Female Adult Mouse Cardiomyocytes Are Protected Against Oxidative Stress

Fangfei Wang, Quan He, Ying Sun, Xiangguo Dai, Xiao-Ping Yang

Abstract—Premenopausal women have less cardiovascular disease and lower cardiovascular morbidity and mortality than men the same age. Our previous studies showed that female mice have lower mortality and better preserved cardiac function after myocardial infarction. However, the precise cellular and molecular mechanisms responsible for such a sex difference are not well established. Using cultured adult mouse cardiomyocytes, we tested the hypothesis that the survival advantage of females stems from activated estrogen receptors and Akt survival signaling pathways. Adult mouse cardiomyocytes were isolated from male and female C57BL/6J mice and treated with hydrogen peroxide (100 μmol/L) for 30 minutes. Cell survival was indicated by rod ratio (rod shaped cells/total cells), cell death by lactate dehydrogenase release, and positive staining of annexin-V (a marker for apoptosis) and propidium iodide (a marker for necrosis). In response to hydrogen peroxide, female adult mouse cardiomyocytes exhibited a higher rod ratio, lower lactate dehydrogenase release, and fewer Annexin-V–positive and propidium iodide–positive cells compared with males. Phospho-Akt was greater in females both at baseline and after hydrogen peroxide stimulation. The downstream molecule of Akt, phosphor–GSK-3β (inactivation), was also higher, whereas caspase 3 activity was lower in females in response to hydrogen peroxide. Bcl-2 did not differ between sexes. Estrogen receptor-α was the dominant isoform in females, whereas estrogen receptor-β was low but similar in both sexes. Our findings demonstrate that female adult mouse cardiomyocytes have a greater survival advantage when challenged with oxidative stress–induced cell death. This may be attributable to activation of Akt and inhibition of GSK-3β and caspase 3 through an estrogen receptor-α–mediated mechanism. (Hypertension. 2010;55:1172-1178.)

Key Words: mice ■ estrogen ■ myocytes ■ oxidative stress ■ apoptosis ■ signal transduction

Premenopausal women have less cardiovascular disease and lower cardiovascular morbidity and mortality than men the same age; however, these cardioprotective benefits disappear after menopause. Experimental and clinical evidence of sex differences in myocardial remodeling and heart failure favor women. We previously showed that female mice had lower mortality, better preserved cardiac function, less inflammation, and augmented healing responses after myocardial infarction (MI), suggesting that females have an inherent cardioprotective advantage. However, the precise underlying mechanisms behind this advantage are not fully understood.

Compelling evidence indicates that cardiomyocyte death via apoptosis and necrosis plays a critical role in a wide range of cardiovascular diseases, including ischemic heart disease, myocarditis, dilated cardiomyopathy, and atherosclerosis. Because adult cardiomyocytes (ACMs) possess minimal capacity to re-enter the cell cycle or divide and/or proliferate, control of myocyte loss through suppression of cell death pathways represents a logical strategy for cardioprotection. Estrogen has also been shown to inhibit apoptosis by blocking transcription factor nuclear factor-κB and caspase activation, which may conserve contractile myocytes and delay or prevent the development of left ventricular dysfunction and heart failure.

Three estrogen receptor (ER) isoforms, ER-α, ER-β, and GPR30, have been identified. ER-α and ER-β are found in the heart and vasculature. The antioxidative and antiapoptotic actions of estrogen have been shown to be mediated by activation of Akt either via ER-α and ER-β or in some ER-independent fashion. Several downstream targets of Akt have been recognized as cell survival regulatory molecules, including glycogen synthase kinase 3β (GSK-3β), caspase 3, and Bcl-2. GSK-3β is a serine/threonine kinase and one of the few protein kinases known to be inactivated by phosphorylation. It has multiple functions in heart tissue and has recently attracted attention because of its association with both cell apoptosis and survival. Caspas, which are cysteine-aspartic proteases, play essential roles in apoptosis, necrosis, and inflammation. Eleven caspas have been found in humans. They are activated by removal of the prodomain on apoptotic stimuli and other caspas. Akt...
(acting via phosphorylation) has been shown to inhibit the initiator caspase 9, which, in turn, blocks the effector caspase 3, suppressing apoptosis. Bcl-2 is an anti-death gene that functions as an intracellular antioxidant. However, we still do not know whether these Akt-associated signaling molecules are involved in the sex difference favoring females in cell survival.

