Estrogen regulates endothelial migration via plasminogen activator inhibitor (PAI-1)

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ABSTRACT: Endothelial plasminogen activator inhibitor (PAI-1) controls vascular remodeling, angiogenesis and fibrinolysis. PAI-1 blood levels in women are related to estrogen. The aim of this study was to characterize the signaling pathways through which estrogen regulates PAI-1 in endothelial cells. Furthermore, we aimed to investigate whether PAI-1 is implicated in the control of endothelial migration by estrogen. Cultured human umbilical vein endothelial cells (HUVECs) and ovariectomized rats were used to test the effects of 17β-estradiol (E₂) on PAI-1 expression and its role on endothelial migration. At physiological concentrations, E₂ increases the expression of PAI-1 in HUVEC within 6–12 h through activation of a signaling cascade initiated by estrogen receptor α and involving G proteins, phosphatidylinositol-3-OH kinase and Rho-associated kinase II. ROCK-II activation turns into an over-expression of c-Jun and c-Fos that is required for E₂-induced expression of PAI-1. Estrogen-induced PAI-1 expression is implicated in HUVEC horizontal migration. PAI-1 regulation is found also in vivo, in female rats, where ovariectomy is associated with reduced PAI-1 expression, while estrogen replacement counteracts this change. In conclusion, E₂ increases PAI-1 synthesis in human endothelial cells and in rodent aorta through a G protein-initiated signaling that targets early-immediate gene expression. This regulatory pathway is implicated in endothelial cell migration. These findings describe new mechanisms of action of estrogens in the vessels, which may be important for vascular remodeling and hemostasis.

Key words: endothelial cells / estrogen / estrogen receptor / ROCK-II/PAI-1

Introduction

Plasminogen activator inhibitor-1 (PAI-1) is a serine protease inhibitor that controls the fibrinolytic system by inhibiting tissue-type and urokinase plasminogen activators (Bergheim et al., 2006; Dimova and Kietzmann, 2008; Zorio et al., 2008; Xu et al., 2010). By controlling the plasminogen cascade, PAI-1 is also implicated in physiological and pathological vascular processes such as thrombosis, inflammation, vascular remodeling and wound healing (Diebold et al., 2008). A prominent source of PAI-1 in these conditions is represented by vascular endothelial cells, which synthesize the protease inhibitor in response to a variety of stimuli (Stiko-Rahm et al., 1990; Marx et al., 1999; Ye et al., 2002; Lyon and Hsueh, 2003; Gramling and Church, 2010).

Beyond being a modulator of the fibrinolytic cascade, PAI-1 has been recently identified as a controller of cell movement and migration. Modulation of cell migration and angiogenesis by PAI-1 appears to be implicated both in vascular remodeling (Diebold et al., 2008) and in promotion of tumor growth, invasion and metastasis (Kwaan and McMahon, 2009). This newly identified set of activities is exerted through interactions with cell surface receptors affecting cell motility as well as through multifaceted interactions with extracellular matrix proteins (Czekay et al., 2011). In addition, signaling events that turn into modifications of the shape of the cytoskeleton and of the cell membrane modulate the expression of PAI-1 (Samarakoon et al., 2010). A link exists between estrogen concentrations and PAI-1 in women. Menopausal estrogen withdrawal is associated with increased blood levels of PAI-1, while a decrease is found with the estrogen therapy (Gebara et al., 1995; Koh et al., 1997; Sowers et al., 2005a). These changes have been interpreted as potential reasons for the beneficial vascular effects of endogenous estrogens in fertile women (Sowers et al., 2005b). In addition, a link between PAI-1 and hormonal status has been identified in the presence of coronary heart disease in women (Grancha et al., 1999) and experimental animals (Chua et al., 2011).
On the other hand, PAI-1 is also a known negative prognostic factor in endocrine-sensitive cancers, such as breast (Manders et al., 2004; Eljuga et al., 2011), and endometrial cancer (Steiner et al., 2008), with higher expression of the protease inhibitor being associated with poorer response to chemotherapy and prognosis. Based on preliminary evidence, estrogen seems to cross talk with growth factors in regulating PAI-1 synthesis in breast cancer cells (Burdette and Woodruff, 2007), but little is known about the signaling mechanisms through which estrogen controls PAI-1 or whether this regulatory action extends to other cell types.

The purpose of the current study was to explore the regulatory actions of estrogen on PAI-1 in human endothelial cells and to characterize the signaling steps through which these actions are enacted.

