Selective estrogen receptor-α agonist provides widespread heart and vascular protection with enhanced endothelial progenitor cell mobilization in the absence of uterotrophic action

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ABSTRACT **The beneficial effects of estrogens on the cardiovascular system are associated with adverse effects on reproductive tissues. On the basis of previous work indicating a major role for estrogen receptor (ER)-α in maintaining cardiovascular health, we evaluated the tissue selectivity of the ERα-selective agonist propyl pyrazole triol (PPT) compared with 17β-estradiol (E2) *in vivo*. Four weeks postovariectomy, equimolar doses of PPT and E2 were administered to rats in subcutaneous implants for 5 d. Both treatments restored rapid vasorelaxation of aortic tissue to estrogenic agents and prevented coronary hyperresponsiveness to angiotensin II in isolated heart preparations. Accordingly, multiple endpoints of myocardial ischemia-reperfusion injury exacerbated by ovariectomy returned to baseline following treatment. These protective effects were linked to increased *in vivo* levels of endothelial progenitor cells (EPCs). Human EPC function was enhanced *in vitro* after PPT treatment. In sharp contrast to E2, PPT treatment had no effect on uterine weight and histomorphology except for vessel density, and failed to up-regulate classic estrogen target genes. Dissection of the effects on vascular reactivity and uterine morphology was also observed following increased exposure to PPT at a higher dose for longer time. These data provide the first *in vivo* evidence for tissue-specific ERα activation. By conferring cardiovascular protection dissected from unwanted uterotrophic effects, ERα-selective agonists may represent a potential safer alternative to natural hormones.—Bolego, C., Rossoni, G., Fadini, G. P., Vegeto, E., Pinna, C., Albiero, M., Boscaro, E., Agostini, C., Avogaro, A., Gaion, R. M., Cignarella, A. Selective estrogen receptor-α agonist provides widespread heart and vascular protection with enhanced endothelial progenitor cell mobilization in the absence of uterotrophic action. *FASEB J.* 24, 2262–2272 (2010). www.fasebj.org**

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THE PROTECTIVE ROLE OF ESTROGEN therapy in cardiovascular health has been challenged by the outcome of large-scale clinical trials showing little or no benefit associated with increased adverse effects. Because 17β-estradiol (E2) and other mammalian estrogens behave as full and nonselective agonists on estrogen receptors (ERs) (1), it is still debated which ER isoform mediates the favorable effects of E2 on vascular function, inflammation, atherosclerosis, hypertension, and stroke that have emerged from experimental studies (2, 3). Whereas it is well established that both ERα and ERβ are expressed to a variable extent in multiple circulating and resident vascular wall cell types (2), the two isoforms govern distinct gene networks (4) and contribute to specific, redundant, or potentially opposing functions in the heart and vasculature. To address the respective role of ERα and ERβ, genetic studies were performed. Although initial studies indicated that both ERα and ERβ mediated the cardiovascular effects of E2 in a vascular injury model (5, 6), it is now clear that the first ERα-deficient model (ERα-NeoKO) (7) was imperfect, as a transcriptional leakage gave rise to natural (46 kDa) and non-natural (55 kDa) AF-1-deficient ERα isoforms (8). Indeed, these vasculoprotective effects persisted in this model but were abrogated in a complete knockout $ER\alpha^{-/-}$ (8–10). Current evidence indicates that ERβ activity manifests mainly in preventing the development of pulmonary hypertension (11, 12), whereas most aspects of vascular, particularly endothelial, function are mediated by ERα activation (13–15). The development of selective ligands for the ERα isoform in place of nonselective hormone agents, however, has been hampered by the fact that ERα is also

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predominant in the uterus and appears to be responsible for unwanted effects associated with estrogen and selective estrogen receptor modulator (SERM) therapy (16, 17). Highly selective ERα agonists, such as propylpyrazole-triol (PPT), have been developed; as expected, this compound evokes responses in classical estrogen target tissues, including uterine weight gain, but with different potency from E2 (18). So far, few studies tested the cardiovascular action of selective ERα agonists following *in vivo* administration (19–22). However, the tissue selectivity of these compounds was not determined simultaneously in reproductive organs.

On the basis of differences in coactivator recruitment and ER isoform selectivity compared with E2 (18, 23), we hypothesized that *in vivo* administration of PPT would allow dissecting the effects on cardiovascular endpoints from uterotrophic response. To test this hypothesis, we measured aortic vasorelaxation, protection from cardiac ischemia-reperfusion injury, and mobilization of endothelial progenitor cells (EPCs), a novel biomarker of cardiovascular health (24), in PPT- and E2-treated ovariectomized rats. These experiments revealed that PPT administration improved all these aspects of cardiovascular function, without causing the changes in uterine weight induced by an equimolar dose of E2. To further investigate this phenomenon, we assessed the differential effects of the two agents on uterine histomorphology and profiled uterine gene expression. Finally, we found that PPT could enhance proliferation, clonogenic expansion, and adhesive capacity of cultured human EPCs, further suggesting that ERα-selective agonists may warrant testing as useful adjuncts in improving cardiovascular health in postmenopausal women.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats (2 mo old; Charles River, Calco, Italy), initial weight 200–225 g, were used. The animals were housed in a conditioned environment (22±1°C, 55±5% relative humidity, 12-h light/12-h dark cycle), with free access to standard laboratory chow and tap water. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (Bethesda, MD, USA; NIH Publication No. 85-23, revised 1996).

Experimental design

Experiments were conducted on 20 rats randomly divided into 4 groups as follows: intact, ovariectomized (OVX), E2-treated OVX, and PPT-treated OVX. Bilateral ovariectomy was performed under ketamine (40 mg/kg i.p.) and xylazine (20 mg/kg i.p.) anesthesia. Four weeks later, OVX animals received a subcutaneous implant of 2 Silastic capsules containing 25 μl of vehicle (peanut oil) or E2 (5.87 μg, 0.86 mM; Sigma-Aldrich, St. Louis, MO, USA) or PPT (8.33 μg, 0.86 mM; Tocris, Bristol, UK) for 5 d. Doses and duration of treatment regimen were selected based on previous studies (13, 25). Selected experiments were performed in OVX animals after treatment with PPT or vehicle by implanting larger capsules for 4 wk, as described elsewhere (26). At the end of treatment, all rats were anesthetized by an intraperitoneal injection of thiopentone sodium (Pentothal, 60 mg/kg body weight); blood was collected from the cava vein, the uterus was removed and weighed, and the heart and thoracic aorta were immediately harvested.

