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Cardioprotection in females: a role for nitric oxide and altered gene expression

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Abstract A number of epidemiological and animal studies have suggested a cardioprotective role for estrogen. This review will focus on the cardioprotective role of estrogen in ischemia-reperfusion injury. Estrogen binding to receptors can lead to altered gene expression and estrogen has been shown to induce expression of a number of genes that have been suggested to be important in cardioprotection. Estrogen is reported to increase expression of the plasma membrane glucose transporter GLUT4 and to increase carbohydrate metabolism. Estrogen has also been reported to increase mitochondrial biogenesis and to alter mitochondrial generation of reactive oxygen species. Estrogen results in upregulation of cardiac eNOS and nNOS, which have been shown previously to be important mediators of cardioprotection. Nitric oxide has been shown to result in S-nitrosylation and inhibition of the L-type calcium channel, thereby reducing calcium loading during ischemia. Nitric oxide has also been reported to inhibit complex I and inhibition of complex I has been reported to reduce activation of the mitochondrial permeability transition pore. Nitric oxide has been shown to result in activation of the mitochondrial K_{ATP} channel, which has been shown to be involved in cardioprotection. Estrogen can also activate rapid non-genomic pathways that activate cardioprotective-signaling pathways such as the phosphatidylinositol-3-kinase (PI-3 kinase) pathway which has also been shown to initiate protection. Taken together, estrogen by genomic and non-genomic pathways can result in the initiation of a number of signaling pathways that enhance cardioprotection.

Keywords Estrogen · Mitochondria · Nitric oxide

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Introduction

Epidemiological studies have suggested that pre-menopausal females have a reduced incidence of cardiovascular disease [1–3]. Much of the cardiovascular protection observed in females have been attributed to beneficial effects of estrogen on lipid profile and endothelial function [4]. The direct effects of estrogen on cardiac myocytes have not been extensively studied. Furthermore, despite reduced cardiovascular disease in pre-menopausal females, in a large clinical trial, hormone replacement therapy did not reduce cardiovascular disease [5]. The lack of estrogen mediated protection in the Women's Health Initiative (WHI) contrast not only with prior epidemiological studies, but also with data from animal studies. Potential reasons for the lack of protection in the WHI study, such as potential differences in the pharmacology between conjugated equine estrogen and 17-β-estradiol and the age of women at the start of treatment have been discussed in detail by others [6–8]. This review will focus on the data in animal models showing a protective effect of estrogen. Perhaps a better understanding of the mechanisms by which estrogen mediates protection in animal studies will provide insight into why hormone replacement therapy was not protective in the WHI study.

Estrogen receptor signaling

Most of the action of estrogen has been attributed to estrogen binding to either estrogen receptor (ER)-α or ER-β, two nuclear hormone receptors that act as ligand-activated transcription factors binding to DNA response elements. ER-α and ER-β are differentially expressed in different tissues of the body. ER-α and ER-β activate some common genes, but they also each activate a unique set of genes. Furthermore, ER-α and ER-β can oppose each other in a ying-yang relationship [9]. For example, ER-α induces and ER-β represses expression of apolipoprotein E in the hippocampus [10]. ER-α and ER-β, which can form homo and heterodimers, bind to DNA resulting in the recruitment of co-activators and co-repressors that modify estrogen mediated transcription. The expression of co-activators and co-repressors is tissue-specific and depends on cell context which will modify the effects of estrogen in a tissue specific manner. For example mitogen activated protein kinases (MAPK) can phosphorylate these co-regulators and modify their activity. Details regarding the complexity of ER interaction with co-regulators have been reviewed elsewhere [6, 11]. In addition, ligand bound ER can bind to DNA indirectly through complexes in association with SP1 (stimulator protein 1) or AP1 (activator protein 1) [12]. IGF and EGF have also been reported to cross talk with ER gene expression. IGF-1 can increase uterine weight in ovariectomized mice by a pathway that requires ER-α, since it does not occur in mice lacking ER-α [13]. Cross talk between ER and other transcription factors such as nuclear factor kappa B has also been reported [14].

