collagen. Lower chambers were filled with M199 supplemented with 100 U/mL heparin and 5% or 15% FBS or estrogenic agents as described. Upper chambers were filled with 50 μL HUVEC suspension (1.6×10^5 cells/mL in M199 supplemented with 1% FBS and 100 U/mL heparin). The GPER antagonist G36 was added to both the upper and lower wells. For assessment of basal motility, M199 supplemented with 1% FBS, 100 U/mL heparin was added in the lower wells. After incubation for 6 h at 37°C, the migrated adherent cells on the underside of the filter were stained with Diff-Quick stain (VWR International, Milan, Italy), and densitometric analysis was performed using ImageJ. Results are reported as arbitrary optical density units.

2.5. Scratch assay

HUVECs (10^5 cells) were seeded in 24-well plates in complete culture medium. Phenol-free medium was added the day after seeding, before cells reached confluence. Once confluence was reached, fresh medium was added, one scratch was made and cells were incubated in fresh medium containing test compounds for additional 6 h. At the end of the experiment, three images of each well were captured at 4 × with a bright field inverted microscope (Nikon Eclipse Ti) equipped with a digital camera, immediately after the scratch was made (time 0) and after 6-h incubation. The wound area of each image was measured using ImageJ 1.47, and the average wound area of three images was measured for each sample. Quantitative analysis of cell migration was performed as the percentage of area change using the following formula: % change = [(average space at t0 − average space at t6) ÷ average space at t0] × 100. Values are expressed as relative migration with respect to negative control.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.02 (GraphPad Software, Inc., San Diego, CA, USA). Student's *t*-test was used to compare means of two independent groups, whereas one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for multiple comparisons. A p value < 0.05 was considered statistically significant. All experiments were performed in at least three independent replicates. The results are presented as mean values, with error bars representing the SEM.

3. Results

3.1. EE and E4 rescued mechanical stress-induced impairment of endothelial migration and enhanced chemotaxis via GPER

Endothelial cell migration is an essential component of angiogenesis. The effect of EE and E4 on HUVEC migration was tested using either wound healing or Boyden chamber migration assays. In particular, to test non-directional integrin-mediated cell migration (haptotaxis), HUVECs were exposed to mechanical stress by performing a scratch on the cell monolayer and afterwards treated with test agents. Treatment with increasing concentrations of EE or E4 significantly increased cell migration with respect to control, thus promoting wound closure

