Estrogen Receptor Regulation of the Heart in an Animal Model of Menopause and a Comparison of the Sex Differences

By

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ABSTRACT

ESTROGEN RECEPTOR REGULATION OF THE HEART IN AN ANIMAL MODEL OF MENOPAUSE AND A COMPARISON OF THE SEX DIFFERENCES

Rosephine Del Fernandes  Advisor:  University of Guelph, 2018  Dr. W. Glen Pyle

No study has examined the chronological myocardial changes associated with menopause. 4-vinylcyclohexene diepoxide (VCD) injection of female CD-1 mice caused accelerated ovarian follicular atresia over 120 d. Contractility declined with 15 min ER-alpha (4,4’,4”-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol, PPT, 100 nM) and ER-beta (diarylpropionitrile, DPN, 1 nM) agonist perfusion in intact hearts. ERα- and ERβ-dependent decreases in contractility were attenuated 60-days post-VCD (analogous to perimenopause). 120-days post-VCD (analogous to end of menopause) ERα-regulation was normalized whereas ERβ-control remained attenuated. Sex differences in ER functional response were not observed. Myofilament function was altered non-linearly during peri-menopause in conjunction with myofilament protein phosphorylation. Ovarian failure promoted a pro-inflammatory and pro-apoptotic state. We are the first to show the rapid effects of ER on myocardial function and their mechanisms of action are altered early in menopause. The non-linear changes in the heart during menopause may help explain the paradoxical findings of hormone replacement therapy clinical trials.
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DECLARATION OF WORK PERFORMED

I declare that all the reported work in this thesis was performed by me, with the exceptions of the items indicated below:

The majority of actomyosin MgATPase assays were run by Dr. Glen Pyle. I generated these samples.

The immunoblots for ERs were run in duplicate with Vishali Balasubramaniam.
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1. INTRODUCTION

1.1 History

According to the 2016 Global Burden of Disease study, cardiovascular disease (CVD) is the leading cause of death in women worldwide, surpassing that of breast cancer (59). Despite the significant impact on women’s health, research in cardiovascular science has historically had an underrepresentation of females. In 1977, the United States Food and Drug Administration restricted the inclusion of women of childbearing ages in early phase clinical trials unless reproductive toxicity studies were able to demonstrate safety (159). The vast majority of basic science and clinical studies have primarily or exclusively used male subjects, which led to extrapolations for women’s health. The application of male-focused knowledge has contributed and exacerbated the lack of understanding about health disparities and CVD in women. Without a solid understanding of CVD in women the female CVD mortality rate has exceeded males from before the 1990s to 2010 (4). As early as 1985, the US Public Health Service Task Force on Women’s Health Issues warned that the historical exclusion of women from research studies compromised the quality of healthcare available to women (227). Systematic action took place in 1997 when the American Heart Association launched its campaign to help close research gaps in women and raise awareness of disparities in CVD. Since then, female mortality rates of CVD have decreased by nearly half, due to educating the public and practitioners about the risks faced by women; better control of major risk factors; and the implementation of sex-diverse evidence-based treatments (140).

1.2 Epidemiological Studies

CVD is responsible for one-third of all deaths of women worldwide and half of all deaths of women aged 50 or older in developing countries (160). CVD is the leading cause of admission
to hospital in North America for both men and women, accounting for 18% of Canadian hospital admissions in 2000 (160). According to Health Canada, CVD is a major economic burden with healthcare costs reaching $5.068 billion in 2008. These economic costs are likely to rise due to the increasing rates of risk factors like obesity and diabetes, along with an aging society (216).

Premenopausal women appear to be relatively protected from CVD compared with men of similar age (7, 62, 160). For example, evidence from the Framingham Heart Study shows premenopausal women are 15 times less likely to develop coronary heart disease (CHD) compared to men of the same age (62). However, the risk for CVD in women significantly increases with age, particularly after menopause. By age 70, the incidence of CVD is equal between the sexes and the rates in women surpass those of age-matched men in later years (7, 8, 65, 207). The Framingham study found a 4-fold increased CVD risk in women in the 10 years following natural menopause. Interestingly, this rise remained true in those with surgical menopause suggesting that the effects were not age-dependent (62) Women who experience premature or early-onset menopause have a higher risk of CHD, CVD mortality, and overall morality (142). In addition, women with premature ovarian insufficiency- natural menopause prior to age 40- have an increased risk of ischemic heart disease and overall CVD (178). These results support the hypothesis that longer exposure to endogenous estrogens protects women against CVD.

