Estradiol Stimulates Capillary Formation by Human Endothelial Progenitor Cells

Role of Estrogen Receptor-α/β, Heme Oxygenase 1, and Tyrosine Kinase

Isabella Baruscotti, Federica Barchiesi, Edwin K. Jackson, Bruno Imthurn, Ruth Stiller, Jai-Hyun Kim, Sara Schaufelberger, Marinella Rosselli, Christopher C.W. Hughes, Raghvendra K. Dubey

Abstract—Endothelial progenitor cells (EPCs) repair damaged endothelium and promote capillary formation, processes involving receptor tyrosine kinases (RTKs) and heme oxygenase 1 (HO-1). Because estradiol augments vascular repair, we hypothesize that estradiol increases EPC proliferation and capillary formation via RTK activation and induction of HO-1. Physiological concentrations of estradiol (10 nmol/L) increased EPC-induced capillary sprout and lumen formation in matrigel/fibrin/collagen systems. Propyl-pyrazole-triol (PPT; 100 nmol/L; estrogen receptor [ER]-α agonist), but not diarylpropionitrile (ER-β agonist), mimicked the stimulatory effects of estradiol on capillary formation, and methyl-piperidino-pyrazole (ER-α antagonist) abolished the effects of estradiol and PPT. Three different RTK activators (vascular endothelial growth factor, hepatocyte growth factor, and stromal derived growth factor 1) mimicked the capillary-stimulating effects of estradiol and PPT. SU5416 (RTK inhibitor) blocked the stimulatory effects of estradiol and PPT on capillary formation. Estradiol increased HO-1 expression by 2- to 3-fold, an effect blocked by SU5416, and PPT mimicked the effects of estradiol on HO-1. The ability of estradiol to enhance capillary formation, increase expression of HO-1, and augment phosphorylation of extracellular signal–regulated kinase 1/2, Akt, and vascular endothelial growth factor receptor 2 was mimicked by its cell-impermeable analog BSA estradiol. Actinomycin (transcription inhibitor) did not alter the effects of estradiol on RTK activity or vascular endothelial growth factor secretion. We conclude that estradiol via ER-α promotes EPC-mediated capillary formation by a mechanism that involves nongenomic activation of RTKs and HO-1 activation. Estradiol in particular and ER-α agonists in general may promote healing of injured vascular beds by promoting EPC activity leading to more rapid endothelial recovery and capillary formation after injury. **(*Hypertension*. 2010;56:397-404.)**

Key Words: hormone replacement therapy ■ estradiol ■ vascular remodeling ■ cardiovascular disease ■ estrogen receptors ■ endothelial progenitor cells

Evidence from epidemiological studies suggests that endogenous human estrogens and estrogen replacement therapy protect women from progression of cardiovascular disease.[1,2] Also, multiple animal studies and some small clinical trials support a cardioprotective action of estrogens.[1,2] In contrast, 2 large randomized clinical trials (Heart and Estrogen/Progestin Replacement Study and Women's Health Initiative) fail to demonstrate that exogenous estrogens protect against cardiovascular disease.[1–4] Although the reasons for these discordant findings remain unclear, a re-evaluation of data from the Heart and Estrogen/Progestin Replacement Study and Women's Health Initiative suggest that, in older participants with established cardiovascular pathology, estrogen replacement therapy is ineffective, whereas in younger healthy women estrogen therapy is protective.[3,4] Similarly, Hodis et al[5] report that estradiol inhibits age-associated increases in intimal thickening in women. These findings have generated a renewed interest in the mechanisms by which estrogens influence the cardiovascular system. To correctly interpret the results of completed and future clinical studies, it is critical to elucidate the mechanisms by which estrogens influence the vessel wall and to identify the independent variables that may influence the vascular actions of estrogens.

Although estrogens induce protective effects on the cardiovascular system via multiple mechanisms,[6] the effects of estrogens on endothelial cell growth and function may play important roles. For example, estradiol promotes endothelial

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cell growth, protects endothelial cells against damage by oxidants and cholesterol, and induces the generation of endothelial-derived vasodilators, such as NO and prostaglandins.[6–8] Because estradiol promotes endothelial function and because recent studies suggest that circulating bone marrow–derived endothelial progenitor cells (EPCs) contribute to tissue repair by inducing angiogenesis and neovasculogenesis,[9] we hypothesize that estradiol may promote endothelial repair in part by stimulating EPC-induced capillary formation.

Estradiol influences cellular growth and differentiation in a variety of tissues in part via estrogen receptors (ERs) α and β.[1,6] Within the cardiovascular system, both ER-α and ER-β mediate the protective actions of estradiol; however, these receptors do not necessarily engage common mechanisms. Indeed, ER-α mediates estradiol-induced NO release, inhibits vascular smooth muscle cell growth and lesion formation, and induces endothelial cell growth.[1,6–10] Arterial blood pressure is increased in ER-β knockout mice,[11] and in Japanese postmenopausal women a specific polymorphism in the ER-β gene is associated with hypertension,[12] suggesting that ER-β plays a critical role in lowering blood pressure. These findings provide evidence that ER-α and ER-β may perform distinct functions and that the endogenous regulation of the expression of these 2 ER subtypes may be important in defining the actions of estradiol on the vessel wall. Because ER-α regulates cell growth, we hypothesize that estradiol may activate EPC-induced capillary formation via ER-α.

Capillary formation (angiogenesis or neovascularization) is a dynamic process regulated by growth factors and signaling pathways. Activators of receptor tyrosine kinases (RTKs), such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and stromal derived growth factor-1 (SDF-1) induce EPCs to form capillaries.[13] Also, angiogenesis appears to depend on heme oxygenase 1 (HO-1), a 32-kDa stress-inducible enzyme that catalyzes the rate-limiting step in the degradation of heme, resulting in the liberation of iron, CO, and biliverdin.[13] Growth factors and cytokines induce HO-1 as an adaptive and beneficial response to tissue injury.[14] Proangiogenic factors, such as VEGF, increase HO-1 expression in endothelial cells in vitro, and inhibition of HO-1 blocks angiogenesis in vivo.[15] Because estradiol is known to induce VEGF synthesis and endothelial cell growth,[2,4,6] we hypothesize that estradiol may induce EPC-induced capillary formation via sequential activation of RTKs and HO-1.

The main purpose of the present study was to investigate whether estradiol promotes EPC-induced capillary formation and whether these effects are mediated via ER-α, ER-β, or both. Because EPC-induced capillary formation likely is regulated by sequential activation of RTKs and HO-1, a second aim of our study was to investigate their roles in mediating the stimulatory effects of estradiol on capillary formation.

Methods

Isolation and Culture of EPCs

EPCs were isolated, cultured, and characterized as described previously.[9] For detailed Materials and Methods, please see the expanded data in the online Data Supplement and Table S1, available online at http://hyper.ahajournals.org.

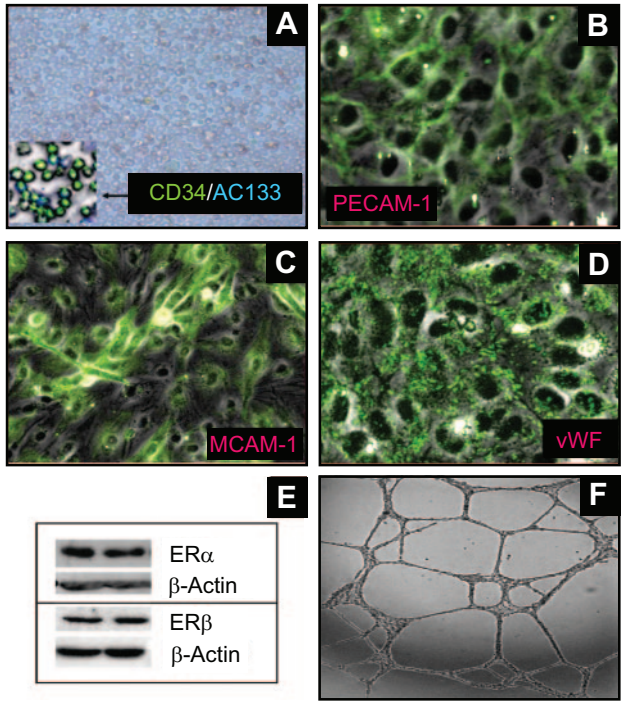

Figure 1. Representative photomicrographs and Western blot showing that circulating CD34$^+$ progenitor cells can transform into endothelial cells and express ERs. A, CD34$^+$ cells isolated after antibody-based magnetic separation. The inset depicts the purity of the cells stained with CD34 and AC133 antibodies. B through D, Progenitor cell–derived endothelial cells express the endothelial cell markers: platelet endothelial cell adhesion molecule 1 (PECAM-1; B); melanoma cell adhesion molecule 1 (MCAM-1; C), and von Willebrand factor (vWF; D). E, Representative Western blots demonstrating that EPCs express both ERs α and β. Photomicrograph in F depicts the functional capability of EPCs to form capillaries in response to serum.