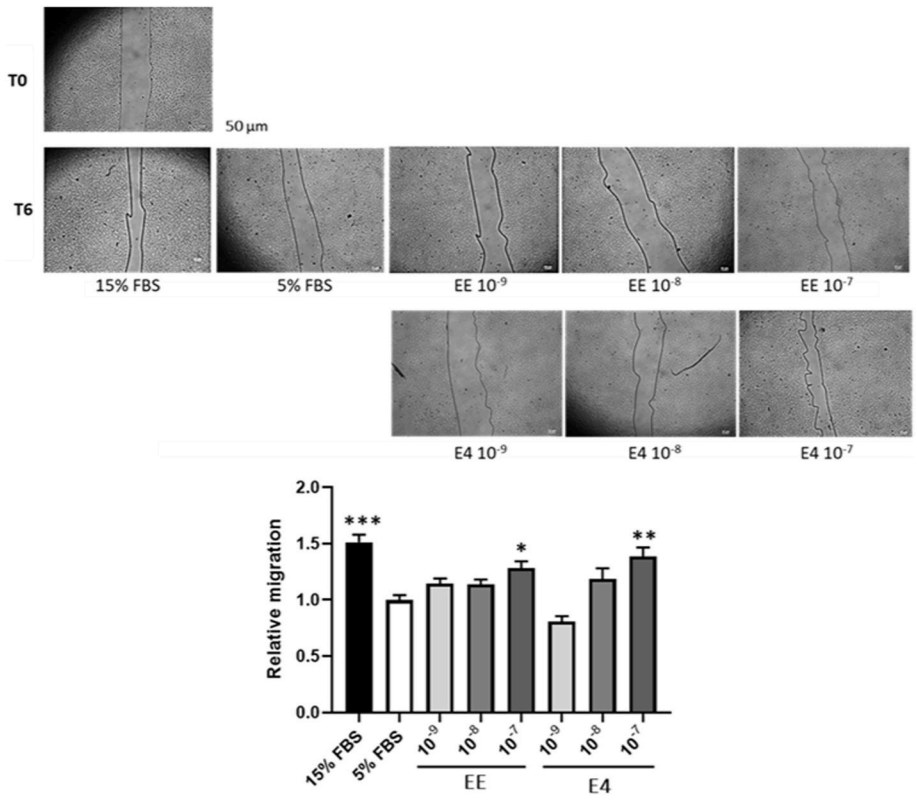

Fig. 1. Scratch assays in HUVECs cultured in 5% FBS phenol red-free M199 medium and treated with increasing concentrations of EE or E4 for 6 h. *Upper panel*: Representative scratch assay images. Areas within blue lines indicate the wound space. For each experimental condition, 3 images were taken with an inverted phase contrast microscope equipped with a 50-µm scale digital camera (40x) immediately after wounding (T0) and after 6 h (T6). Positive control: 15% FBS. *Lower panel*: cell migration expressed as area values relative to negative control (5% FBS). Data are expressed as mean ± SEM of $n = 3$ independent experiments. ANOVA; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* 5% FBS.

(Fig. 1). The two compounds neither induced cellular toxicity nor impaired HUVEC proliferation at the concentration tested (*data not shown*). Treatment with increasing concentrations of either E4 (Fig. 2A) or EE (Fig. 2B) also increased chemotactic FBS-induced HUVEC migration with respect to control.

We previously reported that enhanced endothelial cell migration and capillary network formation upon treatment with ER ligands is mediated by GPER (Trenti et al., 2017; Boscaro et al., 2020a). Therefore, the contribution of GPER to the effects of EE and E4 on HUVEC migration was analysed in Boyden chamber migration assays. A statistically significant decrease in HUVEC migration was observed in the presence of the selective GPER antagonist G36 (Dennis et al., 2011), suggesting that GPER was involved in the angiogenic response to EE and E4 (Fig. 3).

3.2. Regulation of endothelial proteins involved in glycolytic metabolism and cell migration

PFKFB3 is the allosteric activator of phosphofructokinase 1 (PFK1), an important regulator of glycolysis. Moreover, PFKFB3 is widely recognized for its association with the upregulated glycolysis observed in cancer cells (Imbert-Fernandez et al., 2014). We previously reported that oestrogen-induced angiogenesis involves increased endothelial PFKFB3 functional expression via nongenomic mechanisms (Trenti et al., 2017; Boscaro et al., 2020a). Accordingly, the increase in HUVEC chemotactic migration induced by EE and E4 treatment was abolished by the PFKFB3 inhibitor 3PO (Fig. 4). Treatment with increasing concentrations of EE (10^{-9}– 10^{-7} M), but not E4, enhanced PFKFB3 accumulation (Supplemental Fig. 1), suggesting a possible role for EE in regulating endothelial cell glycolytic metabolism.

We previously reported a functional link between PFKFB3 and protein tyrosine kinase 2 (FAK) in HUVEC migration (Boscaro et al., 2020b).

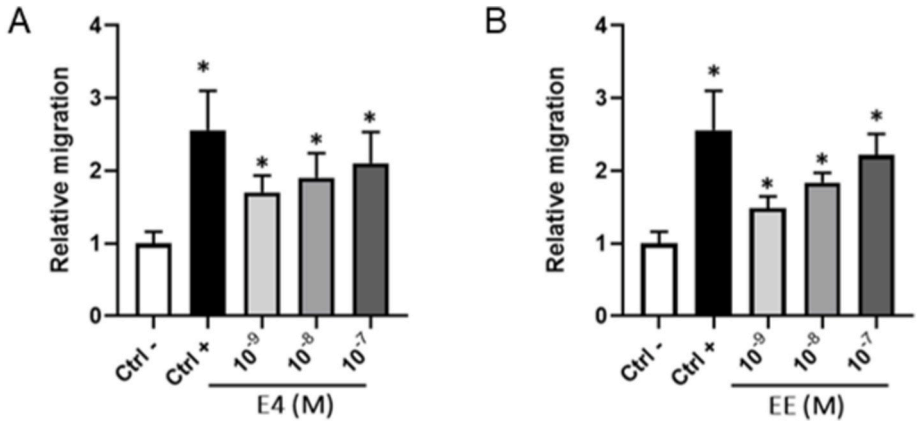

Fig. 2. Effect of the estrogenic ligands EE and E4 on HUVEC migration. Chemotactic migration of HUVECs was measured in response to increasing concentrations of EE (A) and E4 (B) (both 10^{-9}– 10^{-7} M) as assessed by Boyden's functional assay after incubation for 6 h at 37°C. Negative control (Ctrl-): 1% FBS; positive control (Ctrl+): 15% FBS. Cell migration was measured as optical density value and expressed relative to Ctrl-. Data are expressed as mean ± SEM of $n = 4$ independent experiments. One – way ANOVA, $^{*}p < 0.05$ vs. Ctrl-.