**Hormone Replacement Therapy and CVD Risk**

There is a general consensus that hormone replacement therapy (HRT), combined estrogen and progesterone replacement (E+P) or estrogen therapy alone (E), decrease osteoporosis risk and increase risk for breast cancer (27, 65, 66). Whether HRT is beneficial or detrimental for a women’s risk for CVD has been an evolving discussion in the last two decades.
In 1997, the Nurses’ Health Study (NHS) used questionnaires to investigate the impact of all available HRTs (E+P and E) on mortality. According to their observational study, HRT-treated patients with risk factors for CHD have the largest reduction in mortality compared to nonusers (66). Mortality among HRT users was lower than nonusers overall; yet, the benefit of HRT decreased with long-term use and was lower for women at low risk for CHD. Overall, the NHS provided support for the protective role of HRT for women with high risk factors for heart disease. The supplementation with estrogen during menopause through HRT would expect to decrease risk of CVD by maintaining the protective effects of estrogen.

The Heart and Estrogen/progestin Replacement Study (HERS) was a randomized, double-blind, placebo-controlled trial of E+P HRT published in 1998. This study looked at the effects of HRT on the rates of myocardial infarctions (MI) and death due to CHD (78). Secondary cardiovascular disease outcomes that were also studied included congestive heart failure, stroke, and cardiac arrest. Although observational studies, including the NHS, had found lower rates of CHD with HRT, HERS found no significant difference in the rates of MI or CHD between HRT and nonusers. There was a significant time trend with treatment, where HRT users experienced more CHD events in their first year of HRT treatment, but fewer adverse events their fourth and fifth years. HRT users were more likely to experience thromboembolic events overall (78). HERS continued in a subsequent trial (HERS II) to determine if the risk reduction continued past 5 years of HRT on the same outcomes of MI and CHD and found that there was no benefit after 6.8 years of HRT (64). These study findings demonstrate no significant cardiovascular benefit overall with HRT, which led to the recommendation against HRT as a treatment to reduce cardiovascular risk in post-menopausal women (78).
In 2003, the Women’s Health Initiative (WHI) organized a large-scale randomized clinical trial to investigate the impact of E+P HRT on CHD (129). Combined E+P therapy was associated with a significant increase in CHD risk, even in generally healthy postmenopausal women. This large-scale trial contradicts the benefits of HRT reported by NHS.

In 2007 the results from the WHI were reevaluated to determine how timing of HRT initiation may influence its effects on cardiovascular disease (183). For women who started E+P HRT less than 10 years before the onset of menopause, their hazard ratio (HR) for CHD was 0.76; for women who started between 10 and 19 years, HR was 1.10; and starting E+P HRT 20 or more years after menopause was associated with a HR of 1.28. Similar findings demonstrated in the multicenter randomized Danish Osteoporosis Prevention Study (DOPS) found that women receiving E HRT early after menopause also had a reduced risk of mortality, heart failure, and myocardial infarction (189). DOPS also found that the cardiovascular benefits came without any apparent increase in risk of cancer, venous thromboembolism, or stroke (189).

In addition, the majority of clinical trials have used synthetic steroids with hormonal activity so that they have not replaced the natural hormones themselves, leading to contradictory findings of HRT studies. For example, WHI uses medroxyprogesterone, Provera, as a progesterone substitute, which has significantly opposite effects on the coronary arteries than progesterone. Provera and progesterone have opposite effects on coronary reactivity; duration and magnitude of vasoconstriction, minimum coronary diameter during angiography, and myocardial ischemia during angiography (74). Progesterone has anti-glucocorticoid activity and inhibits cortisol-mediated enhancement of adrenergic contraction in vascular arteries (3). Provera is the only progestin with glucocorticoid agonist properties, which can contribute to differences in cardiovascular outcomes (3, 74, 129).
The apparently contradictory findings of HRT studies become less confusing when results are more closely investigated and mitigating factors like delayed treatment are considered. The totality of the evidence suggests the outcome of HRT may be altered by years since menopause, and that there may be irreversible changes to the cardiovascular system after the onset of menopause that estrogen replacement could have provided, that cannot be restored. Without a better understanding of the complexity of estrogen regulation of the heart, including temporal factors, it is difficult to explain the mechanisms by which estrogen might be cardioprotective in premenopausal females and less effective in postmenopausal women.

