17β-Estradiol Reduces Cardiomyocyte Apoptosis In Vivo and In Vitro via Activation of Phospho-Inositide-3 Kinase/Akt Signaling

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Abstract—Female gender and estrogen-replacement therapy in postmenopausal women are associated with improved heart failure survival, and physiological replacement of 17β-estradiol (E2) reduces infarct size and cardiomyocyte apoptosis in animal models of myocardial infarction (MI). Here, we characterize the molecular mechanisms of E2 effects on cardiomyocyte survival in vivo and in vitro. Ovariectomized female mice were treated with placebo or physiological E2 replacement, followed by coronary artery ligation (placebo-MI or E2-MI) or sham operation (sham) and hearts were harvested 6, 24, and 72 hours later. After MI, E2 replacement significantly increased activation of the prosurvival kinase, Akt, and decreased cardiomyocyte apoptosis assessed by terminal deoxynucleotidyltransferase dUTP nick-end labeling (TUNEL) staining and caspase 3 activation. In vitro, E2 at 1 or 10 nmol/L caused a rapid 2.7-fold increase in Akt phosphorylation and a decrease in apoptosis as measured by TUNEL staining, caspase 3 activation, and DNA laddering in cultured neonatal rat cardiomyocytes. The E2-mediated reduction in apoptosis was reversed by an estrogen receptor (ER) antagonist, ICI 182,780, and by phospho-inositide-3 kinase inhibitors, LY294002 and Wortmannin. Overexpression of a dominant negative-Akt construct also blocked E2-mediated reduction in cardiomyocyte apoptosis. These data show that E2 reduces cardiomyocyte apoptosis in vivo and in vitro by ER- and phospho-inositide-3 kinase–Akt–dependent pathways and support the relevance of these pathways in the observed estrogen-mediated reduction in myocardial injury. (*Circ Res*. 2004;95:692-699.)

Key Words: estrogen ■ estrogen receptors ■ myocardial infarction ■ cardiomyocyte ■ apoptosis ■ Akt ■ PI3 kinase

Heart failure is a growing public health problem,[1] with several studies demonstrating that women with heart failure have a better prognosis than men.[2,3,4,5,6,7] Whether endogenous sex hormones contribute to these differences in prognosis remains unknown. However, observational studies have demonstrated that postmenopausal women taking estrogen after a myocardial infarction (MI) have a lower incidence of heart failure.[8,9] Furthermore, retrospective analyses of multicenter heart failure trials have shown that postmenopausal women taking estrogen have a better prognosis than women not on estrogen,[10,11] supporting that estrogen may improve heart failure prognosis.

Several studies have demonstrated increased cardiomyocyte apoptosis in failing hearts,[12,13] and further evidence suggests that apoptosis contributes to heart failure progression.[14,15] Moreover, autopsy studies have shown that female gender is associated with less cardiomyocyte apoptosis in normal and failing hearts compared with males.[16,17,18,19] These observed gender differences in cardiomyocyte survival

provide a plausible explanation for the beneficial effect of female gender on heart failure progression.

We recently showed that physiological estrogen replacement in ovariectomized female mice reduces infarct size both early and late after left coronary ligation.[20] We also explored whether estrogen improves cardiomyocyte survival and reported that physiological estrogen replacement was associated with diminished cardiomyocyte apoptosis in the infarct zone and peri-infarct zone 24 hours after coronary ligation, but the mechanisms that mediate these effects are unknown. The aim of the present study was to characterize the effect of 17β-estradiol (E2) on cardiomyocyte survival after coronary ligation in mice and to explore the molecular pathways involved in these effects.

Materials and Methods

Materials

All chemical agents were obtained from Sigma unless otherwise specified. Anti-phospho-Ser473-Akt1, anti-Akt 1 (rabbit), and anti-
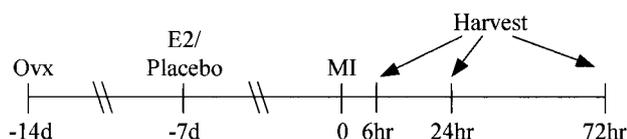
Figure 1. Experimental time-line of procedures used in the in vivo portion of these studies. d indicates day; hr, hour; Ovx, ovariectomy.

glycogen synthase kinase (GSK) 3β (mouse) were obtained from BD Biosciences; anti-phospho-Ser^9-GSK3β (rabbit), anti-phosho-Thr^{242}/Tyr^{248} extracellular signal regulated kinases (ERK1/2) and total ERKs were from Cell Signaling. Anti-sarcomeric α-actinin and anti-desmin antibodies were from Sigma. Horseradish peroxidase–tagged secondary antibodies and enhanced chemiluminescence reagents were from Amersham. Fluorescent-labeled secondary antibodies were from Jackson Immunolabs. Collagenase type II was from Worthington. LY294002 and zVAD-FMK were obtained from Calbiochem and ICI 182,780 (ICI) from Tochris Labs. The selective ER (estrogen receptor) β antagonist, R,R-tetrahydrochrysene (R,R-THC), was kindly provided by Dr Benita S. Katzenellenbogen (Department of Molecular and Integrative Physiology, University of Illinois, Urbana). The replication-deficient andenovirus encoding a dominant negative Akt construct (K179M mutation)[21] was a kind gift of Dr Lewis Cantley (Division of Signal Transduction, Beth Israel Deaconess Medical Center and Department of Systems Biology, Harvard Medical School, Boston, Mass). The adenovirus-GFP was constructed as described previously.[22]

Animals
Fifty-two female C57BL/6 mice (Charles River, NY), 6 to 8 weeks old, weighing 18 to 22 g were studied. Mice were housed at no >5 per cage in an Association for the Assessment and Accreditation of Laboratory Animal Care–approved animal facility and given free access to standard rodent chow (PROLAB, Syracuse, NY) and water. This protocol was approved by the Tufts-New England Medical Center Institutional Animal Care and Use Committee.