Isolated aorta experiments

Changes in vasomotor tone of aortic tissues in response to acute E2 or PPT treatment were recorded in organ chambers (13). The thoracic aorta was carefully removed, cleaned of fat and connective tissue, and cut into 5- to 6-mm rings. Vessels were suspended in 5-ml organ baths containing Krebs-Henseleit solution (KHS) at 37°C, continuously bubbled with 95% O_2 and 5% CO_2. KHS had the following composition (mM): 118 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.1 $MgSO_4$, 2.5 $CaCl_2$, 25 $NaHCO_3$, and 5.5 glucose; pH 7.4. The rings were connected to isometric tension transducers (Fort 10; World Precision Instruments, Sarasota, FL, USA) coupled with a digital recording system (PowerLab 8SP; ADInstruments, Colorado Springs, CO, USA). Vascular tissues were equilibrated for 30 min and contracted with 10^{-5} M noradrenalin (NA) to develop a maximal response. Preparations were then washed with fresh KHS, and the equilibration period was allowed to continue for a further 30 min. Experiments were carried out on tissues precontracted with NA to 60% of maximal contraction ($EC_{60}=10^{-7}$ M). The endothelium was considered functional because relaxation of precontracted vessels to 10^{-5} M acetylcholine was ≥80%. Cumulative concentration-response curves for E2 (nonselective ER agonist) and PPT (ERα agonist) were obtained over the concentration range 10^{-13} to 10^{-7} M. To test vehicle effects, cumulative additions of equivalent ethanol volumes were also performed. Ethanol concentration never exceeded 0.01%, which however has been shown to elicit MAPK and PI3K activation (27). Relaxant responses were expressed as percentage of residual NA-induced contraction.

Indirect systolic blood pressure and heart rate measurements in conscious rats

At the end of the experimental period, the animals were weighed, and systolic blood pressure (SBP) was measured in the conscious state by the tail-cuff method using apparatus from Basile (mod 58500; Basile; Comerio, Italy). Before tail-cuff blood pressure determination, animals were placed into a warming cupboard (35°C) for 30 min. SBP values for individual rats were obtained from the average of 4 consecutive measurements and were considered valid only when these readings did not differ by >5 mmHg. Heart rate (HR) was also calculated from the blood pressure tracing.

Perfused rat heart preparations

Rat hearts were mounted on the experimental setup and perfused as described previously (28). The heart was rapidly excised and placed in cold KHS. The heart was mounted on the experimental setup within 2 min after thoracotomy and perfused at 15 ml/min (Minipuls-3 peristaltic pump; Gilson, Middleton, WI, USA) through the aorta with KHS, maintained at 37°C and aerated with 95% O_2 + 5% CO_2 to stabilize normal pH, pO_2, and pCO_2. Coronary perfusion pressure (CPP) and left ventricular pressure (LVP) were measured with two pressure transducers (HP-1280c; Hewlett-Packard, Palo Alto, CA, USA) connected to a Hewlett-Packard dynograph (HP-7754A). LVP was recorded with a polyethylene catheter, with a small latex balloon on the tip (Hugo

Sachs Elektronik, March-Hugstetten, Germany), inserted into the left ventricular cavity through the mitral valve opening. The volume of the balloon was adjusted to give peak left ventricular systolic pressure (LVSP) 90 ± 5 mmHg with left ventricular end-diastolic pressure (LVEDP) 5–7 mmHg. Hearts that could not achieve this level of contractile performance (8–10%) were excluded. Left ventricular developed pressure (LVDevP; peak LVSP − LVEDP) was also calculated. After 15 min of equilibration, hearts were paced at 300 beats/min with an electrical stimulator (S-88; Grass Instruments, Rockland, Massachusetts, USA) using two silver electrodes attached to the right atrium, and a further 20 min of perfusion was carried out (preischemic period).

Effect of angiotensin II on CPP

To assess the integrity of endothelium-dependent function, at the beginning of each experiment the coronary vasculature reactivity to angiotensin II was evaluated in the same hearts subsequently subjected to ischemia-reperfusion. Angiotensin II (1 μg; Sigma-Aldrich) was injected as a bolus into the perfusion system, and changes in CPP were recorded.

Ischemia-reperfusion in the perfused rat heart

Ischemia was induced by reducing buffer flow rate from 15 to 1 ml/min for 20 min (ischemic period). Normal flow rate (15 ml/min) was then restored, and perfusion was continued for another 30 min (reperfusion period). Throughout the experiment, a thermoregulated chamber held the heart temperature at 37°C to avoid hypothermia-induced cardioprotection. The total duration of each experiment did not exceed 90 min, during which time the experimental preparation remained stable.

Creatine kinase (CK) and lactate dehydrogenase (LDH) assay

The effluent solution obtained from the heart during the preischemic and reperfusion periods was collected in an ice-cold beaker as 2.5-min samples. Each sample was used for the determination of CK and LDH activities according to Bergmeyer (29) and Hohorst (30), respectively. The total activity was measured spectrophotometrically (Lambda-16; PerkinElmer, Wellesley, MA, USA) at 37°C using specific kits, according to the manufacturer's instructions (Sentinel Diagnostic, Milan, Italy).

Determination of circulating rat EPCs

A definite consensus on the best phenotype of rat EPCs is lacking. In this study, generic rat progenitor cells were defined as sca-1$^+$ and Sca-1$^+$c-kit$^+$, while a more specific population of EPCs was defined as Sca-1$^+$CD31$^+$ and determined *ex vivo* by direct 2-color flow cytometry. We have previously shown that the rat Sca-1$^+$c-kit$^+$ cell population is enriched with putative rat EPCs (31). Because of the simultaneous expression of a stem cell marker (sca-1) and an endothelial marker (CD31), sca-1$^+$CD31$^+$ cells can be considered the homologue of human CD34$^+$KDR$^+$ cells, the most credited EPC phenotype (32).

Peripheral blood samples were drawn from the tail vein. After red blood cell lysis, blood cells were washed and stained with PE-conjugated anti-mouse sca-1, FITC-conjugated anti-c-kit, or FITC-conjugated anti-rat CD31 mAbs. The frequency of peripheral blood cells positive for the above reagents was determined by a 2-dimensional sidescatter-fluorescence dot-plot analysis of the samples stained with the different reagents, after appropriate gating to exclude granulocytes. Initially, we gated Sca-1+ peripheral blood cells and then examined the resulting population for dual expression of c-kit or CD31. Data were processed using the Macintosh CellQuest software program (BD Biosciences, Franklin Lakes, NJ, USA). A single trained operator, who was masked to the status of the animal, performed all flow cytometric analyses throughout the study.

Isolation and characterization of human EPCs

Human EPCs were cultured as described previously in detail, according to a standard protocol optimized to yield true late EPCs (32–34). Briefly, mononuclear cells were isolated from peripheral blood of healthy female blood donors aged <35 yr. Cells were plated on 6-well fibronectin-coated dishes (BD Biosciences) at a density of 6×10^6 cells/well, and grown in endothelial growth medium (EGM; Clonetics, Baltimore, MD, USA) supplemented with 20% FCS for 15 d. Culture medium was changed first on d 4 and then every 2 d. Attaching cells rapidly assumed an endothelial-like shape and proliferated in typical endothelial colonies. Two weeks after plating, colonies tend to disappear, leaving a confluent cobblestone monolayer. In preliminary separated experiments, cells were collected at this time point and characterized for binding of FITC-labeled Ulex-Lectin (Sigma-Aldrich), uptake of DiI-acetylated LDL (Molecular Probes, Carlsbad, CA, USA), and expression of typical endothelial markers using immunofluorescence and/or flow cytometry. Nuclei were stained in blue with Hoechst 33258 (Sigma-Aldrich). The following primary antibodies were used for *in situ* immunofluorescence: anti-von Willebrand Factor (Dako Cytomation, Glostrup, Denmark), anti-KDR (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-CD31 (Chemicon International, Temecula, CA, USA). Secondary antibodies for indirect immunofluorescence were Cy2 anti-rabbit and Cy2 anti-mouse (Chemicon). FITC-conjugated anti-human CD34, FITC-conjugated anti-human CD31, FITC-conjugated anti-human CD14, PE-conjugated anti-human CD45 (all from BD Biosciences), and PE-conjugated anti-KDR (R&D Systems, Minneapolis, MN, USA) were used for flow cytometry of cultured cells.

