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ESTROGEN RECEPTOR BETA AND ADIPONECTIN AS TARGETS IN
CARDIOVASCULAR AND METABOLIC DISEASE

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by

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ABSTRACT

Coronary heart disease (CHD) remains the leading cause of morbidity and mortality in aged women, with a 2- to 3-fold increase in incidence following menopause. The loss of estrogen (E₂) has been implicated as a defining factor for reduced cardioprotection, yet estrogen replacement therapy (ERT) fails to reduce cardiovascular morbidity and mortality in postmenopausal women. Moreover, the combined effects of aging and E₂-deficiency contribute to a unique high risk state for metabolic dysregulation which further drives cardiovascular disease risk in aged women. Closer examination of both direct and indirect effects of E₂ and estrogen receptor (ER) signaling in the context of aging and E₂-deficiency is needed to help identify relevant targets for therapeutic intervention in post-menopausal women. The adipokine, adiponectin (APN), has been identified as a biomarker for CHD risk and may be influenced by aging, adiposity, and ERβ. The series of four studies described herein was designed to investigate ER-signaling disruptions in metabolic and cardiac tissues as well as characterize attenuations in APN signaling capacity in a model of aging and E₂-deficiency, the Fischer 344 (F344) female rat.

The aim of the first study (Chapter 3) was to determine the effects of acute ERβ activation on ischemia/reperfusion (I/R) injury in adult, aged, and aged E₂-deficient female rats. Hearts were isolated from adult (6 mo), aged (24 mo), and aged ovariectomized (OVX) rats and subjected to 47 min of global I and 60 min of R. Rats were acutely treated with the ERβ-agonist diarylpropionitrile (DPN) or vehicle 45 min prior to I/R. Acute treatment with DPN had no effect on functional recovery following I/R injury in adult, aged, or aged OVX female rats.

The aim of the second study (Chapter 4) was to determine if age-associated E₂-deficiency is associated with alterations in the ERα/ERβ ratio in visceral adipose tissue and skeletal muscle
of female rats. We also sought to characterize changes in intra-adipocyte and circulating APN. Visceral adipose and skeletal muscle was isolated from adult and aged rats with and without OVX. ERβ protein increased in adipose of aged and aged OVX rats and ERα increased in both adipose and gastrocnemius of aged OVX rats (p<0.05). Intra-adipocyte APN was increased and circulating APN concentration decreased in aged OVX vs adult OVX rats (p<0.05). Downstream APN targets, adiponectin receptor 2 (adipoR2), and the activated AMP-dependant kinase (pAMPK)/total AMPK ratio all decreased with age (p<0.05). Collectively, the data described in Chapter 4 suggests age-associated increases in ERβ in adipose, reduced circulating APN, and alterations in APN downstream effectors might conspire to create a metabolic phenotype associated with reduced cardioprotection.

In the third study (Chapter 5) we employed a direct in vitro approach to assess the effects of ERβ overexpression on APN in an adipocyte-like cell culture model, 3T3-L1 cells. Cells were transfected with ERβ plasmid construct for 24 h. Post-transfection ERβ mRNA was increased 1000-fold, yet protein levels were unchanged. In a second experiment DPN was utilized to activate ERβ in differentiated 3T3-L1 cells. However, due to lack of ER responsiveness, the effects of ERβ activation on APN production or secretion in vitro were not elucidated.

The aim of the fourth study (Chapter 6) was to determine if APN treatment is effective in conferring direct cardioprotection in the female rat heart and also to characterize changes in downstream cardiac APN targets associated with aging, E2-deficiency, and a high fat diet. Hearts were isolated from adult, aged, and aged OVX rats as described in Chapter 3 and were infused with 9 μg of APN or vehicle upon ischemia. APN infusion was successful in improving left ventricular developed pressure (LVDP) and end diastolic pressure (EDP) following I/R injury in all groups (p<0.05). No change in AMPK phosphorylation was observed in APN vs control I/R groups. In a subset of perfused control hearts isolated from rats consuming a high fat diet for 20
weeks total AMPK and AdipoR1 protein was decreased by 20% and increased by 50%, respectively relative to chow fed rats (p<0.05), which suggests APN-resistance.

A summary of the key findings in the collection of four studies described follows. 1) Cardiac ERβ does not likely play a role in cardioprotection in the female F344 myocardium. 2) Defects in APN secretion are associated with increased ERβ in visceral adipose tissue of aged and aged OVX female rats. Unfortunately, a causal link between ERβ and APN could not be evaluated presently in vivo or in vitro models. 3) APN treatment improves post-ischemic function in the F344 female rat heart and APN or downstream APN effectors may be relevant therapeutic targets for combating CHD risk and mortality in aged women. However, high fat consumption may negate the protective effect of APN given the APN-resistant phenotype observed in aged female rats on a high fat diet.
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LIST OF ABBREVIATIONS

ACC ............................................................................................................ acetyl-CoA carboxylase
AdipoR1 ................................................................................................. Adiponectin Receptor 1
AdipoR2 ................................................................................................. Adiponectin Receptor 2
AF ............................................................................................................... activation function
AMPK ........................................................................................................ adenosine monophosphate-activated kinase
AP-1 ............................................................................................................. activator protein 1
APN; acrp30; apM1; GBP 28 ........................................................................ adiponectin
APNK0 ....................................................................................................... adiponectin knock out
APPL1 ....................................................................................................... adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif 1
Ar ................................................................................................................. aromatase
ArKO ........................................................................................................ aromatase knock out
ATCC ........................................................................................................ American Type Culture Collection
BPA ............................................................................................................. bisphenol A
C1q .............................................................................................................. compliment 1q
CDE .......................................................................................................... clatherin dependant endocytosis
CEE .......................................................................................................... conjugated equine estrogen
CHD .......................................................................................................... coronary heart disease
COX .......................................................................................................... cyclooxygenase
DBD .......................................................................................................... DNA binding domain
Dsba-L ...................................................................................................... disulfide oxidoreductase A-like protein
DPN ..................................................................................................................... diarylpropionitrile
E2 ............................................................................................................................ estrogen
ECL ...................................................................................................................................... enhanced chemiluminescence
EDL .......................................................................................................................... extensor digitorum longus
eNOS ............................................................................................................... endothelial nitric oxide synthetase
ER .................................................................................................................................. estrogen receptor
ERE ................................................................................................................................... estrogen response element
ERKO ................................................................................................................... estrogen receptor knock out
ERT ....................................................................................................................... estrogen replacement therapy
Ero1-\(\alpha\) .................................................................................................................. endoplasmic oxidoreductin-1-like
Erp44 ....................................................................................................................... endoplasmic reticulum protein of 44KDa
Erp46 ....................................................................................................................... endoplasmic reticulum protein of 46KDa
EtOH .................................................................................................................................. ethanol
F344 .......................................................................................................................... Fischer 334
FCS .......................................................................................................................... fetal calf serum
GAPDH ...................................................................................................................... glyceraldehyde 3 phosphate dehydrogenase
GLM .......................................................................................................................... general linearized model
GLUT1 .......................................................................................................................... glucose transporter1
GLUT4 .......................................................................................................................... glucose transporter4
gp91\(\beta\)phox .................................................................................................................. superoxide generating subunit of NADPH oxidase
GPCR .......................................................................................................................... G-protein coupled receptor
HEK293 ................................................................................................................... human embryonic kidney 293
HERS ......................................................................................................................... Heart and Estrogen/Progestin Replacement Study
HMW .......................................................................................................................... high molecular weight adiponectin
HRP ............................................................................................................... horseradish peroxidase
Hsp .............................................................................................................. heat shock protein
ICI .................................................................................................................. anti-estrogen
I/R ................................................................................................................ ischemia/reperfusion
IT ................................................................................................................ ischemic tolerance
KEEPS .............................................................................. Kronos Early Estrogen Prevention Study
LBD ............................................................................................................... ligand binding domain
LKB1 ............................................................................................................... AMPK kinase
LMW ........................................................................................... low molecular weight adiponectin
LPL ........................................................................................................................ lipoprotein lipase
LV ............................................................................................................. left ventricle
LVDP .......................................................................................... left ventricular developed pressure
MAPK ............................................................................................ mitogen-activated protein kinase
MMW................................................................................... medium molecular weight adiponectin
MPP .......................................................................... methyl-piperidinyl-pyrazole-dihydrochloride
NADPH ...................................................................... nicotinamide adenine dinucleotide phosphate
N-CoR .................................................................................................. nuclear receptor corepressor
NFκB ...................................................................................................... nuclear factor K B
NHR .......................................................................................................... nuclear hormone receptor
NO ........................................................................................................ nitric oxide
OVX ..................................................................................................... ovariectomy/ovariectomized
PAGE ..................................................................................................... polyacrylamide gel electrophoresis
pAMPK .............................................. (active) phosphorylated adenosine monophosphate-activated kinase
PAQR ................................................................................................ progestin, and adipQ receptor
<table>
<thead>
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<td>PEPI</td>
<td>Postmenopausal Estrogen/Progestin Interventions Trial</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandin</td>
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<td>PGC1α</td>
<td>peroxisome proliferator activated receptor γ co-receptor α</td>
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<td>RIP140</td>
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</tr>
<tr>
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<td>retinoic acid receptor</td>
</tr>
<tr>
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<tr>
<td>SDS</td>
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<tr>
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<td>selective estrogen receptor modulator</td>
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<tr>
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<td>silencing mediator for retinoic acid and thyroid hormone</td>
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<tr>
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<tr>
<td>SRC</td>
<td>steroid receptor coactivator</td>
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<tr>
<td>TIF</td>
<td>transcriptional intermediary factor</td>
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<tr>
<td>TNFa</td>
<td>tumor necrosis factor α</td>
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<tr>
<td>WHI</td>
<td>Women’s Health Initiative</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
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CHAPTER 1

INTRODUCTION TO CARDIOVASCULAR AND METABOLIC DISEASE IN AGING WOMEN

Ischemic heart disease remains the leading cause of morbidity and mortality in aged women, and incidence is increased 2- to 3-fold following menopause (1). The prevalence of CHD increases with aging, and it is clear that post-menopausal women experience greater CHD mortality vs age-matched men (2-7). That E2-deficient post-menopausal women are more vulnerable to myocardial I/R injury vs men suggests a role for E2 in cardioprotection. Concurrently, the prevalence of obesity and metabolic syndrome also increase with age and post-menopausal status which conspire to increase CHD risk in aging women (8-14). Animal models of E2-deficiency provide direct evidence that E2 modulates body weight, adiposity, and cardioprotection (15-18). Moreover, ERT reverses adiposity, weight gain, and the loss of cardioprotection in OVX animal models (15, 17-26). However, while ERT also staves off weight gain in postmenopausal women, clinical trials have failed to demonstrate the cardioprotective benefit observed in adult OVX animals (27-34). ERT is currently contraindicated as a therapy for postmenopausal women not only due to lack of efficacy, but also because some studies indicate ERT increases the incidence of adverse cardiovascular events (30-34). Given that most animal models utilized in ERT studies are adult, and not aged, suggests the adult OVX animal does not properly model menopausal E2-deficiency. The combined effects of aging and E2-deficiency
together increase metabolic and cardiovascular disease risk in aging women and further study is needed to elucidate adequate therapies to treat CHD in the aged E$_2$-deficient heart.

ERs are distributed throughout the body, and positively regulate metabolism in adipose and skeletal muscle in response to E$_2$ (16, 20, 21, 35-49). Moreover, ER polymorphisms are linked to metabolic and CHD risk in women indicating that systemic ER signaling disruptions may contribute to the development of metabolic and cardiovascular disease states (50-54). Studies in animal models have directly shown ERs impact adiposity, metabolism, and promote tolerance to I/R injury in the myocardium (16, 20, 21, 24, 42-47, 55-59). In vivo, E$_2$ mediates the majority of its effects via two receptors, ER$\alpha$ and ER$\beta$. While ER$\alpha$ is relatively well studied, the specific functions of ER$\beta$ are not completely understood. ER$\beta$ acts in adipose tissue and may play a role in modulating metabolism and CHD risk (60, 61). Furthermore it has been proposed that ER$\beta$, like ER$\alpha$, can act directly at the myocardium to confer cardioprotection (55, 57, 58, 62). Gaining a better understanding of the function(s) of ER$\beta$ in metabolism and cardioprotection in a setting of aging and E$_2$-deficiency may lead to the development of relevant therapies for postmenopausal women.

In addition to being E$_2$ responsive, adipose tissue is known to secrete hormonal factors known as adipokines that modulate metabolism and cardioprotection (63-65). APN, is an adipokine that promotes metabolism, and cardioprotection via its anti-diabetic, anti-inflammatory, anti-atherogenic, and anti-apoptotic effects on the body (66-86). However, aging women have paradoxically high APN levels, yet are not protected from ischemic injury (87-92). In animal models of metabolic disease and aging, APN-resistance has been observed and is attributed to both reduced receptor presence and attenuated downstream signaling capacity (93-95). Currently, it is unknown if the tendency toward metabolic dysregulation and reduced ischemic tolerance (IT) observed in aged women is linked to and exacerbated by APN-resistance in cardiac tissue. In the studies contained within Chapters 3, 4, 5, and 6 ER$\beta$ and APN are examined in a female rat
model of aging and OVX to elucidate potential disturbances associated with cardiac and metabolic disease.

Statement of the Problem

The incidence of and mortality due to ischemic heart disease is increased in women following menopause implicating the loss of E₂ as a major cause of reduced ischemic tolerance. The failure of ERT to reduce cardiovascular risk in postmenopausal women, however, necessitates investigation into alternative therapeutic strategies for the treatment of ischemic heart disease in aged women. A greater understanding of the causative alterations surrounding age-associated E₂-deficiency and the loss of cardioprotective reserves is necessary to generate effective therapies for postmenopausal women. Aging women gain weight secondary to increased adiposity with menopause and it is thought that altered metabolism in metabolic tissues may be a driving force in increasing CHD risk in postmenopausal women. The combined effects of aging and E₂-deficiency contribute to a unique high risk state for metabolic dysregulation and cardiovascular mortality in aged women. CHD risk and mortality may be mediated through adverse changes in adipose metabolism associated with ER-signaling disruptions as well as attenuated responses to the cardioprotective adipokine, APN. Targeted studies are needed to better understand the impact of metabolic dysregulation and adipose metabolism on CHD morbidity and mortality.

Specific Aims and Hypotheses

Specific Aim 1. To determine the effects of acute ERβ activation on I/R injury in adult, aged, and aged OVX female rats.
Hypothesis 1.1 ERβ is present in the adult and aged female F344 rat myocardium.

Hypothesis 1.2 Acute ERβ activation will improve cardiac functional recovery following I/R injury in adult, aged, and aged OVX female F344 rats via non-genomic signaling mechanisms.

Specific Aim 2. To determine the singular and combined effects of aging and E2-deficiency on ER isoform distribution in visceral adipose tissue and skeletal muscle isolated from female rats. Also, to characterize alterations in the downstream ER target APN in adipose tissue.

Hypothesis 2.1 ERβ protein abundance is increased, and ERα protein abundance is decreased in the skeletal muscle of F344 female rats with age- and OVX-related weight gain relative to adult ovary-intact controls.

Hypothesis 2.2 ERβ protein abundance is increased, and ERα protein abundance is decreased in the adipose tissue of female F344 rats with age and OVX-related weight gain relative to adult OVX and adult ovary-intact controls. Also the downstream target APN is reduced in aged OVX female F344 rats relative to adult OVX and adult ovary-intact controls.

Hypothesis 2.3 Overexpression of ERβ in vitro will directly reduce APN expression in differentiated 3T3-L1 adipocytes thus providing a mechanistic link between ERβ and APN.

Hypothesis 2.4 AdipoR1 and AdipoR2, and AMPK will be reduced in the adipose tissue of aged OVX rats relative to adult ovary-intact controls thus providing an additional mechanism for metabolic derangement.

Specific Aim 3. To determine if aging and E2-deficiency is associated with APN resistance in the female rat myocardium.

Hypothesis 3.1 APN treatment will improve functional recovery following I/R injury in aged OVX female F344 rats relative to adult ovary-intact controls.
Hypothesis 3.2 Aging and OVX will be associated with compensatory increases in cardiac AdipoR1 and AdipoR2 protein levels in order to promote APN signaling relative to adult ovary-intact controls.

Hypothesis 3.3 Downstream AdipoR target AMPK will be reduced in the aged myocardium relative to adult ovary-intact controls thus suggesting alternative modes of APN action in the aged myocardium.
CHAPTER 2

REVIEW OF THE LITERATURE

The leading cause of death in aged women is CHD, and mortality is greater in post-menopausal women vs age-matched men (1-7). That E2-deficient post-menopausal women are particularly vulnerable to ischemic injury suggests a role for E2 in cardioprotection. Concurrently, the prevalence of obesity and metabolic syndrome also increases following menopause and these co-morbidities conspire to increase CHD risk in aging women (8-14). However, aging and E2-deficiency interact in a complex fashion in aging women and development of adequate therapies to treat CHD has been both challenging and unsuccessful. Case in point, while ERT staves off weight gain and reverses metabolic complications in post-menopausal women, CHD risk is not improved (27-34).

The failure of ERT and lack of alternative therapies has spurred a more detailed investigation of the ERs: ERα and ERβ. Both ERs are directly implicated in modulating metabolism and cardioprotection and often function to antagonize one another. The function of ERβ in the heart is not well understood and targeted activation may reveal a role for ERβ in cardioprotection. The role of adipose and its secreted factors, like the adipokine APN, has also garnered recent attention in modulating CHD risk and may be an additional target for therapeutic intervention. Circulating APN is negatively correlated with CHD risk and confers cardioprotection (66-86). Interestingly, APN and ERβ may be linked via a common signaling pathway in adipocytes. Current evidence indicates both ERβ and APN can act directly at the myocardium to promote IT as well as indirectly modulate CHD risk by impacting metabolism and associated adiposity in peripheral tissues. The efficacy of ERβ activation and APN treatment in
protecting the aging female myocardium is addressed in subsequent chapters. The current body of literature implicating ERβ and APN in cardiac and metabolic disease is reviewed herein.

**Introduction to Estrogen Receptors**

The lack of cardioprotection afforded by ERT has forced a more thorough examination of downstream E₂ signaling mechanisms. To date, extensive research has been conducted on ERs and their roles in metabolism and cardiac function. ERs were originally described as ligand-inducible transcription factors belonging to the family of nuclear hormone receptors (NHR)s (96). Two predominant ER isoforms exist, ERα, the original ER, characterized in 1986 and ERβ which was discovered in 1996 (97, 98). ERα and β have overlapping, yet unique roles in E₂ signaling. Both ERα and β have similar binding affinity for E₂ and are capable of direct transcriptional regulation of target genes containing E₂ response elements (ERE)s as well as modulation of transactivation/repression activity of secondary transcription factors like activator protein-1 (AP-1) and stimulating protein 1 (Sp1) (99-103). In addition to classical nuclear transcriptional regulation, ERs have been shown to exert effects via novel mechanisms including ligand-independent signaling and rapid non-genomic signal transduction (24, 60). The differential expression of ERs in target tissues also explains the wide variety of responses evoked by E₂ in target tissues. In many instances ERβ antagonizes the effects of ERα, however some genes are controlled solely by ERβ (104). For example, while most tissues express both ERs in varying proportions, prostate tissue only expresses ERβ (105). However, despite a clear characterization of its structure and relative tissue distribution, the specific functions of ERβ are not well understood. To date, theories on functional activity of ERβ are derived from insights on ERα, and our knowledge is therefore limited and in some cases flawed. The lack of reliable commercially available antibodies for ERβ in rodent tissue and absence of a dependable ERβ expressing cell
Culture model has made ERβ study challenging. Nevertheless, transgenic animal models and highly selective ER agonists have paved the way for ERβ study which has helped us to begin to understand the function of ERβ in health and disease. Herein we have utilized the selective ERβ agonist, DPN, to acutely activate ERβ in female rats and 3T3-L1 cells. In Chapter 3 we assess the cardioprotective effects of acute ERβ activation prior to I/R injury in the adult, aged, or aged OVX female rat heart. In Chapter 5 we assess the effects of ERβ activation on APN production in adipose-like 3T3-L1 cells.

Estrogen Receptor Structure

ERs share a common structure with members of the NHR family. The basic ER protein structure is divided into six domains designated A-F from N-terminus to C-terminus, respectively (106, 107), see Figure 2-1. ERα has two activation function (AF) domains which are designated AF-1 (A/B domain) and AF-2 (E domain), whereas ERβ contains the AF-2 domain only. The AF-1 domain in ERα is constitutively active and involved in transactivation function, while the corresponding region in ERβ shares less than 20% homology and can function weakly in activation (106). Conversely, some have described the function of A/B domain in ERβ as a repressor eluding to the antagonistic activities of ERα and β observed in some tissues/cell types (108). For both ERα and ERβ the AF-2 is activated via a ligand-dependent mechanism and is important for the recruitment of ER coactivators and/or corepressors. The DNA binding domain (DBD; contained within the C domain) is the most highly conserved region (>90%) between both ERs and thereby explains the overlap in E2-responsive gene regulation (109). The D domain is also known as the hinge domain, and is not well conserved with only 30% homology, while the E domain contains the aforementioned AF-2/ligand binding domain (LBD) and is moderately
conserved at 60%. Finally, the function of the F domain has yet to be defined and the sequence homology between ERs is less than 20% (104).

Additionally, human ERβ has 5 known splice variants which are the result of alternative splicing at the C-terminus affecting exons 7 and 8 (110, 111). The molecular weight of the each ERβ splice variant (1-5) is 59, 56, 54, 54, and 53 kDa, respectively; ERα is 67 kDa. ERβ₁ is also known as the long form, or original ERβ, and has full activity. ERβ₂ also known as ERβ₉ has been shown to act as a dominant negative of ERα (108, 112). ERβ₃ expression appears to be limited to the testis (105, 111). The short forms of ERβ₄ and ERβ₅ are present in great abundance and are as widely distributed throughout the body as ERβ₁ (105). However, due to the truncation at the C-terminus, the ER splice variants ERβ₂, ERβ₄, and ERβ₅ lack complete AF-2/LBDs and therefore cannot function alone or as homodimers (105). Instead, to exert their effects the splice variants must partner with full length ERβ₁ to form functional heterodimers (105). Similar splice variants have also been reported in rodent tissues. Just as in humans, the classical or long form is known as ERβ₁ in rodents and rat ERβ₂ has an insertion in the ligand binding domain which

Figure 2-1: Comparison of domain structures of ERα and ERβ. ERs have six domains, A–F, and the number of amino acids in these domains, as well as the functions associated with these domains, are indicated for ERα and ERβ. The activation function (AF) domains AF-1 and AF-2 are indicated in the A/B and E domains. The degree of homology between ERα and ERβ in the C and E domains is indicated; from Hewitt et al (2005).
differs from the C-terminal deletion in human ERβ2 (113). Two additional rat splice variants have been reported which have deletions in the DNA binding domain and are denoted ERβ1 – δ3, and ERβ2 – δ3 (113). Rat ERβ1 and ERβ2 have similar tissue distributions throughout the body and are likely fully functional, although ERβ2 has a slightly lower binding affinity for E2. ERβ1 – δ3 and ERβ2 – δ3 have lower DNA binding affinity as a result of their deletions, yet like the human splice variants, the rat ERβ1 – δ3, and ERβ2 – δ3 may still be capable of activity if they heterodimerize with the full length rat ERβ1 or ERβ2 (113).

**Estrogen Receptor Activation**

ERs belong to the NHR family and are further sub classified as type I nuclear receptors (96). Upon ligand binding, type I receptors translocate from the cytosol to the nucleus and subsequently modulate transcription of genomic DNA. ER activation modulates a wide array of genomic responses in target tissues and cells. To date, at least eight distinct mechanisms have been described that contribute to the widely diverse function of ERs including: 1) epigenetic events modulating available EREs; 2) expression of coregulators and corepressors; 3) the relative ERα/β ratio; 4) the presence of splice variants; 5) the relative availability of circulating E2; 6) the relative composition of the E2 milieu (i.e. estradiol, vs estrone, or estriol); 7) presence of non-E2 ER agonists/antagonists; and 8) complexity of various ER promoters. Thus, the numerous mechanisms governing ER function partially explain the diverse potential for regulation of ER target genes and also underscores the difficulty associated with the study of ERs. Moreover, following their initial characterization as classical nuclear receptors, ERs have been discovered to signal in additional unpredicted ways atypical of most NHRs. ERs can activate rapid non-genomic signaling cascades and in some cases can signal in the absence of ligand. That ERs are
capable of both genomic and non-genomic signaling with and without ligand adds additional complexity and diversity to the array of responses elicited by E$_2$ in target tissues.

