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Estrogen and the Female Heart

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Abstract

Estrogen has a plethora of effects in the cardiovascular system. Studies of estrogen and the heart span human clinical trials and basic cell and molecular investigations. Greater understanding of cell and molecular responses to estrogens can provide further insights into the findings of clinical studies. Differences in expression and cellular/intracellular distribution of the two main receptors, estrogen receptor (ER) α and β, are thought to account for the specificity and differences in responses to estrogen. Much remains to be learned in this area, but cellular distribution within the cardiovascular system is becoming clearer. Identification of GPER as a third ER has introduced further complexity to the system. 17β-estradiol (E2), the most potent human estrogen, clearly has protective properties activating a signaling cascade leading to cellular protection and also influencing expression of the protective heat shock proteins (HSP). E2 protects the heart from ischemic injury in basic studies, but the picture is more involved in the whole organism and clinical studies. Here the complexity of E2's widespread effects comes into play and makes interpretation of findings more challenging. Estrogen loss occurs primarily with aging, but few studies have used aged models despite clear evidence of differences between the response to E_2 deficiency in adult and aged animals. Thus more work is needed focusing on the effects of aging vs. estrogen loss on the cardiovascular system.

Keywords

estradiol; 17β-estradiol; ERα; ERβ; GPER; cardioprotection; HSF1; heat shock proteins; HSP72; HRT; TNFα; inflammation; heart; cardiovascular; mitochondria; PKC

Introduction

Estrogens are potent steroid hormones present in high levels in women from adolescence to menopause. Estrogens have many properties, both protective and deleterious, and there have been numerous reviews on estrogen and cardiovascular disease.(1–6) The pace and depth of

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estrogen research has steadily increased since the identification of the estrogen receptors and the development of knockout models, facilitating investigation of the many functions of estrogens.

Estrogen Receptors, Genomic/Nongenomic Effects—Estrogen binds two intracellular receptors, estrogen receptor (ER)α and ERβ. These two receptors are primarily found in the cytosol and in the nucleus (figure 1). They are also loosely associated with the plasma membrane, rather than transmembrane like many other receptors. Mitochondrial localization has also been reported. Genomic actions mediated by nuclear ERα are well-described (7;8) and involve ligand binding at E_2 response elements. Non-genomic (rapid) effects of E_2 are thought to be mediated by ERα and/or ERβ localized to the plasma membrane (9–11) and associated functions include Ca^{2+} homeostasis, anti-apoptotic effects and mitochondrial metabolism.(10) Rapid ER signals are also known to regulate ER gene transcription in the myocardium.(12) In this regard, ERs are subject to post-translational modification (PTM) through phosphorylation, acetylation and sumoylation, which not only has the potential to influence ER activity, but may also influence ER stability and localization, particularly with aging (for review see 13–20).

A third receptor, G-protein-coupled estrogen receptor (GPER) or GPR30, has been more recently described. GPER is a classic membrane receptor with 7 transmembrane spanning domains.(21) This receptor is discussed below.

It is thought that at least part of the specificity of E2 signaling is the result of differential expression and cellular localization of the ERs in different tissues and cell types. As ERα and ERβ have been studied the longest, much more is known about their localization than that of GPER. Deconvolution of rat endothelium (cerebral and coronary arteries) images demonstrated that about a third of ERα co-localized with caveolin-1 at the plasma membrane.(22) In this study, ERβ was predominantly nuclear. In studies of the ovine uterine artery endothelial cells, ERα was present in the nucleus and associated with caveolin-1 at the plasma membrane.(23) In contrast, ERβ mRNA levels were much less than those of ERα, and ERβ did not localize with caveolin-1. Thus in adult endothelial cells, ERα is nuclear and membrane associated, co-localizing with caveolin.

Considerably less is known about the ERs in cardiac myocytes and vascular smooth muscle cells (VSMCs), and few studies have investigated their precise subcellular localization in these specific tissues.(24;25) For cardiac myocytes, ERα has been reported to localize to nuclear, cytosolic, mitochondrial, and plasma membrane fractions, respectively.(25) ERβ expression has been reported in human cardiac mitochondria,(10) however controversy exists regarding antibody quality and species dependency.(26) Indeed, we were unable to identify ERβ mRNA in hearts of adult or aged Fisher 344 female rats.(26) Much remains to be defined with regards to expression and localization of ERα and β in cardiac myocytes.

In contrast, more is known about VSMCs. In male SD rat mesenteric artery VSMCs ERα (the ER46 fragment) and ERβ associated with caveolin-1.(13) Both ERα and ERβ are found in adult human aortic VSMCs and both are predominantly nuclear.(13;14) VSMCs from the aortic arch of 8 week old rats expressed ERα and ERβ based on RT-PCR.(15) Similarly,

primary adult human VSMCs contained both receptors, but less ERβ than ERα was found.(16) VSMC cells lines had clearly different ERα and ERβ expression compared primary cells and pathology specimens. Three different VSMCs cell lines expressed either ERα or ERβ, but not both, which makes these cell lines less than desirable models for investigation of ERα and ERβ in VSMCs.(14;15) Multiple studies have shown that ERα and ERβ are both expressed in VSMCs and are primarily in the nucleus, however both ERα and ERβ are also at the plasma membrane and co-localize with caveolin-1. The receptors's relative quantities and intracellular allocation remains to be conclusively defined.

