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Age-Related Differences in Cardiac Ischemia-Reperfusion Injury: Effects of Estrogen Deficiency

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Abstract

Despite conflicting evidence for the efficacy of hormone replacement therapy in cardioprotection of postmenopausal women, numerous studies have demonstrated reductions in ischemia/reperfusion (I/R) injury following chronic or acute exogenous estradiol (E₂) administration in adult male and female, gonad-intact and gonadectomized animals. It has become clear that ovariectomized adult animals may not accurately represent the combined effects of age and E₂ deficiency on reductions in ischemic tolerance seen in the postmenopausal females. E₂ is known to regulate the transcription of several cardioprotective genes. Acute, non-genomic E₂ signaling can also activate many of the same signaling pathways recruited in cardioprotection. Alterations in cardioprotective gene expression or cardioprotective signal transduction are therefore likely to result within the context of aging and E₂ deficiency, and may help explain the reduced ischemic tolerance and loss of cardioprotection in the senescent female heart. Quantification of the mitochondrial proteome as it adapts to advancing age and E₂ deficiency may also represent a key experimental approach to uncover proteins associated with disruptions in cardiac signaling contributing to age-associated declines in ischemic tolerance. These alterations have important ramifications for understanding the increased morbidity and mortality due to ischemic cardiovascular disease seen in postmenopausal females. Functional perturbations that occur in mitochondrial respiration and Ca²⁺ sensitivity with age-associated E₂ deficiency may also allow for the identification of alternative therapeutic targets for reducing I/R injury and treatment of the leading cause of death in postmenopausal women.

Keywords

senescence; estrogen receptors; proteomics; cardioprotection

Introduction

Coronary heart disease (CHD) most commonly presents as an ischemic coronary event such as acute myocardial infarction (MI) or unstable angina (collectively termed *acute coronary syndrome*), and is the single largest killer of American men and women, accounting for one in every five U.S. deaths in 2004 [170]. The estimated annual incidence of myocardial infarction (heart attack) is 865,000 new and recurrent attacks, and the prevalence and mortality due to MI increases with age [170]. In women, longitudinal studies and clinical statistical reports indicate an important influence of the menopausal transition on the determination of cardiovascular risk with advancing age. The incidence of coronary heart disease (CHD) in postmenopausal women is 2–3 folds higher than in premenopausal women

of the same age [105, 170]. Further, 23% of women age 40 and older who experience a first MI will die within one year, compared with 18% of men [170]. These reports implicate the loss of endogenous estradiol (E₂) as an explanation, in part, for the reduced ischemic tolerance (IT) in postmenopausal women.

Despite statistical data suggesting a causative role for E₂ deficiency in the age-associated increase in female cardiovascular risk, studies investigating the efficacy of exogenous hormone replacement therapy (HRT) on cardiovascular risk reduction have produced conflicting results. Observational and epidemiological reports, including the Nurses' Health Study, demonstrated reduced risk for CVD and acute MI in women taking HRT for the management of menopausal symptoms [17, 59, 73, 189]. In contrast, mid-stage analysis of two randomized clinical trials, the Women's Health Initiative (WHI) [131] and the Heart and Estrogen/Progestin Replacement Study (HERS) [87], showed evidence for *increased* MI and stroke risk in postmenopausal women treated with conjugated equine estrogens (CEE) alone or CEE plus medroxyprogesterone acetate. The WHI and HERS trials were terminated early as a result of the increased hazard to women receiving HRT.

One proposed explanation for the conflicting results among these studies is the age of the subjects and the timing of HRT administration in reference to the onset of menopause, in that HRT may be cardioprotective in younger women and those within the first several years of menopause, but ineffective or detrimental in older postmenopausal women [137]. A secondary analysis of the WHI data revealed a trend toward reduced CHD risk in women receiving HRT within 10 years of the onset of menopause and increased risk in women beyond 20 years of menopause, although statistical significance was not demonstrated [173]. The Kronos Early Estrogen Prevention Study (KEEPS), an ongoing clinical intervention trial, aims to identify the effects of early HRT administration in younger menopausal women [80]. Although the timing hypothesis may indeed prove to support short-term cardioprotection with HRT in younger postmenopausal women, the evidence for diminished efficacy and possible detrimental effects of HRT on CHD risk in older women, as well as concerns regarding increased breast and ovarian cancer risk with long-term HRT use [147, 172], demonstrate the need for alternative strategies in the treatment of ischemic heart disease in aging, postmenopausal women. A necessary first step in forging new therapeutic strategies to treat ischemic heart disease in aging women includes identification of the mechanisms which render the aged female heart vulnerable to ischemic insult. A major focus of the current review is to summarize what is known with regard to cardioprotective signaling in the aged, E₂-deficient female heart, with particular focus on salient research challenges associated experimental models to recapitulate human menopause. Notably, the reader is referred to several recent complimentary reviews on mitochondrial aging and mechanisms of cell death [46, 57, 141].

Age, Estrogen Deficiency, and Ischemic Tolerance

Acute MI is caused by coronary occlusion, and current treatment options are focused on reducing the duration of ischemia by initiating reperfusion as quickly as possible. Mechanical (coronary angioplasty) or enzymatic (thrombolytic) interventions, however, are rarely performed soon enough to avert cell death during ischemia, and further are ineffective in preventing the extension of infarction at reperfusion [29, 52]. Extensive and ongoing research has thus focused on the identification of effective treatments for the reduction of ischemia/reperfusion (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. Premenopausal women have reduced risk for CHD relative to age-matched men [16], as well as a lower incidence of LV hypertrophy, coronary artery disease, and cardiac remodeling following MI [83]. The incidence of CHD increases in postmenopausal women,

however, such that aged women have both reduced ischemic tolerance [10, 168] and increased mortality following MI [214] relative to age-matched men. In the paragraphs that follow, information regarding the influence of aging on cardioprotective signals is presented within the context of information gleaned from studies associated with the phenomenon of ischemic preconditioning (IPC). Particular emphasis is also placed on available experimental models of E₂ deficiency.

