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Mechanism of Cardioprotection: What Can We Learn from Females?

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Abstract

This review examines the mechanism of estrogen signaling in cardiomyocytes, with an emphasis on mechanisms that might be important in cardioprotection. It investigates estrogen signaling mediated by the nuclear estrogen receptors alpha and beta and the G-protein-coupled receptor (GPR 30/GPER). Estrogen signaling via nitric oxide and the PI3K pathway are discussed.

Keywords

Cardioprotection; Estrogen signaling; G-protein-coupled receptor; Nuclear estrogen receptors

Considerable interest recently has focused on the role of estrogen in the evolution of cardiovascular disease. Premenopausal women have a significantly lower incidence of cardiovascular disease, but the incidence increases sharply after menopause [1, 2]. This protection in premenopausal women typically has been attributed to estrogen. In addition, a large number of animal studies have shown protection in females or with addition of estrogen [3–9].

It was therefore surprising when the Women's Health Initiative reported that hormone replacement therapy not only failed to reduce cardiovascular disease but actually increased it [10]. The reasons why hormone replacement therapy did not protect in the Women's Health Initiative have been debated [11], but it is clear that we need a better understanding of estrogen-mediated effects and how estrogen mediates protection, as it does in many animal studies.

To understand how estrogen might protect, we need to consider how the effects of estrogen are mediated. Until about 10 years ago, all the effects of estrogen were attributed to the binding of estrogen to its nuclear receptor, which then translocates to the nucleus, where it functions as a ligand-gated transcription factor that alters gene transcription [12]. Estrogen also has been shown to increase myocardial expression of genes such as endothelial nitric oxide synthase (eNOS), which can be cardioprotective.

Recently, findings have shown estrogen signaling to be more complicated. For example, estrogen receptors also associate with the plasma membrane, and estrogen binding to these receptors (especially estrogen receptor- α) has been shown to activate signaling via PI3K [13], which leads to cardioprotection. In addition, findings have shown that estrogen binds to an orphan G-protein-coupled receptor known as GPR30 [14, 15]. Estrogen binding to GPR30 has been shown also to activate the PI3K pathway. Therefore, estrogen can initiate protection by altering gene expression as well as by acute activation of cardioprotective signaling pathways such as PI3K.

The Langendorff-perfused heart model of ischemia–reperfusion is a convenient model for studying cardioprotection. With this model, it is easy to collect samples for biochemical assays, Western blots, and analysis of cell death and dysfunction.

We use two measures of injury. A balloon in the left ventricle (LV) measures pressure development or the difference between diastolic and systolic pressures (left ventricular-developed pressure [LVDP]). The percentage recovery of LVDP is measured as one index of injury. Cell death is measured using tetrazolium tetrachloride (TTC), which stains live cells red. Dead cells are unstained (white).

As mentioned, premenopausal females have a reduced incidence of cardiovascular disease [1, 2]. Much of this protection is attributed to beneficial effects of estrogen on the lipid profile and endothelial cell function, but recent data have suggested that estrogen also can protect cardiomyocytes.

A number of studies have found that acute treatment of animals or hearts perfused with estrogen results in cardioprotection [6, 8, 9, 16]. However, the data are somewhat mixed as to whether females per se show reduced cardiac ischemia–reperfusion injury. Many studies using rats have found reduced cardiac ischemic injury in females compared with males [4, 7], but other studies have not found protection in females [17, 18].

We found that with 20 min of ischemia in a Langendorff-perfused mouse heart model, female hearts had ischemic injury similar to that observed in males [19]. We did find in several genetically altered mouse models associated with increased contractility (e.g., phospholamban knockouts and $\text{Na}^+/\text{Ca}^{2+}$ -exchange overexpressors) that females exhibited less injury than males [19, 20]. We also showed that with increased contractility, associated with brief perfusion with high calcium or isoproterenol, there was less injury in females than in males [21–23].

Recently, Lagranha et al. [3] found that hearts from females subjected to 30 min of ischemia in Langendorff mode have significantly smaller infarcts than males. Lagranha et al. [3] also

found that this protection was lost in ovariectomized females [3]. Taken together, these data suggest that protection in females is significant only with more prolonged or severe ischemia.

