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Regulatory effects of estetrol on the endothelial plasminogen pathway and endothelial cell migration

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A B S T R A C T

Background: Estetrol (E4) is a natural estrogen produced solely during human pregnancy. E4 is suitable for clinical use since it acts as a selective estrogen receptor modulator. In clinical trials E4 has been seen to have little or no effect on coagulation. Hence, it is interesting to investigate whether E4 alters endothelial-dependent fibrinolysis.
Objectives: We studied the effects of E4 on the fibrinolytic system and whether this could influence the ability of endothelial cells to migrate. In addition, we compared the effects of E4 with those of 17β-estradiol (E2).
Study design: Human umbilical vein endothelial cells (HUVEC) were obtained from healthy women. Expression of plasminogen-activator inhibitor-1 (PAI-1), urokinase-type plasminogen activator (u-PA) and tissue plasminogen activator (t-PA) proteins was evaluated by Western blot analysis. Endothelial cell migration was studied by razor-scrape horizontal and multiwell insert systems assays.
Results: E4 increased the expression of t-PA, u-PA and PAI-1 in HUVEC, but less so than did equimolar amounts of E2. The effects of E4 on t-PA, u-PA and PAI-1 were mediated by the induction of the early-immediate genes c-Jun and c-Fos. E4 in combination with E2 antagonized the effects induced by pregnancy-like E2 concentrations but did not impair the effects of postmenopausal-like E2 levels. We also found that the increased synthesis of PAI-1, u-PA and t-PA induced by E2 and E4 is important for horizontal and three-dimensional migration of HUVEC.
Conclusions: These results support the hypothesis that E4 acts as an endogenous selective estrogen receptor modulator (SERM), controlling the fibrinolytic system and endothelial cell migration.
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1. Introduction

Estetrol (E4) is a human-specific natural estrogen produced exclusively during pregnancy by the fetal liver. E4 concentrations increase exponentially during pregnancy and peak at term with fetal levels about 10–20 times higher than maternal ones. Soon after delivery, blood levels of E4 become undetectable [1]. The physiological role and mechanisms of action of E4 during pregnancy are still poorly understood.

E4 is an endogenous selective estrogen receptor modulator (SERM), exerting estrogenic actions on the endometrium and the central nervous system but with antagonistic effects on the breast [2].

Based on the available information clinical studies of E4 [3], oral administration has minimal effects on the liver, and surrogate markers of coagulation and fibrinolysis are compatible with a neutral effect on thromboembolic risk. Due to these characteristics, E4 is currently being developed for a number of clinical applications, including contraception and menopausal hormone replacement therapy (HRT) [4,5]. The Women's Health Initiative (WHI) trial showed that oral estrogen plus progestin – an alternative preparation for such applications – may increase the risk of cardiovascular disease (CVD) among postmenopausal women, especially during the first year after the initiation of HRT [6,7].

Plasminogen-activator inhibitor-1 (PAI-1) is one of the primary regulators of the fibrinolytic system *in vivo*, and over-expression of this inhibitor compromises normal fibrin clearance and promotes fibrin deposition and hence thrombotic events [8]. Menopausal estrogen withdrawal is associated with increased blood levels of PAI-1 [9], while a decrease is found with estrogen therapy [10].

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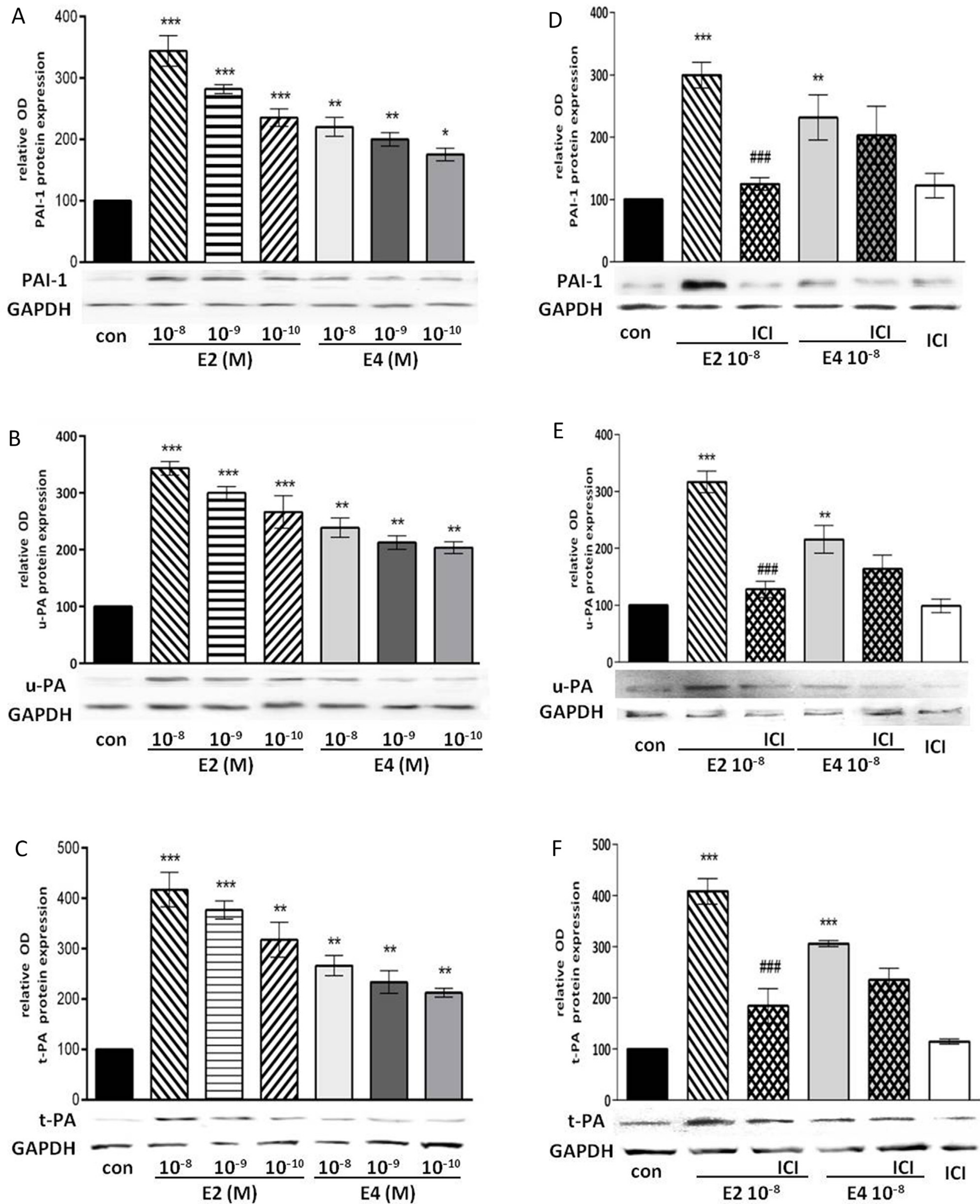