**Estrogen Receptor Activation: Genomic Mechanisms**

Genomic ER activation is the classical mode of NHR activation and results in the transcription or repression of target genes. Specifically, genomic activation is initiated by ligand/agonist binding followed by receptor dimerization. Dimerization triggers ER release from stabilizing heat shock proteins (hsp)70 and hsp90 and nuclear corepressors, nuclear receptor repressor (N-COR), silencing mediator for retinoic acid and thyroid hormone (SMRT), and nuclear receptor interacting protein (RIP140) (114). A variety of coactivator proteins including members of the steroid receptor coactivator (SRC) family SRC1, SRC3, and transcriptional intermediary factor 2 (TIF2), take the place of exiting corepressors and enable ERs to become fully activated. ER$\beta$ interacts with both SRC and TIF2 in adipose tissue to modulate transcriptional activity of metabolic regulatory genes (60). Activated ERs translocate from the cytosol to the nucleus and target EREs containing DNA promoters. To complete classical genomic activation, ERs directly up or downregulate target genes containing EREs.

To date, over 30 coregulators (activators and repressors) have been observed to interact with ERs. Commonly associated cofactors include the aforementioned TIF2 and SRC proteins, as well as members of the p160 family. Proteins belonging to the p160 family bind ER AF-1 and AF-2 domains and bring ERs into contact with other cofactor proteins, thereby leading to enhancement or repression of DNA binding/transcriptional activity (115, 116). Repressors of the AF-2 domain include RIP140 and TIF1 (115). Peroxisome proliferator-activated receptor $\gamma$ coactivator-1$\alpha$ (PGC-1$\alpha$) is a tissue-specific coactivator that strongly enhances the activity of many NHRs including both ERs. Differential cofactor binding is responsible for much of the
temporal and tissue-specific diversity observed with ER activation. In many tissues ERα and β antagonize one another and often ERα upregulates while ERβ downregulates gene expression. For example, approximately 90% of genes downregulated by E2 are ERβ-dependant in murine aorta (117).

Alternatively, ERs can also genomically regulate transcription of genes that do not contain EREs through a process called transcription factor cross-talk. Ligand-bound ERs can couple with c-fos, c-myc, and c-jun adaptor molecules to regulate a broad range of genes including those containing AP-1 or Sp1 sites (101, 103, 118-120). The E2-mediated suppression of lipoprotein lipase (LPL) expression observed in adipose tissue is mediated by an AP-1 dependent mechanism (121).

E2 can also regulate levels of ERs per se. ERs do not have EREs in their promoter regions and they too are regulated by transcription factor cross-talk via stimulation of AP-1 and Sp1 sites (103, 115). In general, E2 upregulates ER expression, but depending on the collective transcription factor milieu and proteosomal degradation rates in a given tissue, the ER response to E2 can vary (40). Specifically, E2 upregulates ER expression in liver and adipose, but can downregulate expression in the uterus and cerebral cortex (40, 122-124). In the heart, short-term (4-5 h) exposure to E2 downregulates ER expression, yet chronic exposure delivered via daily injections increases myocardial ER expression (125, 126).

Finally, phosphorylation status of the ER also modulates transcriptional activity both independent of and in response to ligand activation (127, 128). In the case of classical genomic ligand-dependant activation, enhanced phosphorylation of ERα can occur in response to physiological doses of E2 (127, 129-131). The enzymes responsible for E2-dependent phosphorylation of ER are diverse and include an E2-dependent tyrosine kinase as well as unspecified kinases (129, 132-134).
Estrogen Receptor Activation: Non-genomic Mechanisms

In addition to classical genomic signaling, ERs have also been shown to rapidly activate intracellular signal transduction pathways in cardiac, vascular, skeletal muscle, and adipose tissues (135-137). Rapid, or non-genomic, ER signaling starts with ER activation at the plasma membrane (137-145). Surprisingly, both “nuclear” ERα and β proteins can localize to the plasma membrane and participate in signaling cascades (146-149). For plasma membrane targeting ERα and β both require palmitoylation before anchoring to the membrane protein, cavelolin-1 (137, 140, 142, 150). Stimulation of membrane bound ERs with E2 activates calcium ion channels, nitric oxide (NO) production and protein kinases (151, 152). Specifically, ERs non-genomically activate the phosphoinositol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling cascades and promote protein kinase A (PKA) and protein kinase C (PKC) activation (99, 153). In the heart, rapid ERα activation activates and preserves PKC signaling associated with cardioprotection from I/R injury (24). It has been speculated, but it is unknown, if rapid activation of ERβ in the rat heart can similarly confer cardioprotection via non-genomic signaling. Both ERs were demonstrated capable of rapid non-genomic signaling in the in vitro neonatal rat ventricular myocyte model (154). However, findings are at odds with data from in vivo murine and rabbit models demonstrating no cardioprotective benefit of acute ERβ activation prior to I/R injury (55, 155). Thus it is necessary to determine if there are developmental/species differences regarding the non-genomic signaling capacity of ERβ in the adult myocardium. This issue will be addressed in Chapter 3 utilizing the F344 female rat model.

In addition to the classic ERs, an E2-responsive G-protein coupled receptor (GPCR) 30 has also been described. While GPCR30 was discovered in 1996, it was not until 2005 when its role in E2 signaling was first realized. Studies on GPCR30 are still limited, but like ERα and β GPCR30 is capable of rapid activation of kinases including PI3K and MAPK cascades (156,
GPCR30 has been identified in cardiac and skeletal muscle and has been implicated in cardioprotection in rat models and therefore adds additional complexity to studying the independent and combined effects of each ER (158, 159).

**Estrogen Receptor Activation: Ligand Independent Mechanisms**

ERs are also capable of ligand-independent signal transduction. Microarray studies have revealed that in the absence of ligand, ERβ either stimulated or suppressed the activity of a number of genes that were normally only regulated by ERα in the presence of E₂ (160). Both posttranslational modifications (PTMs) as well as transcription factor availability appear to be two main modulators of ligand independent ER activity. Specifically, PTMs, like phosphorylation, can be an impetus for ER/ERE binding and transcriptional activity (161, 162). Both ERs have multiple PTM sites which modulate ER activity. ERα sites include: serines 102, 104, 106, 118, 154, 167, 236, 305, 559; threonine 311; and tyrosines 52, 219, 537 (128, 129, 134, 161-180). In general, serine phosphorylation is associated with enhanced transcription factor binding (165, 181, 182). Tyrosine 537 phosphorylation on ERα has been implicated in ligand-independent signaling via a MAPK-dependant mechanism (183, 184). ERβ has fewer known PTM sites which include: serines 94, 106, 124, 225 (161, 181, 185, 186). Acetylation and methylation also modulate ER activity by enhancing and suppressing activity, respectively (116, 187).

Transcription factors also influence ER activity independent of ligand binding. In adipocytes, unbound ERβ interacts with the factors SRC and TIF2 promoting downregulation of peroxisome proliferator-activated receptor (PPAR)γ transcriptional activity (60, 188, 189). Along these lines the retinoic acid receptor (RXR) and PPARγ can also work to downregulate ER activity by binding and blocking EREs from ERs (188, 189). Collectively these findings indicate
transcription factor availability differentially modulates NHR activity in adipose tissue. In Chapter 4 ERβ and PPARγ are assessed in adipose tissue of F344 female rats to characterize alterations in NHR signaling capacities associated with aging and OVX.

**Agonists, Antagonists, and SERMS**

Upon discovery of ERβ it became clear that estrogen (17β estradiol) and the synthetic anti-estrogen (ICI; Fulvestrant) alone would not be sufficient to study the individual effects of each ER. The discovery of the GPCR30 has also reinforced the need for additional ER specific modulators. 17β estradiol is the major physiological E2, but it has a similar affinity for both ERs. Thus, a number of selective ERα and β agonists have been created and described; however, only a minority have been evaluated extensively in vivo. ERα targeting compounds include the agonists propyl-pyrazole-triol (PPT) (21, 190) and 16α-LE2 (191), and the antagonist methyl piperidinylethoxyphenol-pyrazole dihydrochloride (MPP). ERβ specific compounds include the agonists DPN (192), ERB-041 (193), WAY-202196 (194), WAY-200070 (193), and 8β-VE2 (191). Currently, ERβ agonists and antagonists are a heavily utilized tool in ER research given the lack of reliable commercially available antibodies for ERβ in rodent tissue and lack of dependable cell culture models.

Selective estrogen receptor modulators (SERMs) are also of great utility and act differentially as agonists and antagonists in a given tissue. Tamoxifen and raloxifene are the most widely used SERMS. Tamoxifen, for example, is an antagonist in breast tissue, but an agonist in bone and thus has utility in treating breast cancer and/or osteoporosis. In skeletal muscle tamoxifen antagonizes ERβ and may be useful in treating metabolic disease in the future (46). An adipose- or cardiac-specific SERM has yet to be developed.
A number of natural compounds also have affinity for ERs and have been utilized in ER research. Phytoestrogens are plant derived estrogens; genistein, an isoflavone, for example has great affinity for ERβ and has been widely utilized (195). Xenoestrogens, also known as environmental estrogens, are found in plastics. Bisphenol A (BPA) is found in polycarbonate plastic used for consumer products and has been suggested as a potential driving force for promoting obesity and diabetes via ER-dependant mechanisms (196). In the heart, BPA is pro-arrhythmic and signals via ERβ in mice (197). Phytoestrogens, E2, SERMs, and the ERα agonist, PPT, but not ERβ agonists positively impact metabolism by modulating energy balance leading to improved lipid profiles and insulin sensitivity (18, 198-203). Chronic DPN administration has been implicated in improving the response to trauma hemorrhage, and reducing hypertension and ischemic damage in rodent models (55, 204-206). In Chapter 3 DPN is utilized to acutely activate ERβ systemically in the F344 female rat. The purpose of the DPN treatment is to characterize ERβ-mediated cardioprotection in the myocardium.

**Distribution of Estrogen Receptors and Expression**

Although ERs were first characterized in both male and female reproductive tissues (97, 98), it is now known that ER expression is distributed throughout the body including, but not limited to the gonadal tract, breast, testis, bone, skeletal muscle, liver, adipose tissue, myocardium, vascular endothelium, smooth muscle, and the central nervous system (35-41, 207). Most tissues express both ERs, but relative levels of each vary thus contributing to the diversity of E2 responses described in target tissues. ERα predominates in the gonadal tract, kidney, bone, adipose, skeletal muscle, and liver. In contrast, ERβ expression is more pronounced in the ovary, prostate, testis, salivary glands, vascular endothelium, smooth muscle, lung, gastrointestinal tract, bladder, immune system, and central and peripheral nervous system (208, 209). Sex, aging, and
menopausal status are associated with varied ER expression, and E\textsubscript{2} is a known modulator of both ER expression as well as tissue distribution (40, 210-214). Myocardial ER\textalpha, but not ER\textbeta, is reduced with aging in female spontaneously hypertensive rats (126). Epidemiological data is conflicting, but reduced ER\textalpha gene expression in the myocardium of female coronary artery bypass patients has been documented (187). In adipose tissue, ER\textalpha is reduced with both E\textsubscript{2}-deficiency and diet-induced obesity in both animal models and obese women (215, 216). ER\textbeta increases in adipose with aging in both women and animal models (40, 207, 217, 218). Tipping the ER\textalpha/\textbeta balance in favor of ER\textbeta is hypothesized to elicit detrimental consequences affecting weight gain, metabolic disease and cardiovascular risk with aging linked to dysregulated signaling in adipose and other tissues.

**Estrogen Receptor Knock Out Mice**

Transgenic whole body knockout mice null for ER\textalpha and/or ER\textbeta have provided additional insight regarding ER tissue localization and key functions. From the phenotypes of the ER null mice, it is clear that ERs play a significant role in metabolism and adiposity as well as impact the myocardial response to I/R injury. Currently, there are four independently constructed and utilized ER\textalpha and ER\textbeta knock out (αERKO and βERKO) mice. The models developed include the αERKO\textsubscript{CH} and βERKO\textsubscript{CH} mice were produced in Chapel Hill by Krege et al. (219); βERKO\textsubscript{KI} mice which were established at the Karolinska Institute; αERKO\textsubscript{ST} and βERKO\textsubscript{ST} mice were produced by Dupont et al. in Strasbourg, France (220); and βERKO\textsubscript{WYE} mice generated by Wyeth (221). Dupont has also produced a double ERKO model, (α/βERKO\textsubscript{ST}) (220, 222). Slight disagreements in ERKO characterizations exist and most are due to the fact that αERKO\textsubscript{CH} mice are incomplete KOs whereas the other αERKO models are completely null for ER\textalpha (220). However, the bulk of cardiac functional studies have been conducted in the Chapel Hill KO mice.
so the cardiac phenotypes and responses discussed herein are consistent. βERKO mice generated in Chapel Hill, Strasbourg, and by Wyeth are completely null for ERβ and descriptions are in agreement (219-221).

In addition to the expected reproductive derangements and infertility experienced by male and female ERKO mice, there are also metabolic derangements. αERKO mice exhibit profound insulin resistance, impaired glucose tolerance, and adipocyte hyperplasia and hypertrophy which worsens with aging (61, 223, 224). In contrast, βERKO mice have a similar body weight and equal fat distribution, as well as normal circulating lipid and insulin levels (60, 225). These findings indicate ERα signaling is critical for maintaining normal metabolic balance. However, a more thorough investigation of the βERKO phenotype has revealed that ERβ acts to maintain a basal level of adiposity and both ERs antagonistically regulate adiposity (61, 224). A detailed description of the role of ERs in regulating metabolism and adiposity is described in the Estrogen and Metabolism section.

ERKO mice have also implicated both ERs in cardioprotection. αERKOCH mice subjected to I/R injury show increased damage compared to wild type (WT) mice (59, 226). Similarly, βERKOCH mice also experience greater I/R injury and additionally develop hypertension and cardiac hypertrophy (55, 57, 227). However, it is important to note that ERKO models are whole body and not tissue specific so developmental abnormalities may confound observations made herein about the integrative function of ERs on metabolism or the cardiovascular system under ischemic stress. Thus, although we have gleaned much from ERKO models, careful interpretation and additional studies are needed to tease apart the specific effects of each ER in a given tissue/system.
Estrogen and Metabolism

It is well established that E$_2$ has a profound effect on food intake, energy balance, body weight, and adiposity and that E$_2$-deficiency predisposes postmenopausal women to weight gain and increased adiposity associated with changes in metabolism (228-232). The metabolic syndrome is a constellation of risk factors including hypertension, impaired fasting glucose, dyslipidemia, and central obesity which all have their own negative implications on health as well as collectively conspire to increase CHD risk. Currently, 25% of the U.S. population is afflicted with metabolic syndrome and the incidence increases with aging such that prevalence is 44% for those 50 years of age and over (233). Moreover, like CHD risk, E$_2$-deficient postmenopausal women are predisposed to metabolic syndrome and more women are afflicted vs age matched men. It is now clear the loss of E$_2$ at the time of menopause has profound implications on whole body health and clearly drives metabolic and cardiovascular disease states.

The first evidence of the effects of E$_2$ on metabolism came from aromatase knockout (ArKO) mice which have a compromised ability to produce E$_2$ from androgens and therefore have whole body E$_2$-deficiency (229-231, 234). ArKO mice exhibit marked glucose intolerance accompanied by increased adiposity and pronounced insulin resistance, but do not demonstrate hyperphagia or reduced energy expenditure (229-231). In addition to ArKO mice, OVX rodent models are utilized to study the effects of E$_2$-deficiency/ERT on metabolism. Postmenopausal E$_2$-deficiency, resulting from reproductive senescence is better modeled by OVX rodents because they retain the capacity to interconvert androgens to E$_2$ in the peripheral tissues, adipose and adrenals. ArKO mice lack any capacity to produce E$_2$ (231, 235). Noteworthy, most rodent chow contains phytoestrogens which may allow for low levels of ER activation and ArKO mice on a phytoestrogen-free diet experience more severe metabolic phenotypes (236). In line with these findings, OVX rodents have less severe metabolic complications vs ArKO mice, but do display
increased body weight secondary to increased fat mass (237, 238). Over time, OVX rats develop dyslipidemia, impaired glucose tolerance, and impaired insulin-mediated glucose uptake which can be improved with ERT (17, 239, 240). Interestingly, increased food intake is sufficient, but not necessary for OVX-induced weight gain; OVX rats pair-fed to match ERT-treated OVX rats still gain as much body weight as ad libitum fed controls (241, 242). The control E2 exerts on body weight and composition appears to be more closely tied to fatty acid metabolism and inhibition of lipid storage, compared to hyperphagia per se. The fact that ArKO mice do not demonstrate hyperphagia with metabolic derangements further supports this idea (230). E2 clearly plays a crucial role in glucose and lipid metabolism in metabolic tissues, yet the molecular mechanisms underlying this protective role of E2 remain incompletely understood. Both ERs are found in adipose and skeletal muscle and their roles in modulating metabolism are discussed herein.

Regarding insulin sensitivity, it is well established that physiological concentrations of E2 preserve normal insulin responses while deficiency results in insulin resistance. The most conclusive evidence of the effects of E2 on insulin actually comes from men with aromatase and ERα polymorphisms. Afflicted men have no capacity to signal via ERα and display insulin resistance and/or glucose intolerance (243, 244). The same insulin resistance is observed in OVX and αERKO animal models (223, 230, 245). Transgenic αERKO mice have a phenotype marked by skeletal muscle insulin resistance, skeletal muscle lipid accumulation, and impaired hepatic glucose regulation (223, 246). Consistent with these findings, ERα activation has been implicated in reversing insulin resistance in both dietary and genetically obese mice via suppression of lipid accumulation (198, 199, 240, 247). OVX in αERKO mice improves insulin signaling by ablation of detrimental unopposed ERβ signaling (61). Although some studies are conflicting, evidence from the Women’s Health Initiative (WHI) trial and other cross-sectional studies indicate that the prevalence of early insulin resistance and glucose intolerance increases with menopausal E2-
deficiency (248-251). ERT given in physiological doses improves metabolic complications in postmenopausal women; curiously though, supraphysiological or high E\(_2\) levels are associated with increased risk of metabolic disease and even diabetes in aged women (252-254).

**The F344 Rat as a Model of Metabolic Disease and Aging**

The F344 rat is characterized as a model of chronic insulin resistance and appropriate for studying aging in a setting of mild metabolic disease (255). In Chapters 3, 4, and 6 we utilize the F344 rat to model aging and postmenopausal E\(_2\)-deficiency with intact and OVX rats. Relative to the Sprague-Dawley strain, the F344 rat gains more adipose mass/g body weight with maturity and significant differences in adiposity are observed as early as 4 months of age (256, 257). Increased adiposity is attributable to both increased caloric consumption and more efficient caloric storage of energy as fat relative to Sprague-Dawley rats (257). The capacity to efficiently store fat with comparatively less weight gain relative to Sprague-Dawley rats is likely the reason F344 rats have been previously described as weight stable. However, with advancing age at 12 and 26 mo weight gain and adiposity is significantly increased vs rats aged 6 mo in both male and female F344 rats (258, 259). Life expectancy of F344 rats is 29 mo, with maximal survival expectancy 36 mo (260, 261).

The metabolic F344 phenotype is observed as early as 4 mo in rats consuming a standard chow diet. Rats demonstrate increased lipid accumulation in skeletal muscle and liver, reduced expression of liver fatty acid oxidation enzymes, and a 15% increase in gonadal fat pad size, vs age-matched Sprague-Dawley rats (256). Moreover, at both 2 and 4 mo F344 rats have 2-3 fold elevations in insulin, glucose, and free fatty acid concentrations during both fasted and fed states relative to Sprague-Dawley rats (256). Interestingly, glucose-stimulated insulin release in whole perfused pancreas is not exacerbated with aging in either gender (258). Thus although F344 rats
are considered an insulin-resistant model relative to other rat strains aging does not exacerbate their condition. The F334 rat is a unique model for studying aging in conjunction with a consistent metabolic phenotype.

**Skeletal Muscle and Estrogen Receptors**

Glucose disposal in response to postprandial insulin secretion is mainly mediated by skeletal muscle. Derangements driving metabolic syndrome and CHD risk may stem from defects promoting aberrant signaling in skeletal muscle glucose handling. Both ERs are present in skeletal muscle and E2 signaling impacts glucose handling and metabolism. ERα activation in skeletal muscle mediates protective actions while ERβ activation is diabetogenic. In addition to metabolic complications linked to skeletal muscle fiber dysfunction at the time of menopause, aging women experience a striking decline in total muscle mass and strength (262, 263). The decline in skeletal muscle function occurs at an earlier age than in men and is reversed with ERT, thereby linking skeletal muscle dysfunction to the menopausal transition. The loss of muscle mass and contractile strength accompanied with aging is associated with decreased fiber size and apoptotic cell death. Apoptosis seems to be greater in glycolytic fibers given that senescent muscle is characterized by a transition to a greater population of oxidative vs glycolytic fibers (264, 265). Loss of individual muscle fibers as well as total muscle mass results in reduced exercise and metabolic capacity and represents an additional risk factor for CHD (266).

At the cellular level both ERs modulate glucose transporter 4 (GLUT4) by regulating its expression and translocation to the cell membrane (267). Defects in GLUT4 translocation, anchoring, or trafficking to the plasma membrane impacts whole body glucose metabolism and directly links ER signaling to insulin sensitivity and metabolism in muscle (268). In skeletal muscle, ERs regulate GLUT4 in an antagonistic fashion; ERα induces whereas ERβ inhibits
GLUT4 expression (41, 46). The loss of functional ERα signaling in αERKO mice markedly reduces GLUT4 expression. However, GLUT4 abundance can be rescued in αERKO mice with tamoxifen treatment (46). Conversely, tamoxifen antagonizes ERβ in skeletal muscle. Along these lines targeted ERβ activation in E2-deficient ArKO mice also decreases GLUT4 expression (41). Collectively, these studies indicate that basal ERα signaling is needed to maintain GLUT4 expression, but also emphasizes the dramatic diabetogenic effects of unopposed ERβ action in skeletal muscle. The specific mechanism governing ER modulation of GLUT4 expression depends on interactions with the transcription factors SP1 and nuclear factor κB (NFκB) (269, 270). ERα augments SP1 activity thereby promoting GLUT4 expression, while ERβ antagonizes (99, 103). With regard to NFκB, a repressor of GLUT4 in skeletal muscle, ERα sequesters NFκB allowing disinhibition of GLUT4 (271, 272). To summarize, ERα-deficiency and or unopposed ERβ activation decreases GLUT4 expression in skeletal muscle which is linked to impaired skeletal muscle glucose utilization. Unopposed ERβ signaling is thought to be the driving force promoting diabetogenic effects leading to metabolic derangements in skeletal muscle.

Regarding ER distribution in skeletal muscle, studies in mice indicate ERα predominates and its abundance is greatest in glycolytic muscle fibers (273). However data are limited and often ER gene expression, but not total protein abundance is reported. In OVX mice, ERα gene expression is upregulated in skeletal muscle, yet OVX rats demonstrate no change in ERα (215, 273). Interestingly, however, diet- and OVX- induced obesity decreased ERβ protein abundance in soleus, but not the extensor digitorum longus (EDL) (215). Aging in general is associated with a loss of fast glycolytic fibers and maintenance of oxidative fibers. Thus, the expression of ERs in soleus and other slow twitch muscles may be the most relevant in metabolism in aging humans. ERα predominates in glycolytic fibers and with aging it is presumable that the decline in glycolytic muscle is also associated with a decline in ERα. A shift in the ERα/β balance in skeletal muscle could explain increased metabolic syndrome incidence in aged E2-deficient
postmenopausal women as well as further link metabolic disease states to CHD risk. In Chapter 4 ERα protein content is assessed in the skeletal muscle of adult and aged F344 female rats with and without OVX to elucidate the independent and combined effects of E2-deficiency on ER abundance.