1.3 Gender Differences

Disparities in CVD may be accountable to sex and gender, and the distinctions between these two forms of categorization are important. According to the World Health Organization, ‘sex’ refers to the biological basis that defines males and females; whereas, ‘gender’ refers to the socially constructed characteristics that assign roles of men and women. While investigations into the role of sex in health and disease have been notoriously neglected, gender studies are even rarer. Ground-breaking work by Elizabeth O’Connor was the first to recognize gender as a non-modifiable risk factor for CHD (7). Since then others have gone on to explore how gender differences in CVD can arise from different lifestyles, environmental exposures, and healthcare biases (140). For example, men and women engage in different behaviours that interact to influence their risk for CVD. Men are more likely to smoke cigarettes than women (23.1% versus 18.1%) (179). However, women smokers are 25% more likely to develop CVD than men smokers (79). Age-adjusted rates of physical inactivity in 2009 were higher in women than men (34.5% versus 30.3%). The gender-dependent differences in CVD risk factors suggest a role for
gender in affecting cardiovascular health, but the general lack of investigation into the role of gender precludes a more definitive link.

Sex differences in cardiovascular physiology and CVD contribute to the differences in CVD outcomes. For example, aspirin is commonly used in the prevention of MI and stroke, yet its effects are different among men and women. In men, aspirin reduces incidence of MI and has no effect on stroke (208); whereas in women aspirin has only a small effect on MI that is age-dependent (175). Some tests used for diagnoses are less accurate for women or require different interpretations. Circulating troponin I, commonly used in the diagnosis of MI, is found in much lower levels in women than that of males (187). Angiograms after an MI tend to appear normal in women, who are more likely to have non-obstructive coronary artery disease (CAD), compared to men (15% vs 8%) (11). Outcomes differ for a variety of treatments in women, including access to treatments such as bypass surgery, angioplasty, and catheter-based aortic valve replacement (TAVR) which tend to be lower in women (81, 241). Unfortunately, these biases may contribute to the higher 30-day mortality post-MI in women compared with men (11).

There are interrelationships between sex and gender that contribute to an individual’s risk for CVD. This study focuses on sex differences, particularly those due to the hormonal changes of estrogen that may contribute to the increased risk of heart disease.

1.4 Estrogen

Estrogens are a group of sex steroid hormones that have broad effects on multiple organs in addition to their well-known role in reproductive function (121). There are three major endogenous estrogens in both humans and mice: estrone (E1), 17β-estradiol (E2) and estriol (E3) (54). The primary source of estrogens in premenopausal women are the ovaries, while other
sources include bone, breast, adipose tissue, and the brain; and the testes and prostate in men (121, 226, 229). Estrogens are synthesized from the androgen precursors androstenedione, testosterone, and 16-hydroxytestosterone by the enzyme aromatase (CYP19a1) (210). Estrogens can be synthesized locally by aromatase in the heart and then activate local ERs (10, 67). The most potent form of circulating estrogen is E2, which binds both ERα and ERβ with the highest and equal binding affinity (6). By contrast, E1 is a weak agonist that binds ERα and ERβ with equal affinity. E3 is a stronger ERβ agonist than estrone but is ~10X less potent than 17β-estradiol (46).

Serum 17β-estradiol concentrations are relatively low in preadolescent girls and rise at menarche. In sexually mature women, the secretion of E2 is pulsatile and its concentration fluctuates according to the menstrual cycle. The serum concentration of E2 ranges from 100 pg/mL in the follicular phase to 600 pg/mL at ovulation (121). E2 rises to nearly 17,000 pg/mL during pregnancy (225). Postmenopausal women have serum E2 values similar or lower than that of men of similar age: 5-20 pg/mL (134). E1 production in postmenopausal women is minimally reduced in comparison to premenopausal women, since most E1 production is from peripheral aromatization (88).

