Estrogen Prevents Cardiomyocyte Apoptosis through Inhibition of Reactive Oxygen Species and Differential Regulation of p38 Kinase Isoforms*

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From human and animal studies, estrogen is known to protect the myocardium from an ischemic insult. However, there is limited knowledge regarding mechanisms by which estrogen directly protects cardiomyocytes. In this report, we employed an in vitro model, in which cultured rat cardiomyocytes underwent prolonged hypoxia followed by reoxygenation (H/R), to study the cardioprotective mechanism of estrogen. 17-β-estradiol (E2) acting via estrogen receptors inhibited H/R-induced apoptosis of cardiomyocytes. Mitochondrial reactive oxygen species (ROS) generated from H/R activated p38α MAPK, and inhibition of p38α with SB203580 significantly prevented H/R-induced cell death. E2 suppressed ROS formation and p38α activation by H/R and concomitantly augmented the activity of p38β. Unlike p38α, p38β was little affected by H/R. Dominant negative p38β protein expression decreased E2-mediated cardiomyocyte survival and ROS suppression during H/R stress. The prosurvival signaling molecule, phosphoinositol-3 kinase (PI3K), has previously been linked to cell survival following ischemia-reperfusion injury. Here, E2-activated PI3K was found to inhibit ROS generated from H/R injury, leading to inhibition of downstream p38α. We further linked these signaling pathways in that p38β was activated by E2 stimulation of PI3K. Thus, E2 differentially modulated two major isoforms of p38, leading to cardiomyocyte survival. This was achieved by signaling through PI3K, integrating cell survival mediators.

Ischemic heart disease with subsequent myocardial infarction and congestive heart failure is the leading cause of mortality in this country (1). The incidence of ischemic heart disease is significantly lower in women than in men until menopause, after which the cardiovascular risk of women accelerates to equal that of men (2, 3). This observation suggests a possibility that female sex hormones, such as estrogen, may have a favorable cardiovascular role. There is evidence from epidemiological, animal, and in vitro studies that estrogen may be cardioprotective (4). For example, postmenopausal women on hormone replacement therapy have a lower mortality than non-users of hormone replacement therapy (5), and meta-analysis shows that women have a better prognosis after myocardial infarction than men after age and other factors are adjusted (6). In animal models, 17-β-estradiol (E2)2 reduces myocardial necrosis in rabbits (7) and prevents global myocardial ischemia/reperfusion (I/R) injury in rats (8). Given such protection of the heart from an ischemic insult, it is possible that estrogen directly acts at the level of individual cardiomyocytes. How estrogen protects the myocardium is not well understood currently.

Considering the reported anti-apoptotic effect of estrogen on diverse cell types (9–13), cardioprotection by estrogen may be due to prevention of cardiomyocyte apoptosis, after ischemic or oxidative stress. Loss of cardiomyocytes by apoptotic death has been associated with myocardial infarction (14), I/R injury (15, 16), or end-stage heart failure (17). Supporting evidence to date suggests that estrogen plays an anti-apoptotic role in the heart. Estrogen replacement in ovariectomized female mice is associated with a smaller infarct size and reduced apoptosis in the peri-infarct zone of the left ventricle after coronary artery ligation and release (18). When cultured neonatal cardiomyocytes were induced to undergo programmed cell death by drugs such as daunorubicin, the presence of E2 reduced myocyte apoptosis by activating pro-survival PI3K/Akt signaling (18).

With numerous modulators involved in cell fate, different stressors are likely to activate different initial signaling pathways leading to cardiomyocytes apoptosis. One model of cardiac apoptosis is that of I/R injury, associated with both the ischemic and the reperfused heart (14, 19–21). Perhaps this is related to the bursts of reactive oxygen species (ROS) that are generated during both stages (15). ROS is thought to be a potent stimulator of apoptosis as part of I/R injury (22).