ER Membrane Complex, Caveolae and Caveolin—As noted above, ERα and β demonstrate both genomic and nongenomic effects, also referred to as nuclear and non-nuclear events. ERα is found in caveolae, where it complexes with caveolin 1, HSP90, eNOS, c-Src, Akt, and PI3K in the plasma membrane (27–29), and this is regulated by palmitoylation.(30–32) ER46, an ERα splice variant, rather than the full length receptor, is present in the caveolar complex.(27;29;30;33) The co-localization of ERα, eNOS and HSP90 within the caveolae facilitates eNOS activation by E2.(30;32;34) The chaperone protein, HSP90, promotes interaction of ER with signaling enzymes, such as eNOS. In contrast, caveolin-1 inhibits eNOS activity. Caveolin-1 knockout leads to increased eNOS activity in mice.(35) The multiple proteins needed for the caveolar-ERα signaling complex have been reviewed in great detail.(36) As would be expected given caveolin-1's inhibitory effects, eNOS activation entails separation from caveolin-1; this separation is usually mediated by calcium/calmodulin, but in special circumstances, including shear stress, eNOS activation does not require calcium. Several excellent papers have reviewed in details the steps involved in regulation of eNOS activation.(37;38)

GPER—There persists some lingering controversy regarding whether GPER is an ER, and more work is necessary to fully delineate the role of GPER in estrogen signaling.(39) Nonetheless, there is increasing acceptance of GPER as key receptor for estrogen signaling in the cardiovascular system.(40) GPER, which is also known as G-protein coupled receptor 30 (GPR30) and membrane estrogen receptor (mER), is the most recently recognized membrane associated ER.(41) RT-PCR and western blotting were used to detect GPER expression in porcine coronary arteries and human coronary artery VSMCs.(42) In addition, GPER is present in all four cardiac chambers with greater expression in the ventricles than the atria.(43) GPER has not been specifically shown to be in cardiac myocytes, but is present in HL-1 cells, which are an atrial tumor derived cell line with many properties found in cardiac myocytes.(44) There is one report that male GPER deficient mice had impaired cardiac function with decreased +dP/dt and −dP/dt, as well as increased LVEDP.(45) Heart weight/tibia length and lung weight/tibia length did not differ from wild type, nor did systolic pressure. Female hearts were normal. As it has yet to be demonstrated conclusively that GPER is expressed in cardiac myocytes, the authors concluded, after ruling out metabolic abnormalities, that the impaired ventricular function in male mice was not a direct cardiac effect of GPER loss, but might reflect vascular changes secondary to GPER loss.

GPER, which has seven transmembrane domains similar to traditional membrane receptors, can mediate E2 driven vasodilation.(21;46) G1, a selective agonist for GPER, relaxed both

porcine aortic rings and human coronary artery VSMCs in endothelium independent manner.(42) G15, a selective inhibitor of GPER prevents G1 mediated relaxation.(42) Further work showed GPER activation of the large-conductance calcium and voltage activated potassium channel (BK_{ca}) in coronary artery VSMCs by GPER, but, interestingly G1 had no direct effect on BK_{ca}.(42) GPER inhibited endothelin-1 mediated vasoconstriction.(47) These findings provide new insights into GPER mediated vascular relaxation. Several groups have also reported activation of GPER by aldosterone.(48–51) Aldosterone increased cardiac vagal tone via activation of GPER.(51) These findings support nongenomic functions for aldosterone, and raise questions about aldosterone/estrogen interactions in regulating GPER activation. The role of aldosterone in GPER activation has recently been reviewed.(48) Thus GPER, unlike ERα and β which are controlled solely by estrogens, has two or possibly more hormones able to activate it.

Clinical Trials, Hormone Replacement Therapy (HRT) and the Timing Hypothesis—Estrogen had been long viewed as cardioprotective based on anecdotal reports over many years. However, double blind, controlled trials of estrogen replacement post-menopause conducted in the 1990's, such as the Women's Health Initiative (WHI) and the Heart and Estrogen/progestin Replacement Study (HERS) demonstrated greater risk of cancer and cardiovascular disease (CHD) with estrogen replacement.(52–54) This was a surprising outcome as premenopausal women have a much lower incidence of CHD than males. Careful consideration of the unexpected results of these clinical trials led to the realization that on average the women enrolled were 10 years post-menopause, and thus were receiving late estrogen replacement long after their ovaries had ceased to make estrogen. This suggested that such a long gap before initiating estrogen could result in unexpected deleterious effects, and this was termed the **timing hypothesis**.(55–57) Recently we have demonstrated that delayed estrogen replacement in aged ovariectomized (OVX) female Norway Brown(NB) rats led to a substantial number of gene changes in the heart including increased expression of CD11b, MIP-1β, STAT3, EMAP II, fibronectin, caspase 6 and MADD.(58) Although neither TNFα or iNOS had increased RNA levels in this model, both had marked increase in their protein levels with late replacement. A striking finding was increased expression of genes that stimulate leukocyte attraction and adhesion, initial steps in atherosclerosis. The most significant finding was the growth in the expression of pro-inflammatory and pro-apoptotic proteins with late estrogen replacement, exemplified by the increase in TNFα and iNOS protein levels with late replacement. Since TNFα enhances the expression of adhesion molecules this would only further promote atherosclerosis.(58)