Clonogenic expansion of cultured EPCs

Proliferation and clonogenic expansion of cultured human EPCs was assessed by counting typical cell colonies at d 15 after plating. EPC colonies were identified as rounded cell clusters of >20 cells, made up of a central core of rounded cells surrounded by radiating spindle-shaped cells. Colony count was performed in 10 randomly selected high-power fields for each experiment (n=3). To assess the effect of PPT and E2 on the clonogenic expansion of cultured EPCs, starting from d 1, the culture medium was supplemented with 100 nM PPT or 100 nM E2. These concentrations were chosen because, in preliminary experiments in which cells were incubated with 1–10-100 nM E2, a significant increase in EPC colonies was found with 100 nM E2.

Adhesive capacity of culture EPCs

We evaluated *in vitro* the functional property of EPCs to adhere to mature endothelium, as described previously (33, 34). For this purpose, a monolayer of human umbilical vein endothelial cells (HUVECs; Clonetics) was prepared 48 h before the assay by plating 2×10^5 cells/cm^2 at early passages. EPCs were labeled with DiI-Ac-LDL as described above, and

1×10^5 cells were added to each well and incubated for 2 h at 37°C. Nonattached cells were gently removed with PBS, and adherent EPCs were fixed with 4% *p*-formaldehyde in PBS and counted in 10 random fields. To assess the effect of PPT and E2 on adhesion of cultured EPCs to HUVECs, starting from d 1, the culture medium was supplemented with 100 nM PPT or 100 nM E2. These concentrations were chosen because, in preliminary experiments in which cells were incubated with 1–10-100 nM E2, a significant increase in adherent EPCs was found with 100 nM E2. Then, equimolar concentrations of PPT were chosen.

Histological specimens and immunohistochemical examinations

Five-micrometer-thick frozen sections of the uterus were made. Hematoxylin-and-eosin staining was performed with a commercially available kit (BioOptica, Milan, Italy), according to the manufacturer's instruction. High-resolution microscopic images were captured, and Adobe Photoshop photoediting software (Adobe Systems, San Jose, CA, USA) was used to reconstruct the whole uterine sections. Glands were then counted in the whole uterine tissue. Glands were defined as tube-like/elongated structures or rounded structures with a clearly visible epithelial lining. Immunohistochemistry was performed using a monoclonal antibody directed against smooth muscle α-actin (Sigma Aldrich), followed by incubation with peroxidase-labeled, rabbit anti-mouse, secondary antibody (Dako Cytomation) to identify the myometrial area. Using Adobe Photoshop, we measured the area of myometrium and endometrium in the whole section to calculate the myometrial/endometrial ratio. To identify blood vessels, immunofluorescence was performed using an antibody against von Willebrand Factor (Dako Cytomation), followed by incubation with Cy2-conjugated, goat anti-rabbit, secondary antibody (Chemicon). Adobe Photoshop was used to measure the signal of Cy2-labeled vessels of high-power fields in the whole section.

Immunofluorescence observations were carried out using a Zeiss Axioplan epifluorescence microscope (Carl Zeiss, Oberkochen, Germany). Optical images were acquired by a Leica DMR microscope connected to a Leica DC300 video camera (Leica Microsystems, Wetzlar, Germany). Each count was repeated for 10 random sections for each sample.

RT-PCR

Uterine tissue was homogenized with a polytron (IKA T10 Ultra Turrax; IKA, Staufen, Germany), and RNA was isolated using RNeasy Mini Kit RNa column (Qiagen, Hilden, Germany), according to the manufacturer's instructions. One microgram of RNA was used for cDNA preparation using the MuMLV reverse transcriptase (Promega, Madison, WI, USA). Control reactions without addition of the enzyme were performed for each sample. PCR reactions were performed using 1 μl cDNA and 0.4 U of DynaZyme DNA polymerase (Finnzymes, Espoo, Finland). The following primers (MWG Biotech, Ebersberg, Germany) were used to amplify the respective rat genes: C3, *a*-5′-CTCAGTGACCAAGTGCCAGA-3′ and *b*-5′-CTCCCACTGTTCGGTCTGAT 3′; pS2, *a*-5′-CCATGGAGCACAAGGTGACCTG-3′ and *b*-5′-GGGAAGCCACAATTTATTCT-3′; lactoferrin, *a*-5′-TGGAGCAGAGTGAGTGTTGG-3′ and *b*-5′-CCACACAATCAGAGCCATTG-3′. Amplification products were run on 3% agarose gel. Amplification of the housekeeping enzyme glyceraldehyde phosphodehydrogenase (*gapdh*) was performed in parallel to assess for RT-PCR efficiency. The following primers were used: *a*-5′-ATGACCCCTTCATTGACC-3′ and *b*-5′-TGCTTCACCACCTTCTTG-3′. PCR reactions were performed on a PerkinElmer Thermal Cycler 480 as follows: C3: 95°C for 5 min, then 30 cycles at 92°C for 1 min, 59°C for 1 min, 72°C for 1 min; pS2: 95°C for 5 min, then 40 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 30 s; lactoferrin: 94°C for 2 min, then 35 cycles at 94°C for 30 s, 62°C for 30 s, 72°C for 30 s; GAPDH: 94°C for 2 min, then 23 cycles at 94°C for 1 min, 53°C for 1 min, 72°C for 1 min.

Western blot analysis

Uterine tissues were minced in ice-cold lysis buffer (20 mM HEPES, pH 7.9; 5 nM $MgCl_2$; 0.1 mM EDTA; 420 nM NaCl; 20% glycerol; 0.1% Triton 100X; 5 mM β-mercaptoethanol; 0.2 mM PMSF; 10 μg/ml aprotinin; and 1 μg/ml leupeptin) and homogenized with a polytron. Protein extracts were centrifuged at 24,000 *g*, 4°C for 30 min. Supernatant was collected, and protein content was determined using the Bradford assay (Pierce Chemical, Rockford, IL, USA). Proteins (10 μg) were separated on a 7.5% SDS-polyacrylamide gel and transferred to a Hybond ECL nitrocellulose membrane (GE Healthcare, Uppsala, Sweden). The membrane was incubated in blocking solution for 1 h with 5% skim milk in TBS-Tween (20 mM Tris, pH 7.5; 150 mM NaCl; and 0.2% Tween-20). The mouse antibody C262 (kindly provided by Dr. Geoffrey Greene, Ben May Institute, Chicago, IL, USA) raised against human progesterone receptors was used at 1:200 dilution at 4°C overnight in TBS-Tween. After extensive washing, a secondary HRP-conjugated anti-mouse IgG (Chemicon) was applied at 1:2000 dilution in TBS-Tween for 60 min; after extensive washing, detection was performed using the ECL kit from GE Healthcare.