It is, therefore, plausible that estrogen may attenuate the generation or actions of ROS resulting from oxidative stress following I/R. Additional mechanisms described in other cell models may be involved in I/R-induced apoptosis (11, 13). This includes p38 mitogen-activated protein kinase (p38 MAPK), generally stimulated by heterogeneous forms of cellular stress. The importance of p38 MAPK in the heart was well displayed in vivo when rats treated with a specific inhibitor of p38 displayed less remodeling and ventricular dysfunction after myocardial infarction (23). In addition, transgenic mice expressing a dominant negative p38 mutant were partially protected from I/R injury (24). There are four isoforms of p38: α, β, δ, and γ. Both p38α and p38β are found in the human heart, although the former is more abundant in ischemic or failing hearts (25–27).

In this study, we describe several molecular mechanisms by which estrogen protects myocytes from I/R-related cell death. Using cultured neonatal rat cardiomyocytes and an established in vitro system of hypoxia followed by reoxygenation (H/R) to simulate I/R, we dissected the relationship among E2-activated estrogen receptor (ER), mitochondrial ROS generation, p38α and p38β MAPK isoforms, and PI3K in the...
net outcome of myocyte survival. We report the novel findings that E2 differentially modulates p38α and β isoforms in the cardiomyocyte, preventing apoptosis during hypoxic/oxidative stress.

MATERIALS AND METHODS

Harvest and Culture of Neonatal Rat Cardiomyocytes—All studies were approved by the Research and Development and Animal Use Committees of the Long Beach Veterans Affairs Medical Center. Myocytes were isolated from the hearts of 1–3-day-old rats (Sprague-Dawley) using a neonatal cardiomyocyte isolation system kit (Worthington) as reported previously (28). Synchronous spontaneously contracting cells in a single layer were plated at 1.5 × 10⁶ cells/ml density after 1 h of differential plating to remove non-myocytes. Greater than 99% of the population was determined to be cardiomyocytes by immunostaining with cardiac myosin heavy chain monoclonal antibody (AbCam). The cells were grown in L-15 Leibovitz culture medium supplemented with 10% fetal bovine serum in a standard tissue culture incubator.

Simulated Ischemia-Reperfusion Model—Neonatal rat cardiomyocytes were synchronized in serum-free L-15 Leibovitz culture medium overnight and treated with 10 nM E2 ± reagents of interest or control vehicle. The cells were placed into an anaerobic chamber (GasPack system, BD Biosciences) for 18 h, which was purged with 95% N₂, 5% O₂. Synchronous spontaneously contracting cells were harvested and cultured in X-Vivo 20 media (Lonza) using a neonatal cardiomyocyte isolation system kit (Worthington). The cells were cultured in X-Vivo 20 media supplemented with 10% FBS and 1% penicillin-streptomycin. After 18 h, the cells were harvested and seeded in a 1:1 ratio with X-Vivo 20 media and 10% FBS in 10-cm dishes for 18 h, which was purged with 95% N₂, 5% O₂. Synchronous spontaneously contracting cells in a single layer were plated at 1.5 × 10⁶ cells/ml density after 1 h of differential plating to remove non-myocytes. Greater than 99% of the population was determined to be cardiomyocytes by immunostaining with cardiac myosin heavy chain monoclonal antibody (AbCam). The cells were grown in L-15 Leibovitz culture medium supplemented with 10% fetal bovine serum in a standard tissue culture incubator.

Cell Viability—At the end of hypoxia-reoxygenation, cells were harvested and assayed for cell viability. Cell viability was determined by the Trypan blue dye exclusion test, which measures the percentage of non-viable cells.

Apoptosis Assays—Early stages of apoptosis were assessed by annexin V-FITC fluorescence microscopy (Pharmingen) according to the manufacturer’s protocol. Briefly, cells were washed with annexin V binding buffer, incubated with annexin V-FITC for 15 min at room temperature, and then counterstained with Hoechst 33342 for nuclei, washed, and visualized by FITC analysis.