In Vivo Study Design
Figure 1 outlines the timeline for the surgical procedures performed. Mice underwent ovariectomy on day −14, and subcutaneous pellets containing either placebo or E2 (0.10 mg 21-day release) were placed 7 days later, as described.[20,23,24] Because of concerns regarding the effects of blood withdrawal on signaling-pathway activation, we were unable to directly measure plasma E2 levels. In multiple previous studies from our laboratory, administration of E2 in this manner resulted in physiological levels of E2 from 80 to 200 pg/mL.[20,23,24] Seven days later (day 0), animals underwent coronary artery ligation to induce MI (n=34) or sham (n=18) operation as described below. Mice were euthanized and hearts harvested at 6, 24, and 72 hours post–coronary ligation. Three shams were included in both the E2 and placebo-treated groups at each time point. Five MIs were included in the E2- and placebo-treated groups at the 6- and 24-hour time points. Seven animals were assigned to each treatment group (placebo-MI; E2-MI) at 72 hours. To confirm treatment assignment, the uterus of each animal was visually inspected for hypertrophy in animals treated with E2 versus atrophy in mice treated with placebo.

Left Coronary Ligation
MIs were induced as described previously[20,25] using inhalation of isoflurane (2.0 to 2.5% v/v) supplemented by intraperitoneal ketamine (45 mg/kg body weight) for anesthesia.

Tissue Harvest
Mice were anesthetized with 2.5% isoflurane gas. Silastic tubing was inserted into the right external jugular vein and 150 μL of 1% Evans Blue dye was infused to define the nonperfused myocardium. The chest was opened, the heart removed and immediately placed in ice-cold phosphate-buffered saline. Only hearts demonstrating in-

farctions that involved the anterior and apical regions were included in the analysis. The great vessels, atria, and right ventricle were removed, and the left ventricle was sectioned transversely into 4 to 5 slices using equally spaced microtome blades (see Supplemental Figure 1 in the online data supplement available at http://circres. ahajournals.org). From each slice, the infarct zone (with no Evans Blue staining), peri-infarct zone (defined as 1 mm of myocardium surrounding the infarct zone), and noninfarct zone were separated, snap frozen in liquid nitrogen (N_2), and stored at –70°C for biochemical assays. One whole transverse section was placed in OCT compound and frozen in liquid N_2-cooled isopentane for TUNEL staining.

Primary Neonatal Rat Cardiomyocyte Cultures
Primary cultures of neonatal rat cardiomyocytes were prepared as previously described.[26,27] Briefly, 1- to 2-day old Sprague-Dawley rats were euthanized, hearts excised, and ventricles minced in dissociation buffer (in mmol/L: 116 NaCl, 20 HEPES, 0.8 Na$_2$HPO$_4$, 5.6 glucose, 5.4 KCl, 0.8 MgSO$_4$, pH 7.35, with 0.6 mg/mL of pancreatin and 0.4 mg/mL collagenase type II). Serial digestions were performed at 37°C; cell pellets were resuspended in Ham's F10 with 10% horse serum and 5% charcoal-stripped fetal bovine serum containing 100 μmol/L bromo-deoxyuridine, 100 units/L penicillin and streptomycin, and preplated for 60 minutes. The cardiomyocyte-enriched fraction (>95% cardiomyocytes as determined by immunofluorescent staining) was plated at a density of 1×10^5/cm^2 on Primaria tissue culture plates (BD Falcon). Cardiomyocytes were grown in serum-containing media for 48 hours and placed in serum-free Dulbecco's modified essential medium (phenol red free) overnight. For adenoviral infection, a multiplicity of infection of 5 to 100 pfu/cell was applied to cells 24 hours after plating in Dulbecco's modified essential medium with 5% fetal bovine serum.

Western Blotting
Frozen segments of myocardium were pulverized in a mortar and pestle, cooled with liquid N_2, and placed in lysis buffer containing (in mmol/L): 50 NaCl, 50 NaF, 20 Tris-HCl, 10 EDTA, 20 Na$_4$P$_2$O$_8$, 1 Na$_3$VO$_4$, 1% Triton, 1 phenylmethylsulfonyl fluoride, 10 β-glycerophosphate, and 10 μmol/L microcystin with protease inhibitors. Cardiomyocytes were lysed in the same buffer. Samples were centrifuged and the protein concentration measured (BioRad, Inc). Lysates were resolved by SDS-PAGE, transferred to nylon membranes that were blocked with 5% nonfat milk in 0.05% Tween in phosphate-buffered saline. After application of primary and secondary antibodies, membranes were incubated with chemiluminescence reagent and exposed to film. Bands were quantified by computerized densitometry. Phospho-isoforms of specific kinases were corrected for total kinase levels measured on the same membranes after stripping. Where appropriate, the ratio of phospho-kinase/total kinase levels for sham or control groups was assigned an arbitrary value of 1, to which all pertinent data were normalized unless otherwise stated.

Terminal Deoxynucleotidyltransferase dUTP Nick-End Labeling
Five micrometer frozen sections were cut from transverse myocardial sections. Sections were postfixed in 1% paraformaldehyde followed by immersion in EtOH/acetic acid (v/v: 1/1) for 5 minutes. Sections were stained via the terminal deoxynucleotidyltransferase dUTP nick-end labeling (TUNEL) method as described.[20] Approximately 1500 nuclei were counted in the non- and peri-infarct zones. For cardiomyocytes, cells were counterstained with an anti-sarcomeric α-actinin antibody, and 500 cells were counted per coverslip. Counting of cells was performed by a blinded investigator.