Capillary Formation Studies

Well-established gel (matrigel, collagen, and Cytodex-3 beads in fibrin)-based assays were used to study capillary, lumen, and sprout formation by EPC-derived endothelial cells. Capillary, lumen, and sprout formations were assessed microscopically by measuring the length of capillaries or counting lumens and sprout formations. For details see the expanded methods in the online Data Supplement at http://hyper.ahajournals.org.

Protein Expression Studies

Western blotting, ELISA assays, and flow cytometry were used to assess the role of various proteins in mediating the angiogenic effects of estradiol on EPCs. For details see the expanded methods in the online Data Supplement at http://hyper.ahajournals.org.

Statistics

Data were analyzed by ANOVA and statistical significance ($P<0.05$) calculated using the Fisher least significant difference test.

Results

Cell Characterization

A homogeneous population of CD34$^+$ cells was isolated after antibody-based magnetic separation (Figure 1A). The purity of EPCs was also confirmed by staining with AC133, a marker for stem cell glycoprotein selectively expressed in CD34$^+$ progenitor cells (Figure 1A). To assess whether progenitor cell–derived endothelial cells were phenotypically

similar to endothelial cells, the cells were immunostained for endothelial cell-specific markers. As shown in the photomicrographs (Figure 1B through 1D), EPCs stained positive for von Willebrand factor, platelet endothelial cell adhesion molecule 1, and melanoma cell adhesion molecule 1. Human aortic smooth muscle cells were used as negative controls and did not show any positive staining. To assess whether EPCs express ERs, EPC lysates were analyzed by Western blotting. Both ER-α and ER-β were highly expressed in EPCs (Figure 1E). To assess whether EPCs were able to form capillaries on matrigel, EPCs were plated on matrigel-coated slides and treated with 10% steroid-free serum. Treatment with serum resulted in robust capillary formation by EPCs (Figure 1F).

Capillary Formation

Treatment of EPCs with estradiol increased capillary formation from 168±16 μm in vehicle-treated control cells to 487±24 μm in cells treated with 10 nmol/L of estradiol ($P<0.05$). The stimulatory effects of estradiol on capillary formation were mimicked by the ER-α agonist propyl-pyrazole-triol (PPT; 100 nmol/L), which stimulated capillary formation by 330±23% ($P<0.05$ versus untreated control; Figure 2A). In contrast to estradiol and PPT, treatment of EPCs with 100 nmol/L of the ER-β agonist diarylpropionitrile (DPN) failed to induce capillary formation (Figure 2A). The stimulatory effects of estradiol on capillary formation were abrogated in EPCs cotreated with the ER-α antagonist methyl-piperidino-pyrazole (MPP; 1 μmol/L). MPP also blocked the stimulatory effects of the ER-α agonist PPT on capillary formation ($P<0.05$ versus EPCs treated with estradiol or PPT alone; Figure 2A). Similar to increasing capillary length, estradiol induced capillary junction formation via ER-α (please see Figure S1 available in the online Data Supplement at http://hyper.ahajournals.org). Moreover, estradiol promoted lumen (Figure 2B) and sprout (Figure 2C) formation in EPCs cultured for 24 hours in collagen gels or Cytodex beads for 7 days in fibrin gels, respectively.

Similar to estradiol and PPT, treatment of EPCs with 100 ng/mL of VEGF, HGF, or SDF-1 stimulated EPC-induced capillary formation (Figure 3A). The stimulatory effects of estradiol on lumen and sprout formation were also mimicked by VEGF and phorbol myristate acetate (Figure 2B and 2C). The efficacies of the RTK activators on capillary formation were comparable to those of estradiol and PPT. Also, the stimulatory effects of VEGF, HGF, and SDF-1 on capillary formation were abolished when EPCs were cotreated with 5 μmol/L of the RTK inhibitor SU5416 (Figure 3A). Treatment of EPCs with SU5416 also abrogated the stimulatory effects of estradiol and PPT on EPC-induced capillary formation by 93% ($P<0.05$ versus EPCs treated with estradiol or PPT alone; Figure 3B). Similar to the effects on capillary length, estradiol and PPT induced junction formation, and these effects were mimicked by RTK activators and blocked by SU5416 (please see Figure S2).

HO-1 Expression

As shown in Figure 4A, treatment of EPCs with 10 nmol/L of estradiol induced HO-1 expression by 2-fold (from 100.0±0.3% to 228.0±7.8%; $P<0.05$ versus untreated control). Treatment of EPCs with PPT, but not DPN, mimicked the stimulatory effects of estradiol on HO-1 expression (Figure 4A). Treatment with PPT induced HO-1 expression by ≈280% ($P<0.05$ versus control). The ER-α antagonist MPP abolished the stimulatory effects of estradiol and PPT on HO-1 expression (Figure 4B). Moreover, the stimulatory effects of estradiol and PPT on HO-1 expression were also abrogated by the RKT inhibitor SU5416 ($P<0.05$ versus EPCs treated with estradiol or PPT; Figure 5A).

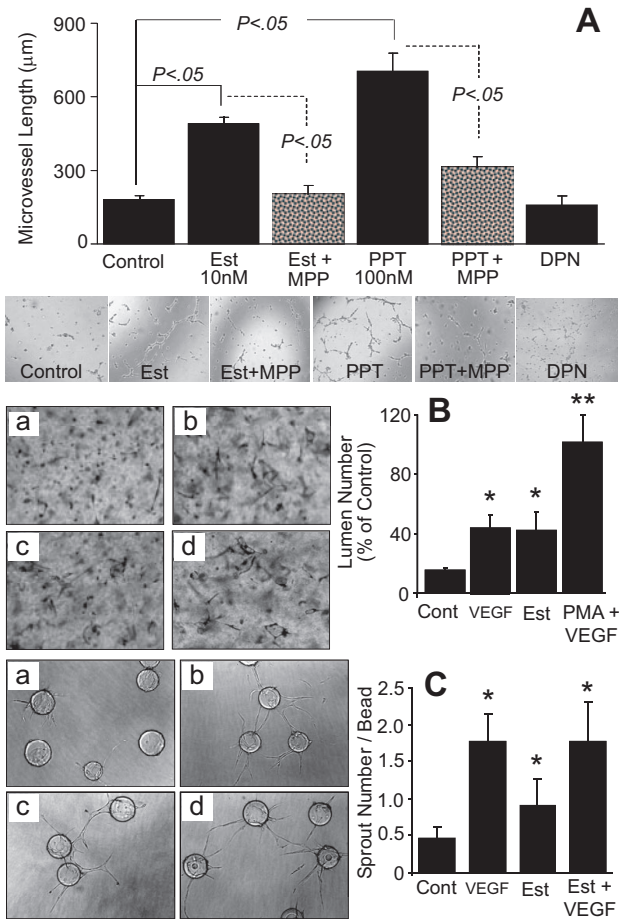

Figure 2. A, Bar graph and representative photomicrographs showing the effects of estradiol (Est; 10 nmol/L), PPT (ER-α agonist; 100 nmol/L), and DPN (100 nmol/L) on capillary formation by EPCs in the presence and absence of the ER-α antagonist MPP (1 μmol/L). Serum-starved EPCs were plated at a density of 50 000 cells per 200 μL per well on matrigel-coated 8-well multichamber slides. After 4 hours the capillary formation was assessed by microscopically measuring the length of capillaries at 5 random locations (×4 magnification). Values represent mean±SEM. B, EPC ECs were seeded into collagen gels (3.75 mg/mL) in EGM2 medium and incubated for 24 hours as follows: (a) control (Cont); (b) VEGF (40 ng/mL); (c) estradiol (Est; 10 nmol/L); or (d) positive control (phorbol myristate acetate [PMA; 50 ng/mL]+VEGF). Cultures were subsequently fixed in 2% paraformaldehyde, stained with toluidine blue, and photographed. Shown are mean number of lumens and SD; *$P<0.05$ and **$P<0.01$ relative to control, by Student t test. C, EPC-EC–coated Cytodex3 beads in fibrin gels were cultured in EGM2 medium as follows: (a) without VEGF; (b) with VEGF (15 ng/mL); (c) with estradiol (Est; 10 nmol/L); or (d) with VEGF+estradiol (Est). Gels were photographed and the number of sprouts counted after 7 days. Shown are mean and SD; *$P<0.05$, **$P<0.01$ relative to control, by Student t test.

