Estradiol increases angiotensin II type 1 receptor in hearts of ovariectomized rats

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Abstract

We tested the hypothesis that 17β-estradiol (E_2) has dual effects on the heart, increasing levels of proteins thought to have beneficial cardiovascular effects (e.g. endothelial nitric oxide (NO) synthase (eNOS)) as well as those thought to have detrimental cardiovascular effects (e.g. type 1 angiotensin II (Ang II) receptor (AT_1R)). Ovariectomized Wistar rats consuming a high-sodium diet received one of four treatments ($n=7$ per group): group 1, placebo pellets; group 2, E_2 (0·5 mg/pellet, 21-day release); group 3, NOS inhibitor, N^{ω}-nitro-L-arginine-methyl-ester (L-NAME; 40 mg/kg per day for 14 days) plus Ang II (0·225 mg/kg per day on days 11–14); group 4, E_2 plus L-NAME/Ang II. E_2 increased cardiac levels of estrogen receptors ESR1 and ESR2, an ESR-associated membrane protein caveolin-3, eNOS, and phosphorylated (p)eNOS, thus, exerting potentially beneficial cardiovascular effects on NO. However, E_2 also increased cardiac levels of proteins associated with cardiovascular injury and inflammation including, AT_1R, protein kinase C delta (PRKCD), phosphorylated PRKC, and phosphorylated extracellular signal regulated kinase (pMAPK)3/1, plasminogen activator inhibitor-1 (PAI-1), osteopontin and ED-1, a monocyte/macrophage-specific protein. E_2 treatment led to similar protein changes in the hearts of L-NAME/Ang II-treated rats except that the increase in peNOS was prevented, and L-NAME/Ang II and E_2 had additive effects in increasing cardiac PRKCD and PAI-1. Thus, the highest levels of cardiac PAI-1 and PRKCD occurred in L-NAME/Ang II-treated rats receiving E_2. In summary, E_2 treatment increased cardiac expression of AT_1R as well as the expression of pro-inflammatory and prothrombotic factors.

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Background

The incidence of cardiovascular disease among women is low before menopause and steadily increases after the onset of menopause (Mendelsohn & Karas 1999). This increase is believed to result in part from the loss of endogenous estrogen and its associated cardioprotective effects (Stampfer *et al.* 1991). In observational human studies, estrogen replacement therapy in postmenopausal women is associated with a reduced risk of cardiovascular disease (Pinto *et al.* 1997). However, the Women's Health Initiative (WHI) study (Rossouw *et al.* 2002) and the Heart and Estrogen/Progestin Replacement Study (Hulley *et al.* 1998) do not support the concept that hormone replacement therapy protects the cardiovascular system and, in fact, suggest the opposite view that such therapy may increase the risk of cardiovascular disease. Further analysis of the WHI data suggests that estrogen plus progesterone therapy was beneficial in healthy, young postmenopausal women, but increased cardiovascular risk when treatment was initiated in older postmenopausal women with established coronary artery disease (Herrington *et al.* 2000, Manson *et al.* 2003). The reasons for the disparate results regarding the cardiovascular effects of estrogen are controversial in part due to an incomplete understanding of the mechanism underlying estrogen's effects on the cardiovascular system.

Many experimental studies in animals and isolated cells support the belief that estrogen protects the cardiovascular system (Huang *et al.* 2000) via activation of estrogen receptors (ESR)-1 and 2 (Mendelsohn & Karas 1999). Animal studies show beneficial effects of 17β-estradiol (E_2) on atherosclerosis (Hayashi *et al.* 1992), inflammation (Koh 2002), and endothelial or vascular function (Gorodeski *et al.* 1995, Crews & Khalil 1999). Studies also demonstrate that estrogen modulation of endothelial nitric oxide (NO) synthase (eNOS) may be a mechanism of cardiac protection (Brunner *et al.* 2003, Khalil 2005).

Other studies suggest that estrogen activates the renin–angiotensin (Ang)–aldosterone system (RAAS), which could be a mechanism of cardiac injury. In humans, estrogen increases circulating levels of Ang II (Schunkert *et al.* 1997) and intrarenal Ang II activity (Seely *et al.* 2004), which is associated with a

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decrease in renal blood flow. In animal models of cardiovascular injury due to an activated RAAS, estrogen increases stroke and renal injury (Stier *et al.* 2003, Oestreicher *et al.* 2006). This increase in renal injury is associated with an increase in renal cortical levels of Ang II type 1 receptor (AT_1R) protein and mRNA (Oestreicher *et al.* 2006).

As estrogen stimulates expression of some proteins that might have beneficial cardiovascular effects as well as others that might have detrimental effects, the goal of this study was to determine the balance of E_2 effects on cardiac proteins involved in the early steps of cardiac injury. We examined the effects of E_2 replacement in ovariectomized (OVX) rats on cardiac levels of eNOS, AT_1R, and AT_1R signaling pathways, and inflammatory and prothrombotic proteins. Furthermore, we tested the hypothesis that the adverse cardiac effects of E_2 would predominate in a rodent model of cardiovascular injury induced by high Ang II and impaired NO production (Rocha *et al.* 2000, Martinez *et al.* 2002, Oestreicher *et al.* 2003). In this rat model, treatment with Ang II and the NOS inhibitor N^{ω}-nitro-L-arginine-methyl-ester (L-NAME) causes cardiac inflammation and increases the prothrombotic factor plasminogen activator inhibitor (PAI-1; Oestreicher *et al.* 2003).

Materials and Methods

Experimental animals

Experiments used 10-week-old female Wistar rats (Charles River Lab, Wilmington, MA, USA) that underwent bilateral ovariectomies (OVX). Rats had ad libitum access to drinking fluid. They were housed in individual metabolic cages in a climate-controlled environment (22 ± 1 °C) with a 12 h light:12 h darkness cycle. All rats received 1% NaCl to drink. Rats were killed at the end of the 14-day treatments without respect to timing of the 4-day estrous cycle and the hearts were collected and frozen immediately. At this time, blood was also collected for determination of E_2 and aldosterone levels. All experimental procedures met guidelines of the Institutional Animal Care and Use Committee at Harvard University.