Akt Kinase Assay
Pulverized myocardium was lysed in buffer containing (in mmol/L) 20 Tris (pH 7.5), 150 NaCl, 1 EDTA, 1 EGTA, 1% Triton, 2.5 sodium pyrophosphate, 1 β-glycerophosphate, 1 Na$_3$VO$_4$, 1 μg/mL Leupeptin, and cleared by centrifugation. Akt was immunoprecipi-
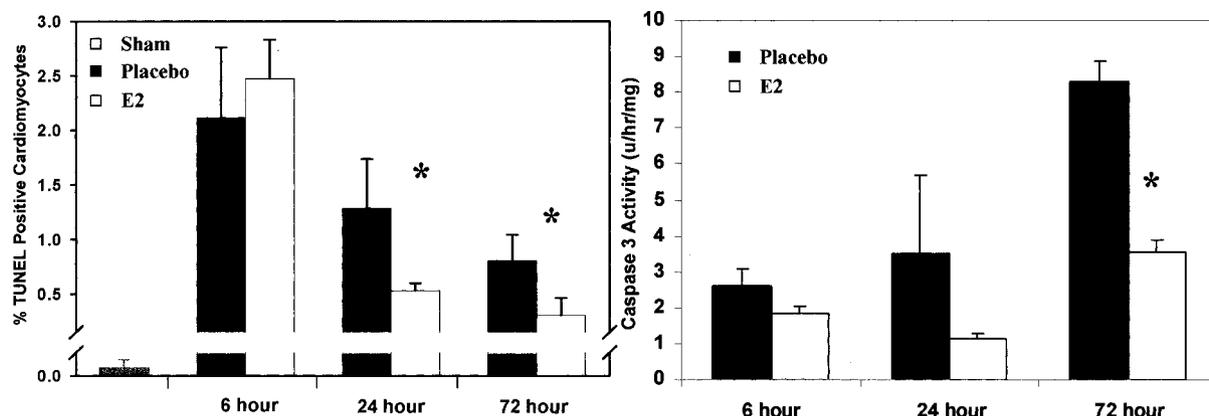
Figure 2. Physiological replacement of E2 reduces cardiomyocyte apoptosis in vivo. Left, Bar graph demonstrating percentage TUNEL-positive cardiomyocytes in the infarct border zone in mice treated with placebo (black bars) or E2 (white bars). Sham data are pooled and indicated in the first bar (gray). Bars represent the means±SEM. Right, Bar graph demonstrating caspase 3 activity in the infarct border zone in mice treated with placebo (black bars) or E2 (white bars). *$P<0.05$ vs placebo.

tated from equal amounts of protein lysate (200 μg) with a mouse monoclonal antibody bound to agarose (Cell Signaling). Reaction buffer containing (in mmol/L) 25 Tris (pH 7.5), 5 β-glycerophosphate, 2 dithiothreitol, 0.1 Na_3VO_4, 10 $MgCl_2$ with 20 μmol/L ATP and 1 μg of an Akt substrate, (GSK3α/β fusion protein - Cell Signaling) was added to the immunopellet and Akt activity quantified by western blot detection of the phosphorylated Akt substrate.

Caspase 3 Activity Assay

Pulverized myocardium (20 mg) was vortexed in ice cold lysis buffer containing (in mmol/L) 50 Pipes/KOH (pH 6.5), 2 EDTA, 0.1% Chaps, 5 dithiothreitol, 20 μg/mL leupeptin, 10 μg/mL pepstatin A, and 10 μg/mL aprotinin. For cells in culture, media were removed and centrifuged to collect detached cells. Lysate from attached cells was then added to the floating-cell pellet. Samples were cleared by centrifugation and protein concentration measured. Caspase activity was assayed as described.[20]

Data Analysis and Statistics

All values are expressed as means±SEM. Data for the estrogen and placebo sham groups were similar and pooled. Where appropriate, mean values from sham/control groups were assigned an arbitrary value of 1, to which all other data were normalized. Multiple-group comparisons were performed using one-way ANOVA and, when significant, the Student–Newman–Keuls post hoc pair-wise test was performed to identify differences among the groups. Probability values of ≤0.05 were considered significant.

Results

In Vivo Studies

All mice, with the exception of 1 E2-treated infarct mouse, survived to tissue harvest. One E2-treated mouse (24 hour) and 2 placebo-treated mice (72 hour) demonstrated infarcts that did not involve the apex and were, therefore, excluded from the analysis. All E2-treated mice had uterine hypertrophy, and placebo-treated mice had uterine atrophy by visual inspection. E2 levels were not directly measured in this study; however, E2 administration in this manner has consistently resulted in physiological levels in multiple previous studies from our laboratory (80 to 200 pg/mL or 0.30 to 0.75 nmol/L).[20,23,24]

Effect of E2 on Cardiomyocyte Apoptosis

Cardiomyocyte apoptosis was quantified 6, 24, and 72 hours after coronary ligation in mice. TUNEL data are shown in

Figure 2. The sham group demonstrated a mean of 0.02±0.02% TUNEL positive cardiomyocytes per heart. Within the E2- and placebo-treated MI groups, there was no increase in TUNEL-positive cells in the noninfarct zone compared with shams (data not shown). In the placebo-MI group, the percentage of TUNEL-positive cardiomyocytes in the peri-infarct zone increased to 2.11±0.64%, 1.29±0.22%, and 0.81±0.12%, at 6, 24, and 72 hours, respectively ($P<0.01$ versus sham group at all time points). In the E2-MI group, the percentage of TUNEL-positive cardiomyocytes in the peri-infarct zone was 2.47±.36%, 0.53±.07%, and 0.31±0.16% at 6, 24, and 72 hours, respectively ($P<0.01$ versus sham group at all time points, and $P<0.05$ versus placebo-MI at 24 and 72 hours). Thus, E2 significantly reduced TUNEL-positive cardiomyocytes in the peri-infarct zone at 24 and 72 hours after coronary ligation.

Results for the caspase 3 assay are also shown in Figure 2. Caspase 3 activity was below the limit of detection in samples from sham animals and from the noninfarct zones of MI groups. In the placebo-MI group, caspase 3 activity in the infarct border zone was 2.6±0.5, 3.5±2.2, and 8.3±0.6 units/h/mg at 6, 24, and 72 hours, respectively. In the E2-MI group, caspase 3 activity was 1.9±0.2, 1.2±0.1, and 3.6±0.3 units/h/mg at 6, 24, and 72 hours, respectively. Thus, E2 treatment resulted in a trend for reduced caspase 3 activity at 24 hours, with a significant reduction at 72 hours compared with placebo-MI ($P<0.05$).