Fig. 1. E4 increases expression of PAI-1, u-PA and t-PA in human endothelial cells. HUVEC were treated during 24 h with vehicle (con) or increasing doses of E2 or E4 (A–C) in the presence or absence of ICI (D–F). Cell lysates were analyses by Western blotting for PAI-1, u-PA and t-PA. Quantitative bar graphs are expressed as mean ± SEM (upper panel) of three independent experiments and representative blots are shown (lower panel). The significance of the observed effects was evaluated using one-way ANOVA followed by Tukey's multiple comparisons post-test (*p < 0.05, **p < 0.01, ***p < 0.001 vs. con) (###p < 0.001 vs. E2).

These changes have been interpreted as potential reasons for the beneficial vascular effects of endogenous estrogens in fertile women. Additionally, estrogen receptor (ER) agonists are known to control endothelial cell migration and the proteolytic activity of PAI-1, urokinase-type plasminogen activator (u-PA) and tissue plasminogen activator (t-PA) [11,12].

In human endothelial cells, E4 mimics the effects of 17β-estradiol (E2), enhancing nitric oxide (NO) synthesis when provided

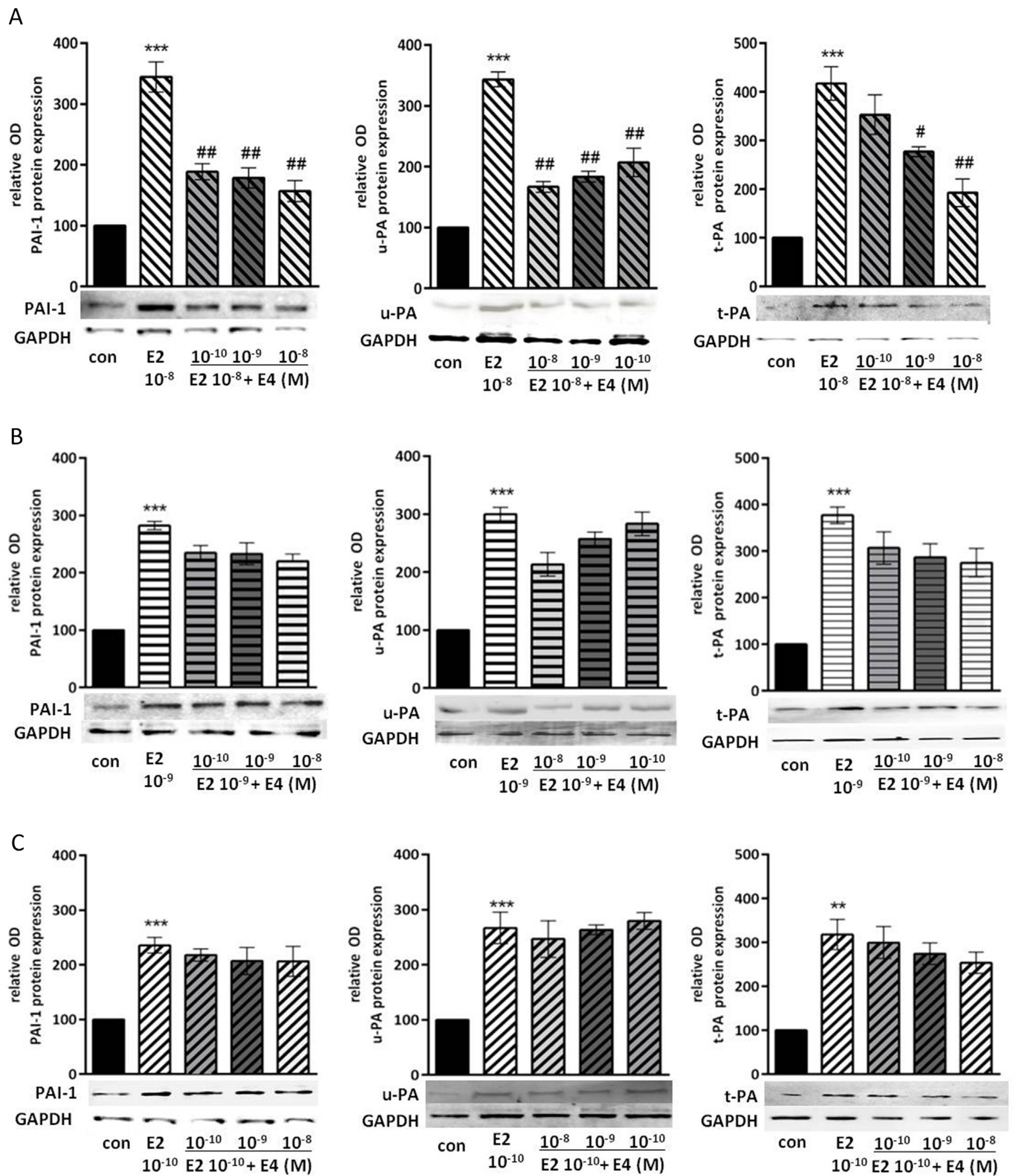

Fig. 2. E4 modulates the effects of E2 in human endothelial cells. HUVEC were co-treated for 24 h with vehicle (con) or increasing concentrations of E4 and E2 (A–C). Cell lysates were analyses by Western blotting for PAI-1, u-PA, t-PA proteins. Quantitative bar graphs are expressed as mean ± SEM (upper panel) of three independent experiments and representative blots are shown (lower panel). The significance of the observed effects was evaluated using one-way ANOVA followed by Tukey's multiple comparisons post-test (**$p < 0.01$, ***$p < 0.001$ vs. control) (#$p < 0.05$, ##$p < 0.01$ vs. E2).

alone, but is an estrogen antagonist when the two steroids are jointly present [13]. We aimed to assess whether E4 exerts modulatory actions on the fibrinolytic system directly at the endothelial level, by controlling the protein synthesis of PAI-1, u-PA and t-PA, and whether this could influence the ability of endothelial cells to migrate. In addition, we compared the effects of E4 with those of E2, and studied the effects of the combination of the two steroids.