In the present study, we treated cultured mouse ACMs with hydrogen peroxide (H₂O₂) to test the hypothesis that improved cardiomyocyte survival in females is mediated by enhanced antiapoptotic and antinecrotic signaling pathways. This involves elevated expression of ER-α, activation of AKT/GSK-3β and AKT/Bcl-2 and inhibition of caspase 3.

**Materials and Methods**

**Animals**

Male and female C57BL/6J mice were antig opened with 50 U of heparin (100 IU/mL, 0.5 mL IP) and anesthetized with pentobarbital sodium (50 mg/kg IP). Hearts were rapidly excised and arrested in Ca⁺⁺-free perfusion buffer consisting of (in millimoles per liter) 120.0 NaCl, 5.4 KCl, 1.2 NaH₂PO₄, 1.2 MgSO₄, 7.0 H₂O, 5.6 glucose, 20.0 NaHCO₃, 10.0 2,3-butanediol monoxide (Sigma), and 5.0 taurine (Sigma; pH 7.2), gassed with 95% O₂-5% CO₂. The aorta was cannulated and the heart perfused retrograde with Ca⁺⁺-free perfusion buffer at a constant flow of 1 mL/min at 37°C for 4 minutes to flush out blood from the vasculature and remove extracellular calcium to stop contraction. The perfusion buffer was then switched to a myocyte digestion buffer consisting of perfusion buffer and 0.50 mg/mL of collagenase type 2 (Worthington) and 0.02 mg/mL of proteinase type D, and maintained for 5 minutes. Calcium concentration was raised to 50 μmol/L in the myocyte digestion buffer for 8 to 10 minutes. The heart appeared swollen, pale, and flaccid. The ventricles were removed and cut into several chunks in the same enzyme solution. Using a wide-open pipette, dispersed myocytes were filtered through a 100-μm mesh and centrifuged for 3 minutes at 500 rpm. The supernatant was removed and the cell pellet promptly resuspended in perfusion buffer containing 125 μmol/L of Ca²⁺ and 5 mg/mL of BSA. The cells were allowed to sediment by gravity in a cell culture incubator for 10 minutes, after which they were washed sequentially with perfusion buffer containing 250 and 500 μmol/L of Ca²⁺. Half of the perfusion buffer from the last wash was replaced by phenol red-free Eagle minimal essential medium (washing medium) containing 2.5% FBS (Gibco) and preincubated at 37°C for 10 minutes. The heart appeared swollen, pale, and flaccid. The ventricles were removed and cut into several chunks in the same enzyme solution. Using a wide-open pipette, dispersed myocytes were filtered through a 100-μm mesh and centrifuged for 3 minutes at 500 rpm. The supernatant was removed and the cell pellet promptly resuspended in perfusion buffer containing 125 μmol/L of Ca²⁺ and 5 mg/mL of BSA. The cells were allowed to sediment by gravity in a cell culture incubator for 10 minutes, after which they were washed sequentially with perfusion buffer containing 250 and 500 μmol/L of Ca²⁺. Half of the perfusion buffer from the last wash was replaced by phenol red-free Eagle minimal essential medium (washing medium) containing 2.5% FBS (Gibco) and 1250 μmol/L of Ca²⁺. ACMs were incubated at 37°C for 10 minutes. Then, half of the medium was replaced by washing medium to gradually increase Ca²⁺ to 1 mmol/L (final concentration). Finally, the cells were counted and plated.