Late stages of apoptosis were also assessed, using the DeadEnd™ Fluorometric TUNEL system (Promega) according to the manufacturer’s instruction. Briefly, cells in culture were washed after H/R, fixed with 4% formaldehyde in phosphate-buffered saline, permeabilized with Triton® X-100, labeled with fluorescein-12-dUTP, and stained with Hoechst 33342 for nuclei, and green fluorescence of apoptotic cells was analyzed by fluorescence microscopy.

Necrotic cells were detected by differential propidium iodide (PI) and Hoechst 33342 staining. Intact membranes of healthy cells exclude PI, whereas necrotic cells are stained brightly with PI after a short incubation. Briefly, cardiomyocytes grown on glass-bottomed, 35-mm culture dishes underwent overnight hypoxia followed by reoxygenation and then were washed, incubated with 1.5 μg/ml Hoechst 33342 in phosphate-buffered saline for 15 min at room temperature, washed again, incubated with 4 μg/ml PI in phosphate-buffered saline for 30 min at room temperature, and then visualized under the fluorescence microscope. From each condition, 500 cells were counted. Experiments were repeated three times and statistically analyzed as below.

ROS Detection—At the end of hypoxia-reoxygenation, cells were assayed for intracellular ROS by using Image-iT™ LIVE green reactive oxygen species detection kit (Molecular Probes), counterstained with Hoechst 33342 for nuclei, and imaged under a microscope, using fluorescent filter sets.

p38 Kinase Detection and Kinase Studies—Cardiomyocytes were synchronized in serum-free medium and then incubated with or without E2 (10 nM), with or without 1 μM ICI182780, and/or with or without 1 μM SB203580. Hypoxia-reoxygenation was then applied as above. Cells were then lysed, and the lysate was immunoprecipitated for p38α or β kinase using polyclonal anti-p38α or anti-p38β antibody (Santa Cruz Biotechnology) conjugated to Sepharose beads, respectively. Cell lysate was then used for in vitro kinase assays with ATF2 as substrate, as described previously (11). The phosphorylated substrate was separated on 10% SDS-PAGE gel and detected by autoradiography, and the bands were quantitated by laser densitometry. Western blotting for total p38α or p38β was performed to demonstrate equal amounts of immunoprecipitated kinase proteins for each condition.

Western Blotting—After H/R was applied, cardiomyocytes were lysed and loaded onto 10% SDS-PAGE gel in the same manner as above. Proteins were transferred to nitrocellulose membrane, which was blocked with 3% nonfat milk and 2% bovine serum albumin in 0.05% Tween-Tris-buffered saline buffer. After binding with primary antibody against p38α, p38β, phosphorylated Akt, or total Akt protein (Santa Cruz Biotechnology), the membrane was washed, and then secondary antibody was applied. The membrane was then incubated with chemiluminescence reagents using a commercially available kit (ECL Plus Western blotting detection system, Amersham Biosciences) and exposed to film.

Adenoviral Infection—Adenoviruses expressing dominant negative p38β MAPK were generously provided by Dr. Jiahui Han (Scripps Research Institute, La Jolla, CA) (29). Viruses were amplified and titrated in 293 cells. The suppression of the p38 activity in cardiomyocytes was confirmed by immunoprecipitation of the kinase from the infected cells and kinase activity assay as described above. Viral stocks at a multiplicity of infection of 25–50 particles/cell were used to infect cardiomyocytes overnight. The cells were fed fresh medium the following day and further incubated for 24 h until ready for experiments. Experiments were performed 36–48 h after infection.

Statistical Analysis—All experiments were repeated at least three times. Data were combined and analyzed by Student’s t test using GB-Stat software for statistical significance at p value < 0.05.