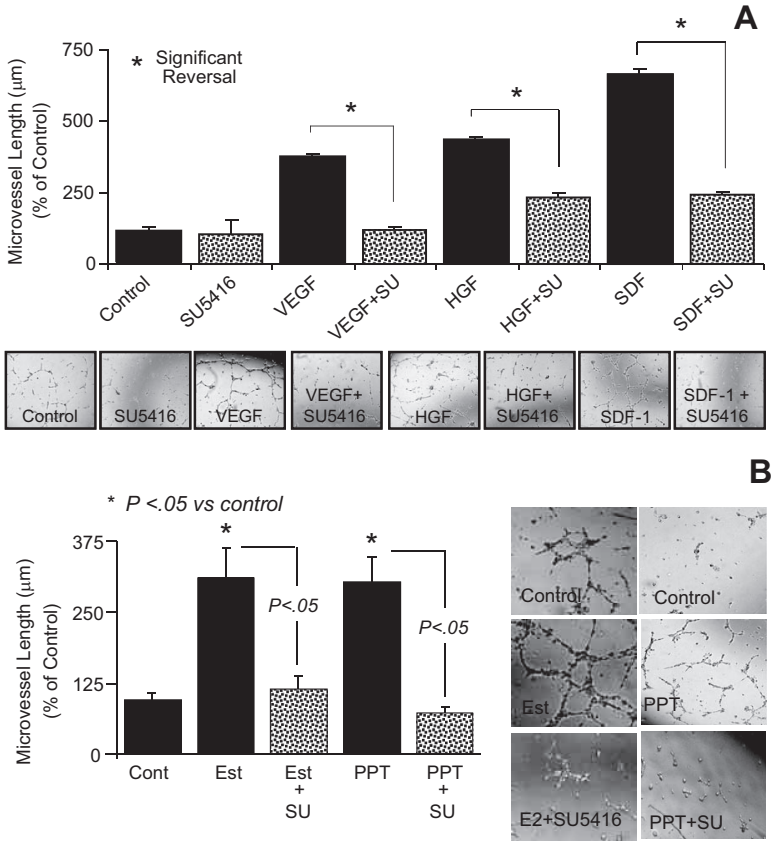

Figure 3. A, Bar graph and representative photomicrographs showing the effects of RTK stimulators VEGF (100 ng/mL), HGF (100 ng/mL), and SDF-1 (100 ng/mL) on capillary formation by EPCs in the presence and absence of the tyrosine kinase inhibitor SU5416 (SU; 5 μmol/L). Serum-starved EPCs were plated at a density of 50 000 cells per 200 μL per well on matrigel-coated 8-well multichamber slides. After 4 hours the capillary formation was assessed by microscopically measuring the length of capillaries at 5 random locations (×4 magnification). B, Bar graph and representative photomicrographs showing the modulatory effects of the tyrosine kinase inhibitor SU5416 (SU; 5μmol/L) on estradiol (Est; 10 nmol/L) and PPT (100 nmol/L)-induced capillary formation in EPCs. Values represent mean±SEM; *$P<0.05$ vs vehicle-treated controls.

Extracellular Signal–Regulated Kinase 1/2 and Akt Phosphorylation

Treatment of EPCs with estradiol (10 nmol/L) significantly upregulated the expression of phosphorylated extracellular signal–regulated kinase (ERK) 1/2 and Akt (Figure 5B). Moreover, these stimulatory effects were abolished by the RTK inhibitor SU5416 (Figure 5B). Treatment with the ERK1/2 pathway inhibitor PD98059 blocked the stimulatory effects of estradiol and PPT on capillary formation (Figure 5C, top). Similarly, the Akt pathway inhibitor LY 294002 blocked estradiol- and PPT-induced capillary formation (Figure 5C, bottom). Both ERK1/2 and Akt pathway inhibitors also blocked the stimulatory effects of estradiol on capillary junction formation (please see Figure S3).

Nongenomic Actions on Capillary Formation

In EPCs treated with fluorescein isothiocyanate–labeled BSA-tagged estradiol, nuclear staining was observed in permeabilized but not intact cells (Figure 6A). Similar to estradiol, treatment of EPCs with impermeable BSA-estradiol (10 nmol/L) significantly induced capillary formation (Figure 6B), and these effects were blocked by ICI182780 (ER-α/β antagonist), MPP (ER-α antagonist), SU5416 (RTK inhibitor), PD98059 (ERK1/2 inhibitor), and LY294002 (Akt pathway inhibitor). Similar stimulatory effects of BSA-estradiol were also observed on capillary junction formation (please see Figure S4). Moreover, like estradiol, treatment with impermeable BSA-estradiol upregulated the expression of HO-1, as well as phosphorylated ERK1/2 and Akt (Figure 6C).

Tyrosine Kinase Activation

Treatment of EPCs with estradiol (10 nmol/L) for 10 minutes significantly induced tyrosine kinase activity, and these effects were not blocked by 100 nmol/L of actinomycin (Figure 6D). Treatment of EPCs with estradiol (10 nmol/L) did not induce VEGF receptor (VEGFR) 2 expression (Figure 6E); however, the levels of phospho–VEGFR-2 were significantly induced in lysates of EPCs treated for 10 minutes with estradiol or its impermeable analog BSA-estradiol (Figure 6F), and these effects of estradiol were not blocked by actinomycin (Figure 6F). Treatment of EPCs with estradiol for 8 hours significantly induced VEGF production, and this effect also was not blocked by actinomycin (Figure 6G). Estradiol did not enhance the stimulatory effects of VEGF on tube formation (Figure 6H).

Discussion

This study demonstrates several important findings in human circulating EPCs: (1) estradiol stimulates capillary formation by EPCs; (2) the capillary-stimulating effects of estradiol are mimicked by a specific agonist for ER-α (PPT) but not for ER-β (DPN); (3) MPP (ER-α–specific antagonist) blocks the stimulatory effects of estradiol and PPT on capillary formation; (4) the stimulatory effects of estradiol and PPT on capillary formation are mimicked by RTK activators (VEGF, HGF, and SDF-1) and blocked by the RTK inhibitor SU5416; (5) expression of the well-known angiogenesis-stimulating enzyme HO-1 is induced by estradiol and by the ER-α agonist PPT but not by the ER-β agonist DPN; (6) the stimulatory effects of estradiol and PPT on HO-1 expression