Effect of E2 on Myocardial Akt Activation

We next analyzed E2-mediated effects on the Akt pathway (Figure 3) because of its known anti-apoptotic role.[28,29,30] The peri-infarct zone of the placebo-MI group demonstrated an increase in phospo-Ser[473]-Akt (phospho-Akt) of 1.8±0.3-, 1.2±0.2-, and 1.8±0.6-fold above shams at 6, 24, and 72 hours, respectively ($P<0.05$ at 6 and 72 hours, $P=$NS at 24 hours versus shams). The E2-MI group developed even greater phospho-Akt within the peri-infarct zone equaling 3.2±0.5-, 1.7±0.2-, and 3.2±0.3-fold above shams at 6, 24, and 72 hours, respectively ($P<0.01$ versus shams and placebo-MI at 6 and 72 hours; Figure 3A and 3B). A similar pattern of Akt phosphorylation was noted in myocardium
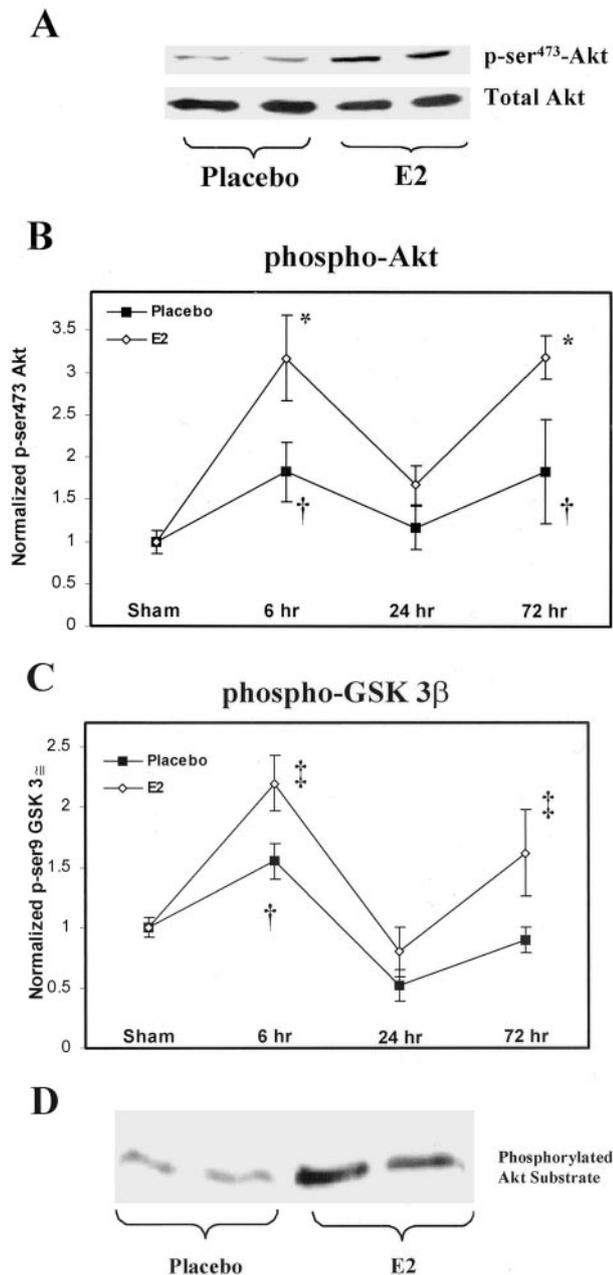
Figure 3. Physiological E2 replacement increases activation of Akt after MI. A, Representative Western blot for phospho (p)-Ser[473]-Akt and total-Akt of peri-infarct zone lysates obtained at 6 hours. B, Line graph demonstrating phospho-Ser[473]-Akt corrected for total Akt; placebo-MI (■) and E2-MI (◇) values represent fold increase above shams. Data shown are means±SEM. C, Line graph demonstrating phospho-Ser[9]-GSK3β corrected for total GSK3β; placebo-MI (■) and E2-MI (◇) values represent fold increase above shams assigned an arbitrary value of 1. *$P<0.01$ vs placebo-MI and shams. †$P<0.05$ vs shams. ‡$P<0.01$ vs shams and $P<0.05$ vs placebo-MI. D, Representative examples of an Akt kinase assay performed on noninfarct zone lysates in which serine-phosphorylation of an Akt substrate is quantified. Normalized p-ser9 GSK3≅ indicates normalized phospho-Ser[9]-GSK3β.

from the noninfarct zone in both E2 and placebo-MI groups (see online Supplemental Figure 2). We next assessed phosphorylation of the downstream Akt target, GSK3β (phospo-Ser[9]-GSK3β), in lysates from the peri-infarct zone. With MI,

the placebo group demonstrated a 1.6±0.2-fold increase in phospho-GSK3β compared with shams at 6 hours ($P<0.05$), but phospho-GSK3β levels were statistically similar to shams at 24 and 72 hours after MI. E2 treatment resulted in a significantly greater increase in phospho-GSK3β at 6 hours (2.2±0.2-fold versus shams; $P<0.01$, and $P<0.05$ versus placebo-MI; see Figure 3C) and 72 hours (1.6±0.4-fold versus shams, $P<0.05$, and $P<0.05$ versus placebo-MI) after coronary ligation. Akt kinase activity measured directly in noninfarct zone lysates from the 6-hour time point was 2.6±0.5-fold higher in the E2-MI compared with placebo-MI hearts ($P<0.05$, Figure 3D). Taken together, these data support that physiological E2-replacement results in greater activation of the Akt signaling pathway in the peri-infarct zone and noninfarct zone compared with placebo after coronary ligation.