Cardioprotection in Females: The Role of Reactive Oxygen Species

In addition to reduced cardiovascular disease, females also have an increased life span in humans and some, but not all, other species. A number of hypotheses have been proposed to account for the increased longevity. According to one current hypothesis, a decrease in reactive oxygen species (ROS) is an important component of increased life span.

In addition to its proposed importance in longevity, ROS also plays an important role in ischemia–reperfusion. Thus, it is of interest to consider whether altered ROS metabolism might be involved in cardioprotection experienced by females. It also is important to consider the mechanism responsible for male–female differences in ROS production and dissipation.

It is generally agreed that mitochondria are the major source of ROS generation in mammalian cells. The electron transport chain is an important source of ROS generation, particularly at complexes 1 and 3. In addition, findings have shown that lipoamide-containing mitochondrial dehydrogenases, such as α -ketoglutarate dehydrogenase (α -KGDH) and pyruvate dehydrogenase (PDH), also are a major source of ROS [24, 25]. Existing data suggest male–female differences in ROS generation [3, 26–30].

In addition, many studies have reported that mitochondria from females have less ROS generation than male mitochondria, although there are data to the contrary [30]. It also should be noted that low levels of ROS also can be involved in estrogen signaling [30]. Borrás et al. [27] reported less H_2O_2 generation and higher levels of antioxidants such as manganese-superoxide dismutase (MnSOD) in mitochondria from female liver and brain.

Table 1 summarizes some of the male–female differences that could be important in cardioprotection. Ovariectomy abolished the male–female differences in H_2O_2 generation [27]. Colom et al. [28] demonstrated that mitochondria from female rats have decreased cardiac mitochondrial content and generate less H_2O_2 . Colom et al. [28] also reported that female cardiac mitochondria have increased levels of glutathione peroxidase (GPx).

Likewise, Razmara et al. [29] showed that mitochondria from female brains have less ROS generation, as indicated by measuring the ratio of aconitase to fumerase activity. Aconitase is sensitive to oxidation, and its activity decreases with ROS. Razmara et al. [29] also reported increased MnSOD activity (no change in levels of MnSOD, but a change in activity) in female mitochondria. Stirone et al. [26] found decreased H_2O_2 levels in cerebrovascular mitochondria from females together with increases in MnSOD and nrf-1 levels.

Lagranha et al. [3] performed a broad-based proteomic study of male and female mitochondria to examine whether any differences in the proteome might contribute to the male–female differences in cardioprotection and ROS handling. Differences were identified in posttranslational modification of lipoamide-containing dehydrogenases (α -KGDH and PDH) and aldehyde dehydrogenase (ALDH), an enzyme involved in detoxification of oxidized lipids [3]. As mentioned, the lipoamide-containing dehydrogenases can be important contributors to mitochondrial ROS production [24].

Although the sex differences in enzyme phosphorylation are of interest, it is important to determine whether there are sex differences in the activity of these enzymes. To address this

issue, Lagranha et al. [3] showed that α -KGDH in permeabilized female mitochondria generated significantly less ROS than in male mitochondria under conditions of elevated nicotinamide adenine dinucleotide (NADH), which occur during ischemia and early reperfusion. Furthermore, female mitochondria generated less ROS than male mitochondria after anoxia and reoxygenation.

Lagranha et al. [3] also found that together with the increase in phosphorylation of ALDH2 in females, an increase in the activity of ALDH2 also was found in females. Phosphorylation of ALDH2 had been shown previously by Mochly-Rosen's group to be cardioprotective [31]. Also consistent with a chronic reduction of ROS in females, Yan et al. [32] reported that aged females have less oxidized proteins than males.

The decreased ROS levels observed in female mitochondria could be due to an increase in the rate of ROS dissipation resulting from elevated antioxidant levels, decreased ROS production, or both. As discussed earlier, both mechanisms appear to be involved. Mitochondria from females have increased levels or activity of MnSOD and GPx, which would break down ROS more rapidly and thereby reduce steady-state ROS levels. As discussed previously, considerable data also suggest less ROS generation in females.