HRT Controversies B—An important issue in HRT is actual type and delivery method of hormone replacement. In HRT estrogen is generally used in combination with progesterone, except in women who have had a hysterectomy, who given the absence of the uterus can receive estrogen alone without risk of endometrial cancer. Estrogen is a powerful steroid hormone with 17β-estradiol (E2) the most active metabolite in humans. There are two important issues in estrogen replacement. Estrogen taken orally leads to high hepatic estrogen levels secondary to first pass metabolism. These high hepatic levels of estrogen can stimulate protein synthesis and are thought to increase the risk of thrombosis compared to transdermal delivery, but this interpretation remains contentious.(59–62) Increasingly studies

support transdermal estrogen as safer with regards to thrombotic events.(63) Estrogen delivery by transdermal patch precludes the first pass surge in hepatic estrogen and may have fewer complications. Indeed, the Kronos Early Estrogen Prevention Study (KEEPS) represents an ongoing clinical intervention trial, which aims to determine the protective efficacy of early transdermal HRT administration in younger menopausal women on cardiovascular outcomes to provide a more definitive test of this hypothesis.(64)

Differences Among Estrogens—The second issue in estrogen therapy is the type of estrogen replacement. The most frequently used estrogen for HRT is conjugated equine estrogen (CEE), which is extracted from the urine of pregnant mares (Premarin). CEE consists of sodium estrone sulfate, sodium equilin sulfate, and sodium 17α-dihydroequilinenin, but no 17β-estradiol, the primary and most potent human estrogen.(65) Various estrogens have disparate binding affinities and specificity for the estrogen receptors (ERs); this will lead to distinct downstream effects. These differences in the estrogen composition of HRT could have a significant impact on the effects of estrogen replacement.

CEE, Estrogens and Thrombosis—Estrone has been connected with thrombin generation, a critical step in the coagulation cascade.(59) In a population based case-control study of HRT in postmenopausal women over six years, CEE were associated with greater risk of venous thromboembolism (odds ratio 2.08).(66) Neither the risk of MI nor stroke reached statistical significance, but the study was underpowered with only 68 subjects. Others have reported less thrombotic events with estradiol vs. CEE.(67) Hence, estrones have the possibility to significantly enhance the chance of thrombosis, and as CEE includes a significant amount of estrones, CEE would be anticipated to increase the probability of thrombotic events. However, there are only limited studies in this area and thus it is too early to make definitive conclusions.

Early Estrogen Replacemen—As a result of the clinical trials of the 1990's and the timing hypothesis, there was a move to initiate HRT early, at the first sign of menopause, rather than waiting. As noted above, the KEEPS trial was initiated in 2005 to investigate the effects of a low dose oral CEE or transdermal patch of combination estrogen and progestin in women with an average age of 52 years (64), and results should be forthcoming. Another approach has been low dose unopposed estrogen, and there is some very limited data to suggest that this is without harm.(68;69) Although the timing hypothesis may prove supportive for short-term cardioprotection with HRT in younger postmenopausal women, the evidence for diminished efficacy and possible detrimental effects of HRT on coronary heart disease (CHD) risk in older women, as well as concerns regarding increased breast and ovarian cancer risk with long-term HRT use remain problematic.

Estrogen and Myocardial Ischemia—Premenopausal women have decreased risk for CHD compared to age-matched men(70), as well as a decreased incidence of LV hypertrophy and cardiac remodeling following myocardial infarction (MI).(71) Post menopause the prevalence of CHD increases, such that aged women have both reduced ischemic tolerance(72;73) and increased mortality following MI(74) relative to age-matched men. In distinction from aging, independent effects of E_2 deficiency on cardiovascular risk

have also been observed. As early as 1953, Wuest *et al.* noted the increased prevalence of coronary artery disease in autopsy studies of premenopausal women who had undergone ovariectomy (OVX) (75), and numerous studies conducted throughout the ensuing five decades have demonstrated increased risk for CHD and MI in both postmenopausal and ovariectomized premenopausal women.(70;76–79) Indeed, E_2 deficiency alone reduces ischemic tolerance in hearts of both adult mice(80) and rats.(81) Epidemiological data also indicate the interaction of gender and aging, and influence of menopausal status on the determination of cardiovascular risk in aging women. Animal and human studies have each identified both functional and cellular alterations in ischemic tolerance and cardioprotection due to the independent and combined effects of aging and E_2 deficiency. A notable limitation in identifying specific mechanistic underpinnings in the adult and aged female heart has been differences in experimental models used to recapitulate postmenopausal E_2 deficiency.(82) In the paragraphs that follow, information regarding the influence of E_2 on cardioprotective signals is presented within the context of information gleaned from studies associated with the protective phenomenon of ischemic preconditioning (IPC). Particular emphasis is also placed on available experimental models of E_2 deficiency and aging.