**Materials and Methods**

**Cell cultures and treatments**

Human umbilical vein endothelial cells (HUVECs) were extracted from umbilical cords of healthy pregnant women by collagenase treatment. HUVECs were seeded in Dulbecco’s Modified Eagle’s Medium (DMEM) containing fetal bovine serum (FBS) for 24 h. Before experiments investigating transcriptional effects, the medium was replaced by DMEM containing 10% FBS stripped with charcoal for 4–6 h. Whenever an inhibitor was used, the compound was added 30 min before starting the active treatments. 17β-Estradiol (E2), pertussis toxin (PTX), (7α,17β)-7-[(4,4,5,5-pentfluoropyrrolonyl)sulfonyl]nonyl]estra-1,3,5(10)-trien-3,17-diol (ICI) 182780 (ICI), 4,4,40-(4-propyl-[1H]-pyrazole-1,3,5-triol) trisphenol and 2,3-bis(4-hydroxyphenyl)-propionitrile, phosphatidylinositol 3-OH kinase (PI3K) inhibitor [wortmannin (WM)], Rho kinase inhibitor (Y-27632) were from Sigma-Aldrich (St Louis, USA). 4-Amino-5-(4-chlorophenyl)-7-(4-hydroxyphenil)-propionitrile, estradiol-1,3,5-triyl) trisphenol and 2,3-phosphatidylinositol 3-OH kinase (PI3K) inhibitor [wortmannin (WM)], Rho kinase inhibitor (Y-27632) were from Sigma-Aldrich (St Louis, USA). 4-Amino-5-(4-chlorophenyl)-7-(4-hydroxyphenil)-propionitrile, phosphatidylinositol 3-OH kinase (PI3K) inhibitor [wortmannin (WM)], Rho kinase inhibitor (Y-27632) were from Sigma-Aldrich (St Louis, USA). 4-Amino-5-(4-chlorophenyl)-7-(4-hydroxyphenil)-propionitrile, phosphatidylinositol 3-OH kinase (PI3K) inhibitor [wortmannin (WM)], Rho kinase inhibitor (Y-27632) were from Sigma-Aldrich (St Louis, USA).

**Animal treatments**

Fertile female Wistar rats, weighing 175–199 g (Harlan, S. Pietro at Natlon, Italy), were kept under 14 h of illumination per day (06.00–20.00 h) and had free access to standard rat chow and tap water. After 14 days, the rats were either sham-operated or ovariectomized in the same estrous cycle stage. Ovariectomized (OVX) rats were treated with E2 at a concentration of 0.01, 0.05 and 0.1 mg/kg. E2 was dissolved in pure ethanol and administered orally after dilution in water. Animals were euthanized by decapitation under pentobarbital anesthesia (30 mg/kg) and abdominal aorta was dissected. Aortas were snap-frozen in dry ice and stored at −80 °C. Animals were maintained in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals (www.nap.edu/readingroom/books/labrats). Eight animals for each experimental group were used.

**Immunoblottings**

After treatments, cells were lysed with lysis buffer containing SDS, glycerol and Tris–EDTA. The concentration of total protein was quantified by bichunoncin acid assay, and 30 μg of proteins were loaded into each well and separated by denaturing SDS–PAGE. Antibodies against the following proteins were used: c-Fos (sc-448), c-Jun (sc-74543), actin (House-keeping protein used as a loading control), all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), PAI-1 (ab82218), estrogen receptor α (ERα) (ab2746) and ERβ (ab288) were purchased from Abcam (Cambridge, UK). All primary antibodies were used at a concentration of 1:1000 and secondary antibodies were used at a concentration of 1:4000. Primary and secondary antibodies were incubated with the membranes, followed by three 5-min washes with tris-buffered saline-Tween-20. Immunodetection was accomplished using enhanced chemiluminescence and was recorded with a quantitative digital imaging system (Quantity One; BioRad, Hercules, CA, USA). Each experiment was repeated three times.

**Gene silencing with RNA interference**

Gene silencing was performed using synthetic siRNAs targeting c-Fos, c-Jun or ERβ purchased from Santa Cruz. ERα siRNAs were from Dharmaco (Lafayette, CO, USA). All siRNAs were used at the final concentration of 30 nM according to the manufacturer’s instructions. HUVECs were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in opti-minimum essential medium without antibiotics or antimycotics. Efficacy of gene silencing was checked by western blotting. Viability of cells was checked throughout transfection experiments. Each experiment was repeated three times.