There also are male–female differences in mitochondrial metabolism that might contribute to differences in ROS production. For example, fatty acid metabolism differs in females, and fatty acids are known to effect uncoupling protein levels, which in turn could alter mitochondrial ROS production.

A decrease in mitochondrial membrane potential typically is associated with a decrease in mitochondrial ROS production. Only a few studies have compared male–female differences in mitochondrial membrane potential in isolated mitochondria, and the data are inconsistent. For example, Borrás et al. [27] found an increase in mitochondrial membrane potential in female mitochondria, whereas others found no difference [33]. Data also exist to suggest that females have slower calcium uptake into mitochondria [33]. However, it is not clear that a reduced calcium influx would reduce ROS. In fact, this study found no male–female difference in ROS production at baseline.

An increase in mitochondrial biogenesis, an increase in mitochondrial components of electron transport, or both are suggested to be involved in reduced ROS levels and enhanced longevity associated with caloric restriction. Interestingly, some data suggest that females have elevated mitochondrial biogenesis, increased mitochondrial components of electron transport, or both [26]. In contrast, reports also describe decreased mitochondrial content in females. These differences could be due to differences in the model or treatment of the animals, and this issue requires additional study.

Cardioprotection in Females: The Role of Nitric Oxide

A number of studies have suggested a role for nitric oxide in the cardioprotection experienced by females [9, 12, 23]. Nitric oxide is generated by several isoforms of the enzyme nitric oxide synthase (NOS). Nitric oxide can signal via activation of guanylyl cyclase or via a posttranslational modification such as S-nitrosylation (SNO), the covalent attachment of an nitric oxide moiety to a protein cysteine group [34, 35].

Sun et al. [23] and Lin et al. [9] reported that female gender and estrogen increase the SNO levels of a number of mitochondrial proteins. Using a proteomics approach, Lin et al. [9] found that treatment of ovariectomized females with estrogen or a β -estrogen receptor agonist increased the SNO of a number of proteins including electron transfer flavoprotein beta; alpha-enolase; heat shock protein 27, 60, and 70; and the alpha-1 subunit of the F₁-

ATPase [9]. However, these broad-based 2D proteomic methods tend to favor proteins in high abundance, so additional low abundant signaling molecules likely exist that are SNO which have not been detected by the 2D gel electrophoresis method used in this study. Sun et al. [23] also reported that females have increased SNO of the L-type Ca^{2+} channel and thus have reduced Ca^{2+} entry into myocytes in the setting of ischemia and reperfusion [23].

Elevated nitric oxide levels via NOS are suggested to enhance mitochondrial biogenesis [36], which is reported to be higher in females [26]. Increased NOS and mitochondrial biogenesis are reported to play a role in increased longevity.

Mechanisms Involved in ROS and Nitric Oxide Sex Differences

The mechanisms responsible for the sex differences in ROS and nitric oxide metabolism are yet to be elucidated. In general, protection in females is attributed to estrogen-mediated signaling mechanisms. Estrogen can bind to two nuclear receptors (estrogen receptor-alpha and estrogen receptor-beta). Estrogen binding to these estrogen receptors can alter gene expression (via classical nuclear receptor-mediated mechanisms), or alternatively, estrogen binding to an estrogen receptor localized to the plasma membrane can acutely activate the PI3K pathway.

Estrogen also can activate a G-protein-coupled receptor, GPR30, which was recently shown to bind estrogen [14, 15]. Deschamps et al. [37] showed that GPR30 is present in the heart and that a selective GPR30 activator, G1, results in cardioprotection via activation of the PI3K- and ERK-signaling pathway. Activation of GPR30 also is reported to reduce hypertension [38, 39].

Thus, two important issues need to be considered regarding estrogen signaling: estrogen can bind to three different receptors, and these associations can lead to alterations in protein levels over the long term, initiate acute signaling pathways, or both. In turn, the estrogen can alter levels of proteins and signaling pathways, leading to posttranslational modifications that can alter protein activity. How these signaling pathways are integrated is just beginning to be understood.