2. Methods

2.1. Cell culture and treatments

Human umbilical vein endothelial cells (HUVEC) were obtained from umbilical veins from healthy women at the time of delivery. Under a laminar flow hood, the umbilical vein was cannulated

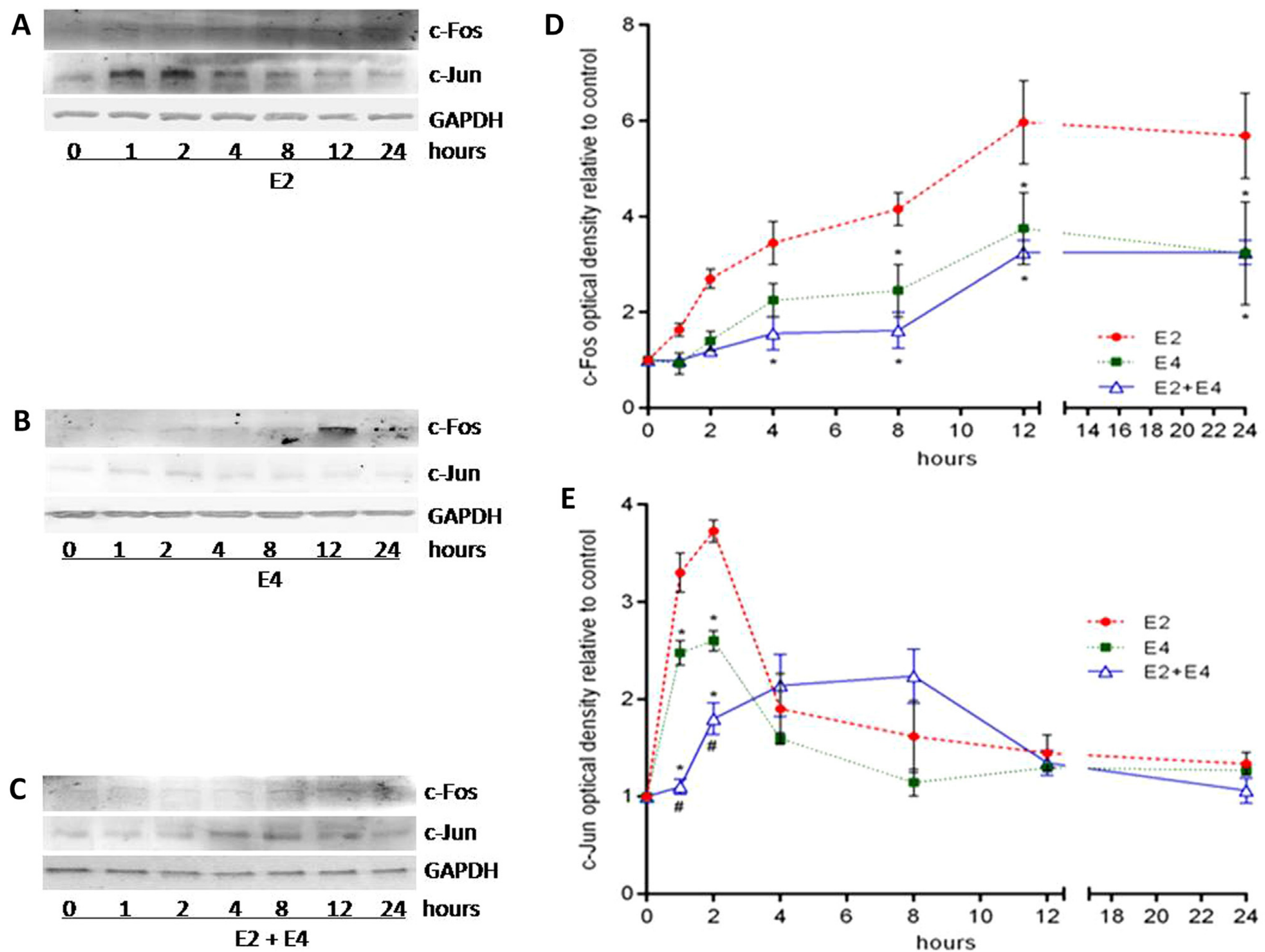

Fig. 3. E4 modulates c-Fos and c-Jun expression. HUVEC were treated with vehicle (con), or E2, E4 or the combination of both steroids (10^{-9}M) for different times (0–24 h). Cell lysates were analyzed by Western blotting for c-Fos and c-Jun. Representative blots are shown (A–C) and quantitative time responses are expressed as mean ± SEM of three independent experiments (D–E). The significance of the observed effects was evaluated using two-way ANOVA followed by Tukey's multiple comparisons post-test. (E2 vs. E4 or E2 + E4: *p < 0.05, **p < 0.01, ***p < 0.001) (E4 vs. E2 + E4: #p < 0.05, ###p < 0.001).

and rinsed with sterile saline solution. After clamping one extremity, the vein was filled with pre-warmed 0.1% type IA collagenase (Sigma-Aldrich, USA) for 30 min at 37 °C. The action of collagenase was blocked with DMEM 10% FBS. The collected solutions were centrifuged at 4 °C for 30 min at 1300 rpm, and the pellet resuspended in Medium 200PRF supplemented with Low Serum Growth Supplement Kit (LSGS, Gibco, Invitrogen). HUVEC were plated on culture plates pre-coated with Attachment Factor (AF, Gibco, Invitrogen) and maintained at 37 °C in a humidified 5% CO_2 atmosphere. Cells were used up to passage 5. Twenty-four hours before experiments, medium was replaced with steroid-deprived FBS. The ER antagonist ICI 182,780 (ICI 10^{-7}M) (Tocris Cookson, UK) was used 30 min before the active treatments. Control cells were treated with ethanol (vehicle) at a final concentration of 0.01% (solvent for E2/E4/ICI).