**Transfection experiments**

Dominant negative Gα13 (Gα13Q226L/D294N) was obtained from the Guthrie DNA Resource Center (www.cdna.org). The insert was cloned in pcDNA3.1+. The plasmid (10 μg) was transfected into HUVEC using Lipofectamine (Invitrogen). Cells were treated 48 h after transfection. Each experiment was repeated three times.

**Cell migration assays**

Cell migration was assayed with razor scrape assays as previously described (Fu et al., 2008). Briefly, a starting line was scored through the confluent HUVEC monolayer into the plastic plate using a razor blade. HUVECs were swept away from one side of that line. Cells were washed, and 2.0 ml of DMEM containing steroid-deprived FBS and gelatin (1 mg/ml) were added. Cytosine b-D-arabinofuranoside hydrochloride (Sigma) (10 mM), a selective inhibitor of DNA synthesis, which does not inhibit RNA synthesis, was used 1 h before the test substance was added to prevent cell proliferation, while allowing migration. Migration was monitored for 24 h. Every 12 h fresh medium and treatments were replaced. Cells were digitally imaged and migration distance was measured by using phase-contrast microscopy in five different areas of the culture well. Each experiment was repeated three times.

**Statistical analysis**

All values are expressed as mean ± SD. Statistical differences between mean values were determined by analysis of variance followed by the Fisher’s protected least significance difference.

**Results**

E2 induces PAI-1 expression in endothelial cells via ERα

HUVECs were treated with 17β-E2 for different times and protein lysates were obtained. As shown by western immunoblotting, expression of PAI-1 gradually increased during exposure to E2 and peaked at 24–48 h (Fig. 1A). Endothelial PAI-1 expression was increased in the presence of physiological concentrations (Fig. 1B).

In order to check which ER isoform mediates the induction of PAI-1, we silenced ERα or ERβ with small interfering RNAs. Silencing of ERα blocked PAI-1 induction by E2 (Fig. 1C), whereas silencing of ERβ was ineffective (Fig. 1D).
E2 induces PAI-1 through up-regulation of c-Fos and c-Jun

PAI-1 gene promoter region is controlled by c-Jun and c-Fos through two dedicated consensus sequences. We checked whether E2 regulates PAI-1 through these transcription factors performing time course experiments with 1 nM E2 and analyzing expression of c-Fos and c-Jun in HUVEC. E2 induced the expression of c-Fos and c-Jun as early as after 2 h, with a peak at 8 h (Fig. 2A and B). In parallel, silencing of c-Fos and c-Jun with siRNAs blocked PAI-1 induction by E2 (Fig. 2C and D).

E2 regulates c-Jun, c-Fos and PAI-1 expression via signaling to G proteins, PI3K and Rho-associated kinase II

To search for the signaling pathways involved in E2-mediated c-Fos and c-Jun induction, we blocked different signaling intermediates that have an established role in regulating c-Jun and c-Fos. As shown by immunoblot analysis, E2 failed to induce c-Fos expression in HUVEC when G proteins, PI3K or ROCK-II were inhibited with PTX, WM or Y-27632, respectively (Fig. 2E). Similar results were found with c-Jun (Fig. 2F).

In parallel, the increased expression of PAI-1 associated with E2 treatment was prevented by PTX, WM or PP2, which blocks the activity of the PI3K controller, c-Src (Fig. 3A). As a control, the
PP2-related, inactive compound, negative control for the src kinase inhibitor PP2 (PP3), was ineffective (Fig. 3A). In addition, transfection of HUVEC with a dominant negative Ga13 construct indicated that E2 uses Ga13 to induce PAI-1 expression (Fig. 3B).

E2 controls PAI-1 via ROCK-II
Administration of either normal E2 or of a membrane-impermeable form of E2 (E2 covalently cross-linked to bovine serum albumin, E2-BSA) to HUVEC resulted in increased expression of ROCK-II and its expression was blocked by the ER antagonist (ICI 182 780). As a control, the ROCK-II inhibitor Y-27632 did not decrease significantly the expression of ROCK-II associated with E2 (Fig. 4A). Consistent with this result, E2 as well as E2-BSA induced PAI-1 expression through ROCK-II, as shown by the loss of effect in the presence of Y-27632 (Fig. 4B).

E2 regulates PAI-1 expression in vivo
To check whether estrogen also regulates PAI-1 in an in vivo setting, we administered E2 at different doses for 14 days to OVX rats, and compared the expression of PAI-1 in the abdominal aorta versus that of intact or of placebo-treated OVX rats. PAI-1 expression was present in the aorta of fertile rats and was decreased in OVX rats. OVX rats treated with oral E2 showed a dose-related increase of PAI-1 expression in the abdominal aorta (Fig. 5).

