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Novel Mitochondrial and Nuclear Targets of Acute PKCε Activation in the Aged Female Rat Heart

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ABSTRACT

Mortality due to myocardial infarction (MI) increases as women experience menopause, implicating a protective role for estradiol (E_2). However, studies have produced conflicting results on the effects of hormone replacement therapy (HRT) on occurrence of MI. This points to the need for development of therapeutic methods for reducing ischemia/reperfusion (I/R) injury in the aged, E_2-deficient heart. This study focused on the cardioprotective effects of PKCε and its mechanism of action in the mitochondria as well as its effects on gene expression. Using a high-throughput proteomics approach, we aimed to characterize altered expression levels of mitochondrial proteins contributing to both reduced ischemic tolerance in the aged, E_2-deficient female rat heart and cardioprotection in PKCε-activated hearts. We also aimed to characterize changes in mRNA level of two proteins involved in PKCε-mediated cardioprotection. A Langendorff-perfused heart model was utilized to measure functional recovery in adult (5 mo) and aged (23 mo) female F344 ovary-intact or ovariectomized (OVX) rats administered a PKCε-activator (ψεRACK) prior to 47 min ischemia and 60 min reperfusion. mRNA was isolated from the LV's of these hearts and subjected to RT-PCR. Proteomic analysis was conducted on the LV mitochondrial fractions of hearts undergoing control perfusions with or without ψεRACK, utilizing isobaric tags for relative and absolute quantitation (iTRAQ) 8-plex labeling and tandem mass spectrometry. The aged OVX hearts showed reduced functional recovery following I/R when compared with the adult ovary-intact hearts. Administration of ψεRACK prior to I/R increased functional recovery, measured by recovery of LVDP, in both adult intact and aged OVX groups. Proteomic analysis revealed three possible targets of PKCε cardioprotective signaling, the antioxidant enzymes glutathione peroxidase and superoxide dismutase 2, as well as the heat shock protein Hsp 10. Finally, decreased levels of Cx43 and RACK2 mRNA were seen with age, while administration of ψεRACK prior to I/R at least partially abrogated this effect. These results indicate a protective role for PKCε in the aged, E_2-deficient heart and identify novel nuclear and mitochondrial targets of acute PKCε activation.

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Chapter 1

Introduction

Coronary heart disease (CHD) was reported to have caused 35.3% of deaths in the United States in 2009, making it the leading cause of death for men and women alike (46). Also, one in every three American adults is estimated to suffer from some form of CHD. Although men and women are both subject to increased risk for CHD with age, women tend to see a sharp increase in risk following menopause. The incidence of CHD is 2-3 times higher in post-menopausal women than in their pre-menopausal counterparts(39). Post-menopausal women also show greater incidence and severity of CHD than males of the same age, with 43% of women over 40 dying within five years of their first myocardial infarction (MI), as compared to 33% of men (46). These findings suggest that the loss of estrogen at menopause may contribute to the increased risk of post-menopausal women for CHD and indicate a possible protective role of estrogen.

Despite the observational evidence that estrogen may play a protective role against CHD, clinical trials using hormone replacement therapy have demonstrated mixed results. While the Nurses' Health Study showed reduced risk for CHD in women receiving hormone replacement therapy, the Women's Health Initiative actually demonstrated an increased risk for MI and stroke following estrogen replacement(47). These results emphasize the importance of finding alternative methods for reducing the risk of post-menopausal women for CHD.

One well-studied target for limiting ischemic damage in the heart and thus decreasing cell death due to MI is a novel protein kinase known as protein kinase Cε (PKCε). Dorn and colleagues used the PKCε activating peptide ψεRACK to show that activation of PKCε prior to ischemia reduces ischemic damage in mice. Ping and colleagues also showed that activated

PKCε translocates from the cytosol to the mitochondria(60). Finally, Korzick *et al* have demonstrated that acute PKCε activation prior to ischemia reduces ischemia/reperfusion (I/R) injury in the aged male rat heart and that mitochondrial PKCε decreases with age and E_2-deficiency in the female rat heart(31). It is still unknown whether activation of PKCε prior to ischemia is sufficient to induce cardioprotection in the aged, E_2-deficienct female rat heart.

Statement of the Problem

Post-menopausal women suffer from an increased risk for mortality due to MI when compared to pre-menopausal women and men of the same age. Although this observation suggests a protective role of estrogen in the heart, clinical studies have been unable to produce consistent results regarding the effects of hormone replacement therapy. Thus, it is necessary to investigate alternative therapeutic targets for the treatment of CHD in post-menopausal women. One protein that has been well studied for its role in cardioprotection, PKCε, has shown decreased expression in the mitochondria with age and estrogen-deficiency. This reduction in mitochondrial PKCε could contribute to impaired ischemic tolerance through changes in PKCε-mediated signaling at the mitochondria.

Specific Aims and Hypotheses

Specific Aim #1: To determine the role of acute PKCε activation in reducing ischemia-reperfusion injury in the aged and estrogen-deficient female rat heart. Reduced levels of PKCε have been observed in the aged, E_2-deficient female rat heart, offering a possible explanation for the reduced ischemic tolerance seen in this model. Surgical ovariectomy will be used to create an E_2-deficient model in both young and aged female rats. A Langendorff isolated perfused heart model will be used to assess left ventricular function following ischemia in the presence and absence of an acute PKCε activator peptide ψεRACK, reversibly conjugated to the carrier peptide Tat.

Hypothesis: Acute PKCε activation will improve cardiac functional recovery following ischemia/reperfusion injury in the aged, E_2-deficient female rat heart.

Specific Aim #2: To determine identities of mitochondrial proteins that are altered by acute PCKε activation in the aged, estrogen-deficient female rat heart. Proteins that demonstrate increased expression in the mitochonria following acute PKCε activation could represent downstream signaling targets of PKCε, which translocates to the mitochondria upon activation. Homogenized heart tissue from aged, ovariectomized female rats treated with the PKCε activator peptide, ψεRACK, will be labeled using isobaric tags for relative and absolute quantitation (iTRAQ) reagents and subjected to LC/MS/MS. These results will be compared to those of adult, ovary-intact female rats to determine the identities of proteins that show significantly increased abundance in the aged, estrogen-deficient rat heart.

Hypothesis: Mitochondrial proteins upregulated by PKCε are those involved in the cellular response to oxidative stress, including antioxidants and regulators of apoptosis.

Chapter 2

Review of Literature

Coronary heart disease (CHD), a classification that encompasses both myocardial infarction (MI) and angina pectoris (AP), is the most common cause of death for both men and women in the United States(46). CHD was the cause of 1 in every 5 deaths in the United States in 2005. MI accounted for 31.5% of the total deaths due to heart disease, and the incidence and mortality due to MI increases with advancing age. Women in general face a slightly lower level of mortality due to heart disease than men, with 47% of women dying from their first MI as compared to 53% of men(46). However, post-menopausal women in particular are more likely to die from their first MI than men, with 43% of women over 40 dying within five years of their first MI, as compared to 33% of men. Postmenopausal women also experience a 2-3 fold increase in incidence of CHD when compared with premenopausal women. These data suggest a cardioprotective function of endogenous estradiol (E_2), which accounts for the increased incidence of CHD in postmenopausal women.

Although the increased risk of postmenopausal women for CHD points to a protective role for E_2 in the heart, research addressing the effects of hormone replacement therapy (HRT) on CHD has produced conflicting results. The Nurses' Health Study demonstrated a 50% lower risk of major coronary disease among women currently taking HRT as compared to their age-adjusted counterparts who had never used HRT(24). However, the Women's Health Initiative clinical trial indicated an *increased* risk for MI and stroke in women receiving conjugated equine estrogens (CEE) and HRT is now contraindicated in post-menopausal women(47). These conflicting results point to the need to identify alternative therapeutic targets for reducing the risk of CHD in postmenopausal women.

Protein Kinase C

Structure

Protein kinase C (PKC) includes a family of serine/threonine kinases including at least 10 isozymes that contribute to intracellular signaling in a tissue-specific manner(64). Three subfamilies of PKC isozymes exist, as classified by cofactors required for activation(51). The classical PKC's (α, βI, βII, and γ) are dependent on both diacylglycerol (DAG) and Ca^{2+} for activation. The novel PKC's (ε, δ, θ, and η) require only DAG. Atypical PKC's (ζ and ι /λ) need neither DAG nor Ca^{2+} for activation. PKC exists in all tissues, notably the brain, heart and kidneys(13). PKC isozymes have many functions and may even oppose each other within the same tissue.

PKC isozymes contain both a variable N-terminal region and a highly conserved C-terminal region (Fig. 1). These are separated by a flexible hinge region that allows for changes in conformation of the enzyme. The C-terminal region contains C3 and C4 domains, which are conserved among all PKC isozymes(15, 57). The C3 domain represents the ATP binding site while the C4 domain constitutes the substrate-binding site.

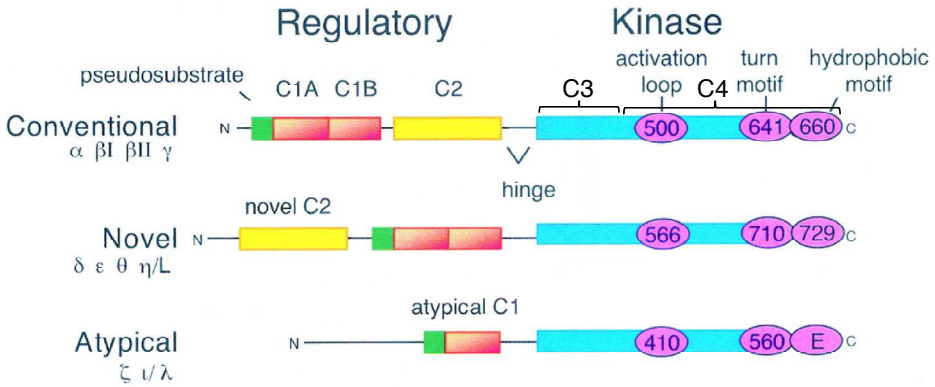

Figure 1: Structure of PKC isozymes.

The N-terminal domain contains several regions that are conserved among all PKC isozymes, including a pseudosubstrate site, a receptor for activated C kinase (RACK) binding site, and C1 domains. The C1 domain functions as a binding site for diacylglycerol (DAG), phorbol esters, and phosphatidylserine (PS) in the classical and novel PKC's(52). The C1 domain of atypical PKC's, however, lacks the sequence critical for binding these molecules. Novel and classical PKC's both contain a C2 domain as well, but these domains differ significantly between the two subfamilies and are completely absent in the atypical PKC's. In classical PKC's the C2 domain binds membrane phospholipids in a Ca^{2+}-dependent manner. However, the C2-like domain present in novel PKC's lacks the aspartate residues that confer this Ca^{2+}-dependency, rendering novel PKC's interactions with membrane phospholipids independent of Ca^{2+} binding(55).

The pseudosubstrate domain and RACK binding site are both required for the autoregulation of PKC activity through intramolecular binding. In the inactive conformation, the pseudosubstrate domain binds the substrate-binding site in the C4 domain while a pseudo-RACK sequence in the C2 domain interacts with the RACK binding site(29, 50).

Mechanism of Activation

Activation of classical PKC's occurs through the action of phopholipase C (PLC), which cleaves phosphatidylinositol (4,5)-bisphosphate into inositol triphosphate (IP_3) and DAG. IP_3 production leads to an increase in intracellular Ca^{2+} concentration, which binds the C2 domain of classical PKC's and increases the affinity of the C2 domain for DAG at the membrane(40). The binding of DAG to the C2 domain of PKC causes the pseudosubstrate to be released from the substrate-binding site and the active site to be exposed(67).

Activated PKC's have been demonstrated to interact with specific anchoring proteins, known as RACK's, at the various membranes within the cell(40). These RACK's are specific to each PKC isozyme and they anchor the isozymes to specific subcellular locations, allowing PKCs to interact with their individual substrates. RACK1 specifically binds PKCβII while RACK2 specifically interacts with PKCε(16).

Regulators of PKC

Several regulators of PKC isozymes have been developed by Mochly-Rosen and colleagues, who have designed small modulator proteins based on their knowledge of the intramolecular regulation of PKC(66). Most of these regulators work by either promoting interaction of PKCs with their RACKs, in the case of activators, or by inhibiting the PKC-RACK interaction (PKC inhibitors)(17).

Activators, such as ψεRACK, the activator of the ε PKC isozyme, are derived from the ψRACK sequence of the PKC enzyme (Fig. 2) (17). These activators work by binding to the RACK binding site, keeping the enzyme in the active conformation so that it is able to translocate to the membrane portion of the cell and bind its specific RACK(11).

Inhibitors of PKC (ie. εV1-2 for PKCε) act by competitively inhibiting the binding of PKC to RACK(11). Inhibitory peptides are derived from the RACK-binding site in the N-terminal domain of PKC (Fig. 2). They prevent translocation, binding of PKC to RACK, and subsequent activation of PKC.