Experimental procedures

We examined the following groups of rats receiving Purina Lab Chow 5001 (Ralston Purina Co., St Louis, MO, USA) and 1% NaCl to drink: 1) OVX rats implanted with pellets containing placebo and minipumps containing saline, $n=7$; 2) OVX rats implanted with E_2 pellets, $n=7$; 3) OVX rats implanted with placebo pellets and receiving L-NAME/Ang II treatment, $n=7$; and 4) OVX rats implanted with E_2 pellets and receiving L-NAME/Ang II treatment, $n=7$. Pellets containing E_2 (#E121, Innovative Research of America, Sarasota, FL, USA, 0·5 mg/pellet, 21-day release) or placebo (#C111, Innovative Research of America) were implanted subcutaneously in each rat 7–10 days after ovariectomy. These E_2 pellets were designed to achieve plasma estradiol levels in the high–normal physiological range for cycling female rats (100–150 pg/ml). One week after implantation of the pellets, animals were treated with L-NAME/Ang II as described previously (Oestreicher *et al.* 2006). Briefly, rats received drinking water containing 1% NaCl. L-NAME (Sigma, 40 mg/kg per day) was administered for 14 days via a subcutaneously implanted pellet (Innovative Research of America). Saline or Ang II (Sigma, 0·225 mg/kg per day) was administered via Alzet osmotic minipumps (Model 2001, Durect Corporation, Cupertino, CA, USA; 1·0 µl/h, 7 days) for the final 3 days. Pellets and minipumps were implanted under general anesthesia using isoflurane. On day 14, death was induced by administration of isoflurane followed by the immediate collection of blood and hearts.

Histological evaluation

The heart tissue for histological evaluation was processed into paraffin blocks. The heart sections (5 µm) were stained with hematoxylin and eosin and examined using light microscopy by a pathologist unaware of the treatment group assignment. The histologic sections of the hearts were scored for myocardial damage on a scale from 0 to 4 as follows: 0, normal histology; 1, focal interstitial inflammatory infiltrates without myocyte injury; 2, a single focus of interstitial inflammatory infiltrate associated with myocyte injury; 3, two or three foci of interstitial inflammatory infiltrates associated with myocyte injury; and 4, four or more foci of inflammatory infiltrates associated with myocyte injury.

Measurements and assays

Daily food intake, water intake, body weight, and urine output were recorded. Systolic blood pressure (SBP) was measured in conscious animals by tail-cuff plethysmography (Blood Pressure Analyzer, Model 179, IITC Life Science, Woodland Hills, CA, USA). Plasma estradiol was measured with the DPC Double Antibody Estradiol (analytical sensitivity of 1·4 pg/ml) as described previously (Oestreicher *et al.* 2006). Aldosterone was measured using the DPC Coat-A-Count Aldosterone RIA as described previously (Turchin *et al.* 2006; DPC Diagnostic Products, Los Angeles, CA, USA).

Western blot analysis

The heart tissues were homogenized in 1 ml ice-cold lysing solution (Bio-Rad cell lysis kit – catalog #171-304012). The ground tissue was transferred to a clean microcentrifuge tube and frozen at −70 °C. Homogenates were then thawed and sonicated on ice (Fisher Sonic Dismembrator, model 300, Fisher Scientific, Pittburgh, PA, USA). Samples were then centrifuged at 4800 ***g*** for 4 min at 4 °C. Supernatant was collected without disturbing the pellets. Protein concentration in the supernatant was determined using modified Lowry assay (RC DC protein assay, Bio-Rad catalog #500-0119, Bio-Rad). Supernatants (20 µg of protein concentration) were combined

at least 1:2 with sample buffer (62·5 mM Tris, pH 6·8, 2% SDS, 25% glycerol, 0·01% bromophenol blue, 200 mM β-mercaptoethanol), heated at 95 °C for 4 min, and size fractionated by electrophoresis on 12·5% SDS-polyacrylamide gels using 1× of the following 10× buffer: 250 mM Tris base, 1·92 M glycine, 34·7 mM SDS. Proteins were electrophoretically transferred to Hybond-ECL nitrocellulose membranes (Amersham Bioscience) using the following transfer buffer: 25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8·3. The membranes were blocked in 5% nonfat dried milk in PBS-T (80 mM Na_2HPO_4 anhydrous, 20 mM NaH_2PO_4, 100 mM NaCl, and 0·1% Tween 20) for 1 h at room temperature on an orbital shaker. Primary antibody incubation was incubated overnight at 4 °C with antibody diluent consists of 1% nonfat dried milk in PBS-T. Equal loading was assessed by reprobing membranes with an antibody to β-actin (1:20 000; Clone AC-15, Sigma). After overnight incubation, the bound antibody was detected by enhanced chemiluminescence (Western Lightning Reagent Plus, Perkin–Elmer Life Sciences, Boston, MA, USA) with HRP-conjugated goat anti-rat IgG (sc-2006, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, dilution 1:3000), or goat anti-mouse IgG (sc-2005, Santa Cruz, dilution 1:5000), or goat anti-rabbit IgG (sc-2004, Santa Cruz, dilution 1:5000). Developed X ray films were scanned and densitometric analysis was performed with the ImageQuant 5.2 software (Molecular Dynamics, Piscataway, NJ, USA). To control for inter-gel variations we used the following procedure. On each 15-well mini-gels, we analyzed three to four samples from each of the four treatment groups; two samples were used for normalization between mini-gels. All western blots were re-probed once with anti-β-actin antibody and the protein of interest was normalized to β-actin to correct for loading variability. Samples were re-analyzed on a separate western blot to confirm results. All values were expressed relative to the average of the OVX rats receiving control treatment.