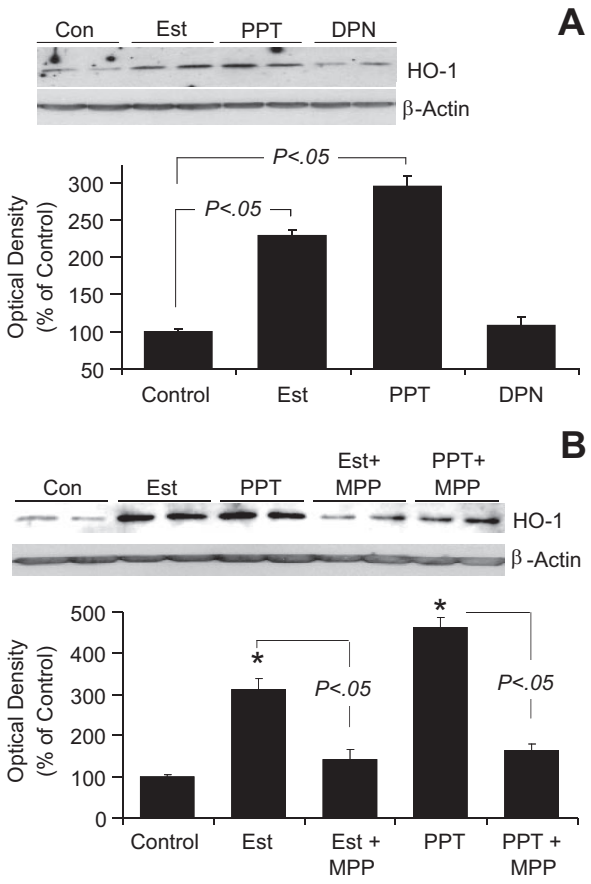

Figure 4. Bar graph and representative Western blots demonstrating that estradiol induces HO-1 expression in EPCs via ER-α. A, Effects of estradiol (Est; 10 nmol/L), PPT (ER-α agonist; 100 nmol/L), and DPN (ER-β agonist; 100 nmol/L) on HO-1 expression in EPCs treated for 4 hours. B, Effects of Est (10 nmol/L) and PPT (100 nmol/L) on HO-1 expression in EPCs in the presence and absence of the ER-α antagonist MPP (1 μmol/L). Cells were pretreated for 15 minutes with MPP, and subsequently estradiol or PPT was added for an additional 4 hours. Values represent mean±SEM.

are blocked by the ER-α antagonist MPP, as well as by the RTK inhibitor SU5416; (7) treatment with estradiol induces Akt and ERK1/2 phosphorylation, and inhibition of the ERK1/2 and Akt pathways by PD98059 and LY294002, respectively, abrogates the capillary stimulatory effects of both estradiol and PPT; and (8) estradiol stimulates tyrosine kinase activity and phosphorylation of the RTK VEGFR-2. Together, our findings provide strong evidence that estradiol promotes EPC-induced capillary formation, that these effects are ER-α mediated, and that the stimulatory effects of estradiol and PPT are mediated via activation of RTKs leading to induction of HO-1 with involvement of the Akt and ERK1/2 signal transduction pathways. Importantly, our findings that the stimulatory effects of estradiol on tube formation, HO-1 expression, and phosphorylation of Akt, ERK1/2, and VEGFR-2 are mimicked by the membrane-impermeable BSA-estradiol and that the stimulatory effects of estradiol on tyrosine kinase activity and VEGF production are not blocked by actinomycin indicate that estradiol induces tube formation via a nongenomic mechanism involving membrane ERs.

Some of the actions of estradiol would be expected to improve vascular health. For example, estradiol induces growth of endothelial cells and speeds the recovery of denuded endothelium,[4,7] thus enabling the vessel wall to recover more rapidly after injury. Alternative sources of endothelial cells, for example, bone marrow–derived circulating EPCs,[9] may also participate in vascular repair, and studies show that estradiol promotes EPC growth and recruits EPCs to sites of injury. The present study shows for the first time that estradiol actually activates EPC-induced capillary formation. Our finding that estradiol promotes EPC-induced capillary formation suggests that estradiol may facilitate tissue repair in part via this mechanism.

The biological effects of estradiol are largely mediated via ER-α and ER-β.[1,6] Our finding that the stimulatory effect of estradiol on capillary formation is mimicked by the ER-α agonist PPT, but not by the ER-β agonist DPN, provides evidence that the effects are indeed ER-α mediated. It is important to note that, because selective ER modulators are being developed for safer and more effective hormone replacement therapy in postmenopausal women, the use of PPT as a selective ER modulator to promote endothelial and tissue recovery may be of therapeutic importance.[16] This concept is further supported by the facts that, via ER-α, estradiol inhibits injury-induced neointima formation,[10] induces NO release, and prevents bone loss and hot flushes.[16]

The mechanisms involved in EPC-induced capillary formation or angiogenesis remain unclear. However, it is well established that activation of the RTK pathway by ligands such as VEGF and HGF enhances EPC-induced capillary formation.[17] Our findings that RTK activators mimic the effects of estradiol on capillary formation and that the RTK inhibitor SU5416 blocks the effects of estradiol and PPT on capillary formation suggest that the stimulatory effects of estradiol on capillary formation are mediated by activation of RTKs. Also, our observations that estradiol induces ERK1/2 and Akt phosphorylation and that the Akt pathway inhibitor LY294002 and the ERK1/2 pathway inhibitor PD98059 attenuate the stimulatory effects of estradiol and PPT on capillary formation suggest that, via ER-α, estradiol can stimulate capillary formation via activation of these signal transduction mechanisms. It is likely that the Akt and ERK1/2 pathways are downstream of RTK activation because it is well known that RTKs activate these classic signal transduction pathways.[17]

Previous studies implicate a role for HO-1 in angiogenesis.[13] Pharmacological or genetic manipulations that increase HO-1 expression enhance proliferation and tube formation in human microvascular endothelial cells in vitro,[18] whereas inhibition of HO-1 decreases tube formation, a phenomenon independent of HO-2.[18] RTKs and the Akt and ERK1/2 signal transduction pathways activate HO-1,[19] and HO-1 mediates their effects on angiogenesis.[17] Our finding that estradiol and the ER-α agonist PPT, but not the ER-β agonist DPN, induce HO-1 expression links the proangiogenic effects of estradiol to HO-1. This notion is further supported by the fact that the stimulatory effects of estradiol and PPT on both capillary formation and HO-1 expression are blocked by the ER-α antagonist MPP and by the RTK inhibitor SU5416. Although