Statistical analysis

All data are presented as the means ± SE for *n* experiments. Differences between means were tested for statistical significance using the unpaired *t* test or 1-way ANOVA with Newman-Keuls multiple test, as appropriate. From such comparisons, differences yielding $P \leq 0.05$ were judged to be significant.

RESULTS

Morphometric and hemodynamic data

At 4 wk postovariectomy, OVX rats appeared generally healthy. As shown in **Table 1**, there was no significant difference in body weight among groups. SBP in OVX + vehicle rats was significantly higher ($P<0.05$) than in intact animals. This increase in SBP caused by ovariectomy was fully prevented by treatment with the ERα-selective agonist PPT, but not with the nonselective ER

TABLE 1. *Body weight, mean arterial pressure, and heart rate at the end of treatment in control and ovariectomized rats*

Treatment	Body weight (g)	Systolic blood pressure (mmHg)	Heart rate (beats/min)
Control	289 ± 10	128 ± 6	315 ± 16
OVX + vehicle	299 ± 6	150 ± 5*	278 ± 12
OVX + E_2	303 ± 13	135 ± 6	314 ± 15
OVX + PPT	300 ± 9	126 ± 4†	308 ± 12

Data are expressed as means ± SE; $n = 5$ rats/group. *$P < 0.05$ *vs.* control; †$P < 0.05$ *vs.* OVX + vehicle

agonist E2. No differences in heart rate were observed among groups.

Vasorelaxant responses to ER agonists

The functional capacity of the aortic endothelium *ex vivo*, which was tested by obtaining concentration-response curves to the physiological endothelium-dependent vasodilator acetylcholine, was comparable among groups (data not shown). We previously reported that addition of E2, as well as PPT, to the organ chamber induces a rapid vasorelaxant response in aortic tissues from intact animals and from E2-replaced OVX animals (13, 25). Similar experiments performed in aortic tissues from OVX rats that had been administered PPT or vehicle are shown in **Fig. 1**. The addition of both PPT and E2 rapidly relaxed in a concentration-dependent manner NA-precontracted aortic rings from OVX rats that had been treated with PPT (Fig. 1*A*), whereas no changes in vascular tone were observed in tissues from vehicle-treated OVX rats (Fig. 1*B*). Acute responses to PPT and E2 did not significantly differ in terms of efficacy [E_{max}, 28.3±0.8 and 26.5±1.6, respectively; not significant (NS)] and potency (pD_2, 10.6±0.4 and 10.8±0.6, respectively; NS). The basal NO release after NOS blockade by L-NAME was comparable in tissues from vehicle- and PPT-replaced animals (Supplemental Fig. 1*A*). The improvement in rapid vasorelaxation to ERα agonist was observed also in tissues from OVX animals exposed to a higher dose of PPT (100 μg/kg) compared with vehicle for 4 wk (Supplemental Fig. 1*B*). In the latter set of experiments, we tested the response to acetylcholine in vessels from vehicle- and PPT-treated OVX animals and found no significant difference (Supplemental Fig. 1*C*), consistent with previous results (25). Hence, the activation of rapid signaling mediated by ERα agonists was restored in the aorta of PPT-treated OVX rats.

Isolated perfused rat heart studies

Angiotensin II activity on CPP

A bolus injection of 1 μg angiotensin II into the perfusion system of hearts from control rats induced a prompt increase in CPP, which reached a peak of 22 ± 4 mmHg above basal values (**Fig. 2**). In hearts from OVX rats, the angiotensin II-induced CPP increase was nearly twice as large as that measured in control hearts ($P<0.05$). Such hyperresponsiveness of coronary vasculature to angiotensin II, an index of vascular endothelium dysfunction, was prevented in heart preparations from E2- or PPT-treated rats (Fig. 2).

Ischemia-reperfusion in the perfused rat heart

At baseline, cardiac parameters were similar and not significantly different in the 4 experimental groups (**Table 2**). When the perfusion of electrically paced isovolumic left heart preparations from control animals was reduced from 15 to 1 ml/min for 20 min, LVEDP progressively rose, indicating that an ischemic process was occurring after the standstill (**Fig. 3*A***). During reperfusion, left ventricular function was impaired, LVDevP and maximum rate of rise and fall of left ventricular pressure ($+dP/dt_{max}$) being significantly reduced, and CPP considerably increased over baseline (**Fig. 4*A–C***). The hearts from OVX rats subjected to ischemia-reperfusion showed marked worsening of

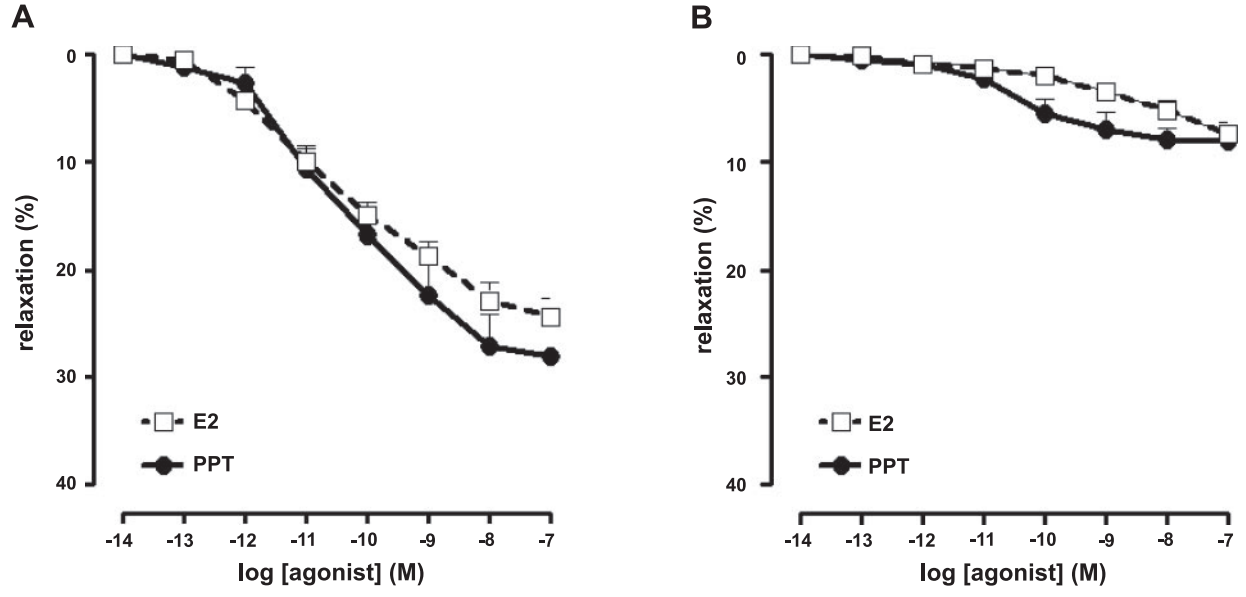

Figure 1. Acute vasorelaxation in response to estrogen receptor agonists PPT or E2. Graphs show concentration-response curves of aortic rings isolated from ovariectomized female rats that had been administered PPT (*A*) or vehicle (*B*) for 5 d. Tissues were precontracted with 0.1 μM noradrenalin and then incubated with increasing concentrations of PPT or E2. Data are shown as means ± SE ($n=5$).