We next analyzed the degree of ERK1/2 phosphorylation (Thr[242]/Tyr[248]) by Western blotting of peri-infarct zone lysates obtained 6 hours after coronary ligation. MI induced similar increases in ERK1/2 phosphorylation in hearts of placebo-treated mice (2.48±0.05-fold above shams, $P<0.01$; online Supplemental Figure 3), and in E2-treated mice (2.35±0.48-fold, $P<0.01$ versus shams, $P=NS$ versus placebo-MI). The lack of effect of E2 on MI-induced increases in ERK1/2 activation contrasts with our findings with Akt, supporting the specificity of the E2 effects on Akt activation described above.

In Vitro Studies

Effect of E2 on Cardiomyocyte Signaling
The effect of E2 on cardiomyocyte signaling was also studied in cultured neonatal rat cardiomyocytes in vitro (Figure 4). Estrogen induced a significant 2.7±0.6-fold increase in phospho-Akt by 5 minutes ($P<0.05$ versus 0-time point), which was sustained at 60 minutes (2.9±0.1-fold, $P<0.05$ versus 0 time point; Figure 4A) and persisted for 5 hours (1.5±0.1-fold increase in phospho-Akt with 1 nmol/L E2, $P<0.01$; not shown). Pretreatment of cells with the ER antagonist ICI blocked the increase in phospho-Akt after E2 stimulation, supporting the hypothesis that Akt activation occurs through an ER-dependent mechanism (Figure 4B).

The relative contribution of the 2 known ERs, ERα and ERβ, in estrogen-mediated activation of Akt was explored. Cardiomyocytes were pretreated with either the nonselective ER antagonist, ICI, or with the selective ERβ antagonist, R,R-THC[31] before E2 stimulation. In separate experiments, ICI again blocked the rapid activation of Akt by E2, but R,R-THC had no effect on E2-induced Akt activation, supporting the hypothesis that ERα mediates E2-induced activation of Akt in cardiomyocytes (Figure 4C). Estrogen is known to activate phospho-inositide-3 kinase (PI3-kinase) in non-myocardial cells[32,33,34] and PI3 kinase is a proximal element of Akt activation pathways.[35] We, therefore, tested whether E2 activates Akt via PI3 kinase in cultured neonatal rat cardiomyocytes. Myocytes pretreated with the PI3 kinase inhibitor, LY294002 (10 μmol/L), before E2 stimulation no longer demonstrated E2-mediated activation of Akt (Figure 4D). These data support the hypothesis that E2 activation of
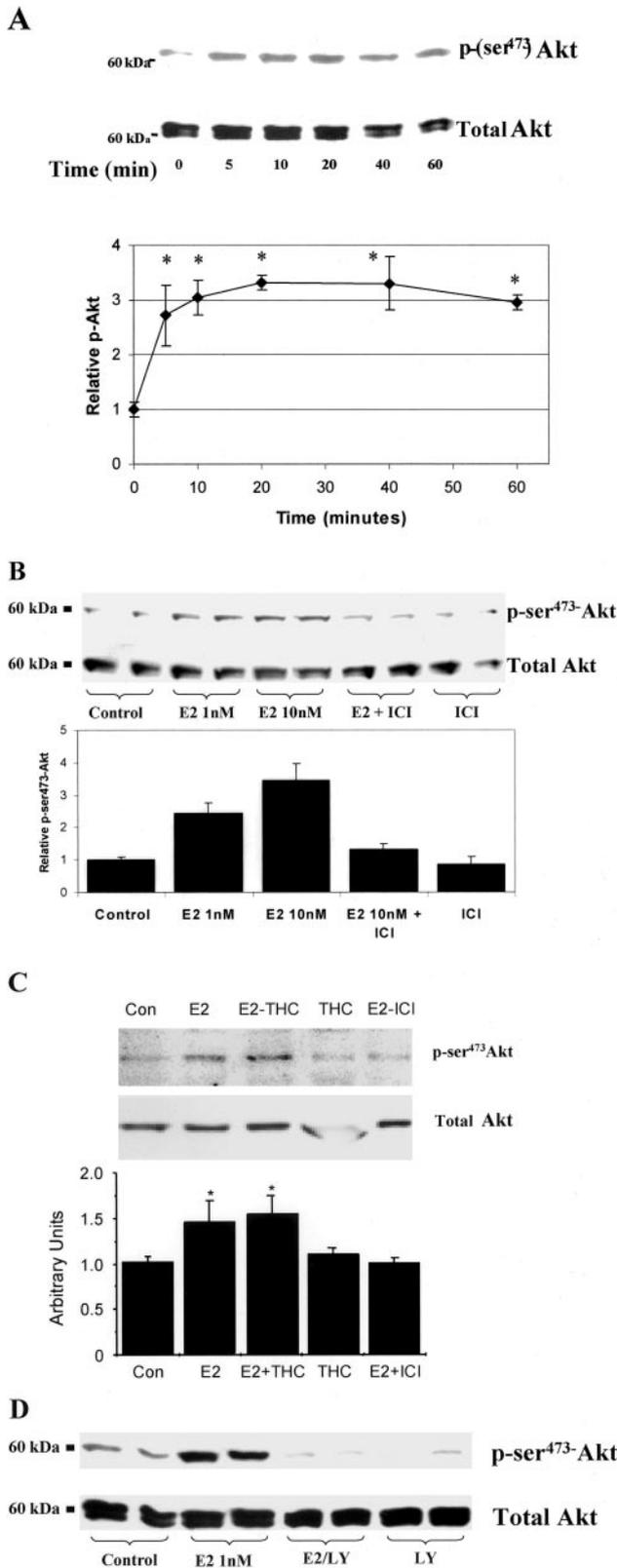
A