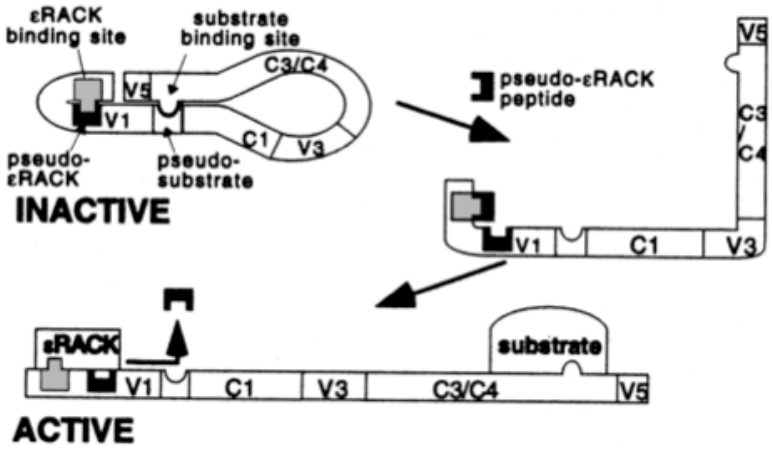

Figure 2: Mechanism of the PKCε activator, ψεRACK

Regulators of PKC have been delivered to the desired cells in a number of ways. First, the regulators were delivered by transient cell permeabilization using detergents(37) or by expression of the peptides as transgenes. However, delivery of peptides is now achieved both *in vitro* and *in vivo* by reversible conjugation to a carrier peptide derived from the HIV protein Tat, via cysteine-cysteine bond at the N-terminus(70). The peptide cargo is delivered to the cell interior where the disulfide bond is readily cleaved, releasing the functionally active regulatory peptide. The intracellular delivery of Tat-conjugated cardioprotective peptides to the myocardium, including the cytosolic and mitochondrial fractions, was demonstrated in the isolated perfused rat heart by Gustaffson *et al*.(25) using fluorescence-tagging and western blotting, and was confirmed for Tat-conjugated PKC regulatory peptides by Begley *et al.(4)*

PKC in Ischemia/Reperfusion Injury

Ischemic Preconditioning

An important form of cardioprotection is seen in the phenomenon of ischemic preconditioning (IPC), which is defined as brief periods of ischemia and reperfusion that reduce damage to the myocardium during subsequent prolonged periods of I/R(48). Murry *et al*. (49) first observed IPC in a canine model of coronary occlusion. Murry's group found that several cycles of 5 minutes of ischemia followed by 5 minutes reperfusion rendered hearts resistant to damage caused by subsequent prolonged periods of ischemia.

The occurrence of IPC has clinical significance in that the protective signaling pathways associated with IPC can be manipulated to reduce ischemic injury. At reperfusion, these signaling pathways appear to converge at the mitochondria, where necrotic and apoptotic cell death are regulated(20). PKCε specifically has been implicated as a critical player in the protective signaling that occurs at the mitochondria during IPC89(33).

Role of PKCε in Cardioprotection

The first study that investigated the role of PKCε in IPC was pioneered by Downey, *et al* (18). They showed that the reduced infarct size caused by IPC could be abrogated by administration of the general PKC inhibitor, staurosporine, in isolated rabbit cardiomyocytes as well and *in vivo* rat heart model(2, 72)They also delivered the general PKC activator phorbol 12-myristate 13-acetate (PMA) prior to ischemia and discovered that the protection afforded by PMA was equivalent to that of IPC.

Further research has identified PKCε as a key isozyme in the signaling which underlies the protection associated with IPC. Utilizing a model of rabbit cardiomyocytes, Ping and colleagues administered a preconditioning stimulus and observed the translocation of the PKCε isozyme from the cytosolic to membrane fraction without a change in total PKC activity(60).

The PKCε regulatory peptides, εV1-2 and ψεRACK, have been utilized in various studies to confirm the importance of PKCε in cardioprotection. In neonatal rat cardiomyocytes, Gray *et al*. (23) administered the PKCε antagonist εV1-2 and demonstrated a reduction in IPC-induced protection. Using the PKCε activating peptide ψεRACK, Dorn and colleagues (17) next showed that activation of PKCε prior to ischemia reduced ischemic damage and that this reduction was comparable to that caused by IPC. They also demonstrated translocation of PKCε from the cytosolic to the particulate fraction and that this translocation could be blocked using εV1-2 or the general PKC inhibitor chelerythrine (17). The same study also utilized a transgenic mouse model in which ψεRACK was expressed specifically in the myocardium. Following I/R in an isolated perfused heart model, the ψεRACK transgenic mice showed improved recovery of left ventricular dP/dt and decreased creatine phosphokinase release.

Similarly, Chen and colleagues (11)delivered the ψεRACK peptide through conjugation to the Tat carrier peptide prior to ischemia in an isolated perfused rat heart model and an *in vivo* rat model of coronary artery occlusion. They found reduced ischemic damage in the hearts receiving Tat-ψεRACK as measured by decreased infarct size, improved functional recovery and decreased release of creatine kinase(11). Delivery of Tat-ψεRACK during reperfusion, as demonstrated by Ingaki *et al*. (32) in an isolated perfused rat heart model, did not confer the same protective effect. Saurin *et al*. also observed a loss of IPC-induced protection in an isolated perfused mouse heart model using PKCε null transgenic mice (63).

The Korzick laboratory has previously described that decreased ischemic tolerance with age is associated with reduced PKCε at the mitochondria and nucleus in male rats, and that ischemic tolerance could be improved through administration of Tat-ψεRACK prior to ischemia (31). Abete and colleagues (1)used a male rat model to demonstrate that the protection afforded by IPC does not extend to the aged heart. Age and E_2-deficiency have also been shown to decrease ischemic tolerance in female animals. Hunter *et al*. (31) described an increase in infarct size following I/R in aged relative to adult female rats and measured decreased PKCε expression at the mitochondria with age in this model. Song *et al*. (65) demonstrated an increase in infarct size following I/R and the loss of IPC-induced cardioprotection in adult OVX relative to adult ovary-intact female mice. However, the efficacy of acute PKCε activation in the aged female heart is unknown.

Cardioprotective Signaling at the Mitochondria

Although activated PKCε has been shown to translocate to the various membrane fractions of the cell, the critical role of the mitochondria in cellular energetics, oxidative stress and cell death suggests that the mitochondria may be an important site of PKCε protective signaling (Fig. 3). IPC signaling converges at the mitochondria through the phosphatidylinositol 3-kinase (PI3-K) pathway, which involves activation of AKT, endothelial nitric oxide synthase (eNOS), and guanylyl cyclase (GC). This pathway results in the opening of the mitochondrial ATP-sensitive K^+channels (mitoK_{ATP})(14, 53). The influx of K^+ into the mitochondria results in generation of reactive oxygen species (ROS), which in turn activate PKCε (42). This pathway plays an important role during reperfusion, when PKCε activates the PI3-K/Akt and MEK1/2-

ERK1/2 survival pathways. Both Akt and ERK1/2 phosphorylate mitochondrial glycogen synthase kinase-3β (GSK-3β), thus inactivating it(3, 69). This leads to inhibition of the mitochondrial permeability transition pore (MTPT)(35), which represents the hypothesized final target of IPC.

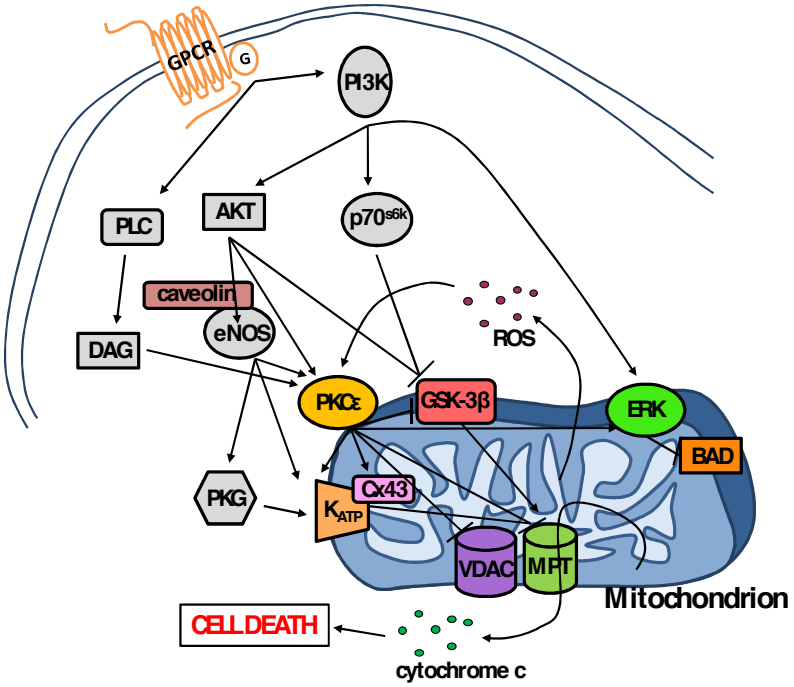

Figure 3: Simplified schematic of cardioprotective cellular signaling

The MPTP is a large-conductance multiprotein complex that connects that mitochondrial matrix to the cytosol. PKCε has been established as a potential regulaor of this complex along with voltage-dependent anion channel 1 (VDAC1), hexokinase II (HKII), cyclophilin D, and adenine nucleotide translocator (ANT). Although these proteins were once thought to be components of the MPTP, recent evidence suggests that they are either regulators or, in the case of VDAC1, not essential for MPTP function (74). Although the mechanism of MPTP formation has recently been questioned, it is known that pore opening dispels the mitochondrial membrane potential, resulting in the inability to produce ATP, cell lysis and necrosis.

PKCε activation attenuates pore opening through phosphorylation of VDAC1, activation of mitoK_{ATP} channels(22), and phosphorylation of GSK-3 β102(38). PKCε also phosphorylates connexin 43 (Cx43) at the inner mitochondrial membrane. Cx43 is a gap junction protein that has been implicated in cardioprotection. Interestingly, mitochondrial Cx43 is decreased with age and associated with a loss of cardioprotection due to IPC (6). More recently, Rottlaender *et al*. (62) demonstrated that Cx43 can stimulate mitoK_{ATP} channels in mouse cardiomyocytes.

Another proposed intra-mitochondrial substrate of PKCε is aldehyde dehydrogenase 2 (ALDH2). Mitochondrial ALDH2 was first identified using a high-throughput proteomics search by Chen *et al*. (10)as a molecule whose activation correlated with a reduction in ischemic damage in rats, probably through inhibition of cytotoxic aldehyde formation. Further research by Budas *et al*. (9)demonstrated that ALDH2 is phosphorylated and activated by PKCε. The same group recently conducted a study to determine the mechanism by which PKCε is imported into the mitochondria to reach targets for phosphorylation, such as Cx43, ALDH2 and the cytochrome c oxidase subunit IV (COIV). Heat shock protein 90 (Hsp 90) was identified as a mediator of mitochondrial import of PKCε (8). Moreover, inhibition of Hsp 90 reduced mitochondrial import of PKCε and increased necrotic cell death.

Use of Proteomics in Identification of PKCε Targets

Proteomics techniques have been used to investigate the mechanisms which underlie PKCε-mediated cardioprotection. Ping *et al*.(59) immunoprecipitated total cardiac tissue lysates of transgenic mice overexpressing PKCε and nontransgenic controls. A two-dimensional gel electrophoresis (2D-GE) approach in conjunction with matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) was used to identify 36 proteins that physically associated

with PKCε(59). These proteins validated known targets of PKCε-mediated cardioprotection, including PI3-K, Akt, ERK1/2, Cx43, eNOS, MAPKs, and heat shock proteins (HSPs) 27/70.

An additional 57 proteins that form complexes with PKCε in PKCε-overexpressing mice were identified by the Ping laboratory using 1D and 2D gel electrophoresis with in-gel trypsin digest as well as in-solution trypsin digest followed by nanoscale liquid chromatography (LC) and electrospray ionization quadrupole time-of-flight (Q-TOF) tandem MS(19). Proteins identified included ATP synthase subunits and proteins involved in mitochondrial transport such as ANT1/2 and VDAC.

iTRAQ Proteomics Approach

Although the previously mentioned studies successfully utilized gel electrophoresis techniques combined with MS analysis to identify possible targets of PKCε, these approaches possess certain disadvantages including the inability to detect extremely basic, acidic, hydrophobic, or low-abundance proteins. Additional disadvantages include the need to excise and digest specific bands from gels for MS analysis(73). Several high-throughput methods for protein quantitation have since been developed, including isotope coded affinity tags (ICAT) and stable isotope labeling by amino acids in cell culture (SILAC)(26, 56). These approaches involve in-solution labeling and digestion and minimize problems with detecting low-abundance, hydrophobic and acidic/basic proteins. However, these methods do not allow for multiplex comparisons and their utilization of mass-difference labeling, while useful in identifying proteins based on a shift in the MS spectrum, leads to great complexity of MS spectra (61).

Recently, a method for high-throughput protein quantitation that allows for multiplex, or multiple sample, comparisons has been developed. Isobaric tags for relative and absolute

quantitation (iTRAQ) differ from the previously mentioned methods in that they utilize isobaric, or mass-equal, peptide labeling (61). The tags contain a reporter group and a balance group created using stable isotopes (Fig. 3). The use of these two groups in combination allows the total mass of all tags to be equal. The tags also contain an amine-specific peptide reactive group that binds peptides at the N-terminus and ε-amino groups of lysine side-chains through amide bond formation. This allows iTRAQ tags to bind all peptides in a mixture.

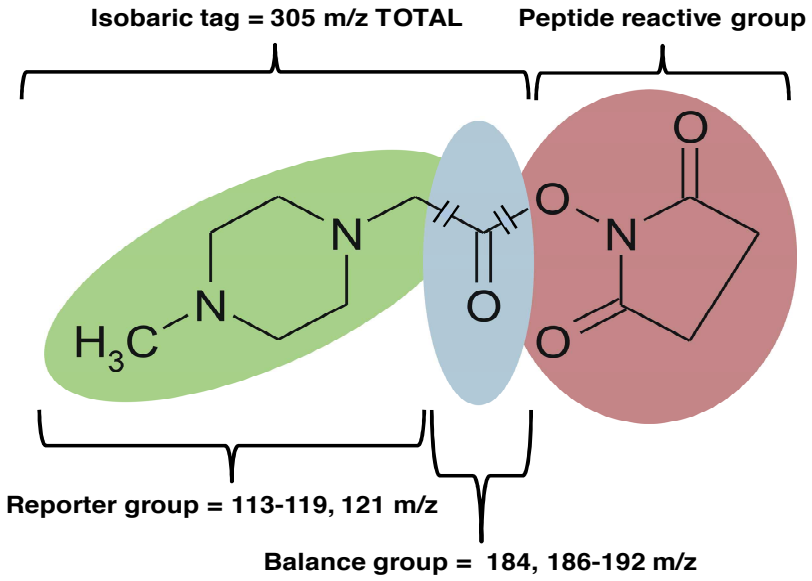

Figure 4**:** Chemical structure of the iTRAQ isobaric tags.