2.2. Immunoblottings

After treatments, cells lysates were separated by SDS-PAGE. Antibodies against u-PA (ab8473), PAI-1 (sc-5297), t-PA (sc-5239), c-Fos (sc-447), c-Jun (sc-1694) and GAPDH (sc-59540, a housekeeping protein used as loading control) were used. Primary and secondary antibodies were incubated with a standard technique. Immunodetection was accomplished with a quantitative digital imaging system (Quantity One; BioRad, USA). Densitometric analysis of the proteins bands was performed using NIH ImageJ 1.40 g software. The values shown in each quantitative bar graph in the Results section below are the ratio of each protein band vs. a loading reference (GAPDH).

2.3. Gene silencing with RNA interference

Gene silencing was performed using synthetic small interfering RNAs (siRNAs) targeting PAI-1 (sc-36179), u-PA (sc-36705) and t-PA (sc-36779). Each siRNAs is a pool of 3 target-specific 19–25nt which are designed to knock down gene expression. All siRNAs were used at a final concentration of 25 and 50 nM according to the manufacturer's instructions. HUVEC were transfected with Lipofectamine RNAiMAX (Invitrogen, USA) in opti-MEM without ATB. The efficacy of gene silencing was checked after 24, 48 and 72 h by Western analysis and found to be optimal with 50 nM from 24 h. HUVEC active treatments were done 24 h after siRNA transfection.

2.4. Cell migration assays

Cell migration assays with razor-scrape were performed as previously described [14]. Briefly, a razor blade was pressed through the confluent HUVEC monolayer to mark the starting line and cells were swept away on one side of that line. Cells were washed and a medium was added that contained steroid-deprived FBS and ARA-C

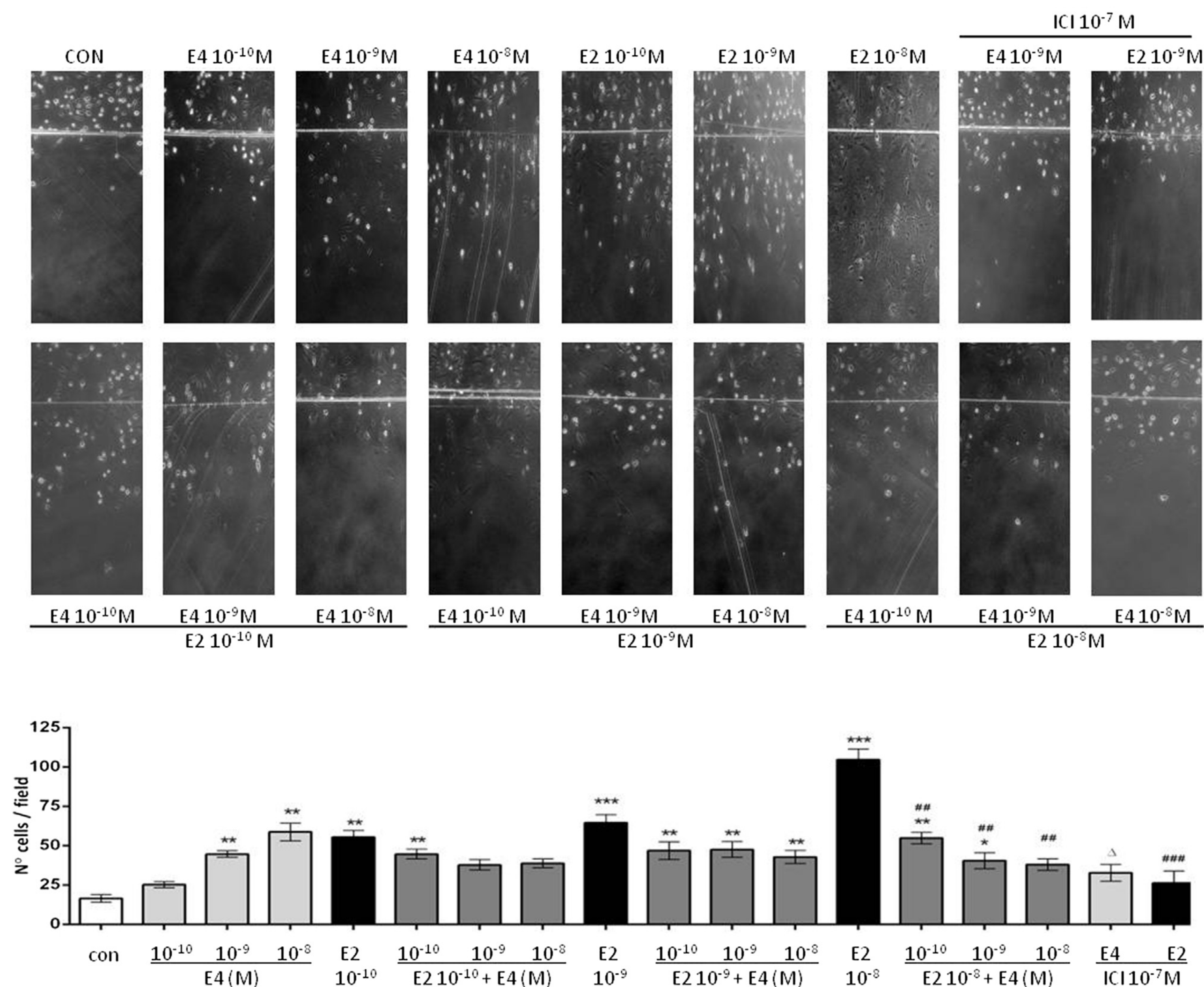

Fig. 4. E4-modulated HUVEC horizontal migration. HUVEC were scraped out from the culture dish and the number of cells crossing the starting line was analysed after 48 h. HUVEC were treated with vehicle (con) or increasing doses of E4, E2 (10^{-10} to 10^{-8}M) or with the combination of both steroids in the presence or absence of ICI. Representative images are shown of three independent experiments. The significance of the observed effects was evaluated using one-way ANOVA followed by Tukey's multiple comparisons post-test (*p < 0.05, **p < 0.01, ***p < 0.001 vs. control), (##p < 0.01, ###p < 0.001 vs. E2), (Δp < 0.05 vs. E4).