Estrogen Receptors (ER) and Cardioprotection—As noted above, effects of E_2 in the heart are primarily mediated by ERα and ERβ, although the precise subcellular distribution of cardiac ER receptors remains to be fully elucidated. Evidence linking ERα and ERβ polymorphisms to adverse cardiac outcomes in women(83–85) suggest that ERα and ERβ may each play distinct roles in cardioprotection. While the importance of cardiac ER subtypes in ischemic injury remains controversial, studies employing ERα and ERβ deficient mice have each demonstrated reductions in ischemic tolerance.(86;87) However, it is important to note that ER deficiency in these models is not cardiac-specific, and some results are confounded by use of mice which encode a truncated ERα, as well as a metabolic phenotype which develops with age.(88;89) Nevertheless, in mice completely null for ERα, greater I/R (ischemia/reperfusion) injury and more impaired mitochondrial function(90;91) were observed vs. wild type mice. Further, activation of ERα with the specific agonist, propyl pyrazole triol (PPT), protects the *in vivo* rabbit heart from I/R injury, while the specific ERβ activator, diarylpropiolnitrile (DPN) was without effect.(92) Recent studies also suggest a greater role for ERα vs. ERβ in the modulation of endothelial progenitor cells and cardiac repair.(93;94) Taken together, these data support a dominant role for ERα as the cardioprotective ER involved in I/R injury.(92)

In contrast, acute ERβ activation does not appear to impact functional recovery following I/R injury in either adult or aged female rats, and ERβ mRNA was not detected.(26) The lack of measurable ERβ in the F344 rat myocardium(26) was surprising given results gleaned from past studies utilizing the ERβ knockout mouse model (86;95;96) mentioned heretofore. In this regard, ERβ expression in the rodent myocardium remains controversial (10;97–102), and the protein signal produced by ERβ antibodies in cardiac homogenates may be the result of cross-reactivity with ERα.(26) Combined with these previous findings, either ERβ signaling varies substantially between rat, rabbit and murine models or, cardioprotection observed in mouse models may be mediated indirectly through extra-cardiac ERβ signaling. For instance, DPN injection at the rostral ventrolateral medulla, an

area associated with autonomic cardiovascular control, has been shown to reduce systemic arterial pressure in rats.(103) That ERβ activation can reduce systemic arterial pressure via autonomic influence indicates that additional autonomic cardioprotective mechanisms attributed to E_2 may be mediated through ERβ. Indeed, E_2-linked cardioprotection has been associated with reduced sympathetic input to the heart and vasculature during ischemia in female rats, resulting in reduced heart rate, mean arterial pressure, arrhythmia frequency, and overall improved ischemic tolerance vs. males.(104;105) Therefore it is plausible that hypertension and vascular dysfunction observed in whole body ERβ knockout mice as well as cardioprotection observed in chronic DPN treated mice may be explained by indirect ERβ effects on autonomic cardiac control and not direct effects on the myocardium.(106;107) Definitive studies on ER subtype distribution in adult myocardium are needed.

GPER may also activate the nonnuclear protective response via a truncated 36 kDa ERα (108) GPER activation by G1 reduced infarct size in both the isolated perfused male mouse heart and the isolated perfused male Wistar rat heart.(43;109) Opening of the mitochondrial permeability transition pore (mPTP) was also inhibited by GPER activation in the same study. Treatment with the ERK inhibitor, PD98059, eliminated the protective benefits of GPER activation. GPER activation can attenuate diastolic dysfunction and ventricular remodeling after OVX.(40) The demonstration that rapid ERα and/or GPER activation reduces I/R injury in the female heart supports a key role for non-genomic ER signaling in the maintenance of cardioprotection. In this regard, 17-β estradiol is the major physiological E_2, but it has a similar affinity for ERα and ERβ. As noted, a number of selective ERα and ERβ agonists have been created and described; however, only a minority of these compounds has been evaluated extensively *in vivo*. The discovery of the GPER has also reinforced the need for additional ER specific modulators. Selective estrogen receptor modulators (SERMs) may be of great utility and in understanding the role of ERs in ischemic tolerance with aging.

PI3K-Akt-GSK-3β Signaling and Estrogen—Interestingly, many of the protective actions mediated by rapid ER signaling involve downstream effectors known to be associated with IPC, such as PI3K-Akt, eNOS and PKCε (figure 2, for review see 110). Increased levels and/or activity of Akt have also been observed in female (vs male) animal and human myocardium.(111;112) ERα-mediated nuclear transcription is also affected by Akt, and nuclear accumulation of Akt in human cardiocytes is increased 5.8-fold in adult women over men and reduced in postmenopausal women.(111) Collectively, these data suggest that the PI3K-Akt pathway is acutely activated by E_2. Urata and colleagues(113) recently demonstrated that E_2 administration (18 hrs.) in H9c2 cells leads to a reduction in hydrogen peroxide (H_2O_2)-induced apoptosis through upregulation of glutaredoxin (GRX), which was abolished by the ER inhibitor ICI-182,780. Effects were presumed ERβ-mediated since these cells do not express ERα.