Ischemia/Reperfusion Injury in Aging

Reduced IT and increased susceptibility of the heart to I/R injury is a hallmark adaptation of both aged human and animal hearts [2, 30, 93, 120, 128, 132, 158, 180, 192, 215]. The aged heart is also refractory to endogenous protection from interventions like IPC (described below), verifying inadequate protective cellular reserves [63, 64, 99]. 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 [99, 120]). 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 [39, 79, 102, 159, 174].

In distinction from aging, independent effects of E₂ 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 oophorectomy [219], and numerous studies conducted throughout the ensuing five decades have demonstrated increased risk for CHD and myocardial infarction in both postmenopausal and oophorectomized premenopausal women [16, 42, 44, 60, 171]. Epidemiological data indicate the interaction of gender and aging, and the influence of menopause on the determination of cardiovascular risk in aging women. Animal and human studies have identified both functional and cellular alterations in ischemic tolerance and cardioprotection due to the independent and combined effects of aging and E₂ 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 E₂ deficiency.

Ovariectomy as a Model of E₂ Deficiency in the Aged Rat

Given the discrepancies in observational and epidemiological data indicating the effects of menopause and HRT on cardiovascular risk in aging women, an animal model suitable for the experimental study of age- and E₂-related cellular changes has presented significant challenges. Although the study of nonhuman primates has been purported as the model perhaps most applicable to the menopausal transition in humans [107, 217, 218], the feasibility of this approach is extremely limited, especially in the context of aging and in the physiological study of myocardial infarction. The feasibility of aging studies in other animal models commonly used to study I/R injury, such as the canine and porcine models, is also reduced by the relatively long lifespan of these animals and the limited availability of aged supply colonies. The relevance to the human heart of I/R studies performed in the rabbit, especially in aging, has also been questioned [4].

The clinical definition of menopause is the cessation of spontaneous menstrual cycling for at least one year, and occurs in women at an average age of 51 years [202]. In human and nonhuman primates, the cessation of menstruation is preceded by a gradual decline in the function of the hypothalamic-pituitary-gonadal (HPG) axis [218]. Plasma E₂ concentrations in postmenopausal women have been reported to average about 30 pg/ml [26], compared

with a cyclic variation from ~80 to 800 pg/ml in healthy, premenopausal women [190]. The menopausal transition in the rat is incompletely understood, and exhibits important differences from menopause in humans. Notably, the onset of senile anestrus is variable in rats [1, 33, 140, 179, 187], resulting in a state of persistent estrous followed by persistent diestrus, whereby sustained E₂ levels are similar in magnitude to diestrus in adult animals. The age of ovarian decline and the timing of this progression may also vary between mice and rats, and also between different strains of the same species. Nevertheless, important similarities between menopause and 'estropause' (for recent review see [33, 140, 227]) include cessation of estrous cyclicity (~16 mo in F344 rats) and a progressive deterioration in HPG axis function thereafter [187] until senile anestrus. Interestingly, the menopausal transition in humans is also characterized by elevated E₂ levels [74, 76, 213].

Given the complicated nature of the menopausal transition in rodents, surgical ovariectomy (OVX) has been used to create a model of menopause to more closely approximate the dramatic E₂ deficiency observed in menopausal women. Ovariectomized *adult* rats represent, in fact, the most commonly used animal model of postmenopausal changes. However this model does not reflect the possible interactions between aging and E₂ deficiency occurring in natural menopause [179]. Indeed, studies from our laboratory suggest a highly selective myocardial response to E₂ deficiency in adult vs aged female rats with regard to alterations in mitochondrial protein targets [121]. Additional considerations when using the adult OVX model include the time course of changes in plasma E₂ following OVX. Adult OVX animals reveal dramatic reductions in plasma E₂ initially, which is followed by significant increases at 4, 5, and 6 months post-surgery (~30 pg/ml) [234]. The increase in plasma E₂ post-OVX has been attributed to increased extragonadal aromatization of testosterone to E₂. Other studies have demonstrated increased adiposity following OVX in rats [108]. The increases in extragonadal aromatization with time after OVX, as well as increased adiposity and potential metabolic alterations in the OVX rat, have important implications for the validity and applicability of the adult OVX model to age-associated E₂ deficiency. An alternative approach for the study of menopause and cardioprotection includes use of age-appropriate rats in conjunction with OVX [90, 121, 122, 151, 198], which represents an often overlooked but critical design consideration of rodent studies to recapitulate postmenopausal E₂ deficiency and reproductive senescence. At the very least, experimental design limitations should be acknowledged with regard to the interpretation of research findings and extent of the conclusions drawn. Given the cyclic nature of protein turnover and potential influence of circulating E₂, some standardization of estrous cycle activity in rodents should also be considered. With regard to studies employing E₂ replacement, assessment of circulating E₂ levels should also be performed at routine intervals throughout the entire duration of replacement, to determine the physiological relevance and potential impact of dosages employed on observed responses.

Ischemic Preconditioning as a Model of Cardioprotection

IPC, in which brief intermittent periods of ischemia (I) and reperfusion (R) reduce myocardial damage during subsequent prolonged I/R injury [145], represents the most powerful and reproducible form of cardioprotection identified to date [144]. Two phases of cardioprotection have been characterized: an early or acute phase that lasts for two to three hours following the preconditioning stimulus, and a late phase that is effective beginning 24 hours following the stimulus and can last for three to four days [223]. Although still incompletely understood, much has been learned about the mechanisms by which acute IPC renders the heart resistant to I/R injury (for review, see [53, 144]), and by default, glean potential insight into aged-associated mechanisms of reductions in ischemic tolerance. While the direct therapeutic relevance of IPC is limited simply by the requirement that it must be invoked prior to the onset of an ischemic event, which is rarely foreseeable, the

endogenous cellular pathways of IPC have come to be used as a model by which to study cardioprotective signaling and to identify targets for clinically-applicable interventions. These efforts have been encouraged by recent findings that I/R injury can be reduced by the activation of signaling pathways immediately prior to ischemia or at the beginning of reperfusion [236]. While initially thought to minimize infarction during ischemia, current thinking states that the protection afforded by IPC is realized primarily *at reperfusion* through a reduction in necrotic, apoptotic and potentially autophagic cell death, which are normally responsible for the extension of infarct size and are critically regulated by the mitochondria [58, 81, 237]. Substantial experimental evidence has promoted the mitochondria as the convergence point for the protective cellular signaling pathways of IPC, and has established the role of protein kinase C (PKC; and the PKC ϵ isozyme in particular) as a critical mediator of this convergence [91]. In the paragraphs that follow, particular emphasis is placed on the potential role of PKC ϵ modulation as a potential therapeutic strategy to improve ischemic tolerance with age-associated E₂ deficiency.