According to blood samples from the Framingham Heart Study, serum estrone and 17β-estradiol concentrations in men are positively associated with age, yet free estradiol levels decline with age, suggesting higher serum hormone binding globulin (SHBG) levels in older men (83). However, this remains inconclusive because some studies have found higher total estradiol levels in older men than young men, or no age-related changes (49, 150).
1.5 Estrogen Receptors

There are three known ERs expressed in the heart: ERα, ERβ, and G protein-coupled estrogen receptor, GPER, previously known as GPR30. They are coded by the genes ESR1, ESR2, and GPR30, respectively (146, 152, 217). ERα and ERβ are both part of the superfamily of steroid hormones and they share high sequence homology (128). Varying ERα isoforms have been reported to be expressed in the heart. The 66 kDa ERα isoform is considered the full-length protein and the 46 kDa ERα isoform is the N-terminal truncated form (181). A 50 kDa ERα isoform was also identified in hypothalamic neurons and a 36 kDa ERα isoform was identified in breast carcinomas (94). These different isoforms have different biological activities, broadening the range of responses elicited by estrogen in the heart. GPER is a 7-transmembrane spanning protein receptor that lacks ligand-binding transcription activation. Each ER isoform has distinct, non-overlapping physiological functions and are expressed in a cell and tissue specific manner (94).

ERα and ERβ are unique steroid hormone receptors in that they mediate their actions through a variety of ways once activated. It is classically known that ERα and ERβ act as transcription factors that alter gene expression once complexed to estrogen (104). Once activated, ERs dimerize in E2-estrogen-receptor complexes and bind to specific DNA sites known as estrogen-response-elements (ERE) to regulate target genes (71). This process can offer complexity in many ways. In addition to forming homodimers within receptors, ERα and ERβ form heterodimers with each other to alter the regulation of gene expression and exert transcriptional regulation by association with other DNA bound elements (29). In addition, the regulation of ERα and ERβ have been shown to interact, altering regulational outcomes of the
cell. ERβ has the potential to inhibit the transcriptional effects of ERα and vice versa (29, 71). Thus, the ratio of ERα and ERβ expression and regulation is important in determining responses.

ERα and ERβ associate with coactivator and corepressor proteins to regulate gene expression. As estrogen binds its receptor, the ER changes its conformation in the ligand-binding domain to enable recruitment of co-activators such as SRC-1, TIF2, and TRIP1 (196). Once bound to ER, coactivators induce conformational changes within the complex to permit interaction with transcriptional machinery more efficiently. Corepressors, such as SMRT and N-CoR bind ERs to promote an antagonistic effect on gene transcription. Corepressors bind and induce a conformational change in the estrogen-ER complex that results in a negative interaction with transcriptional machinery and reduces efficiency of transcription activation (107, 196).

In addition to the classic genomic pathways of ER signaling it has more recently been discovered that estrogens can also act like typical protein ligands that bind receptors at the plasma membrane to initiate fast, non-genomic signaling responses. Increasing evidence shows that estrogens can act at the cell surface of a variety of target tissues and cell types to initiate rapid, non-genomic responses by binding ERs at the membrane (152). About 5% to 10% of α- and β- ERs are located in the plasma membrane, although the relative amount of each isoform at the plasma membrane varies according to cell type (157).

ERα and ERβ expression and distribution is well studied in endothelial cells (ECs) and vascular smooth muscle cells. There is a large body of evidence showing their localization at the plasma membrane (69). ERα was identified in the plasma membranes of ECs using three different ERα antibodies and plasma membrane fraction isolation from the cell. Both ERα and ERβ colocalize with eNOS in caveolae near the plasma membrane. Both classic ER isoforms are found in the nucleus (23, 69).
ERα expression and distribution has been well-studied in cardiomyocytes. ER isoforms are generated from multiple ERα mRNA transcripts by alternative promotor usage, splicing, and translation initiation (94). As previously mentioned, there are two predominant isoforms of ERα: a 66 kDa full-length (long) isoform and an N-terminal truncated 45 kDa (short) isoform. These isoforms distribute differentially between the plasma membrane and the nucleus in cardiomyocytes. The ERα short isoform was mainly detected in the membrane fraction while the ERα long isoform was mainly detected in the cytosolic fraction (181). In addition, ERα was prominently expressed in T-tubular membranes and tends to associate with α-actinin when in a continuous distribution pattern. ERβ distribution in the heart is less well studied, but evidence of expression in myocardium and cardiomyocytes is demonstrated (67, 134). When comparing sex differences in expression of ERα, there is no difference between LV cardiomyocyte male and female ERα mRNA expression (166).