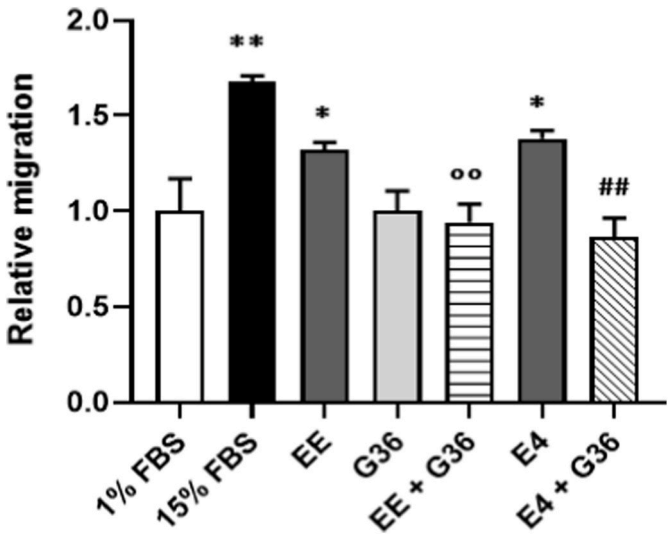

Fig. 3. Chemotactic migration of HUVECs in response to treatment with EE (10^{-7} M) and E4 (10^{-7} M) in the presence or absence of the GPER antagonist G36 (10^{-6} M) as assessed by Boyden's functional assay after 6 h incubation at 37°C. G36 was added 30 min before test agents. Negative control: 1% FBS; positive control: 15% FBS. Cell migration was measured as optical density value and expressed relative to negative control (1% FBS). Data are expressed as mean ± SEM of $n = 5$ independent experiments. One-way ANOVA; $^{*}p < 0.05$ $^{**}p < 0.01$ *vs* 1% FBS, $^{\circ}$ $p < 0.01$ *vs* EE, $\#\#p < 0.01$ *vs* E4.

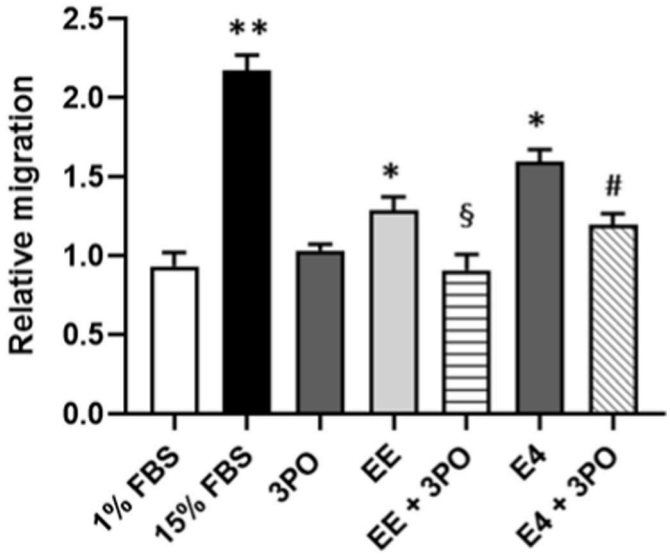

Fig. 4. Chemotactic migration of HUVEC in response to treatment with EE and E4 (both 10^{-7} M) in the presence or absence of the PFKFB3 inhibitor 3PO (40 μM). Boyden functional assays were run for 6 h incubation at 37°C. Negative control: 1% FBS; positive control: 15% FBS. Cell migration was measured as optical density value of migrated cells and expressed relative to negative control (1% FBS). Data are expressed as mean ± SEM of $n = 5$ independent experiments. *t*-test; $^{*}p < 0.05$, $^{**}p < 0.01$ vs 1% FBS, $\#p < 0.05$ *vs* E4, §$p < 0.05$ *vs* EE.