Working Model of Cardioprotective Signaling

Although the molecular mechanisms of cardioprotection have yet to be fully elucidated, years of extensive study into IPC have characterized many cell signals associated with reductions in I/R injury in adult animals (Figure 1; adapted from [144]). Brief preconditioning cycles of I/R cause the release of agonists including adenosine [126], bradykinin [207], and opioids [182] from the ischemic myocardium, which act through G-protein coupled receptors to trigger multiple signaling cascades. The protection provided by each of these agonists can be blocked by inhibition of PKC [13, 71, 136, 175], illustrating the central importance of PKC as a common target in this signal transduction. Moreover, PKC ϵ has been directly implicated in infarct sparing following global ischemia [92]. Low-level activation of PKC ϵ has consistently been found to reduce hypoxic injury [167], and Mochly-Rosen and colleagues [37, 92] have provided direct evidence that isoform-specific activation of PKC ϵ utilizing cell-permeating peptides prior to global ischemia is sufficient to reduce infarct size in adult male rats. Bradykinin and opioids stimulate PKC ϵ by way of a complex phosphatidylinositol 3-kinase (PI3-K) pathway that involves activation of Akt, endothelial nitric oxide synthase (eNOS), guanylyl cyclase (GC), protein kinase G, and the opening of mitochondrial ATP-sensitive K⁺ channels (mitoK_{ATP}) [43, 153]. Subsequent K⁺ influx to the mitochondria leads to the generation of ROS, which act as a second messenger to activate PKC ϵ [110]. Adenosine, in contrast to bradykinin and opioids, activates PKC ϵ during IPC by a distinct pathway, since PI3-K inhibition does not block adenosine-stimulated cardioprotection. Although critically important to IPC, PKC ϵ acts not as an effector of cardioprotection, but rather as a crucial intermediate in linking protective signaling to the mitochondria and initiating cellular protection at reperfusion. PKC ϵ activates both the PI3-K/Akt and MEK1/2-ERK1/2 survival kinase cascades at reperfusion. Akt and ERK1/2 both phosphorylate and inactivate mitochondrial glycogen synthase kinase-3 β (GSK-3 β) [15, 199], which has been shown to result in strong inhibition of the mitochondrial permeability transition pore (MPTP) [95, 100], the hypothesized end-effector of IPC [82, 95].

The MPTP is a large conductance pore directly connecting the mitochondrial matrix to the cytosol. While its identity has not been firmly established [53], induction of the MPTP results in dissipation of the mitochondrial membrane potential which compromises the cell's capacity for ATP production and hence volume regulation by Na⁺/K⁺ ATPase pumps, which leads to cellular swelling, lysis, and necrosis [75]. Mitochondrial swelling is also encouraged by MPTP formation, and lysis of the outer mitochondrial membrane results in cytochrome c release and the initiation of apoptosis [125].

PKC ϵ prevents MPTP formation at reperfusion not only by activation of the survival kinases Akt and ERK1/2, but also by direct phosphorylation and inhibition of GSK-3 β [100], activation of mitoK_{ATP} channels [70], and phosphorylation of possible MPTP regulatory proteins such as the voltage-dependent ion channel (VDAC) and the adenine nuclear transporter (ANT) [14]. Additionally, PKC ϵ phosphorylates mitochondrial connexin43 (Cx43), which may cooperate with the mitoK_{ATP} channel in mitochondrial volume regulation and ROS production [183]. Further roles for PKC ϵ in the regulation of cellular redox status through association with eNOS [164], and in the regulation of myocardial ATP synthesis by targeting of the mitochondrial F₁ATPase [112] and cytochrome *c* oxidase subunit IV [154], have been demonstrated. The central role of PKC ϵ in IPC suggests that cardioprotection is likely mediated by additional mitochondrial PKC ϵ binding partners that have yet to be identified. How these signals may be influenced by age-associated E₂ deficiency and reductions in ischemic tolerance is discussed in the next sections.

Aging and Protective Signaling

Animal models of I/R injury demonstrate impaired functional recovery and larger infarct size following I/R in the aged heart [3, 11, 193]. In addition, many studies demonstrate the reduced or abolished efficacy of IPC to reduce infarct size in the aged heart [6, 64, 130, 181, 186], although the majority of studies have been conducted in males. Nevertheless, clinical studies have also suggested a diminished capacity for cardioprotection following IPC or persistent angina in the aged human heart [5, 18, 123, 129]. At least in male animals and men, age-related declines in ischemic tolerance appear to correlate with alterations in cellular protein expression related to cardioprotective signal transduction. For instance, increased evidence of apoptosis and reduced induction of HSP70 was reported following ischemia in the aged rat heart [128, 150], and the loss of IPC-induced cardioprotection in the aged mouse heart was associated with reductions in gap junctional and mitochondrial Cx43 [21]. Tani *et al.* found in the middle-aged rat heart that the loss of IPC-induced cardioprotection was associated with altered PKC translocation, and that cardioprotection was achieved less effectively by PKC activation than by mitoK_{ATP} activation, suggesting that disruptions downstream of PKC signaling contributed to the loss of IPC with age [192]. Our laboratory previously demonstrated that impaired ischemic tolerance in aged male rats was associated with 1) increased basal PKC δ expression and could be improved by acute PKC δ inhibition (by Tat- δ V1-1 administration) [114], and 2) reduced PKC ϵ and increased GSK-3 β at the mitochondria during ischemia and could be improved by acute PKC ϵ activation (by Tat- ψ eRACK administration) [112]. Impaired responsiveness to IPC in elderly patients undergoing coronary angioplasty has also been attributed to attenuated activation of K_{ATP} channels, since the K_{ATP} channel agonist nicorandil restored IPC-induced cardioprotection [123]. Chakravarti *et al.* described the altered expression of numerous proteins, primarily relating to cellular energetics at the mitochondria, in the aged male mouse heart through proteomic profiling experiments [34]. Examples of these include age-related reductions in mitochondrial aconitase 2, mitochondrial F₁ATP synthase β , and NADH dehydrogenase subunit expression. Evidence also support a role for post-translational modifications or proteolysis, however the exact nature and quantity of these modifications was not further examined [35].

