Estrogen Effects on Endothelial Progenitor Cell Activation and Angiogenic Responses

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**Background:** Gender differences in the incidence of coronary heart disease have driven investigation of estrogen's effects on the vascular wall. Recent clinical trials have raised concerns about the cardiovascular effects of hormone replacement therapy, though. Likely estrogen's (E2) most favorable effect on the vasculature is in the prevention of atherogenesis, rather than in modulation of atherosclerosis. Estrogen receptors (ER), in addition to functioning as ligand-activated transcription factors, mediate rapid signaling pathways through engagement of plasma membrane (PM) bound ER form, truncated 46-kDa ER46 isoform. E2-induced increase in nitric oxide (NO) production by the endothelium may be protective of the vasculature. The Bender laboratory has shown that E2 rapidly induces NO release from human endothelial cells *in vitro* via a c-Src/ PI3-kinase/Akt pathway in the absence of modulated gene expression, suggesting direct engagement of PM ER. Estrogen has been shown to have a proangiogenic effect following ischemia and vascular injury, potentially via the effect of estrogen on bone marrow-derived endothelial progenitor cells (EPCs). EPCs are present in the systemic circulation and have been shown to be important in neovascularization, homing to sites of ischemia.

**Specific Aims:** 1. Demonstrate E2-stimulated, ER-mediated rapid activation of the c-Src/PI3-kinase/Akt/eNOS pathway in human EPCs; and 2. Document the importance of E2-stimulated, rapid EPC signaling in angiogenesis *in vitro*.

**Hypothesis:** Via engagement of the membrane-localized ER46, E2 stimulates the c-Src/PI3-kinase/Akt-dependent activation of eNOS, which contributes to angiogenic responses.

**Methods:** Cultured EPCs were E2-deprived for 48 hours, E2 (1-50nM)- or control for 5-60 min, following which lysates were subjected to immunoblotting with antibodies to phospho-specific activation sites on c-Src, Akt and eNOS with additional samples that included the use of the ER antagonist ICI 182,780, the PI3-kinase inhibitor LY294002 and the Src family kinase inhibitor PP2. Late EPCs were mixed with growth factor-depleted matrigel and plated in suspension at 37°C, allowing formation of vascular cords in 3 dimensions in E2- or control conditions as above with or without the above inhibitors.

**Results:** E2 stimulated activation of c-Src, Akt, and eNOS in late EPCs with similar kinetics as those described for mature ECs. Free E2 was used in these experiments, which can passively diffuse across the membrane to stimulate cytosolic/nuclear receptors, in addition to engaging PM-bound ER. Given the short timeframe, this activation was transcription independent and likely resulted from engagement of receptors at the PM. ER inhibitor assays with ICI 182,780 showed significant inhibition of *in vitro* Matrigel assays showed that E2 stimulation results in formation of vascular cords, with increased cord number, width and branchpoints. The ER antagonist also abrogated E2-stimulated cord formation.

**Conclusions:** We believe that a major mechanism through which E2 confers vascular protection and promotes angiogenesis is via eNOS activation and NO production, both in mature endothelium and EPCs. Our data show that, likely through PM-localized receptors, E2 activates a c-Src/PI3-kinase/Akt pathway in late human EPCs. Additionally, via the same pathway, E2 stimulates the incorporation of human EPCs into correlates of neovessels *in vitro*, suggesting an angiogenic effect of E2 through this mechanism. Future studies will include the use of animal models to determine whether E2 does enhance angiogenesis through its signaling effects in EPCs *in vivo*.