Digested samples can be labeled with unique iTRAQ tags, pooled and subjected to LC/MS/MS. Because of the isobaric nature of iTRAQ tags, the samples behave identically in LC and single-stage MS, showing the same chromatographic characteristics and m/z values (61). During tandem MS, the reporter ions are fragmented from the peptide and yield peaks at 113-119 and 121 m/z depending on the mass of the reporter. These peaks are used to quantify relative

amounts of peptide in different samples and the y- and b- ion series created by peptide fragmentation are used to identify the peptides(5). iTRAQ labeling kits have been sold by Applied Biosystems as both 4-plex and 8-plex kits, which contain reporter ions of 114-117 m/z and 113-119, 121 m/z respectively. These kits allow for multiplex comparisons of either four or eight samples at a time.

Summary

Despite observational evidence for the protective effect of E_2 in reducing I/R injury following MI, clinical studies have yielded conflicting results on the efficacy of HRT. This finding points to the need for a better understanding of the cellular pathways that contribute to ischemic tolerance in the aged, E_2-deficient heart. Much evidence exists for the protective role of PKCε in the heart in numerous animal models, but given the decreased mitochondrial levels of PKCε observed in aged, E_2-deficient hearts, it is unknown whether PKCε activation through ψεRACK is sufficient to produce cardioprotection in this model. PKCε has been shown to translocate to the nuclear and mitochondrial membranes upon activation. Although many mitochondrial signaling partners of PKCε have been described, identification of novel or altered mitochondrial targets as well as altered gene expression in response to PKCε activation will provide further evidence for the mechanisms by which PKCε confers cardioprotection.

Chapter 3

Novel Mitochondrial and Nuclear Targets of Acute PKCε Activation in the Aged Female Rat Heart

Introduction

Although women generally face lower mortality due to myocardial infarction (MI) than men, postmenopausal women show a 2-3 fold increase in incidence of coronary heart disease (CHD) when compared to premenopausal women(46). These facts suggest a protective role for endogenous estradiol (E_2). However, clinical studies on the effects of hormone replacement therapy (HRT) in postmenopausal women suggest an *increased* risk for MI, indicating the need for exploration of alternate therapies to combat the decreased ischemic with the E_2-deficient aged heart.

A mediator of cardioprotection that has been the subject of many recent studies is the protein kinase C isozyme PKCε. Activation of PKCε prior to ischemia through the isozyme-specific regulatory peptide ψεRACK has been shown to reduce ischemia/reperfusion (I/R) injury in multiple model systems through a mechanism that resembles ischemic preconditioning (IPC). IPC is a cardioprotective phenomenon in which several brief periods of ischemia and reperfusion decrease cell death in the heart following a prolonged period of I/R. IPC involves signaling cascades that converge on the mitochondria, the site to which PKCε also translocates upon activation. Korzick *et al* have shown that mitochondrial PKCε levels are decreased in the aged, E_2-deficient female rat heart(31), but it is not known whether acute activation of PKCε prior to ischemia can produce cardioprotection in this model system.

The hypothesized final target of IPC is the mitochondrial permeability transition pore (MPTP), which opens during I/R leading to a loss of mitochondrial membrane potential, the

halting of ATP production, and cell death. This large-conductance multiprotein complex also represents a target of PKCε-mediated cardioprotection. PKCε directly phosphorylates the MPTP component voltage-dependent anion channel 1 (VDAC) and interacts with mitochondrial signaling targets such as glycogen synthase kinase-3β (GSK-3β), aldehyde dehydrogenase 2 (ALDH2) and connexin 43 (Cx43), thus inhibiting the opening of the MPTP. This stabilizes mitochondrial membrane potential and limits cell death. Other known targets of PKCε in the mitochondria include the mitochondrial ATP-sensitive K^+ channel (mitoK_{ATP})(22), endothelial nitric oxide synthase (eNOS)(58), and cytochrome *c* oxidase subunit IV (COIV) (54).

PKCε translocates to the nuclear membrane upon activation and decreased levels of nuclear PKCε with aging have been associated with a loss of ischemic tolerance (31). It is possible that activated PKCε is therefore involved in the regulation of cardioprotective gene expression. One protein that is necessary for PKCε-mediated cardioprotection is the receptor for activated PKCε, RACK2. RACK2 is an anchoring protein to which activated PKCε binds at the various membranes within the cell(40). A decrease in particulate (membrane) RACK2 with age has been associated with a decrease in PKCε translocation(43), suggesting a role for activated PKCε in regulating RACK2 abundance. Another cardioprotective protein that decreases with age is connexin 43 (Cx43). PKCε phosphorylates Cx43 at the inner mitochondrial membrane, which turn can stimulate mitoK_{ATP} channels, thus inhibiting the opening of the MPTP and subsequent cell death. Decreased Cx43 levels with aging correspond to a decrease in PKCε translocation, suggesting a possible regulatory role for PKCε on Cx43 abundance.

The purpose of the current study was to determine the effects of acute PKCε activation on I/R injury in the aged, E_2-deficient female rat heart. We hypothesized that delivery of the PKCε-activating peptide ψεRACK prior to ischemia would reduce I/R injury and increase

translocation of PKCε to the mitochondria. We also sought to characterize changes in mitochondrial protein expression involved in decreased ischemic tolerance and to identify mitochondrial targets of PKCε using a high-throughput quantitative proteomics approach. We hypothesized that impaired ischemic tolerance in the aged, E_2-deficient female rat heart would lead to altered expression of proteins involved in cell death, oxidative stress and cellular metabolism and that these proteins represent potential signaling targets of PKCε, as well as potential therapeutic targets for reducing cell death during MI in postmenopausal women.

Methods

Note: detailed methods are provided in **Appendix A.**

Animal Care

Adult (5 mo, *n*=16) and aged (23 mo, *n*=15) female Fisher 344 rats were supplied by Harlan Sprague Dawley (Indianapolis, IN) and Taconic (Hudson, NY). Rats were housed using a 12:12 hr light:dark cycle and received food and water *ad libitum*. Bilateral ovariectomy surgeries were performed by the supplier on adult (*n*=10) and aged (*n*=8) animals in order to create a model of E_2 deficiency. Rats were allowed to recover for 4-6 weeks following surgery before experimental study.

PKCε Regulatory Peptide

An isozyme-specific protein kinase Cε (PKCε) activator peptide (ψεRACK; C-HDAPIGYD) derived from the PKCε pseudo-RACK sequence (PKCε amino acids 85 to 92) and a PKCε inhibitory peptide (εV1-2; C-EAVSLKPT) derived from the PKCε RACK-binding sequence (PKCε amino acids 14-21), each conjugated reversibly to a Tat-derived carrier peptide [Tat amino acids 43–58 (C-YGRKKKRRQRRR)] by disulfide bond at the N-terminus, were provided by KAI Pharmaceuticals (San Francisco, CA).

Isolated Heart Preparation

Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (40 mg/kg body wt). Hearts were rapidly removed by midline thoracotomy and rinsed with ice-cold saline. The hearts were attached by the aorta to a Langendorff apparatus within one minute of excision and were perfused at constant pressure (85 mmHg), temperature (37°C) and pH (7.4) using a modified Krebs-Henseleit (KH) bicarbonate buffer (95% O_2/5%CO_2) containing (in mM): 1.75 $CaCl_2$, 117.4 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.3 KH_2PO_4, 24.7 $NaHCO_3$, 11 glucose, 0.5 pyruvate, 0.5 EDTA, and 1.2 USP units/mL heparin. The hearts were paced at 260 beats/min and a latex balloon filled with water was inserted into the left ventricle to yield an end diastolic pressure (EDP) of 5-6 mmHg. LV function was measured using the Ponemah Physiology platform (Gould Instrument Systems, Valley View, OH). Following perfusion, the LVs were isolated, weighed, halved and snap-frozen in liquid N_2. The LVs were stored at -80°C until homogenization.

Protocol of the Isolated Heart Study

Hearts were perfused as described above for an equilibration period of 30 min. duration (Fig. 4). Control hearts were perfused with KH buffer alone for the entire equilibration period, while drug-treated hearts received either 1) Tat-ψεRACK (500 nM in KH buffer) via the perfusate for the final 10 min of equilibration or 2) Tat-εV1-2 (1 μM in KH buffer) for 10 min immediately followed by co-administration of Tat-εV1-2 (1 μM) and Tat-ψεRACK (500 nM) for the final 10 min of equilibration. For baseline biochemistry and proteomics studies, LVs were isolated, weighed, halved, and snap-frozen in liquid N_2 following the initial 30 min. equilibration period. For functional studies in hearts undergoing ischemia/reperfusion (I/R), global no-flow ischemia of 47 min duration was induced immediately following the initial 30 min. perfusion. Pacing was resumed 1.5 min after the restoration of flow, and hearts were reperfused for either 60 min. (for

biochemistry studies) or 120 min (for infarct size analysis). All isolated LV sections were stored at -80°C until tissue preparation.

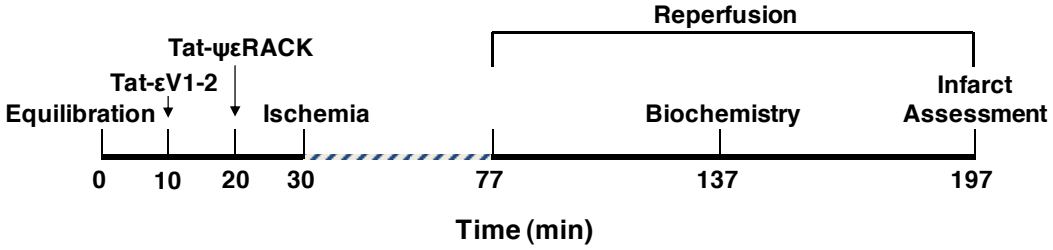

Figure 5: Protocol of the isolated heart studies.

Infarct Size Assessment

A subset of hearts (n=3-5 per group) underwent 47 min. ischemia followed by 120 min. reperfusion, and the area of infarction was assessed as described previously by Downey138. Briefly, after reperfusion LVs were isolated, weighed, and frozen at -20°C for 30 min. LVs were then sliced transversely and stained with 1% triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4) for 20 min at 37°C, followed by overnight incubation in 10% formalin at room temperature. Heart slices were mounted onto glass plates at 1.5 mm thickness, and both sides of each slice were digitally photographed using a camera mounted to a SZX9 microscope (Olympus). Digital images of infarcted LV were analyzed using National Institutes of Health (NIH) ImageJ software.

Tissue Sample Preparation

Frozen LV samples were homogenized using glass-glass grinding in 10 vol buffer A containing (in mM) 250 sucrose; 10 HEPES, pH 7.4; 1 EDTA, pH 7-8; 1 orthovanadate; 1 NaF; 0.3 PMSF; 0.5 μg/mL pepstatin A, and 5 μg/mL of both leupeptin and aprotinin. A portion of the homogenate was subjected to serial centrifugations. The pellet (mitochondrial fraction) was washed in 5 vol buffer A and centrifuged. The pellet was then resuspended in 5 vol buffer B containing (in mM): 150 NaCl; 20 HEPES, pH 7.4; 10 EDTA, pH 7-8; 1 orthovanadate; 1 NaF; 0.3 PMSF; 0.5 μg/mL pepstatin A; 5 μg/mL each of leupeptin and aprotinin; and 1% NP-40. The homogenate was subjected to centrifugation for 10 min. The supernatants was collected and defined as the mitochondrial fraction. Protein concentration was determined by the Bradford method.

iTRAQ Sample Preparation

Mitochondrial fractions of LV protein homogenates were aliquoted, concentrated and resuspended in 20 µL triethylammonium bicarbonate (TEAB) buffer. They were then denatured using 0.1% SDS, reduced with 5 mM tris-(2-carboxyethyl) phosphine (TCEP), and alkylated with 3.5 mM iodoacetamide. 5% Trifluoroethanol was added and samples were digested overnight at 48°C using 10 μg sequencing grade modified trypsin (Promega). Each sample was labeled with its respective iTRAQ reagent and samples were washed and resuspended in SCX buffer (12mM ammonium formate in 25% acetonitrile, pH 2.6). Liquid chromatography and tandem mass spectrometry (MS/MS) were performed at the Hershey Proteomics Core facility.

Western Blotting

Western blotting was performed according to well-established procedures in our laboratory114. Briefly, equal amounts of total, cytosolic, nuclear, or mitochondrial protein sample were electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels (BioRad) and transferred to polyvinylidene difluoride (PVDF) membranes for 3 hr. Following incubation in blocking buffer (6% non-fat milk) for 2 hr, membranes were probed with primary antibody against PKCε (Santa Cruz Biotechnology, 1:1500 for 3 hr at room temp.). Membranes were incubated with anti-rabbit (1:25000) or anti-mouse (1:10000), respectively, horseradish peroxidase (HRP)-linked secondary antibody for 1 hr., and immunoreactive bands were visualized by enhanced chemiluminescence (GE Amersham). Densitometry analysis was performed using Scion Image (NIH).

Minor differences in protein loading were corrected for by SYPRO Ruby staining (Invitrogen), and densitometry values were adjusted as described previously175. All western blot analyses were expressed relative to the adult control group.

iTRAQ Data Analysis

ProteinPilot 3.0 software (ABI-MDS) was used for protein identification and quatitation from MS/MS spectra. Proteins identified at a false discovery rate (FDR) <%5 and unused protein scores of >1.3 were considered positive identifications. Protein Analysis Through Evolutionary Relationships (PANTHER) was also utilized to assign biological functions according to UniProt IDs. This required the conversion of protein GI numbers to UniProt ID numbers using the ID Mapping function of the UniProt beta website (http://beta.uniprot.org).