(Sigma-Aldrich) (10 μM), a selective inhibitor of DNA synthesis that does not inhibit RNA synthesis. Active treatments were done one hour later. Migration was monitored for 48 h. Cells were digitally imaged and migration distance was measured with phase-contrast microscopy in five different areas of the culture. Quantification of migrated cells was performed using NIH ImageJ 1.40 g software.

Cell migration assays with multiwell insert systems were performed using inserts (8 μm pores/cm^2) (1185-HTS, BD Biosciences Labware). In brief, 40000 cells were plated in the upper well (insert) with medium containing steroid-deprived FBS, and in the lower chamber medium containing the active treatment. HUVEC were allowed to expand for 24 h. Subsequently cells were fixed in 3% paraformaldehyde phosphate buffered saline for 20 min and stained in Giemsa solution. Non-migrated cells were removed using a cotton-tipped swab. Cells were digitally imaged using phase-contrast microscopy in four different areas. Quantification of migrated cells was performed using NIH ImageJ 1.40 g software.

2.5. Statistical analysis

Each test condition was reproduced in three independent experiments. All data are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism 6 software, using one- or two-way ANOVA followed by Tukey multiple comparisons post-test, depending on the outcomes analyzed. Differences at $p < 0.05$ were considered significant.

3. Results

3.1. E4 increases the expression of PAI-1, u-PA and t-PA in human endothelial cells

HUVEC were exposed for 24 h to a range of E4 concentrations, spanning those selected to treat climacteric complaints in postmenopausal women (10^{-10}M) to those that are found during pregnancy (maternal side 10^{-9}M, fetal side 10^{-8}M). Parallel amounts of E2 were tested, mimicking the span in reproductive life: pregnancy-like (10^{-8}M), follicular phase-like (10^{-9}M) or postmenopause-like E2 (10^{-10}M).

E4 treatment resulted in a significant increase in PAI-1, u-PA and t-PA expression in a dose-dependent manner, although E4 was less effective than equimolar amounts of E2 (Fig. 1A–C). The effects of E4 were reduced by the addition of ICI, suggesting that E4 acts via estrogen receptor recruitment (Fig. 1D–F).

To evaluate whether E4 interferes with E2 signaling, we performed co-treatments with the two steroids. We observed a