Adipose and Estrogen Receptors

The sexually dimorphic pattern of fat distribution observed in men vs women has long implicated a role for sex hormones in adipose biology. OVX/ERT studies in rodent models have clearly shown E2 controls the relative distribution of adipose between subcutaneous vs visceral depots. E2-replete premenopausal women have more subcutaneous fat whereas aging E2-deficient postmenopausal women and men accumulate more visceral fat (248, 249, 274, 275). The mechanism by which E2 controls fat partitioning is not completely understood. Wade and Gray provided the first evidence of ERs in adipose tissue in 1978 by demonstrating E2-binding in cytosolic extracts from OVX rats (276). This work was corroborated by several studies demonstrating E2 binding in both whole tissue and isolated adipocytes, then was expanded by the identification of both ERα and β in adipose tissue (20, 40, 44, 49, 61, 277-281). Studies in humans indicate women have a higher concentration of ERs in adipose tissue than men and it is ERβ that accounts for this difference. The sexually dimorphic variability in ERβ concentration may explain some of the divergence in E2 responses and distribution of adipose observed between the sexes (214, 279). Moreover, human adipose tissue contains all five ERβ splice variants and abundance increases with aging which may provide for additional variability in E2 signaling in adipose of aging women (40, 111, 279). However, ERα predominates over ERβ in adipose tissue and OVX influences ERα, but not ERβ expression in adipose (40, 199). Investigations regarding the combination of aging plus E2-deficiency on ERs, however, is unfortunately lacking in much
of the animal literature. We address this issue in Chapter 4 and additionally attempt to characterize changes downstream ERβ targets in adipose associated with metabolism and CHD.

The abundance of ERα in adipose paired with the enhanced adiposity observed in αERKO mice suggests ERα plays an important role in basic adipocyte physiology (223). Interestingly, it was originally thought that ERβ played no significant function in adipose tissue. That βERKO mice appear identical to WT mice in terms of weight and adiposity supported this notion (224). However, when metabolically challenged with a high fat diet, βERKO mice are resistant to weight gain and adiposity compared to WT mice (224). Moreover, OVX in αERKO mice attenuates adiposity which indicates unopposed signaling via ERβ, thereby contributing to increases in adipose mass (61). Collectively, these studies not only confirm functional ERβ signaling in adipose tissue, but link enhanced ERβ signaling to adiposity. A clearer indication of the ERβ mechanism of action in adipose was established following the discovery that ERβ negatively regulates PPARγ, a key regulator of metabolism and adipogenesis (60, 188, 189). Disruption of the normal ER balance in favor of augmented ERβ signaling has clear implications for promoting adiposity and should be investigated further in obesity and aging models.

**Estrogen and Obesity**

Ovarian E2-deficiency in OVX animals, like ER dysfunction, is associated with enhanced lipid accumulation, adiposity, and weight gain (17, 20, 238, 241, 282). According to epidemiological reports in humans, E2-deficient postmenopausal women are predisposed to accrue additional weight and adiposity with aging (283). Interestingly, it is visceral fat which predominates in men and E2-deficient postmenopausal women that is most closely tied to risks for adipocyte pathology, obesity, and metabolic disorders (284, 285). Left unchecked, enhanced adiposity and associated weight gain can progress to overweight and obesity which is currently a
serious global health problem (286, 287). In the U.S. alone, 60% of the population are overweight and 30% are obese (288). Obesity is not only characterized by increased adiposity and fat mass, but also pathophysiological changes in adipose tissue. Closer characterization of obesity models indicates that ERα levels are reduced in adipose tissue in association with both OVX-induced weight gain and diet-induced obesity (40, 207, 215, 217, 218).

E\textsubscript{2} signaling at the cellular level has a profound impact on adipose metabolism and lipid storage. E\textsubscript{2} suppresses the expression and catalytic activity of lipoprotein lipase (LPL), the rate limiting enzyme controlling lipid storage in adipocytes (121, 282, 289). Reducing lipid storage likely prevents excessive adiposity and weight gain in E\textsubscript{2}-replete women and it has been similarly shown that ERT in postmenopausal women specifically reduces LPL activity (290).

Changes in adipose and other metabolic tissues due to E\textsubscript{2}-deficiency induced weight gain can drive metabolic disease and has also been linked to increased CHD disease risk. CHD is a leading cause of mortality associated with obesity and better understanding the links between the obesity, associated adipocyte pathology, and CHD may lead to therapies for those at risk. In postmenopausal women, ERT reverses abdominal obesity which is also associated with improvements in insulin sensitivity and staving off metabolic disease (248). However, ERT use as a therapeutic has been limited in postmenopausal women due to controversy surrounding its potential to increase the risk of heart disease.

**Estrogen and Heart Disease**

Like its risk factors, obesity and associated metabolic disease, CHD is also clearly linked to E\textsubscript{2}-deficiency. Prior to menopause the female heart is protected from CHD risk and associated mortality (2-7). However, CHD mortality rates in aged women actually surpass that observed in men following the menopausal transition (2-7, 291). According to the American Heart
Association, since 1984 more women than men have died of heart disease in the US and 53% of total cardiovascular disease deaths occur in women. Thus, endogenous E_2 not only functions to limit CHD risk by promoting normal metabolism and adipocyte function, but also directly protects the heart from fatal ischemic injury. Studies in rodent models have indicated that ERα and possibly even ERβ are present and functional in the myocardium. Acute E_2 treatment or ERα activation augments IT in adult animals (292-298). Studies utilizing OVX rodents have confirmed that the E_2-deficient female heart is more vulnerable to ischemic injury than gonad-intact controls. In line with these findings, ERT or a high phytoestrogen diet can rescue the E_2-deficient heart from ischemic damage (17, 22, 297, 299-303).

**Estrogen Replacement Therapy and Heart Disease**

Encouraging results from animal studies as well as initial epidemiological reports from the Nurses’ Health Study and The Postmenopausal Estrogen/Progestin Interventions (PEPI) Trial indicated ERT as a therapeutic treatment for decreasing CHD risk and acute myocardial infarction in aged women (31, 291, 304-308). In contrast, mid-stage analysis of two randomized clinical trials, the WHI and the Heart and Estrogen/Progestin Replacement Study (HERS), revealed that conjugated equine estrogens (CEE) alone or CEE plus progesterone did not protect postmenopausal women from infarction, but increased stroke risk (32, 33). Currently, ERT is contraindicated for the treatment of heart disease in postmenopausal women as a consequence of the untoward outcomes of the WHI and HERS trials.

The disparity between the outcomes of the ERT clinical trials prompted a secondary analysis of the WHI trial by partitioning subjects into perimenopausal and aged postmenopausal categories. These data indicate ERT may serve as a cardioprotective agent for women at the menopausal transition, but not aged women (31, 309, 310). The utility for ERT as a CHD therapy
in perimenopausal women is under current investigation in The Kronos Early Estrogen Prevention Study (KEEPS) (34, 311, 312). Regardless of the outcome of this trial, prior evidence for diminished efficacy and the possible detrimental effects of ERT on CHD risk in aged women demands the need for alternative therapies.

Part of the failure for ERT to protect the aged E₂-deficient myocardium is confounded by the fact that aging occurs concurrently with the menopausal transition. It is well known that the prevalence of CHD increases with aging in both men and women and even normal ‘healthy’ aging is associated with reduced IT. Animal models have revealed the senescent myocardium is more vulnerable to ischemic damage and demonstrates compromised recovery following an insult which is attributed to greater cell death and damage than seen in the adult heart (313-321). Morphological, biochemical and genetic changes manifest in the aged heart and collectively compromise the capacity to tolerate ischemic insult including, myocyte cell loss, fibrosis, hypertrophy, impaired intracellular calcium handling, and alterations in contractile protein isoforms (322, 323). The changes in the myocardium associated with aging render the senescent heart refractory to treatments proven efficacious in improving function or IT in adult animals. ERT, for example, reverses hypertrophy, a risk factor for CHD mortality, in adult OVX, but not aged OVX spontaneously hypertensive rats (126). Ischemic preconditioning is also ineffective in rescuing the aged heart from I/R injury despite its beneficial effects on the adult myocardium (315, 324-326). Most concerning, however, is that animal models of menopause, like the aged OVX female rat, experience even greater ischemic damage and worsened functional recovery following an ischemic insult than aged gonad-intact animals (24, 299). Congruent with animal models, aged women also experience increased damage following acute myocardial infarction. A better understanding of specific E₂ signaling mechanisms in heart is clearly needed to properly understand and protect the aged female heart and reduce CHD mortality.
Estrogen Receptors, Estrogen Receptor Knock Out Mice and the Myocardium

ER polymorphisms have been linked to increased CHD risk in aged women, demonstrating that ERs and not just E₂-deficiency are associated with disease risk (50-54, 327, 328). Given the links to ERs and heart disease paired with the disappointing failure of ERT, the study of ERs has emerged as the new focus for developing CHD therapies. Although there is no cardiac-specific ERKO model, use of whole body ERKO mice have greatly increased understanding of ER signaling in the myocardium and their roles in cardioprotection (55, 61, 226, 227, 301).

ERα is clearly implicated in modulating ischemic tolerance in both male and female animal models. Following I/R injury male αERKO mice experience greater ischemic damage and lose more viable myocytes than WT mice (59). Although female αERKO mice similarly experience reduced recovery following I/R injury, damage is associated with reduced levels of cardioprotective signaling proteins (226). However, preserving ERα signaling is critical for maintaining IT in adult animals of both sexes, although, the mechanisms of action are clearly divergent. The apparent sex differences in ER signaling underscore the importance in utilizing appropriate models to address ER signaling derangements in the aged female myocardium. In Chapter 3 the F344 female rat model is utilized to assess the effects of aging and OVX on the myocardial response to acute ERβ activation prior to I/R injury.

Acute ERα activation in aged OVX female rats rescues IT via activation of the cardioprotective protein, PKCε (24). Although studies in aging models are limited, other models of cardiac disease including the spontaneously hypertensive rat have also responded with increased IT following ERα activation (155, 329). Collectively, current evidence from transgenic mouse and ERα agonist studies strongly support a role for ERα in cardioprotection; the role of ERβ is less clear, and discussed herein.
Like αERKO mice, female βERKO mice experience greater cardiac damage following I/R injury than WT mice (55, 57). However, these results must be interpreted with caution as they were conducted under the stress of hyper-contractile conditions and may not reflect normal physiological responses (55, 57). One study indicated that ERβ activation with E₂ in αERKO mice limited ischemic damage following I/R, however hypertrophy and associated mortality was also increased thus negating the cardioprotective benefit (330). Adding to the uncertainty surrounding the capacity of ERβ to directly protect the myocardium, no improvement in IT was observed after acute ERβ activation in a rabbit model (155). Moreover conflicting reports question the presence of ERβ in rodent myocardium (38, 98, 300, 331)(227).

Estrogen Receptor Beta and the Myocardium

It has been 16 years to date since the discovery of ERβ in 1996, and there is still no consensus regarding ERβ presence in the myocardium. Kuiper, who is credited with cloning ERβ, demonstrated weak ERβ mRNA signal with PCR and in situ hybridization detection methods in the rat heart (98). Saunders corroborated the presence of ERβ by demonstrating localization with immunohistochemistry in male rat ventricles (38). In 2000 and 2004 ERβ presence was confirmed in the human heart with both immunohistochemical and proteomic approaches (332). These findings were later discounted by Schwend and Gustafsson in 2006 who demonstrated Yang et al. had over-interpreted mass spectrometry data (333). Specifically, mitochondrial hydrogen-ATPase peptide fragments with mass to charge ratios similar to ERβ had been misidentified (332). Replication of experimental conditions and a more stringent analysis confirmed ERβ is not detectable in mitochondrial lysates (333). Unfortunately, Schwend and Gustafsson utilized murine, not human tissue to challenge the previous report (333). Thus as of 2006 ERβ presence in the human and rat myocardium is still questionable (334). Additional
studies providing compelling evidence of ERβ expression in the human heart are lacking. Moreover, inconsistencies in later studies on rodent myocardium have spurred a reevaluation of past data and more stringent requirements for confirmation of ERβ detection in animal models. Non-specific antibodies demonstrating ERα cross reactivity are partially to blame. While multiple groups have confirmed ERβ existence in neonatal rat ventricular myocytes (36, 332, 335) there is equal evidence confirming and refuting ERβ presence in adult and aged rodent hearts (300) (36, 37, 62, 155, 227). In Chapter 3 we add to the current body of literature by assessing ERβ mRNA and protein presence in the aged female F344 rat myocardium.

Estrogen Receptor Beta and Cardioprotection

Regardless of the presence of ERβ in the myocardium, human ERβ gene polymorphisms and βERKO knockout mice indicate a clear role for ERβ in cardioprotection. By five months of age βERKO mice become hypertensive and develop cardiac hypertrophy by eight months (227, 336). In a model of heart failure βERKO mice also experience greater hypertrophy than either both WT or αERKO mice (330, 337, 338). Taken together, these data indicate the presence of ERβ does impact the myocardium although the mechanism of cardioprotection may be via indirect vascular mechanisms. Multiple studies have confirmed ERβ presence in vascular tissue (117, 139, 339-343). In further support of indirect ERβ cardioprotection, chronic ERβ activation studies demonstrate improved IT in OVX mice and amelioration of hypertension in salt sensitive rats (62, 155, 344). Central delivery of ERβ agonists to the autonomic cardiovascular control center in the brain also reduces systemic arterial pressure in rats (345). Thus, if ERβ is indeed absent from the myocardium, a likely explanation for its role in cardioprotection may occur via indirect modulation of neural and vascular mechanisms associated with hypertension resulting in reduced complications and risk factors for ischemic heart disease (227). Further studies are
needed to determine if acute or chronic ERβ activation will prove to be a viable therapy for CHD risk reduction or treatment in the aged female heart. The capacity of the F344 female rat heart to mount a cardioprotective response following acute ERβ activation is assessed in Chapter 3.

**Adiponectin in Cardiovascular and Metabolic Disease**

It is well established that CHD is linked to metabolic disease given that the symptoms of metabolic syndrome—hypertension, dyslipidemia, impaired glucose tolerance, and central obesity—are all factors which increase heart disease risk (346, 347). Moreover, the severity of metabolic syndrome complications correlates with worsened myocardial infarction outcomes (348, 349). APN is a hormone produced in adipose tissue which circulates in plasma at concentrations of 3-30 μg/mL and has anti-diabetic, anti-atherogenic, anti-inflammatory, and cardioprotective effects (66-86). Low APN, or hypoadiponectinemia, is thought to play a role in the etiology of metabolic disease given that patients with reduced circulating APN concentrations are at increased risk for insulin resistance, glucose intolerance, type II diabetes, obesity-linked inflammation, and CHD (67-86, 350). Additionally, APN receptors are downregulated with obesity and metabolic disease, and downstream signaling activity is reduced with aging (83, 93-95, 351-353). Reduced receptor availability and/or signaling capacity can lead to APN resistance, thus further propagating metabolic disease progression and increasing CHD disease risk. Therapeutic interventions aimed at increasing circulating APN concentrations and/or APN receptors/downstream targets may reverse symptoms of metabolic syndrome and reduce CHD morbidity and mortality in at risk populations. Currently, APN supplementation shows promise as a new therapy for patients with ischemic heart disease. A bolus injection of APN reduces infarct size and limits the decline in functional recovery following ischemic insult in animal models (354-356). However, diabetic rodents consuming a western diet display APN-resistance, and APN treatment is less efficacious
in attenuating ischemic injury in these models (93, 353). Thus, the relevance of APN as a cardioprotective agent is questionable given the prevalence of metabolic co-morbidities, like diabetes in CHD patients. However, whereas metabolic and CHD incidence increases with aging, APN efficacy in an aged population has yet to be assessed. Aging women represent a unique population which demonstrates increased adiposity, reduced IT, and progression toward metabolic dysregulation following the onset of E2-deficiency at the menopausal transition. Elucidating APN efficacy in treating CHD morbidity and mortality in models of aging and E2-deficiency could help develop relevant therapies for postmenopausal women. In Chapter 6 we assess the efficacy of acute APN treatment on the isolated female rat myocardium in the context of both aging and OVX.

**Adiponectin**

APN is an adipokine secreted from adipose tissue that has profound implications in cardiovascular and metabolic health. APN was independently characterized in 1995 by four groups, and is therefore also known as adipose most abundant gene transcript 1 (apM1), adipocyte complement–related protein of 30 kDa (acrp30), adipoQ, and gelatin-binding protein of 28kDa (GBP28) (63, 69, 357, 358). APN has been classified as a member of the complement 1q (C1q) family based on common structural properties (359, 360). APN and other C1q family members also have structural homology with tumor necrosis factor (TNF) proteins (361). Members of the TNF family are involved in a range of biological processes such as inflammation, adaptive immunity, apoptosis, and energy homeostasis and tissue regeneration (360, 362). APN has been described as an anti-diabetic, anti-atherogenic, anti-apoptotic, and anti-inflammatory cytokine and thus not only shares structural homology, but also functional commonality with C1q and TNF families. The paradoxical downregulation of APN with increased fat mass and obesity
in conjunction with TNFα upregulation has been attributed to complex cross-regulatory interactions between APN and its inflammatory TNF cytokine relatives (363-366). The specific structure of APN consists of a signal peptide at the N-terminus followed by a short variable region, a collagenous domain with 22 triple-helical Gly-X-Y repeats and a globular head at the C-terminus (361, 362); see Figure 2-2. Individual units of C1q family members can assemble and multimerize into higher-order levels of organization. APN homomultimerizes into three forms: trimers, hexamers, and multimers (18 or more units) of low, medium, and high molecular weight (LMW, MMW, and HMW), respectively (76, 79, 367-369). Multimerization occurs intracellularly and all forms of APN, have been detected in human and animal serum (70, 76, 367, 370, 371).

![Figure 2-2: Schematic of adiponectin intracellular assembly; from Wang and Scherer (2008).](image)

Adipocyte processing and assembly of APN into LMW, MMW, and HMW forms is relatively complex, involves multiple posttranslational modifications, and requires numerous cofactors and chaperones. The initial APN assembly steps are achieved via hydrophobic interactions between monomers which unite to form trimers (LMW APN) (367, 368). Two trimers then can associate to produce a heximer (MMW APN) (76, 367). This process requires disulfide bond formation which is achieved with support from several endoplasmic reticulum chaperones: disulfide oxidoreductase A-like protein (DsbA-L), endoplasmic reticulum oxidoreductase 1-Lα (Ero1-Lα) and endoplasmic reticulum protein of 44kDa (Erp44) (368, 370,
372, 373). Specifically, Erp44, a member of the thioredoxin family, localizes to the endoplasmic reticulum and Golgi intermediate compartments where it binds thiol-containing residues of Ero1-Lα or APN (370, 372). Erp44 can bind and retain APN for posttranslational processing at the endoplasmic reticulum until Ero1-Lα displaces APN from Erp44 (370, 372, 374). HMW assembly requires at least three heximers to come together to make an 18 unit complex. Hydroxylation and glycosylation on conserved lysines is critical for HMW assembly, yet the specifics of this process have not yet been elucidated (372, 375, 376); see figure 2-2. Finally, the expression of DsbA-L is correlated with secretion of HMW APN in 3T3-L1 adipocytes which suggests DsbA-L is required for the completion of HMW assembly/secrezione into the circulation (368). Relative imbalances in APN chaperones could impact the intra-cellular production/secrezione of L, M, and HMW APN as well as change the APN profile in circulation. Imbalances could have serious implications on metabolic health given that it is HMW APN, not total APN that best correlates with disease risk (76, 79, 350, 367, 377-383).

**Modulation of Adiponectin Multimers by Estrogen Receptor β**

Adipose tissue is E₂ responsive and expresses both ERs (40, 44, 49, 61, 278-281, 384). In adipose tissue ERβ interacts with the APN regulatory protein PPARγ (60, 188, 189). PPARγ is a master regulator of metabolism and upregulates transcription of numerous genes involved in APN processing and secretion, including APN (385-388). ERβ negatively regulates the activity of PPARγ via competition for common transcriptional cofactors like TIF2 and SRC (60). Relative changes in ERβ/PPARγ balance in favor of ERβ may attenuate APN expression resulting in an insufficient intracellular APN protein supply for the support maintenance of normal circulating APN concentrations. Moreover, ERβ regulation of PPARγ can additionally impact the intracellular assembly of existing intracellular APN affecting multimer distribution. PPARγ also
transcriptionally regulates the APN processing chaperones Erp44, Ero1-Lα, and DsbA-L (368, 370, 372, 373, 389). Lack of sufficient assembly chaperones impacts the relative distribution of APN multimers favoring production of LMW vs the more complex MMW and HMW APN (368, 370, 372, 373, 389). It is important to note, much of this work has been conducted in vitro and it is unknown if physiological changes in ER expression in vivo can similarly impact APN intracellular assembly and/or the relative distribution of APN multimers in circulation. ER expression is known to change with adiposity and E₂ status. Specifically, ERβ increases in visceral adipose of obese and aging women (40, 390). Theoretically, postmenopausal changes in ER expression may alter APN processing in aged women, which could impact total/HMW APN levels, thus providing an additional link explaining changes in E₂ status and CHD risk factors. In Chapter 4 we characterize changes in ERβ protein abundance in association with alterations in downstream signaling proteins associated with APN processing and secretion in adipose tissue. In Chapter 5 we attempt to discern a direct link between ERβ overexpression and APN assembly/secretion in the adipose-like 3T3-L1 cell culture model.

**Adiponectin and Gender**

Gender differences in circulating APN concentrations indicate that estrogens and androgens may play a role in APN regulation (11, 74, 78, 80, 82, 391-401). Testosterone has been shown to negatively correlate with circulating APN, and men consistently have reduced circulating levels vs women (86, 393, 395, 399, 400, 402). Men with very low APN (< 4 μg/mL) are at greatest risk for CHD (74, 75). Lower APN concentrations in men in general, may in part explain the sexual dimorphism observed with protection from CHD in premenopausal women vs age-matched men. Postmenopausal women have similar or even greater APN concentrations than premenopausal women (11, 363, 391, 393, 396, 397, 399, 403-407); see table 2-1. This similarly
may indicate that E$_2$, like testosterone, may also have an inhibitory effect on APN, albeit a lesser one. OVX rodent models demonstrate increases circulating APN, which further supports that E$_2$ may have a negative effect on APN concentration (408, 409). However, data are conflicting regarding the direct relation between circulating APN concentrations and E$_2$. Circulating APN does not vary with normal menstrual cycling in premenopausal women and few studies exist which show a compelling E$_2$ and APN correlation (392, 393, 398, 410). It has been proposed that the effects of sex hormones on APN are indirect. Testosterone has been shown to inhibit HMW APN secretion (400). However, Horenburg et al. showed no effect of direct application of testosterone or E$_2$ on APN production in vitro (411). Currently, it is hypothesized that testosterone and estrogens exert the majority of their suppressive effects on APN by preventing adipocyte proliferation and maturation. Testosterone directly inhibits adipocyte differentiation and E$_2$ stimulates LPL and adipocyte catabolism (412).

That the sexual dimorphism in APN concentration persists even following reproductive senescence in aged individuals is curious (399, 413, 414). Sex hormones clearly play a role in maintaining the balance between adequate adiposity for APN production, and obesity mediated suppression of APN. However, the exact tipping point where E$_2$-deficiency promotes appropriate adiposity for enhanced APN production vs excessive adiposity in postmenopausal women is unclear; see Table 2-1.

Greater concentrations of APN in non-overweight and non-obese aged women suggests that they should be protected from metabolic and CHD, yet as heart disease is the number one killer of women, a significant proportion are still afflicted. A possible explanation for these findings is that although total APN increases with age the relative APN multimer distribution may be altered. Relative to total APN, HMW APN is a better predictor of disease risk in men (78). However, circulating total and HMW APN remain closely correlated in aged women and assessment of both provides no greater insight to predicting disease risk (415, 416). Finally, it
may be that APN-resistance rather than hypoadiponectinemia is more relevant issue in aged women. Compromised signaling at the receptor level could explain increased disease risk in the presence of adequate or even overabundant APN. Further studies are needed to address the complex control that sex hormones, reproductive senescence, and aging exert on APN.

<table>
<thead>
<tr>
<th></th>
<th>Decreased</th>
<th>Increased</th>
<th>No Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premenopausal overweight or obese</td>
<td>(363)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postmenopausal normal weight</td>
<td>(11)</td>
<td>(393, 396, 397, 399, 403-407)</td>
<td>(391, 417, 418)</td>
</tr>
<tr>
<td>Postmenopausal overweight or obese</td>
<td>(396, 404, 407, 417)</td>
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</table>

**Adiponectin Receptors**

While the specifics of APN intra-cellular assembly are in part elucidated, the details regarding APN/receptor interactions are not yet clearly understood. APN associates with multiple receptors: adiponectin receptor (AdipoR)1, AdipoR2, AdipoR3, and T-cadherin (378, 419-422). AdipoR1, R2, and R3 belong to the progestin and adipoQ family of receptors (PAQR) (423). AdipoR1 and R2 were cloned in 2003, and are integral membrane proteins with 7-transmembrane spanning domains which are functionally and structurally distinct from GPCRs (421). Specifically, the AdipoR N-terminus is internal and C-terminus is external and this topology is converse to GPCRs (421). AdipoR3 was not characterized until 2011. Little is known about this receptor other than it binds APN and is structurally homologus to PAQR family members (422). T-cadherin was identified as an APN receptor in 2004, and is found in endothelial and smooth muscle cells, yet as peripheral membrane protein it likely cannot independently transduce APN.
signals and has been redefined as a co-receptor (378, 419). AdipoR1 and AdipoR2 knockout animals display severe metabolic phenotypes indicating the majority of the effects of APN are mediated via AdipoR1 and AdipoR2 (424). AdipoR1 KO mice demonstrate increased adiposity, insulin resistance, and glucose intolerance, while AdipoR2 KO mice are insulin resistant, but glucose tolerant (424). This compelling evidence is further strengthened by the fact that both AdipoR1 and R2 are ubiquitously expressed throughout the body (85, 421, 425-430).