PAI-1 is required for E2-induced endothelial cell horizontal migration
Estrogen is a known controller of endothelial cell horizontal migration (Simoncini et al., 2006; Sanchez et al., 2011). Endothelial cell movement is controlled by estrogen through the regulation of a series of cytoskeletal and membrane components. To check whether migration induced by E2 also involves PAI-1, we silenced PAI-1 with siRNAs and performed horizontal migration assays. E2 (1 nM) significantly increased endothelial cell migration (Fig. 6). Silencing of PAI-1 blocked E2-induced HUVEC migration (Fig. 6).
Discussion

This paper identifies a direct regulation of PAI-1 expression by E2 in human endothelial cells, which is exerted through an extra-nuclear signaling to the early-immediate genes c-Jun and c-Fos.

Regulation of PAI-1 by estrogen has been suggested in a variety of clinical settings; however, it is unclear how this hormone directs PAI-1 expression in tissues. A recent paper indicates that in endothelial cells estrogen has a double-sided effect on PAI-1, triggering its expression through ER\alpha while repressing it through ER\beta (Smith et al., 2004). We here extend this concept showing that estrogen drives ER\alpha to enact rapid, extra-nuclear signaling involving G proteins and the Rho-A-associated kinase (ROCK-II). The cross talk between this cytoplasmic recruitment of ROCK-II and the subsequent activation of c-Jun and c-Fos expression mediates PAI-1 increase in endothelial cells. This proposed regulatory pathway is consistent with the idea of a prominent role of ER\alpha as positive regulator of PAI-1. Furthermore, it provides possible ways to selectively modulate PAI-1 in endothelial cells by either using preferential ER\alpha or ER\beta ligands, or by associating pharmacological tools that interfere with the rapid signaling of ER\alpha to ROCK-II. Such strategies may be helpful in conditions where estrogen-related induction of PAI-1 may either be desirable or undesirable.

Endocrine-sensitive cancers, such as breast or endometrial cancer, represent a straightforward situation where PAI-1 induction linked to estrogen may be unwanted (Manders et al., 2004; Steiner et al., 2008; Eljuga et al., 2011). Inactivation of ER\alpha-mediated signaling could turn into reduced ability to remodel the extracellular matrix and decreased cancer cell motility, thus possibly leading to less efficient cancer progression. If this hypothesis holds, our findings may suggest a role of endothelial cell-derived PAI-1 as a possible target of estrogen to support cancer progression. However, our unpublished data (Gopal et al., personal communication) as well as work by others (Burdette and Woodruff, 2007) suggest that estrogen also increases PAI-1 expression directly in breast cancer cells. This fits with the mounting evidence that sex steroids are effective enhancers of cancer cell motility and invasion through a multi-modal control of cytoskeletal remodeling, membrane shaping and, now, extracellular matrix turnover (Sanchez et al., 2010; Flamini et al., 2011).

It is less clear how our results fit with the large body of evidence that endogenous or exogenous estrogens decrease PAI-1 blood levels (Gebara et al., 1995; Koh et al., 1997). Our findings suggest that, contrary to what previously thought, estrogen-related decrease in circulating PAI-1 is not explained by an endothelial effect, but may...
rather rely on other phenomena, such as modified metabolic clearance or reduced production in other tissues (maybe because of local prevalent ERβ activity).

However, increased endothelial-derived PAI-1 may still represent a biologically important mean through which estrogens regulate vascular function. Indeed, this action may result in locally reduced fibrinolysis, which may contribute to the pro-thrombotic actions of estrogens in veins (Mendelsohn, 2002). On the other side, PAI-1-mediated extracellular matrix remodeling seems to add to the previously identified stimulatory effects of estrogens on endothelial cell motility (Simoncini et al., 2006), thus helping arterial re-endothelialization processes, and may thus affect the incidence of arterial events in women.

In conclusion, this paper provides new molecular cues on how estrogens control PAI-1 in endothelial cells. These findings may help to develop new therapeutic strategies to reduce unwanted side effects of estrogens in the cardiovascular system or on endocrine-sensitive cancers.

**Authors’ roles**

S.Go. and S.Ga. carried out the majority of the experiments and drafted the manuscript. L.G. performed endothelial cell extraction and cell culturing. K.P., G.P. and S.S. performed in vivo treatments in rats and extracted relevant tissues for later analysis. A.R.G. and A.D.G. were instrumental in funding the study and participated to the writing of the manuscript. T.S. planned and funded the project, supervised the experiments and wrote the paper.

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**Conflict of interest**

None declared.

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