Antibodies

We used the following antibodies to detect the proteins and receptors of interest by western blot: ESR1 (#GR17, Calbiochem, San Diego, CA, USA, dilution 1:1000); ESR2 (#sc-8974, Santa Cruz Biotechnology Inc., dilution 1:500); and AT_1R (#sc-1173, Santa Cruz, dilution 1:1000). The specificity of the antibody to AT_1R was confirmed by receptor binding assays as described previously (Oestreicher *et al.* 2006); CAV3 (#RDI-CAVEOL3abrx, Research Diagnostics, Concord, MA, USA, dilution 1:10 000); eNOS (#N30030/L14, BD Transduction Laboratories, San Jose, CA, USA, dilution 1:2500); PRKCD (#610397BD Transduction Laboratories, dilution 1:1000); PAI-1 (#612024, BD Transduction Laboratories, dilution 1:2500); ED-1 (#554954, BD Transduction Laboratories, dilution 1:1000); phosphorylated protein kinase C (pPRKC; #9371S, Cell Signaling, Danvers, MA, USA, dilution 1:1000); and pMAPK3/1 (#9101S, Cell Signaling, dilution 1:2000); peNOS (9571S, Cell Signaling, dilution 1:1000. The peNOS antibody is directed against phosphorylated serine 1177. This site is specific for eNOS activation.); and OPN (#ab8448, Abcam, Cambridge, MA, USA, dilution 1:5000).

Statistical analysis

The statistical significance of the differences between the group means for the data were determined by one-way ANOVA followed by Newman–Keuls *post hoc* test for multiple comparisons. $P \leq 0{\cdot}05$ was considered statistically significant. Values are expressed as mean ± S.E.M.

Results

L-NAME, Ang II, and E_2 effects on cardiac histology

Treatment with L-NAME and Ang II caused a significant increase in cardiac damage compared with control treatment (Figs 1 and 2). Damaged hearts showed inflammatory infiltrates associated with myocyte injury (Fig. 1C and D). E_2 treatment had no significant effect on cardiac histology in the control NaCl-treated OVX rats or in the L-NAME/Ang II-treated OVX rats (Figs 1B, D and 2).

E_2 effects in healthy young OVX rats

OVX rats receiving 1% NaCl in the drinking water were implanted with subcutaneous pellets containing either placebo or E_2. After 14 days, E_2 levels were significantly higher in the OVX rodents receiving E_2 when compared with those not receiving E_2 (Table 1). Consistent with the known effects of E_2 in rodents, OVX rats receiving E_2 had lower body weights, higher uterine weights, and higher uterine/body weight ratios than OVX rats not receiving E_2 (Table 1). SBP and heart weights were similar in the E_2- and placebo-treated OVX rats (Table 1).

Protein levels of ESR1 (Fig. 3A) and ESR2 (Fig. 3B) were increased in the heart tissues of OVX rats receiving E_2 treatment when compared with OVX rats not treated with E_2 (1·3-fold increase for ESR1, $P<0{\cdot}05$ and 1·6-fold increase for ESR2, $P<0{\cdot}001$). Furthermore, E_2 treatment increased cardiac levels of eNOS ($P<0{\cdot}05$) and phosphorylated peNOS ($P<0{\cdot}05$), the active form of eNOS, when compared with estrogen-deficient OVX rats (Fig. 4A and B), a result consistent with the known effects of estrogen. E_2 treatment also resulted in higher cardiac levels of CAV3 (1·55-fold increase, $P<0{\cdot}01$, Fig. 4C), a caveolae protein that is part of the E_2 signaling pathway in cardiomyocytes.

Plasma aldosterone levels were significantly elevated in rats receiving E_2 when compared with animals that did not receive E_2 (Table 1). Furthermore, E_2 treatment increased protein levels of AT_1R in the heart homogenates when compared with OVX rats not receiving E_2 (Fig. 5A, $P<0{\cdot}05$).

Cardiac levels of PRKCD were increased by 2·5-fold (Fig. 5B, $P<0{\cdot}01$), pPRKC by 2·2-fold (Fig. 5C, $P<0{\cdot}05$), and pMAPK3/1 by 1·3-fold (Fig. 5D, $P<0{\cdot}05$) in rats implanted with E_2 pellets versus those implanted with placebo pellets.

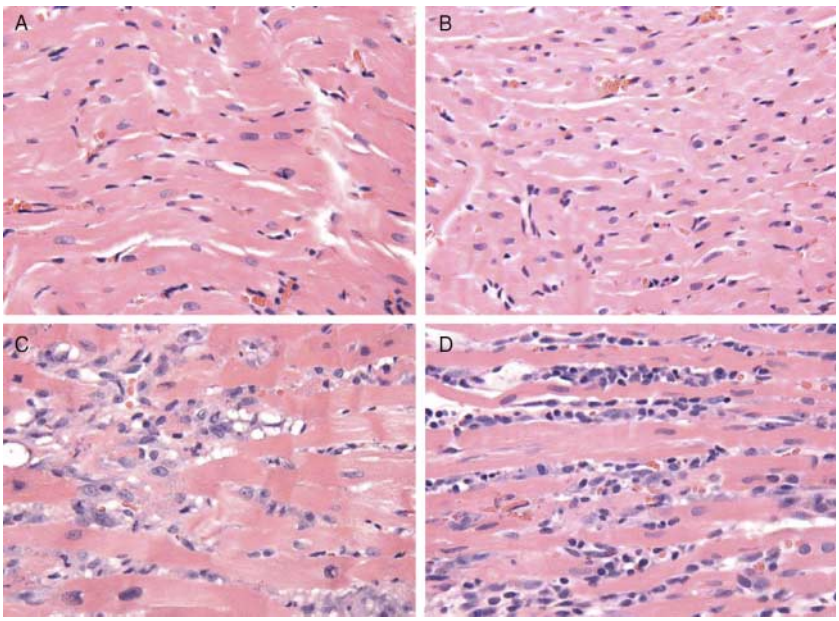

Figure 1 Pathologic assessment of myocardial injury. Shown are representative histological sections of the myocardium stained with hematoxylin and eosin at 400× magnification. In the OVX rats treated with placebo (A) or estrogen (B), there is essentially normal histology. In the L-NAME/Ang II-treated rats treated either with placebo (C) or estrogen (D) there are inflammatory infiltrates associated with myocyte injury. Full colour version of this figure available via http://dx.doi.org/10.1677/JOE-08-0199.