Elucidating the unique and overlapping functions of each receptor and APN/receptor interaction is a current area of research. T-cadherin responds only to MMW and HMW APN (378). AdipoR1 and R2 can bind monomeric or even cleaved globular LMW APN; AdipoR1 has high affinity for globular domains and AdipoR2 has high affinity for full length uncleaved monomeric, or L, M, and HMW multimeric APN (379, 420). That AdipoR1 and AdipoR2 can be activated with individual monomers or even APN cleavage products still has yet to be reconciled with the fact that greater concentrations of multimeric HMW APN are predictive of and protective against the development of metabolic and cardiovascular disease (431, 432). Nevertheless, following ligand binding AdipoR1 and R2 interact with several intracellular proteins including: adaptot protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif 1 (APPL1), endoplasmic reticulum protein of 46KDa (Erp46), receptor of activated C kinase protein 1 (RACK1) and protein kinase CK2β subunit (433-435). It is likely these proteins act as internal scaffolding for signaling cascades; however the specific interactions have not yet been determined. Finally, it is well established that the fatty acid oxidation regulators, PPARα and AMPK, as well as the modulator of inflammation, cyclooxygenase (COX)2, are the end effectors following AdipoR1 and R2 activation in metabolically active tissues such as liver, adipose, skeletal vascular, and cardiac muscle (354, 420, 421, 436). Specifically in the heart, APN protects the ischemic myocardium by limiting
inflammation, apoptotic cell death, and nitrate cytotoxicity via COX2-, AMPK-, and NO-
dependent mechanisms (95, 354).

Finally, cessation of APN/AdipoR signaling is mediated by clathrin-dependent endocytosis (CDE) via a Rab5 dependent mechanism (437). Rab5 is a small GTPase which regulates early endosome fusion and has been reported to associate with APPL1, a potential AdipoR scaffolding protein (435, 438). Rab5 and its other family members regulate the intracellular trafficking of endocytosed membrane proteins including a number of transmembrane receptors (439-441). It is well established that GPCRs are known to internalize via Rab5-positive vesicles, recycle to the membrane via Rab4-positive vesicles, or are sorted for degradation via Rab7-positive vesicles. It remains to be determined whether AdipoRs cycle through the endosomal pathways in a similar fashion. It has been shown that CDE inhibition not only increases AdipoR levels at the plasma membrane, but also enhances APN binding and downstream activation of AMPK (437). Thus, it is likely that AdipoR receptor internalization may be a critical determinant in attenuation of APN signaling the subsequent development APN-resistance in at risk populations. The mechanisms at work are still being investigated and regulation of AdipoR membrane localization poorly understood. AdipoR1 internalizes both in response-to and independent-of APN stimulation (437).

**Adiponectin and Cardioprotection**

It is well established that hyperglycemia and hyperlipidemia accompanying metabolic disease consequently cause vascular injury and complications in the myocardium. Thus, it was originally thought that the links between hypo adiponectinemia and CHD were secondary to the fact that obesity and the symptoms resultant of metabolic disease also promote CHD risk. While metabolic disease does link APN and CHD, studies utilizing APN knockout mice (APNK0) have
revealed APN has direct effects on the myocardium. APNKO mice have reduced IT as evidenced by greater infarct size and increased apoptotic damage relative to wild type controls following I/R injury (354, 442, 443). Supplementing APNKO mice with APN attenuates injury due to I/R in a dose-dependent manner (354, 443). These data further strengthen the conclusion that circulating APN concentration not only correlates with CHD risk, but is also predictive of I/R injury severity. APN supplementation in wild type rodents and pigs also improves IT following I/R injury (354-356, 444). Moreover, APN treatment is effective in reducing infarct size when administered before, during, or post ischemia (354, 356). APN accumulates in the infarcted heart following I/R injury and binding during injury may be a critical adaptation of the myocardium to promote protective APN signaling. (355, 356, 445-449).

Although these data suggest APN therapy is promising, studies in diabetic mice have revealed APN efficacy is substantially reduced in models of metabolic disease (93, 94, 353). Three times the dose (6 μg/g) of APN was required to elicit the same degree of cardioprotection in very high fat diet-induced diabetic mice vs controls (93). Streptozotocin-induced diabetes blocks APN-mediated cardioprotection in early, but not late, phases of disease progression linked to fluctuations in AdipoR1 protein levels (94). Clearly, APN-resistance is a potential issue further limiting the treatment for metabolic disease. Careful characterization of each disease state to determine the extent of hypoadiponectinemia and/or APN-resistance will be necessary to provide adequate therapy for afflicted individuals.

**Adiponectin Mechanisms of Cardioprotection**

APN acts on both the myocardium and vascular endothelium to modulate cardioprotection. In the vasculature, APN phosphorylates and activates endothelial nitrous oxide synthetase (eNOS) and prostaglandin (PG) I₂ which prevents systolic hypertension as well as
pressure overload induced hypertrophy (434, 450-454). APN acts directly on the myocardium to limit inflammation, apoptotic cell death, and cytotoxic nitrate production following I/R injury via COX-2, AMPK, and anti-nitrative mechanisms (95, 354, 434, 455-457); see Figure 2-3. Noteworthy, cardiomyocytes also produce APN, however it is unclear if the quantity (10% of that produced in adipocytes) is sufficient to elicit significant physiological effects on the myocardium (428, 458).

Adenosine Monophosphate-Dependent Kinase Dependent Mechanisms

APN directly increases AMPK activity in perfused hearts resulting in cardioprotection characterized by reduced infarct size and limited apoptosis (95, 351, 354, 377, 433, 459-462). In
conjunction with increased apoptotic cell death both basal and post-ischemic AMPK phosphorylation is reduced in APN-resistant diabetic rats (93, 94). Similarly, APN-KO mice have reduced cardiac AMPK phosphorylation (354). Moreover, expression of dominant negative AMPK directly blocks APN-mediated cardioprotection and confirms a critical role for AMPK (354, 436). The downstream mechanisms linking APN-mediated AMPK activation and reduced apoptotic damage have not been fully elucidated. A description of APN-AMPK signaling and cardioprotection is reviewed herein.

In general, AMPK is activated by phosphorylation on Thr172 in response to a shift in the AMP/ATP ratio in favor of AMP. Activated AMPK, or pAMPK directly acts to enhance fatty acid oxidation via phosphorylation and inhibition of ACC activity as well as enhance insulin-independent glucose uptake via GLUT1 (463, 464). Specifically, in the healthy heart under aerobic conditions, the majority of energy required for contractile function is derived from fatty acids while the remainder (~30%) is obtained via metabolism of glucose (465). Controlled fatty acid metabolism prevents triglyceride accumulation and associated lipotoxic effects like apoptosis and insulin resistance (466). Under normoxic conditions, APN-AMPK signaling promotes normal cardiac function by enhancing fatty acid and glucose uptake and utilization in isolated working hearts (433, 467, 468).

In response to APN, AMPK is phosphorylated on Thr172 by LKB1 (an AMPK kinase), which additionally is associated with anti-apoptotic signaling pathway activation (469, 470). LKB1 primarily resides in the nucleus and translocates to the cytosol via an APN-dependant mechanism (433, 471). Specifically, APN binding to AdipoR1 promotes binding of APPL1, a scaffolding protein which then recruits LKB1 to the plasma membrane (433, 471). Although APPL1 also binds AdipoR2, AMPK phosphorylation in the myocardium is thought to be exclusive to AdipoR1 activation (433, 435, 472).
Initially in cardiomyocytes, APN-treatment directs glucose metabolism, but by 24 h of treatment glucose oxidation levels are reduced (468). Fatty acid oxidation then increases as glucose oxidization wanes at 24 and 48 h of APN treatment in vitro (468). Long term control of fatty acid metabolism does prevent triglyceride accumulation and lipotoxicity associated apoptosis and insulin resistance (466). These findings indicate that transient administration of APN may be effective in improving glucose oxidation, and prolonged treatment effective in enhancing fatty acid utilization and reducing chronic lipotoxicity.

The stress of ischemia also independently triggers AMPK activation and subsequent glucose and fatty acid utilization (354, 473, 474). Interestingly, AMPK stimulated glucose utilization maintains energy homeostasis and confers protection following I/R injury (462, 475). However, in the presence of high fatty acid concentrations following I/R, AMPK activation results in increased cardiac damage (476-480). It is now accepted that following ischemia switching from the prevailing excessive fatty acid metabolism toward glucose oxidation is indeed beneficial (465, 481). The dual role of activating both glucose and fatty acid metabolism makes AMPK an important target in the ischemic myocardium. In Chapter 6 the APN-mediated activation of AMPK is characterized in female rat hearts isolated from adult, aged, and aged OVX rats and subjected to I/R injury.

Ablation of AMPK signaling with expression of dominant negative AMPK prevents APN-mediated cardioprotection and results in greater apoptotic damage following I/R injury (354). Currently, it is unknown if anti-apoptotic APN-AMPK signaling is linked to glucose oxidation, fatty acid oxidation, or a completely novel pathway. Enhanced AMPK and ACC phosphorylation is observed in vivo coronary artery ligation experiments at 3, 24, and 48 h following APN-treatment relative to vehicle treated animals (93, 354). It is unknown if ACC activation and associated fatty acid metabolism is critical for anti-apoptotic APN effects or if events are simply coincident. That transient APN treatment is associated with glucose oxidation,
suggests that acute APN administration may be an effective therapy linked to AMPK glucose utilization. Indeed as soon as 60 min following ischemia, AMPK phosphorylation is increased by 60% and associated with cardioprotection in isolated APN-treated Langendorff-perfused male rats vs controls (482).

Anti-Inflammatory and Anti-Nitrative Effects of Adiponectin

Independent of AMPK, COX-2 also activates cardioprotective signaling pathways in response to APN treatment during I/R injury (354, 483). COX-2 is a key enzyme in regulating the production of PGs and inflammation. In the ischemic myocardium, COX-2-mediated synthesis of PGE$_2$ reduces inflammation via TNF$\alpha$ suppression in response to APN treatment (354, 483). Several in vivo models have confirmed APN-mediated suppression of inflammation (354, 355, 483, 484). APN also confers protection by suppressing cytotoxic peroxynitrite production (93, 443). APN inhibits, the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase responsible for producing super oxide during I/R injury, (gp91$^{	ext{phox}}$) in the myocardium see Figure 2-3 (443). Super oxide combines with NO produced from iNOS to yield the cytotoxic peroxynitrite (443). Anti-nitrative actions of APN are active in the presence of dominant negative AMPK and thus clearly mediated via a divergent signaling pathway (93, 436, 443). Finally, it is important to note APN can promote production of eNOS derived NO in the vasculature which is both cardioprotective and distinct from the cytotoxic iNOS derived NO/peroxynitrite (482).

APN Resistance

Similar to patients presenting with hypoadiponectinemia, patients with gene polymorphisms of AdipoR1 and AdipoR2 have increased risk for diabetes and CHD (424, 485-
Moreover, AdipoR1/2 double knockout mice actually exhibit more severe metabolic complications than APNKO mice and overexpression of both AdipoR1 and AdipoR2 reverses adiposity and improves metabolic markers in mice (424, 485-488). Taken together these findings underscore the importance of AdipoR1 and AdipoR2 in mediating APN signaling. Interestingly, reductions in AdipoRs in skeletal muscle are observed prior to pathological lipid accumulation and insulin resistance in diet induced obese/hyperlipidemic rats. These findings suggest AdipoRs are not only associated with, but may be a causal agent for metabolic dysregulation (92). Reduced receptor abundance has recently been proposed as a potential cause of APN-resistance. In Chapter 6 AdipoR1 and AdipoR2 protein levels are characterized in the F344 female rat myocardium.

Curiously, animal models of obesity and metabolic disease demonstrate varied AdipoR expression levels in adipose, skeletal muscle, liver, heart, and the aorta. Variability has been attributed to timing of sample collection, length of dietary interventions, and the relative age of animals. In normal fasted and fed states, AdipoRs fluctuate inversely with insulin levels (83). Consistent timing of sample collection is critical for accurately characterizing relative AdipoR changes (489). Disagreements reported in AdipoR abundance in obese and metabolically challenged animal models where time of sample collection is controlled is likely a function of disease progression on AdipoR regulation. A pattern of characteristic changes in APN and AdipoRs in metabolic tissues occurs in high fat diet-induced obese mice, diabetic Zucker rats, and streptozotocin-induced diabetic rodents over time (Table 2-2). At the onset of metabolic disease, reduced AdipoR levels in conjunction with attenuated downstream signal activation have been observed in liver, adipose tissue, aorta, heart, and skeletal muscle (83, 353, 426, 460, 489-491). While reductions in AdipoR2 in adipose tissue appear to be stable over time, AdipoR1 increases in cardiac, liver and skeletal muscle in diabetic animal models after 7 weeks and obese animal models after 16 weeks of treatment (94, 425, 489, 491-493). APN also fluctuates and often increases in early stages of metabolic disease before decreasing (83, 94, 489).
<table>
<thead>
<tr>
<th>Adipose</th>
<th>Skeletal Muscle</th>
<th>Liver</th>
<th>Heart</th>
<th>Circulating Adiponectin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>c57BL6/6J mice consuming chow (13% fat) for 10 weeks</td>
<td>AdipoR1 mRNA ↑</td>
<td>AdipoR1 mRNA ↔ AdipoR2 mRNA ↔</td>
<td>Increased</td>
<td>(489)</td>
<td></td>
</tr>
<tr>
<td>c57BL6/6J mice consuming chow (13% fat) for 18 weeks</td>
<td>AdipoR1 mRNA ↑</td>
<td>AdipoR1 mRNA ↑</td>
<td>No Change</td>
<td>(489)</td>
<td></td>
</tr>
<tr>
<td>c57BL6/6J mice consuming high fat (42%) for 10 weeks</td>
<td>AdipoR1 mRNA ↓</td>
<td>AdipoR1 mRNA ↑</td>
<td>Increased</td>
<td>(489)</td>
<td></td>
</tr>
<tr>
<td>c57BL6/6J mice consuming high fat (42%) for 18 weeks</td>
<td>AdipoR1 mRNA ↑</td>
<td>AdipoR1 mRNA ↑</td>
<td>Reduced</td>
<td>(489)</td>
<td></td>
</tr>
<tr>
<td>c57BL6/6J mice consuming high fat high sucrose for 16 weeks</td>
<td>AdipoR1 mRNA ↓</td>
<td>AdipoR2 mRNA ↓</td>
<td>Increased</td>
<td>(460)</td>
<td></td>
</tr>
<tr>
<td>A/J (obesity resistant) mice consuming chow (16% fat) for 10 weeks</td>
<td>AdipoR1 mRNA ↑</td>
<td>AdipoR1 mRNA ↔ AdipoR2 mRNA ↔</td>
<td>No Change</td>
<td>(489)</td>
<td></td>
</tr>
<tr>
<td>A/J (obesity resistant) mice consuming high fat (42%) for 10 weeks</td>
<td>AdipoR1 mRNA ↑</td>
<td>AdipoR1 mRNA ↑</td>
<td>Increased</td>
<td>(489)</td>
<td></td>
</tr>
<tr>
<td>Ob/ob (leptin deficient) 15 weeks of age</td>
<td>AdipoR1 mRNA ↓</td>
<td>AdipoR2 mRNA ↓</td>
<td>Reduced</td>
<td>(83)</td>
<td></td>
</tr>
<tr>
<td>Condition</td>
<td>AdipoR1 mRNA</td>
<td>AdipoR2 mRNA</td>
<td>Change</td>
<td></td>
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<td>--------------------------------------------------------------------------</td>
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<td></td>
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<tr>
<td>Obese Zucker 18 weeks of age</td>
<td>↓</td>
<td>↓</td>
<td>Increased (490)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obese Zucker consuming HF diet for 6 weeks</td>
<td>↑</td>
<td>↑</td>
<td>(425)</td>
<td></td>
<td></td>
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<tr>
<td>Obese Zucker 7 weeks of age</td>
<td>↓</td>
<td>↓</td>
<td>(426)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Db/db (leptin resistant) mice 13 weeks of age</td>
<td>↑</td>
<td>↑</td>
<td>(492)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic mice (streptozotocin induced)</td>
<td>↑</td>
<td>↑</td>
<td>(492)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic mice (streptozotocin induced) diabetic for 1 week</td>
<td>↓</td>
<td></td>
<td>(94)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic mice (streptozotocin induced) diabetic for 7 weeks</td>
<td>↑</td>
<td></td>
<td>(94)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic rats (streptozotocin and high fat/sugar induced)</td>
<td>↓</td>
<td></td>
<td>(493)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic rats (streptozotocin induced) diabetic for 8 weeks</td>
<td>↑</td>
<td></td>
<td>(491)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wistar rats (42% fat) diet for 3 weeks</td>
<td>↓</td>
<td>↑</td>
<td>(494)</td>
<td></td>
<td></td>
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</table>
The APN decrease often precedes the AdipoR1 increase in metabolic tissues, yet the specific mechanism governing these events is unknown. Whereas reduced AdipoR1 abundance may contribute to APN-resistance in early stages of obesity and metabolic disease progression, hypoadiponectinemia or downstream signaling defects are a more likely cause in later stages.

The F344 rat is characterized as a model of chronic insulin resistance (255). By 4 mo of age F344 rats have 2-3 fold elevations in insulin, glucose, and free fatty acid concentrations during both fasted and fed states which is sustained throughout their lifespan. (257). Although insulin resistance does not worsen with age, weight gain and adiposity does increase in female F344 rats (258). Currently, it is unknown if the F344 phenotype becomes severe enough to evoke changes in cardiac AdipoR1 and AdipoR2. In Chapter 6 AdipoR1 and AdipoR2 abundance is characterized in aged F344 female rats on both a chow and a high fat diet.

Alternatively, attenuations in downstream APN targets could contribute to APN-resistance. Diabetic mice and rats demonstrate reduced AMPK phosphorylation in response to APN treatment both under basal and ischemic conditions (94, 495). Reduced basal pAMPK translates to a reduced capacity to upregulate pAMPK during I/R injury and these animals experience greater infarct size and ischemic damage (95, 351). Aging also reduces basal AMPK phosphorylation in cardiac and skeletal muscle of male rats (95, 351, 352, 496, 497). It remains to be tested, but it is likely aging animals also demonstrate APN-resistance. In Chapter 6 alterations in total and phosphorylated AMPK are assessed in the myocardium of F344 rats both on a chow and high fat diet.

Currently, it is unknown if the tendency toward metabolic dysregulation and reduced IT observed in aged women is linked to APN-resistance in cardiac tissue. Normal weight aging women demonstrate normal or even elevated circulating APN concentrations, yet are not protected from I/R injury (11, 363, 391, 393, 396, 397). High APN levels in conjunction with reduced IT in aging women superficially resembles the APN resistance state preceding
hypoadiponectinemia during metabolic disease progression in rodent models (93, 94, 353). Further investigation is needed to determine if APN resistance in association with a moderate metabolic phenotype truly explains CHD risk and reduced IT in aged women. In Chapter 6 we assess IT in the in adult, aged, and aged OVX F344 female rat with an APN infusion. Moreover we also assess the downstream APN target phosphorylated AMPK in the myocardium following I/R injury.
CHAPTER 3

ESTROGEN RECEPTOR BETA DOES NOT INFLUENCE ISCHEMIC TOLERANCE IN THE AGED FEMALE RAT HEART

Introduction

Ischemic heart disease remains the leading cause of morbidity and mortality in aged women, with a 2- to 3-fold increase in incidence following menopause (1). Postmenopausal women are less likely to survive a myocardial infarction compared to age matched men and clinical trials have failed to demonstrate a cardioprotective benefit from chronic ERT (33, 498). Protective effects of E₂ in the myocardium have been demonstrated in adult animal models and are mediated, in part, by ERα and ERβ (24, 36, 55-59). Mouse ER knockout models have provided further insight into the apparent links between ERs and cardiovascular disease. Specifically, βERKO mice become hypertensive by five months of age and develop cardiac hypertrophy (336). Furthermore, female βERKO mice experience greater cardiac damage following I/R injury relative to wild type female mice indicating that ERβ also impacts IT in the female heart (55, 57). Moreover, chronic activation with the ERβ-specific agonist DPN upregulates proteins associated with cardioprotection and improves IT in adult female mice rendered E₂-deficient by OVX (62). These data indicate that chronic ERβ signaling can promote genomic upregulation of cardioprotective proteins over time and contribute to the maintenance of IT in females.
In addition to classical genomic ER signaling, ER subtypes are also capable of rapid, non-genomic signaling following acute activation. Non-genomic signaling through membrane ER subtypes does not require classical nuclear transcription of proteins, but can rapidly activate signaling cascades by triggering kinase activation and increasing Ca^{2+} or NO levels (152). Acute activation of ERα has been shown to improve cardiac IT in aged female rats with varying degrees of E2-deficiency following I/R injury (24, 152). Existence of non-genomic, ERβ-mediated, cardioprotective signaling mechanisms in the female heart have yet to be confirmed. A single study in adult OVX rabbits failed to demonstrate any reduction in infarct size following I/R with acute ERβ activation (155). Furthermore, while both ER subtypes have been observed in neonatal rat cardiac myocytes (36), conflicting reports exist on the presence of ERβ in the adult or aged rat myocardium (38, 98, 300, 331). Therefore, the purpose of the present investigation was to examine the effects of acute ERβ activation on IT in the adult, aged, and aged OVX female F344 rat heart. Here, we tested the hypothesis that acute ERβ activation with the agonist DPN would improve cardiac functional recovery following I/R injury in aged rats.

Methods

Animal Care

Certified specific pathogen free female adult (5–6 months, n = 18) and aged (23 months, n = 40) F344 rats were obtained from Harlan Sprague Dawley (Indianapolis, IN). Rats were singly housed in filter-top cages containing corn-cob bedding and provided with a nylabone or wood block for enrichment. Rats were maintained on a 12 h light/dark cycle in an environment maintained at 22 °C with 20% humidity. Water and chow were supplied ad libitum and chow consisted of a standard laboratory rodent diet, LabDiet 5001 (PMI Nutrition International, St.
Louis, MO) with a caloric breakdown of 28.5% protein, 13.5% fat, and 58% carbohydrate. Noteworthy, the LabDiet 5001 chow is soy-based and contains 810 μg/g of phytoestrogens, which are compounds known to have weak estrogenic activity in vivo. All animal experimentation described was conducted in accordance with accepted standards of the Institutional Animal Care and Use Committee of the Pennsylvania State University.

Ovariectomy

To yield a significantly controlled level of E2-deficiency, a subset of aged F344 rats underwent surgical OVX (n=20). Remaining aged and adult rats underwent sham surgery and all surgeries were performed by the supplier. Following surgery animals were transferred to the Pennsylvania State University and were allowed to recover for four weeks prior to experimental use. Uterine weight was used to confirm E2-deficiency. We have previously documented significantly reduced circulating E2 concentrations in aged (14.6 ±1.4 pg/mL), and aged OVX (7.6 ± 1.1 pg/mL) vs adult intact rats (21.4 ± 1.0 pg/mL) through radioimmuno-assay (24, 499).

Study Design

Animals were randomly assigned to receive either the ERβ agonist, DPN (Tocris, Ellisville, MO; 5 μg/kg body weight; n=9 adult, n=10 aged and aged OVX) or vehicle (10% dimethylsulfoxide; n=9 adult, n=10 aged and aged OVX) in corn oil subcutaneously 45 min prior to euthanasia. The DPN dose was chosen based on efficacy demonstrated in past studies utilizing a trauma-hemorrhage model (206). Study of adult, aged, and aged OVX rats was alternated each day. DPN- and vehicle treated-rats of the same group (adult, aged, and aged OVX) were paired such that n=2 experiments were completed per day. Order of DPN and vehicle treatments were
alternated to control for any influence of circadian rhythm on cardiac function and all experiments were conducted between 9:00am and 2:00pm.