GPER is a 7-transmembrane spanning G-protein receptor that acts through rapid, nongenomic signalling (164). In the presence of estrogens GPER rapidly activates signalling pathways in cells that do not express the classic α- or β- ERs but did express GPER (50). Two research groups independently found that recombinant GPER had the same binding characteristics of a membrane ER and activated multiple signaling pathways upon exposure to estrogens (51, 173, 220). Similar to other G-protein membrane receptors, GPER activates a G-protein (G₃) leading to activation of adenylyl cyclase and an increase in intracellular cAMP levels (51). GPER has signalling roles in calcium mobilization and synthesis of phosphatidylinositol 3,4,5-trisphosphate (PIP₃) (165, 173).
1.6 Estrogens in Preclinical Studies

A number of large, wide-scale clinical trials produced contradictory findings about the effectiveness of HRT in cardiovascular disease outcomes (64, 78, 129, 183, 189). Yet, in preclinical studies, there is overwhelming evidence for the protective effects of estrogens on the cardiovascular system. The majority of preclinical studies on estrogen focused on its regulatory effects on vasculature. Chronic E2 treatment induces genomic changes that promote protective circulating lipoproteins, decrease oxidization of LDL (198), and reduce atherosclerotic plaque development (2). Long-term E2 treatment decreased plasma concentrations of renin, ACE, endothelin-I, vascular expression of ANG II receptor type I, and increased the ratio of NO to endothelin I: all these changes promote vasodilation and lower blood pressure (134). Estrogens also cause rapid nongenomic vasodilation within minutes after treatment (231).

Estrogen regulation in the vasculature has been well studied; however, there is increasing evidence that estrogens have direct effects on the myocardium, independent of vascular hormone action (201). Estrogens protect against ischemia-reperfusion (I/R) injury. Short-term (20 min) E2 treatment before I/R reduces myocardial necrosis in male rabbits (70). In a separate study, short-term E2 treatment for 30 min before I/R decreased infarct size in OVX female rabbits (16). Similarly, a decline in infarct size was seen in both female and male rabbits with only 15 min of acute E2 treatment prior to I/R, which suggests cardioprotection by the estrogens can be mediated in both sexes through rapid, non-genomic effects (70). Finally, treatment with E2 and an ER antagonist significantly inhibited protected effects of E2 on infarct size, which provides evidence of E2 mediating its protection through ER (15).

There is significant evidence suggesting that estrogens acutely mediate protection against I/R injury through ERα and not ERβ. I/R studies with acute treatment of E2 and the ERα agonist
4,4’,4”-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) decreased infarct size, reduced circulating cardiac troponin I levels, and reduced inflammation compared to controls in an in vivo rabbit heart model. The ERβ agonist diarylpropionitrile (DPN) did not offer cardioprotection (17). In addition, chronic ERα agonist treatment prior to I/R reproduced the response by E2 treatment in reducing cardiomyocyte death, neutrophil infiltration, and oxygen-free radical availability. These cardioprotective effects were not inhibited by an ERβ antagonist (84). Acute ERβ activation that induces rapid nongenomic effects may not contribute to myocardial protection, but one study has shown chronic treatment of the ERβ agonist DPN for 2 weeks prior to ischemia is cardioprotective in OVX female mice. Long-term ERβ activation increases LVDP recovery and upregulates protective genes such as heat shock protein (hsp) 70 and antiapoptotic proteins which help the heart respond to injury (145). These results suggest that the cardioprotective effects of estrogens may depend on ovarian function and production of estrogens.

Sex differences exist in the way ERα affects heart recovery from I/R injury. In mice a post-I/R increase in vascularization was seen in both sexes with ERα overexpression; but only females displayed reduced levels of fibrosis and higher phosphorylation of the JNK signalling pathway (125). Another study showed abolished protection by E2 after treatment with a nitric oxide synthase (NOS) inhibitor and ER antagonist in separate cases (57). This evidence suggests ER-dependent JNK signalling and NO production contributes to the beneficial effects of E2 treatment post-ischemia (57, 125), and that different ERs are important for the chronic versus acute effects of E2. The observation that acute addition of E2 just before ischemia reduces infarct size suggests that acute signalling pathways play an important role in cardioprotection (143).
Several preclinical studies support the protective effects of estrogens on the development of cardiac hypertrophy. Chronic E2 treatment reduced cardiac hypertrophy by 31% four weeks after pressure overload by transverse aortic constriction (TAC) and 26% eight weeks after TAC in OVX mice. The mechanism in which E2 induces its antihypertrophic effects include inhibition of the MAPK pathway and upregulation of atrial natriuretic peptide (ANP). Chronic E2 treatment increased expression of ANP and attenuated the increased phosphorylation of MAPK in OVX mice under TAC (42). In a separate study, at 2 weeks post-TAC in OVX female mice, E2 offered cardioprotection by reducing cardiomyocyte hypertrophy and attenuating decreased LV systolic function and contractility.