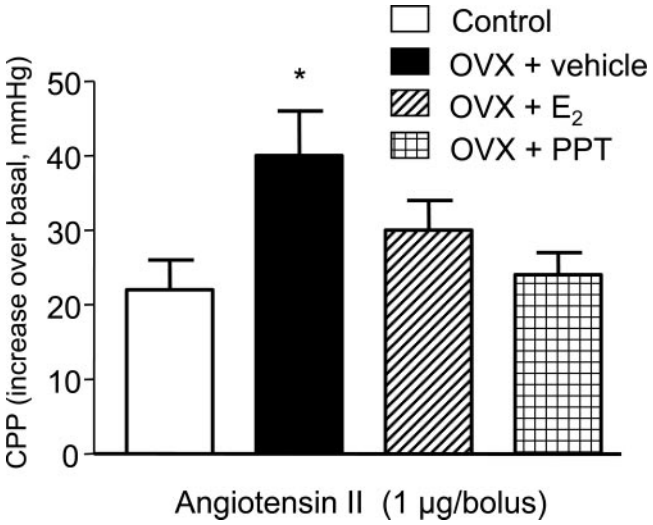

Figure 2. Changes in CPP induced by angiotensin II injected into perfused rat heart preparations during the preischemic period. Hearts were obtained from intact (control) and OVX rats treated with vehicle, E2, or PPT for 5 d. Values are expressed as means ± SE (n=5). *$P < 0.05$ *vs.* control.

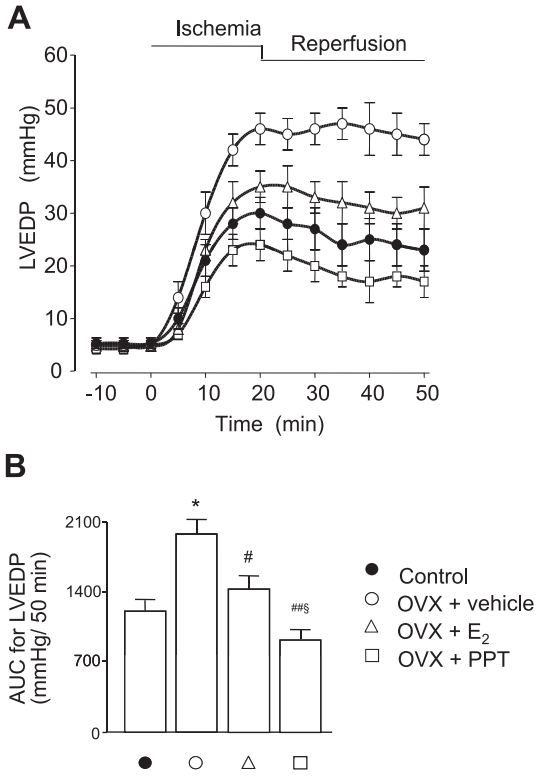

Figure 3. *A*) Time course of LVEDP in perfused heart preparations subjected to ischemia-reperfusion from intact (control) and OVX rats treated with vehicle, E2 or PPT for 5 d. *B*) Area under the curve (AUC) related to LVEDP curves. All data are shown as means ± SE (n= 5). *$P < 0.01$ *vs.* control; #$P < 0.05$, ##$P < 0.001$ *vs.* OVX + vehicle; §$P < 0.05$ *vs.* OVX + E2.

postischemic ventricular dysfunction. At the end of reperfusion, the area under the LVEDP-time curve was 1.7-fold higher (P<0.01) than that obtained in hearts from controls (Fig. 3*B*). Consequently, LVDevP (Fig. 4*A*) and $+dP/dt_{max}$ (Fig. 4*B*) were further depressed, with minimal recovery of myocardial contractility. Treatment of OVX animals with E2 and PPT induced myocardial protection against mechanical changes due to ischemia-reperfusion. The characteristic ventricular contracture observed during the 20 min of ischemia was reduced, fostering recovery of LVDevP and $+dP/dt_{max}$ on reperfusion (Fig. 4*A, B*). In line with the improvement of postischemic ventricular dysfunction, E2 and PPT significantly reduced the elevated CPP during reperfusion measured in OVX + vehicle rats (Fig. 4*C*).

CK and LDH activity in heart perfusates

During reperfusion, activity of CK and LDH, two biomarkers of myocardial damage, in the heart perfusates from control rats were 4.3- and 6.6-fold higher than those found in the preischemic period, respectively (Fig. 4*D, E*). The increased severity of postischemic ventricular dysfunction caused by ovariectomy was associated with a marked increase in CK and LDH activities in heart effluents. During reperfusion, CK and LDH release rates from heart preparations of OVX + vehicle rats were nearly twice as high as in control animals. Treatment with E2 or PPT significantly reduced the amount of CK and LDH compared with OVX + vehicle rats (Fig. 4*D, E*).

Circulating EPC levels

EPCs have been identified as circulating precursors for adult neovasculogenesis and vascular homeostasis and are mobilized to the peripheral blood on many stimuli, including vascular damage and tissue ischemia (24, 35). We assessed the impact of ovariectomy and subsequent

TABLE 2. *Baseline cardiac parameters in isolated perfused hearts from control and OVX rats*

Treatment	CPP (mmHg)	LVEDP (mmHg)	LVDevP (mmHg)	$+dP/dt_{max}$ (mmHg/s)	$-dP/dt_{max}$ (mmHg/s)
Control	62.1 ± 4.4	4.7 ± 0.7	97.8 ± 6.1	3070 ± 252	2275 ± 171
OVX + vehicle	68.3 ± 4.7	6.0 ± 0.8	88.9 ± 5.0	2815 ± 221	2050 ± 155
OVX + E2	63.7 ± 5.2	5.2 ± 0.4	93.5 ± 6.2	2916 ± 178	2107 ± 202
OVX + PPT	61.3 ± 5.0	4.8 ± 0.6	98.4 ± 5.2	2985 ± 213	2216 ± 181

Values are expressed as means ± SE; n = 5 rats/group. Cardiac parameters were evaluated immediately before ischemia. CPP, coronary perfusion pressure; LVEDP, left ventricular end-diastolic pressure; LVDevP, left ventricular developed pressure; $\pm dP/dt_{max}$, maximum rate of rise and fall of left ventricular pressure.