I/R Protocol

Animals were anesthetized with pentobarbital (40 mg/kg body weight; intraperitoneal) and hearts were excised via midline thoracotomy. Pentobarbital does not interfere with cardioprotective cell signaling pathways and was thusly chosen for euthanasia. Hearts were secured to a modified Langendorff perfusion apparatus via the aorta, perfusion pressure was set at 85 millimeters of mercury (mmHg), and hearts were paced at 260 beats/min. LVDP, and positive and negative developed pressure with respect to time (+/- dP/dt_{max}; indices of contractility and relaxation, respectively), were assessed utilizing a pressure transducer attached to a water-filled latex balloon inserted into the left ventricle. EDP was adjusted to 5–6 mmHg according to established procedures in our laboratory (24, 299, 500). After 30 minutes of equilibration, hearts were subjected to 47 min global, isothermic, ischemia as described previously (24). Pacing was re-initiated 1.5 min after the start of reperfusion and hearts were reperfused for 60 min. After reperfusion, left ventricle (LV) tissue was isolated, weighed, halved, and snap frozen in liquid N2. Due to the duration of the isolated heart protocol, LV weights are necessarily increased due to time-dependent edema. All hearts were treated similarly and therefore would not be expected to affect group comparisons. All LV sections were stored at -80°C until tissue preparation.

RNA Extraction and Real-Time PCR

RNA was obtained from frozen LV tissue through acid guanidinium thiocyanate-phenol-chloroform extraction with TriReagent (Sigma Chemical Co., St. Louis, MO) as described by
Chomczynski and Sacchi (501). Extracted RNA was reverse transcribed to cDNA and subjected to quantitative real-time-PCR using the TaqMan 2x Universal Mix (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol (ABI 7300 Real-Time Sequence Detection System). Gene expression levels of ERβ were analyzed by three different sets of primers. Two sets of ERβ primers were manually designed by the Penn State Genomics Core Facility (forward and reverse primers 5’-CACTGCACCTCCCAGGAGTCA-3’ and 5’-AACTTGGCATTCGTTACATAT-3’ as well as forward and reverse primers 5’-TCCCGGCAGCACCAGTAA-3’ and 5’-CCATAGCACATTTGGGCTTGT-3’). A third primer and probe sequence was obtained from Applied Biosystems. The PCR cycling parameters were as follows: 95 °C for 10 min, and 45 cycles of 95 °C for 15 s, 60 °C for 1 min and 25 °C for 2 min. Each sample was analyzed in triplicate and normalized to the housekeeping gene, cyclophilin, using the following equation: \( \Delta C_t_{ER\beta} = C_t_{ER\beta} - C_t_{cyclophilin} \) where \( C_t \) is the linear part of the curve. The fold change in mRNA expression was calculated using the following equation: \( 2^{(\Delta\Delta C_t)} \) where \( \Delta\Delta C_t = \text{mean } \Delta C_t \text{ of ERβ in F344 heart or ovary tissue homogenates.} \)

**Protein Sample Preparation and Western Blotting**

ERβ-transfected human embryonic kidney 293 (HEK293) cells were produced using HEK293 cells obtained from ATCC and transfected using lipofectamine (Invitrogen) with a mouse-ERβ plasmid obtained from the Korach laboratory (502). Total cardiac and ERβ-transfected HEK293 protein lysates were separated using sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE; 10%). Samples were transferred to polyvinylidene fluoride (PVDF) membranes and blocked in 5% non-fat dry milk for 2 hours at room temperature as described, by us, previously (24, 503). Membranes were probed overnight at 4°C with ER (54 kDa, 1:1000; Millipore 07-359). Membranes were then incubated with
horseradish peroxidase (HRP)-linked anti-rabbit secondary antibody 1:20,000 for 1 h at room temperature and visualized using Enhanced Chemiluminescence (ECL; GE Healthcare). Membranes were then stripped in a buffer containing 100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl and reprobed with ERα (67 kDa, Santa Cruz sc-542; 1:1000). Membranes were then re-processed with HRP-linked anti-rabbit secondary and visualized with ECL. Representative films were scanned using an Epson V700 scanner.

**Statistical Analysis**

All data are presented as means ± SE and analyzed using the Statistical Analysis System (SAS) General Linear Model (GLM) procedure. Because DPN treated animals did not differ from untreated animals, data were pooled and a one-way ANOVA was used to analyze morphological characteristic data for adult, aged, and aged OVX animals. A one-way ANOVA was also used to analyze all functional and mRNA data for adult, aged, and aged OVX animals with and without DPN treatment. The Tukey test was used for post hoc analysis. An α-level of $p < 0.05$ was considered statistically significant.

**Results**

**Baseline Characteristics**

Rats with baseline LVDP below 130 mmHg, unstable EDP (> 6 mmHg), or notable pathology/weight loss were excluded from the study and subsequent analysis. Final data analysis was conducted on n=4 adult, n=7 aged, and n=8 aged OVX rats treated with vehicle and n=5 adult, n=6 aged, and aged OVX treated with DPN.
Morphological and baseline characteristics for adult, aged, and aged OVX animals are displayed in Table 3-1. Characteristics did not differ between DPN and vehicle treated rats and baseline data were pooled. Body and LV weight was significantly greater in aged and aged OVX animals compared with adults ($p < 0.05$ and $p < 0.0001$, respectively). A difference in body weight between aged and aged OVX groups was not observed. Uterine weight was significantly reduced with OVX ($p < 0.0001$). No significant group differences were observed in LV weight to body weight ratio, LVDP, end diastolic pressure (EDP), or $\pm/dP/dt$.

### Table 3-1: Morphological and baseline characteristics for adult, aged, and aged OVX female rats.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Adult</th>
<th>Aged</th>
<th>Aged OVX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>200 ± 3</td>
<td>274 ± 5*</td>
<td>282 ± 6*</td>
</tr>
<tr>
<td>LV weight (mg)</td>
<td>546 ± 19</td>
<td>752 ± 14*</td>
<td>758 ± 14*</td>
</tr>
<tr>
<td>LV weight/Body weight (mg/g)</td>
<td>2.73 ± 0.09</td>
<td>2.76 ± 0.07</td>
<td>2.70 ± 0.05</td>
</tr>
<tr>
<td>Uterine weight (g)</td>
<td>0.44 ± 0.04</td>
<td>0.52 ± 0.02</td>
<td>0.28 ± 0.03†</td>
</tr>
<tr>
<td>EDP (mmHg)</td>
<td>5.6 ± 0.1</td>
<td>5.7 ± 0.1</td>
<td>5.7 ± 0.1</td>
</tr>
<tr>
<td>LVDP (mmHg)</td>
<td>136.3 ± 1.6</td>
<td>139.1 ± 1.2</td>
<td>140.2 ± 1.5</td>
</tr>
<tr>
<td>$+dP/dt$ (mmHg/s)</td>
<td>3917 ± 131</td>
<td>3741 ± 88</td>
<td>3848 ± 54</td>
</tr>
<tr>
<td>$-dP/dt$ (mmHg/s)</td>
<td>2484 ± 24</td>
<td>2421 ± 54</td>
<td>2441 ± 43</td>
</tr>
</tbody>
</table>

**Functional Recovery Post I/R Injury**

Significant reductions in LVDP functional recovery were observed following ischemic insult in both aged and aged OVX groups relative to adult intact rats (LVDP ($p < 0.0001$)). Post-hoc analysis revealed significant differences occurred during early reperfusion at min 1-3 in both
aged and aged OVX rats ($p < 0.05$; Figure 3-1). Importantly, acute DPN treatment (5μg/kg) 45 min prior to heart isolation had no effect on LVDP during 60 minutes of reperfusion following ischemia in adult, aged, or aged OVX animals (Figure 3-1). Although not significant, DPN has a tendency to attenuate rather than improve LVDP recovery in adult rats (Figure 3-1 Panel A).

Figure 3-1: DPN treatment does not improve post-ischemic LVDP in the female rat heart. Recovery of LVDP following a 47 min ischemia in adult (Panel A), aged (Panel B), and aged OVX (Panel C). * denotes different from adult; $p<0.05$.

Similar to LVDP, ± dP/dt during reperfusion was significantly reduced in aged and aged OVX female rats (Figure 3-2). Significant differences were observed at min 1-3 of reperfusion for both positive and negative dP/dt. Also in line with LVDP recovery DPN had no significant effect on ±dP/dt recovery during 60 min of reperfusion. The tendency for DPN to attenuate functional recovery in adult, but not aged or aged OVX female rats is also apparent in ±dP/dt recovery (Figure 3-2 Panel A and D).

To bolster our findings indicating no effect of DPN on the F344 female rat heart we assessed an additional 1 mg/kg dose of DPN. Even at 200 times the 5 μg/kg concentration, DPN still had no effect on LV functional recovery in the female rat heart (data not shown).
ERβ mRNA Expression and Protein Abundance

A total of three primer sets were used to assess ERβ mRNA levels in adult and aged female rat myocardium. Ovary highly expresses ERβ mRNA (504-506), and rat ovary homogenate served as a positive control for ERβ mRNA expression. Independent of the primer set utilized, ERβ mRNA was detected in ovary homogenates, but not in cardiac samples. Also, ERβ protein in total LV homogenates could not be detected with western blotting (Figure 3-3).

Figure 3-2: DPN treatment does not improve post-ischemic +/−dP/dt_{max} in the female rat heart. Recovery of dP/dt_{max} following a 47 min ischemia in adult (Panel A and D), aged (Panel B and E), and aged OVX (Panel C and F). * denotes different from adult; p<0.05.
Current evidence suggests that the ischemic adult myocardium may be protected, in part, by activation of ERβ signaling pathways (55, 57, 62). We sought to determine the efficacy of acute ERβ activation on IT in the aged female rat heart following I/R injury. Ischemic heart disease remains the primary cause of morbidity and mortality in post-menopausal women in North America and we hypothesized that acute ERβ activation (via engagement of non-genomic signaling pathways) would improve cardiac functional recovery following ischemic insult in aged female rats. Notably, IT was not augmented by acute treatment with the ERβ-activator, DPN, in hearts isolated from either adult or aged rats. Furthermore, we were unable to confirm the presence of ERβ mRNA or protein, which suggests that ERβ is not expressed in the F344 female rat myocardium.

Figure 3-3: Western blot for ERα and β with total heart homogenate and ERβ-transfected HEK293 cells. ERβ (Panel A) reprobe of stripped ERβ membrane with ERα (Panel B). Lane 1 total cardiac homogenate 30 µg; lane 2 ERβ-transfected HEK293 cells 20 µg.

Discussion

Current evidence suggests that the ischemic adult myocardium may be protected, in part, by activation of ERβ signaling pathways (55, 57, 62). We sought to determine the efficacy of acute ERβ activation on IT in the aged female rat heart following I/R injury. Ischemic heart disease remains the primary cause of morbidity and mortality in post-menopausal women in North America and we hypothesized that acute ERβ activation (via engagement of non-genomic signaling pathways) would improve cardiac functional recovery following ischemic insult in aged female rats. Notably, IT was not augmented by acute treatment with the ERβ-activator, DPN, in hearts isolated from either adult or aged rats. Furthermore, we were unable to confirm the presence of ERβ mRNA or protein, which suggests that ERβ is not expressed in the F344 female rat myocardium.
Body weight increased significantly in aged and aged OVX rats vs adult intact controls, but there was no significant weight gain in aged vs aged OVX female rats. The lack of OVX-induced weight gain was surprising given that we and others have previously documented contrary findings (24, 299). Noteworthy, the particular cohort of rats studied in this investigation has a greater frequency of cardiac tumors than previously observed in our laboratory. Although animals with obvious pathology, neoplastic tumor growth, and associated weight loss ( > 10 g/week) were excluded (7 aged, and 6 aged OVX rats) it is possible OVX-induced weight gain was masked by confounding asymptomatic diseases in the cohort of rats studied.

Consistent with previous findings from our laboratory (500), we observed attenuated functional recovery following I/R injury in aged hearts, with greatest impairment occurring immediately upon reperfusion when activation of cell death programs are known to occur. Contrary to our hypothesis, however, we observed no significant effects of acute ERβ activation on IT in either adult or aged rats with varying degrees of E2-deficiency. Booth and colleagues (155) were also unable to demonstrate improved IT in E2-deficient rabbits with DPN doses as high as 3 mg/kg. Collectively, these findings are at odds with studies utilizing βERKO mice or chronic DPN treatment which suggest that ERβ is a mediator of cardioprotection (55, 57, 58, 62). A logical interpretation of our results is that while classical genomic ERβ activation via chronic stimulation is possible, rapid, non-genomic signaling mechanisms downstream of ERβ may not be operative in the female rodent myocardium. Upon further examination, however, we were unable to detect ERβ mRNA levels in either the adult or aged F344 rat myocardium. Although this is the first study examining ERβ in the F344 female rat, two studies in Sprague Dawley rats have provided evidence indicating ERβ may be present in the myocardium (38, 300). In this regard, ERβ expression in the rodent myocardium remains controversial (38, 98, 227, 300, 331-333). Here, we utilized both commercially available and manually designed ERβ mRNA primer sets to assess ERβ mRNA in the female F344 rat myocardium. Manually designed primers were
based on previously reported sequences (98, 507, 508), and truncated to increase stringency and limit PCR product size to 70-80 base pairs in length. Several ERβ antibodies including those used by Xu (300) were also utilized to substantiate the absence of ERβ in our model. We determined that the signal produced by ERβ antibodies in cardiac homogenates was likely due to cross-reactivity with ERα (see Figure 3-2).

The lack of measureable ERβ in the F344 rat myocardium was surprising given results gleaned from past studies utilizing the βERKO mouse model (55, 57, 58). Combined with these previous findings, our results suggest that either ERβ signaling varies substantially between rat, rabbit and murine models or, more likely that cardioprotection observed in mouse models may be mediated indirectly through extra-cardiac ERβ signaling. Interestingly, DPN injection at the rostral ventrolateral medulla, an area associated with autonomic cardiovascular control, has been shown to reduce systemic arterial pressure in rats (345). That ERβ activation can reduce systemic arterial pressure via autonomic influence indicates that additional autonomic cardioprotective mechanisms attributed to E₂ may be mediated through ERβ. Indeed, E₂-linked cardioprotection has been associated with reduced sympathetic input to the heart and vasculature during ischemia in female rats, resulting in reduced heart rate, mean arterial pressure, arrhythmia frequency, and overall improved IT vs males (509, 510). Therefore, it is plausible that hypertension and vascular dysfunction observed in whole body βERKO mice as well as cardioprotection observed in chronic DPN treated mice may be explained by indirect ERβ effects on autonomic cardiac control and not direct effects on the myocardium (56, 62). Future studies are indicated to address this important issue.

In conclusion, acute ERβ activation had no impact on functional recovery following I/R injury in our model. That we were unable to discern detectable ERβ mRNA or protein in either the adult, aged, or aged OVX female rat heart suggests that direct cardioprotective ERβ signaling is not operative in the female rat heart. Future studies examining extra-cardiac effects of chronic
ERβ stimulation, including vascular and neural mechanisms, may prove useful in elucidating possible therapeutic interventions with aging.
CHAPTER 4

INCREASED ESTROGEN RECEPTOR BETA IN ADIPOSE TISSUE IS ASSOCIATED WITH INCREASED INTRACELLULAR AND REDUCED CIRCULATING ADIPONECTIN PROTEIN LEVELS IN AGED FEMALE RATS

Introduction

The prevalence of obesity and associated metabolic risk in women increases significantly after menopause, such that 40-50% of women aged 50-59 develop metabolic complications according to the most recent report from the National Health and Nutrition Examination Survey (511). However, the signaling alterations by which E2-deficiency increases obesity risk with aging are poorly understood, and development of therapeutic interventions have been stalled by the detrimental effects of hormone replacement on CHD morbidity and mortality in women (33, 498). Interestingly, ER polymorphisms have been linked to increased metabolic and cardiovascular risk in post-menopausal women, indicating a potential important role for ER isoforms α and/or β in regulating adiposity, metabolic derangements and cardiovascular risk (51, 53, 54). In this regard, both ERα and ERβ are present in adipose tissue and appear to reciprocally regulate adiposity (375).

Increased adiposity associated with the development of metabolic derangements such as insulin resistance are observed in αERKO mice (41, 223, 224, 246), thus indicating that ERα plays an important role in positively regulating adipose metabolism. In contrast, selective activation of ERβ exacerbates metabolic phenotypes (41), and ovariectomy of αERKO mice
ameliorates increases in adiposity and development of insulin resistance (61). Thus it is plausible that alterations in ER levels, specifically reductions in ERα and increases in ERβ, not only contribute to increased adiposity with advancing age, but may also play a critical role in increasing metabolic and cardiovascular disease risk in post-menopausal women.

Although the specific mechanisms by which ERs regulate adiposity and adipocyte metabolism are incompletely understood, however, it is known that ERβ inhibits transcriptional activity of PPARγ, a master regulator of insulin sensitivity (60). PPARγ mediates its effects, in part, by regulating the intracellular processing and secretion of APN. Low plasma levels of APN are associated with obesity and metabolic syndrome and predictive of CHD risk (70, 71, 512). It is unknown whether and to what extent age-associated E2-deficiency disrupts the balance of ERα and β in adipose tissue and if APN is the causal link between dysregulated ER signaling and disease.

Similarly, skeletal muscle also expresses both ERα and ERβ and compromised metabolism observed in ERKO mice may also stem from derangements in skeletal muscle. Targeted ERα activation in skeletal muscle has been deemed protective while ERβ activation is pro-diabetogenic (41, 46). Studies describing relative changes in ER content in skeletal muscle are conflicting and one study indicates an increase in ERα with OVX-induced E2-deficiency in mice (273). However, in female rats both the independent and combined effects of diet- and OVX-induced obesity resulted in no change in ERα (215). ERβ protein levels are decreased in soleus, a primarily oxidative muscle, but not in glycolytic muscles in OVX female rats (215). Further studies are clearly needed to better characterize potential changes in ERs associated with aging and E2-deficiency.

Accordingly, the purpose of the present study was to determine the singular and combined effects of aging and E2-deficiency on ER isoform distribution in visceral adipose tissue and skeletal muscle isolated from female rats. We also sought to characterize alterations in the
downstream adipose ER targets PPARγ and APN, which are likely to influence metabolic and cardiovascular disease risk in this model. We hypothesized that increased ERβ protein in the adipose tissue of aged animals would not only be associated with age, and E2-deficiency related weight gain, but also reduced protein levels of PPARγ and circulating APN. As similarly hypothesized in adipose tissue, alterations in the ERα/β balance in skeletal muscle may also point to a mechanism for the development of metabolic and cardiovascular disease risk in association with aging and E2-deficiency. Finally, to gain an appreciation of adipocyte autocrine signaling derangements in a setting of aging-associated E2-deficiency, we assessed AdipoR1 and AdipoR2, as well as downstream regulators of lipid metabolism PPARα and AMPK in adipose tissue.

**Methods**

**Animal Care**

Certified specific pathogen free female adult (5–6 months, n = 11) and aged (23 months, n = 11) F344 rats were obtained from Harlan Sprague Dawley (Indianapolis, IN). Rats were singly housed in filter-top cages containing corn-cob bedding and provided with a nylabone or wood block for enrichment. Rats were maintained on a 12 h light/dark cycle in an environment maintained at 22 °C with 20% humidity. Water and chow were supplied *ad libitum* and chow consisted of a standard laboratory rodent diet, LabDiet 5001 (PMI Nutrition International, St. Louis, MO) with a caloric breakdown of 28.5% protein, 13.5% fat, and 58% carbohydrate. Noteworthy, the LabDiet 5001 chow is soy- based and contains 810 μg/g of phytoestrogens, which are compounds known to have weak estrogenic activity *in vivo*. All animal experimentation described was conducted in accordance with accepted standards of the Institutional Animal Care and Use Committee of the Pennsylvania State University.
**Ovariectomy**

To yield a significantly controlled level of E$_2$-deficiency, a subset of adult (4 mo, n=6) and aged (22 mo, n=6) F344 rats underwent OVX. All OVX surgeries were performed by the supplier. Following surgery animals were transferred to the Pennsylvania State University and were allowed to recover for four weeks prior to experimental use and thus were age-matched to intact animals. Uterine weight was used to confirm E$_2$-deficiency. We have previously documented significantly reduced circulating E$_2$ concentrations in aged (14.6 ±1.4 pg/mL), and aged OVX (7.6 ± 1.1 pg/mL) vs adult intact rats (21.4 ± 1.0 pg/mL) through radioimmuno-assay (24, 499).

**Study Design**

Rats were anesthetized using a sodium pentobarbital intra-peritoneal injection, sacrificed, and tissue collected. Perimtrial and retroperitoneal adipose depots were pooled and utilized for adipose studies. The left gastrocnemius and soleus muscle were utilized for skeletal muscle studies. All tissues were collected between 9:00am and 2:00pm and study of adult, adult OVX, aged, and aged OVX rats were alternated each day. Tissue was snap frozen in liquid N$_2$ and stored at -80°C until further analysis.

**Serum Adiponectin Levels**

Serum concentration of total APN was assessed using an enzyme-linked immunosorbent assay (ELISA) (EZRADP-62K; Millipore) according to manufacturer’s instructions.
Tissue Homogenization

Adipose and skeletal muscle tissue (~1 gm) was homogenized with a Polytron (Kinematica) in 3.5 volumes of buffer containing (in mM): 40 HEPES, pH 7.4; 4 EGTA; 100 NaF; 50 KCl; 1 EDTA; 100 β-glycerol phosphate; 2 benzamidine; 1 Na-orthovanadate; 0.4 aminoethyl benzenesulfonyl fluoride; 6.5 CHAPS; 1% Triton X-100, and 0.2μg/ml of microcystin. Homogenates were centrifuged at 4°C for 10 min at 10,000 X g and the supernatant was assessed for protein concentration using the method of Bradford (513).

Western Blotting

Protein lysates were subject to separation using SDS-PAGE, transferred to PVDF membranes and blocked in 6% non-fat dry milk for 2 hours at room temperature as described, by us, previously (24, 500, 514). Membranes were probed overnight at 4°C with primary antibodies purchased from Santa Cruz Biotechnology Inc. at a concentration of 1:1000 for APN (30 kDa, sc-26497), ERα (66 kDa, sc-542), ERβ (56 kDa, sc-8974), PPARγ (67 kDa, sc-7196); and 1:300 for PPARα (55 kDa, sc-9000). Cell Signaling antibodies were used at a concentration of 1:500 for Thr-172 phosphorylated AMPK (pAMPK) (62 kDa, 2531) and 1:1000 for Erp44 (44 kDa, 2886S). A concentration of 1:1000 was used for both AdipoR2 (42 kDa, AdipoR2-1; α-Diagnostics) and AMPK (63 kDa, 07-350; Millipore). AdipoR1 (42 kDa), a gift from R. Ramachandran, was used at a concentration of 1:1000. Membranes were then incubated with either HRP-linked anti-rabbit 1:20,000 for ERα, PPARα, PPARγ, Erp44, AMPK, pAMPK, AdipoR1, and AdipoR2 or HRP-linked anti-goat at 1:25,000 for ERβ and APN for 1 h at room temperature and visualized using ECL (GE Healthcare). Densitometry was performed using Scion Image (NIH). To correct for potential protein loading errors all membranes were stained.
with SYPRO Ruby Blot Stain or Ponceau S (100 μg protein/lane) and densitometry performed as previously described (24, 299, 500, 514).

Non-Reducing Western Blotting for Adiponectin Multimers

Protein lysates were subject to separation under non-reducing conditions. Electrophoresis was performed on a gradient gel 4-20% (BioRad) without the reducing agent, β-mercaptoethanol in the sample buffer, and without SDS in the sample and electrophoresis buffers. Samples were transferred and blotted for APN as indicated in standard western protocol.

Statistical Analysis

All data are presented as means ± SE and analyzed using the SAS GLM procedure. A two-way ANOVA was used to analyze all western data with age x E\textsubscript{2} status as the interaction term. Post hoc analysis was performed on significant interactions using a Tukey test. An α-level of \( p<0.05 \) was used for all comparisons and considered statistically significant.