In addition to estrogen action via binding to ER and activation of gene expression, there are data suggesting that estrogen can bind to an ER localized to the plasma membrane and acutely activate PI3-kinase and other signaling pathways [15]. There are also data suggesting that estrogen can bind to and signal via a G protein coupled receptor (GPR 30) [16]. Thus depending on the relative mix of receptors that are present in a target tissue, the response of the tissue to estrogen can differ. Additional diversity is provided by polymorphisms in ER as well as post-translational modification of ER such as S-nitrosylation and *N*-acetyl-glycosylation.

Estrogen and cardioprotection in animal models

A number of different models and approaches have been used to examine the effect of estrogen on ischemia-reperfusion injury. One approach has been to examine whether there are male–female differences in ischemia-reperfusion injury. Another approach is to examine the effect of addition of exogenous estrogen.

Male–female differences in I/R

Some studies have reported that females have reduced ischemia-reperfusion injury [17, 18]. Other studies have failed to observe a male–female difference in ischemia-reperfusion injury [19–21]. However, females have reduced ischemia-reperfusion injury in a number of transgenic mouse models characterized by increased contractility and in wild-type hearts with addition of isoproterenol or elevated extracellular calcium [21–26]. These data raise the possibility that the discrepancies regarding endogenous protection in females may be related to the sympathetic tone or contractile state in the different models.

Treatment with estrogen

There also are many studies showing that acute treatment of animals or perfused hearts with exogenous estrogen can reduce ischemia-reperfusion injury [20, 27–30]. Hale et al. reported that bolus IV administration of beta estradiol (10 μg), but not administration of 1 mg of alpha estradiol (which does not bind to ER) reduced infarct size in rabbits [29]. Booth et al. [30] showed that treatment of rabbits with 20 μg of estradiol prior to coronary occlusion reduced infarct size (19%) compared to vehicle (48%). Similarly, Das and Sarkar reported [28] that pretreatment of rabbits with estradiol (10 μg/kg iv) prior to coronary artery ligation significantly reduced infarct size (19% versus 40%). Sbarouni et al. showed that 4 week treatment with estrogen reduces infarct size in oophorectomized female rabbits on a normal [31], and cholesterol-enriched diet [32]. However, raloxifene treatment did not reduce infarct size [32]. It has also been reported that the inhibitors of the mitochondrial K_{ATP} channel block the protection afforded by estrogen [33]. Taken together the data suggest that estrogen can be protective.

Which ER is involved and are the effects via gene expression or acute signaling?

As discussed above, estrogen mediates most of its effect by binding to the estrogen receptor. A number of studies have examined whether the protection afforded by estrogen is mediated by ER-α or ER-β. These studies have been carried out using either genetically altered mice that lack either ER-α or ER-β or by addition of an ER-α or ER-β selective agonist. Unfortunately there is no consensus regarding which estrogen receptor mediates protection against ischemia-reperfusion injury. There are data suggesting a role for both ER-α [18, 27, 34] and ER-β [25, 35–37] in mediating cardioprotection. Possible reasons for

the discrepancy include different models of ischemia-reperfusion and different end-points. Another variable, particularly in the studies using ER-α and ER-β agonists, is the dose and the timing of the addition of the agonist. In some studies, high levels of an ER-α agonist given for a short time were protective [27]; this protection may be mediated by acute-signaling pathways rather than gene induction. Another study showed protection by addition of an ER-β agonist after long-term (2 week) addition [36]; the protection observed in this study is likely mediated by altered gene expression. Thus both ER-α and ER-β may mediate protection, but by different mechanisms. Furthermore, altered gene expression is likely to be important, but acute effects of estrogen may also be involved and these acute and chronic effects may be mediated by different estrogen receptors. Also, some of the effects of estrogen are associated with high non-physiological levels and the mechanisms involved may not be mediated by binding to ER.