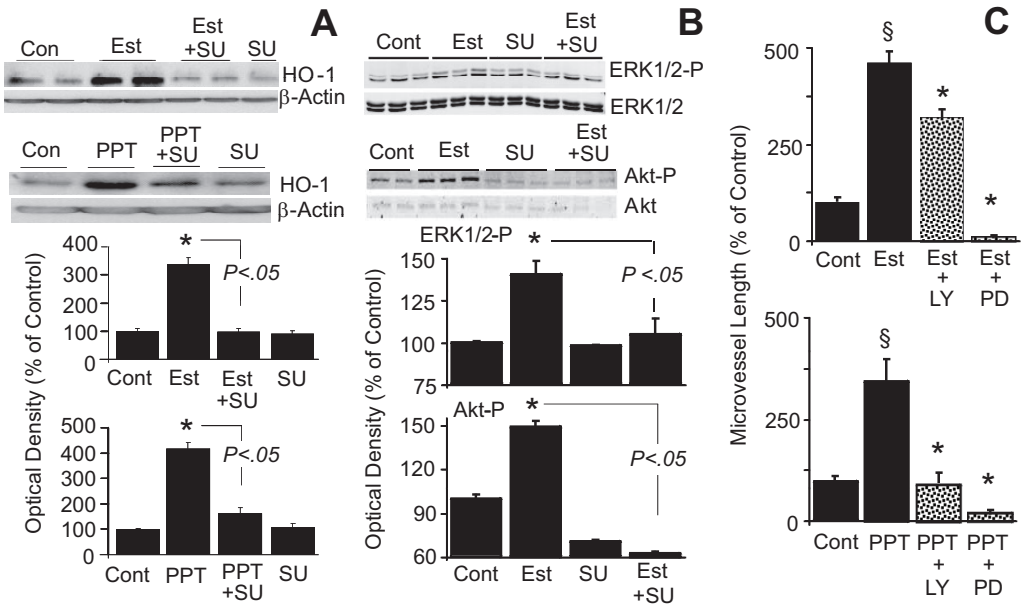

Figure 5. A, Bar graph and representative Western blots demonstrating that inhibition of tyrosine kinase with SU5416 (SU) abrogates the stimulatory effects of estradiol and PPT on HO-1 expression in EPCs. EPCs were pretreated with SU (5 μmol/L) for 15 minutes, and subsequently estradiol (Est; 10 nmol/L) or PPT (100 nmol/L) was added for an additional 4 hours. HO-1 expression was assayed in the lysates with β-actin as a control. Bar graph depicts the densitometry analysis of the HO-1 bands, which were normalized to β-actin. **P*<0.05 vs vehicle-treated control. B, Modulatory effects of SU5416 (SU) on estradiol-induced ERK1/2 and Akt phosphorylation in EPCs. Cells were pretreated with SU for 15 minutes, and subsequently estradiol (100 nmol/L) was added for an additional 10 minutes. Cell lysates were prepared and expression of phosphorylated ERK1/2 and Akt was analyzed by Western blotting. For normalization, nonphosphorylated ERK1/2 and Akt were used. Bar graph depicts the densitometry analysis of the phosphorylated ERK1/2 (ERK1/2-P) and Akt (Akt-P) bands, which were normalized to ERK1/2 and Akt, respectively. **P*<0.05 vs vehicle-treated control. Values represent mean±SEM. C, Bar graph showing the inhibitory effects of LY294002 (Akt inhibitor; LY; 10 μmol/L) and PD98059 (ERK1/2-P inhibitor; PD; 10 μmol/L) on estradiol (Est; 10 nmol/L) and PPT (100 nmol/L)-induced capillary formation by EPCs. EPCs were pretreated for 15 minutes with either LY or PD, and subsequently Est or PPT was added for another 4 hours and capillary formation analyzed microscopically at ×4 magnification. **P*<0.05 vs estradiol or PPT alone.

our findings implicate HO-1 as a likely intermediate in estradiol-induced capillary formation, the mediators downstream from HO-1 remain unknown. In this regard, detailed studies are required to determine whether HO-1–derived CO or another molecule, such as vasodilator-stimulated phosphoprotein, which is known to stimulate capillary formation by EPCs,[13,17] is involved in transducing the angiogenic effects of estradiol.

Estradiol induces multiple vascular actions via nongenomic mechanisms[20] and increases VEGF synthesis and mobilizes the RTK VEGFR-2.[21] Our finding that the effects of estradiol are mimicked by its impermeable analog BSA-estradiol suggests the participation of a nongenomic mechanism in estradiol-induced tube formation. The observations that estradiol induces RTK, that these effects are not blocked by actinomycin, and that the membrane-impermeable BSA-estradiol induces VEGFR-2 phosphorylation suggest that estradiol can activate this key pathway responsible for capillary induction. Because the effect of estradiol to increase VEGF secretion is not affected by actinomycin, it is likely that estradiol stimulates the release of VEGF from intracellular stores via a nongenomic mechanism.[22] Based on the above findings it is feasible that estradiol induces capillary formation by stimulating the release of VEGF, which, in turn, activates RTKs, ERK1/2, Akt, and HO-1. Although our findings suggest that estradiol can stimulate capillary formation via a nongenomic mechanism, the participation of nuclear receptors or genomic activation cannot be ruled out and requires further investigated. Because the TRK family constitutes >58 receptors, it is feasible that receptors other than VEGFR-2 also participate in mediating estradiol's tube-forming effects.

Our observation that estradiol stimulates capillary formation via a nongenomic mechanism suggests that estradiol may repair endothelial damage within vascular beds. Consistent with this concept, in vivo studies provide evidence that estradiol promotes accumulation of progenitor endothelial cells at sites of injury.[23] Our finding that estradiol promotes capillary formation within hours would imply that estradiol could facilitate tissue repair by re-establishing capillary formation to improve perfusion within the damaged vascular sites.

In summary, the present study provides evidence that estradiol induces capillary formation by human EPCs, and these effects are ER-α mediated. The stimulatory effects of estradiol are likely attributed to RTK-mediated induction of HO-1. The Akt and ERK1/2 signal transduction pathways are also involved, perhaps as intermediates between RTK activation and HO-1 induction.

Perspectives

Here we provide strong evidence that estradiol promotes EPC-mediated capillary formation via ER-α and that the stimulatory actions of estradiol are mediated via RTK and