Results

Animal Characteristics

As demonstrated by us previously (24), rat weight was significantly increased with age (Table 4-1; \( p<0.01 \)). OVX resulted in a significant weight gain (14%) in aged OVX, animals Adult OVX rats experienced a 5% increase in body weight, but the change failed to reach
significance. Uterine weight decreased significantly with OVX in both adult and aged groups (Table 4-1; p<0.0001), confirming successful OVX surgery and relative E2-deficiency.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Adult</th>
<th>Adult OVX</th>
<th>Aged</th>
<th>Aged OVX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Weight (g)</td>
<td>202.7</td>
<td>213.3</td>
<td>270.8*</td>
<td>307.8*†</td>
</tr>
<tr>
<td>Uterine Weight (g)</td>
<td>0.6</td>
<td>0.1†</td>
<td>0.6</td>
<td>0.3†</td>
</tr>
</tbody>
</table>

**Estrogen Receptors, PPARγ, and Erp44 Protein Levels**

Immunoblotting for ERα, ERβ, PPARγ, and Erp44 was performed in visceral adipose tissue isolated from gonadal and retroperitoneal depots of adult and aged rats and group differences were expressed relative to levels in adult ovary-intact animals. ERα was additionally examined in skeletal muscle. ERα levels showed no significant change with age or E2-deficiency relative to adult control in adipose or soleus tissue (Figures 4-1A and 4-2A). However, aged OVX animals demonstrated a significant increase in ERα relative to adult OVX (Figure 1A). In gastrocnemius muscle there is a significant increase in ERα in aged OVX samples only (Figure 4-2B). We were unable to detect ERβ in skeletal muscle, but we did observed a two-fold increase in ERβ with age when compared to both adult intact and OVX animals (Figure 4-1B).
Figure 4-1: The effects of aging and E2-deficiency on estrogen receptor α (ER)α and ERβ protein levels in adipose. Representative blots and adipose protein levels for ERα (Panel A); and ERβ (Panel B). *denotes age effect, ‡ denotes significantly different from adult OVX; (p < 0.05; n=5-6/group). Values are means ± SEM; data is presented relative to adult intact, and corrected with Sypro Ruby blot stain.
Figure 4-2: The effects of aging and $E_2$-deficiency on estrogen receptor $\alpha$ (ER)$\alpha$ protein levels in skeletal muscle. Representative blots and adipose protein levels for ER$\alpha$ in soleus (Panel A); and ER$\alpha$ in gastrocnemius (Panel B). † denotes significantly different from adult intact, ‡ denotes significantly different from adult OVX, § denotes significantly different from aged intact; ($p < 0.05$; $n=5-6$ group). Values are means ± SEM; data is presented relative to adult intact, and corrected with Sypro Ruby blot stain.
Interestingly, PPARγ, which plays a significant role in adipose metabolism, decreased with age and OVX (Figure 4-3A). Finally, there was a tendency for Erp44 protein levels to increase with E2-deficiency in adipose tissue isolated from both adult and aged female rats (p=0.09; Figure 4-3B).

Figure 4-3: Peroxisome proliferator activated receptor γ (PPAR)γ is reduced with age and E2-deficiency while endoplasmic reticulum protein of 44 kDa (Erp44) is unchanged. Representative blots and adipose protein levels for PPARγ (Panel A) and Erp44 (Panel B). * denotes age effect, † denotes OVX effect; (p < 0.05; n=5-6/group). Values are means ± SEM; data is presented relative to adult intact, and corrected with Sypro Ruby blot stain.
Adiponectin, Adiponectin Receptors, and Downstream Targets AMPK and PPARα Protein Levels

To investigate potential dysregulated autocrine signaling related to ERs in adipose tissue with advancing age and E2-deficiency, we assessed serum APN, intra-adipocyte APN, and its receptors, AdipoR1 and AdipoR2. Circulating APN levels increased by 50 percent with OVX in adult, while reductions of 35 percent and 40 percent were observed in aged and aged OVX, respectively, relative to adult and adult OVX (Figure 4-4A; p<0.05). In contrast, immunoblotting revealed that total APN protein levels were increased in aged OVX animals (Figure 4-4B; p<0.05). AdipoR1 levels increased in aged OVX (Figure 4-4C p=0.056). AdipoR2 levels were significantly decreased with age (Figure 4-4D; p<0.05).
Figure 4-4: Total intra-adipocyte adiponectin (APN) is significantly increased with age-associated E2-deficiency while circulating APN is significantly increased with OVX and reduced with age. Adiponectin Receptor 1 (AdipoR) 1 is unchanged with age or E2-deficiency while AdipoR2 is decreased with age. Circulating APN (Panel A), representative blots and adipose protein levels for APN (Panel B), AdipoR1 (Panel C), and AdipoR2 (Panel D). * denotes age effect, † denotes significantly different from adult intact, ‡ denotes significantly different from adult OVX, § denotes significantly different from aged intact; (p < 0.05; n=5-6/group). Values are means ± SEM; data is presented relative to adult intact. APN and AdipoR2 are corrected with Sypro Ruby Blot Stain and AdipoR1 is corrected with Ponceau S.
Under non-reducing conditions we assessed the relative abundance of each APN multimer (Figure 4-5). HMW APN increased in all groups relative to adult controls (Figure 4-5; Panel A). MMW, LWM and monomeric APN is increased in aged OVX animals only (Figure 4-5; Panels B-D).

Figure 4-5: Representative blots and adipose protein levels for adiponectin (APN) multimers processed under non-reducing conditions. High molecular weight (HWM) APN (Panel A), MMW APN (Panel B), LWM APN (Panel C), monomeric (Panel D); representative non-reducing blot (Panel E). * denotes significantly different from adult intact, † denotes significantly different from adult OVX, ‡ denotes significantly different aged; (p < 0.05; n=5-6/group). Values are means ± SEM; data is presented relative to adult intact.
PPARα was unchanged with age or E₂-deficiency (Figure 4-6). Similarly, AMPK and pAMPK levels were unchanged with age-associated E₂-deficiency (Figure 4-7A and B; p=0.39 and p=0.29, respectively). However, the ratio of active pAMPK to AMPK was significantly decreased with age (Figure 4-7C; p<0.05).
Figure 4-7: Adenosine monophosphate-dependent protein kinase (AMPK) and activated AMPK (pAMPK) protein levels are unchanged, yet the pAMPK to total AMPK ratio is decreased with age-associated E2-deficiency. Representative blots for AMPK and pAMPK and adipose protein levels for AMPK (Panel A), pAMPK (Panel B), pAMPK to AMPK ratio (Panel C). * denotes age effect; (p < 0.05; n=5-6/group). Values are means ± SEM; data is presented relative to adult intact, AMPK and pAMPK are corrected with Sypro Ruby Blot Stain.
Discussion

The primary focus of the current investigation was to examine, for the first time, links between increased adiposity known to occur with age-associated E2-deficiency, ER protein levels and APN processing and secretion signaling in visceral adipose tissue of female rats. We also characterized ERα protein changes associated with aging and estrogen in skeletal muscle. Given the interesting increases in ERβ protein observed in adipose with aging we further investigated potentially linked APN autocrine signaling derangements in aged and E2-deficient adipose tissue, including downstream regulators of lipid metabolism PPARα and AMPK. Collectively, the results presented herein suggest increased ERβ protein levels are associated with compromised APN secretion, as well as reduced AdipoR2 levels thus providing potentially important observations regarding increased metabolic risk in aged women.

Evidence from αERKO models indicate that unopposed ERβ signaling promotes weight gain and a metabolic phenotype in adult mice (61, 224, 246). Previous data also show that ERα is reduced with OVX induced E2-deficiency in adipose of adult rats, and we observed a similar phenomenon in the current study (Figure 4-1A) (515). We also observed a significant increase in ERα in adipose of aged OVX vs adult OVX animals which underscores the importance of using an aging model to elucidate the combined effects of age and E2-deficiency to study metabolic phenotypes. ERα was increased in aged OVX animals in gastrocnemius, but not soleus muscle. ERα is known to be more abundant in muscles containing glycolytic muscle fibers like the gastrocnemius, and likely plays a more important role in regulating metabolism here rather than in oxidative muscle like the soleus where we observed no significant change in ERα. With aging, a decline in glycolytic fibers (and presumably ERα protein) occurs (264, 265). However, as demonstrated by our model as well as others, OVX exerts a significant effect on ERα and the overall result is an increase in relative protein (273). Regardless of changes in ERα, the most
striking finding we observed was a two-fold increase in ERβ in adipose of both aged and aged OVX rats. That we observe greatest weight gain in aged E2-deficient animals co-incident with a robust increase in ERβ suggests that ERβ alone, or perhaps the shift in the ERα/β ratio favoring ERβ, may play a role in the development of age-associated increases in adiposity. We were unable to detect ERβ in either soleus or gastrocnemius muscle.

Because ERβ is known to negatively regulate PPARγ transcriptional activity in 3T3-L1 cells and PPARγ activity is also enhanced in βERKO mice (60), we assessed PPARγ levels as a potential indicator of aberrant ERβ signaling in adipose. Indeed, we observed significant reductions in PPARγ protein levels with age-associated E2-deficiency, which may limit the downstream signaling capacity of PPARγ. Because PPARγ can upregulate APN, and in turn, positively affect glucose and lipid metabolism in insulin sensitive tissues (388, 516-518), we also assessed a PPARγ-responsive protein associated with intra-adipocyte APN retention, Erp44 (Figure 4-2B).

In this regard, we observed increased levels of circulating APN in OVX rats which are similar to findings observed in adult mice (408). Previous findings also indicate reduced circulating APN levels with aging in association with metabolic and cardiovascular disease status (70, 71, 512). Here, reduced circulating APN levels in aged OVX rats were associated with elevated intra-adipocyte APN protein levels. In fact, HMW, MMW, LMW, and monomeric APN were all increased in aged OVX animals relative to adult animals in adipose tissue (Figure 4-5 Panel E). Collectively, these findings suggest the possibility of APN retention in adipocytes of aged E2-deficient animals, thereby providing a mechanism to increase metabolic risk. Indeed, Erp44, which acts to retain and prevent secretion of APN during post-translational processing (370), showed a tendency to increase in both adult and aged OVX animals (p=0.09). It is also noteworthy that Erp44 is transcriptionally downregulated by PPARγ in adipocytes (370). The observed reductions in PPARγ protein levels and/or negative regulation by ERβ in aged OVX rats
could promote disinhibition of Erp44 and subsequently increase APN retention. Although speculative at this time, our results provide evidence for an ERβ-driven mechanism explaining APN retention and/or secretion failure in aged and E2-deficient female rats (summarized in Figure 4-8), although additional studies are indicated to investigate this important issue.

Finally, reduced autocrine signaling in adipose may also play a role in further exacerbating adiposity and metabolic disease in aged E2-deficient animals. Thus we examined protein levels for both AdipoR1 and 2 in adipose tissue. While no group differences were observed for AdipoR1, AdipoR2 was significantly decreased with age. Reduced AdipoR2, with unchanged R1 levels has also been observed in diabetic rats indicating, as expected, aged E2-deficient animals are likely progressing to a metabolic phenotype (519). Metabolic dysregulation associated with age and E2-deficiency in our model is further evidenced by the significant decrease in the pAMPK/AMPK ratio in aged and aged OVX animals, and while speculative, may result in impaired pAMPK-driven processes like glucose uptake and fatty acid mobilization in adipocytes (424, 520) (model summarized in Figure 4-8).

Figure 4-8: Proposed signaling model for effects of age-associated E2-deficiency on ERβ signaling in adipose tissue and potential links to increased obesity, metabolic, and cardiovascular disease risk through dysregulated APN signaling.
Conclusions

We have demonstrated that the unique state of E2-deficiency in aged rats is associated with an altered ERα/β ratio in favor of ERβ in adipose tissue. In skeletal muscle we observed significantly increased ERα in gastrocnemius of aged OVX rats relative to aged intact, adult OVX, and adult intact controls. In adipose tissue with increased ERβ protein levels we also observed decreased PPARγ and decreased circulating APN levels which set the stage for increased adiposity and subsequent metabolic dysregulation in aged E2-deficient animals. Compromised APN processing and/or secretion to the circulation may be driven by reductions in PPARγ signaling and subsequent disinhibition of Erp44, thus providing a potential explanation for the apparent disconnect between increased intra-adipocyte and reduced circulating APN protein levels in aged E2-deficient rats. In conjunction with decreased circulating APN levels we also observed decreases in AdipoR2 and the pAMPK/AMPK ratio. Decreased autocrine signaling in adipose tissue mediated by APN and its downstream targets may result in a reduced capability to metabolize lipids in adipose and may, in part, explain increased weight gain in aged animals. Further studies within the context of aging and E2-deficiency are required to determine if post-menopausal weight gain, metabolic syndrome, and CHD risk are linked through reduced APN secretion, and ERβ-driven derangements in adipose tissue.
CHAPTER 5

EXAMINING THE EFFECTS OF ESTROGEN RECEPTOR BETA ON ADIPONECTIN ON 3T3-L1 CELLS

Introduction

Aberrant ER β signaling has been implicated in promoting adiposity and indirectly increasing obesity-associated CHD risk (46, 51, 55, 60, 61, 245, 289, 328, 521). Previously we have shown ERβ protein abundance is increased in adipose tissue of aged female rats, a model demonstrating reduced myocardial ischemic tolerance (409). However, the functional implication of ERβ abundance and/or activation in adipocytes on CHD risk is not completely understood. Interestingly, APN, a cardioprotective protein produced in adipose tissue is reduced in aging female rats. We propose that increased ERβ expression and/or ligand-dependant activation reduces APN in adipose tissue, thereby contributing to CHD risk in association with increased adiposity.

The interaction between ERβ and APN in adipose tissue has been linked through the master regulator of metabolism, PPARγ (60, 522). ERβ overexpression negatively regulates PPARγ transcriptional activity, which in turn likely prevents positive regulation of PPARγ’s downstream target, APN (60). While the relation between ERβ overexpression and PPARγ suppression has been clearly established, the exact impact of ERβ on APN remains unstudied.

The purpose of this study was to determine the role of ERβ on intracellular APN synthesis and secretion mechanisms. APN is a complex protein and undergoes multiple
posttranslational modifications prior to intra-adipocyte assembly into its three biologically active forms: LMW, MMW, and HMW APN (361). PPARγ upregulates APN via several mechanisms including direct transcriptional activation as well as regulation of APN processing and assembly via modulation of the APN chaperone proteins, Erp44 and Ero1-Lα (369, 370, 372, 374, 389). In order to directly determine a mechanism by which ERβ suppresses APN we selected the adipocyte-like 3T3-L1 cell line to overexpress ERβ in vitro. The 3T3-L1 cell line is a well established model for studying adipocyte maturation and function (523). Herein, we sought to develop a suitable model to characterize the effects of ERβ overexpression and activation on APN.

**Methods**

**Cell Culture**

Mouse 3T3-L1 fibroblasts and HEK293 cells were purchased from American Type Culture Collection (ATCC). Cells were maintained in 10 cm plates (3X10^5 cells/plate) in a humidified atmosphere with 5% CO_2_ at 37°C. Phenol red is a weak ER agonist, therefore cells were maintained in phenol-free basal culture media (high glucose Dulbecco’s Modified Eagle Media; Invitrogen) supplemented with 10% fetal calf serum (FCS; ATCC), 10 µg/mL ciprofloxacin (Sigma), 1 mM sodium pyruvate (Invitrogen), and 1X non-essential amino acids (Invitrogen). Ciprofloxacin was utilized to prevent mycoplasma and bacterial infection in cultures (524).
**3T3-L1 Differentiation**

Three days post-confluence, differentiation was induced in 3T3-L1 cells with basal medium supplemented with 500 μM 3-isobutyl-1 methylxantine, 250 nM dexamethasone, and 330 nM insulin (differentiation medium) as previously described (525). Cells were maintained with differentiation medium for 48 h, and then treated with basal medium supplemented with 330 nM insulin for an additional 48 h. To complete the differentiation process, cells were maintained with basal medium and differentiated for an additional 6-8 days; total differentiation time was 10-12 days. To confirm 3T3-L1 cell differentiation, representative samples from each experiment were stained with Oil Red O staining and assessed for lipid accumulation (0.5% Oil Red O in 60% isopropanol).

**Plasmid Purification and Linearization**

Human and mouse ERβ plasmids (Figure 5-1) were a gracious gift from Dr. Kenneth Korach, (NIEHS, Research Triangle Park, NC). Plasmids were prepared using the Qiagen Plasmid Purification Maxi kit according to manufacturer’s instructions (526, 527). Following purification, an aliquot of both mouse and human ERβ plasmids were digested with restriction enzymes (New England Biolabs) for 1 h at 37°C to yield linearized plasmid products see Figure 5-2 for restriction enzyme cut sites. To generate potentially easier to transfect products, circular plasmids were digested with Scal (R0122S) to yield a linearized plasmid 7 kb in size. SSpI and PciI (R0132S; R0655S) were used to excise the ampicilin sequence and yield a 5 kb product. BciVI (R0596S) was used to digest and separate the ERβ encoding region and promoter containing sequence to yield a 3 kb product. Digested plasmid products were separated electrophoretically using 1.5% agarose gel, and stained with ethidium bromide to visualize bands.
Linearized plasmid products were excised and isolated with Qiaex II Gel extraction kit (Qiagen) per manufacturer’s instructions.

Figure 5-1: Korach ERβ mouse construct.
Figure 5-2: ERβ mouse construct with restriction enzyme cut sites identified.
Determination of Optimal Transfection Conditions

**Lipofectamine LTX**

3T3-L1 and HEK293 cells were plated in 6-well plates (1X10^5 cells/well) in basal medium. After 24h, cells were 70-80% confluent and transfected with either mouse or human ERβ constructs. A pcDNA3 vector without an ERβ insertion was utilized as a negative control. Intact circular construct as well as linearized plasmid products of 7, 5, and 3 kb were utilized for all transfection studies and 50-200 ng (for HEK293 cells) or 1-1.5 μg (for 3T3-L1 cells) of DNA construct was transfected. On the day of transfection, plasmids were incubated for 30 min with Lipofectamine LTX (Invitrogen) in serum free media according to manufacturer’s instructions. Basal medium was changed immediately prior to addition of plasmid/Lipofectamine mixture to 3T3-L1 and HEK293 cells. Basal media was changed 6 h post-transfection. Cells were assayed for mRNA expression or protein abundance 24-48 h post transfection with q-RT PCR and western blotting, respectively. Detailed methods describing mRNA isolation and protein homogenate preparation are described in the respective sections contained herein.

**FuGene6**

A subset of 3T3-L1 cells was transfected with FuGene6 reagents according to manufacturer’s instructions. Cells and plasmids were prepared in the same conditions and concentrations as described in the Lipofectamine LTX section, but FuGene6 reagents were substituted for Lipofectamine LTX reagents.
AMAXA

A subset of 3T3-L1 cells (1 × 10⁶ cells / well) was transfected with 2 μg of plasmid DNA using AMAXA nucleofactor solution (Lonza) following manufacturer’s instructions. Cells were assayed 24-48 h following transfection for mRNA expression.

Stable Transfection

The Lipofectamine protocol was utilized for all stable transfection assays. However, in place of basal media, media supplemented with 500 μg/mL neomycin (G418; Sigma) was added to cells 6 h post transfection. 3T3-L1 cells were maintained on G418 containing selection media for four weeks until 80% confluence was achieved. Cell lysates were assayed immediately for ERβ mRNA and protein abundance.

RNA Isolation and qRT-PCR

Following two washes in ice cold PBS mRNA was isolated from 3T3-L1 and HEK293 cells using an RNeasy kit (Qiagen) per manufacturer’s instructions. RNA concentration was determined spectrophotometrically (Beckman DU60) using the 260/280 ratio method. Complementary DNA (cDNA) was synthesized from mRNA using a high capacity cDNA reverse transcription kit (Applied BioSystems) under the following conditions; 25°C for 10 min, 37°C for 120 min, then 85°C for 5 min using an iCycler (BioRad). From this reaction, 100 μg of cDNA and 25 ng of mouse or human ERβ primers were used per qRT-PCR reaction with SensiMix SYBR master mix (Bioline). Primers were designed for mouse and human ERβ using Geneious Basic software version 5.0 and targeted to the conserved n-terminal domain to detect all splice variants. Primers were generated by the Penn State genomics core facility (Table 5-1).
amplification conditions were 95°C for 10 min, then 40 cycles of 95°C, 55°C, and 72°C for 15 sec using the BioRad iCycler.

Table 5-1 Primers for detection of human and mouse ERβ

<table>
<thead>
<tr>
<th>ERβ mouse primer</th>
<th>Forward (5’→3’)</th>
<th>Reverse (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GAAGCTTGCGGGAGCGGTT</td>
<td>TGCAGACGGCGACAGAAGTGA</td>
</tr>
<tr>
<td>ERβ human primer</td>
<td>CTGCCAGGCCCTGCGACCTTC</td>
<td>CGCACAAGGGCGGTACCCACA</td>
</tr>
</tbody>
</table>

Protein Homogenate Preparation

Cells were washed with ice cold phosphate buffered saline and homogenized in lysis buffer (150 mM NaCl, 40 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM NaF, 0.5 μg/mL pepstatin A, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 1 mM PMSF, 1mM sodium ortho-vanadate, and 1% Triton X100; Sigma). Cells were lysed during one freeze/thaw cycle (-80°C/4°C) and then centrifuged at 10,000Xg at 4°C for 10 min. The supernatant was assessed for protein concentration using the method of Bradford prior to western blotting (513).

Estrogen Receptor Activation

For all ER activation studies, differentiated 3T3-L1 cells were treated with PPT, DPN (Tocris Bioscience), 17-β estradiol (E_2; Sigma), or vehicle (0.01% ethanol). To control for any non-specific ER activation, cells were maintained in basal media containing charcoal stripped FBS (Invitrogen) for 12 h prior to ER agonist treatment. Charcoal stripping not only reduces E_2 – content, but also the concentration of all other hormonal/growth factors in serum. Pre-incubation of 3T3-L1 cells in serum-free media resulted in notable cell death during experimentation and
cells were thusly maintained in the low-hormone, low-growth factor containing charcoal-stripped FBS for the duration of the experiment. Cells were then dosed with 10μM PPT, DPN, E₂ or vehicle for 24 h, and then acutely activated for 3 minutes with 100 nM insulin. Cells were immediately harvested in ice cold lysis buffer, and assessed for protein kinase B (Akt) phosphorylation with western blotting.

**Western Blotting**

Protein lysates were subject to separation using SDS-PAGE, transferred to PVDF membranes and blocked in 5% non-fat dry milk for 2 h at room temperature as previously described (24, 500, 514). Membranes were incubated overnight at 4°C with primary antibodies at a dilution of 1:1000. For ERβ detection three commercially available antibodies were used: ERβ H-150 (Santa Cruz; sc-8974), and ERβ Y-19 (Santa Cruz; sc-6831); and ERβ clone 68-4 (Millipore; 05-824). For ER activation studies, Akt (Cell signaling; 9272) and serine 473 phosphorylated Akt (pAkt; Cell Signaling 9271) antibodies were assessed. All membranes were then incubated with either HRP-linked anti-rabbit secondary antibody at a dilution of 1:20,000 except for ERβ Y-19 which was incubated with HRP-linked anti-goat secondary antibody at a dilution of 1:25,000 for 1 h at RT and proteins visualized by ECL (GE Healthcare). To control for loading errors membranes were then stripped and reprobed with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Sigma) primary antibody and HRP-linked anti-mouse secondary antibody at a dilution of 1:20,000. Densitometry was performed using Scion Image (NIH) as previously described (24, 299, 500, 514).
Total and HMW Adiponectin Assessment

Differentiated 3T3-L1 cells were treated with DPN or ethanol vehicle for 12 and 24 h. Time points were chosen based on previous ER activation studies (528-530). Cells were immediately homogenized in lysis buffer and assessed for APN content utilizing a total/HMW APN ELISA kit (47-ADPHU-E01; ALPCO) according to manufacturer’s instructions. Culture media was also assessed for total and HMW adiponectin. APN abundance was assessed at a range of DPN doses (100 pM to 1 µM) at both 12 and 24 h time points; n=3/treatment/time point.

Statistics

For ER activation and APN assessment studies a two way ANOVA was conducted for time, drug, and time X drug interactions. A Tukey test was used for all post hoc analysis and a P-value < 0.05 was considered statistically significant.

Results

Estrogen Receptor β Overexpression in 3T3-L1 Cells

ERβ mRNA increased 1000-fold in 3T3-L1 cells 24 h following transfection with ERβ constructs compared with non-transfected controls (Figure 5-3A). However, induction of mRNA did not result in concomitant increase in ERβ protein abundance. In an attempt to optimize transfection efficiency in 3T3-L1 cells, three transfection reagents were utilized Lipofectamine LTX, FuGene6, and AMAXA. The lipid-based transfection reagents Lipofectamine LTX and FuGene6 did not differ in transfection efficacy in 3T3-L1 cells (data not shown). The AMAXA electroporation system resulted in substantial cell death and was deemed too harsh a transfection
method for 3T3-L1 cells. Lipofectamine LTX reagents were utilized for the remainder of transfection studies presented herein. Finally, excluding ciprofloxacin from the culture media during transfection did not improve efficiency.