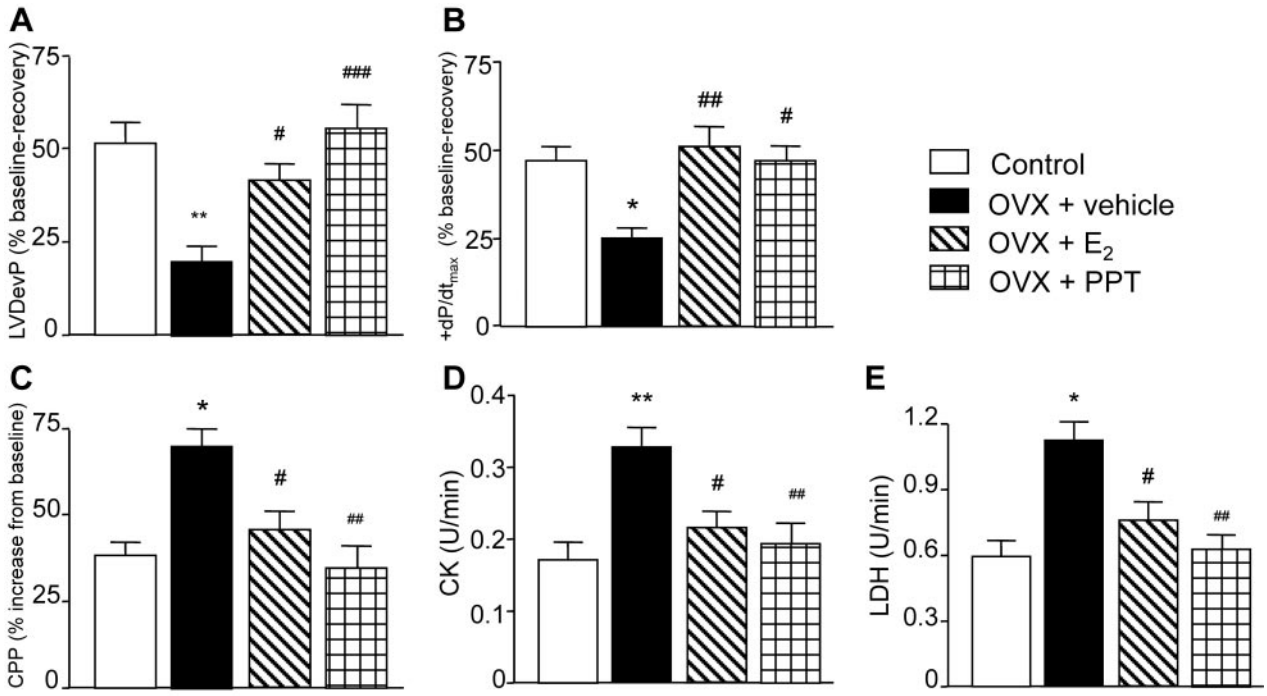

Figure 4. Multiple endpoints of ischemia-reperfusion injury are restored by E2 or PPT treatment. Recovery of LVDevP (*A*), $+dP/dt_{max}$ (*B*), CPP reperfusion (*C*), CK (*D*), and LDH (*E*) activities in perfused heart preparations subjected to ischemia-reperfusion from intact (control) and OVX rats treated with vehicle, E2, or PPT. LVDevP, +dP/d*t*max, and CPP were calculated at the end of 30-min reperfusion and expressed as percentage of preischemic values. CK and LDH were measured during the 30-min reperfusion and expressed as increase over preischemic values (CK, 0.04±0.02 U/min; LDH, 0.09±0.02 U/min); $n = 5$. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* OVX + vehicle.

pharmacological treatment on progenitor cell release after ischemia by measuring sca-1$^+$, sca-1$^+$c-kit$^+$, and sca-1$^+$CD31$^+$ cells in the different groups. While sca-1 and sca-1$^+$c-kit$^+$ cells are to be considered mainly hematopoietic, sca-1$^+$CD31$^+$ cells are the rat homologue of human CD34$^+$KDR$^+$ endothelial progenitors. Ovariectomy tended to reduce EPCs, being the change significant for sca-1$^+$ CD31$^+$ EPCs, whereas 5-d treatment with E2 and even more so with PPT significantly raised EPC counts above control levels across the board (**Fig. 5**).

Effects of ER agonists on isolated human EPC function

The relevance of the above findings to the human setting was tested in human EPCs, which were isolated according to a validated protocol (33). The genuine phenotype of these cells is based on lectin binding, AcLDL uptake, and expression of typical endothelial and stemness surface markers (**Fig. 6*A, B***), consistent with previous studies (33, 34). Both EPC colony formation 15 d after plating and the adhesive properties of EPCs to a mature endothelial layer were significantly enhanced by supplementation with E2 or PPT in culture (Fig. 6*C*).

Differential effects of E2 and PPT on uterine morphology

As expected, uterus weight decreased in OVX *vs.* intact animals (n=5; P<0.001; **Fig. 7*A***). Administration to

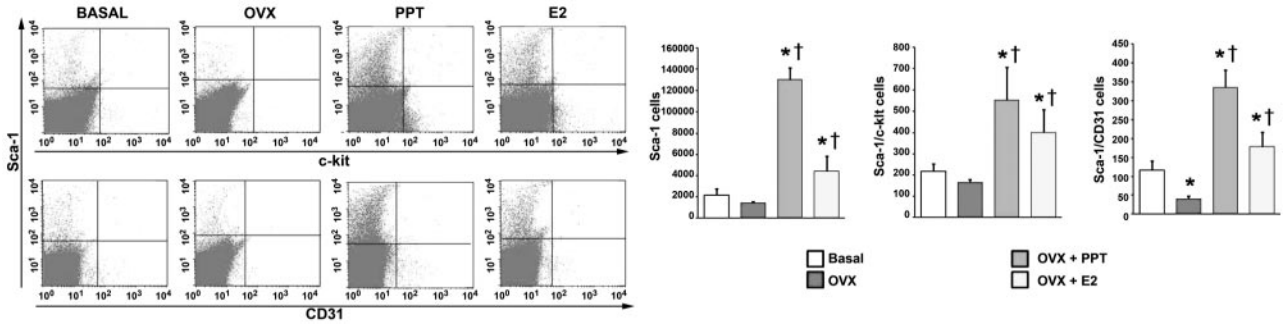

Figure 5. Gating strategy used to enumerate total sca-1$^+$, total c-kit$^+$ and sca-1$^+$ c-kit$^+$, and sca-1$^+$ CD31$^+$ cells by flow cytometry in peripheral blood of intact (basal) and OVX rats treated with vehicle, PPT, or E2. sca-1$^+$ CD31$^+$ cells significantly decreased after ovariectomy, whereas treatment with E2 and even more so PPT increased all cell types. $n = 5$. $^{*}P < 0.05$ *vs.* basal; $^{\dagger}P < 0.05$ *vs.* OVX.