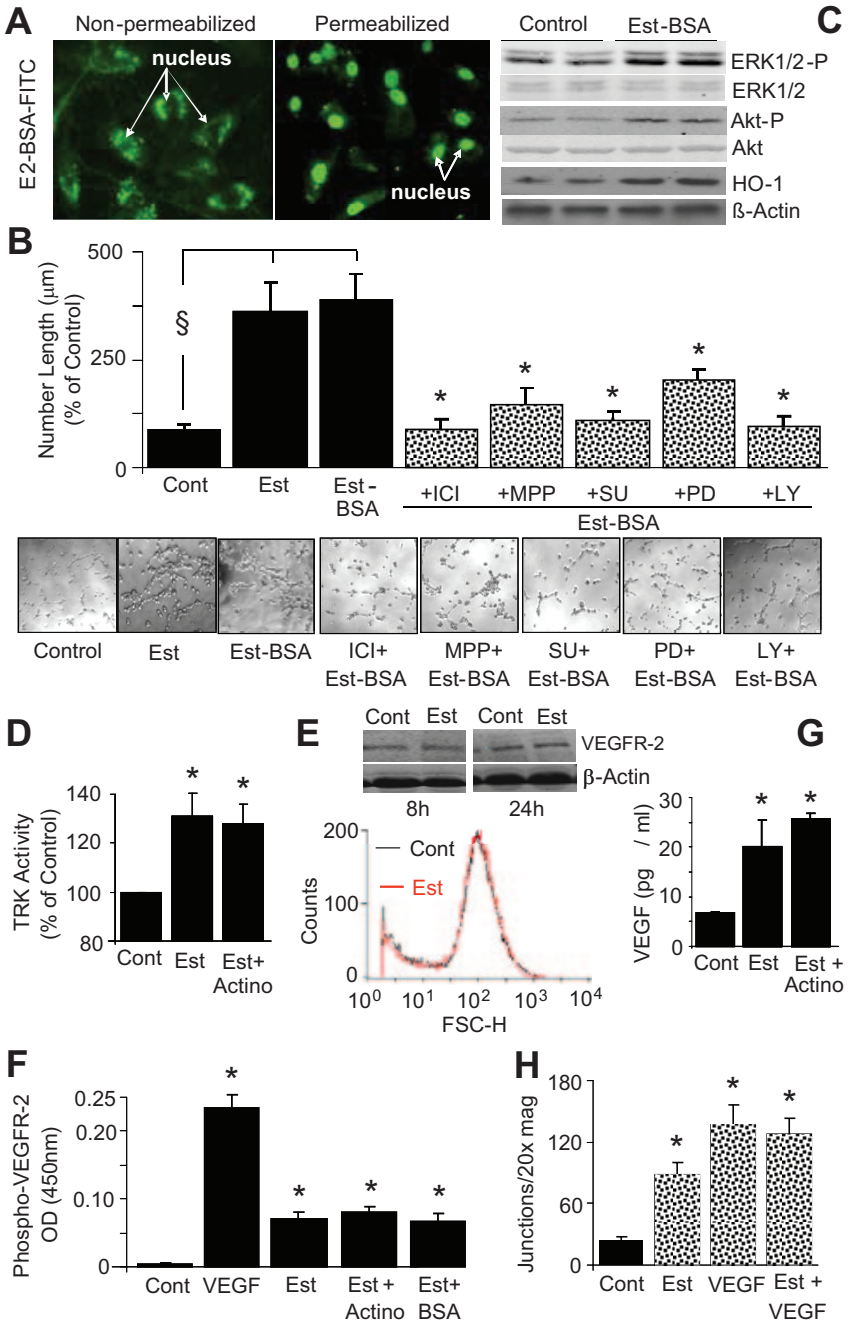

Figure 6. A, Representative photomicrographs providing evidence that BSA-estradiol is impermeable and stains the nucleus of permeabilized, but not intact, EPCs (original magnification, ×20). B, Bar graph and representative photomicrographs showing the modulatory effects of RTK inhibitor SU5416 (SU; 5 μmol/L), LY294002 (Akt pathway inhibitor; LY; 10 μmol/L), PD98059 (ERK1/2 pathway inhibitor; PD; 10 μmol/L), ICI182780 (non-specific ER antagonist, ICI; 1 μmol/L), and MPP (ER-α antagonist; 1 μmol/L) on BSA-estradiol (Est-BSA; 10 nmol/L)–induced capillary formation in EPCs. Serum starved EPCs were plated at a density of 50 000 cells per 200 μL per well on matrigel-coated 8-well multichamber slides. Cells were pretreated for 15 minutes with ICI, MPP, SU, LY, or PD, and subsequently BSA-estradiol was added for an additional 4 hours and capillary formation was assessed by microscopically measuring the length of capillaries at 5 random locations (×4 magnification). Values represent mean±SEM; §$P<0.05$ vs vehicle-treated controls; *$P<0.05$ vs Est-BSA. C, Representative Western blots demonstrating that BSA-estradiol (Est-BSA) induces the expression of HO-1, phosphorylated ERK1/2, and Akt. Cells were treated with Est-BSA (10 nmol/L) for 4 hours or 10 minutes, lysates prepared, and the expression of HO-1, phosphorylated ERK1/2 and Akt analyzed using Western blots. β-Actin, non-phosphorylated Akt, and ERK1/2 were used for normalization. D, Bar graph showing the stimulatory effects of estradiol on tyrosine kinase activity in lysates prepared from EPCs treated for 10 minutes with estradiol (Est; 10 nmol/L) and estradiol+actinomycin (Actino; 100 nmol/L). *$P<0.05$ vs vehicle-treated control (Cont). Values represent mean±SEM. E, Representative flow cytometry histogram and Western blots showing the effects of estradiol on VEGFR-2 expression in EPCs treated for 8 and 24 hours. F, Bar graph showing the stimulatory effects of VEGF (100 ng/mL), 10 nmol/L of estradiol (Est), estradiol plus actinomycin (100 nmol/L), and BSA-estradiol (Est-BSA) on phosphorylated VEGFR-2 in lysates prepared from EPCs treated for 10 minutes. *$P<0.05$ vs vehicle-treated control (Cont). Values represent mean±SEM. G, Bar graph showing the extracellular levels of VEGF in EPCs treated for 8 hours with estradiol (10 nmol/L) in the presence and absence of actinomycin (100 nmol/L). *$P<0.05$ vs vehicle-treated control (Cont). Values represent mean±SEM. H, Bar graph showing the capillary-forming effects of VEGF (10 ng/mL) in the presence and absence of estradiol (10 nmol/L). Values represent mean±SEM. *$P<0.05$ vs vehicle-treated control (Cont).

HO-1 activation. Because HO-1 induction occurs as an adaptive and beneficial response to tissue injury, estradiol in particular and ER-α agonists in general may protect the vascular system by promoting EPC activity, leading to more rapid endothelial recovery and capillary formation after injury. Apart from repairing the vascular endothelium, estradiol-induced capillary formation would also facilitate tissue repair in the heart after myocardial infarction by re-establishing capillaries and blood supply.

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Disclosures

None.

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Estradiol Stimulates Capillary Formation by Human Endothelial Progenitor Cells: Role of ER-α/β, Heme Oxygenase-1 and Tyrosine Kinase

Supplementary Materials and Methods

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Running Title: Estradiol promotes EPC angiogenesis via ER-α

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Isolation and Culture of Endothelial Progenitor Cells (EPCs): EPCs were isolated and cultured as previously described[1]. Sample collection for the study was approved by the institutional ethics commission at University Hospital Zurich and written consent was obtained prior to blood collection. Briefly, peripheral blood (10ml) was collected in heparinized tubes from women undergoing in vitro fertilization treatment. White blood cells were isolated by gradient centrifugation using Biocoll (1.077 g/ml; Biochrom AG, Germany) and washed twice with buffer (2 mM EDTA in phosphate-buffered saline). CD34 positive cells were selectively separated using magnetic beads coated with CD34 antibodies. The purity of the isolated CD34 positive cells was confirmed by staining with antibodies to AC133, a marker for stem cell glycoprotein that is selectively expressed in CD34 positive progenitor cells. CD34 positive cells were resuspended in phenol red-free endothelial basal medium (Lonza Group Ltd, Switzerland) supplemented with EGM-2 SingleQuot Kit (Lonza Group Ltd, Switzerland) and 20% fetal calf serum, plated at a density of $2.5x10^6$ cells/well in 24-well fibronectin-coated plates (BD Biosciences, USA) and cultured under standard tissue culture conditions. Following 4-6 weeks in culture, EPCs were plated in 75 cm^2 flasks, and upon confluency cells were characterized for specific endothelial cell markers (von Willebrand factor, PECAM-1 and MCAM-1) using endothelial cell characterization kit (Chemicon–Millipore, Billerica, US) and re-plated at split ratios of 1:4 **(for antibody details see Table-1 below).**

Capillary Formation Studies: To assess EPC-induced microvessel formation, EPCs in 3rd passage were serum starved overnight in DMEM-F12, phenol red-free medium (Gibco, Invitrogen Corporation, USA) supplemented with 0.4% steroid-free serum. Subsequently, 200 µl aliquots containing 50,000 cells and the various experimental agents were layered on 8-well chamber slides coated with matrigel (BD Biosciences, USA) and incubated under standard tissue culture condition.[2,3] After 4 hours, microvessel formation was analyzed using an Olympus inverted microscope (4x magnification) and photomicrographs. The capillary length was randomly measured at 10 separate locations and the average compared to the untreated control. DMSO at a final concentration of 0.1% was used as a vehicle-treated control.

Lumen and Sprout Formation: To investigate whether estradiol induces lumen and sprout formation in EPC derived endothelial cells we utilized collagen gel and Cytodex3 beads in fibrin gels, respectively and as previously described[4,5,6]. Briefly, for lumen formation studies, EPC-EC were seeded into collagen gels (3.75mg/ml) in EGM2 medium and incubated for 24-hrs as follows: (a) control; (b) VEGF (40ng/ml); (c) estradiol (E2,10nmol/L); or (d), positive control (phorbol myristate actetate [PMA;50ng/ml] + VEGF). Cultures were subsequently fixed in 2%paraformaldehyde, stained with toluidine blue, photographed and the number of lumens counted microscopically (original magnification 100x). For sprout formation assays, EPC-EC-coated Cytodex3 beads in fibrin gels were cultured in EGM2 medium as follows: (a) without VEGF; (b) with VEGF (15ng/ml); (c) with estradiol (E2,10nmol/L); or (d) with VEGF+E2. After 7 days the gels were photographed and the number of sprouts counted under a microscope (original magnification 100x).

Protein Expression Studies: Western blotting was employed to assess the role of various proteins in mediating the angiogenic effects of estradiol on EPCs. Briefly, EPCs in 3rd to 5th passages were grown to sub-confluence in 35mm^2 culture dishes and were serum starved for 12

hours. Subsequently, EPCs were treated with different agents for either 15 minutes for HO-1 expression studies, 10 minutes for tyrosine kinase receptor (TRK) activity and phosphorylated vascular endothelial growth factor recetor-2 (p-VEGFR-2), 30 minutes for phosphorylted Akt (Akt-P) and phosphorylated ERK (ERK-P), or 8 and 24 hours for VEGFR-2 . Cells were washed with ice cold buffer and lysed in 50 μl of lysis buffer (Cell signalling Technology Inc., USA). Protein was assayed using the BCA protein assay kit (Pierce, USA), and the expressions of ERs, HO-1, Akt-P, ERK-P and VEGFR-2 were analyzed by Western blotting. TRK activity and phosphorylated-VEGFR-2 in cell lysates were measured using Antibody Beacon tyrosine kinase assay kit (Molecular Probes, Eugene, OR) and DuoSet sandwich ELISA kit (Abingdon, OX14 3NB, UK), respectively. VEGFR-2 expression on EPCs was also analyzed by flow cytometery and Western blotting. VEGF levels in supernatants of EPCs treated with estradiol for 8 hours were assayed using ELISA kit (Invitrogen Corp., Camarillo, CA, USA).

Statistics: Data were analyzed by analysis of variance, and statistical significance ($p<0.05$) calculated using Fisher's LSD test.

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Table S1: List of antibodies used for EPC and endothelial cell characterization.