**Culture of Adult Mouse Cardiomyocytes**

The entire culture process was performed under a biological safety hood. Culture plates were precoated with 8 to 10 μg/mL of mouse laminin in PBS (Hyclone) with 1% penicillin-streptomycin (Gibco) and preincubated at 37°C for 1 hour. Freshly isolated ACMs were plated at 0.5×10⁶ cells per centimeter squared in washing medium and incubated at 37°C with 5% CO₂ and saturated water temperature for 1 hour. Then, the medium was gently changed to culture medium (CTL), which was washing medium without FBS, and maintained for 1 hour.

**Cell Viability and H₂O₂-Induced Cell Death**

After 1 hour of starvation, ACMs on 24-well plates in 500 μL of medium per well were treated with either CTL or 100 μmol/L of H₂O₂ for 30 minutes to induce cell death. Cell viability was determined by the ratio of rod-shaped (typical viable ACMs) with length:width >1.5) to total number of cells. Apoptosis and necrosis were detected by an annexin-V (AV)-fluorescein isothiocyanate (FITC)/propidium iodide (PI) stain detection kit (BioVision). Briefly, ACMs were incubated with AV-FITC and PI for 10 minutes at room temperature in the dark. Plates were photographed under both phase-contrast and fluorescence microscopes, and rod-shaped, sphere-shaped (length:width <1.5), and total cells were counted. Apoptotic myocytes were defined as AV-FITC positive (A⁻V⁺; green-stained cells) and necrotic myocytes as PI positive (PI⁺; red-stained cells) plus A⁻V⁻. Images were processed and the surface area of freshly isolated ACMs measured using MicroSuite software (Biological Suite). For each parameter, 200 to 300 myocytes were counted in randomly selected fields by 3 independent researchers. Each experiment was conducted in triplicate and averaged as 1 experiment.

**Caspase 3 Activity Assay**

Caspase 3 activity was measured using an ApoTarget caspase 3 protease assay kit (BioSource International). Briefly, at the end of the experiment the medium was centrifuged and the supernatant removed to collect detached cells as a pellet. Attached cells were scraped and combined with the pellet in ice-cold lysis buffer (from the kit). Samples were centrifuged and the supernatant collected. Protein concentration was determined with a BCA assay kit (Pierce). The cell lysate (30 μg) from each sample was combined with 200 μmol/L of DEVD-pNA substrate and diluted with 2X reaction buffer containing 10 mmol/L of dithiothreitol. After incubation at 37°C in the dark for 1 hour, samples were read at 405 nm in a microplate reader. Measurements were repeated after 1 hour. Values were compared with known standards to determine enzymatic activity. Caspase 3 activity was expressed as picomoles per microgram (protein) per hour.

**Cytotoxicity**

Cytotoxicity was measured by lactate dehydrogenase (LDH) release using an LDH assay kit (Bio Vision). Briefly, ACMs were plated at 2×10⁴ cells per well in 24-well plates. Cells were treated with CTL or 100 μmol/L of H₂O₂ for 30, 60, and 90 minutes. At the end of each time point, the plates were gently shaken to make sure that LDH was evenly distributed in the medium. Media were centrifuged and the supernatant transferred to a 96-well plate (10 μL per well). We added 100 μL of LDH WST substrate mixtures (4-[3-(4-iodophenyl]-2-(4-nitrophenyl]-2H-5-tetrazolol-1,3-benzen disulfonate) and incubated for 30 minutes at room temperature. Absorbance was measured with a plate reader at 450 nm. Cytotoxicity (expressed as a percentage) was calculated as an index of cell death using the following formula: cytotoxicity (%) = [test sample − low control)]/ [low control − high control]×100, where “test sample” is the experimental sample (cells) with or without H₂O₂ treatment; the “low control” is a sample taken from CTL containing freshly isolated cells without any treatment, and “high control” is sample taken from CTL containing cells treated with lysis solution. Each experimental sample had its own low and high controls.

**Western Blot**

Cells were harvested in lysis buffer containing 20.0 mmol/L of Tris (pH 7.5), 150.0 mmol/L of NaCl, 1.0 mmol/L of EDTA, 1.0 mmol/L of EGTA, 1% Triton X-100, 2.5 mmol/L of sodium pyrophosphate, 1.0 mmol/L of β-glycerophosphate, 1.0 mmol/L of Na₃VO₄, 1.0 μg/mL of leupeptin, and 1.0 mmol/L of PMSF. After 20 minutes of incubation at 4°C, the lysate was centrifuged at 13 500 rpm for 15 minutes and protein concentrations in the supernatant determined as...
described above (for details please see the Methods section in the online Data Supplement at http://hyper.ahajournals.org).