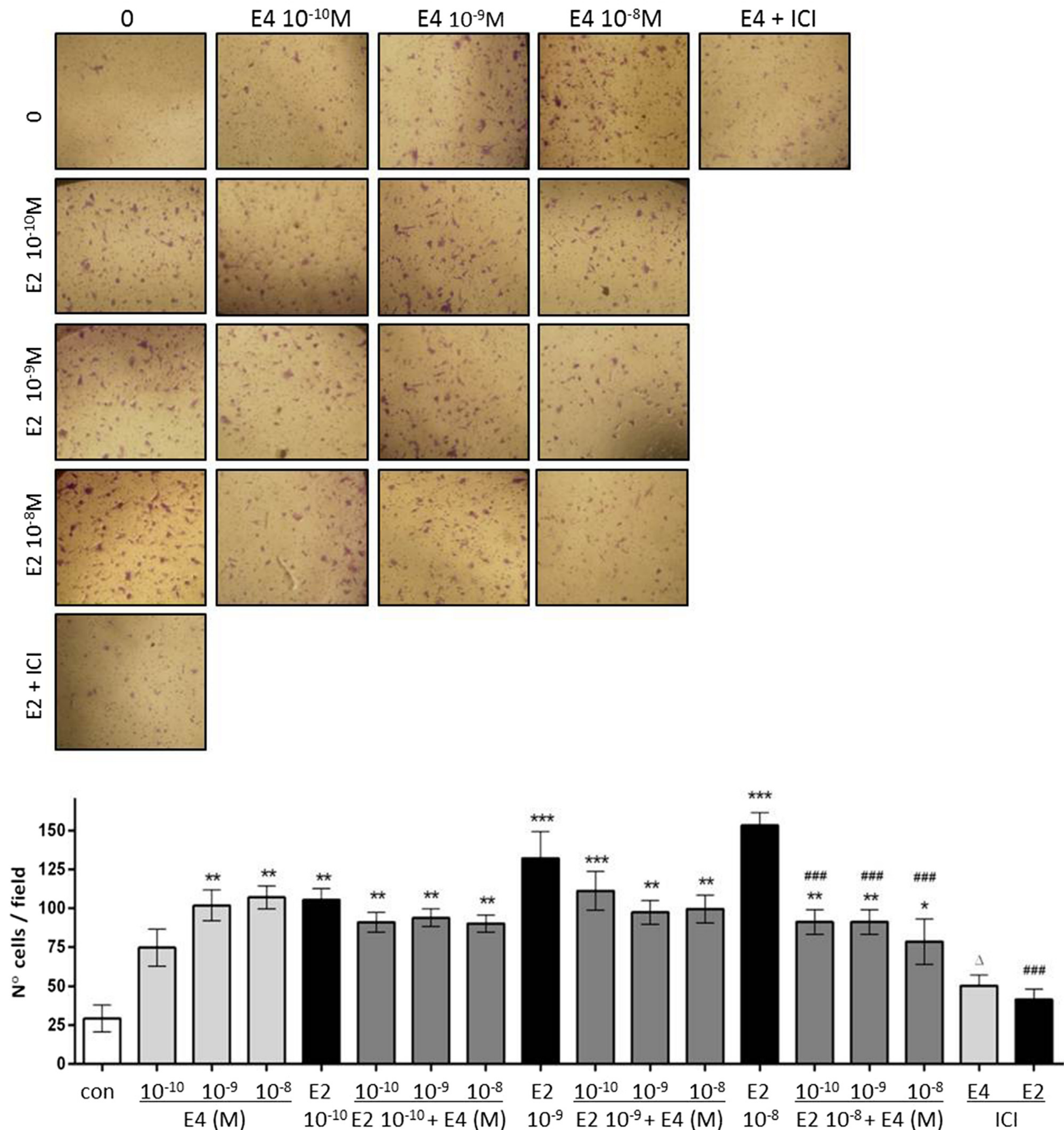

Fig. 5. E4-modulated HUVEC insert migration. HUVEC were plated in the upper well (insert) and in the lower chamber medium with vehicle (con) or increasing doses of E4, E2 (10^{-10} to 10^{-8}M) or with the combination of both steroids during 24 h, in the presence or absence of ICI. Representative images are shown of three independent experiments. The significance of the observed effects was evaluated using one-way ANOVA followed by Tukey's multiple comparisons post-test (*p < 0.05, **p < 0.01, ***p < 0.001 vs. control), (##p < 0.01, ###p < 0.001 vs E2), (Δp < 0.05 vs E4).

significant reduction of the pro-stimulatory effects of E2 when E4 was added to 10^{-8}M E2 (Fig. 2A). This reduction was related to the amount of E4 added. However, co-treatment of E4 with 10^{-9}M E2 produced a non-significant reduction in the expression of PAI-1, u-PA and t-PA (Fig. 2B), while co-treatment of E4 with 10^{-10}M E2 did not affect the pro-stimulatory effects of E2 (Fig. 2C).

3.2. E4 modulates c-Fos and c-Jun expression

A previous study by our group showed that estradiol induces PAI-1 expression through up-regulation of c-Fos and c-Jun [12]. PAI-1, u-PA and t-PA genes are regulated by the Activator Protein-1 (AP-1) complex, which is composed of a heterodimer of c-Fos and c-Jun [15,16]. We therefore checked whether E4 regulates these transcription factors, in HUVEC, performing time-course experiments with E4 alone or in combination with E2.

Both E4 and E2 administration increased c-Fos and c-Jun expression (Fig. 3A and B, D and E). The co-treatment E2-E4 resulted in c-Fos or c-Jun delayed expression, and was less efficient than E2 alone (Fig. 3C–E).

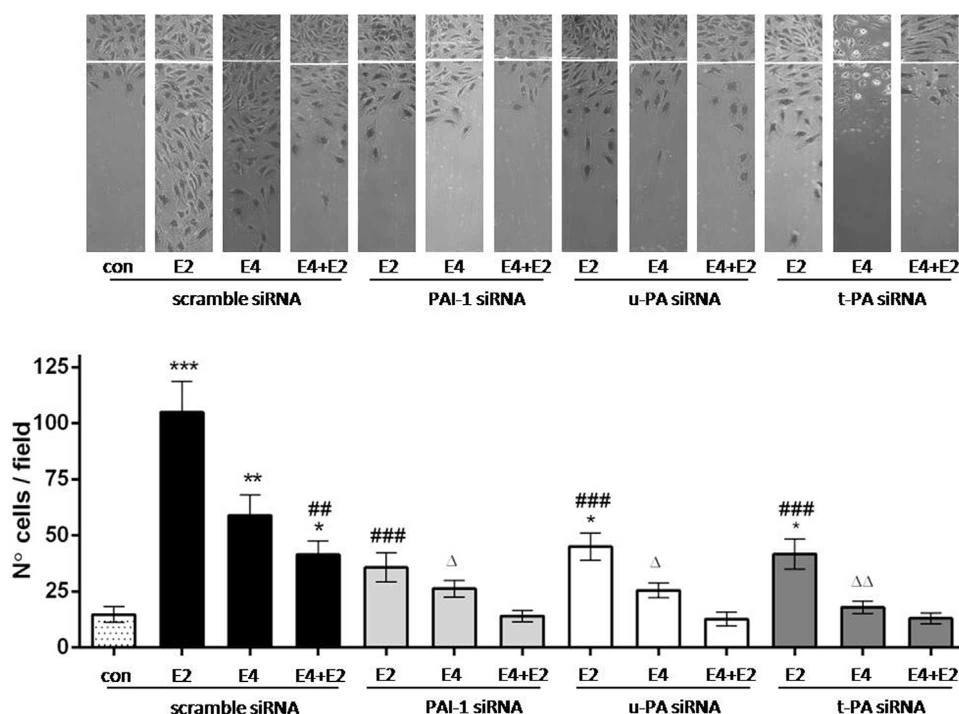

Fig. 6. PAI-1, u-PA and t-PA are required for E4-induced endothelial cell horizontal migration. HUVEC were transfected with siRNA versus PAI-1 (siRNA PAI-1), u-PA (siRNA u-PA) and t-PA (siRNA t-PA) or scramble siRNA as control (scr). Horizontal migration assay was performed 24 h after cells transfection. HUVEC were treated with vehicle (con) or E4, E2 (10^{-8}M) and with the combination of both steroids for 48 h. Representative images are shown of three independent experiments. The significance of the observed effects was evaluated using one-way ANOVA followed by Tukey's multiple comparisons post-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. scramble), (##$p < 0.01$, ###$p < 0.001$ vs. E2), (Δ$p < 0.05$, ΔΔ$p < 0.01$ vs. E4).

3.3. E4 modulates HUVEC migration

We tested whether E4 administration modified migration in HUVEC. Two different migration assays obtained similar results: a razor-scrape horizontal migration and a multiwell inserts migration assay.

E4 treatment significantly increased cell migration in a dose-dependent manner, although E4 was less effective than equimolar amounts of E2. Once more, the effects of E4 were blunted by ICI, suggesting that E4 regulates endothelial cell migration via estrogen receptors (Figs. 4 and 5).

The pro-migratory effects of 10^{-8}M E2 were significantly reduced when increasing amounts of E4 were added. This reduction was related to the amount of E4 added. Again, co-treatment of E4 with 10^{-9}M or 10^{-10}M E2 did not affect the pro-migratory effects of E2 (Figs. 4 and 5).