Senescence is also associated with enhanced cytochrome *c* release in male rats [113, 162] and expression of the proapoptotic proteins Bad, Bax and caspases [12, 32, 127] [233]. Age-related increases in reactive oxygen species (ROS) [72, 120, 146, 177] are also likely to contribute to increased apoptotic signaling. While ROS can be cytoprotective through activation of known survival signals such as PKC ϵ [101] [138], increased ROS production in the face of declines in antioxidant defenses [222] is likely to contribute to increased I/R injury in the aged heart. Elevated nitric oxide (NO) production during I/R through NOS-

dependent processes can also result in formation of more reactive oxidant species like superoxide (O_2^-) and peroxynitrite ($ONOO^-$) [197, 212, 226] (i.e. NOS uncoupling) [239]. In females, links between E_2 deficiency, aging, ROS and I/R injury are unclear, as studied in OVX younger animals may not accurately mimic the aged state [222]. In aged male rats, elevated O_2^- production does occur during early reperfusion [155], however effects in aged females are unknown and are currently under investigation in a number of laboratories, including our own.

In female animals, reduced ischemic tolerance has been observed and is attributed to both the independent and combined effects of aging and E_2 deficiency. Willems *et al.* demonstrated increased infarct size and impaired functional recovery following I/R in aged relative to adult female mice [216], and Hunter *et al.* showed that the increase in infarct size following I/R in aged relative to adult female rats was associated with decreased Akt and mitochondrial PKC ϵ levels, as well as increased mitochondrial GSK-3 β [89]. E_2 deficiency alone reduces ischemic tolerance in the female heart, as Song *et al.* demonstrated increased infarct size following I/R and the loss of IPC-induced cardioprotection in adult OVX relative to adult ovary-intact female mice [188]. Kam *et al.* also showed, under hypercontractile conditions of elevated Ca^{2+} , similar results using adult OVX rats [104]. 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 [89]. In support of a protective role for PKC ϵ targeting in the aged, E_2 -deficient female rat heart, acute activation of PKC ϵ prior to ischemia by local delivery of ψ ERACK peptide has been associated with 1) improved functional recovery and reduced infarct size, 2) increased mitochondrial targeting of PKC ϵ , and 3) candidate downstream signaling targets suggesting a role for activation of antioxidant enzymes as a mechanism of PKC ϵ -mediated protection [122]. Specifically, mitochondrial Hsp10, GPX, and SOD2 (MnSOD) abundance are significantly increased with ψ ERACK administration in aged OVX hearts (by ~10, 20, and 30%, respectively). Due to the brief time period of PKC ϵ activation in these hearts (10 min), changes observed in this analysis are likely attributable to PKC ϵ -stimulated mitochondrial translocation or import of identified proteins. Following ischemia, it is likely that improved levels of mitochondrial Hsp10, GPX and MnSOD2 observed in PKC ϵ -treated aged OVX hearts are further influenced by protective effects limiting protein degradation. It is clear from the work of Zhang and colleagues [232] that I/R-induced alterations to the mitochondrial proteome of adult mice occur and are dependent upon severity of ischemia and specific protein abundance. How specific mitochondrial proteins are targeted for lysosomal and/or proteosomal degradation in the aged heart, and the dynamic regulation of these processes, is poorly understood and a necessary focus of future studies. Moreover, results are confounded by differing models of I/R injury including varying amounts of ischemic insult (i.e. duration of ischemia).

Nevertheless, it is likely that increases in GPX and SOD2 immediately following PKC ϵ activation may serve to combat increased ROS production in the aged female heart [124]. In contrast, Hsp10 is a stress-response and chaperone protein shown to regulate mitochondrial pro-caspase-3 activation, and thus the initiation of apoptosis, through the formation of a complex with Hsp60 in the intermembrane space [176]. HSPs have recently been implicated in mitochondrial import of PKC ϵ during I/R [28], and thus may contribute to observed increases in mitochondrial PKC ϵ localization following acute PKC ϵ activation in the aged female heart. Identification of candidate downstream PKC ϵ signaling targets in mitochondria suggests a role for the regulation of oxidative stress as a mechanism of PKC ϵ -mediated cardioprotection in the aged female heart. Studies are clearly needed to quantify the extent of interplay between ROS production and cell death in the aged female myocardium.

Estrogen Receptors (ER) and Cardioprotection

Effects of E₂ in the heart are primarily mediated by two ER subtypes, ER α and ER β , although the precise subcellular distribution of cardiac ER receptors remains to be elucidated. Recent evidence linking ER α and ER β polymorphisms to adverse cardiac outcomes in women [160, 163, 206] suggest that ER α and ER β may each play distinct roles in cardioprotection. Genomic actions mediated by nuclear ER α are well-described [50, 54] and involves ligand binding at E₂ response elements. Non-genomic (rapid) effects of E₂ are thought to be mediated by ER α and/or ER β localized to the plasma membrane [8, 224, 230], and associated functions include Ca²⁺ homeostasis, anti-apoptosis and mitochondrial metabolism [224]. With regard to the latter, the recent demonstration that mitochondrial ER β s are present in human myocardium [224] have positioned ER β as a potential regulator (or regulated target) of mitochondrial function and cell survival, perhaps through mitochondrial gene regulation [86]. Rapid ER signals are also known to regulate ER gene transcription in the myocardium [134]. 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 [36, 62, 67, 231]).