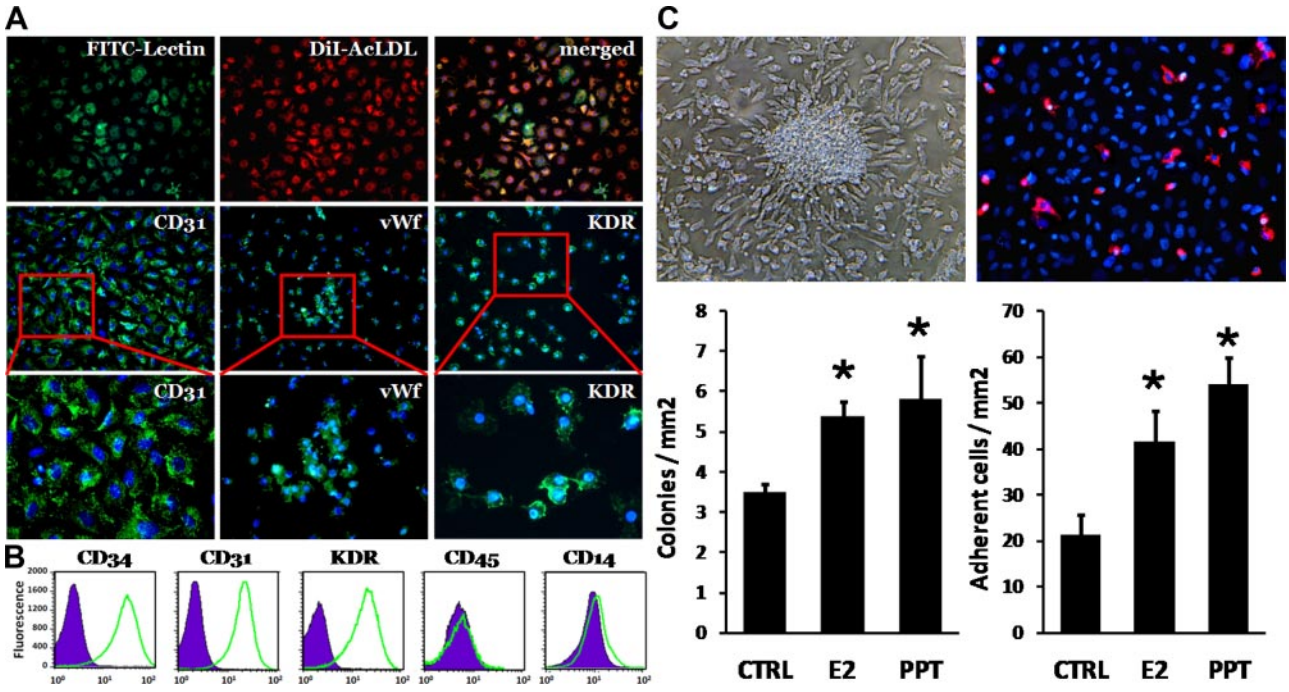

Figure 6. Characterization of cultured human EPCs and response to ER agonists. *A*) Immunofluorescence for lectin, acLDL, CD31, vWF, and KDR. *B*) Expression of CD34, CD31, KDR, CD45, and CD14 by flow cytometry. *C*) Colony formation and adhesion to a HUVEC monolayer in response to 100 nM E2 or PPT. $*P < 0.05$ *vs.* control (CTRL).

OVX rats of E2, but not of an equimolar PPT dose, for 5 d restored uterine weight (OVX+E2, $P<0.001$ *vs.* OVX+vehicle; OVX+PPT, NS; $n=5$). We next investigated in more detail the effect of the two ER agonists on uterine morphology in OVX rats. Compared with tissues from vehicle-treated animals, E2 but not PPT treatment increased the myometrium to endometrium thickness ratio, as shown by specific smooth muscle α-actin immunostaining (Fig. 7*B*). Furthermore, E2 but not PPT treatment sharply reduced gland density, as measured by hematoxylin-and-eosin staining, as a likely reflection of the changed myometrial/endometrial ratio. By contrast, vessel density was significantly increased following treatment with both ER agonists as measured by von Willebrand factor (vWF) immunostaining (Fig. 7*B*). The lack of significant effects on uterine morphology other than those on vessel density was confirmed in tissues from OVX animals exposed to

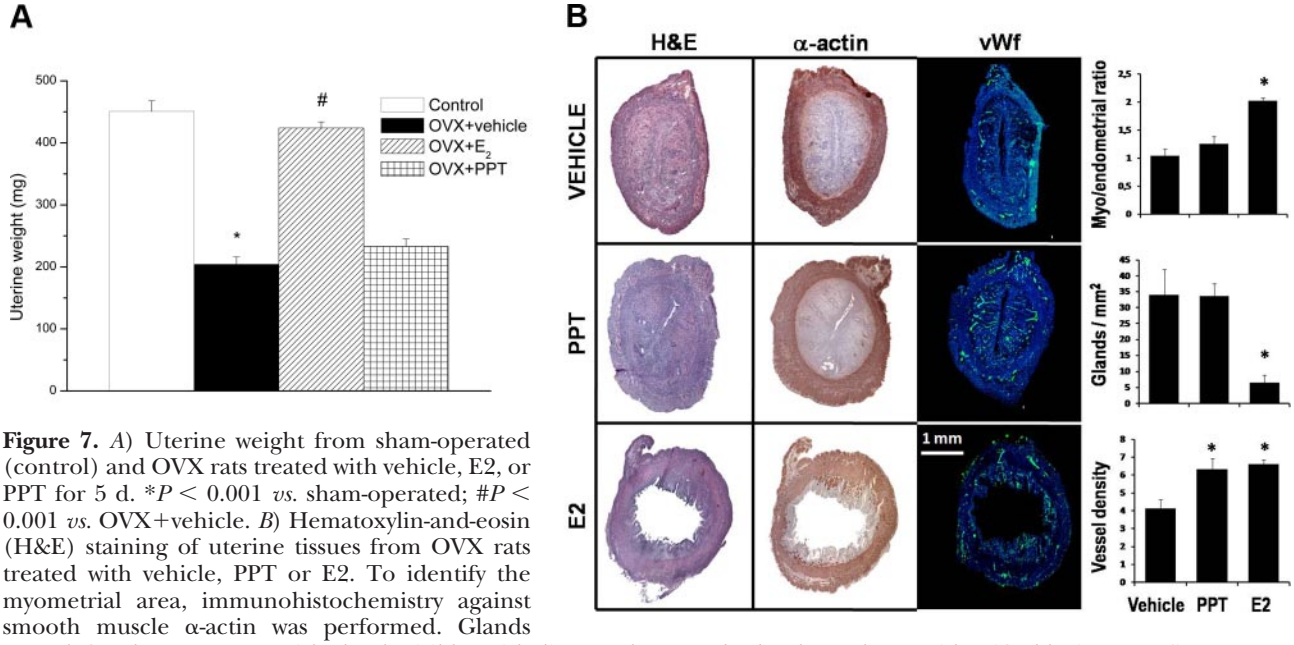

Figure 7. *A*) Uterine weight from sham-operated (control) and OVX rats treated with vehicle, E2, or PPT for 5 d. $*P < 0.001$ *vs.* sham-operated; $\#P < 0.001$ *vs.* OVX+vehicle. *B*) Hematoxylin-and-eosin (H&E) staining of uterine tissues from OVX rats treated with vehicle, PPT or E2. To identify the myometrial area, immunohistochemistry against smooth muscle α-actin was performed. Glands were defined as structures with clearly visible epithelium and counted. Blood vessels were identified by immunofluorescence using an antibody against von Willebrand factor (vWF). Images are representative sections. $*P < 0.05$ *vs.* vehicle.

an higher dose of PPT (100 μg/kg) for 4 wk compared with vehicle (Supplemental Fig. 2). Finally, the expression of genes known to be positively regulated by estrogens was assessed in the uterus of OVX rats. In contrast to E2, treatment with PPT had no effect on the mRNA levels of complement component C3, pS2, and lactoferrin (**Fig. 8*A***). The same pattern could be shown for the expression of progesterone receptors A and B at the protein level (Fig. 8*B*).