B

C

D

Figure 4. E2 rapidly activates the prosurvival kinase, Akt, in cultured neonatal rat cardiomyocytes. A, Time course of Akt activation by E2 determined by Western blotting for phospho-Ser^{473}-Akt, normalizing for total Akt. The line graph demonstrates the quantification of 3 separate experiments. *$P<0.05$ vs control. B, Representative Western blot of duplicate samples representative of 5 separate experiments in which
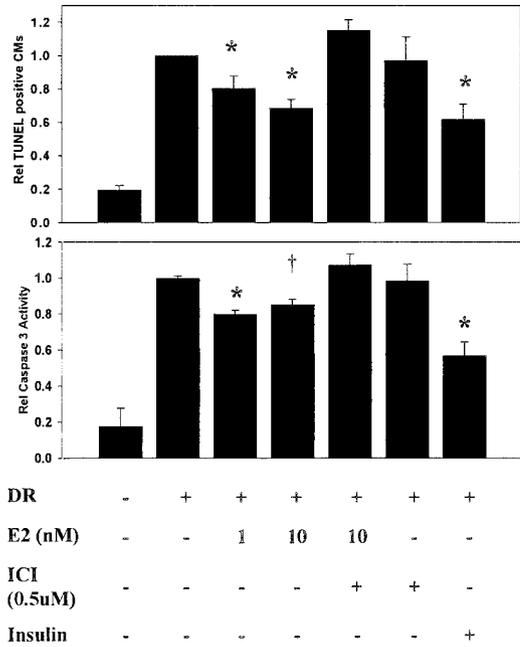

Figure 5. E2 inhibits DR-induced apoptosis in primary neonatal rat cardiomyocytes in vitro. Bar graphs representing results of TUNEL staining (upper graph) and caspase 3 assay (lower graph) from cells pretreated with vehicle or E2 (1 to 10 nmol/L) for 2 hours followed by the addition of DR (0.5 μmol/L for cells on coverslips and 1.0 μmol/L for cells on tissue culture plates). DR-only–treated cells were assigned an arbitrary value of 1. Bars represent means±SEM. *$P<0.05$ vs DR plus vehicle control; †$P=0.065$ vs DR plus vehicle control.

Akt in cardiomyocytes occurs through a PI3-kinase–dependent mechanism.

Effect of E2 on Cardiomyocyte Apoptosis: In Vitro Studies

To explore whether E2 inhibits cardiomyocyte apoptosis in vitro, the effect of E2 on cardiomyocyte apoptosis induced by the anthracycline, daunorubicin (DR) was studied (Figures 5 and 6). DR increased the percentage of TUNEL-positive cardiomyocytes from 6.6±1.5% to 31.5±1.9% after 24 hours ($P<0.01$). E2 reduced the TUNEL-positive cells after DR treatment to 26.1±4.2 ($P<0.05$) and 21.6±2.0% ($P<0.05$) at 1 and 10 nmol/L, respectively (Figure 5A). The decrease in TUNEL-positive cells with E2 was blocked completely by ICI, supporting the hypothesis that the E2-mediated reduction in apoptosis was ER-dependent. DR also increased caspase 3

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serum-deprived cardiomyocytes were harvested 30 minutes after stimulation with E2 (1 to 10 nmol/L), with or without the ER antagonist ICI (0.5 μmol/L); *$P<0.01$ vs control. C, Representative Western blot of 3 separate experiments in which cardiomyocytes were pretreated with vehicle (0.1% EtOH), ICI, or the ERβ specific antagonist, R,R-THC (1 μmol/L) and harvested 30 minutes after E2 stimulation. Bar graphs represent means±SEM. *$P<0.05$ vs control (Con). D, Representative Western blot of 3 separate experiments in which cardiomyocytes were pretreated with vehicle or the PI3 kinase inhibitor, LY294002 (LY) (10 μmol/L) followed by stimulation with vehicle or E2 (1 nmol/L) for 30 minutes. LY blocked the E2-mediated increase in phospho-Ser^{473}-Akt. Because of the near absence of phosphorylated Akt in the LY-treated samples, these data were not quantified.
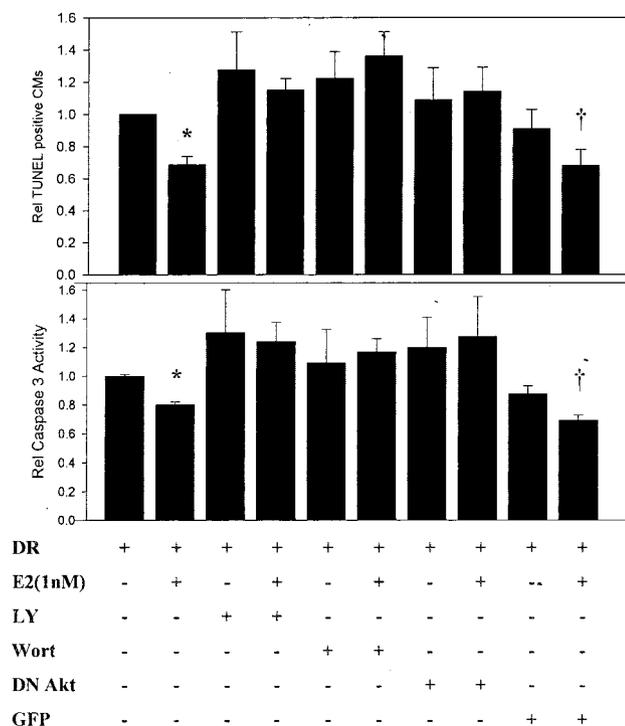
Figure 6. The reduction in apoptosis by E2 is PI3 kinase and Akt dependent. Bar graphs representing the results of TUNEL staining (upper graph) and caspase 3 assay (lower graph) in cells pretreated with vehicle or E2, followed by addition of DR. The PI3 kinase inhibitors, LY294002 (LY) (10 μmol/L) and Wortmannin (Wort) (100 nmol/L) and overexpression of the dominant negative Akt (DN-Akt), K179M, abolished the reduction in apoptosis by E2. Bars represent the means±SEM. *$P<0.05$ vs control; †$P<0.05$ vs vehicle-treated GFP expressing cells.