A target of Akt which has been proposed as a convergence point for many cardioprotective signals is inactivation of mitochondrial GSK-3β and associated apoptotic signaling. This model is supported by I/R- and OVX-dependent changes in mitochondrial pGSK-3β which mirror changes in pAkt in adult but interestingly *not* aged rats (114), suggesting dysregulated Akt-GSK-3β interactions in aged. Additional mechanisms by which rapid E_2

signaling may influence GSK-3β and subsequent ischemic tolerance are worth noting. Recent studies suggest that GSK-3β can enhance ERα-mediated transcription (115) implicating the nuclear compartment as an potentially important site of regulation in the female heart. If this is so, several cardioprotective or apoptotic proteins that are modulated by E_2, such as heat shock proteins (116), ANT-1(117), or Cx43 (118) may show altered expression or activity, thus contributing to reduced ischemic tolerance in aging. Future studies are indicated to determine the role, if any, of altered gene expression in relation to cell survival with age-associated E_2 deficiency.

Age, Estrogen, and Ischemic Tolerance

Ischemia/Reperfusion Injury in Aging—Reduced ischemic tolerance and increased susceptibility of the heart to I/R injury is a hallmark adaptation of both aged human and animal hearts.(119–129) The aged heart is also refractory to endogenous protection from interventions like IPC, verifying inadequate protective cellular reserves.(129–131) The precise cellular mechanisms underlying this dysfunction, however, are incompletely understood. The problem is further exacerbated by the **paucity** of studies using females, limiting extrapolation of results. Reversal of cardioprotection with senescence is likely to involve aberrations in both intrinsic (i.e. excitation-contraction coupling) and extrinsic (adrenergic) inotropic regulatory mechanisms (for review see 122;131). However, alterations in cell signaling pathways related to metabolic and oxidative stress may also shift the balance from cell survival to cell death regulating pathways.(132–136) Extensive and ongoing research has thus focused on the identification of effective treatments for the reduction of I/R injury (termed *cardioprotection*), which may be implemented in a clinical setting of acute MI to limit infarct size and minimize loss of cardiac function.

With regard to mechanistic underpinnings, increases in infarct size following I/R in aged relative to adult female rats have been associated with decreased Akt and mitochondrial PKCε levels, as well as increased mitochondrial GSK-3β.(114) Hunter *et al.* further found that aged OVX rats exhibited more severely impaired functional recovery and greater infarct size following I/R than was seen with aging or OVX alone, suggesting an additive detriment of aging and OVX in the female rat heart.(114) In support of a protective role for PKCε targeting in the aged, E_2-deficient female rat heart, acute activation of PKCε prior to ischemia by local delivery of ψεRACK peptide has been associated with 1) improved functional recovery and reduced infarct size, 2) increased mitochondrial targeting of PKCε, and 3) candidate downstream signaling targets suggesting a role for activation of antioxidant enzymes as a mechanism of PKCε-mediated protection.(137) Specifically, mitochondrial HSP10, GPX, and SOD2 (MnSOD) abundance are significantly increased with ψεRACK administration in aged OVX hearts (by ~10, 20, and 30%, respectively) and likely attributable to PKCε-stimulated mitochondrial translocation or import of identified proteins.

With regard to the potential cardioprotective role of non-genomic ER activation in reducing I/R injury in aged female hearts, our work has also implicated a possible role for selective ERα activation as follows(25): 1) effectively reduced infarct size, 2) resulted in greater mitochondrial and particulate ERα localization coordinate with a protective pattern of PKCε activation, and 3) enhanced gene expression of the PKCε anchoring protein RACK2.

Collectively, these results demonstrate a protective role for non-genomic ERα signaling in the aged female rat heart, the cellular basis of which may involve two distinct PKCε-dependent mechanisms. What are less clear are the mechanisms which underlie altered cardiac ER translocation. As noted above, PTMs such as phosphorylation, acetylation and sumoylation (for review see 17–20;138) are known to effect ER targeting, the effects of which are unstudied in aging. Since some non-genomic ER effects are specific to aged animals(25), it will be important that future studies incorporate true models of aging in conjunction with E_2 deficiency to fully characterize the non-genomic ER response.

Mitochondrial Mechanisms of Cell Death—Mitochondria are the main source of both ATP and reactive oxygen species (ROS) in the heart ideally positioning them as mediators of, and therapeutic targets for, ischemic CHD. Because of the pivotal role played by the mitochondria in the maintenance of cell survival and cardioprotection, it is logical that age-associated reductions in ischemic tolerance might arise from alterations in mitochondrial proteins. Given the estimate that 1000 to 2000 proteins are expressed in the mitochondria(139), it is likely that the adaptation of additional mitochondrial proteins in aging and/or E_2 deficiency may contribute to the reductions in ischemic tolerance and increased I/R injury associated with advancing age and menopause. While correlational relationships between age-dependent declines in ischemic tolerance and altered expression and localization of cardioprotective signaling proteins have been noted in the female heart, the breadth and extent of protein changes have only recently been addressed. Notably, the reader is referred to several recent complimentary reviews on mitochondrial aging and mechanisms of cell death.(6;140–142)