RNA Isolation

RNA was isolated as previously described by Chomczynski *et al* (12). Briefly, LV samples were homogenized in RNA-Bee (Tel-Test) using a Polytron homogenizer. Samples were phase separated using chloroform and vigorous shaking. The homogenate was centrifuged for 20 min at 4°C. The supernatant was subjected to phenol/chloroform extraction and shaken vigorously for 1 min. Samples were centrifuged again for 20 min. at 4°C. Samples were separated again with chloroform, vigorously shaken (1 min), and were spun for 20 min at 4°C. RNA was isolated from the aqueous phase with isopropanol and shaken for 15 sec. Samples were placed at -20°C for 20 minutes after which they were centrifuged at 4°C for 20 minutes. Pellets were air-dried and dissolved in DEPC-treated ddH_2O. Sodium acetate (3M) and chilled ethanol were added to precipitate the RNA pellet further and samples were stored at -80°C for 25 minutes. Samples were then centrifuged for 20 minutes at 12,000*g* at 4°C. Pellets were briefly air dried and RNA was resuspended in DEPC-treated ddH_2O. RNA concentrations were determined via spectrometer (Beckman-Coulter) using 260/280 ratios.

Real-Time PCR

RNA samples were reverse transcribed (RT) into cDNA. The RT mix (ABI High Capacity RT kit) was immediately added to samples and incubated at 25°C for 10 min and then 37° C for 2 hr. The cDNA was probed for expression of RACK2 and CX43 genes. TaqMan 2X Universal Mix, forward and reverse primers and probe were added to cDNA. Samples were amplified (ABI Model 7300) with the reference gene cyclophilin (CyP), using the following protocol: 50°C for 2 min, 95°C 10 min and 40 repeats of 95°C 15 sec, 60°C 1 min. Forward and reverse primers for each gene of interest were as follows: RACK2, 5'GCAAATTGAAGGAAGCAGAATTG 3',

5'GCTGTGACGATG TTCCAAACA3'; CX43, 5'GCTCCTCACCAACGGCT, 3' TTGCGGCAGGAGGAATTG.

Statistical Analysis

With the exception of proteomics results, all data are presented as means ± standard error and were analyzed using the Statistical Analysis System (SAS) general linear models (GLM) procedure. Baseline morphological and functional data were analyzed by two-way analysis of variance (ANOVA; age x ovary-status). Group comparisons of functional recovery, infarct size, Western blotting data, and mRNA changes were analyzed by three-way ANOVA (age x ovary-status x drug). All *post hoc* comparisons were analyzed using the Tukey-Kramer or Duncan method.

iTRAQ quantitative proteomics data are presented as protein expression ratios and error factors. Statistical analysis of iTRAQ quantitative protein data was automated by the Paragon algorithm of the ProteinPilot v2.0 software package and is based on the contributing peptide ratios. An α-level of $p<0.05$ was defined as statistically significant for all comparisons.

Results

Baseline Morphology and Function

Baseline morphology and LV functional characteristics were compiled for adult and aged, ovary-intact and OVX rats (Table 1). Tabulated LV functional values were recorded during baseline equilibrium perfusion of the isolated heart prior to drug administration and ischemia. Rat body weight (BW) and LV weight were significantly increased by both age and OVX ($p<0.0001$). LV developed pressure (LVDP) and -dP/dt were significantly reduced with aging ($p<0.0001$). No significant differences were observed in LV weight/BW ratio, end diastolic pressure (EDP), +dP/dt, and time to peak pressure development (TTPK).

Table 1: Baseline morphology and functional characteristics. OVX, ovariectomized; LV, left ventricle; BW, body weight; EDP, end diastolic pressure; LVDP, left ventricular developed pressure; dP/dt, first derivative of LVDP curve; TTPK, time to peak pressure development; * effect of age, † effect of OVX; $p<0.0001$.

Characteristic	Adult	Adult OVX	Aged	Aged OVX
N	19	19	17	16
Body weight (g)	183 ± 3	218 ± 4 †	260 ± 7 *	283 ± 10 * †
LV weight (mg)	0.50 ± 0.02	0.61 ± 0.02 †	0.81 ± 0.02 *	0.81 ± 0.02 *
LV weight/BW (mg/g)	2.73 ± 0.01	2.80 ± 0.01	3.12 ± 0.03	2.86 ± 0.01
EDP (mmHg)	5.59 ± 0.03	5.65 ± 0.05	5.61 ± 0.09	5.66 ± 0.07
LVDP (mmHg)	139.7 ± 2.9	145.1 ± 3.2	131.5 ± 2.4 *	133.7 ± 4.1 *
+dP/dt (mmHg/s)	3741 ± 146	3992 ± 51	3664 ± 133	3850 ± 195
-dP/dt (mmHg/s)	2392 ± 52	2596 ± 52	2344 ± 100 *	2353 ± 107 *
TTPK (ms)	70.0 ± 1.6	69.1 ± 0.9	66.8 ± 3.1	66.9 ± 1.8

Recovery from I/R following acute PKCε activation

Acute activation of PKCε by administration of ψεRACK prior to ischemia improved functional recovery in the adult ovary-intact female rat heart, as indicated by improved percentage recovery of baseline LVDP during reperfusion ($p<0.0001$, Fig. 6). Functional recovery following 47 min ischemia was reduced in the aged OVX rat heart, as indicated by reduced percentage recovery of baseline LVDP, reduced absolute recovery of LVDP, and reduced recovery of positive and negative dP/dt compared to adult ovary-intact rat hearts (all $p<0.0001$). These reductions in functional recovery in the aged OVX rat heart were improved by administration of ψεRACK prior to ischemia ($p<0.0001$, Fig.6). Additionally, a greater increase in EDP during reperfusion was observed in aged OVX compared to adult ovary-intact rat hearts, and this increase was attenuated by ψεRACK treatment ($p<0.0001$, Fig.7). Following 47 min. ischemia and 120 min. reperfusion, infarct size was also greater in the aged ovary-intact and aged OVX rat heart compared to the adult ovary-intact rat heart. Infarct size was reduced in all three groups by ψεRACK administration prior to ischemia (Fig. 8).

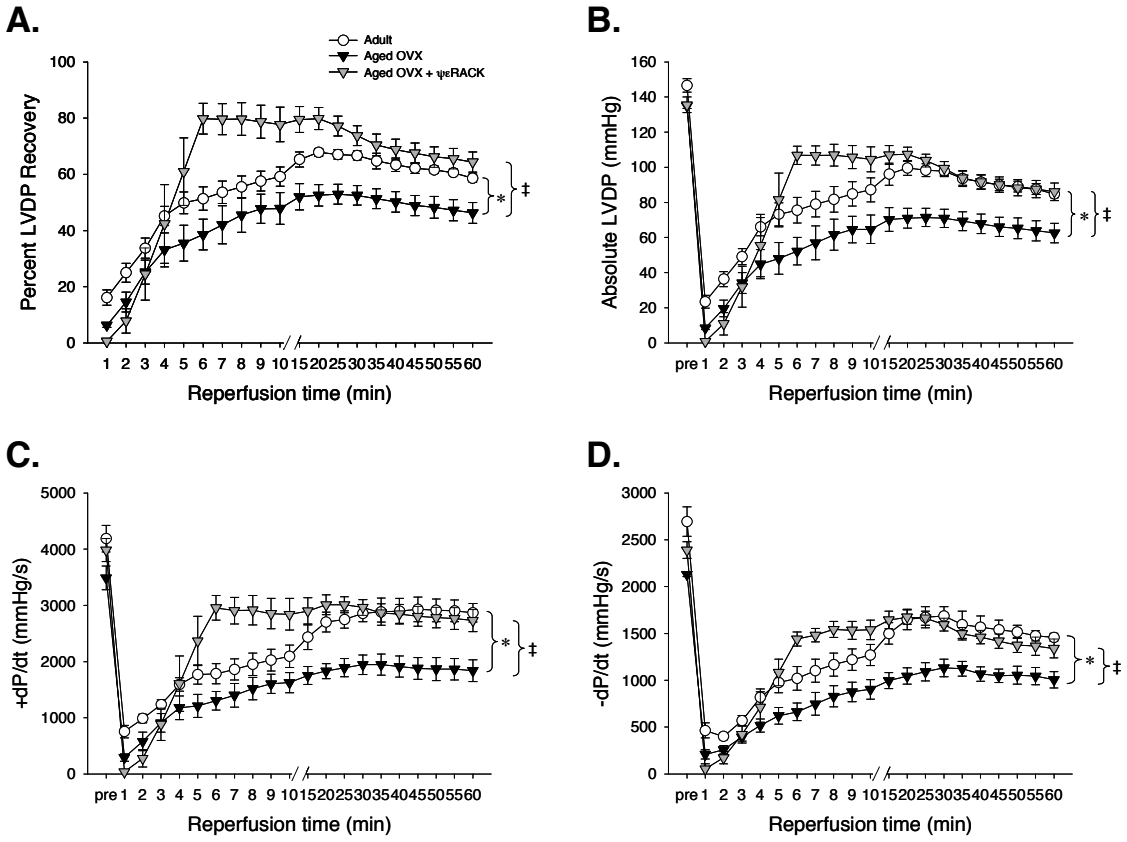

Figure 6: Reduced functional recovery in the aged, E_2-deficient rat heart is reversed by acute PKCε activation. *A)* Percentage recovery of left ventricular developed pressure (LVDP), *B)* absolute recovery of LVDP, *C)* recovery of +dP/dt, and *D)* recovery of -dP/dt were reduced in the aged OVX rat heart following 47 min ischemia, and were improved by ψεRACK administration prior to ischemia. * effect of age + OVX, ‡ effect of drug; $p<0.0001$.

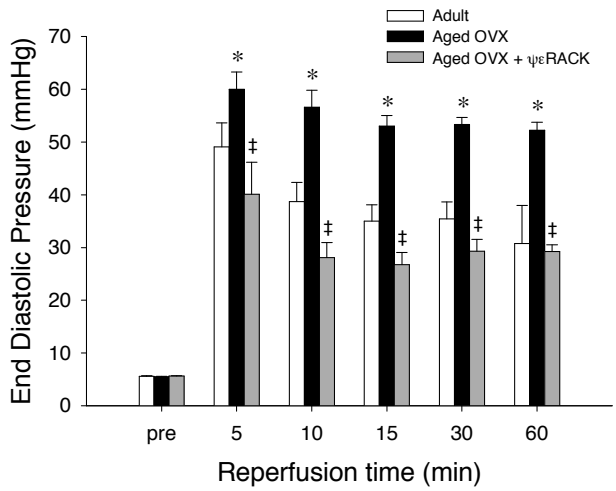

Figure 7: Increased end diastolic pressure (EDP) in the aged, E_2-deficient rat heart is attenuated by acute PKCε activation. The increase in EDP following 47 min. ischemia was greater in the aged OVX rat heart, and this increase was reduced by ψεRACK administration prior to ischemia. * effect of age + OVX, ‡ effect of drug; $p<0.0001$.

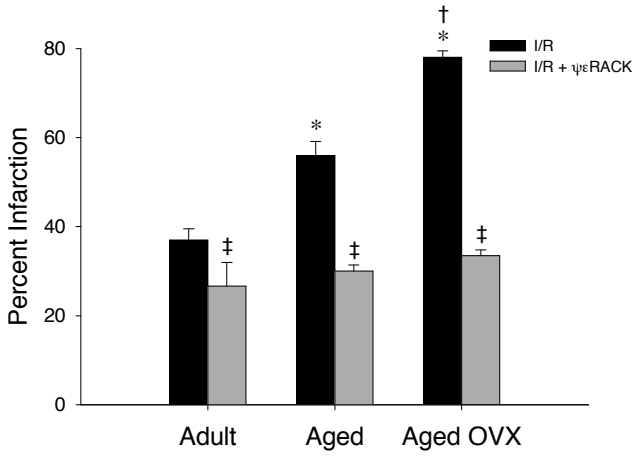

Figure 8: Increased infarct size in the aged, E_2-deficient rat heart is reduced by acute PKCε activation. Following 47 min. ischemia and 120 min. reperfusion, infarct size (expressed as percentage of LV mass infarcted) was greater in the aged and aged OVX rat heart compared to the adult rat heart. Infarct size was reduced in all groups by ψεRACK administration prior to ischemia. I/R, ischemia/reperfusion; * effect of age, † effect of OVX, ‡ effect of drug; $p<0.0001$

Left ventricular PKCε

Western blotting was used to measure relative protein levels of mitochondrial, cytosolic, and total PKCε. All western blotting values are expressed relative to the adult ovary-intact group at baseline (pre-ischemia). At baseline, mitochondrial PKCε demonstrated a tendency to be reduced in adult OVX hearts, and was significantly reduced by ~50% in aged OVX hearts ($p=0.0238$; Fig. 9A). Mitochondrial PKCε was further reduced by ~30% following I/R in aged OVX, and was increased with ψεRACK treatment in both adult ovary-intact and aged OVX following I/R ($p<0.0003$, Fig. 9B). Cytosolic and total PKCε levels were not significantly altered in the experimental groups (data not shown).