While the importance of cardiac ER subtypes in I/R injury remains controversial, studies employing ER α and ER β deficient mice have each demonstrated reductions in ischemic tolerance [69, 208]. 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 ER α , as well as a metabolic phenotype which develops with age [27, 84]. Nevertheless, in mice completely null for ER α , greater I/R injury and impaired mitochondrial function [210, 229] is observed vs non-transgenics. Further, activation of ER α with the specific agonist, propyl pyrazole triol (PPT), protects the *in vivo* rabbit heart from I/R injury, while the specific ER β activator, diarylpropionitrile (DPN) was without effect [24]. Recent studies also suggest a greater role for ER α vs ER β in the modulation of endothelial progenitor cells and cardiac repair [48, 77]. Taken together, these data support a dominant role for ER α as the cardioprotective ER involved in I/R injury [24]. In contrast, Murphy and colleagues [69, 149] have provided equally compelling evidence that ER β mediates gender differences in I/R injury using ER β deficient mice under hypercontractile conditions or with DPN.

With regard to the potential cardioprotective role of non-genomic ER activation in reducing I/R injury in aged hearts, several recent findings implicate a possible role for selective ER α activation as follows [151]: 1) effectively reduced infarct size, 2) resulted in greater mitochondrial and particulate ER α localization coordinate with a protective pattern of PKC ϵ activation, and 3) enhanced gene expression of the PKC ϵ anchoring protein RACK2. Collectively, these results demonstrate a protective role for non-genomic ER α signaling in the aged female rat heart, the cellular basis of which may involve two distinct PKC ϵ -dependent mechanisms. What is less clear are the mechanisms which underlie altered cardiac ER translocation. As noted above, post-translational modifications such as phosphorylation, acetylation and sumoylation (for review see [36, 38, 62, 67, 231]) 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 [151], it will be important that future studies incorporate true models of aging in conjunction with E₂ deficiency to fully characterize the non-genomic ER response.

In contrast, acute ER β activation does not appear to impact functional recovery following I/R injury in either adult or aged rats with varying degrees of E₂-deficiency [198]. A logical interpretation of these results is that while classical genomic ER β activation via chronic stimulation is possible, rapid, non-genomic signaling mechanisms downstream of ER β may

not be operative in the female rodent myocardium. However in this study, ER β mRNA was not detected in either the adult or aged rat myocardium [198]. The lack of measureable ER β in the F344 rat myocardium was surprising given results gleaned from past studies utilizing the ER β knockout mouse model [68, 209, 211] mentioned heretofore. In this regard, ER β expression in the rodent myocardium remains controversial [66, 94, 115, 178, 184, 221, 225], and the protein signal produced by ER β antibodies in cardiac homogenates may be the result of cross-reactivity with ER α [198]. Combined with these previous findings, either ER β signaling varies substantially between rat, rabbit and murine models or, cardioprotection observed in mouse models may be mediated indirectly through extra-cardiac ER β 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 [185]. That ER β activation can reduce systemic arterial pressure via autonomic influence indicates that additional autonomic cardioprotective mechanisms attributed to E₂ may be mediated through ER β . Indeed, E₂-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 [55, 56]. Therefore it is plausible that hypertension and vascular dysfunction observed in whole body ER β knockout mice as well as cardioprotection observed in chronic DPN treated mice may be explained by indirect ER β effects on autonomic cardiac control and not direct effects on the myocardium [148, 157]. Future studies examining extra-cardiac effects of chronic ER β stimulation, including vascular and neural mechanisms, may prove useful in elucidating possible therapeutic interventions with aging. Definitive studies on ER subtype distribution in adult and aged human myocardium are needed.

The demonstration that rapid ER α activation reduces I/R injury in the aged female heart supports a key role for non-genomic ER signaling in the maintenance of cardioprotection. A better understanding of the non-genomic actions of E₂ may 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 E₂ observed in adult women without increased cardiovascular risk observed from chronic HRT. In this regard, 17- β estradiol is the major physiological E₂, but it has a similar affinity for both ERs. As noted, a number of selective ER α and ER β agonists have been created and described; however, only a minority of these compounds has been evaluated extensively *in vivo*. The discovery of the GPCR30 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-3 β 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 PKC ϵ (for review see [143]). Increased levels and/or activity of Akt have also been observed in female (vs male) animal and human myocardium [31, 235]. ER α -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 [31]. Collectively, these data suggest that the PI3K-Akt pathway is acutely activated by E₂ and could be subject to modulation by aging. Urata and colleagues [204] recently demonstrated that E₂ administration (18 hrs) in myocardial H9c2 cells leads to a reduction in hydrogen peroxide (H₂O₂)-induced apoptosis through upregulation of glutaredoxin (GRX), which was abolished by the ER inhibitor ICI-182,780. Effects were presumed ER β -mediated since these cells do not express ER α .

A target of Akt which has been proposed as a convergence point for many cardioprotective signals is inactivation of mitochondrial GSK-3 β and associated apoptotic signaling. This model is supported by I/R- and OVX-dependent changes in mitochondrial pGSK-3 β which mirror changes in pAkt in adult but interestingly *not* aged rats [88], suggesting dysregulated Akt-GSK-3 β interactions in aged. Additional mechanisms by which rapid E₂ signaling may influence GSK-3 β and subsequent ischemic tolerance are worth noting. Recent studies suggest that GSK-3 β can enhance ER α -mediated transcription [135], implicating the nuclear compartment as a potentially important site of regulation in the aged female heart. If this is so, several cardioprotective or apoptotic proteins that are modulated by E₂ (such as heat shock proteins [78], ANT-1 [201], or Cx43 [228]) may show altered expression or activity, thus contributing to reduced ischemic tolerance in aged. Future studies are indicated to determine the role, if any, of altered gene expression in relation to cell survival with age-associated E₂ deficiency.