Using a high throughput proteomics approach targeting the cardiac mitochondrial subproteome in adult and aged F344 female rats, we observed significant directional changes in **67** proteins with aged and/or aged OVX, and **32** were unique to aged OVX.(143) Notably only **6** proteins were similarly altered in adult OVX (voltage-dependent ion channel 1, adenine nucleotide translocator 1, cytochrome c oxidase subunits VIIc and VIc, catalase, and myosin binding protein C), highlighting the specificity of the E_2 deficiency response in adult vs. aged female rats. Proteins affected by aging were primarily related to cellular metabolism, oxidative stress and cell death, with the largest change seen in monoamine oxidase-A, a potential source of oxidative stress. About 50% of the identified proteins altered in aged OVX were associated with mitochondrial ATP production.(143) In aged, E_2-deficient female hearts, reduced quantity of protein subunits of electron transport chain complex I (NADH dehydrogenase), II (succinate dehydrogenase), III (cytochrome bc_1 complex), IV (cytochrome *c* oxidase), and V (F_0F_1 ATPase), and bidirectional changes in proteins involved in fatty acid substrate metabolism (acyl Co-A synthetase subunits) have been observed. In contrast, increases were primarily observed for proteins involved in carbohydrate and amino acid metabolism (pyruvate dehydrogenase subunits) and enzymes of the tricarboxylic acid cycle.(143) Increased levels of HSP60 and mtHSP70 in aged OVX are consistent with previous studies in aged male hearts(144) and may be related to alterations in mitochondrial matrix protein import of nuclear-encoded enzymes, which may or may not be balanced by changes in proteolysis. Measurement of the activity and/or

phosphorylation status(145) of these proteins is indicated for a more comprehensive characterization of metabolic alterations and substrate utilization in the aged female heart.

Estrogen, Cardiac Myocytes and the Heat Shock Response

E2 indirectly regulates cardiac HSP 72 expression in both male and female adult rat cardiac myocytes.(116;146;147) Cardiac HSP72 levels in female Sprague Dawley (SD) rats are twice that of males.(147) OVX reduces HSP72 to the levels of males over a period of 9 weeks, supporting an indirect estrogen effect. Besides its facilitating function in protein folding, HSP72 has cardioprotective effects, and reduces apoptosis by stabilizing the mitochondrial membrane and preventing apoptosome formation.(148–150) Preventing the normal increase in HSP72 in response to hypoxia/reoxygenation with antisense increases cardiac myocyte injury.(151) In isolated adult SD male and female cardiac myocytes, pharmacologic concentrations of E2 increased HSP 72 expression through consecutive activation of NFκB and HSF-1.(figure 2;116;146) Pre-treatment with E2 reduced injury after hypoxia/reoxy-genation.(116;146) E2 had similar effects in human coronary artery endothelial cells (HCAEC) from young donors.(152)

In young adult cardiac myocytes and in HCAEC, E2 can increase HSP 72 expression. In HCAEC this occurs through simultaneous activation of P38, JNK and Akt leading to ERK 1/2 activation followed by activation of NFκB.(153) The SERMS, raloxifine and tamoxifen activated the same pathways, except they did not activate JNK.(153) However in cardiac myocytes from aged OVX (studied 9 weeks post-OVX) NB rats with and without immediate E2 replacement, the activation of NFκB and HSF1 by E2 was lost.(24) Similarly, treatment with hypoxia and reoxygenation, which readily induces expression of HSP72, had no effect on HSP72 in aged cardiac myocytes, but did increase HSP72 in cardiac myocytes from adult (5–6 mo.) rats with OVX and with/without E2 replacement.(24) Thus, two different activators of HSP expression had no effect in aged female myocytes, regardless of estrogen status. HSP expression is controlled primarily by HSF(heat shock factor)1. In male hearts and skeletal muscle there is a reduction in activation of HSF1 and a loss of the heat shock response (upregulation of HSPs).(154;155) Similarly, aged male hepatocytes have loss of activation of HSF1 with stress.(156) In skeletal muscle there was no difference in HSF1 binding by EMSA, but no increase in HSP72 with exercise.(157) Electromobility shift (EMSA) measures binding of HSF1 to the heat shock element, but this needs to be followed by increased transcription. Phosphorylation of serines 303/307 on HSF1, as we saw in the aged female heart, does not interfere with binding, but prevents initiation of transcription.(24;158) Different causes for the loss of the heat shock response have been found in male tissues including reduced binding of HSF1 to the heat shock element (HSE) and loss of stability of the HSF1 trimer, which forms prior to nuclear translocation and binding of HSF1 to the HSE.(156) As the normal heat shock response reduces inflammation, stabilizes protein structure and facilitates removing irreversibly denatured proteins, loss of this response with aging is quite detrimental.