Estrogen mediated mechanisms of cardioprotection

Regardless of which ER mediates the protection, the mechanisms by which estrogen elicits cardioprotection in females are poorly understood. Given the lack of protection observed by HRT in the WHI, it is important to better understand the mechanisms by which estrogen might elicit cardioprotection. In this review we will focus on the role of estrogen and its well-established target nitric oxide synthase in cardioprotection. There are considerable data suggesting that alterations in nitric oxide synthase expression and signaling are important for protection in females [24, 38–40]. Altered expression of other genes are also likely to be involved and we will also review estrogen-mediated changes in gene expression that might play a role in cardioprotection.

Nitric oxide and cardioprotection in females

It has been well-established that estrogen results in increased expression of several nitric oxide synthase (NOS) isoforms [4, 39, 40]. The increase in basal NOS levels can lead to an increase in baseline nitric oxide generation in females [38, 40]. The increase in NOS in females has been shown to result in improved endothelial function. An increase in eNOS, mediated by ER-β, has been reported in cardiac myocytes [39]. Chen et al. have reported an increase in nNOS in cardiac myocytes [41] and Sun et al. have reported an increase in eNOS in the heart associated with caveolin 3 [40], the cardiomyocyte specific caveolin. The increase in eNOS and nNOS in cardiomyocytes in females is interesting in light of the well established role for increased NOS in cardioprotection [42]. Nitric oxide is suggested to mediate expression of cyclooxygenase 2 (COX-2), which has also been shown to be involved in cardioprotection [42]. Furthermore an increase in nitric oxide has been shown to be involved in cardioprotection via activation of protein kinase G (PKG), which leads to activation of mitochondrial pathways including activation of an ATP regulated mitochondrial channel that allows transport of K^+ into the mitochondria (mito K_{ATP} channel) [43]. Opening of the mitochondrial K_{ATP} channel has been reported to induce cardioprotection. An increase in nitric oxide has also been shown to result in increased S-nitrosylation of complex I of mitochondria [44]; it is suggested that this might alter ROS generation during ischemia and/or reperfusion. An increase in NOS and nitric oxide in females has also been shown to cause increased S-nitrosylation of the L-type Ca^{2+} channel, which results in less Ca^{2+} loading during ischemia [40]. There are also a number of studies showing that nitric oxide can alter cell metabolism [45–47]. Nitric oxide is also reported to increase mitochondrial biogenesis and reduce mitochondrial generation of ROS [47]. An increase in NOS in females can thus alter gene expression and activate acute nitric oxide signaling pathways. Given the pleiotropic effects of nitric oxide, it is likely that the increased NOS in female cardiomyocytes has an important role in the male-female differences in cardioprotection.

Estrogen regulated gene expression

There are a large number of genes which are regulated by estrogen in a tissue specific manner. Otsuki et al. examined gene changes in hearts from ovariectomized females treated for 3 weeks with estradiol compared to vehicle [48]. They reported an induction of seven genes and decreased expression of nine genes [48]. The induced genes included lipocalin-type prostaglandin D synthase and dipeptidase I. The repressed genes included thymosin beta10 and several types of procollagen. Gabel el al. performed gene profiling to determine genes that are differentially expressed in hearts from mice lacking ER-β (compared to WT and αERKO mice) [25]. Loss of ER-β was found to lead to an induction of solute carrier 4 (member 1) and decreased expression of a number of metabolism genes including SPOT14 homolog, lipoprotein lipase, ATP citrate lyase, stearoyl CoA desaturase and fatty acid synthase [25]. These data suggest that estrogen via ER-β results in induction of a number of genes involved in metabolism. The effect of an increase in these genes by ER-β is unclear but worthy of study. It is interesting that mice lacking eNOS were also reported to have an increase in solute carrier family 4 (member 1) and a decrease in stearoyl-CoA desaturase [49]. A cardiac specific stearoyl CoA desaturase has been