To further optimize transfection efficiency, both mouse and human ERβ plasmids were linearized with restriction enzymes prior to transfection. The circular human and mouse ERβ plasmids are 7 kb in size and intact constructs may be too large to transfect into 3T3-L1 cells. Three different digests were completed to yield a full length 7 kb plasmid, a 5 kb product lacking ampicilin resistance genes, and a 3 kb fragment containing only the ERβ coding sequence and promoter for both mouse and human ERβ constructs. No difference was observed in relative transfection efficiency between mouse and human ERβ constructs in 3T3-L1 cells (data not shown). Moreover, linearizing plasmids prior to transfection did not improve transfection efficiency in 3T3-L1 cells; ERβ mRNA expression was not different in linearized vs circular plasmid transfected (Figure 5-3A).

To determine if it was indeed possible to achieve concomitant increases in mRNA expression and protein abundance following ERβ transfection, we additionally utilized HEK293 cells. HEK293 cells are a readily transfectable cell line and following transfection ERβ mRNA expression was increased 20,000 fold relative to non-transfected controls. More importantly we successfully demonstrated ERβ protein presence in transfected HEK293 cells with western blotting (Figure 5-3 B). The migration pattern of ERβ did differ between transfected HEK293 cells and the F344 rat ovary homogenate positive control. The transfected ERβ construct derived from murine ERβ has a predicted Mr of 53 kDa and rat ovary contains three ERβ splice variants with predicted Mr of 54.2, 59.2 and 61 kDa (506, 531, 532). We also determined that 100 ng of plasmid DNA was sufficient for ERβ mRNA overexpression in HEK293 cells, which is 10-fold less than the 1 µg necessary for maximal transfection 3T3-L1 cells.
A. 

B. 

C. 

D. 

E. 

75- 

50- 

37- 

Non 

Trans 

Stable 

3T3-L1 

HEK293 

Ovary
Figure 5-3: ERβ mRNA expression and protein abundance in transfected 3T3-L1 and HEK293. ERβ mRNA expression in transiently transfected 3T3-L1 cells (Panel A), stably transfected in 3T3-L1 cells (Panel B), transiently transfected HEK293 cells (Panel C), stably transfected HEK293 cells (Panel D). Representative blot of ERβ protein in transfected 3T3-L1 and HEK239 cells (Panel E); non-transfected (non) transient transfection (trans), stable transfection (stable).
We additionally explored stable transfection to increase ERβ expression in 3T3-L1 cells. However, instead of increasing transfection efficiency, stable transfection reduced mRNA expression in both 3T3-L1 and HEK293 cells (Figure 5-3C and D). Moreover no ERβ protein could be detected in either 3T3-L1 or HEK293 cells (Figure 5-3E).

**Total and High Molecular Weight Adiponectin Assessment**

Based on our inability to significantly overexpress ERβ we explored an alternative approach examining ligand-dependant ERβ activation. Differentiated 3T3-L1 cells express endogenous ERs. We activated ERβ with the agonist DPN in differentiated 3T3-L1 cells to examine ligand-dependant ERβ activation on APN intracellular processing and secretion. To determine if ERβ could affect the relative distribution of APN multimers, HMW and total APN were assessed in cell lysates and culture media. Neither total nor HMW APN abundance changed in DPN treated cells relative to vehicle treated control cells. DPN doses ranging from 100 pM to 1 µM did not affect APN protein secretion; representative data for the 100 pM DPN dose is shown in Figure 5-4. HMW APN did decrease in cell lysates and increase in media after 24 vs 12 h in both control and DPN treated cells.
Finally, we assessed activation Akt in 3T3-L1 cells to determine ER responsiveness. Lack of APN response to DPN treatment in 3T3-L1 cells may be due to a defect in endogenous ER signaling. As a positive control for ER responsiveness we repeated the ER activation experiments of Muraki et al (530). As previously shown, we did observe insulin-activated Akt

**Figure 5.4:** Total and high molecular weight (HMW) adiponectin (APN) obtained from cell lysates (intracellular) and culture media (secreted) after 12 or 24 h of ERβ activation in differentiated 3T3-L1 cells. Intracellular total APN (Panel A), intracellular HMW APN (Panel B), secreted total APN (Panel C), and secreted HMW APN (Panel D) are not affected by 100pM DPN treatment. *denotes effect of time; (p<0.05; n=3 samples/group; all experiments were repeated twice). Values are means ± SEM; data is presented relative to 12 h vehicle treated control cells.

**Estrogen Receptor Activation**

Finally, we assessed activation Akt in 3T3-L1 cells to determine ER responsiveness.
phosphorylation in differentiated 3T3-L1 cells (530). However, unlike Muraki et al, treatment of differentiated 3T3-L1 cells with supraphysiological concentrations of DPN, PPT, or E2 prior to insulin treatment did not yield expected E2-mediated attenuation of Akt phosphorylation (Figure 5-5).

Figure 5-5: Insulin increased phosphorylated Akt (pAkt) in differentiated 3T3-L1 cells. Representative blot and protein levels for pAkt, Akt, and GAPDH. Representative blots (Panel A) and relative protein expression of the relative pAkt/Akt ratio (Panel B); * denotes significantly different from vehicle (EtOH) treated control (n=4/group; all experiments were repeated twice). Values are means ± SEM; data is presented relative to EtOH control; estrogen (E2), propyl pyrazole-triol (PPT), diarylpropionitride (DPN).


**Discussion**

The purpose of this study was to determine the effect of ERβ on intracellular APN synthesis and secretion mechanisms utilizing an *in vitro* model. We encountered numerous challenges attempting to overexpress ERβ in 3T3-L1 cells and were unsuccessful in stimulating endogenous ER activation in differentiated 3T3-L1 cells. It is still unknown if ERβ plays a role in modulating APN in adipocytes.

In our efforts to maximize ERβ overexpression we gleaned valuable information regarding the 3T3-L1 cell line. We determined that transiently transfected 3T3-L1 cells overexpress ERβ mRNA. However, increased mRNA expression was independent of ERβ protein presence in 3T3-L1 cells. Similarly transfected HEK293 cells demonstrated both increased ERβ mRNA expression and protein was detected thus confirming that the ERβ construct utilized can yield detectable ERβ protein *in vitro*. We attempted to optimize transfection in 3T3-L1 cells by using lipid based transfection reagents, Lipofectamine LTX and FuGene6, which resulted in the most successful ERβ mRNA induction in 3T3-L1 cells. Electroporation was unsuccessful because it resulted in extensive cell death. It is unknown why 3T3-L1 cells were unable to produce detectable quantities of ERβ protein following transfection. Others have had success transfecting the 3T3-L1 cell line with various plasmid constructs using Lipofectamine LTX (60, 368, 530, 533). It may be that ERβ protein in transfected 3T3-L1 cells is just under the detection limits of western blotting. Differentiated 3T3-L1 cells do express endogenous ERs and expression increases during the differentiation process (530) (533). The upper band detected in 3T3-L1 cells may represent endogenous ERβ (Figure 5-3). A faint signal at the same molecular weight appears in ovary homogenates providing further evidence that the signal is likely a larger ~60 kDa ER splice variant. HEK293 cells do not express endogenous ERs and the ~55 kDa signal is likely the result of non-specific binding.
We further attempted to modify transfection conditions to enhance transfection in 3T3-L1 cells via plasmid linearization and stable selection. The ERβ construct utilized in the current investigation was large (7 kb; Figure 5-1) relative to other ERβ constructs (4 kb) used in 3T3-L1 transfection studies (60). Using a restriction enzyme digest regime, we modified the ERβ plasmid construct to yield linearized (7 kb), smaller (5 and 3 kb), and potentially easier to transfect ERβ encoding products. The smaller ERβ linearized plasmids (5 and 3 kb) did not increase mRNA expression compared with non-linearized circular constructs. After determining plasmid size was not a limiting factor for ERβ expression, we attempted stable transfection techniques. Cell proliferation was reduced 10-fold during stable selection, suggesting proliferation of non-transfected cells was successfully inhibited. Curiously, after four weeks of stable selection both 3T3-L1 and HEK293 cells demonstrated reduced mRNA expression vs transiently transfected cells. Stable transfection was determined to be ineffective toward improving transfection efficiency.

In lieu of ERβ overexpression, we attempted an alternative approach to assess ERβ activation on APN processing and secretion in differentiated 3T3-L1 cells. Following differentiation 3T3-L1 cells endogenously express ERs (121, 530). In the current investigation HMW APN decreased in cell lysates and increased in culture medium at 24 vs 12 h in both control and DPN-treated cells which suggests APN secretion. Similar to our findings HMW APN secretion has been reported to occur between 12-17 h in rat adipocytes (400). The culture medium change prior to DPN treatment may have provided an impetus for HMW APN secretion. Regarding ERβ activation, others have shown no effect on APN, or APN regulatory proteins in 3T3-L1 cells (60, 530). At odds with these findings, another study demonstrated ERβ activation reduces APN regulatory proteins in adipose tissue (522). After 12 and 24 h of ERβ activation in differentiated 3T3-L1 cells, neither total nor HMW APN abundance changed in cell lysates or culture medium relative to vehicle treated controls. These data indicate ERβ activation does not
inhibit total APN production, HMW assembly, or secretion. However, these findings must be interpreted with caution as we were unable to confirm ER responsiveness in differentiated 3T3-L1 cells following E2 treatment. Pretreatment with supraphysiological E2 concentrations for 24 h is known to reduce insulin-mediated Akt phosphorylation in differentiated 3T3-L1 cells (533). This response is also observable in clinical studies in individuals with high concentrations of E2 such as pregnancy, trans-sexuality, and irregular menstrual cycling (534-537). Lack of response to E2 casts doubt on the validity of ERβ activation studies conducted herein.

In conclusion it is still unknown if ERβ overexpression or ligand-dependent activation regulates APN in adipocytes. A limitation of the study is that we only used one adipose-like cell line. The human Simpson-Golabi-Behmel syndrome (SGBS) pre-adipocyte cell is not as widely used as the 3T3-L1 cell line, but is an alternative model which similarly develops an adipose-like phenotype following differentiation (411, 538). Also, a more aggressive method of transfection, like adenoviral infection, was not attempted. Future studies should include assessing the SGBS cell line, or primary adipocyte cultures as well as employ adenovirus-mediated transfection methods.
CHAPTER 6
ADIPONECTIN IS CARDIOPROTECTIVE IN THE AGED FEMALE RAT HEART

Introduction

Postmenopausal E₂-deficiency is clearly linked to the loss of IT in the female heart. Moreover, the aged female heart is refractory to many cardioprotective interventions proven efficacious in rescuing the adult heart from I/R injury (315-317, 319, 321). The lack of available treatments is of great concern given that heart disease is the number one killer of women (7). Recently APN has garnered attention as a new treatment to limit the infarct size and ischemic damage following myocardial I/R injury (93, 94, 354-356, 443, 444, 482).

APN is a factor secreted from adipose tissue and in addition to directly acting at the myocardium to promote IT, reduced circulating levels are predictive of adverse cardiac events associated with CHD risk (80, 350, 354, 383, 442, 443, 512). Moreover, APN is particularly promising as a treatment since delivery prior to, during, and following ischemia all result in enhanced cardioprotection (354-356). APN acts directly on the myocardium to limit inflammation, apoptotic cell death, and nitrate production following I/R injury via three independent mechanisms (95, 354, 434, 455-457). Specifically, in the ischemic myocardium, APN treatment promotes COX2–mediated, suppression of TNFα thereby reducing inflammation (354, 483). APN also inhibits peroxynitrite formation via inhibition of the superoxide producing subunit of NADPH, gp91phox in the ischemic myocardium (443). Additionally, APN also reduced apoptotic cell death via and AMPK-dependant mechanism (354).
AMPK is of particular interest in the ischemic myocardium given its dual role in promoting cardioprotection and damage. When activated by phosphorylation on site Thr172, AMPK promotes both fatty acid and glucose utilization. Following I/R injury AMPK stimulated glucose utilization confers protection, yet enhanced fatty acid utilization associated with ACC phosphorylation promotes damage (462, 475-480).

Transient APN treatment is associated with glucose oxidation in vitro which suggests that cardioprotective effects may be linked to AMPK-associated glucose utilization. However, enhanced AMPK and ACC phosphorylation is also observed in vivo coronary artery ligation experiments at 3, 24, and 48 h following APN-treatment relative to vehicle treated animals in association with improved cardiac function (93, 354). Currently, it is unknown if anti-apoptotic APN-AMPK signaling is linked to glucose oxidation, fatty acid oxidation, or simply coincident and mediated via a completely novel pathway.

Cardioprotection conferred by APN has been demonstrated in multiple species including mice, rats, and pigs; however, animal models of diabetes show resistance to APN treatment (93, 94, 354, 355, 482). The dose of APN necessary for cardioprotection from I/R injury in diet-induced Type II diabetic rats is three times that required in wild type controls (93). In streptozotocin-induced diabetic rats, APN efficacy is differentially modulated by transient reductions in AdipoR1 during early (1 week), but not late stages (7 weeks) of disease progression (94). While F344 female rats are not diabetic, they are characterized at insulin resistant and with aging do demonstrate increased adiposity and reduced IT similar to diabetic animal models (24, 93, 255, 499, 539).

The primary focus of this investigation was to determine if APN treatment is efficacious in protecting the female rat myocardium from I/R injury in aged and aged OVX rats. Also, given the phenotype of AdipoR1 expression associated with obese/diabetic states (94, 489), we also sought to characterize AdipoR1 and AdipoR2 protein abundance in cardiac muscle of the aged
and aged OVX female rat both on a standard chow as well as a high fat diet. Finally, to elucidate potential therapeutic targets for CHD in postmenopausal women we examined a downstream modulator of APN signaling, AMPK in aged APN treated hearts.

Methods

Animal Care

Certified specific pathogen free female adult (6-7 mo; n = 14) and aged (23 mo; n =14) F344 rats were obtained from Harlan Sprague Dawley (Indianapolis, IN). Rats were singly housed in filter-top cages containing corn-cob bedding and provided with a nylabone or wood block for enrichment. Rats were maintained on a 12 h light/dark cycle in an environment maintained at 22 °C with 20% humidity. Water and chow were supplied *ad libitum* and chow consisted of a standard laboratory rodent diet, LabDiet 5001 (PMI Nutrition International, St. Louis, MO) with a caloric breakdown of 28.5% protein, 13.5% fat, and 58% carbohydrate. Noteworthy, the LabDiet 5001 chow is soy- based and contains 810 μg/g of phytoestrogens, which are compounds known to have weak estrogenic activity *in vivo*.

Ovariectomy

To yield a consistent level of E2-deficiency, a subset of F344 rats were ovariectomized by Taconic (n=11 at 22 mo). Following surgery animals were transferred to the Pennsylvania State University and were allowed to recover for four weeks prior to experimental use or start of dietary intervention. Uterine weight was used to confirm E2-deficiency as previously validated in
our laboratory (24, 499). All animal experimentation was conducted with approval from the Institutional Animal Care and Use Committee of the Pennsylvania State University.

**Study Design for APN Treatment**

Animals were randomly assigned to receive a 9 µg infusion of either APN (Biovendor; adult n=5, aged n=7, aged OVX n=5) or vehicle (Krebs-Henseleit buffer) delivered in 2mL during the isolated heart protocol. The APN dose and timing of delivery was chosen based on efficacy demonstrated in past studies with male Sprague-Dawley rats (482). Study of adult, aged, and aged OVX rats was alternated each day. Vehicle and APN treated-rats of the same group (adult, aged, and aged OVX) were paired such that n=2-3 experiments were completed per day. Order of APN and vehicle treatments were alternated to control for any influence of circadian rhythm on cardiac function and all experiments were conducted between 9:00am and 2:00pm.

**Dietary Intervention**

A second group of F344 female rats were subjected to a 20 week dietary intervention. Adult (n=9), aged (n=12), and aged OVX (n=8) rats were assigned to receive either the standard laboratory rodent diet, LabDiet 5001 or the Lieber-DeCarli liquid diet (Bio-Serv, Frenchtown, NJ) for 20 weeks. The Lieber-DeCarli diet is considered to be a high fat diet and has a caloric breakdown of 15.1% protein, 35.9% fat, and 49% carbohydrate. Rats were obtained at 3 and 18 months of age at the start of the dietary intervention and were thus age-matched to rats utilized for I/R studies.
Protocol of the Isolated Heart

Female adult, aged, and aged OVX F344 rats were anesthetized with pentobarbital (40 mg/kg body weight; intraperitoneal), and hearts were excised via midline thoracotomy. Isolated hearts were secured to a modified Langendorff perfusion apparatus via the aorta, perfusion pressure was set at 85 mmHg, and hearts were paced at 260 beats/min. LVDP, and positive and negative developed pressure with respect to time (+/- dP/dt_max; indices of contractility and relaxation, respectively), were assessed by a pressure transducer attached to a water-filled latex balloon inserted into the left ventricle. EDP was adjusted to 5–6 mmHg according to established procedures in our laboratory (24, 299, 500). After 30 minutes of equilibration, all Lieber-DeCarli fed rat hearts and a subset of chow fed rat hearts (n=4/group) were isolated, LV tissue weighed, halved, and snap frozen in liquid N2.

All other hearts were subjected to 47 min global, isothermic, ischemia as described previously (24). Animals received either 9 µg dose of APN (Biovendor) or vehicle (Krebs-Henseleit buffer) infused in a volume of 2 mL for 1 min upon initiation of global ischemia (see Figure 6-1). Pacing was re-initiated 1min 30 sec after the start of reperfusion and hearts were reperfused for 60 min. After reperfusion, LVs were isolated weighed, halved, and snap frozen in liquid N2. All LV sections were stored at –80ºC until tissue preparation.

Figure 6-1: Schematic of the isolated heart protocol and adiponectin (APN) infusion.
**Tissue Homogenization**

LV tissue was minced and homogenized by glass-glass grinding in buffer containing: 250 mM sucrose; 10 mM Tris-HCl, pH 7.4; 1 mM EDTA, pH 7; 1 mM ortho-vanadate; 1 mM NaF; 0.3 mM PMSF; 5 µg/ml each of leupeptin and aprotinin; 0.5 µg/ml pepstatin A, and 1% Triton-X100. LV tissue was then subjected to a 100,000 X g centrifugation for 60 minutes. The supernatant was assessed for total protein concentration using the Bradford method (513).

**Western Blotting**

Protein lysates were subject to separation using SDS-PAGE, transferred to PVDF membranes and blocked in 5% non-fat dry milk for 2 hours at room temperature as described, previously (24, 500, 514). Membranes were probed overnight at 4°C with primary antibodies at a dilution of 1:1000 for AdipoR1 (42 kDa, AdipoR1-2; Alpha Diagnostics), 1:2000 for AdipoR2 (42 kDa, AdipoR2-1; Alpha Diagnostics), 1:500 for phosphorylated AMPK (pAMPK, Thr-172; 62kDa; 2535; Cell Signaling), and 1:1000 for AMPK (63 kDa, 07-350; Millipore). Membranes were then incubated with HRP-linked anti-rabbit secondary antibody at a dilution of 1:20,000 for 1 h at RT and visualized using ECL (GE Healthcare). Densitometry was performed using Scion Image (NIH). To correct for potential protein loading errors all membranes were stained with Sypro Ruby blot stain (Invitrogen) and densitometry was performed as previously described (24, 299, 500, 514).

**Adiponectin ELISA**

Rat serum was assessed for total APN concentration utilizing a kit (EZRADP-62K; Millipore) per manufacturer instructions.
**Statistical Analysis**

All data are presented as means ± SE and analyzed using the SAS GLM procedure. A one-way ANOVA was used to analyze data for morphological characteristics and circulating APN data for adult, aged, and aged OVX animals. Protein levels for adult, aged, and aged OVX rats on chow or Lieber-DeCarli dietary intervention were assessed with a one-way ANOVA. A two-way ANOVA (group X drug) was used to analyze all functional data for adult, aged, and aged OVX animals with and without APN treatment. A three way ANOVA (group X drug X ischemia/reperfusion) was used to analyze protein levels for adult, aged, and aged OVX animals subject to control perfusion, or I/R with or without drug. The Tukey test was used for all post hoc analysis. An α-level of $p < 0.05$ was considered statistically significant.

**Results**

**Baseline Morphological Characteristics**

For the I/R study rats with pre-ischemic LVDP below 130 mmHg, unstable pre-ischemic EDP (> 6 mmHg), or notable pathology/weight loss were excluded from analysis. Final data analysis was conducted on n=4 adult, n=5 aged, and n=3 aged OVX rats treated with vehicle and n=5 adult, n=6 aged, and n= 4 aged OVX treated with APN.

Baseline characteristics did not differ between APN and vehicle treated rats and data were pooled. Body, LV, and gonadal adipose depot weights were significantly greater in aged and aged OVX rats vs adult control rats (Table 6-1). There was no difference in body, LV, or gonadal adipose weights between aged and aged OVX rats. Uterine weight decreased significantly with OVX which suggests relative E$_2$-deficiency in aged OVX rats. Pre-ischemic LVDP, but not ± dP/dt, was significantly reduced in aged and aged OVX vs adult.
Table 6-1: Baseline morphological and functional characteristics. OVX, ovariectomized; LV left ventricle; EDP end diastolic pressure; LVDP left ventricular developed pressure; dP/dt first derivative of LVDP. *denotes different from adult; † denotes different from aged; p<0.05.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Adult; n=11</th>
<th>Aged; n=9</th>
<th>Aged OVX; n=7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight, g</td>
<td>208 ± 1</td>
<td>296 ± 2 *</td>
<td>299 ± 3 *</td>
</tr>
<tr>
<td>LV weight, mg</td>
<td>581.6 ± 5.8</td>
<td>779.6 ± 6.1 *</td>
<td>761.0 ± 8.7 *</td>
</tr>
<tr>
<td>LV/body weight, mg/g</td>
<td>2.8 ± 0</td>
<td>2.6 ± 0</td>
<td>2.5 ± 0</td>
</tr>
<tr>
<td>Uterine Weight, g</td>
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<td>0.67 ± 0.01</td>
<td>0.34 ± 0.01 †</td>
</tr>
<tr>
<td>Gonadal Adipose Weight, g</td>
<td>3.41 ± 0.16</td>
<td>7.30 ± 0.27 *</td>
<td>7.17 ± 0.11 *</td>
</tr>
<tr>
<td>EDP, mmHg</td>
<td>5.5 ± 0.1</td>
<td>5.8 ± 0.1</td>
<td>5.9 ± 0.1</td>
</tr>
<tr>
<td>LVDP, mmHg</td>
<td>145.7 ± 1.3</td>
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<td>139.1 ± 1.4 *</td>
</tr>
<tr>
<td>+dP/dt, mmHg/sec</td>
<td>3907 ± 66</td>
<td>3865 ± 33</td>
<td>3757 ± 47</td>
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<tr>
<td>-dP/dt, mmHg/sec</td>
<td>2541 ± 40</td>
<td>2328 ± 21</td>
<td>2543 ± 37</td>
</tr>
</tbody>
</table>

**Circulating Adiponectin**

Circulating APN was significantly reduced in aged, but not aged OVX groups relative to adult intact rats (Figure 6-2). These findings recapitulate previously published data represented in Figure 4-4 (409).
Adiponectin Infusion and Left Ventricular Function

Aged and aged OVX rats demonstrated reduced functional recovery following I/R vs adult controls. However, APN infusion was successful in improving functional recovery following I/R injury in all groups. Specifically, LVDP was improved in all groups with APN infusion by 10-20% (Figure 6-3). Post-ischemia, EDP was significantly increased by 100 fold but the rise in EDP was significantly attenuated by 15-35 mmHg in all APN- treated groups (Figure 6-4). Similar to LVDP, ± dP/dt\textsubscript{max} was attenuated following I/R injury in all groups, but decrements in function were significantly attenuated with APN treatment (Figure 6-5).

Figure 6-2: Circulating adiponectin in adult aged and aged OVX female rats, * denotes different from adult; p < 0.05; n=7-11/group.
**Figure 6-3:** Adiponectin (APN) delivery improves functional recovery in the female rat heart. Recovery of left ventricular developed pressure (LVDP), expressed as percent of pre-ischemic LVDP. Krebs-Henseleit buffer (vehicle) or 9μg of APN was infused during the first minute of a 47 min ischemia period at a flow rate of 2mL/min in adult (Panel A), aged (Panel B), aged OVX (Panel C) female rats. * denotes different from adult; ‡ denotes main effect of drug; p<0.05; n=3-6/group.