FAK activation, as determined by phosphospecific antibody recognition of the FAK Y397 autophosphorylation site, is a key step in endothelial cell motility (Sieg et al., 2000). Levels of FAK Y397 were increased in response to treatment with EE (10^{-8} and 10^{-7} M) and 10^{-7} M E4 (Fig. 5), consistent with enhanced HUVEC migration capacity as described above (Figs. 1–4).

4. Discussion

The mechanism(s) via which E2 mediates growth (proliferation and migration) and endothelial healing have been widely explored. Recently, membrane ERs have been shown to mediate the endothelial regenerative actions of E2 (reviewed in Zahreddine et al., 2021). In the present study, we investigated the effects of two estrogenic agents used in hormone contraceptive medications, EE and E4, on endothelial migration *in vitro* and the signalling pathways involved.

Our findings indicate that both EE and E4 triggered HUVEC migration via GPER. In a previous study using razor-scrape and multiwell inserts migration assays, E4 was found to interfere with the effects of E2 in HUVECs, depending on the amount of E2 present (Montt-Guevara et al., 2017). In that study, E4 treatment significantly increased cell migration in a concentration-dependent manner, which was blunted by ICI 182,780/fulvestrant. Of note, fulvestrant not only behaves as an ERα and ERβ antagonist (Schmidt et al., 2003), but also as a GPER *agonist* (Thomas et al., 2005; Trenti et al., 2017). To the best of our knowledge, we here report for the first time that EE- and E4-induced increase in HUVEC motility was mediated substantially by GPER, as suggested by experiments with the highly selective antagonist G36. While EE binds to both ERα and ERβ in the nM range (Jain et al., 2006), the profile of ERα activation by E4 is characterised by the uncoupling of nuclear and membrane activation (Smith and O'Malley, 2004). Thus, the apparent concentration-dependence of E4 effects in the scratch assay may be due to a more selective pharmacological profile as well as to a different relative contribution of ERs to cell migration in the two assays. Although E4 is unable to elicit major endothelial actions mediated by membrane ERα (Abot et al., 2014), a contribution of nuclear ERα to EE and E4 pro-migratory responses within the 6-h experimental time frame cannot be ruled out. A recent *in vivo* study suggests that ERα in vascular smooth muscle cells is required to accelerate endothelial healing in response to E4 (Davezac et al., 2023). Accordingly, absence of nuclear ERα activity in the hematopoietic compartment abrogates E4-mediated protection against thromboembolism in mice (Valéra et al., 2018). E4 has tissue-selective actions (Visser et al., 2008); for instance, E4 appears to induce less effect than EE on hepatic derived haemostatic biomarkers, which may be beneficial in terms of reduced thromboembolic risk (Adlanmerini et al., 2022). The distinctive uterine and vascular actions of E4 (Abot et al., 2014) are consistent with those shown by our group for the selective ERα agonist PPT as opposed to the nonselective agent E2 (Bolego et al., 2010).

E4 C_{max} is 17.9 ng/mL (Mayne Pharma, 2021). As this corresponds to 5.9×10^{-8} M, the concentrations used in our study are achievable with E4-containing contraceptives. Due to higher potency of EE compared to E4 (Coelingh Bennink et al., 2008), lower EE doses are administered, resulting in lower C_{max} values compared to E4. However, during repeated oral administration, substantial accumulation of EE can be expected (Stanczyk et al., 2013). Hence, the concentrations used in our study may be achievable with EE-containing contraceptives, also considering that: (i) endothelial cells are directly exposed to drugs at plasma concentrations, and (ii) selected progestins included in combined oral contraceptives such as norethindrone are converted to EE (Chu et al., 2007). In addition, because 2-methoxy-estradiol, an endogenous metabolite of E2 and EE but not of E4, has been reported to inhibit endothelial cell proliferation (Yue et al., 1997), the effect of long-term exposure to EE-containing medications on vascular remodelling and regeneration deserves further investigation.