recently reported [50]. Stearoyl CoA desaturase catalyzes the synthesis of monounsaturated fatty acids from saturated fatty acids; monounsaturated fatty acids are important components in membrane phospholipids. As reviewed elsewhere [51] leptin has been reported to repress hepatic stearoyl CoA desaturase and expression of stearoyl CoA desaturase is increased in leptin deficiency. Mice with global deletion of stearoyl CoA desaturase have decreased body fat. It is therefore not clear that increased levels of this enzyme per se would enhance cardioprotection in females, although the role of this enzyme in cardiac and hepatic tissue could be different. ATP citrate lyase converts citrate to oxaloacetate and acetyl CoA; acetyl CoA is then converted to malonyl CoA which is an inhibitor of carnitine-palmitoyl transferase 1 (CPT-1) and thereby inhibits fatty acid oxidation. Interestingly, many of the genes decreased in the βERKO females are regulated by sterol receptor element binding protein (SREBP). SREBP-1α is reported to bind directly to the ERE(1/2) motifs and enhance ER binding when both ER subtypes are present [52]. A recent study by Nikolic et al. [36] examined cardiac genes induced by treatment of ovariectomized females with an ER-β selective agonist, DPN (2,3-bis(4-hydroxyphenyl)-propionitrile). DPN was reported to increase expression of over 100 genes, including COX2 and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, an enzyme important for regulating glycolysis [53].

Other studies have used a candidate gene approach and have identified a number of genes regulated (directly or indirectly) by estrogen, including, peroxisome proliferator-activated receptor gamma-coactivator 1α (PGC-1α), connexin 43 [54, 55], adenine nucleotide translocator [56], heat shock proteins [57], mitochondrial complex IV [58], GLUT4 [59], and MCIP1 an inhibitor of calcineurin [60]. Many of these proteins have been suggested to be important in cardioprotection [61, 62].

Estrogen and metabolism

As discussed, ER-β increases the level of several key enzymes involved in substrate selection. In addition to altered regulation of metabolism genes, estrogen could regulate metabolism by alterations in signaling pathways such as nitric oxide signaling which is also upregulated in females, due to induction of NOS. Since estrogen results in altered expression of a large number of metabolism genes, coupled with the estrogen induction of NOS which can also regulate metabolism, the relationship between estrogen and metabolism and its potential role in cardioprotection will be discussed.

Mitochondria and mitochondrial biogenesis

Mitochondria from females are reported to have increased maximum rates of electron transport and increased oxygen consumption [63]. Females also have increased mitochondrial biogenesis and reduced generation of mitochondrial ROS [64]. However, there are also reports that estrogen can increase mitochondrial ROS generation resulting in activation of redox sensitive transcription factors. Mitochondrial generation of ROS is complex and may depend on the levels of estrogen as well as other signaling pathways that are activated in the cell. Additional studies will be needed to resolve the role of estrogen on mitochondria and ROS generation. Increased mitochondrial generation of ROS has been a popular theory of aging. It has been suggested that with aging there is increased ROS mediated damage to electron transport chain components, which causes increased ROS production leading to a downward spiral. It has also been suggested that the increased longevity in females is related to altered mitochondrial electron transport and reduced ROS [64, 65]. NO is reported to be involved in increased mitochondrial biogenesis and increased longevity associated with caloric restriction [47, 66]. Nisoli et al. also reported that an increase in NO results in an increase in Sirt1 [66], a transcription factor associated with an increase in longevity and cardioprotection [67]. It is tempting to speculate that perhaps these mitochondrial alterations in females are mediated by nitric oxide.