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 [133], it is likely that the adaptation of additional mitochondrial proteins in aging and/or E₂ 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.

Using a high throughput proteomics approach targeting the cardiac mitochondrial subproteome in adult and aged female rats, significant directional changes were observed in **67** proteins with aged and/or aged OVX, and **32** were unique to aged OVX [121]. Notably only **6** proteins were similarly altered in adult OVX, highlighting the specificity of the E₂ 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 (MAO-A), a potential source of oxidative stress. About 50% of the identified proteins altered in aged OVX were associated with mitochondrial ATP production [121]. Age-associated reductions in cardiac mitochondrial ATP production have been previously reported in male rodents, including declines in the rate of oxidative phosphorylation and the activity of electron transport chain (ETC) complexes III and IV [124]. A recent report on age-associated alterations in male rat cardiac mitochondrial gene transcripts also noted widespread downregulation of ETC complex RNA as well as decreased complex I and IV activity [165], while proteomic profiling in aged male mouse hearts demonstrated reduced expression of several mitochondrial ETC complex subunits [34]. In aged, E₂-deficient female hearts, reduced quantity of protein subunits of ETC complex I (NADH dehydrogenase), II (succinate dehydrogenase), III (cytochrome *bc₁* complex), IV (cytochrome *c* oxidase), and V (F₀F₁ 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, in contrast, for proteins involved in carbohydrate and amino acid metabolism (pyruvate dehydrogenase subunits) and enzymes of the tricarboxylic acid cycle [121]. Increased levels of Hsp60 and mtHsp70 in aged OVX are consistent with previous studies in aged male hearts [45] 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 [49] of these enzymes is indicated for a more comprehensive characterization of metabolic alterations and substrate utilization in the aged female heart.

Nevertheless, dysregulated mitochondrial metabolism has been suggested as a contributory mechanism underlying impaired ischemic tolerance in the aged heart [99, 119, 165] and associated I/R injury (see Figure 2 for model summary). First, the reduced capacity for ATP production upon reperfusion leads to swelling, lysis, and initiation of necrotic and apoptotic cell death [53]. Observed reductions in the Na⁺/K⁺ ATPase and Ca²⁺ ATPase pumps in aged OVX hearts may further contribute to these detrimental events. Additionally, metabolic dysregulation is thought to contribute to cellular injury through increased mitochondrial ROS production in the aged heart [124]. Complex III, for example, has been identified as a major source of age-associated increases in mitochondrial superoxide radical (O₂[·]) production both at baseline and in response to I/R [124]. High levels of ROS are generated during I/R from additional sources both within and outside the mitochondria, including ETC complex I [19, 203], the xanthine oxidase system [220], and vascular NADPH oxidase [7], and contribute to cellular injury through lipid peroxidation, protein oxidation, enzyme inactivation, and DNA damage [23]. Further, ROS can induce opening of the MPTP and therefore initiation of cell death by the facilitation of mitochondrial Ca²⁺ overload and/or the oxidation of thiol groups of ANT, a possible MPTP regulatory protein [106, 109, 238].

Indicative of possible increased ROS production in the aged, E₂-deficient heart, altered expression of several mitochondrial proteins involved in the oxidative stress response have also been observed. A large increase (>90%) MAO-A, which is found in the outer mitochondrial membrane and represents a potent source of hydrogen peroxide (H₂O₂) during I/R [20, 200], has been noted in both aged and aged OVX hearts but not adult OVX [121]. Given recent evidence that MAO-A inhibition can reduce I/R injury in adult hearts (for review see [51, 103]), studies from our laboratory addressed the effects of acute MAO-A inhibition on mitochondrial respiration and subsequent I/R injury in the aged, E₂-deficient rat heart [121]. While we observed a protective pattern of mitochondrial respiration in isolated mitochondria following MAO-A inhibition with clorgyline (predictive of mild mitochondrial uncoupling), acute MAO-A inhibition at varying doses and durations of exposure prior to I/R injury *in vivo* was unable to produce an infarct sparing effect in the aged female rat heart. We observed a similar lack of efficacy in isolated perfused hearts when clorgyline was delivered 15 min prior to I/R, suggesting that the aged female heart is refractory to protection by MAO-A inhibition. The mechanism of reduced cardioprotective efficacy of MAO-A inhibition in aged animals previously demonstrated in adult animals [20] is not immediately evident, but combined with the well-characterized refractoriness of the aged heart to ischemic intervention [99], likely includes an inability of age-associated changes in antioxidant machinery to combat overproduction of ROS associated with senescence.

In this regard, SOD2 (MnSOD), the mitochondrial SOD isoform that catalyzes the conversion of the strongly reactive O₂[·] radical to less reactive H₂O₂ and molecular O₂ [96], was increased by nearly 40% in aged OVX. It is likely that these increases represent compensatory adaptations to chronically increased ROS production in the aged female heart [124], and interestingly, our observation of increased SOD2 expression is in contrast to studies in *male* F344 rats demonstrating age-related increases in cardiac SOD2 *activity* [97, 162] but unaltered SOD2 *expression* [7, 205]. Increased levels in aged and aged OVX hearts of mitochondrial proteins involved in the initiation of cell death, including cytochrome *c* and possible MPTP regulatory proteins VDAC1 and ANT1 (Figure 2). The increased quantity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme that has been reported to play a pro-apoptotic role in the mitochondria through induction of the MPTP

[195], was also noted. In this regard, direct measures of ROS are indicated in the aged female rat heart under conditions of I/R injury.