We determined the effect of E_2 on cardiac expression of PAI-1 (an E_2-responsive prothrombotic factor (Smith *et al.* 2004), the chemokine osteopontin (OPN), and ED-1 (a protein expressed by monocytes/macrophages)). The cardiac levels of PAI-1 protein were increased in rats receiving E_2 when compared with those not receiving E_2 (Fig. 6A, $P<0{\cdot}01$). Furthermore, E_2 treatment significantly increased cardiac levels of ED-1 (Fig. 6B, $P<0{\cdot}01$) and OPN (Fig. 6C, $P<0{\cdot}05$). Thus, E_2 increases prothrombotic and inflammatory factors in cardiac tissue in OVX female rats that were otherwise healthy.

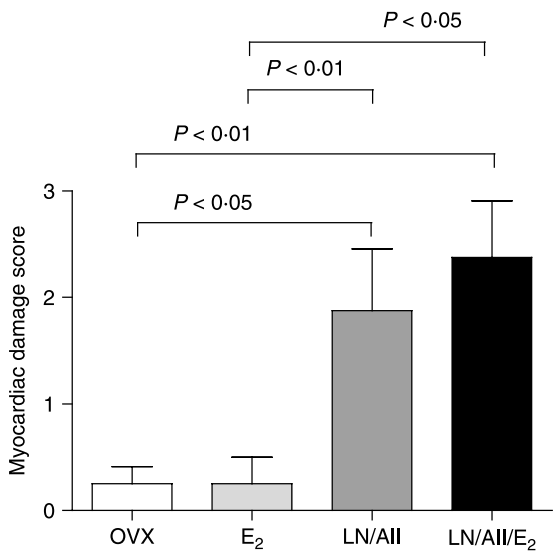

Figure 2 Myocardial damage scores in ovariectomized rats receiving placebo (OVX), estrogen (E_2), L-NAME/Ang II (LN/AII), and L-NAME/Ang II plus E_2 (LN/AII/ E_2).

E_2 effects in OVX rats receiving L-NAME/Ang II

OVX rats treated with L-NAME/Ang II plus E_2 had higher blood levels of E_2, decreased body weight, increased uterine weight, and increased uterine/body weight ratio compared with OVX rats receiving L-NAME/Ang II (Table 1). E_2 treatment did not affect the heart weight or SBP of L-NAME/Ang II-treated rats. As occurred in rats drinking 1% NaCl, E_2 treatment increased cardiac levels of ESR1 and ESR2 in rats receiving L-NAME/Ang II, a treatment that itself did not affect ESR levels (data not shown).

L-NAME/Ang II treatment alone did not alter cardiac levels of eNOS, peNOS, or AT_1R, nor plasma levels of aldosterone. By contrast, E_2 treatment increased protein levels of eNOS (Fig. 7A) in the hearts from L-NAME/Ang II-treated rats. The magnitude of this E_2 effect in the L-NAME/Ang II/NaCl-treated rats was similar to that observed in OVX rats drinking 1% NaCl. However, E_2 treatment did not increase peNOS levels in rats receiving L-NAME/Ang II (Fig. 7C). As occurred in OVX rats drinking 1% NaCl, E_2 treatment increased plasma aldosterone and cardiac AT_1R levels in rats receiving L-NAME/Ang II/NaCl.

Table 1 Effects of E_2 treatment on body, uterine and heart weights, blood pressure, and E_2 and aldosterone levels. All values are mean ± S.E.M.; $n=7$. The statistical significance of the differences between group means for the data was determined by one-way ANOVA followed by Newman–Keuls *post hoc* test for multiple comparisons

	OVX		OVX/L-NAME/Ang II	
	Placebo	E_2	Placebo	E_2
E_2 (pg/ml)	11·2±2·1	212·6±130·8*	10·3±0·9	168·0±85·7†
ALDO (ng/dl)	15·3±4·8	39·0±5·0*	11·51±5·3	33·5±5·7†
SBP (mmHg)	111·0±5·0	110±4·0	153±7·0‡	158±5·0§
BW (g)	324·0±6·6	255±11·0*	308±3·4	251±7·9†
HW (g)	1·1±0·1	1·14±0·03	1·14±0·02	1·09±0·04
UW (g)	0·10±0·01	0·70±0·09*	0·10±0·01	0·70±0·09†
UW/BW (mg/g)	0·31±0·01	2·70±0·3*	0·33±0·01	2·72±0·32†

E_2, estradiol; ALDO, aldosterone; SBP, systolic blood pressure; UW, uterine weight. *$P\leq0{\cdot}05$ for OVX/placebo versus OVX/E_2, †$P\leq0{\cdot}05$ for OVX/L-NAME/Ang II placebo versus OVX/L-NAME/Ang II/E_2. ‡$P\leq0{\cdot}05$ for OVX/placebo versus OVX/L-NAME/Ang II/placebo, §$P\leq0{\cdot}05$ for OVX/E_2 versus OVX/L-NAME/Ang II/E_2.

Both E_2 and L-NAME/Ang II treatment increased cardiac levels of PRKCD and PAI-1, and these effects were additive (Fig. 8A and B). Finally, while E_2 increased cardiac levels of pPRKC, pMAPK3/1, ED-1, and OPN in L-NAME/Ang II/NaCl-treated animals as occurred in the rats receiving 1% NaCl alone, L-NAME/Ang II treatment did not affect these factors and there was no additive effect of these two treatments (data not shown).