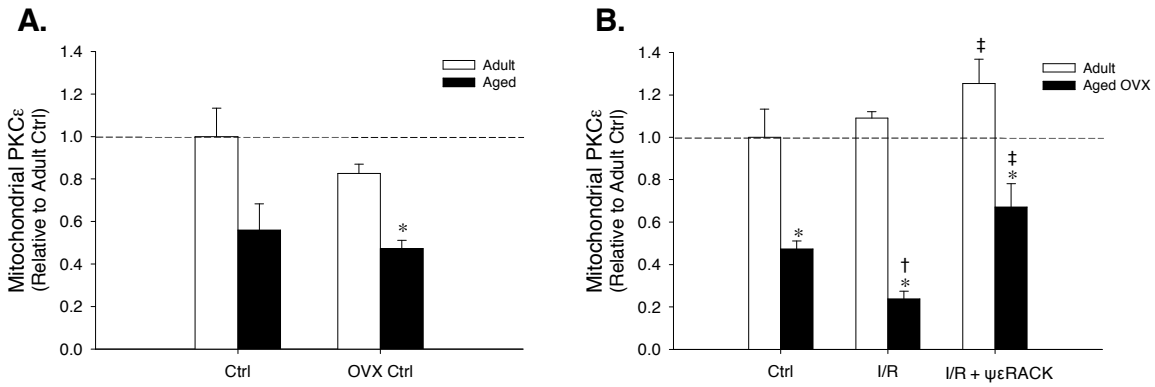

Figure 9: Western blotting demonstrating effects of age, ovariectomy (OVX), and ψεRACK on mitochondrial PKCε in hearts *A)* at baseline (Ctrl) and *B)* following ischemia/reperfusion (I/R). Values are means ± SE (n=3-4/group). * effect of age + OVX, † effect of I/R, ‡ effect of drug; $p<0.03$.

iTRAQ analysis of LV mitochondrial proteins

A preliminary assessment of possible downstream signaling targets of PKCε in the aged, E_2-deficient rat heart was conducted by identifying proteins with altered mitochondrial abundance after administration of the PKCε-activating peptide ψεRACK. In this analysis, proteins of interest were those that differed significantly in aged OVX + ψεRACK relative to aged OVX, and that had functional relevance to I/R injury (Table 2).

Table 2: Possible downstream signaling targets of PKCε. Cardiac mitochondrial proteins demonstrating altered expression following ψεRACK treatment in the aged OVX rat heart were identified from three iTRAQ 8plex analyses and are grouped according to associated biological processes. Bold indicates that the difference in expression was significant relative to aged OVX control.

Unused Score	Total Score	% Coverage	Accession #	Protein Identified	Quantitation Ratio Aged OVX + ψεRACK/Aged OVX	p-Value	Error Factor
17.3	17.3	82.4	gil461731	10 kDa heat shock protein, mitochondrial (Hsp10)	**1.11**	**0.0134**	**1.08**
5.6	5.6	37.6	gil68138297	glutathione peroxidase	**1.21**	**0.0073**	**1.09**
15.3	15.3	50.9	gil8394331	superoxide dismutase 2, mitochondrial (SOD2)	**1.28**	**0.0001**	**1.07**

mRNA Quantitation

RNA isolated from LVs was reverse transcribed into cDNA and subjected to real time PCR using primers for RACK2 and Cx43. RACK2 mRNA decreased significantly with age and ovariectomy. Levels of RACK2 mRNA were significantly increased with administration of ψεRACK in aged I/R aged OVX I/R hearts subjected to I/R (Fig. 10) and was sufficient to return RACK2 mRNA levels to those seen in adult hearts. Cx43 mRNA levels also decreased significantly with age, but no additional decrease in Cx43 levels were seen with ovariectomy (Fig. 11). Administration of ψεRACK increased Cx43 mRNA levels in the aged OVX I/R group, but not in the aged, ovary-intact I/R group.

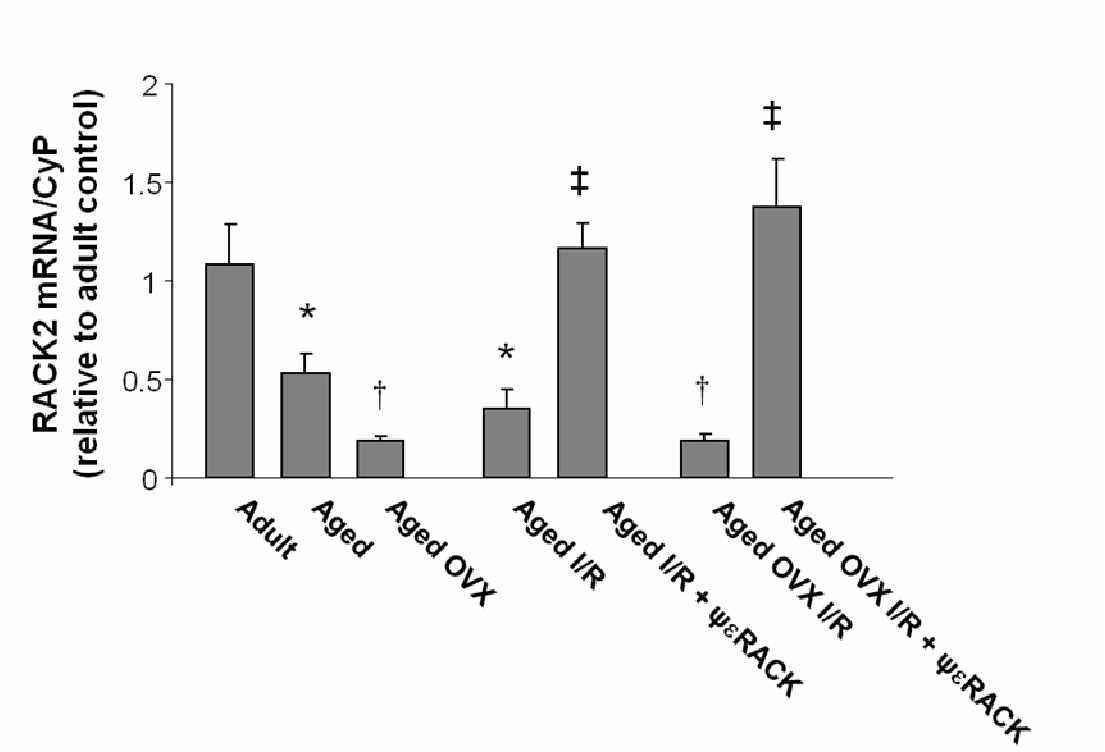

Figure 9: RT-PCR of RACK2 mRNA shows effects of age, ovariectomy (OVX), and ψεRACK on levels of RACK2 mRNA in the left ventricle. Data are mean ± SE,* effect of age, †effect of OVX, ‡ effect of drug; p <0.05.

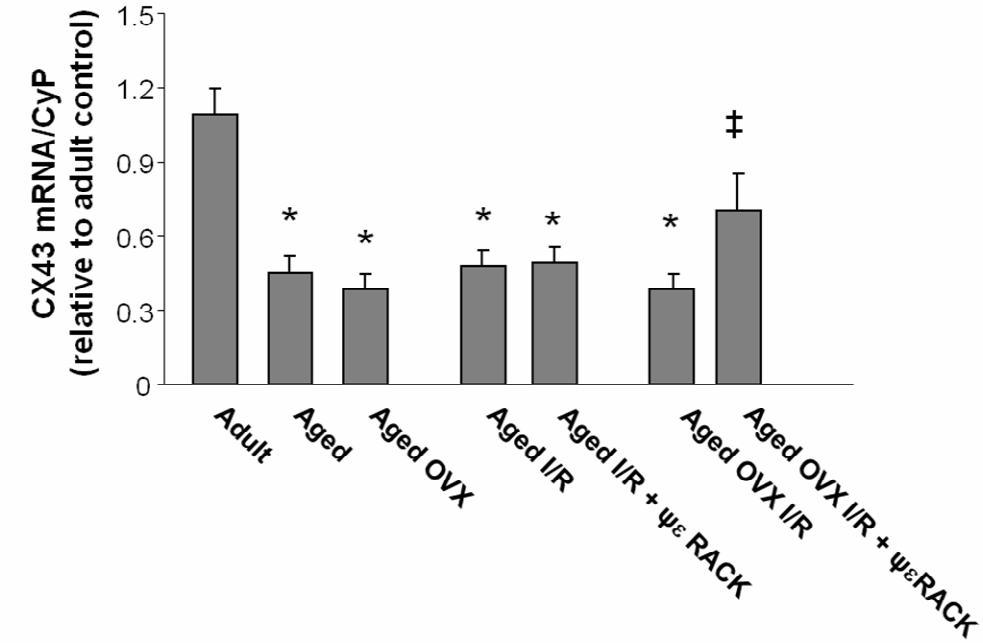

Figure 10: RT-PCR of Cx43 mRNA shows effects of age, ovariectomy (OVX), and ψεRACK on levels of Cx43 mRNA in the left ventricle. . Data are mean ± SE,* effect of age, †effect of OVX, ‡ effect of drug; p <0.05.

Discussion

The incidence and mortality due to acute MI is increased in women after menopause (46), and clinical and experimental evidence implicate the loss of E_2 in the reduced ischemic tolerance of the aged female heart (31). Acute activation of PKCε prior to ischemia is known to mimic IPC and improve ischemic tolerance through mechanisms critically involving the mitochondria (33), however the efficacy of this intervention in the aged, E_2-deficient heart was unknown given observed reductions in mitochondrial PKCε (31) and evidence for the loss of cardioprotection in the aged heart101. PCKε has also been shown to translocate to the nuclear membrane upon activation, suggesting that activated PKCε may increase transcription of genes associated with cardioprotection. A primary aim of the current study was to determine the efficacy of acute PKCε activation in protection of the aged, E_2-deficient rat heart from I/R injury. A second aim of this study was to identify, using a high-throughput quantitative proteomics approach, alterations in mitochondrial proteins contributing to a) reduced ischemic tolerance and b) cardioprotection downstream of acute PKCε activation in the aged, E_2-deficient rat heart observed herein. It was also the aim of this study to determine the effects of acute PKCε activation on nuclear mechanisms implicated in the loss of cardioprotection seen with aging, including RACK2 and Cx43 gene transcripts.

In support of our hypotheses, key findings of the current study are as follows: 1) acute activation of PKCε prior to ischemia by local delivery of ψεRACK peptide improved functional recovery and reduced infarct size after I/R in the aged, E_2-deficient rat heart, 2) improved ischemic tolerance resulting from acute PKCε activation by ψεRACK was associated with increased mitochondrial targeting of PKCε following I/R, 3) identification of candidate downstream PKCε signaling targets suggests a role for the regulation of oxidative stress through

the activation of antioxidant enzymes as a mechanism of PKCε-mediated cardioprotection in the aged female heart and 4) increased mRNA levels of proteins involved in PKCε-mediated cardioprotection with acute PKCε activation by ψεRACK following I/R suggest a role for PKCε in regulation of cardioprotective gene transcription.

Small alterations in baseline morphological data were seen with aging and E_2-deficiency in female rats. These included increased body weight and LV weight with both age and ovariectomy (Table 1). These findings are consistent with previous reports of increased adiposity in ovariectomized rats (41). Reductions in LVDP and -dP/dt were also observed at baseline in aged hearts, which is consistent with age-associated reductions in myocardial contractility and relaxation118. Reduced functional recovery (Fig. 6 and 7) and increased infarct size (Fig. 8) were both observed in the aged, E_2-deficient heart following I/R. This decrease in ischemic tolerance was associated with reductions in mitochondrial PKCε at baseline in aged and aged OVX hearts (Fig. 9A) and following I/R in aged OVX hearts. This could indicate that PKCε is degraded by the proteosome during I/R stress, compounding the basal reductions in cardioprotective reserves of the aged heart(8, 45).

Acute activation of PKCε by local delivery of ψεRACK peptide prior to ischemia mimicked IPC and reduced I/R injury in the adult heart, as indicated by improved recovery of LVDP during reperfusion (Fig. 6A). PKCε activation by ψεRACK administration was sufficient to produce cardioprotection in the aged, E_2-deficient heart, restoring functional recovery and reducing infarct size to similar levels as those seen in adult control hearts. The protection afforded by ψεRACK was associated with increased mitochondrial targeting of PKCε following I/R in both adult and aged OVX hearts. No differences were observed in cytosolic or total PKCε expression in the experimental groups (data not shown).

A search for possible mitochondrial signaling partners of PKCε involved in cardioprotection was conducted using aged, E_2-deficient female rat hearts perfused with the PKCε-activating peptide ψεRACK followed by iTRAQ 8-plex analysis. A significant increase in mitochondrial heat shock protein 10 (Hsp10), glutathione peroxidase (GPX), and SOD2 abundance was observed following ψεRACK administration in aged OVX hearts (by ~10, 20, and 30%, respectively; Table 2). Since these hearts were perfused with ψεRACK for only 10 min., altered abundance of these proteins is most likely due to mitochondrial translocation or import rather than alterations in transcription or translation, since rapid changes in protein expression due to transcription and translation have only been noted in a small subset of proteins with very short half-lives. Most mitochondrial proteins have half-lives in the range of 20-100 hours(27).

Hsp10 is a chaperone protein that, alone or in complex with Hsp60, has been shown to protect against ischemic injury. These heat shock proteins also form a complex that regulates activation of mitochondrial pro-caspase-3 and thus initiation of apoptosis191. SOD2 and GPX are antioxidant enzymes involved in the reduction and subsequent removal of O_2^- and H_2O_2 respectively. Some evidence already points to a role for GPX and SOD2 in I/R injury. Small increases in Ca^{2+} concentration, such as those seen during brief hypoxia and reoxygenation (as in IPC) have been shown to increase activity of GPX and SOD2 in rat liver mitochondria198. IPC has been demonstrated to increase abundance of mitochondrial SOD2 in male rat hearts with reduced cytochrome c release upon I/R (36). The SOD2-mimetic, HO-3538, has also been shown to inhibit the MPTP and protect the ischemic heart against apoptotic and necrotic cell death in Langendorff-perfused male rat hearts. Also, the SOD-mimetic HO-3538 was cardioprotective in isolated male rat hearts and inhibited MPTP opening and the release of pro-apoptotic proteins30,

while the SOD-mimetic EUK-8 was cardioprotective in isolated hearts from adult ovariectomized female rats (7). Perhaps of greatest interest is the report of cardioprotection and increased SOD activity following pharmacological preconditioning with the adenosine A_1 receptor agonist CCPA in isolated rat hearts(28), as PKCε is known to be activated in cardioprotection downstream of the adenosine receptor(18). An increase in SOD2 activity in response to signaling through the adenosine receptor fits with the observed increase in SOD2 abundance following acute PKCε activation observed in this study. The above findings suggest that signaling through the adenosine receptor activates PKCε, which in turn causes an increase in SOD2 abundance.