Although preserving ATP and limiting ROS production by inhibiting MPTP formation is a common strategy for cardioprotection, as noted above these strategies are often less effective in the aged [22, 64, 194]. While several groups have investigated changes in basal mitochondrial function with age, the results are variable, likely due to differences in isolation, mitochondrial subfractions, and measurement protocols. Respiration rates have been reported to increase [40, 47], decrease [41, 116, 161, 166, 196], or have variable effects depending on individual complexes or mitochondrial subpopulation [61, 85, 97, 117]. It is important to note that with the exception of Davies *et al* [47], all of these aging studies were performed on male heart mitochondria which may not necessarily extrapolate to aging in females. Given reported reductions of subunits in all five complexes of the ETC, subsequent studies [90] revealed that: 1) age significantly reduced the respiratory control index (RCI) at Complexes I and II, 2) Estrogen deficiency and age sensitized the mitochondria to Ca^{2+} -overload, and 3) PPT increased mitochondrial RCI but did not improve Ca^{2+} sensitivity. No significant age-dependent changes in state 2 or state 3 respiration of Complexes I, II, IV (state 3 only) were observed, which are in agreement with oxygen consumption studies of similar mitochondrial populations in adult and 24 mo male rats [41, 61, 85]. However, in contrast to these studies, age-dependent decreases in the RCI's for Complexes I and II were observed in females, which is consistent with increased mitochondrial uncoupling with aging. Although inhibition of ATP/ADP exchange and/or ATP synthase may also account for decreased RCI, ADP-induced respiration was not significantly decreased. Furthermore, it has been proposed that mitochondrial uncoupling may be a compensatory mechanism sacrificing ATP production efficiency to combat increased ROS production seen with aging in tightly-coupled mitochondria [25].

Cardiac calcium handling is perturbed with senescence and the aged myocardium is more sensitive to ROS- and Ca^{2+} - induced MPTP opening [98, 118]. Similar to studies in male hearts, increases in Ca^{2+} -sensitivity with aging occur in the female myocardium as evidenced by Ca^{2+} -induced decreases in Complex I respiration and swelling [90]. Interestingly, the age-dependent reductions in Complex I respiration with Ca^{2+} are mirrored in adult mitochondria with OVX, suggesting that E_2 may play a protective role with respect to Ca^{2+} -sensitivity in adult animals. This hypothesis is supported by the observation that female mitochondria accumulate Ca^{2+} more slowly than do male [9], and that E_2 supplementation reduces mitochondrial calcium accumulation [139]. That OVX does not worsen the age-dependent sensitization to Ca^{2+} suggests that age and OVX are sufficient to sensitize the mitochondria to Ca^{2+} to the same degree. Given discrepant results in respiration studies of mitochondria isolated from aged animals, more studies addressing measures in both subsarcolemmal and interfibrillar mitochondrial populations are indicated. Moreover, studies in true models of female aging are also sorely needed to reconcile the impact of associated changes in mitochondrial protein levels and functional outcomes.

Conclusion

The increased prevalence of cardiovascular diseases in women following menopause coupled with the failure of HRT to demonstrate cardioprotection, has led many researchers to reexamine mechanisms of cardioprotection and subsequent loss of this cardioprotection in advancing age with E_2 deficiency. It has become clear that ovariectomizing adult animals may not accurately represent the combined effects of age and E_2 deficiency seen in postmenopausal females. E_2 is known to regulate the transcription of several cardioprotective genes by action through $\text{ER}\alpha$ and $\text{ER}\beta$, including eNOS and Akt, and females exhibit increased association of eNOS with the myocardial-specific caveolin-3 [152,

156, 191]. Acute, non-genomic signaling downstream of ER activation or E₂ action at GPCRs, in addition, can activate many of the same signaling pathways recruited in cardioprotection, including PI3-K, Akt, and eNOS pathways [65, 142]. Alterations in cardioprotective gene expression or acute cardioprotective signal transduction are therefore likely to result in the context of aging and E₂ deficiency, and may help explain the reduced ischemic tolerance and loss of cardioprotection in the senescent female heart. The assessment of cardioprotective signal transduction downstream of PKC ϵ activation in aging and E₂ deficiency may, further, allow for the identification of alternative therapeutic targets for reducing I/R injury in postmenopausal women. In this regard, recent findings [111, 113] demonstrating improved ischemic tolerance in aged male and female rats following acute PKC modulation extends the protective reach of PKC therapeutics to a model of senescence.

It is also clear that mitochondria play a central role in cardioprotection and research elucidating the mechanisms of this protection in the aged female heart is ongoing. Importantly, the vast majority of this research is being performed in adult models, rather than the population at risk for a cardiovascular event, i.e. the aged. Indeed, recent proteomic screens of mitochondria isolated from aged and E₂-deficient rat hearts have revealed a highly selective response to E₂ deficiency in aged vs adult, and perturbations of several ETC proteins may upset the stoichiometry of the ETC and contribute to increased ROS production. Importantly, quantification of ROS and characterization of the mitochondrial subproteome as it adapts to advancing age and E₂ deficiency is indicated, which will allow for the identification of proteins and possible post-translational modifications associated with cardiac signaling disturbances contributing to age-associated declines in ischemic tolerance. Evidence-based medical treatments and therapies have helped to drastically reduce deaths due to CHD with $\approx 47\%$ of the reduction in deaths in the US from 1980 to 2000 being attributed to their increased use [169]. This finding emphasizes the necessity of further research into the field of I/R injury to enable the continued development of these treatments and therapies particularly for aged, postmenopausal women.