Discussion

These studies determined the cardiac effects of E_2 treatment in OVX female rats. In OVX, but otherwise healthy female rats, E_2 increased cardiac expression of eNOS and peNOS, which would be expected to enhance NO production and, thus, have a beneficial cardiac effect. However, E_2 also increased cardiac levels of AT_1R and other factors (PAI-1, OPN, ED-1, and PRKCD) known to induce inflammation, thrombosis, and/or cardiac damage. In the model of cardiovascular injury induced by Ang II and NOS inhibition, the E_2-mediated increase in peNOS was lost, while E_2-mediated increases in cardiac AT_1R, PAI-1, OPN, ED-1, and PRKCD were maintained. Furthermore, E_2 acted additively with L-NAME/Ang II treatment to increase cardiac levels of PAI-1 and PRKCD. Thus, these data demonstrate that E_2 increases the expression of cardiac proteins that have beneficial cardiac effects (peNOS) as well as those that have detrimental cardiac effects (e.g. AT_1R and PAI-1). When the beneficial effects of E_2 on peNOS were blocked with a NOS inhibitor, the detrimental effects of E_2 dominated.

Our results are consistent with the well-known beneficial effect of E_2 to increase peNOS leading to increased NO and improved vasodilation (Reis *et al.* 1994, Collins *et al.* 1995). We also demonstrated an increase in pMAPK3/1 with E_2 treatment. This latter result is consistent with studies demonstrating the activation of the MAPK3/1 pathway by estrogen in multiple cell types and tissues, including endothelial cells (Gorodeski *et al.* 1995), neuronal cells (Alexaki *et al.* 2006), smooth muscle cells (Keyes *et al.* 1996), and myocardium (Patten *et al.* 2004, Pedram *et al.* 2005). These non-genomic effects of estrogen are mediated via its two receptors, ESR1 and ESR2 (Mendelsohn & Karas 1999). We demonstrated that E_2 increased expression of both ESR1 and ESR2 in the heart tissues, consistent with other reports showing that E_2 replacement increases expression of ESR1, and ESR2 in the heart tissues from aged rats (Xu *et al.* 2003). In our study, the increase of ESR2 was greater than that of ESR1, possibly due to differential effects of estradiol on synthesis and/or degradation of ESR subtypes (Barchiesi *et al.* 2004). ESRs interact with the caveolae anchoring protein CAV3 to mediate the rapid, non-genomic effects of estrogen and other steroids, and increasing CAV3 levels tends to inhibit eNOS activation (Hisamoto & Bender 2005). Estrogen treatment increased CAV3 in our studies, raising the possibility that estrogen-mediated changes in caveolin levels modulate the effects of estrogens and other steroids on intracellular signaling pathways (Feron & Kelly 2001, Damy *et al.* 2004, Williams & Lisanti 2004).

The observation that E_2 increases protein levels of AT_1R in the hearts of healthy, OVX rats and L-NAME/Ang II-treated rats is consistent with the reports that E_2 increases AT_1R expression in the uteri of healthy rats (Krishnamurthi *et al.* 1999) and in the renal cortex of rats receiving L-NAME/Ang II (Oestreicher *et al.* 2006). In the latter study, the level of AT_1R expression correlated with proteinuria (Oestreicher *et al.* 2006). Similarly, estrogen has been shown to increase cardiac and renal injury in other animal models characterized by an activated RAAS. In the stroke prone spontaneously hypertensive rat, ovariectomy reduced stroke and renal injury, while estrogen replacement increased this injury (Stier *et al.* 2003). The hypertensive mRen2.Lewis rat is a transgenic rat strain carrying the mouse ren-2 renin gene backcrossed into the inbred Lewis rat. In the hypertensive mRen2.Lewis female rat, ovariectomy reduced proteinuria, renal injury, and blood levels of the inflammatory marker C-reactive protein in older, 64-week-old mRen2.Lewis rats on a high-salt diet (Yamaleyeva *et al.* 2007). Our observation that E_2 and L-NAME/Ang II treatment have additive effects on cardiac PAI-1 is consistent with these studies, and together these

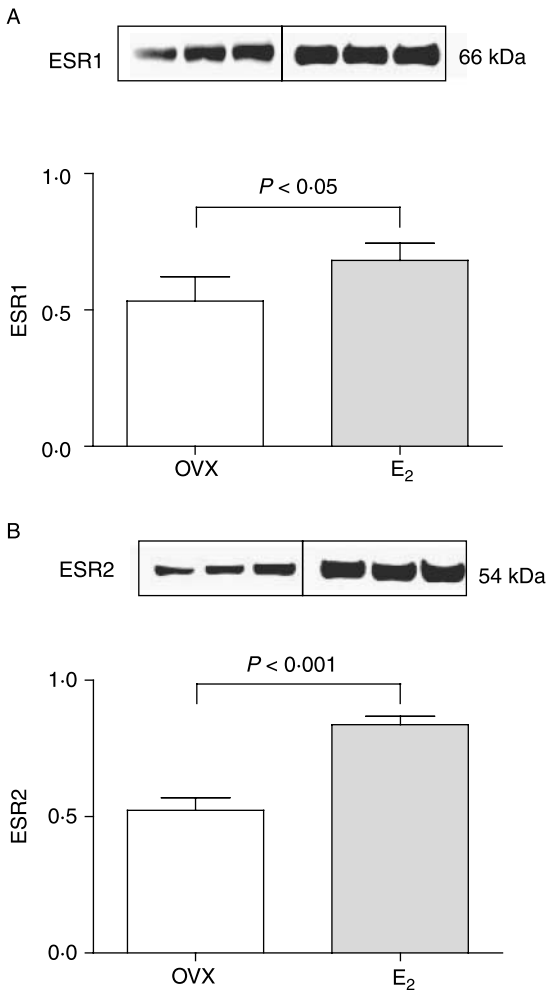

Figure 3 E_2 treatment increases ESR1 and ESR2 protein expression in the heart tissue of OVX rats. Western blot of heart tissue showing results for three representative animals (20 µg of total protein per lane, each lane represent an individual animal) from OVX rats receiving placebo (OVX) and OVX E_2-treated rats (E_2). (A) 66 kDa band for ESR1 and (B) 54 kDa band for ESR2.