Cardiac Myocyte Changes with Aging—Basic studies are important to understand differences that occur with aging and loss of estrogen, so that we can better comprehend the mechanisms and extent of cardiovascular changes in aging females. Studies of cardiac

myocytes in aging and estrogen loss are remarkably limited, as most studies of estrogen loss using very young rodent models. We have found that cardiac myocytes from aged (22 mo.) OVX NB rats with and without immediate E2 replacement had differences in the expression of inflammatory cytokines.(24) Although plasma levels of IL-6 and TNFα did not differ amongst the groups in the study, the aged OVX cultured cardiac myocytes had increased expression of IL-6 and TNFα mRNA compared to all other groups, indicating that cytokines tissue levels may be more informative than plasma levels.(24) IL-6 and TNFα levels were decreased by treating the cardiac myocytes with E2 for 6 h. The aged OVX-derived cardiac myocytes also had a greatly reduced ability to handle reactive oxygen species (ROS), which increase with age.(24;159) E2 treatment in culture did not reduce ROS levels, even though it had reduced inflammatory cytokine levels. In other work, Ross and Howlett found that cardiac myocytes isolated from adult (3 mo.) Fisher 344 female rats had better recovery after simulated I/R and better resistance to injury compared to cardiac myocytes from male Fisher 344 rats.(160) Cardiac myocytes from adult OVX (5 mo. age) and aged Fisher 344 rats (24 mo.) lost the gender benefits seen in cardiac myocytes from young intact females. Overall, aged OVX derived cardiac myocytes had loss of the heat shock response, increased inflammatory cytokines and markedly reduced ability to handle ROS. E2 replacement at the time of OVX improved many of the changes, but inactivation of HSF1 by phosphorylation of serines 303/307 persisted.

Summary/Conclusions

Much has been learned with regards to the ERs, the cardioprotective effects of estradiol, and the nongenomic, downstream signaling cascade. However, many questions remain unanswered and/or incomplete. Further knowledge of the distribution of the three receptors is needed, and to what degree these receptors mediate functional responses to different estrogen compounds in different cell types/cellular locations. There is also a substantial literature supporting the use of 17β-estradiol to ameliorate traumatic injury.(161) A better understanding of the non-genomic actions of estrogen can potentially lead to improved clinical therapeutic interventions for treating acute coronary syndrome in aged women, specifically selective modulation of cardiac ERs and non-genomic ER signaling in an attempt to harness the protection associated with E_2 observed in adult women without increased cardiovascular risk observed from chronic HRT.

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Abbreviation

ANT-1	Adenine Nucleotide Translocator 1
CEE	Conjugated Equine Estrogen
CHD	Coronary Heart Disease
Cx43	Connexin 43

EMSA	Electrophoretic Mobility Shift Assay
eNOS	Endothelial Nitric Oxide Synthase
ER	Estrogen Receptor
GPER	G-Protein-Coupled Estrogen Receptor
HSF	Heat Shock Factor
HSP	Heat Shock Protein
iNOS	Inducible Nitric Oxide Synthase
IPC	Ischemic Preconditioning
IR	Ischemia Reperfusion
MI	Myocardial Infarction
NB	Norway Brown
OVX	Ovariectomy
PTM	Post translational modification
VSMC	Vascular Smooth Muscle Cell

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Highlights

- Cardiac non-genomic and genomic estrogen receptor signaling is reviewed.
- Hormone replacement controversies and cardiac outcome are identified.
- Experimental models of age-associated estrogen loss are described.
- Defective estrogen signaling contributes to cardiac ischemic injury with aging.
- Cardioprotective targets in the aged heart include heat shock proteins and PKC.

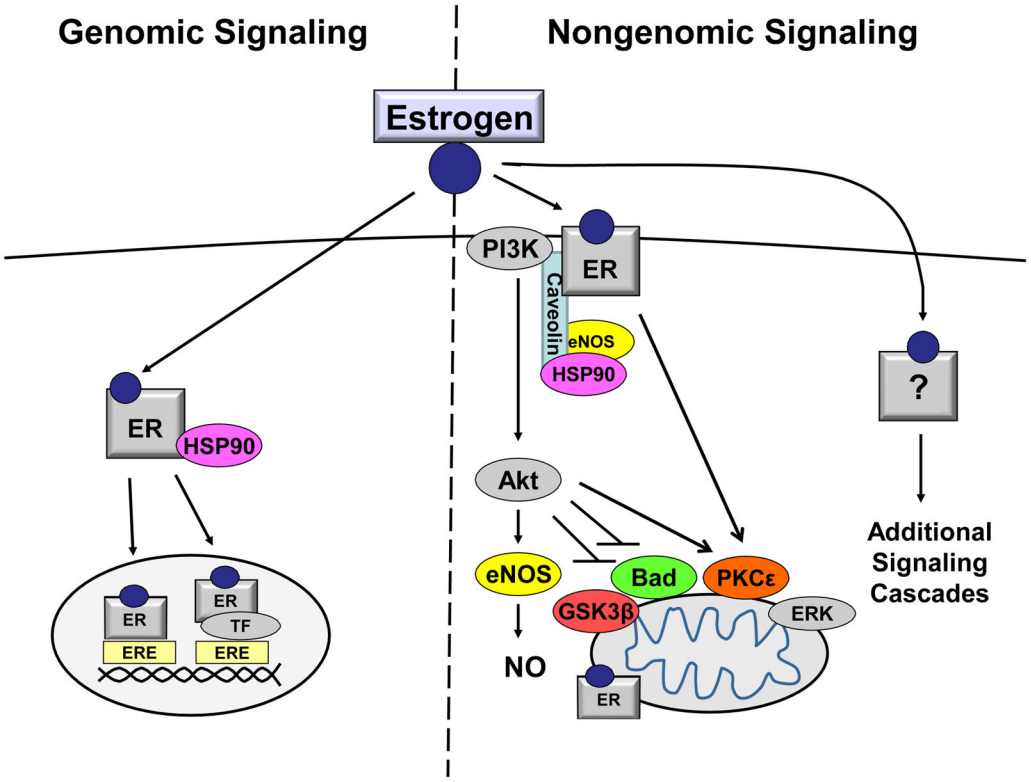

Figure 1.