RESULTS

ER protects Cardiomyocyte from Apoptosis by Suppressing p38α Following Simulated I/R—Approximately 60% of cardiomyocytes were found to be non-viable after undergoing overnight hypoxia followed by reoxygenation (Fig. 1A). Cell death was significantly reversed in the presence of 10 nM E2. ICI182780, a specific inhibitor of ER, demonstrated involvement of ER in the cell survival actions of the steroid hormone. A large portion of cell death was due to apoptosis, as evidenced by positive annexin V staining (measuring the early stages of apoptosis), significantly prevented by E2 (Fig. 1B, left panel). The anti-apoptotic action of E2 was prevented by ICI182780. A second method, the deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay (assessing late stages of DNA fragmentation), also illustrated E2 prevention of cardiomyocyte apoptosis (Fig. 1B, right). Death due to necrosis was less than 5% of the total cell population in our H/R system (data not shown), as determined by strong uptake of PI in necrotic cells, in contrast to PI-negative cells (30).

We then sought to delineate molecular mechanisms by which E2 reduced apoptosis. Prominent signaling molecules activated under a variety of cellular stresses include the p38 MAPK family. Here, blocking p38 kinase activity with a specific inhibitor, SB203580, resulted in a reduced number of apoptotic myocytes (Fig. 2). This

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occurred in a similar magnitude to survival induced by E2, and the effects of SB203580 + E2 were not different from either alone. Although SB203580 is a soluble inhibitor of both p38β (31, 32), our finding is consistent with a previously published report that showed, by using a SB203580-resistant p38α mutant, that the anti-ischemic effect of SB203580 was through inhibition of p38α (33). We examined whether the improved cell survival due to E2 correlated with suppression of p38α by the hormone (Fig. 3). Following H/R, p38α kinase activity increased. E2 efficiently blocked the activity of this pro-apoptotic kinase, and antagonizing ER with ICI182780 restored the activity of p38α. Therefore, inhibition of p38α by E2/ER contributes to cardiomyocyte survival.

Estrogen Blocks the Generation of ROS Upstream of p38α in Apoptotic Signaling—We then examined at which steps of the apoptotic process the E2-p38 interaction interceded. Apoptosis in general can be either death receptor-mediated (extrinsic pathway) or via cytochrome c release from mitochondria (intrinsic pathway). Human cardiomyocytes have the highest density of mitochondria of all mammalian cells (34). Mitochondria play a key role in apoptosis, releasing apoptotic factors into the cytosol. Ischemia/reperfusion generates ROS, potent inducers of cardiac apoptosis (22), and a majority of ROS originates from mitochondria during oxidative stress. We postulated that E2 would affect ROS-driven apoptosis and tested whether attenuation of apoptosis by E2 was linked to reduction of ROS. After myocytes underwent H/R, intracellular ROS was detected using 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate-based assay (Fig. 4A). Considerably more ROS was observed following H/R, when compared with the normoxic condition, as expected. Most of ROS were generated from mitochondria, as rotenone suppressed ROS following H/R. Rotenone inhibits the mitochondrial electron transport complex I and is known to inhibit mitochondrial ROS generation in cardiac cells (35, 36). We also used another specific inhibitor of mitochondrial ROS, Mito-Q (37), to confirm that ROS detected in our system were from mitochondria (data not shown).
blocks ROS generation during H/R and 2) ROS may act upstream of p38α.

To confirm that ROS is upstream of p38α, we examined the kinase activity in the presence of ROS inhibitors, rotenone and N-acetyl-cysteine (NAC). The p38α activity decreased drastically in the presence of rotenone or NAC (Fig. 4B), consistent with a previous report demonstrating that mitochondrial ROS can initiate p38 MAPK phosphorylation (38). Blockade of ROS generation and subsequent inhibition of p38α, by rotenone or NAC, led to better survival of cardiomyocytes, as shown in Fig. 4C. Also implied from the marked reversal of apoptosis by rotenone is that a significant stimulus for cell death during H/R arises from mitochondrial ROS production. Taken together, an important mechanism for the survival of cardiomyocytes conferred by E2/ER during H/R stems from inhibition of both ROS generation and subsequent p38α kinase activation.