As an example, we focus on the estrogen-signaling mechanisms that might lead to altered ROS and NO signaling. Estrogen is known to alter the levels of a number of proteins in the heart, including eNOS (Fig. 1). This NOS increase in female myocytes is via altered gene expression. Nuedling et al. [40] reported that estrogen receptor-beta is responsible for the increase in eNOS levels in myocytes. In addition, estrogen via acute signaling pathways can lead to activation of the PI3K pathway as well as phosphorylation and activation of eNOS. Thus estrogen can lead to both an increase in the NOS level and a further increase in the activity of NOS.

As discussed earlier, females also are reported to have altered levels of antioxidants as well as differences in ROS generation. Some of these changes could be related to altered nitric oxide signaling. For example, S-nitrosylation of mitochondrial proteins might lead to changes in ROS generation. It also is of interest that nitric oxide is reported to alter mitochondrial biogenesis. It is possible that estrogen acting as a ligand-gated transcription factor can alter the level of several antioxidant proteins. It also is likely that estrogen acting via rapid signaling pathways, such as PI3K, can influence the activity of these enzymes. For example, Lagranha et al. [3] found male-female differences in phosphorylation of a number of proteins, including α -KGDH and ALDH2, which were blocked by an inhibitor of PI3K, suggesting a role for this signaling pathway. Activation of the PI3K pathway leads to downstream activation of protein kinase C (PKC), which in turn phosphorylates and

activates ALDH2, resulting in cardioprotection. Furthermore, PKC also can phosphorylate α -KGDH and reduce its production of ROS.

Taken together, these studies demonstrate that estrogen increases levels of signaling proteins such as eNOS and proteins regulating ROS. Estrogen also acutely activates the PI3K pathway, leading to increased activation of NOS and the PKC pathway, which leads to posttranslational modifications that also contribute to cardioprotection.

Future Directions

It is too simplistic to classify a change in protein level or change in phosphorylation as protective or detrimental. These changes can be protective, detrimental, or neutral depending on the conditions. However, in the setting of acute ischemia–reperfusion, the overall balance of estrogen signaling seems to be beneficial. It is important to consider that estrogen signaling is composed of signaling by estrogen receptor-alpha, estrogen receptor-beta, and GPR30. It appears that estrogen receptor-alpha and estrogen receptor-beta can regulate different genes and can even regulate some genes in opposite directions.

Consideration also must be given to the acute effects of estrogen, which acts via an estrogen receptor tethered to the plasma membrane, GPR30, or both. The complexity of estrogen signaling raises several questions that require further study. Are tissue levels of estrogen receptor-alpha, estrogen receptor-beta, and GPR30 differentially regulated? If so, would this allow for altered signaling as a result of changes in receptor makeup?

Interestingly, estrogen receptor-alpha can be posttranslationally modified, and this can alter its function. For example, estrogen receptor-alpha is reported to be SNO, which interferes with its binding to DNA such that the SNO of estrogen receptor-alpha enhances the acute signaling of the estrogen receptor relative to its transcriptional activation. Estrogen receptor-alpha also has a number of phosphorylation sites, which can alter its activity. Future studies are needed for better elucidation of the mechanism by which estrogen alters cardiovascular function.

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Fig. 1.
Synergistic signaling by the different estrogen receptors

Table 1

Male–female differences that may play a role in cardioprotection

Factor	Effect	Reference
P- α KGDH	F have increased phosphorylation	[3]
P-ALDH	F have increased phosphorylation	[3]
ROS production	F have less ROS	[26–29]
NOS levels	F have increased NOS	[21, 40]
GPx	F have increased levels	[28]
MnSOD activity	F have increased activity	[27, 29]
S-nitrosylation	F have increased levels	[9, 23]

α KGDH α -ketoglutarate dehydrogenase; *F* females; *ALDH* aldehyde dehydrogenase; *ROS* reactive oxygen species; *NOS* nitric oxide synthase; *GPx* glutathione peroxidase; *MnSOD* manganese-superoxide dismutase