**Figure 6-4:** Adiponectin delivery improves post-ischemic end diastolic pressure (EDP) in female rat heart. Recovery of EDP following a 47 min ischemia in adult (Panel A), aged (Panel B), aged OVX (Panel C) female rats. ‡ denotes main effect of drug; p < 0.05; n=3-6/group.
Adiponectin Receptors and AMPK in Perfused Hearts

Although there was no significant change in AdipoR1 protein abundance, AdipoR2 levels decreased in both aged and aged OVX groups compared to adult intact rats (Figure 6-6). Due to variability in AdipoR1 data, samples were assessed three times with western blotting and representative images are shown in Figure 6-6. AMPK phosphorylation increased in response I/R injury in adult and aged female rats (Figure 6-7). No additional increased in AMPK phosphorylation was observed with APN infusion in any group. In fact, APN treatment resulted in a decrease in pAMPK in adult rats. There was no significant difference in total AMPK protein levels among groups.
Figure 6-6: Adiponectin Receptor 1 (AdipoR1) and AdipoR2 in isolated hearts perfused for 30 min (Perfused Control), or subjected to ischemia/reperfusion (I/R; 47/60 min) with vehicle, or adiponectin (APN) infusion (9μg) delivery upon ischemia. Representative blots and protein levels for AdipoR1 (Panel A), and AdipoR2 (Panel B). § denotes different from adult groups; p < 0.05; n=4-5/group). Values are means ± SEM; data is presented relative to adult perfused control and corrected with Sypro Ruby Blot Stain.
A.

B.

C.

D.
Figure 6-7: Adenosine monophosphate-dependent protein kinase (AMPK), activated AMPK (pAMPK), and the pAMPK/AMPK ratio in isolated hearts perfused for 30 min (Perfused Control), or subjected to ischemia/reperfusion (I/R; 47/60 min) with vehicle, or adiponectin (APN) infusion (9μg) delivery upon ischemia. Representative blots for pAMPK and AMPK (Panel A), protein levels for pAMPK (Panel B), protein levels for AMPK (Panel C) and the ratio of pAMPK/AMPK (Panel D). * denotes different from adult perfused control, † denotes different from aged perfused control; # denotes different from adult I/R; p < 0.05; n=4-5/group). Values are means ± SEM; data is presented relative to adult perfused control and corrected with Sypro Ruby Blot Stain.
Effect of High Fat Diet on Body Weight and Caloric Consumption

Randomly divided F344 rats assigned to consume standard chow (13% calories from fat) or the Lieber-DeCarli diet (35% calories from fat) showed no difference in initial body weight among groups. Aged and aged OVX female rats had significantly greater body weight relative to adult control rats (p<0.05). In aged (n=4) and aged OVX (n=2) Lieber-DeCarli diet consuming groups six rats died during the dietary intervention due to age-associated complications. All rats consuming the Lieber-DeCarli diet that survived the 20 week intervention had significantly greater final body weight vs chow consuming rats (Table 6-2; p<0.05). Greater body weight in Lieber-DeCarli fed rats is attributable to increased total calorie consumption and fat consumption relative to chow fed rats (p<0.05).

Table 6-2: Body weight and food consumption in chow and Lieber-DeCarli fed rats. OVX, ovariectomized; *denotes different from adult chow; † denotes different from adult Lieber-DeCarli; p<0.05.

<table>
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<th>Adult Chow</th>
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<th>Aged OVX Chow</th>
<th>Adult Lieber-DeCarli</th>
<th>Aged Lieber-DeCarli</th>
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<tr>
<td>N</td>
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<td>5</td>
<td>4</td>
<td>5</td>
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<td>2</td>
</tr>
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<td>Initial Body Weight, g</td>
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<td>260 ± 1*</td>
<td>200 ± 2</td>
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<td>264 ± 5†</td>
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<tr>
<td>Final Body Weight, g</td>
<td>218 ± 7</td>
<td>303 ± 4*</td>
<td>304 ± 7*</td>
<td>262 ± 2†</td>
<td>410 ± 3†</td>
<td>397 ± 6†</td>
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<tr>
<td>Δ Body Weight, g</td>
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<td>44 ± 3</td>
<td>62 ± 6</td>
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<td>Total Kcal consumed /100 g body weight /day</td>
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<td>7 ± 1</td>
<td>17 ± 0*</td>
<td>14 ± 0*</td>
<td>14 ± 1*</td>
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<tr>
<td>Total fat consumed /100 g body weight / day</td>
<td>2 ± 1</td>
<td>1 ± 0</td>
<td>1 ± 0</td>
<td>6 ± 0*</td>
<td>5 ± 0*</td>
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Effect of High Fat Diet on Adiponectin Receptors and AMPK

AdipoR1 was significantly increased in aged and aged OVX female vs adult rats consuming the Lieber-DeCarli diet for 20 weeks (Figure 6-8). AdipoR2 did not differ among groups.

A.

B.

Figure 6-8: Adiponectin Receptor (AdipoR) 1 and AdipoR2 in isolated perfused control hearts of rats consuming the Lieber-DeCarli diet. Representative blots and protein levels for AdipoR1 (Panel A), and AdipoR2 (Panel B), *denotes different from adult; p<0.05 ; n=3-5/group). Values are means ± SEM; data is presented relative to adult intact and corrected with Sypro Ruby Blot Stain.

Basal AMPK phosphorylation was unchanged with age in Lieber-DeCarli consuming female rats. Total AMPK abundance was significantly reduced in aged and aged OVX groups vs adult and the pAMPK/total AMPK ratio was increased in aged rats only (Figure 6-9).
**A.**

pAMPK

AMPK

Sypro

**B.**

<table>
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<tr>
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<th>Adult Perfused Control</th>
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<th>Aged O VX Perfused Control</th>
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**C.**

<table>
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**D.**

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<th>Aged Diet</th>
<th>Aged O VX Diet</th>
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<td>pAMPK (Thr-172)/Total AMPK (Relative to Adult Ctrl)</td>
<td><img src="chart.png" alt="Graph" /></td>
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</table>
Figure 6-9: Adenosine monophosphate-dependent protein kinase (AMPK) and activated AMPK (pAMPK) protein levels in isolated perfused control hearts of rats consuming the Lieber-DeCarli diet. Representative blots for pAMPK and AMPK (Panel A), protein levels for pAMPK (Panel B), protein levels for AMPK (Panel C) and the ratio of pAMPK/AMPK (Panel D) * denotes different from adult control; p < 0.05; n=3-5/group. Values are means ± SEM; data is presented relative to adult intact, and corrected with Sypro Ruby Blot Stain.
**Discussion**

APN has direct cardioprotective effects in response to I/R injury in adult male mice, rats, and pigs (354-356, 443, 482). However, no previous studies have addressed APN-mediated cardioprotection in the aged E2-deficient female heart. The aged female myocardium is refractory to many cardioprotective interventions proven efficacious in adult animals (315-317, 319, 321) and therapies are needed to treat aged post-menopausal women. The current study demonstrates APN is effective in improving LV functional recovery in isolated hearts from adult, aged, and aged OVX female rats. Moreover, we determined that AdipoR1 abundance is unchanged with aging alone, but increased with aging in conjunction with a high fat diet. Finally, we characterized the phosphorylation status of the APN target AMPK, and demonstrated pAMPK is reduced with APN treatment in adult, but sustained in aged hearts following I/R injury.

Body and gonadal adipose depot weights increased significantly in aged and aged OVX groups vs adult intact controls, but there was no significant weight gain in aged vs aged OVX female rats. The lack of OVX-induced weight and adipose mass gain was surprising given that we and others have previously documented contrary findings (24, 299). Although animals with obvious pathology, neoplastic tumor growth, and associated weight loss (> 10 g/week) were excluded (7 aged, and 6 aged OVX rats) it is possible OVX-induced weight gain was masked by confounding asymptomatic diseases in the cohort of F344 rats studied. Rats studied in the investigation described in Chapter 3 also did not demonstrate significant OVX-induced weight gain. Gonadal adipose mass did not differ between aged and aged OVX rats which may be a function of similar total body weight in both groups or due to dissection errors in adipose collection. In aged rats adiposity also increases in the retroperitoneal, mesenteric, and omental depots. These depots can obscure the gonadal fat pad making it more challenging to dissect.
Similar to findings in adult male Sprague-Dawley rats (482), LVDP and ± dP/dt\textsubscript{max}, were significantly improved with a single 9 µg dose of APN delivered upon ischemia in adult, aged, and aged OVX female rat hearts subjected to I/R injury (Figures 6-3 and 6-5). APN also attenuated the post-ischemic rise in EDP in all groups (Figure 6-4). Thus, our findings extend previous knowledge demonstrating APN-mediated cardioprotection in adult males to the adult, aged, and aged OVX female heart. These data also support the assertion that APN may be a relevant therapy for CHD in aged postmenopausal women.

Previously, we have shown circulating APN is reduced with age, but increased with OVX (409). The interaction of age and OVX results in unchanged circulating APN concentrations in aged OVX rats vs adult controls and was observed previously and confirmed in the current study (Figure 6-2 and 4-4) (409). That APN levels are reduced in aged female rats suggests they, like obese and diabetic animals, may require greater doses of APN to confer protection vs adult or aged OVX animals (93). That we saw protection in all groups suggests adult animals (which have the highest endogenous APN concentrations) may be protected with lower doses of APN during I/R injury. Further studies are needed to determine the threshold dose for APN efficacy in females and aging models.

We characterized AdipoR1 and AdipoR2 receptor abundance in our model to better understand the mechanism(s) of APN action in a setting of aging and E\textsubscript{2}-deficiency. As expected we observed no significant effect of I/R injury on AdipoR1 or AdipoR2 abundance. Noteworthy, the AdipoR1 data exhibited variability which may be attributed to the timing of sample collection. Insulin reciprocally regulates AdipoR levels and significant changes in receptor abundance have been observed during fasted and fed states in mice (83). Although we limited data collection time to the hours between 9:00am and 2:00pm daytime fasting may explain the variability observed.
Interestingly, aging and high fat diet differentially regulated AdipoR1 and AdipoR2 abundance. Specifically, AdipoR1 was significantly increased in the myocardium of aged and aged OVX female rats consuming the Lieber-DeCarli diet, but not in age-matched chow fed rats. The Lieber-DeCarli diet contains 35% of calories derived from fat and rats had significant weight gain vs chow fed animals consuming an 13% fat diet. Since both total calorie consumption as well as calories derived from fat increased in Lieber-DeCarli fed rats it is impossible to determine if the change in AdipoR protein levels is attributable to increased total food intake or high fat diet alone. Increased AdipoR1 mRNA and protein abundance in liver, skeletal, and cardiac muscle is commonly observed in animal models of obesity and diabetes (83, 94, 425, 489, 491, 492). Fluctuations in AdipoR1 mRNA and protein levels have been reported during early stages of metabolic disease progression and decreased AdipoR1 has been reported during short (10 week) high fat dietary interventions (94, 489). The increase in AdipoR1 after 20 weeks of high fat feeding observed in the present study is in line with findings of similar durations or more extreme (42% fat content) dietary interventions (94, 489, 491). Both aged and diabetic rats are models predisposed to reductions in IT and upregulation of AdipoR1 under metabolic stress may be a compensatory mechanism to preserve APN signaling.

A role for AdipoR2 in the heart has yet to be discovered (433) and previous findings indicate AdipoR2 does not change in cardiac or skeletal muscle with metabolic disease (426, 491). We observed a significant age-related decrease with age in AdipoR2 in chow fed rats, but no change in Lieber-DeCarli fed rats. Previously, we have shown depressed AdipoR2 protein levels in adipose tissue with aging (409). Decreased AdipoR2 abundance may be a unique phenomenon associated with aging in F344 female rats or may be linked to altered metabolism associated with aging.

We also assessed the phosphorylation status of the downstream target AMPK in chow and Lieber-DeCarli fed female rats. Basal AMPK phosphorylation was unchanged in aged female
rats consuming chow (Figure 6-7). However, total AMPK and the pAMPK/AMPK ratio in aged rats on the Lieber-DeCarli diet were decreased and increased, respectively (findings for aged OVX rats failed to reach significance; Figure 6-9). Similar findings were observed in aged male F344 and high fat diet consuming mice in conjunction with reduced IT (93, 94, 352). Increased pAMPK levels in Lieber-DeCarli fed rats may be consequent of increased myocardial fatty acid utilization. This phenomenon represents an adaptation to reduce the risk of lipotoxicity associated with increased circulating free fatty acids (466). We did not assess I/R injury in aged female rats on a high fat diet. However, a similar phenotype observed between our model and others on a high fat diet suggests APN-resistance and a predisposition for reduced IT.

All APN-treated rats subjected to I/R injury were protected from decrements in functional recovery vs control hearts. In accordance with previous reports pAMPK increased in response to I/R injury in adult and aged rats (93, 94, 354, 482). In aged OVX rats the post-ischemic increase in pAMPK failed to reach significance. This may suggest the interaction of aging and E2-deficiency is associated with a diminished capacity for AMPK activation.

Interestingly, with APN infusion, there was no additional increase in pAMPK after 60 min of reperfusion. Moreover, pAMPK was surprisingly downregulated after 60 min of reperfusion in adult, but not aged animals. The response in female adults is curious given that in vivo coronary artery ligation studies report sustained pAMPK levels even after 24 and 48 h of reperfusion following APN treatment (93, 354, 482, 540). The divergent results observed in the current study vs in vivo models may be consequent of the absence of fatty acids in the perfusion buffer as well as timing of sample collection. However, in isolated Sprague-Dawley male rat hearts perfused in a similar Langendorff preparation, pAMPK is increased by 60% with APN treatment relative to vehicle treated hearts after 60 min of reperfusion (482). Sex or strain differences may account for divergent AMPK responses associated with APN treatment. Further studies will be needed to address this question. Regardless of the cause of divergent results, the
data suggest that AMPK activation may not be a critical mediator of APN-mediated cardioprotection in the female rat heart.

That functional recovery is still improved in APN-treated aged OVX females suggests an AMPK-independent cardioprotective mechanism may be operational. COX2-mediated suppression of inflammation or attenuation of cytotoxic nitrate production may be more relevant mechanisms of APN action in the ischemic aged OVX female rat heart. We attempted to assess TNFα accumulation in hearts following I/R injury to determine the impact APN treatment has on the COX2-TNFα inflammatory pathway in aged hearts. However, cardiac-specific TNFα was below detection limits and could not be assessed.

In conclusion, we have demonstrated that the aged female rat heart is responsive to APN treatment. This suggests APN treatment may be a relevant therapy for aged post-menopausal women. A limitation of the study is that we only assessed one dose of APN. Animal models of obesity and diabetes demonstrate resistance to APN treatment (94, 460, 489). Type II diabetic mice require triple the dose needed for cardioprotection in adult controls. This suggests a threshold for APN-mediated cardioprotection exists and endogenous APN concentrations may dictate the supplemental quantity needed to protect the myocardium during I/R injury.

Additionally, we observed divergent changes in pAMPK activation in response to I/R and APN treatment in adult, aged and aged OVX female rats. This discordance suggests alternative mechanisms of APN action involving suppression of inflammation or nitrate production may be differentially important in E2-replete and deficient animals. We also extended findings that a moderate high fat diet can evoke derangements in AdipoR1 and pAMPK levels at baseline in female rats indicative of APN-resistance.
CHAPTER 7

SUMMARY AND CONCLUSIONS

The aim of the four studies contained herein was to investigate ER-signaling disruptions in metabolic and cardiac tissues as well as characterize attenuations in APN signaling capacity in female aged and OVX F344 rats. The collective purpose of the work was to elucidate relevant targets for therapeutic intervention in a setting of aging and E₂-deficiency with the ultimate goal of reducing CHD morbidity and mortality in aged women.

The aim of the first study (Chapter 3) was to determine the effects of acute ERβ activation on I/R injury in adult, aged, and aged OVX female rats. It was found that:

1. Acute ERβ activation does not affect cardiac functional recovery following I/R injury in adult, aged, and aged OVX female F344 rats.

2. ERβ is not detectable in the adult and aged female F344 rat myocardium.

Thus ERβ is not likely a relevant target for influencing direct cardioprotection in the F344 female rat heart. Any influence of ERβ on the rat myocardium is likely via indirect genomic, or ligand-independent mechanisms in non-cardiac tissues. This work provides additional evidence refuting the controversial existence of ERβ in the myocardium.

The aim of the second study (Chapter 4) was to determine the singular and combined effects of aging and E₂-deficiency on ER isoform distribution in visceral adipose tissue and skeletal muscle in F344 female rats. An additional aim was to also characterize alterations in
APN and its receptors and downstream targets PPARα and AMPK in adipose tissue. It was found that:

1. ERα protein abundance is increased in gastrocnemius of aged OVX F344 female rats relative to adult ovary-intact controls. ERα protein abundance is unchanged in soleus of F344 female rats.

2. ERα protein abundance is decreased in the adipose tissue of adult OVX relative to aged OVX female F344 rats.

3. ERβ protein abundance is increased in the adipose tissue of aged and aged OVX female F344 rats relative to adult OVX and adult ovary-intact controls.

4. APN is increased in adipose-tissue in conjunction with reduced PPARγ protein levels, yet circulating APN is increased with OVX, and decreased with age such that concentrations are reduced in aged OVX vs adult OVX and aged vs adult F344 female rats.

5. AdipoR2, but not AdipoR1 is reduced with aging in adipose tissue such that aged and aged OVX female rats demonstrate reduced levels vs adult intact rats. PPARα levels are unchanged. The pAMPK/AMPK ratio is reduced in the adipose tissue of aged and aged OVX rats relative to adult ovary-intact thus providing a mechanism for metabolic derangement.

Thus the unique state of aging plus E2-deficiency is associated with an altered ERα/β ratio in favor of ERβ in adipose tissue. Compromised APN processing and/or secretion to the circulation is linked to enhanced ERβ presence in adipose tissue. Unfortunately, cell culture studies (Chapter 5) failed to reveal conclusive evidence regarding ERβ and APN intracellular actions. The increase in ERα in gastrocnemius, but not soleus muscle suggests there is differential regulation of ERα in glycolytic vs oxidative muscle fibers associated with aging.

In Chapter 4 we additionally conclude the aged-associated reductions in AdipoR2 and the pAMPK/AMPK ratio may translate to decreased autocrine signaling in adipose tissue. This
phenomenon may contribute to a reduced capability to metabolize lipids in adipose and may, in part, explain increased weight gain in aged animals.

The aim of the study described in Chapter 6 was to determine if aging and OVX is associated with APN resistance in the female rat myocardium. It was found that:

1. **APN treatment improves functional recovery following I/R injury in adult, aged, and aged OVX female F344 rats.**

2. I/R injury increased pAMPK levels in adult and aged female rats, but APN treatment did not result in a further increase in pAMPK levels indicating APN-mediated cardioprotection is likely via an AMPK-independent mechanism.

3. Aging and OVX alone did not affect basal cardiac AdipoR1, AdipoR2, or AMPK protein levels. However, in conjunction with high fat diet AdipoR1 and AMPK protein levels are increased in the myocardium of aged and aged OVX female rats indicating an APN-resistant phenotype.

Increased adiposity and reduced circulating APN in aged female rats suggests progression toward a metabolic phenotype which may lead to APN-resistance. Fortunately, adult, aged and aged OVX female rats respond to APN treatment with improved post-ischemic function following I/R injury. However, consumption of a high fat diet in conjunction with aging produces changes in AdipoR1 protein abundance and basal AMPK phosphorylation that resemble an APN-resistant phenotype. In conclusion, APN or downstream APN signaling targets may be a relevant therapy for combating CHD risk and mortality in aged women.

A summary of all key findings is presented in Figure 7-1.
Strengths and Limitations

Aged OVX Female Rat Model

Use of age-appropriate rats in conjunction with OVX represents an overlooked but critical design consideration of rodent studies to recapitulate post-menopausal E\(_2\)-deficiency in women (541). The adult OVX rat was erroneously relied upon as a model for post-menopausal E\(_2\)-deficiency in past studies by others (23, 300, 301, 303, 542). We have previously shown divergent responses to I/R injury and cardiac signaling proteins in adult OVX and aged OVX female rats (24, 299, 543). Aged rats were utilized in studies presented herein to appropriately mimic the point at which cardiovascular morbidity and mortality is likely to become manifest in
aged women (7). Also important, aged female rats have variable E₂ concentrations due to unpredictable estrous cycling that occurs with aging and therefore rats must be OVX to ensure consistent E₂-deficiency. In Chapter 4 adult intact, adult OVX, aged intact, and aged OVX rats were utilized to emphasize the independent and combined effects of aging and E₂-deficiency. Now that clear differences have been established between the four groups, only the clinically relevant aged OVX, and adult ovary intact control need be studied in the future.

**Langendorff Isolated Heart Model**

The Langendorff isolated heart preparation is a reliable model which has been successfully utilized and cited in the literature for over one hundred years. The isolated heart preparation enables the assessment of the ventricular function in the absence of confounding neural and hormonal influences. Examining the heart in isolation provides the advantage of allowing for a tightly controlled setting for directly comparing LV function. Global cardiac ischemia is extreme and less representative of an acute myocardial infarction *in vivo*; however it is required as a caveat for ensuring sufficient ischemic damage in this preparation. A clear disadvantage of studying the heart in isolation is the loss of circulating factors that may differentially modulate the response to I/R injury. An *in vivo* coronary artery ligation approach may be relevant for future studies focusing on titration of an appropriate APN dose for cardioprotection based on relative endogenous circulating APN. However, use of the Langendorff is preferred at this time given the cost prohibitive nature of APN and the increased dosage required for whole animal vs isolated heart studies.
Future Directions

A suitable ERβ model of overexpression is necessary to extend the primarily descriptive findings demonstrating derangements in ERβ and other adipose proteins associated with metabolic disease in Chapter 4. We attempted to produce such a model in Chapter 5, however, were unsuccessful. Future efforts should include adenoviral transfection of a functional ERβ plasmid in an APN expressing cell line. A more physiologically relevant approach, albeit more challenging, would employ use of adipocyte explants in primary culture for the study of ERβ interactions, in vitro.

To extend the findings in Chapter 6 regarding age and diet-induced APN-resistance, functional recovery following I/R injury of aged female rats on a high fat diet must be completed. We demonstrated that aged OVX female rats on a high fat diet exhibit changes in AdipoR1 and AMPK protein levels indicative of APN-resistance. At this time these findings are descriptive and IT must be assessed. Additionally, at least three pathways of APN-mediated cardioprotection have been described. To gain a better understanding of which APN signaling pathway(s) is relevant in the female rat heart markers of apoptotic (caspase 3), inflammatory (TNFα) and nitrative (superoxide; peroxynitrite) stress should be assessed. The candidate downstream targets of APN thought to mediate these processes in the myocardium currently include AMPK, COX2, and gp91phox, respectively. Pretreatment with activators, inhibitors, and dominant negative forms of AMPK, COX2 and gp91phox in conjunction of APN delivery in aged female rats will help elucidate which pathways are relevant in conferring cardioprotection. Both Langendorff and in vivo coronary artery ligation approaches may be needed to determine the respective relevance of each signaling pathway as well as a threshold dose of APN for adult and aged OVX female rats in the presence and absence of endogenous circulating APN.
APPENDIX

Preliminary ACC data in the female rat myocardium is presented herein. Samples were prepared as described in Chapter 6 methods. Briefly, hearts were subjected to I/R injury and treated with adiponectin or vehicle infusion upon ischemia. Perfused control hearts (no ischemia or adiponectin treatment) of chow or Lieber-DeCarli fed rats were also assessed. Samples were assessed once with western blotting, but transfer efficiency controls and loading controls have yet to be completed.
Figure A-1: Acetyl CoA carboxylase (ACC), phosphorylated ACC (pACC), and the pACC/ACC ratio in isolated hearts perfused for 30 min (Perfused Control), subjected to 47 min of ischemia and 60 min reperfusion (I/R; 47/60) with control Krebs-Henseleit infusion, or subjected to I/R with adiponectin (APN) infusion (9ug/mL). Protein levels for pACC (Panel A), protein levels for total ACC (Panel B), and the ratio of pACC/ACC (Panel C). * denotes different from adult perfused control, † denotes different from aged perfused control; $ denotes different from aged OVX perfused control; p < 0.05; n=4-5/group and values are means ± SEM.
Figure A-2: Acetyl CoA carboxylase (ACC), phosphorylated ACC (pACC), and the pACC/ACC ratio in isolated hearts perfused for 30 min (Perfused Control) from rats consuming Lieber-DeCarli diet. Protein levels for pACC (Panel A), protein levels for pACC (Panel B), and the ratio of pACC/ACC (Panel C). Values are means ± SEM.


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