**Estrogen Up-regulation of p38β Is Essential in Cardiomyocyte Survival**—Among the four known p38 isoforms (α, β, δ, and γ), mainly p38α and p38β are expressed in the heart (26). The two kinases also have opposing effects on apoptosis. In contrast to the pro-apoptotic property of p38α, p38β can enhance cell survival (11, 29). We hypothesized that anti-apoptotic actions of E2/ER could involve inverse regulation of the two p38 subtypes during the H/R-related injury. E2 alone was found to up-regulate p38β significantly during normoxia or H/R (Fig. 5A). Additionally, p38β kinase activity was not significantly altered by hypoxic/oxidative stress in the absence of E2.

To confirm that p38β is essential for the E2-mediated rescue of cardiomyocytes, we suppressed endogenous p38β function and determined the extent of the cell death. This was accomplished by expressing a dominant negative p38β construct (p38β DN) (Fig. 5B) containing a TGY → AGF mutation in the activating phosphorylation site. This mutation renders the kinase inactive (29). When the p38β DN protein was expressed, E2 no longer stimulated p38β activity. Upon application of H/R to these p38β DN-expressing cells, the anti-apoptotic effect of E2 was substantially reduced (Fig. 5C). We inferred from these results that a large part of E2 protection of cardiomyocytes under ischemic/oxidative stress was due to activation of p38β.

As shown above, an important trigger for the myocyte to undergo H/R-induced apoptosis was the generation of ROS. Given the interaction of E2 and p38β necessary for myocyte survival above, we postulated that E2-activated p38β might decrease the oxidative stress. Thus, we examined ROS production in cells expressing p38β DN mutant (Fig. 5D). As before, H/R significantly increased the generation of ROS, which was strongly suppressed by E2. Expressing dominant negative p38β kinase, however, led to a large percentage of cells producing ROS in the presence of E2. Counteraction of steroid-mediated ROS suppression by the dominant negative kinase suggests a novel mechanism for E2 and p38β in abrogating the oxidative stress response in cardiomyocytes.

**E2 Regulates Multiple Points of Signaling to Cell Survival**—A previously published report suggests that E2 stimulates PI3K/Akt, reducing apoptosis in the heart during H/R (18). Furthermore, MAPK family proteins and PI3K may form an integrative signaling network important for regulation of apoptosis (39). We speculated that PI3K might interact with p38α and/or p38β in our experimental setting. Alternatively, PI3K activity may represent a parallel pathway to cell survival.

We therefore determined whether the rescue from oxidative stress was a mechanism behind E2-PI3K protection. We performed H/R experiments in the presence and absence of a PI3K inhibitor, wortmannin, and examined intracellular ROS (Fig. 6A). In the presence of wortmannin, reduction of ROS by E2 was reversed, suggesting that E2 signaling through PI3K prevents ROS generation. Blocking PI3K with
wortmannin resulted in a higher level of ROS even at normoxia. This observation raised an interesting possibility that PI3K may be involved in baseline control of ROS turnover in a cardiomyocyte. Since we showed that ROS was upstream of p38α, our results suggest an interactive signaling pathway modulated by E2.

To further probe the relationship between PI3K and E2 in ROS-induced apoptosis, we assessed p38α activity in the presence of the PI3K inhibitor (Fig. 6B). As expected, p38α activity was increased in H/R and blocked by E2. However, E2-mediated inhibition of p38α was significantly reversed by blocking PI3K. Wortmannin alone did not affect p38α in the presence or absence of H/R. This illustrates that PI3K plays a novel intermediary role in E2 inhibition of p38α, in the setting of cardiomyocyte stress.