Simplified schematic of cardioprotective cellular signaling mediated by genomic and non-genomic actions of estrogen receptor (ER) activation. Genomic actions mediated by nuclear ERs involve nuclear translocation and activation of protective gene transcription. Non-genomic ER actions include activation of downstream cardioprotective signaling pathways and possible mitochondrial localization. See text for specific details. Abbreviations: BAD, Bcl-2-associated death promoter; diacylglycerol, DAG; eNOS, endothelial nitric oxide synthase; ERE, estrogen response element; ERK, extracellular signal-regulated kinases; GSK-3β, glycogen synthase kinase-3β; HSP90, heat shock90; PI3K, phosphoinositide 3-kinase; PLC, phospholipase C; PKCε, protein kinase Cε; TF, transcription factor. Updated from Stice et al.(162)

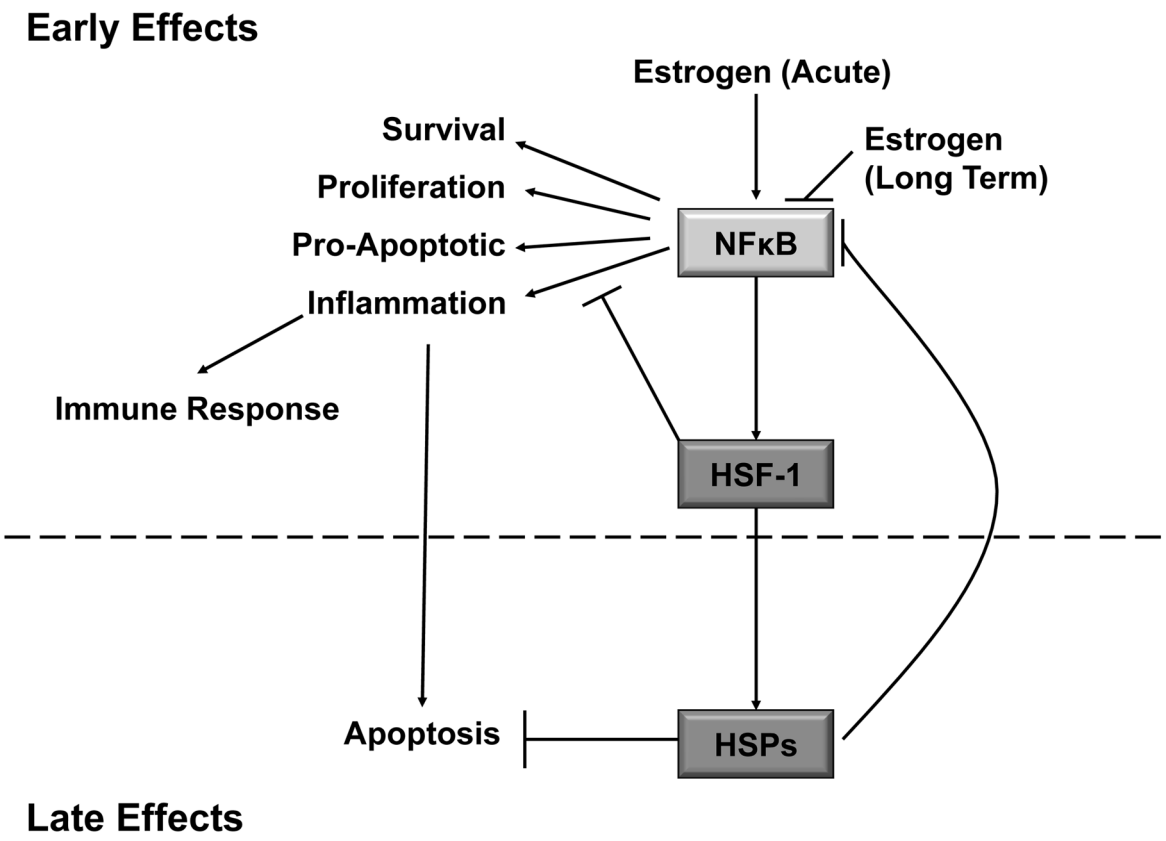

Figure 2.

Diagram summarizes interactions of NFκB, estrogen and the heat shock proteins. Treatment of cardiac myocytes or endothelial cells with estrogen leads to activation of NFκB followed by HSF1 activation and increased expression of HSP72. HSPs and HSF1 have feedback loops leading to suppression of inflammation and NFκB. Longer term estrogen treatment inhibits NFκB and increases HSP72 expression in the heart. See text for more detail. Updated from Sticee t al.(162)