Statistics
Data are expressed as mean±SE. All of the parameters were analyzed using Student t test, taking \( P<0.05 \) as significant. The number of experiments (n) refers to independent cultures from different hearts.

Results

Sex Difference in Cell Survival in Response to \( \text{H}_2\text{O}_2 \)
Freshly isolated viable ACMs were rod shaped with rectangular “stepped” ends and clear cross-striations. The surface area of female ACMs was smaller than in males; however, this difference disappeared when it was corrected by body weight (Figure S1 of the online Data Supplement). In contrast to rod-shaped viable ACMs, nonviable cells were spherical. Viability was indicated by the rod ratio (rod-shaped cells:total cells). Under CTL conditions, the rod ratio was similar between female and male ACMs. \( \text{H}_2\text{O}_2 \) decreased the rod ratio in both sexes; however, the reduction was significantly greater in males (Figure 1). We further confirmed the survival differences between male and female ACMs by measuring LDH released into the medium. As shown in Figure 2, \( \text{H}_2\text{O}_2 \) increased LDH release in a time-dependent manner, and the increase was significantly greater in male ACMs at all of the time points.

Sex Difference in \( \text{H}_2\text{O}_2 \)-Induced Apoptosis and Necrosis
Apoptotic and necrotic cell death were shown, respectively, by A-V\( ^+ \) (green stained) and PI\( ^+ \) (red stained) cells (Figure 3A). Under control conditions, there were a few apoptotic and necrotic cells, and no sex difference was found (Figure 3B). \( \text{H}_2\text{O}_2 \) increased apoptosis and necrosis (as indicated by more numerous A-V\( ^+ \) and PI\( ^+ \) cells) in both male and female ACMs; however, the increase was significantly greater in males (Figure 3B and 3C).

Sex Difference in Akt and GSK-3\( ^\beta \) Phosphorylation
Under basal conditions, activated Akt (phosphor-Akt) was significantly higher in female ACMs. \( \text{H}_2\text{O}_2 \) reduced Akt phosphorylation in both males and females; however, the reduction was significantly greater in males (Figure 4A). Basal phosphor-GSK-3\( ^\beta \) (inactivated form) tended to be higher in females, although the difference did not reach statistical significance. \( \text{H}_2\text{O}_2 \) reduced phosphorylation of GSK-3\( ^\beta \) in both male and female ACMs, and this reduction was significantly greater in males (Figure 4B).

Sex Difference in Caspase 3 Activity and Bcl-2 Expression
As shown in Figure 5, basal caspase 3 levels were similar between male and female ACMs. \( \text{H}_2\text{O}_2 \) activated caspase 3 in males but not in females. Bcl-2 protein expression was similar in both males and females. \( \text{H}_2\text{O}_2 \) reduced Bcl-2 expression to a similar extent in both males and females (data not shown).

ER-\( \alpha \) and -\( \beta \) Expression
We found that ER-\( \alpha \) and ER-\( \beta \) were expressed by both male and female ACMs. ER-\( \alpha \) was the dominant isoform in females, 4-fold higher than in males (Figure 6A). The much higher level of ER-\( \alpha \) may be responsible for the improved survival of female ACMs challenged with \( \text{H}_2\text{O}_2 \)-induced cell

![Figure 1](image_url)

**Figure 1.** Sex difference in ACM viability, as indicated by the rod ratio in response to \( \text{H}_2\text{O}_2 \). A, Representative images showing viable ACMs (rod-shaped cells, indicated by arrows) and dead cells (spherical, indicated by sparkles) under CTL conditions or treated with \( \text{H}_2\text{O}_2 \) (100 \( \mu \)mol/L). B, Quantitative analysis of the rod ratio (rod-shaped/total cells, left) and change from CTL in response to \( \text{H}_2\text{O}_2 \) (right). n=5.