Analysis of mRNA levels of two proteins implicated in PKCε-mediated cardioprotection was conducted to determine the effects of age, E_2-deficiency and ψεRACK on transcription of these proteins. The decrease in mRNA for the receptor for activated PKCε, RACK2, observed with both age and ovariectomy corresponds with decreased functional recovery and increased infarct size observed herein. Levels of nuclear PKCε have been found to decrease with age (31), indicating a possible role for PKCε in regulating transcription of RACK2. We observed a significant increase in RACK2 mRNA levels with administration of ψεRACK prior to I/R in both aged and aged OVX animals, returning RACK2 mRNA to at least the level seen in adult hearts. This finding provides further evidence for a regulatory role of PKCε in transcription of RACK2, suggesting that PKCε activation contributes to a positive feedback mechanism in which activation of PKCε causes an increased abundance of RACK2, thus amplifying the cardioprotective effects of PKCε. Connexin 43 mRNA levels were shown to decrease with age, but no additional decrease was observed with ovariectomy. This finding is consistent with previous studies that show a decrease in Cx43 protein abundance in the ventricle with aging (6).

Interestingly, we observed an increase in Cx43 mRNA levels with administration of ψεRACK prior to I/R in the aged OVX hearts, but not in the aged ovary-intact group. It is possible that regulation of Cx43 transcription differs in the presence and absence of E_2, just as a recent study showed that dephosphorylation of Cx43 decreases in response to E_2 replacement(71). In fact, Schulz *et al (62)* suggest that preservation of Cx43 phosphorylation following I/R may enhance its association with kinases such as PKC, suggesting that the phosphorylation status of Cx43 could dictate interactions with PKC and perhaps PKC-mediated gene regulation. In the absence of E_2, however, PKCε may exert its cardioprotective effects by increasing transcription of Cx43.

Conclusions

1. Acute activation of PKCε prior to ischemia by local delivery of ψεRACK improved functional recovery and reduced infarct size after I/R in the aged, E_2-deficient rat heart. While decreased functional recovery and increased infarct size were observed in aged, E_2-deficient animals without ψεRACK when compared with adult controls, PKCε activation by ψεRACK administration was sufficient to produce cardioprotection in the aged, E_2-deficient heart, restoring functional recovery and reducing infarct size to similar levels as those seen in adult control hearts.
2. Improved ischemic tolerance resulting from acute PKCε activation by ψεRACK was associated with increased mitochondrial targeting of PKCε following I/R. While mitochondrial expression of PKCε was increased following treatment with ψεRACK, no differences were observed in cytosolic or total PKCε expression.
3. Identification of candidate downstream PKCε signaling targets suggests a role for the regulation of oxidative stress through the activation of antioxidant enzymes as a mechanism of PKCε-mediated cardioprotection in the aged female heart. Antioxidant

enzymes GPX and SOD2, as well as mitochondrial Hsp10, showed increased abundance in the mitochondria following with ψεRACK administration in aged OVX hearts.

4. Increased levels of RACK2 and Cx43 mRNA in response to acute PKCε activation by ψεRACK prior to I/R suggest a role for PKCε in regulation of cardioprotective gene transcription. Interestingly, PKCε activation only increased levels of Cx43 mRNA in aged OVX hearts whereas RACK2 mRNA was increased in both aged ovary-intact and aged OVX hearts.

Limitations of the Study

One limitation of the study is the use of a global ischemia model of I/R. Although this model has provided many clinically relevant findings, a local model of ischemia could more closely mimic the physiology of a typical myocardial infarction. Using an *in vivo* model of local ischemia, such as coronary artery ligation, could further confirm the effects of ψεRACK on infarct size in the aged, E_2-deficient rat heart. Another possible limitation of the study can be seen in the iTRAQ analysis of mitochondrial proteins. If the mitochondrial fraction contained any contaminants from other fractions, then the altered levels of SOD2, Hsp10 and GPX would not be specific to the mitochondria.

Future Directions

1. Western blotting could be used to confirm the altered levels of SOD2, Hsp10 and GPX seen in the iTRAQ analysis.

2. PKCε could be co-immunoprecipitated with binding partners in the nucleus and mitochondria followed by proteomic analysis to further characterize PKCε-mediated signaling.
3. Amount of phosphorylated Cx43 could be measured by Western blot following ψεRACK administration and I/R in the aged, E_2-deficient heart to determine whether activation of PKCε increases the amount of active Cx43. This analysis could also compare ovary-intact to ovariectomized animals to determine whether mitochondrial levels of Cx43 differ with E_2 status.

Appendix A

Detailed Methods

Animal Care and Preparation

Adult (5 mo.) and aged (23 mo.) female Fisher 344 (F344) rats were supplied by Harlan Sprague Dawley (Indianapolis, IN) and Taconic (Hudson, NY). Use of the F344 strain was decided upon as a result of this strain's general acceptance as a model of cardiovascular aging123. Rats were housed under a 12:12 hr. light:dark cycle and received food and water *ad libitum*. A total of 52 animals were used to complete the functional assessment arm of this study (Specific Aim 1; *n*=28 adult and *n*=24 aged). An additional 19 animals were used to complete the proteomics arm of the study (Specific Aim 2, *n*=10 adult and *n*=9 aged). All animal handling and utilization protocols were reviewed and approved by the Penn State University Institutional Animal Care and Use Committee.

To create a model of E_2 deficiency, bilateral ovariectomy (OVX) surgeries were performed by the supplier on both adult and aged rats according to standard procedures. Access to the abdominal cavity of anaesthetized animals was attained by dorsal incision, and each ovary and part of the oviduct was removed following ligation of the oviduct. Animals were allowed to recover for 4-6 weeks prior to the date of experimental study. Successful surgery was confirmed at the time of sacrifice by measuring uterine weight, which was used as an indication of circulating estradiol concentrations. Ovary-intact animals were used as age-matched controls for comparison to the surgery groups.

PKCε Regulatory Peptides

An isozyme-specific protein kinase Cε (PKCε) activator peptide [pseudo ε-receptor for activated C kinase (ψεRACK; C-HDAPIGYD)] derived from the PKCε pseudo-RACK sequence (PKCε amino acids 85 to 92) conjugated reversibly to a Tat-derived carrier peptide [Tat amino acids 43–58 (C-YGRKKKRRQRRR)] by disulfide bond at the N terminus was provided by KAI Pharmaceuticals (San Francisco, CA). ψεRACK promotes binding of PKCε to its specific RACK adaptor protein, RACK2, thus promoting PKCε translocation and activation.

A Tat-conjugated isozyme-specific PKCε inhibitory peptide (εV1-2; C-EAVSLKPT) derived from the PKCε RACK-binding sequence (PKCε amino acids 14-21) was also provided by KAI Pharmaceuticals. εV1-2 binds to the PKCε binding site on RACK2, competitively inhibiting PKCε binding and thereby preventing translocation and activation.

Isolated Heart Preparation

In vitro assessment of left ventricular (LV) function was performed using a modified Langendorff isovolumic heart preparation115. Animals were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg body wt.), and hearts were rapidly excised by midline thoracotomy and immediately rinsed in ice cold (4°C) saline. Within 60 sec. of excision, hearts were secured via aortic cannulation to a Langendorff apparatus and perfused at constant pressure (85 mmHg), temperature (37°C), and pH (7.4) with a modified Krebs-Henseleit (KH) bicarbonate buffer (constantly gassed with 95%O_2/5%CO_2) containing (in mM): 1.75 $CaCl_2$, 117.4 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.3 KH_2PO_4, 24.7 $NaHCO_3$, 11 glucose, 0.5 pyruvate, 0.5 EDTA, and 1.2 USP units/ml heparin. Hearts were paced at 260 beats/min with a pulsatile

electric stimulus delivered via a platinum wire placed on the right ventricle, and a fluid-filled latex balloon in line with a pressure transducer (BD Medical Systems) was inserted into the LV to allow measurement of LV functional parameters. Balloon volume was adjusted to yield an LV end diastolic pressure (LVEDP) of 5–6 mmHg. Functional data [LV developed pressure (LVDP; calculated as LV systolic pressure - LVEDP), LVEDP, and positive and negative change in pressure over time (+dP/d*t* and -dP/d*t*)] were assessed beat to beat with the Ponemah Physiology platform (Gould Instrument Systems, Valley View, OH).

Protocol of the Isolated Heart Study

Hearts were perfused as described above for an equilibration period of 30 min. duration. Control hearts were perfused with KH buffer alone for the entire equilibration period, while drug-treated hearts received either 1) ψεRACK peptide conjugated to Tat (Tat-ψεRACK, 500 nM in KH buffer) via the perfusate for the final 10 min. of equilibration or 2) εV1-2 peptide conjugated to Tat (Tat-εV1-2, 1 μM in KH buffer) for 10 min. immediately followed by co-administration of Tat-εV1-2 (1 μM) and Tat-ψεRACK (500 nM) for the final 10 min. of equilibration. For baseline biochemistry and proteomics studies, LVs were isolated, weighed, halved, and snap-frozen in liquid N_2 following the initial 30 min. equilibration period. For functional studies in hearts undergoing ischemia/reperfusion (I/R), global no-flow ischemia of 47 min. duration was induced immediately following the initial 30 min. perfusion by clamping of the perfusion line and cessation of external pacing. Isothermic conditions (37°C) were maintained during ischemia by containment of the heart in a jacketed glass cup. Pacing was resumed 1.5 min. after the restoration of flow, and hearts were reperfused for either 60 min. (for biochemistry studies) or 120 min. (for infarct size analysis). LVs for biochemistry studies were isolated and snap-frozen

as above following 60 min. reperfusion, and all isolated LV sections were stored at -80°C until tissue preparation. Administration of the PKCε regulatory peptides prior to global ischemia is based on previous studies in which Tat-ψεRACK was shown to be cardioprotective and to readily distribute throughout cardiac tissue with this exposure duration and concentration23,90(4, 34).

Infarct Size Assessment

A subset of hearts (n=3-5 per group) underwent 47 min. ischemia followed by 120 min. reperfusion, and the area of infarction was assessed as described previously by Downey138. Briefly, after reperfusion LVs were isolated, weighed, and frozen at -20°C for 30 min. LVs were then sliced transversely and stained with 1% triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4) for 20 min at 37°C, followed by overnight incubation in 10% formalin at room temperature. Heart slices were mounted onto glass plates at 1.5 mm thickness, and both sides of each slice were digitally photographed using a camera mounted to a SZX9 microscope (Olympus). Digital images of infarcted LV were analyzed using National Institutes of Health (NIH) ImageJ software.

Tissue Sample Preparation

For western blotting, total LV protein homogenates as well as isolated cytosolic, particulate, nuclear, and mitochondrial subcellular protein fractions were prepared as follows85: frozen LV samples were homogenized by glass-glass grinding in 10 vol of buffer A containing (in mM) 250 sucrose; 10 Tris-HCl, pH 7.4; 1 EDTA, pH 7–8; 1 orthovanadate; 1 NaF; 0.3 PMSF; 0.5 μg/ml

pepstatin A, and 5 μg/ml each of leupeptin and aprotinin. The resulting homogenate was divided and one portion was, after addition of Triton X-100 (to 1% concentration by volume) and 30 min. agitation on ice, subjected to 100,000 x *g* centrifugation at 4°C. The supernatant was taken as the total homogenate sample. The second portion of the original homogenate was subjected to serial centrifugations of 1,000 x *g*, 10,000 x *g*, and 100,000 x *g*. The 1,000 x *g* pellet (nuclear fraction) and the 10,000 x *g* pellet (mitochondrial fraction) were washed in 5 vol buffer A and recentrifuged at 10,000 x *g*. The resulting pellets were resuspended in 5 vol buffer B containing (in mM) 150 NaCl; 20 Tris-HCl, pH 7.4, 10 EDTA, pH 7–8, 1 orthovanadate; 1 NaF; 0.3 PMSF, 0.5 μg/ml pepstatin A, 5 μg/ml each of leupeptin and aprotinin, and 1% NP-40, and subjected to 21,000 x *g* centrifugation for 10 min. The resulting supernatants were defined as the nuclear and mitochondrial fractions. The 100,000 x *g* supernatant was defined as the cytosolic fraction, while the pellet was resuspended in 5 vol buffer B and defined as the particulate fraction. All protein concentrations were determined by the method of Bradford34.

For proteomics studies, isolated mitochondrial protein homogenates were prepared exactly as described above, except that 10 and 20 mM Tris-HCl (pH 7.4) in buffers A and B, respectively, was replaced with equivalent concentrations of HEPES (pH 7.4). This substitution was required because iTRAQ labeling reagents are incompatible with sample buffers containing free amines such as Tris. Peptide labeling with the iTRAQ isobaric tags is achieved via the formation of an amide bond between the peptide reactive group of the iTRAQ tag and amine groups of the target peptides (namely the NH_2 terminus and lysine side chains). The presence of free amines in the sample buffer could therefore result in the competitive binding of the iTRAQ tags, thereby reducing peptide-labeling efficiency and consistency. Qualitative **mu**lti-**d**imensional **p**rotein **i**dentification **t**echnology (MudPIT) proteomics comparisons of the protein

isolation protocols were performed to ascertain that this substitution did not alter the distribution or number of protein identifications obtained.