Consistent with reduced ROS damage in females, Yan et al. using a proteomics approach reported age-dependent differences between male and female monkey hearts in glycolytic and mitochondrial electron-transport pathways [68]. They note that the changes in the old male monkeys are similar to changes that occur in disease and suggest that the lack of these changes in old female hearts is consistent with delayed cardiovascular risk in females. Also consistent with estrogen mediated changes in mitochondrial proteins, Stirone et al. [65] have shown that in blood vessels, estrogen increases levels of the nuclear coded cytochrome c, subunit IV of complex IV and manganese SOD, and increases the level of subunit I of complex IV that is coded in the mitochondria. Stirone et al. [65] further showed that hydrogen peroxide levels were decreased in estrogen treated animals. These changes were inhibited by the ER antagonist ICI-182, 780, but not by inhibitors of nitric oxide or the PI3-kinase pathway. Mitochondrial DNA contains estrogen receptor response elements. ER has been detected in the mitochondria [69, 70]; however these data are controversial [71].

Diabetes and body weight

A number of studies have suggested a link between estrogen and metabolism. Data from the Heart and Estrogen/Progestin Replacement Study (HERS) showed that there was significantly less type II diabetes in women on HRT [72]. Furthermore, the αERKO mice develop type II diabetes and exhibit an increase in body fat [73]. Interestingly ovariectomy of αERKO mice decreases body fat and body weight [74]. A human male lacking ER-α was also reported to have glucose intolerance [75], suggesting that the phenotype observed in mice is also relevant to humans. Whether the increase in type II diabetes associated with loss of ER-α is due to lack of ER-α signaling or due to unopposed ER-β signaling is unresolved. It is also interesting that eNOS-KO mice were reported to have an increase in body weight [47], suggesting a possible link between estrogen, NOS and altered body weight and metabolism. Thus there are considerable data suggesting that estrogen can regulate whole body metabolism; however, there are very limited data examining the effect of estrogen on myocardial metabolism.

Cardiac metabolism

We have recently found that substrate selection in heart is different in males versus females [25]. Using 13C NMR, labeled fatty acid, and carbohydrates, and measuring incorporation of the label into the C4 of glutamate, Gabel et al. [25] reported that in the C4 of glutamate, the ratio of 13C label from carbohydrate relative to fatty acid was significantly higher in females compared to males. The increase in carbohydrate relative to fatty acid utilization in females is consistent with the estrogen induction of ATP citrate lyase and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. How might this alteration in substrate selection result in cardioprotection? Numerous studies have shown that stimulation of glucose oxidation is protective. It has been proposed that increasing glucose oxidation during ischemia-reperfusion will result in pyruvate oxidation by pyruvate dehydrogenase (PDH) rather than pyruvate conversion to lactate; the latter can increase acidosis and result in calcium overload during ischemia and early reperfusion [76, 77]. Drugs that increase glucose oxidation, such as activators of PDH, have been shown to reduce ischemia-reperfusion injury [78, 79]. An increase in glucose oxidation in females might therefore be cardioprotective. In contrast to the observation by Gabel et al. [25] that females have increased glucose oxidation relative to fatty acid oxidation, Saeedi et al. [80] reported that relative to males, females have reduced glucose oxidation; however, in this study females also exhibited significantly poorer recovery of function than males. The reason for this discrepancy, particularly the enhanced ischemia-reperfusion injury in females is unclear.

In contrast to the concordant increase in mitochondrial biogenesis by nitric oxide and estrogen, an increase in nitric oxide is suggested to decrease carbohydrate oxidation and increase fatty acid oxidation, an effect opposite of that suggested for estrogen by Gabel el al. It has been shown in an in vivo model that acute inhibition of NOS causes an increase in glucose oxidation in heart [45, 46] This would suggest that NO results in a decrease in glucose oxidation. Since females have an increase in NO, if the metabolic effects observed in females are due solely to the effect of NO, one would expect a decrease in glucose oxidation in females. This suggests that perhaps estrogen alters expression of genes involved in metabolism resulting in increased glucose oxidation, but at the same time estrogen increases NO which counters the gene changes. It is also interesting that in a perfused heart model, inhibition of NOS does not cause an increase in carbohydrate metabolism [81]. Nitric oxide is also reported to alter glucose metabolism in the heart, but the results are somewhat conflicting. Lei et al. report that exogenous nitric oxide reduces glucose transporter translocation in heart [82]. However, Li et al. report that AMP kinase stimulation of GLUT4 translocation is at least partially mediated by an increase in NO [83]. Additional experiments will be necessary to unravel the complex role of estrogen and NO in metabolism.