![Figure 2](image_url)

**Figure 2.** Sex difference in \( \text{H}_2\text{O}_2 \)-induced cytotoxicity as indicated by LDH release. LDH was calculated using the following formula: \([\text{test sample-lower control}/\text{higher control-lower control}]\times100\) and expressed as the percentage of LDH release. n=4.
death. ER-β was low but similar in both males and females (Figure 6B).

**Discussion**

Using cultured ACMs, we demonstrated a significant sex difference in favor of females in \( \text{H}_2\text{O}_2 \)-induced cell death. ACMs from female mice showed a clear survival advantage over males, as indicated by a greater rod ratio, lower LDH release, and fewer apoptotic and necrotic cells in response to \( \text{H}_2\text{O}_2 \). We also showed that female ACMs treated with \( \text{H}_2\text{O}_2 \) had less reduction of Akt and GSK-3\( \beta \) phosphorylation and less activation of caspase 3, whereas Bcl-2 protein expression did not differ between sexes. We further demonstrated that ER-\( \alpha \) was the dominant form in females, whereas ER-\( \beta \) was low and similar in both sexes. Although our culture system is estrogen free, the freshly isolated ACMs from mice have been exposed to the estrogen environment during their lifetime, and their ERs may have already become activated. Our findings support the hypothesis that higher ER-\( \alpha \) and activated Akt survival signaling pathways play significant roles in protecting female ACMs against oxidative stress-induced cell death. They also support the cardioprotective advantage of females found in human and animal models.

Survival advantage and functional recovery that favor females in response to cardiac ischemic/hypoxic stimuli have been evidenced both in vivo and in vitro\(^1\); however, the molecular mechanisms responsible for such protection are not entirely known. In addition, there are limitations to in vivo experiments with animal models and in vitro studies of cultured neonatal cardiomyocytes (NCMs) conducted to uncover sex-dependent signaling pathways. For example, in the whole animal it is difficult to define whether cardiomyocytes exposed to the estrogen environment during their lifetime, and their ERs may have already become activated. Our findings support the hypothesis that higher ER-\( \alpha \) and activated Akt survival signaling pathways play significant roles in protecting female ACMs against oxidative stress-induced cell death. They also support the cardioprotective advantage of females found in human and animal models.
are the essential cell types being protected. However in the NCM ERs are inactivated in situ because NCM has not been exposed to the endogenous estrogen environment, making NCM a less than ideal tool for studying sex-dependent mechanisms. The advantage of using ACMs is that they have been exposed to sexual hormones during adulthood and express mature ERs, which help overcome the above limitations. Indeed, we found that female ACMs exhibited higher levels of ER-α associated with a better survival rate and activated Akt survival signaling pathways, consistent with published studies showing that female hearts are protected against cell death and is responsible for the cardioprotective effect of estrogen.

Estrogen and ER signaling pathways have not been fully elucidated. In the classic pathway, estrogen was thought to diffuse into the cell and bind to nuclear ERs, which are referred to as transcription factors, interacting with cis elements of the estrogen response gene to activate gene transcription. Accumulated data support a second estrogen signaling pathway in which estrogen binds to G protein-coupled membrane-bound ERs and generates a rapid signaling response via a nongenomic mechanism; for example, estrogen causes isolated coronary artery relaxation within 10 minutes. In our study, we used freshly isolated ACMs, and experiments were completed within 2 hours. We observed a significant sex difference in Akt and GSK3β phosphorylation and caspase 3 activation in response to H2O2, supporting the possibility that these effects may be mediated through a rapid nongenomic mechanism.