3.4. PAI-1, u-PA and t-PA are required for E4-induced endothelial cell migration

To study whether E4 migration involves the proteins of the fibrinolytic system, we silenced PAI-1, u-PA and t-PA with siRNA and performed horizontal migration. First we tested the efficacy of gene silencing by Western blot (Fig. S1 in Supplementary Material). After down-regulation of the mentioned proteins, we performed a cell migration assay. E4, E2 and the co-treatment E2-E4 significantly increased horizontal migration. Silencing PAI-1, u-PA and t-PA significantly blocked endothelial cell migration in all conditions (Fig. 6).

4. Discussion

The main finding of our paper is the identification of a direct regulation by E4 of endothelial fibrinolysis. E4 modulates endothelial levels of PAI-1, u-PA and t-PA. These proteins could mediate the effects of estrogen and estetrol on endothelial migration. Additionally, E4 can interfere with the effects of E2 in HUVEC, depending on the amount of estradiol present.

In the presence of pregnancy-like amounts of estradiol, estetrol is a potent antagonist, decreasing E2-induced migration and PAI-1, u-PA and t-PA expression. This is consistent with our previous work on the regulation of NO synthesis by estetrol in human endothelial cells [13]. The implications of this phenomenon may reside in the hypothetical role of estetrol during pregnancy, where it may be a key actor in dissecting the vasodilatory actions of estradiol in the maternal and fetal circulations. Concentrations of E4 are 10-fold lower in the maternal circulation than in the fetal one. This may result in a lower inhibition of the fibrinolytic and NO systems [13], therefore favoring efficient blood flow towards the fetus. In contrast, high amounts of estetrol on the fetal side may help to prevent excessive vascular dilatation or may interfere more effectively with those processes that are modulated by the components of the fibrinolytic system, such as blood clotting or endothelial remodeling. Indeed, a correct function of the plasminogen pathway and NO system is crucial in the placenta, umbilical vessels and in the fetus to promote normal fetal development and to allow fetal preparation for the stress of delivery [17,18].

The vascular actions of estetrol are particularly relevant in the context of its possible use as a post-menopausal chronic treatment. Estrogen therapy exerts a strong vascular protective action after the menopause [19]; thus it is a relevant safety issue to assess whether estetrol treatment shares this positive effect or whether it may be harmful. In this setting, it is important to understand whether the addition of E4 to concentrations of E2 similar to those found after menopause will result in modifications of the biological actions of estradiol. Our results suggest that this is not the case, since the stimulatory effect on PAI-1, u-PA and t-PA expression or on cell migration induced by postmenopausal-like amounts of estradiol

(10^{-10}M) was not modified by high or low amounts of estetrol (10^{-8}-10^{-10}M).

Furthermore, as E4 appears to be a naturally occurring SERM [20,21], it is valuable to characterize better its signaling mechanism, particularly in view of its potential development for clinical use [22]. E4 binds to both ERα and ERβ with a 100-fold lower affinity than E2. A competitive inhibition of ERs may in part explain its action as an estrogen antagonist [20]. On the other hand, estetrol may also act via G protein-coupled estrogen receptor 1 (GPER1), which is a newer receptor mediating some of the actions of estrogens [23]. Our results, along with the evidence that ER inhibitors prevent the effects of estetrol in a variety of settings [13,14,21,24], support the notion that ER is the main mediator of estetrol's actions. We now add a piece of information to the characterization of E4 signaling, highlighting its link to the expression of the early-immediate genes c-Jun and c-Fos, which are involved in a variety of genomic actions by hormones and growth factors.

In conclusion, based on our current and previous results, we suggest that the endothelial cells are a target of estetrol. Specifically, we found that the regulation of the fibrinolytic protein system in endothelial cells may be one of the key actions of estetrol in the vascular system, with potential implications for the local control of blood clotting and for vascular remodeling. This information helps characterize this interesting steroid and its potential for clinical use.

Contributors

M.M.M.G. performed the molecular studies, bioinformatics statistical analysis and drafted the manuscript.

JES participated in the data interpretation and helped to draft the manuscript.

G.P., S.S., G.B., E.C. and A.E.C. performed the molecular studies.

A.C. participated in the data interpretation and helped to draft the manuscript.

T.S. designed and participated in the data interpretation, helped to draft the manuscript and supervised the project.

All authors saw and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Ethical approval

Human umbilical cords were obtained from healthy women; written informed consent was obtained from each subject in accordance with the Declaration of Helsinki.

Provenance and peer review

This article has undergone peer review, coordinated by Professor Margaret Rees independently of Tommaso Simoncini, an author and *Maturitas* editor, who was blinded to the process.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.maturitas.2017.02.005.

References

[1] F. Coelingh Bennink, C.F. Holinka, M. Visser, H.J. Coelingh Bennink, Maternal and fetal estetrol levels during pregnancy, Climacteric 11 (Suppl. 1) (2008) 69–72.

[2] H.J. Coelingh Bennink, C.F. Holinka, E. Diczfalusy, Estetrol review: profile and potential clinical applications, Climacteric 11 (Suppl. 1) (2008) 47–58.

[3] M. Visser, H.J. Coelingh Bennink, Clinical applications for estetrol, J. Steroid Biochem. Mol. Biol. 114 (1–2) (2009) 85–89.

[4] H.J. Coelingh Bennink, S. Skouby, P. Bouchard, C.F. Holinka, Ovulation inhibition by estetrol in an in vivo model, Contraception 77 (3) (2008) 186–190.

[5] M. Visser, C.F. Holinka, H.J. Coelingh Bennink, First human exposure to exogenous single-dose oral estetrol in early postmenopausal women, Climacteric 11 (Suppl. 1) (2008) 31–40.

[6] H.M. Boardman, L. Hartley, A. Eisinga, C. Main, I.F.M. Roque, X. Bonfill Cosp, R. Gabriel Sanchez, B. Knight, Hormone therapy for preventing cardiovascular disease in post-menopausal women, Cochrane Database Syst. Rev. 3 (2015), CD002229.

[7] H.N. Hodis, W.J. Mack, D. Shoupe, S.P. Azen, F.Z. Stanczyk, J. Hwang-Levine, M.J. Budoff, V.W. Henderson, Methods and baseline cardiovascular data from the early versus late intervention trial with estradiol testing the menopausal hormone timing hypothesis, Menopause 22 (4) (2015) 391–401.