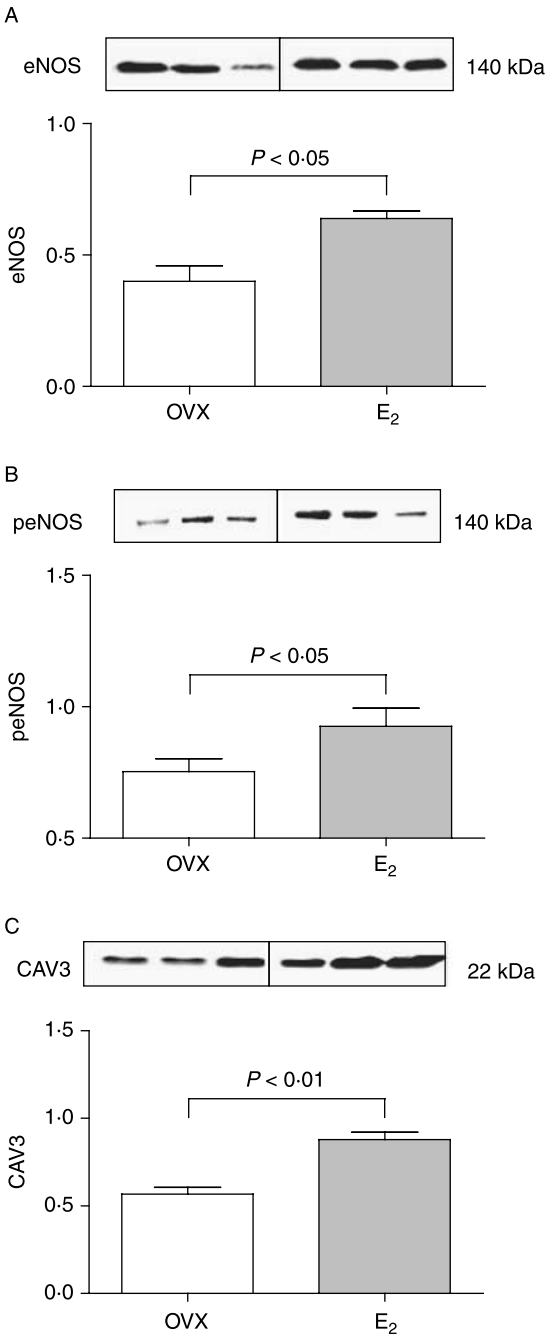

Figure 4 E_2 treatment increases eNOS, peNOS, and CAV3 protein expression in the heart tissue of OVX rats. Western blot of heart tissue showing results for three representative animals (20 µg of total protein per lane, each lane represent an individual animal) from OVX rats receiving placebo (OVX) and OVX E_2-treated rats (E_2). (A) 140 kDa band for eNOS, (B) 140 kDa band for peNOS, and (C) 22 kDa band for CAV3.

animal studies demonstrate that E_2 promotes Ang II-mediated cardiovascular injury. AT_1R is expressed in endothelial cells, vascular smooth muscle cells, and cardiomyocytes (Bueno *et al*. 2000). Additional studies are needed to determine which cell types within the heart demonstrate altered AT_1R expression with estrogen treatment. Our observation that E_2 increases AT_1R protein provides a potential mechanism for the increase in intrarenal Ang II activity leading to a reduction in renal blood flow in postmenopausal women treated with estrogen (Seely *et al*. 2004).

In contrast to our observations, estrogen replacement was reported to decrease cardiac AT_1R, increase cardiac Ang II type 2 receptor, and improve heart remodeling in 1-year-old OVX rats (Xu *et al*. 2003). Estrogen also decreased AT_1R levels in the adrenal and pituitary glands of OVX rats

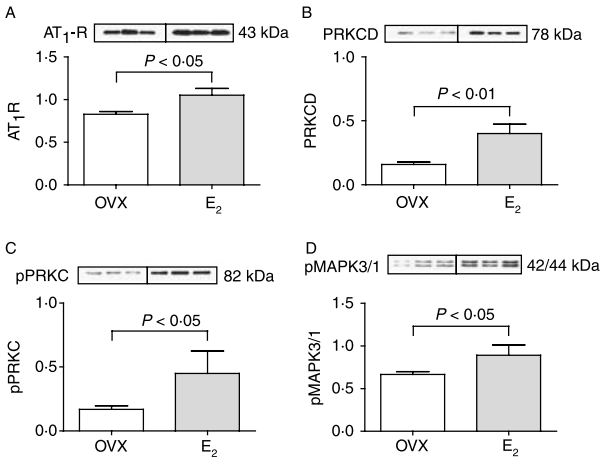

Figure 5 Effect of estrogen on AT_1R and intracellular signaling pathways. Western blot of heart tissue showing results for three representative animals (20 µg of total protein per lane, each lane represent an individual animal) from OVX rats receiving placebo (OVX) and OVX E_2-treated rats (E_2). (A) 43 kDa band for AT_1R, (B) 78 kDa band for PRKCD, (C) 82 kDa band for pPRKC, and (D) 42/44 kDa band for pMAPK3/1.

(Wu *et al.* 2003). It is likely that the effects of estrogen on AT_1R expression and Ang II-mediated injury differ depending on the experimental animal model. Factors such as age, genotype, dietary sodium intake, and underlying activity of the RAAS or NO system may modify the effects of estrogen. In our study, L-NAME treatment blocked the beneficial effects of E_2 on peNOS levels. Additionally, as E_2 increased cardiac levels of the Ang II receptor AT_1R, the co-administration of Ang II in our rat model further amplified the adverse cardiac effects of E_2 treatment.