With regard to the mechanisms governing HUVEC motility, we previously reported that treatment with 3PO, a PFKFB3 inhibitor, inhibits FBS-induced migration (Boscaro et al., 2020b). In the present study, the EE- and E4-mediated increase in chemotactic migration was abolished by 3PO, further supporting the role of PFKFB3 enzyme activity in the process. However, endothelial PFKFB3 accumulation was increased by 3-h treatment with EE but not with E4. The fact that E4 failed to elicit a response that is mediated at least in part by GPER

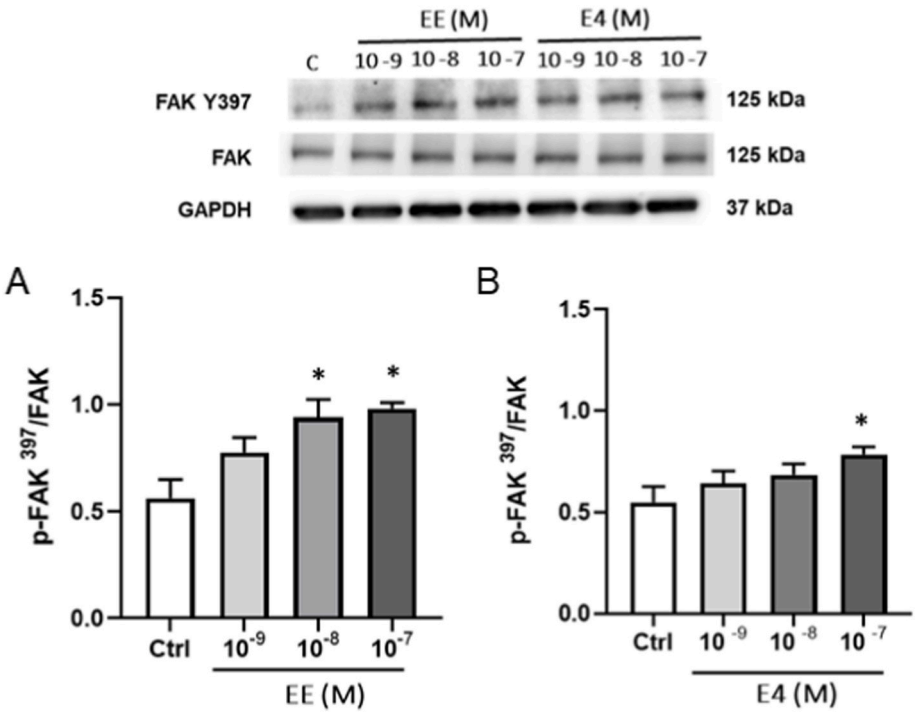

Fig. 5. Effect of estrogenic ligands on the accumulation of FAK Y397. HUVECs (3×10^5 cells/well) were grown in 35-mm plates in M199 medium and subsequently treated with EE or E4 (10^{-9} – 10^{-7} M) for 30 min. *Upper panel*: representative Western blot showing detection of FAK and FAK Y397; *lower panel*: densitometric analysis of bands normalized to GAPDH levels and p-FAK/FAK ratio relative to control (Ctrl) after treatment with EE (**A**) and E4 (**B**). Data are expressed as mean ± SEM of $n = 4$ independent experiments. One-way ANOVA, $^*p < 0.05$ *vs.* control.

suggests that E4 has lower binding affinity to or intrinsic activity on GPER than EE. Levels of p-FAK Y576/577 as well as total FAK are also significantly decreased by 3PO treatment (Boscaro et al., 2020b), in line with the observation that glycolytic rates are coupled to actin cytoskeleton architecture (Park et al., 2020). Accordingly, treatment with both EE and E4 led to increased FAK Y397 levels. Since E4 enhanced HUVEC migration without an apparent upregulation of glycolytic enzymes, EE and E4 possibly activated HUVEC migration via parallel intracellular signalling pathways including, *e.g.*, PI3K/Akt. In fact, the p85 regulatory PI3K subunit binds to the FAK Y397 autophosphorylation site, thereby leading to Akt activation and enhanced cell migration (Reiske et al., 1999; Zhao and Guan, 2011), whereas the PI3K inhibitor LY294002 abrogates FBS-induced HUVEC migration (Boscaro et al., 2020b).

In conclusion, treatment with the ER ligands EE and E4 increased HUVEC migration through GPER-dependent mechanisms, possibly involving distinct signalling pathways. These findings suggest that the two main estrogenic agents used in combined oral contraceptives are endowed with comparable vascular remodelling and regeneration capacity.

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CRediT authorship contribution statement

Aida Dama: Investigation, Writing – original draft, preparation. **Chiara Baggio:** Validation, Formal analysis, Visualization. **Lucia Trevisi:** Validation, Resources. **Chiara Bolego:** Conceptualization, Validation, Supervision. **Andrea Cignarella:** Conceptualization, Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2023.175591.

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