Antibody	Company	Cat #	Concentration	Dilution used
Anti-HO-1	Abcam plc, Cambridge, UK	Cat # ab13248	1 mg/ml	4 μg/ml
IRDye 680 conjugated Goat Anti-Mouse IgG	Li-Cor Biosciences, Lincoin, US	Cat # 926-32220	1 mg/ml	1:10000
Anti-β-actin	Sigma-Aldrich Chemie GmbH, Buchs, CH	Cat # A-5441		1:5000
Anti-ERα	Alexis Biochemicals	Cat #210-201-C050	1 μg/μl	1:1000
Anti-ERβ	Alexis Biochemicals	Cat #210-180-C050	1 μg/μl	1:1000
IRDye 680 conjugated Goat Anti-Rabbit IgG	Li-Cor Biosciences, Lincoin, US	Cat # 926-32221	1 mg/ml	1:10000
Anti-AC133-APC	Miltenyi Biotec, Bergisch Gladbach, D	Cat # 130-090-826	100 μg/ml	1:10
Anti-CD34-FITC	Miltenyi Biotec, Bergisch Gladbach, D	Cat # 130-081-001		1:10
Endothelial cell characterization kit	Chemicon (Millipore), Billerica, US	Cat # SCR023	CD146: 1 mg/ml VWF: 1 mg/ml CD31: 0.1 mg/ml	CD146 1:500 vWF 1:5000 CD31 1:50
Anti-Mouse IgG – FITC	Sigma-Aldrich Chemie GmbH, Buchs, CH	Cat # F-2012		1:64
Anti-Rabbit IgG – FITC	Sigma-Aldrich Chemie GmbH, Buchs, CH	Cat # F-0382		1:80
Anti-Phospho-Akt (Thr 308)	Cell Signaling Technology Inc.,Beverly, US	Cat # 9275		1:1000
Anti-Akt	Cell Signaling Technology Inc., Beverly, US	Cat # 9272		1:1000
Anti-phospho-MAP Kinase 1/2 (Erk1/2)	Upstate Biotechnology, Lake Placid, US	Cat # 07-467	0.28 mg/ml	1:1000
Anti-MAP Kinase/Erk1/2-CT	Upstate Biotechnology, Lake Placid,US	Cat # 06-182	0.55 mg/ml	1:1000
Anti VEGFR-2	Calbiochem, Merck KgaA, Darmstadt, D	Cat # 676486	1 mg/ml	1:1000

Figure S1

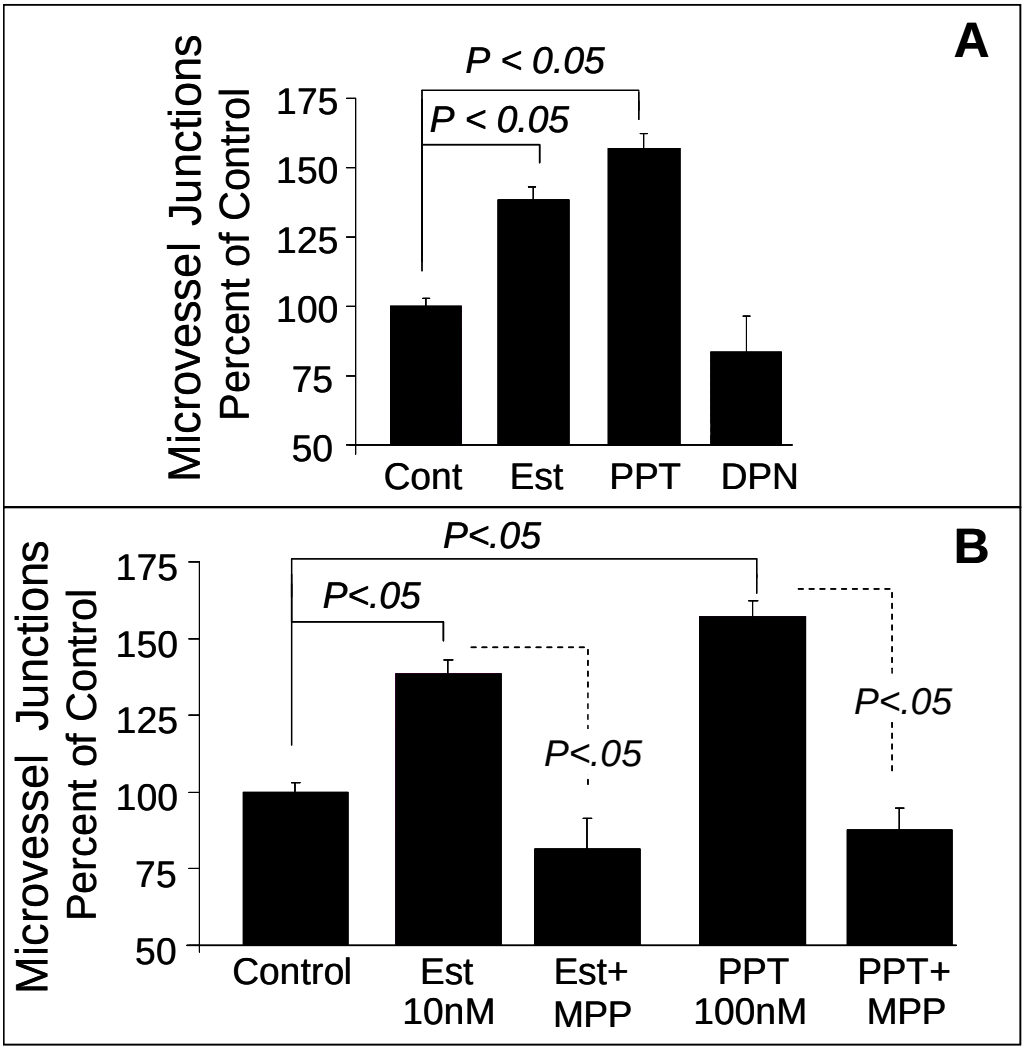

Figure S1: (A) Bar graph showing the effects of estradiol (Est; 10nmol/L), PPT (ERα agonist; 100nmol/L) and DPN (100nmol/L) on capillary formation by EPCs. Serum starved EPCs were plated at a density of 50'000 cells/200µl / well on matrigel coated 8-well multichamber slides. After 4-hrs the capillary formation was assessed by randomly counting the number of sprouting junctions at 4 different locations under a microscope and 4x magnification. (B) Bar graph showing the effects of estradiol (10nmol/L) and PPT (100nmol/L) on EPC-induced capillary formation in the presence and absence of ERα antagonist MPP (1µmol/L). Values represent means±SEM.