Akt is an anti-apoptotic protein whose phosphorylation and activation depends on PI3K (10, 18). We detected p-Akt by Western blotting to assess the functional activity of PI3K in the presence of p38 inhibition (Fig. 6C). Akt phosphorylation was not significantly altered in H/R but was enhanced in the presence of E2. PI3K inhibition with wortmannin reversed E2-mediated phosphorylation of Akt. Blocking p38 MAPK with SB203580, on the other hand, did not affect p-Akt stimulated by E2. This is consistent with the result from Fig. 6A, suggesting that PI3K/Akt lies proximal to p38 in the anti-apoptotic actions of E2. We then studied specifically the relationship between PI3K and p38β as both were associated with E2-mediated suppression of ROS. Fig. 6D demonstrated that E2 stimulation of p38β was mostly inhibited in the presence of wortmannin.

The data in total suggest that PI3K/Akt activated by E2 suppresses ROS and subsequently p38α. Activation of the proximal PI3K/Akt pathway by E2 led to increased p38β activity. p38β then suppressed ROS, with subsequent inhibition of pro-apoptotic p38α and apoptosis.

**DISCUSSION**

Understanding the pathophysiology of I/R injury and how endogenous molecules such as estrogen mitigate the resulting cardiomyocyte apoptosis is important. In the present study, we report novel mechanistic details of how estrogen protects cardiomyocytes from apoptosis. We found that E2 modulates two distinct and opposite functions of p38 isoforms in its protection of cardiomyocytes. In the H/R-induced apoptotic pathway, E2 inhibition of mitochondrial ROS is vital to cell survival, and protection by E2 is exerted through an integrated signaling network involving PI3K, ROS, p38α, and p38β. By using specific inhibitors and dominant negative protein expression, we defined the order of the signaling cascade among these key molecules. E2-induced PI3K inhibited ROS, which is generated by I/R to activate p38α kinase. This is consistent with previous reports that redox intermediates mediate p38 MAPK activation in an angiotensin II-signaling system (40–42) and that exogenous addition of ROS stimulates activity of p38 (38, 43).

Of the four p38 MAPK family members, p38α is the predominant...
form in the cardiac tissue (26). We found in cultured cardiomyocytes that blocking this isoform with SB203580 resulted in almost complete reversal of apoptosis, attesting to the fact that p38α plays a major role in stress-induced apoptosis. Estrogen inhibited p38α via PI3K signaling to the suppression of ROS during simulated I/R. Part of the cellular insult is related to the mitochondrial ROS, which is a potent stimulator of apoptosis, especially in the setting of I/R (22). p38β, on the other hand, is often pro-survival in function, thus acting opposite of p38α (29). Here, we showed that a dominant negative p38β significantly reversed the survival effects of E2. Interestingly, hypoxic stress did not elicit a compensatory increase in p38β activity. E2 acted to modulate the balance between the two isoforms favoring cell survival during stress.

We propose a working model of how estrogen protects cardiomyocytes from ischemia-reperfusion injury, pictured in Fig. 7. Upon hypoxic and oxidative insult, ROS is generated from mitochondria. The increased ROS stimulates activation of pro-apoptotic p38α, which exists in balance with the opposing p38β until cellular stress occurs. However, in the presence of the E2-ER complex, anti-apoptotic signals are activated, probably at the cell membrane. This includes PI3K activation, subsequent inhibition of ROS generation, and suppression of...
p38α activity. E2-ER also augments pro-survival p38β kinase. This activation is in part through PI3K/Akt activation stimulated by E2. p38β and PI3K activation results in ROS suppression, removing a stimulus for p38α activation and apoptosis.

One potential drawback of our model is that the results derive from neonatal myocytes. I/R injury is a pathological process most commonly associated with coronary artery disease, thus observed in adults. Although there are obviously differences between neonatal and adult cardiomyocytes, intracellular distribution and differential localization of ER isoforms upon E2 stimulation are identical between them.3 In cardiomyocytes, intracellular distribution and differential localization of ER isoforms was mainly due to suppression of ROS, thus reducing apoptotic death.4 In conclusion, we report new findings in which E2 directly promotes cardiomyocyte survival during ischemic stress in women.

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