DISCUSSION

The major finding of this study is that the ERα-selective agonist PPT, when administered to OVX rats, restored endothelial function and afforded protection from ischemia-reperfusion injury along with increased circulating EPCs in the absence of uterotrophic action. To the best of our knowledge, this is the first demonstration of cardiovascular as opposed to uterine selectivity of ERα-selective agonists *in vivo* and represents a clear advance over selectivity studies *in vitro* (36). Conversely, known nonselective hormonal agents, such as mammalian estrogens, behave as full agonists on both ER isoforms, thereby inducing adverse effects in classic target tissues such as the uterus (16).

The endothelium is recognized as a biologically active dynamic organ involved in a number of pathophysiological processes. Herein, we show that PPT administration restored endothelial function, as measured by acute vasorelaxant response to E2/PPT and hyperresponsiveness to angiotensin II, and provided cardioprotection from ischemia-reperfusion injury. Although both effects have been reported to occur in response to ERα activation in separate studies (13, 19, 22), the present study depicts an integrated view of the endothelium as a primary target of PPT action. As the basal NO production was unaffected (Supplemental Fig. 1*A*), PPT replacement likely improved endothelial function by enhancing eNOS phosphorylation and activation. Beyond the disruption of the ERα/eNOS signaling network (25), the present findings bring to light additional manifestations of loss of cardiovascular function subsequent to endogenous steroid hormone deprivation in rats, including hypertension, increased biomarkers of myocardial damage, and slower recovery from ischemia. Notably, all of these events were prevented by treatment with the selective ERα agonist. While the observed protection may arise from reduced inflammatory response during reperfusion, as proposed by some authors (19), the present findings suggest an associated relationship between improved endothelial function/angiogenic response to ischemia and enhanced release of EPCs in response to PPT treatment. Although we did not evaluate the specific recruitment of the circulating EPCs at the cardiac site after injury in this study, the *bona fide* integration of labeled EPCs into blood vessels after ischemia-reperfusion (31), and the beneficial effects of E2 treatment on EPCs (34, 37) are well established. Thus, ERα-mediated enhancement of EPC function may be a unifying mechanism underlying the widespread impact of PPT on the serious cardiovascular dysfunction associated with hypoestrogenicity. It is also conceivable that selective ERα activation bypasses the concomitant activation of ERβ by E2, which can antagonize the actions of ERα (38). This would be consistent with the superior activity on specific cardiovascular parameters, such as LVEDP (Fig. 3*B*), EPC counts (Fig. 5) and SBP (Table 1), shown by PPT with respect to E2 in the present study. The latter effect could be due to an effect of ERα activation in the central nervous system.

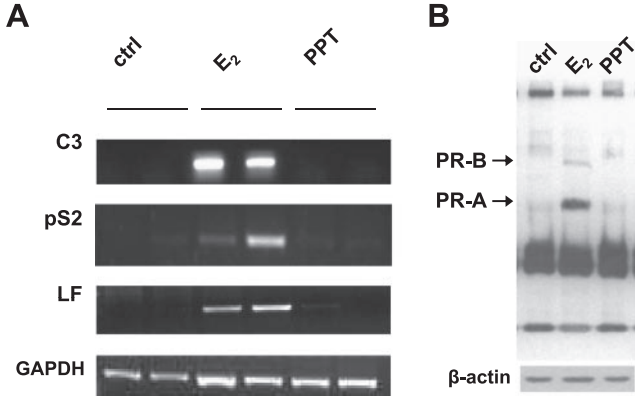

Figure 8. Expression level of estrogen target genes in the uterus. *A*) mRNA levels for complement 3 (C3), pS2 and lactoferrin (LF) genes were analyzed by endpoint PCR on uterine tissue collected from ovariectomized animals treated for 5 d with vehicle (ctrl), E2, or PPT. PCR amplification products were run on agarose gel using 2 animals/experimental group. *GAPDH* gene was analyzed as control. *B*) Western blot analysis of progesterone receptors in uterine protein extracts from ovariectomized animals treated for 5 d with vehicle, E2, or PPT. Detection of β-actin was performed as a loading control.

The potential relevance of the present findings to the human setting is supported by increased *in vitro* adhesion and proliferation of human EPCs in response to PPT treatment. Thus, the ERα-selective agonist shares the ability of E2 to promote generation and adhesion of EPCs, as demonstrated in a recent study using analogous EPC isolation and culture conditions (34). Human EPCs express both ER isoforms, and the selective ERα antagonist methyl-piperidinethoxyphenol-pyratole (MPP) was shown to prevent E2 action. This is consistent with our results, although a role for ERβ in EPC regulation cannot be ruled out (37). Further translation of the present findings to human settings remains to be performed.

Uterus weight gain is regarded as a bioassay for ERα selectivity and target engagement. Although ligands with preferential binding affinity for ERβ have shown some vasculoprotective effects in the absence of uterus stimulation (39, 40), our study shows that widespread protective cardiovascular responses to ERα activation following PPT treatment *in vivo* also occurred in the absence of uterotrophic response. In previous studies, PPT doses of 1 or 2 mg/kg were associated with

uterotrophic effects (18, 41). By contrast, we administered 16.7 μg PPT/rat (~67 μg/kg) in Silastic capsules implanted subcutaneously for 5 d in parallel with an equimolar dose of E2 known to be both uterotrophic and vasculoprotective (13, 25). On the basis of published data (18, 42), this treatment regimen is expected to yield plasma levels of PPT and E2 in the nanomolar range. The mechanisms underlying the uterus-sparing action of PPT as opposed to E2 may include differential regulation of coactivator activity (18, 23), potency separation between different estrogenic endpoints *in vivo*, and favorable pharmacokinetics featuring preferential targeting of the vascular tree, as confirmed by the comparable effects of PPT and E2 on uterine vessel density (Fig. 7). Remarkably, PPT elicited no significant effects on uterine gland density and the myometrial-to-endometrial ratio, nor affected the expression profile of classical target genes, consistent with the compartmentalization of uterine effects of ER ligands in OVX rats (43). Because PPT could still affect vascular function without a uterotrophic effect when administered at a higher dose (~100 μg/kg) for longer time (4 wk), as shown in Supplemental Figs. 1 and 2, the therapeutic window might be of clinical relevance.

In summary, our study shows that selective ERα activation by PPT recapitulates a wide range of beneficial effects of E2 treatment on the heart and vasculature in the absence of untoward uterotrophic action in estrogen-deprived rats. Whether this scenario applies to postmenopausal women is worth further investigation. FJ

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