Western Blotting

Assessment of myocardial protein levels in LV subcellular fractions was performed by western blotting according to well-established procedures in our laboratory(44). Equal amounts of total, cytosolic, nuclear, or mitochondrial protein sample were electrophoresed on precast sodium dodecyl sulfate (SDS)-polyacrylamide gels (BioRad) and transferred to polyvinylidene difluoride (PVDF) membranes for 3 hr. Following incubation in blocking buffer (6% non-fat dry milk in 0.1% TBS-Tween20) for 2 hr., membranes were probed with primary antibody against PKCε (Santa Cruz Biotechnology, 1:1500 for 3 hr. at room temp). After washing three x 10 min. in 0.1% TBS-Tween20, membranes were incubated with anti-rabbit (1:25000) or anti-mouse (1:10000), respectively, horseradish peroxidase (HRP)-linked secondary antibody for 1 hr., and then washed again. Protein expression was visualized by enhanced chemiluminescence (ECL; GE Amersham) and quantified by densitometric analysis using Scion Image (NIH). Additional candidate proteins identified through proteomics analyses will be confirmed by western blotting in the future.

Following western blotting and analysis, minor differences in protein loading across lanes were corrected for by SYPRO Ruby staining. Membranes were air-dried and incubated for 15 minutes in SYPRO Ruby blot stain (Invitrogen), and were then washed three x 1 min. in ddH_2O and again allowed to air-dry. The fluorescence was visualized and imaged using a Fluor-S Multi-Imager (BioRad), and a nonspecific band around 75 kDa was selected for densitometric analysis using Scion Image (NIH). The average density of this band was calculated across all

lanes, and a correction ratio was calculated for each lane by dividing the density of the nonspecific band in that lane by the average density of the nonspecific band. The density of the band of interest in each lane was then corrected by multiplying by the correction ratio for that lane.

iTRAQ Sample Preparation

LV mitochondrial protein homogenates were prepared for proteomics analysis using the **i**sobaric **t**ags for **r**elative and **a**bsolute **q**uantitation (iTRAQ) 8plex reagent kit (Applied Biosystems) according to modified protocols of the Penn State Proteomics/Mass Spectrometry Core facility (Dr. Bruce Stanley, Director)212. These sample preparation protocols were optimized in a series of preliminary, qualitative MudPIT proteomics experiments. Minimal cytosolic contamination of the isolated mitochondrial fraction has been previously demonstrated for the protein homogenization techniques employed here17,85(3, 30), however known limitations of the current study design remain.

Three individual iTRAQ 8plex analyses were conducted and samples were prepared and analyzed as follows: from each of up to eight mitochondrial samples, 100 µg of protein was aliquoted and concentrated using a centrifugal vacuum concentrator (SpeedVac) to a remaining volume of about 10-20 µl. Each sample was then resuspended in 20 µl 0.5 M triethylammonium bicarbonate (TEAB) buffer. Proteins were denatured in 0.1% SDS (by adding 1 µl of 2% SDS stock), and disulfide bonds were reduced by incubation in 5 mM tris-(2-carboxyethyl) phosphine (TCEP) for 60 min. at 60°C (by adding 2 µl of 50 mM TCEP stock). Cysteine residues were reversibly blocked by alkylation with 3.5 mM iodoacetamide for 30 min. at room temperature (by adding 1 µl of freshly prepared 84 mM iodoacetamide stock). Trifluoroethanol (TFE) was

then added to 5% concentration by volume. Vials of sequencing grade modified trypsin (Promega) were resuspended at ~1 µg/µl in 21 µl reconstitution buffer, and 10 µl solution (~10 µg trypsin) was added to each sample (protein:trypsin ratio = 10:1). Trypsin digestion was carried out overnight (12-16 hr.) by incubation at 48°C.

Following trypsin digestion, the samples were concentrated to a volume of ~25 µl by SpeedVac. The eight vials of iTRAQ reagents (113-119 and 121 *m/z*) were brought to room temp., and each reagent was reconstituted with 50 µl isopropanol. After verifying that the iTRAQ reagent pH was between 7.8 and 8.5 by aliquoting 0.5 µl reagent solution onto a pH strip, the contents of each iTRAQ reagent vial were transferred to its corresponding sample tube. Following incubation for 2 hr. at room temp., the iTRAQ labeling reaction was quenched by addition of 100 µl Nano-Pure ddH_2O to each sample tube and incubation for 30 min. The combined sample was then washed three times with Nano-Pure ddH_2O, being dried completely by SpeedVac between each wash. After the final drying, the sample was resuspended in 500 µl strong cation exchange (SCX) loading buffer (12 mM ammonium formate in 25% acetonitrile, pH 2.5-3.0), and it was verified that the sample pH was between 2.5 and 3.3 by aliquoting 0.5 µl sample onto a pH strip. The sample was stored at -80°C until being shipped to the Hershey Proteomics Core facility for two-dimensional liquid chromatography (2D LC) separation and tandem mass spectrometry (MS/MS). Specifically, LC/MS/MS analysis utilized SCX LC separation (separation by charge), 15X nanoflow C18 reversed-phase LC separation (separation by hydrophobicity), matrix-assisted laser desorption/ionization (MALDI) plate spotting, and tandem time-of-flight (TOF/TOF) mass analysis.

iTRAQ Data Analysis

Protein identifications (IDs) and protein quantitation were determined from MS/MS spectra using the Paragon algorithm202 of the ProteinPilot v2.0 software package (ABI-MDSSciex), searching the NCBInr (National Center for Biotechnology Information) protein database concatenated to a reversed version of itself (as a Decoy database). Only those proteins identified at an instantaneous (local) false discovery rate (FDR) < 5%, which was determined by analyzing the number of Decoy (reversed) database hits using the PSPEP (**P**roteomics **S**ystem **P**erformance **E**valuation **P**ipeline) beta software tool (ABI-MDSSciex), were considered positive identifications(21). Additionally, PANTHER (**P**rotein **An**alysis **Th**rough **E**volutionary **R**elationships) analysis(68) was conducted to classify identified proteins by linking their UniProt IDs to specific biological processes (using the Batch ID Search function of the PANTHER web application; http://www.pantherdb.org/batchIdSearch.jsp). PANTHER analysis first required the conversion of protein GI numbers (the NCBI database identifiers provided in the ProteinPilot data output) to UniProt ID numbers, which was accomplished using the ID Mapping function of the UniProt beta web application (http://beta.uniprot.org). Although these techniques are designed to give an unbiased examination of potential changes in all identified proteins, rather than targeting known proteins of interest, candidate proteins having established links to I/R injury and demonstrating significant differences between experimental groups were of particular interest.

Statistical Analysis

With the exception of proteomics results, all data are presented as means ± standard error and were analyzed using the Statistical Analysis System (SAS) general linear models (GLM) procedure. Baseline morphological and functional data were analyzed by two-way analysis of variance (ANOVA; age x ovary-status). Group comparisons of functional recovery, infarct size, mRNA analysis, and Western blotting data were analyzed by three-way ANOVA (age x ovary-status x drug). All *post hoc* comparisons were analyzed using the Tukey-Kramer or Duncan method.

iTRAQ quantitative proteomics data are presented as protein expression ratios and error factors. Statistical analysis of iTRAQ quantitative protein data was automated by the Paragon algorithm of the ProteinPilot v3.0 software package and is based on the contributing peptide ratios. An α-level of $p<0.05$ was defined as statistically significant for all comparisons.

References

1. **Abete P, Ferrara N, Cioppa A, Ferrara P, Bianco S, Calabrese C, Cacciatore F, Longobardi G, and Rengo F.** Preconditioning does not prevent postischemic dysfunction in aging heart. *J Am Coll Cardiol* 27: 1777-1786, 1996.
2. **Armstrong S, Downey JM, and Ganote CE.** Preconditioning of isolated rabbit cardiomyocytes: induction by metabolic stress and blockade by the adenosine antagonist SPT and calphostin C, a protein kinase C inhibitor. *Cardiovasc Res* 28: 72-77, 1994.
3. **Baines CP, Zhang J, Wang GW, Zheng YT, Xiu JX, Cardwell EM, Bolli R, and Ping P.** Mitochondrial PKCepsilon and MAPK form signaling modules in the murine heart: enhanced mitochondrial PKCepsilon-MAPK interactions and differential MAPK activation in PKCepsilon-induced cardioprotection. *Circ Res* 90: 390-397, 2002.
4. **Begley R, Liron T, Baryza J, and Mochly-Rosen D.** Biodistribution of intracellularly acting peptides conjugated reversibly to Tat. *Biochem Biophys Res Commun* 318: 949-954, 2004.
5. **Boehm AM, Putz S, Altenhofer D, Sickmann A, and Falk M.** Precise protein quantification based on peptide quantification using iTRAQ. *BMC Bioinformatics* 8: 214, 2007.
6. **Boengler K, Konietzka I, Buechert A, Heinen Y, Garcia-Dorado D, Heusch G, and Schulz R.** Loss of ischemic preconditioning's cardioprotection in aged mouse hearts is associated with reduced gap junctional and mitochondrial levels of connexin 43. *Am J Physiol Heart Circ Physiol* 292: H1764-1769, 2007.
7. **Bognar Z, Kalai T, Palfi A, Hanto K, Bognar B, Mark L, Szabo Z, Tapodi A, Radnai B, Sarszegi Z, Szanto A, Gallyas F, Jr., Hideg K, Sumegi B, and Varbiro G.** A novel SOD-mimetic permeability transition inhibitor agent protects ischemic heart by inhibiting both apoptotic and necrotic cell death. *Free Radic Biol Med* 41: 835-848, 2006.
8. **Budas GR, Churchill EN, Disatnik MH, Sun L, and Mochly-Rosen D.** Mitochondrial import of PKC{varepsilon} is mediated by HSP90: a role in cardioprotection from ischaemia and reperfusion injury. *Cardiovasc Res*, 2010.
9. **Budas GR, Disatnik MH, and Mochly-Rosen D.** Aldehyde dehydrogenase 2 in cardiac protection: a new therapeutic target? *Trends Cardiovasc Med* 19: 158-164, 2009.
10. **Chen CH, Budas GR, Churchill EN, Disatnik MH, Hurley TD, and Mochly-Rosen D.** Activation of aldehyde dehydrogenase-2 reduces ischemic damage to the heart. *Science* 321: 1493-1495, 2008.
11. **Chen L, Hahn H, Wu G, Chen CH, Liron T, Schechtman D, Cavallaro G, Banci L, Guo Y, Bolli R, Dorn GW, 2nd, and Mochly-Rosen D.** Opposing cardioprotective actions and parallel hypertrophic effects of delta PKC and epsilon PKC. *Proc Natl Acad Sci U S A* 98: 11114-11119, 2001.
12. **Chomczynski P and Sacchi N.** Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156-159, 1987.
13. **Churchill E, Budas G, Vallentin A, Koyanagi T, and Mochly-Rosen D.** PKC isozymes in chronic cardiac disease: possible therapeutic targets? *Annu Rev Pharmacol Toxicol* 48: 569-599, 2008.
14. **Cohen MV, Baines CP, and Downey JM.** Ischemic preconditioning: from adenosine receptor to KATP channel. *Annu Rev Physiol* 62: 79-109, 2000.
15. **Coussens L, Parker PJ, Rhee L, Yang-Feng TL, Chen E, Waterfield MD, Francke U, and Ullrich A.** Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signaling pathways. *Science* 233: 859-866, 1986.

16. **Csukai M, Chen CH, De Matteis MA, and Mochly-Rosen D.** The coatomer protein beta'-COP, a selective binding protein (RACK) for protein kinase Cepsilon. *J Biol Chem* 272: 29200-29206, 1997.
17. **Dorn GW, 2nd, Souroujon MC, Liron T, Chen CH, Gray MO, Zhou HZ, Csukai M, Wu G, Lorenz JN, and Mochly-Rosen D.** Sustained in vivo cardiac protection by a rationally designed peptide that causes epsilon protein kinase C translocation. *Proc Natl Acad Sci U S A* 96: 12798-12803, 1999.
18. **Downey JM, Davis AM, and Cohen MV.** Signaling pathways in ischemic preconditioning. *Heart Fail Rev* 12: 181-188, 2007.
19. **Edmondson RD, Vondriska TM, Biederman KJ, Zhang J, Jones RC, Zheng Y, Allen DL, Xiu JX, Cardwell EM, Pisano MR, and Ping P.** Protein kinase C epsilon signaling complexes include metabolism- and transcription/translation-related proteins: complimentary separation techniques with LC/MS/MS. *Mol Cell Proteomics* 1: 421-433, 2002.
20. **Eefting F, Rensing B, Wigman J, Pannekoek WJ, Liu WM, Cramer MJ, Lips DJ, and Doevendans PA.** Role of apoptosis in reperfusion injury. *Cardiovasc Res* 61: 414-426, 2004.
21. **Elias JE and Gygi SP.** Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nat Methods* 4: 207-214, 2007.
22. **Garg V and Hu K.** Protein kinase C isoform-dependent modulation of ATP-sensitive K+ channels in mitochondrial inner membrane. *Am J Physiol Heart Circ Physiol* 293: H322-332, 2007.
23. **Gray MO, Karliner JS, and Mochly-Rosen D.** A selective epsilon-protein kinase C antagonist inhibits protection of cardiac myocytes from hypoxia-induced cell death. *J Biol Chem* 272: 30945-30951, 1997.
24. **Grodstein F, Manson JE, Colditz GA, Willett WC, Speizer FE, and Stampfer MJ.** A prospective, observational study of postmenopausal hormone therapy and primary prevention of cardiovascular disease. *Ann Intern Med* 133: 933-941, 2000.
25. **Gustafsson AB, Sayen MR, Williams SD, Crow MT, and Gottlieb RA.** TAT protein transduction into isolated perfused hearts: TAT-apoptosis repressor with caspase recruitment domain is cardioprotective. *Circulation* 106: 735-739, 2002.
26. **Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, and Aebersold R.** Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* 17: 994-999, 1999.
27. **Hare JF and Hodges R.** Turnover of mitochondrial inner membrane proteins in hepatoma monolayer cultures. *J Biol Chem* 257: 3575-3580, 1982.
28. **Hochhauser E, Kaminski O, Shalom H, Leshem D, Shneyvays V, Shainberg A, and Vidne BA.** Role of adenosine receptor activation in antioxidant enzyme regulation during ischemia-reperfusion in the isolated rat heart. *Antioxid Redox Signal* 6: 335-344, 2004.
29. **House C and Kemp BE.** Protein kinase C contains a pseudosubstrate prototope in its regulatory domain. *Science* 238: 1726-1728, 1987.
30. **Hunter JC and Korzick DH.** Age- and sex-dependent alterations in protein kinase C (PKC) and extracellular regulated kinase 1/2 (ERK1/2) in rat myocardium. *Mech Ageing Dev* 126: 535-550, 2005.
31. **Hunter JC, Kostyak JC, Novotny JL, Simpson AM, and Korzick DH.** Estrogen deficiency decreases ischemic tolerance in the aged rat heart: Roles of PKCdelta, PKCepsilon, Akt, and GSK3beta. *Am J Physiol Regul Integr Comp Physiol* 292: R800-809, 2007.