Summary and conclusions

Estrogen binding to receptors can lead to altered gene expression, which can alter the response of the cell to ischemia-reperfusion. Figure 1 illustrates mechanisms by which estrogen might mediate cardioprotection. Nitric oxide synthase isoforms have been shown in many tissues to be increased in response to estrogen. Estrogen also results in altered expression of many additional genes. As discussed elsewhere, estrogen mediated gene expression is different in different tissues and can vary depending on co-repressors, co-activators, and other signaling pathways active in the tissue. We are just beginning to identify how estrogen alters cardiac gene expression. How does this all result in protection in females? Estrogen results in upregulation of cardiac eNOS and nNOS which have been shown previously to be important mediators of cardioprotection. An increase in NO has been shown in females to increase S-nitrosylation of the L-type Ca channel and thereby reduce Ca loading during ischemia and reperfusion [40]. An increase in NO has also been reported to enhance cardioprotection by activation of the mitochondrial K_{ATP} channel [84]. Estrogen also induces expression of a number of other

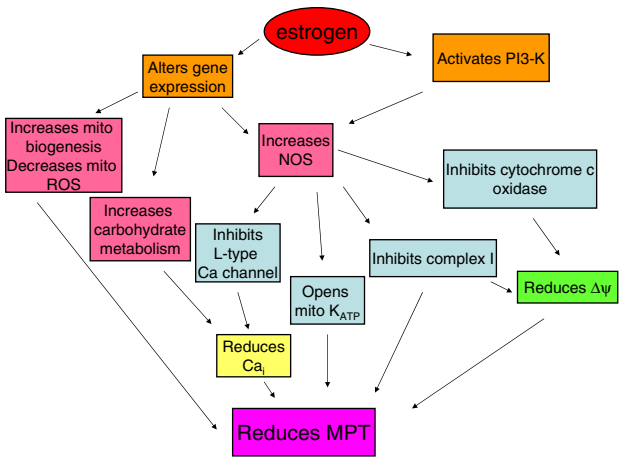

Fig. 1 The figure illustrates mechanisms involved in estrogen-mediated cardioprotection. Estrogen alters expression of a number of cardioprotective genes such as nitric oxide synthase (NOS). An increase in NOS activity results in stimulation of nitric oxide (NO) production, which can activate the mitochondrial K_{ATP} channel [84]. NO can also lead to S-nitrosylation of the L-type calcium channel [40] which would reduce calcium loading and thereby reduce opening of the mitochondrial permeability transition pore (mPTP). NO also results in S-nitrosylation of complex I of the mitochondria [44], which inhibits opening of the mitochondrial permeability transition pore (mPTP) [85]. NO also inhibits cytochrome c oxidase which would reduce $\Delta\psi$ and thus reduce calcium uptake into the mitochondria and opening of the mPTP. Estrogen is also reported to reduce mitochondrial generation of reactive oxygen species (ROS) [65] which would also reduce mPTP. Furthermore estrogen increases glucose oxidation, which, during ischemia-reperfusion, would reduce lactate production and thus reduce cytosolic increases in sodium and calcium. Finally estrogen can act via rapid non-genomic mechanisms to increase signaling pathways, such as the PI3-kinase pathway, to enhance protection in females

genes that have been suggested to be important in cardioprotection.

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