activation 5.6-fold above vehicle-treated control cells (Figure 5B) and E2 reduced the DR-induced activation of caspase $3\times20.1\pm2.0\%$ (1 nmol/L, $P<0.05$) and $14.8\pm3.2\%$ (10 nmol/L, $P=0.065$). The E2-mediated reduction in caspase 3 activation was blocked completely by ICI (Figure 5B). DR also induced internucleosomal DNA fragmentation and this was inhibited by 10 nmol/L E2 in the absence, but not in the presence of ICI (see online Supplemental Figure 4). To explore the potential role of the PI3 kinase–Akt signaling pathway in inhibition of cardiomyocyte apoptosis by E2, we studied cardiomyocytes pretreated with the PI3 kinase inhibitors, LY294002 (10 μmol/L) and Wortmannin (100 nmol/L). Both PI3 kinase inhibitors blocked E2-mediated inhibition of cardiomyocyte apoptosis assessed by TUNEL staining and caspase 3–activity assay (Figure 6). Overexpression of a dominant negative, kinase inactive mutant of Akt (K179M)[21] also abolished the anti-apoptotic effect of E2 (Figure 6), whereas infection of cardiomyocytes with Adv-GFP virus (control) had no effect on the anti-apoptotic effects of E2 assessed by either assay.

Discussion

The data presented here support that physiological E2 replacement reduces cardiomyocyte apoptosis after MI in ovariectomized female mice. E2 treatment in vivo increased activation of the prosurvival, serine-threonine kinase, Akt,

which preceded the reduction in cardiomyocyte apoptosis at 24 and 72 hours post-MI. In vitro studies in cardiomyocytes demonstrated that E2 rapidly activates Akt in an ERα- and PI3 kinase–dependent manner. In anthracycline treated cells, E2 also attenuated apoptosis in an ER-dependent manner that involves activation of the PI3 kinase–Akt signaling pathway.

The mechanism by which E2 activates PI3 kinase–Akt signaling in cardiomyocytes is not known. Our results support the hypothesis that ERα specifically mediates this E2 effect, which is consistent with data in vascular endothelial cells in which E2 rapidly stimulates endothelial nitric oxide synthase activity, in part, via activation of PI3 kinase–Akt pathways[32,34,36] through a nongenomic mechanism. Simoncini et al reported a direct interaction of ligand-activated ERα and the p85 regulatory subunit of PI3 kinase coinciding with activation of PI3 kinase in endothelial cells.[34] Furthermore, activation of PI3 kinase–Akt is required for the E2-mediated inhibition of apoptosis in both MCF-7 breast cancer cells and in cultured retinal neurons.[37,38] Our laboratory is now engaged in exploring the mechanism of E2-ERα induced PI3 kinase–Akt activation in cardiomyocytes.

Previous reports support the findings from the present study. E2 treatment of neonatal rat cardiomyocytes has been shown to result in nuclear accumulation of phospho-Ser[473]-Akt.[16] Another study conducted in cultured neonatal rat cardiomyocytes showed inhibition of staurosporine-induced apoptosis in cells treated with E2.[39] The role of cardiomyocyte apoptosis in the pathophysiology of progressive heart failure remains controversial.[14] Animal and human studies have demonstrated the presence of apoptotic cardiomyocytes within both the infarct and peri-infarct zones after coronary occlusion.[14,20,40–44] Abbate et al showed that the degree of cardiomyocyte apoptosis correlated directly with LV chamber enlargement after MI.[44] Although the role of estrogen in cardiomyocyte survival in response to ischemic injury is not well understood, estrogen has been shown to reduce infarct size in several experimental models of ischemia-reperfusion injury.[45,46] Whether protection from cardiomyocyte apoptosis was important to the reduction in myocardial injury by E2 in these models is unknown. However, cardiomyocyte preservation in response to a potent apoptotic stimulus, such as reperfusion after a period of ischemia, provides a plausible explanation for these E2-mediated effects.

Despite generally positive observational studies published in the previous decade regarding the cardioprotective effects of hormone-replacement therapy, recent double-blind, randomized clinical trials have demonstrated that therapy with conjugated equine estrogens and progesterone or with conjugated equine estrogens alone has no beneficial impact on cardiovascular events in postmenopausal women.[47,48,49–51] However, the major end points in these clinical studies were actually vascular end points including MI and stroke. The pathophysiology of these end points differs substantially from the processes being explored here. Thus, it is possible that potentially adverse systemic effects of hormone-replacement therapy on the vasculature may offset favorable effects taking place within the myocardium. In our chronic-MI model, physiological estrogen-replacement administered to ovariectomized female mice increased mortality, despite a reduction

in infarct size and cardiomyocyte apoptosis. Clearly, further investigation is needed to understand the complex effects of sex-steroid hormones on cardiomyocyte biology.

Summary

In this study, we demonstrate that E2 increased activation of Akt and improved survival in murine cardiomyocytes both in vivo and in vitro. These experiments support the importance of the PI3 kinase–Akt signaling pathway in the prosurvival effects of estrogen that may, in part, account for observed gender differences in the myocardial responses to injury and improved survival in female heart failure patients.

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There are no conflicts to disclose.

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Circulation Research

JOURNAL OF THE AMERICAN HEART ASSOCIATION

17β-Estradiol Reduces Cardiomyocyte Apoptosis In Vivo and In Vitro via Activation of Phospho-Inositide-3 Kinase/Akt Signaling

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Supplemental Material:

Methods:

DNA Fragmentation: Internucleosomal DNA fragmentation was determined using a previously published method.[1] Culture medium was removed and centrifuged at 3000 × g for 5 min to collect detached cells. Adherent cells were lysed with a hypotonic lysis buffer (10 mM Tris-HCl, pH 8.0) containing EDTA (10 mM) and Triton X-100 (0.5%) and then pooled with detached cells. RNA was digested using Rnase A (0.1 mg/ml at 37 °C for 1 h) followed by proteinase K treatment for 2 hr at 50°C. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated with isopropyl alcohol, stored overnight at -20 °C, and centrifuged at 12,000 × g for 15 min at 4 °C. The pellet was resuspended in 20 µl Tris-acetate EDTA buffer supplemented with 2 µl of sample buffer (0.25% bromphenol blue, 30% glyceric acid), and electrophoretically separated on a 2% agarose gel containing 1 µg/ml ethidium bromide and visualized under ultraviolet transillumination.