[8] Y. Aso, Plasminogen activator inhibitor (PAI)-1 in vascular inflammation and thrombosis, Front. Biosci. 12 (2007) 2957–2966.

[9] A.K. Ganti, A. Potti, R. Yegnanarayan, Plasma tissue plasminogen activator and plasminogen activator inhibitor-1 levels in acute myocardial infarction, Pathophysiol. Haemost. Thromb. 32 (2) (2002) 80–84.

[10] E. Shahar, A.R. Folsom, V.V. Salomaa, V.L. Stinson, P.G. McGovern, T. Shimakawa, L.E. Chambless, K.K. Wu, Relation of hormone-replacement therapy to measures of plasma fibrinolytic activity. Atherosclerosis Risk in Communities (ARIC) Study Investigators, Circulation 93 (11) (1996) 1970–1975.

[11] T. Simoncini, C. Scorticati, P. Mannella, A. Fadiel, M.S. Giretti, X.D. Fu, C. Baldacci, S. Garibaldi, A. Caruso, L. Fornari, F. Naftolin, A.R. Genazzani, Estrogen receptor alpha interacts with Galpha13 to drive actin remodeling and endothelial cell migration via the RhoA/Rho kinase/moesin pathway, Mol. Endocrinol. 20 (8) (2006) 1756–1771.

[12] S. Gopal, S. Garibaldi, L. Goglia, K. Polak, G. Palla, S. Spina, A.R. Genazzani, A.D. Genazzani, T. Simoncini, Estrogen regulates endothelial migration via plasminogen activator inhibitor (PAI-1), Mol. Hum. Reprod. 18 (8) (2012) 410–416.

[13] M.M. Montt-Guevara, M.S. Giretti, E. Russo, A. Giannini, P. Mannella, A.R. Genazzani, A.D. Genazzani, T. Simoncini, Estetrol modulates endothelial nitric oxide synthesis in human endothelial cells, Front. Endocrinol. 6 (2015).

[14] M.S. Giretti, M.M. Montt Guevara, E. Cecchi, P. Mannella, G. Palla, S. Spina, G. Bernacchi, S. Di Bello, A.R. Genazzani, A.D. Genazzani, T. Simoncini, Effects of estetrol on migration and invasion in T47-D Breast cancer cells through the actin cytoskeleton, Front. Endocrinol. 5 (2014) 80.

[15] D. D'Orazio, D. Besser, R. Marksitzer, C. Kunz, D.A. Hume, B. Kiefer, Y. Nagamine, Cooperation of two PEA3/AP1 sites in uPA gene induction by TPA and FGF-2, Gene 201 (1–2) (1997) 179–187.

[16] T. Kooistra, P.J. Bosma, K. Toet, L.H. Cohen, M. Griffioen, E. van den Berg, L. le Clercq, V.W. van Hinsbergh, Role of protein kinase C and cyclic adenosine monophosphate in the regulation of tissue-type plasminogen activator, plasminogen activator inhibitor-1, and platelet-derived growth factor mRNA levels in human endothelial cells. Possible involvement of proto-oncogenes c-jun and c-fos, Arterioscler. Thromb. Vasc. Biol. 11 (4) (1991) 1042–1052.

[17] V.E. Murphy, R. Smith, W.B. Giles, V.L. Clifton, Endocrine regulation of human fetal growth: the role of the mother, placenta, and fetus, Endocr. Rev. 27 (2) (2006) 141–169.

[18] E.A. Herrera, B. Krause, G. Ebensperger, R.V. Reyes, P. Casanello, M. Parra-Cordero, A.J. Llanos, The placental pursuit for an adequate oxidant balance between the mother and the fetus, Front. Pharmacol. 5 (2014) 149.

[19] A.R. Genazzani, T. Simoncini, Pharmacotherapy: benefits of menopausal hormone therapy–timing is key, Nat. Rev. Endocrinol. 9 (1) (2013) 5–6.

[20] M. Visser, J.M. Foidart, H.J. Coelingh Bennink, In vitro effects of estetrol on receptor binding, drug targets and human liver cell metabolism, Climacteric 11 (Suppl. 1) (2008) 64–68.

[21] A. Abot, C. Fontaine, M. Buscato, R. Solinhac, G. Flouriot, A. Fabre, A. Drougard, S. Rajan, M. Laine, A. Milon, I. Muller, D. Henrion, M. Adlanmerini, M.C. Valera, A. Gompel, C. Gerard, C. Pequeux, M. Mestdagt, I. Raymond-Letron, C. Knauf, F. Ferriere, P. Valet, P. Gourdy, B.S. Katzenellenbogen, J.A. Katzenellenbogen, F. Lenfant, G.L. Greene, J.M. Foidart, J.F. Arnal, The uterine and vascular actions of estetrol delineate a distinctive profile of estrogen receptor alpha modulation, uncoupling nuclear and membrane activation, EMBO Mol. Med. 6 (10) (2014) 1328–1346.

[22] C.F. Singer, H.J. Bennink, C. Natter, S. Steurer, M. Rudas, F. Moinfar, N. Appels, M. Visser, E. Kubista, Antiestrogenic effects of the fetal estrogen estetrol in women with estrogen-receptor positive early breast cancer, Carcinogenesis 35 (11) (2014) 2447–2451.
[23] C. Gérard, M. Mestdagt, E. Tskitishvili, L. Communal, A. Gompel, E. Silva, J.-F. Arnal, F. Lenfant, A. Noel, J.-M. Foidart, C. Péqueux, Combined estrogenic and anti-estrogenic properties of estetrol on breast cancer may provide a safe therapeutic window for the treatment of menopausal symptoms, Oncotarget 6 (19) (2015) 17621–17636.
[24] C. Gerard, S. Blacher, L. Communal, A. Courtin, E. Tskitishvili, M. Mestdagt, C. Munaut, A. Noel, A. Gompel, C. Pequeux, J.M. Foidart, Estetrol is a weak estrogen antagonizing estradiol-dependent mammary gland proliferation, J. Endocrinol. 224 (1) (2015) 85–95.