In the present study, E_2 treatment increased plasma aldosterone levels in OVX rats receiving either placebo or L-NAME/Ang II. It is unlikely that E_2 increased systemic aldosterone levels through increases in adrenal AT_1R as other investigators have shown that E_2 decreases AT_1R levels in adrenal tissue (Wu *et al.* 2003). However, this increase may result from E_2-mediated increases in angiotensinogen leading to increases in Ang II, and thus increased adrenal aldosterone production (Klett *et al.* 1992, Gallagher *et al.* 1999).

It is now well established that aldosterone causes cardiovascular injury with activation of the mineralocorticoid receptor causing increases in PAI-1, vascular injury, and inflammation, as well as myocardial necrosis, inflammation, and fibrosis (Rocha *et al.* 2000, 2002, Oestreicher *et al.* 2003). Blockade of the mineralocorticoid receptor markedly reduces cardiovascular injury caused by L-NAME/Ang II treatment (Rocha *et al.* 2000, Oestreicher *et al.* 2003). Given the effects of E_2 on AT_1R and aldosterone, it would be of interest to determine whether mineralocorticoid receptor blockade prevents the adverse cardiovascular effects of E_2.

There are some limitations to these studies. Consistent with previous studies (Rocha *et al.* 2000), we did not detect a significant effect of Ang II on aldosterone levels in animals

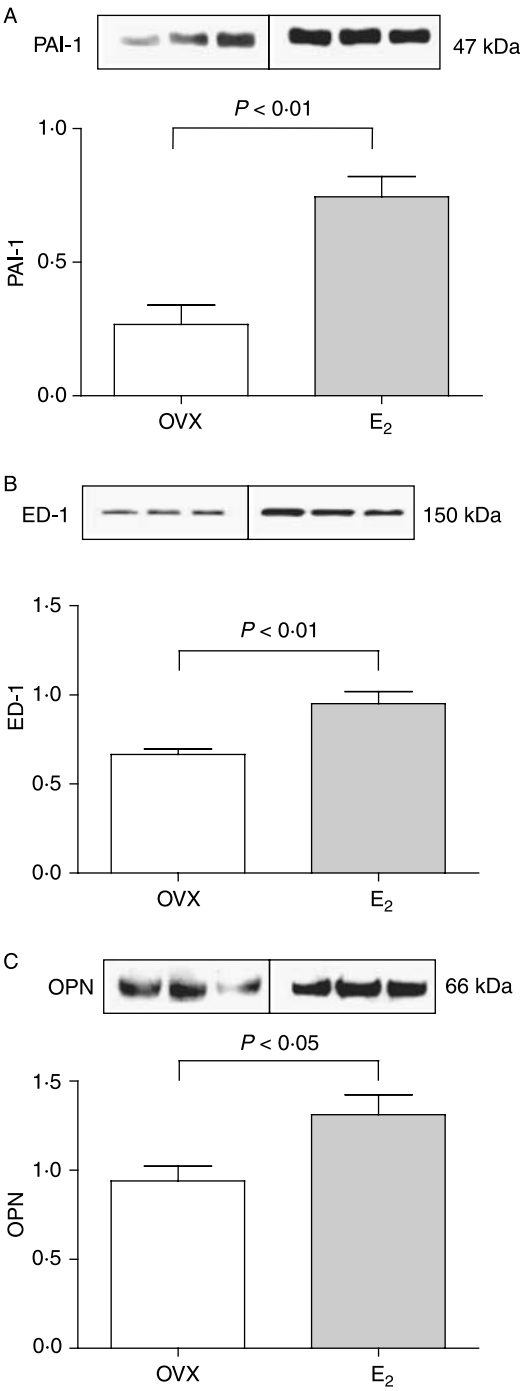

Figure 6 E_2 effect on fibrinolytic and inflammatory proteins in the heart. Western blot of heart tissue showing results for three representative animals (20 µg of total protein per lane, each lane represent an individual animal) from OVX rats receiving placebo (OVX) and OVX E_2-treated rats (E_2). (A) 47 kDa band for PAI-1, (B) 150 kDa band for ED-1, and (C) 66 kDa band for OPN.