32. **Inagaki K, Begley R, Ikeno F, and Mochly-Rosen D.** Cardioprotection by epsilon-protein kinase C activation from ischemia: continuous delivery and antiarrhythmic effect of an epsilon-protein kinase C-activating peptide. *Circulation* 111: 44-50, 2005.
33. **Inagaki K, Churchill E, and Mochly-Rosen D.** Epsilon protein kinase C as a potential therapeutic target for the ischemic heart. *Cardiovasc Res* 70: 222-230, 2006.
34. **Inagaki K, Hahn HS, Dorn GW, 2nd, and Mochly-Rosen D.** Additive protection of the ischemic heart ex vivo by combined treatment with delta-protein kinase C inhibitor and epsilon-protein kinase C activator. *Circulation* 108: 869-875, 2003.
35. **Javadov SA, Clarke S, Das M, Griffiths EJ, Lim KH, and Halestrap AP.** Ischaemic preconditioning inhibits opening of mitochondrial permeability transition pores in the reperfused rat heart. *J Physiol* 549: 513-524, 2003.
36. **Jin ZQ, Zhou HZ, Cecchini G, Gray MO, and Karliner JS.** MnSOD in mouse heart: acute responses to ischemic preconditioning and ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* 288: H2986-2994, 2005.
37. **Johnson JA, Gray MO, Karliner JS, Chen CH, and Mochly-Rosen D.** An improved permeabilization protocol for the introduction of peptides into cardiac myocytes. Application to protein kinase C research. *Circ Res* 79: 1086-1099, 1996.
38. **Juhaszova M, Zorov DB, Kim SH, Pepe S, Fu Q, Fishbein KW, Ziman BD, Wang S, Ytrehus K, Antos CL, Olson EN, and Sollott SJ.** Glycogen synthase kinase-3beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. *J Clin Invest* 113: 1535-1549, 2004.
39. **Kannel WB, Hjortland MC, McNamara PM, and Gordon T.** Menopause and risk of cardiovascular disease: the Framingham study. *Ann Intern Med* 85: 447-452, 1976.
40. **Kheifets V and Mochly-Rosen D.** Insight into intra- and inter-molecular interactions of PKC: design of specific modulators of kinase function. *Pharmacol Res* 55: 467-476, 2007.
41. **Kimura M, Irahara M, Yasui T, Saito S, Tezuka M, Yamano S, Kamada M, and Aono T.** The obesity in bilateral ovariectomized rats is related to a decrease in the expression of leptin receptors in the brain. *Biochem Biophys Res Commun* 290: 1349-1353, 2002.
42. **Korichneva I, Hoyos B, Chua R, Levi E, and Hammerling U.** Zinc release from protein kinase C as the common event during activation by lipid second messenger or reactive oxygen. *J Biol Chem* 277: 44327-44331, 2002.
43. **Korzick DH, Holiman DA, Boluyt MO, Laughlin MH, and Lakatta EG.** Diminished alpha1-adrenergic-mediated contraction and translocation of PKC in senescent rat heart. *Am J Physiol Heart Circ Physiol* 281: H581-589, 2001.
44. **Korzick DH, Hunter JC, McDowell MK, Delp MD, Tickerhoof MM, and Carson LD.** Chronic exercise improves myocardial inotropic reserve capacity through alpha1-adrenergic and protein kinase C-dependent effects in Senescent rats. *J Gerontol A Biol Sci Med Sci* 59: 1089-1098, 2004.
45. **Lakatta EG and Sollott SJ.** Perspectives on mammalian cardiovascular aging: humans to molecules. *Comp Biochem Physiol A Mol Integr Physiol* 132: 699-721, 2002.
46. **Lloyd-Jones D, Adams R, Carnethon M, De Simone G, Ferguson TB, Flegal K, Ford E, Furie K, Go A, Greenlund K, Haase N, Hailpern S, Ho M, Howard V, Kissela B, Kittner S, Lackland D, Lisabeth L, Marelli A, McDermott M, Meigs J, Mozaffarian D, Nichol G, O'Donnell C, Roger V, Rosamond W, Sacco R, Sorlie P, Stafford R, Steinberger J, Thom T, Wasserthiel-Smoller S, Wong N, Wylie-Rosett J, and Hong Y.** Heart disease and stroke

statistics--2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 119: e21-181, 2009.
47. **Manson JE, Hsia J, Johnson KC, Rossouw JE, Assaf AR, Lasser NL, Trevisan M, Black HR, Heckbert SR, Detrano R, Strickland OL, Wong ND, Crouse JR, Stein E, and Cushman M.** Estrogen plus progestin and the risk of coronary heart disease. *N Engl J Med* 349: 523-534, 2003.
48. **Murphy E and Steenbergen C.** Preconditioning: the mitochondrial connection. *Annu Rev Physiol* 69: 51-67, 2007.
49. **Murry CE, Jennings RB, and Reimer KA.** Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 74: 1124-1136, 1986.
50. **Newton AC.** Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. *Chem Rev* 101: 2353-2364, 2001.
51. **Newton AC.** Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. *Biochem J* 370: 361-371, 2003.
52. **Nishizuka Y.** Discovery and prospect of protein kinase C research: epilogue. *J Biochem* 133: 155-158, 2003.
53. **O'Rourke B.** Evidence for mitochondrial K+ channels and their role in cardioprotection. *Circ Res* 94: 420-432, 2004.
54. **Ogbi M and Johnson JA.** Protein kinase Cepsilon interacts with cytochrome c oxidase subunit IV and enhances cytochrome c oxidase activity in neonatal cardiac myocyte preconditioning. *Biochem J* 393: 191-199, 2006.
55. **Ohno S and Nishizuka Y.** Protein kinase C isotypes and their specific functions: prologue. *J Biochem* 132: 509-511, 2002.
56. **Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, and Mann M.** Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol Cell Proteomics* 1: 376-386, 2002.
57. **Parker PJ, Coussens L, Totty N, Rhee L, Young S, Chen E, Stabel S, Waterfield MD, and Ullrich A.** The complete primary structure of protein kinase C--the major phorbol ester receptor. *Science* 233: 853-859, 1986.
58. **Ping P, Takano H, Zhang J, Tang XL, Qiu Y, Li RC, Banerjee S, Dawn B, Balafonova Z, and Bolli R.** Isoform-selective activation of protein kinase C by nitric oxide in the heart of conscious rabbits: a signaling mechanism for both nitric oxide-induced and ischemia-induced preconditioning. *Circ Res* 84: 587-604, 1999.
59. **Ping P, Zhang J, Pierce WM, Jr., and Bolli R.** Functional proteomic analysis of protein kinase C epsilon signaling complexes in the normal heart and during cardioprotection. *Circ Res* 88: 59-62, 2001.
60. **Ping P, Zhang J, Qiu Y, Tang XL, Manchikalapudi S, Cao X, and Bolli R.** Ischemic preconditioning induces selective translocation of protein kinase C isoforms epsilon and eta in the heart of conscious rabbits without subcellular redistribution of total protein kinase C activity. *Circ Res* 81: 404-414, 1997.
61. **Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlet-Jones M, He F, Jacobson A, and Pappin DJ.** Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. *Mol Cell Proteomics* 3: 1154-1169, 2004.
62. **Rottlaender D, Boengler K, Wolny M, Michels G, Endres-Becker J, Motloch LJ, Schwaiger A, Buechert A, Schulz R, Heusch G, and Hoppe UC.** Connexin 43 acts as a

cytoprotective mediator of signal transduction by stimulating mitochondrial KATP channels in mouse cardiomyocytes. *J Clin Invest* 120: 1441-1453, 2010.
63. **Saurin AT, Pennington DJ, Raat NJ, Latchman DS, Owen MJ, and Marber MS.** Targeted disruption of the protein kinase C epsilon gene abolishes the infarct size reduction that follows ischaemic preconditioning of isolated buffer-perfused mouse hearts. *Cardiovasc Res* 55: 672-680, 2002.
64. **Schechtman D and Mochly-Rosen D.** Isozyme-specific inhibitors and activators of protein kinase C. *Methods Enzymol* 345: 470-489, 2002.
65. **Song X, Li G, Vaage J, and Valen G.** Effects of sex, gonadectomy, and oestrogen substitution on ischaemic preconditioning and ischaemia-reperfusion injury in mice. *Acta Physiol Scand* 177: 459-466, 2003.
66. **Souroujon MC and Mochly-Rosen D.** Peptide modulators of protein-protein interactions in intracellular signaling. *Nat Biotechnol* 16: 919-924, 1998.
67. **Sutton RB and Sprang SR.** Structure of the protein kinase Cbeta phospholipid-binding C2 domain complexed with Ca2+. *Structure* 6: 1395-1405, 1998.
68. **Thomas PD, Campbell MJ, Kejariwal A, Mi H, Karlak B, Daverman R, Diemer K, Muruganujan A, and Narechania A.** PANTHER: a library of protein families and subfamilies indexed by function. *Genome Res* 13: 2129-2141, 2003.
69. **Tong H, Imahashi K, Steenbergen C, and Murphy E.** Phosphorylation of glycogen synthase kinase-3beta during preconditioning through a phosphatidylinositol-3-kinase--dependent pathway is cardioprotective. *Circ Res* 90: 377-379, 2002.
70. **Wadia JS and Dowdy SF.** Modulation of cellular function by TAT mediated transduction of full length proteins. *Curr Protein Pept Sci* 4: 97-104, 2003.
71. **Wang Y, Wang Q, Zhao Y, Gong D, Wang D, Li C, and Zhao H.** Protective effects of estrogen against reperfusion arrhythmias following severe myocardial ischemia in rats. *Circ J* 74: 634-643, 2010.
72. **Ytrehus K, Liu Y, and Downey JM.** Preconditioning protects ischemic rabbit heart by protein kinase C activation. *Am J Physiol* 266: H1145-1152, 1994.
73. **Zieske LR.** A perspective on the use of iTRAQ reagent technology for protein complex and profiling studies. *J Exp Bot* 57: 1501-1508, 2006.
74. **Zorov DB, Juhaszova M, Yaniv Y, Nuss HB, Wang S, and Sollott SJ.** Regulation and pharmacology of the mitochondrial permeability transition pore. *Cardiovasc Res* 83: 213-225, 2009.

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Education

The Pennsylvania State University, State College, PA **Schreyer Honors College**
Bachelor of Science in Microbiology Honors in Kinesiology
Expected Graduation: August 2010

Honors

Phi Eta Sigma Honors Society
National Merit Scholarship Finalist
APS Undergraduate Summer Research Fellowship Awardee
David S. Bruce Award Finalist
Dean's List every semester

Experience

Pennsylvania State University, State College, PA
Undergraduate Research Assistant, May 2009-Aug 2009; May 2010-Aug 2010

- Conducted research for an honors thesis in Kinesiology
- Studied phosphorylation targets of a cardioprotective protein kinase in the mitochondria utilizing iTRAQ labeling, immunoprecipitation and LC/MS/MS methods

Pennsylvania State University, Hershey, PA
Undergraduate Research Assistant, June 2008-August 2008

- Assisted a graduate student in the Cellular and Molecular Physiology Department
- Performed cell culture work, PCR, Western blotting, plasmid construction, gel electrophoresis, DC protein and luciferase assays

American Physiological Society, State College, PA
Summer Research Fellowship Awardee, May 2007-December 2007

- Conducted research under grant from American Physiological Society
- Performed isolated heart studies, TTC staining, and RNA isolation

Activities

Student Red Cross Club, 2006-2007
Centre Volunteers in Medicine, 2010
Students for Barack Obama, 2008

- Precinct leader
- Election day line manager

Publications

Tuckow AP, Jefferson SJ, Kimball SR and Jefferson LS. Simvistatin represses eukaryotic initiation factor 2B (eIF2B) expression and activity in C2C12 myoblasts with a concomitant reduction in protein synthesis. Amer. J. Physiol. Endo Met, in press, (E-00383-2010).

Lancaster TS, Jefferson SJ, Stanley BA, Van Eyk J, Lakatta EG and Korzick DH. Quantitative proteomic analysis reveals novel mitochondrial targets of estrogen deficiency in the aged female rat heart. Cardiovascular Research, in press.

Skills

Isolated heart preparations, RNA isolation, TTC staining, use of NIH ImageJ software, PCR, Western blotting, gel electrophoresis, cell culture work, plasmid construction, immunoprecipitation, iTRAQ labeling