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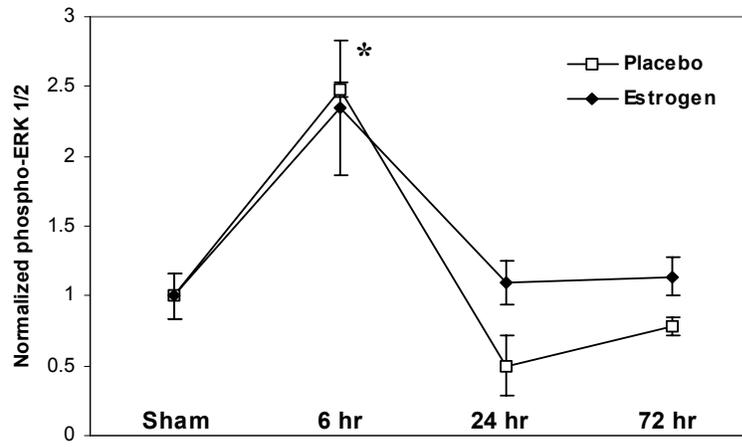
Supplemental Figure 1
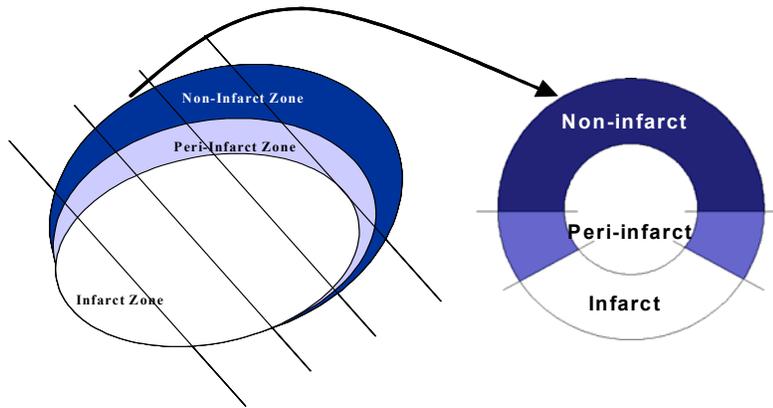
Supplemental Figure 1. Procedure for Tissue Harvesting: Schematic drawing demonstrating the manner in which the LV was sectioned and infarct zones, peri-infarct zones and non-infarct zones delineated. The peri-infarct zone was arbitrarily defined as a 1mm segment juxtaposed to the non-Evan's Blue – staining area representative of the infarct or non-perfused zone.
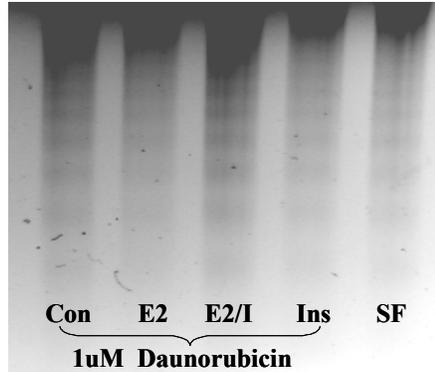
Supplemental Figure 2
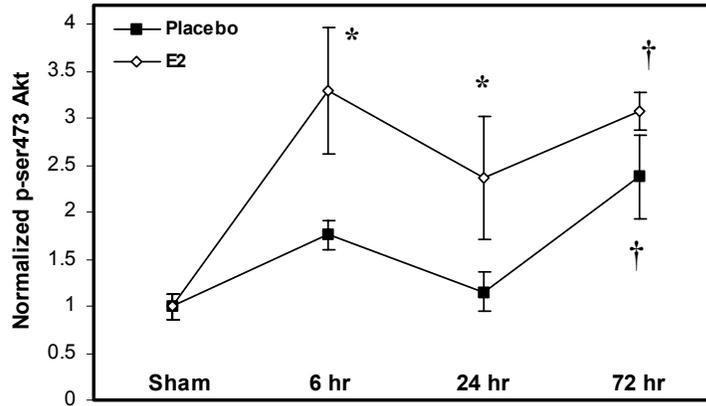
Supplemental Figure 2. Akt activation within the Non-Infarct Zone. Line graph demonstrating p-ser[473]Akt corrected for total Akt with values for the Sham group assigned an arbitrary value of 1. Placebo-MI are shown by solid squares and E2-MI, open diamonds. Values represent fold increase above Shams. Data shown are means±sem. * $p < 0.01$ vs. Sham and Placebo-MI. † $p < 0.01$ vs. Shams. E2=17β-estradiol; hr=hour; MI=myocardial infarction

Supplemental Figure 3

Supplemental Figure 3. Activation of Extracellular Signal Regulated Kinase 1/2 (ERK1/2) in the Peri-Infarct Zone. Line graph demonstrating p-thr^{242}/tyr^{248}ERK1/2 corrected for total ERK1/2 with values for the Sham group assigned an arbitrary value of 1. Placebo-MI are shown by solid squares and E2-MI, open diamonds. Values represent fold increase above Shams. Data shown are means±sem. * p<0.05 vs. Shams E2=17β-estradiol; hr=hour; MI=myocardial infarction.

Supplemental Figure 4

Supplemental Figure 4. E2 Reduces Daunorubicin Induced Internucleosomal DNA Fragmentation: DNA fragmentation in cardiomyocytes pre-treated for 2 hours as labeled, followed by daunorubicin (DR) 1.0uM. DR increases DNA fragmentation above Serum Free (SF) conditions alone. 17β-estradiol (E2 1nM) reduces DNA fragmentation compared to Vehicle Control (Con), and the non-selective ER antagonist, ICI 182,780 (I), reverses the E2-mediated reduction in DNA fragmentation; Insulin (Ins-4ug/ml) serves as a positive control for apoptosis inhibition.