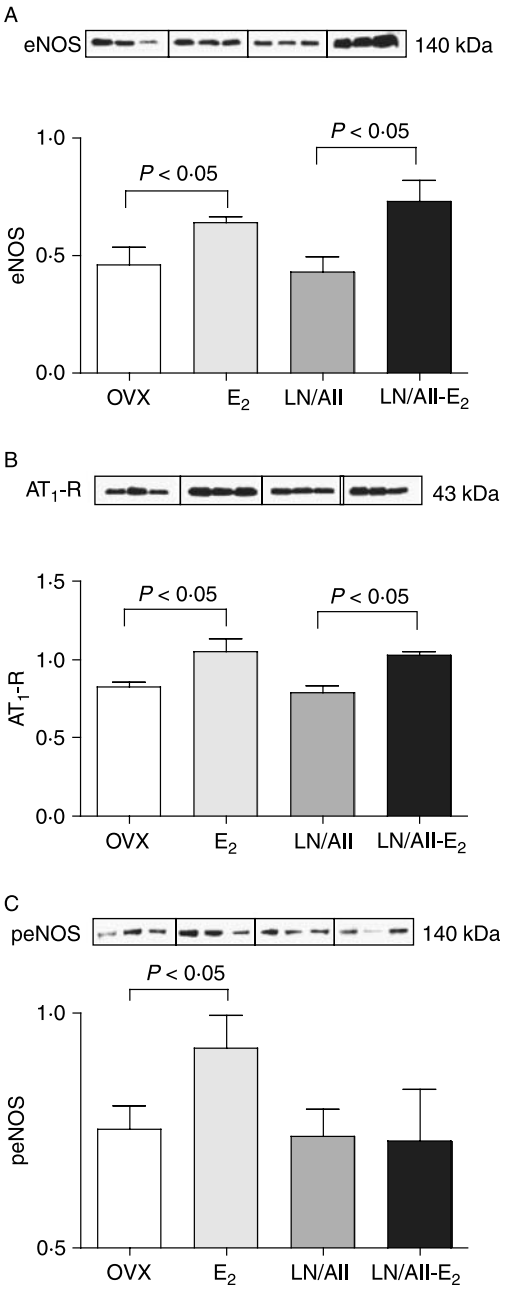

Figure 7 Effect of E_2 on eNOS, peNOS, and AT_1R expression in the hearts of OVX animals treated with L-NAME/Ang II when compared with E_2 effects in absence of L-NAME/Ang II. Western blot of heart tissue showing results for three representative animals (20 μg of total protein per lane, each lane represent an individual animal) from OVX rats receiving placebo (OVX), OVX E_2-treated rats (E_2), L-NAME/Ang II and placebo (LN/AII), and L-NAME/Ang II and E_2 treatments (LN/AII/E_2). (A) 140 kDa band for eNOS, (B) 43 kDa band for AT_1R, and (C) 140 kDa band for peNOS. For ease of comparison, OVX and E_2 data from Figs 4A, B and 5A are reproduced in this figure.

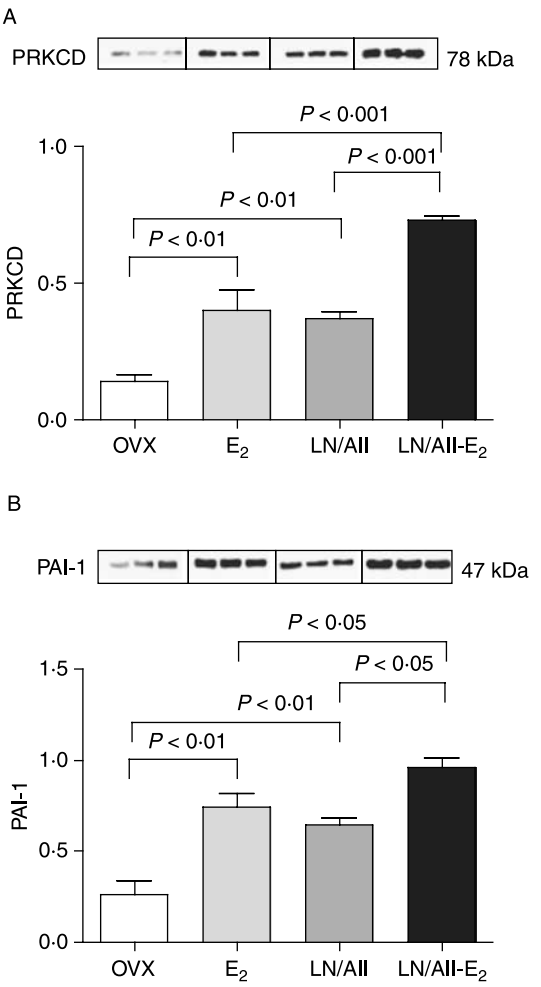

Figure 8 Effect of E_2 on PRKCD and PAI-1 expression in the hearts of OVX animals treated with L-NAME/Ang II when compared with E_2 effects in absence of L-NAME/ Ang II. Western blot of heart tissue showing results for three representative animals (20 μg of total protein per lane, each lane represent an individual animal) from OVX rats receiving placebo (OVX), OVX E_2-treated rats (E_2), L-NAME/Ang II and placebo (LN/AII), and L-NAME/Ang II and E_2 treatments (LN/AII/E_2). (A) 78 kDa band for PRKCD and (B) 66 kDa band for PAI-1. For ease of comparison, OVX and E_2 data from Figs 5B and 6A are reproduced in this figure.

receiving L-NAME and a high-salt diet. This is likely due in part to our using a low dose of Ang II that is a subpressor dose in the absence of L-NAME treatment. However, this Ang II dose suppresses plasma renin activity (Rocha *et al.* 2000) and it is possible that increases in aldosterone production would be detected using a more sensitive method such as 24-h urinary aldosterone levels. Our studies used 0·5 mg, 21-day E_2 pellets that are commonly used to assess effects of E_2 and are designed to raise E_2 levels into the range observed in pro-estrus (50–150 pg/ml; Klett *et al.* 1992). This experimental design did not allow us to determine whether there are different E_2 dose response characteristics for the beneficial and detrimental

cardiac effects of E_2. The wide range of E_2 values in the E_2-treated rats may have introduced variability and the presence of low E_2 levels of ~10–12 pg/ml in the OVX rats may have limited our ability to detect E_2 effects. These levels are consistent with the published reports of E_2 (11·3±3·6 pg/ml) in OVX rats and are likely attributable to non-ovarian sources of E_2 (Hugel *et al*. 1999). In addition, our experimental approach did not allow us to determine whether the cardiac effects of E_2 are due to direct effects of E_2 or are mediated through other factors. For example, E_2 treatment increased the cardiac AT_1R levels and the activation of either ESRs or AT_1R can increase PRKC pathways and PAI-1 (Smith *et al*. 2004, Alexaki *et al*. 2006). In our study, we used cardiac PAI-1 levels as a marker of early cardiovascular injury as described previously (Oestreicher *et al*. 2003). While E_2 treatment increased PAI-1 and other mediators of cardiovascular injury, the increase in these factors were not associated with a detectable increase in cardiac injury histopathology, possibly due to relative insensitivity of this method and to the relatively short duration of treatment.

These findings indicate that E_2 has diverse effects on the heart, some of which are beneficial (increases in eNOS and peNOS), and others of which are detrimental (increases in AT_1R, PAI-1, and cardiac inflammation). The relative balance of these effects may determine whether the overall effect of E_2 is beneficial or detrimental. Further elucidations of the factors that modify this balance are needed. The finding that E_2 increases cardiac expression of AT_1R has relevance to the mechanisms underlying the adverse cardiac effects of estrogen therapy in postmenopausal women.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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