Cell survival has been shown to be mediated in part by an Akt signaling pathway. Estrogen protects against oxidative stress-induced cell death via activation of Akt in cardiac H9c2 cells. It also reportedly reduces cardiomyocyte apoptosis, preserves cardiac function, and enhances cardiac myocyte survival via activation of Akt in MI, myocardial ischemia-reperfusion injury, and cultured NCMs treated with daunorubicin. Akt-enhanced cell survival by inhibition of apoptosis can be mediated by transcription-independent and -dependent mechanisms. In the transcription-independent mechanism, Akt phosphorylates Bad, which prevents Bad from interacting with Bcl-2 to release cytochrome C, thereby preventing apoptosis. Akt also phosphorylates the initiator caspase 9 and, in turn, prevent activation of the effector caspase 3, blocking apoptosis. In the transcription-dependent mechanism, Akt phosphorylates Forkhead box transcription factors of the class O subfamily,
reducing apoptosis. Camper-Kirby et al reported that young women have higher levels of nuclear phosphor-Akt than age-matched men and postmenopausal women. Consistent with these findings, our study showed that female ACMs inherently had high levels of phosphor-Akt. When stimulated with H₂O₂, Akt phosphorylation was reduced much less in female ACMs than in males, associated with better survival and diminished apoptotic and necrotic cell death, supporting the role of Akt activation in the survival advantage observed in females. GS3 does not mediate acute myocardial protection in females. This research was supported by National Institutes of Health grant HL-078951 and HL-28892, PPG Project II (to X.-P.Y.) and Henry Ford Health System institutional funds (to X.-P.Y.).

Disclosures

None.

References


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Female Adult Mouse Cardiomyocytes Are Protected Against Oxidative Stress

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**METHODS**

**Western blot.** Cells were harvested in lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na$_3$VO$_4$, 1 µg/ml leupeptin, and 1 mM PMSF. After 20 min incubation at 4°C, the lysate was centrifuged at 13,500 rpm for 15 min and protein concentrations in the supernatant was determined with a BCA assay kit (Pierce). Aliquots of protein (30µg/lane) were subjected to electrophoresis on 10 or 12% Tris-glycine gel under reducing conditions. The proteins in the gels were transferred onto nitrocellulose membranes, immersed membranes in the 2% I-block (Promega) dissolved in PBS containing 0.05% (v/v) Tween 20 (PBS-T) for 1 hr and then incubated with primary antibodies overnight at 4°C. After several washes with PBS-T, the membranes were incubated with a secondary antibody which conjugated with HRP (Horseradish Peroxidase) at room temperature for 1 hr. The Western blot signals were visualized with ECL Western blot detection reagents and an enhanced chemiluminescence detection kit (Amersham Pharmacia, NJ). Films were scanned with an Epson Perfection 3200 scanner (Epson America, Long Beach, CA), and band density analyzed with Image J software (NIH). Phospho-isoforms of specific kinases were corrected by total kinase, β-actin or GAPDH, which was detected on the same membranes after stripping.
RESULTS

Figure S1. Characteristics of adult cardiomyocytes (ACM) from male and female mice. 400 cells were measured in each experiment. Freshly isolated viable ACMs were rod-shaped with rectangular “stepped” ends and clear cross-striations (Fig. 1A). Quantitative analysis of surface area of is shown in Fig. 1B. Surface area of female ACMs was smaller than males (2812 ± 62 vs 3692 ± 129 µm²; p < 0.05; n = 6); however, this difference disappeared when it was corrected for body weight.
**Figure S2.** Effect of 17β-extradiol (E2) on adult cardiomyocytes (ACM) viability (rod ratio) in response to H₂O₂. Freshly isolated ACMs were treated with H₂O₂ (100 µM) in the presence or absence of E2 (10⁻⁸ M). The effect of E2 was assessed as per cent from H₂O₂ alone. E2 increased rod ratio similarly in both males and females.
Figure S3. Effect of the ERα agonist propylpyrazole triol (PPT, 10^-8 M) on H₂O₂-induced cell death, expressed as rod ratio. We further determined whether the pro-survival effect of PPT was blocked by the ER antagonist fulvestrant (ICI 182780, 10^-8 M) in male ACMs treated with H₂O₂. Data were presented as per cent from control (CTL). PPT significantly increased rod ratio and this effect was abolished by fulvestrant, indicating that the cell survival advantage in females is most likely mediated via ERα.