Figure S2

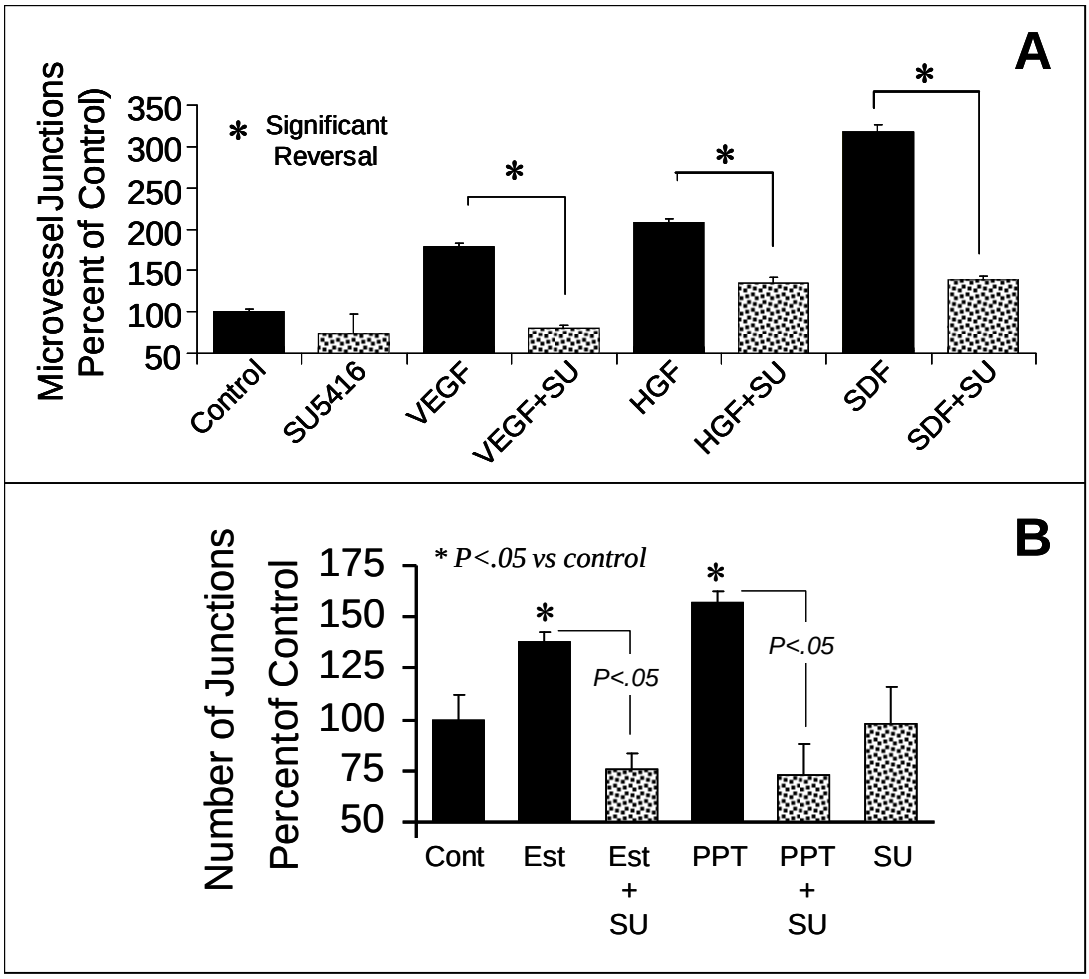

Figure S2: (A) Bar graph showing the effects of tyrosine kinase stimulators vascular endothelial growth factor (VEGF**;**100ng/mL), hepatocyte growth factor (HGF, 100ng/mL) and stromal cell-derived growth factor (SDF-1 100ng/mL) on capillary junction formation by EPCs in the presence and absence of tyrosine kinase inhibitor SU5416 (SU; 5µmol/L). Serum starved EPCs were plated at a density of 50'000 cells/200µl / well on matrigel coated 8-well multichamber slides. After 4 hrs the capillary junction formation was assessed by randomly counting the number of sprouting junctions at 4 different locations under a microscope and 4x magnification. (B) Bar graph showing the modulatory effects of tyrosine kinase inhibitor SU5416 (SU; 5µmol/L) on estradiol (10nmol/L) and PPT (100nmol/L) induced capillary junction formation in EPCs. Values represent means±SEM. * $p<0.05$ versus vehicle treated controls.

Figure S3

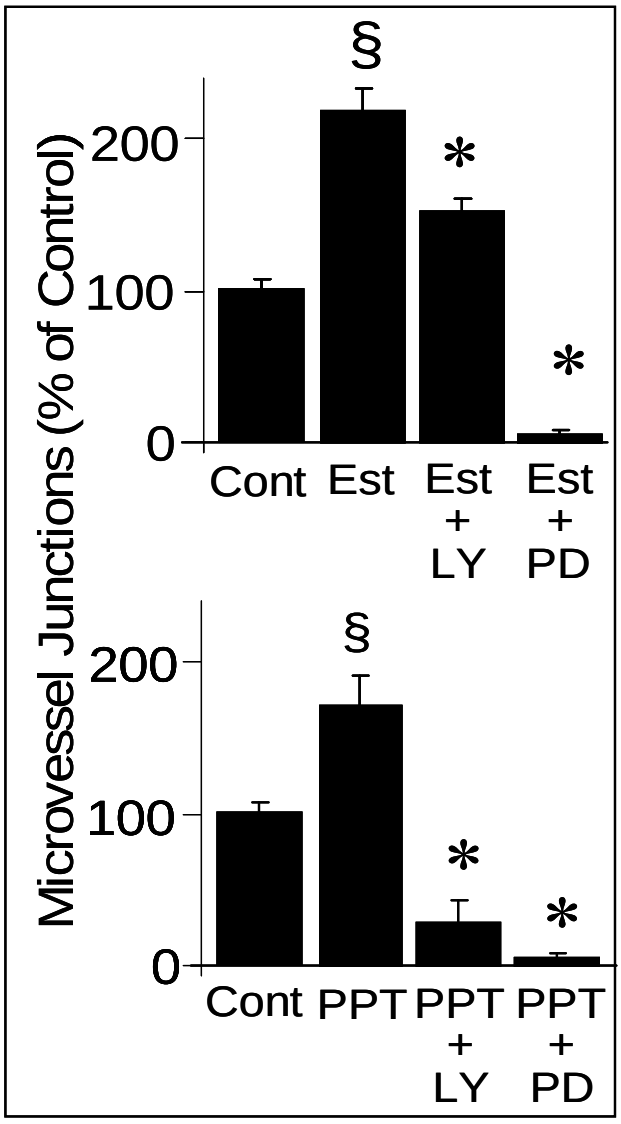

Figure S3: Bar graph showing the inhibitory effects LY294002 (Akt inhibitor; LY; 10μmol/L) and PD98059 (ERK1/2-P inhibitor; PD; 10μmol/L) on estradiol (Est; 10nmol/L) and PPT (100nmol/L) induced capillary junction formation by EPCs. EPCs were pretreated for 15-minutes with either LY or PD and subsequently Est orPPT added for another 4-hrs and capillary formation analyzed microscopically at 4x mag. * $p<0.05$ vs Estradiol or PPT alone.

Figure S4

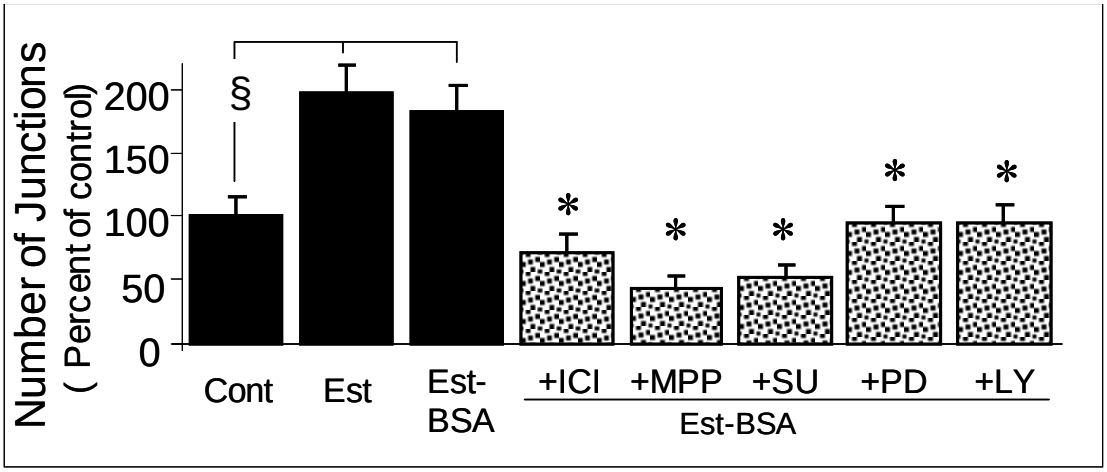

Figure S4: Bar graph showing the modulatory effects of tyrosine kinase inhibitor SU5416 (SU; 5μmol/L), LY294002 (Akt pathway inhibitor; LY, 10μmol/L) and PD98059 (ERK1/2 pathway inhibitor; PD, 10μmol/L) and MPP (ERα antagonist, 1μmol/L) on BSA-estradiol (Est-BSA, 10nmol/L) induced capillary formation in EPCs. Serum starved EPCs were plated at a density of 50,000 cells/200μl/well on matrigel coated 8-well multichamber slides. Cells were pretreated for 15-minutes with MPP, SU, LY, or PD and subsequently BSA-estradiol was added for an additional 4-hours and capillary junction formation assessed by randomly counting the number of sprouting junctions at 4 different locations under a microscope at 4x magnification. Values represent means±SEM. $^{\S}p<0.05$ versus vehicle treated controls; *$p<0.05$ versus Est-BSA.

Estradiol Stimulates Capillary Formation by Human Endothelial Progenitor Cells: Role of Estrogen Receptor- α/β, Heme Oxygenase 1, and Tyrosine Kinase

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