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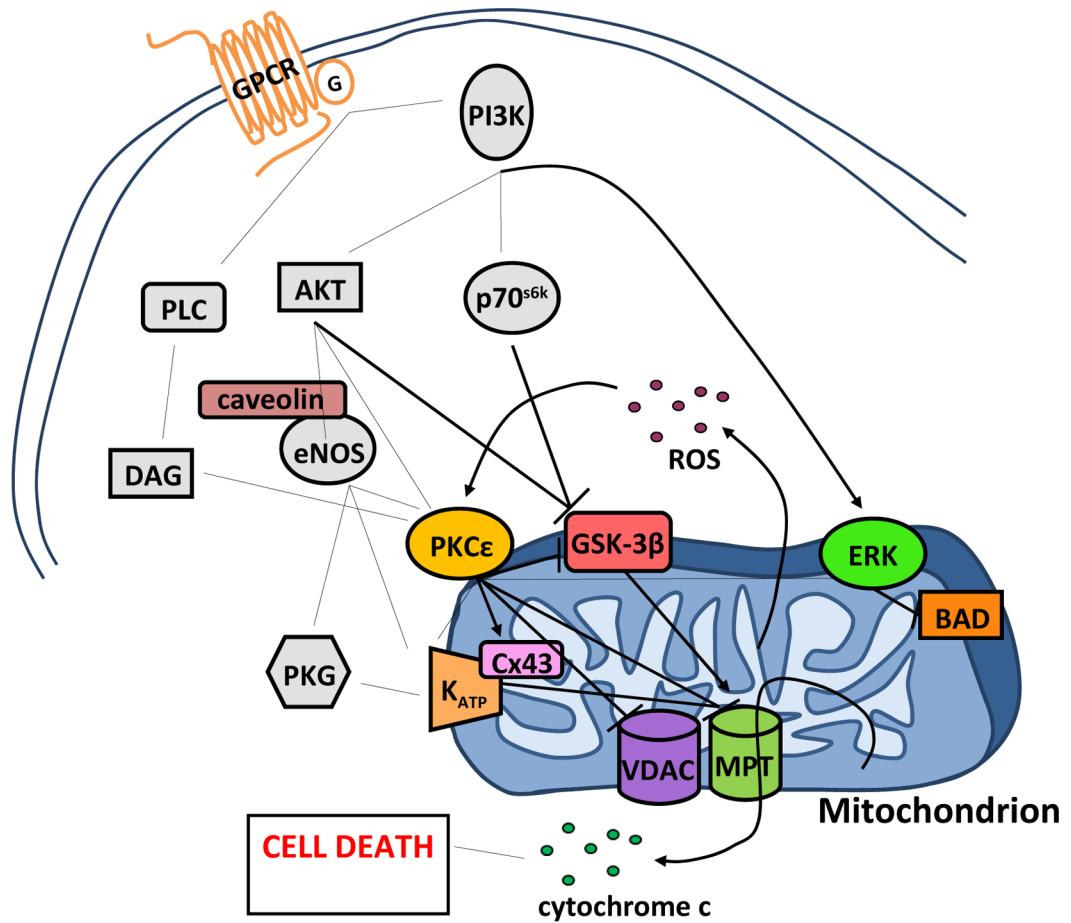


Figure 1.

Simplified schematic of cardioprotective cellular signaling. Abbreviations: BAD, Bcl-2-associated death promoter; Cx43, connexin 43; diacylglycerol, DAG; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinases; GSK-3 β , glycogen synthase kinase-3 β ; MPTP, mitochondrial transition pore; PI3K, phosphoinositide 3-kinase; PLC, phospholipase C; PKC ϵ , protein kinase C ϵ ; PKG, protein kinase G; VDAC, voltage-dependent anion channel. Adapted from [144]).

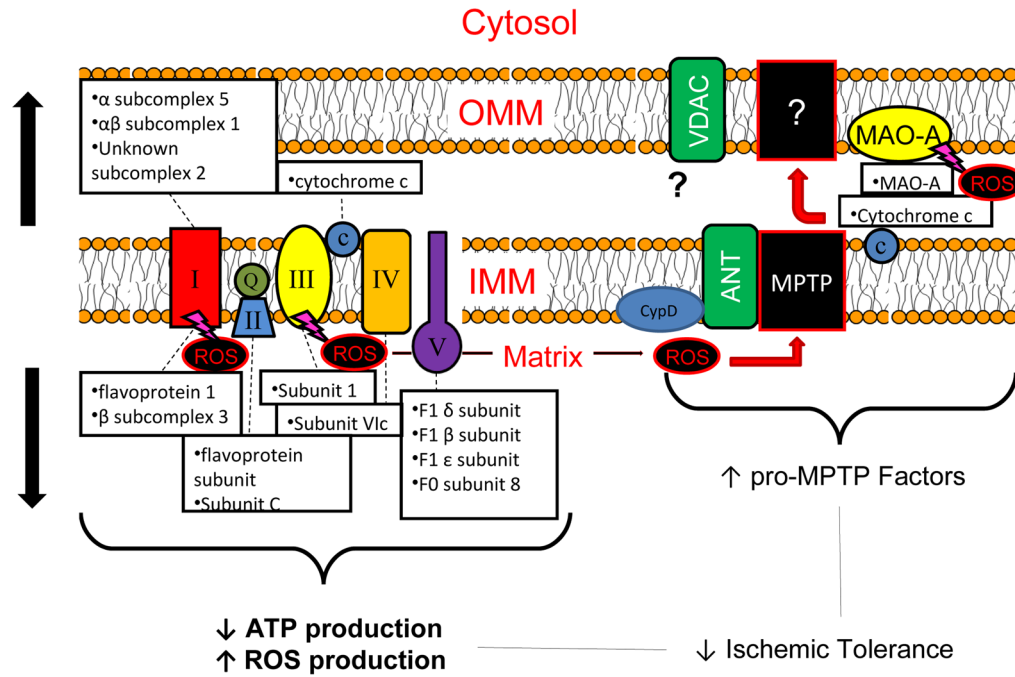


Figure 2. Summary of proposed changes in mitochondrial electron transport complex and pro-apoptotic proteins which may contribute to reductions in ischemic tolerance with age-associated E₂ deficiency. Abbreviations: ANT, adenine nucleotide translocator 1; ATP, adenosine triphosphate; CypD, cyclophilin D; IMM, inner mitochondrial membrane; MAO-A, monoamine oxidase-A; MPTP, mitochondrial permeability transition pore; OMM, outer mitochondrial membrane; ROS, reactive oxygen species; VDAC, voltage-dependent anion channel.