Another mechanism that contributes to the reduction of LVH by E2 involves an ER-dependent pathway that increases calcineurin A (CnA) degradation and its downstream targets, nuclear factor of activated T-cell (NFAT) family of transcription factors. These effects were reversed in both CnA knockout mice and treatment with an ER antagonist (37).

ERα and ERβ knockout models were used to specify which ER mediates LVH protection. E2 reduced LVH, blocked increased phosphorylation of MAPK and increased ANP only in ERα knock out and WT mice, but not ERβ knock out mice. Despite ERα being the predominant receptor involved in I/R protection, studies demonstrate E2 acts through ERβ to protect the heart against LVH (5). ERα and ERβ differentially regulate the heart in MI protection and remodeling.

Overall, E2 offers cardioprotection and the loss of estrogens have been damaging in preclinical animal studies. However, how soon after estrogen loss causes damage has not been fully investigated. In addition, it is unknown how long after estrogen deficiency results in irreversible effects with estrogen therapy.
1.7 Calcium and Action Potential Regulation

Calcium handling is central to the development of contractile force in cardiac muscle. During a normal cycle of calcium handling in a cardiac myocyte, a change in the membrane electrical potential depolarizes the sarcolemma, including transverse (T) tubules, and opens voltage-gated L-Type Ca\(^{2+}\) channels (LTCC), allowing an influx of Ca\(^{2+}\) into the cardiac myocyte (13, 43, 209). A rise in intracellular Ca\(^{2+}\) induces the ryanodine receptor (RyR) to release additional Ca\(^{2+}\) from the sarcoplasmic reticulum (SR), an intracellular store of calcium (25). The process whereby Ca\(^{2+}\) from extracellular influx activates the release of Ca\(^{2+}\) intracellularly is known as calcium induced calcium release (CIRC) (13, 209). The combination of Ca\(^{2+}\) influx via LTCC and its release from the SR results in an increase of free intracellular Ca\(^{2+}\) levels, providing the Ca\(^{2+}\) necessary to bind and activate myofilaments (13, 205). After contraction, Ca\(^{2+}\) is removed from the cytoplasm to prepare for relaxation. Ca\(^{2+}\) is pumped back into the SR by the SR Ca\(^{2+}\)-ATPase (SERCA) (36) or is removed from the cell by the sodium-calcium exchanger (NCX) and sarcolemmal Ca\(^{2+}\)-ATPase (197). There is also uptake of Ca\(^{2+}\) by the mitochondrial Ca\(^{2+}\) uniporter into mitochondria although this is insignificant under physiological conditions (13).

There are fundamental differences in calcium regulation in cardiac myocytes of males and premenopausal females (176): LTCC density and LTCC current (I\(_{Ca,L}\)) in cardiomyocytes are higher in adult female rabbits than males at prepubertal ages and reversed in adult rabbits (199). ERs may downregulate LTCC (primarily Cav1.2\(\alpha\)) and reduce I\(_{Ca,L}\), which affects calcium transients, cardiac excitability, and contractility. Male ER knock out mice have an increased membrane density of LTCC channels, increased calcium current, I\(_{Ca,L}\), and longer action potential duration as compared to age-matched females (87). In addition, expression and activity
of LTCC increases after a large decline in circulating estrogens by OVX (89). This was reversed with E2 replacement and inhibition of protein kinase A (PKA).

Calcium entry into cardiomyocytes can also be regulated by rapid, nongenomic effects of E2. Acute treatment of E2 decreases $I_{\text{Ca,L}}$ and has a negative inotropic effect in isolated cardiac muscle preparations and myocytes (135, 200). Antagonistic effects on $I_{\text{Ca,L}}$ only appeared at levels above the physiological range. However, when myocytes were pretreated with isoprenaline, a β-adrenergic agonist, a significant decrease in $I_{\text{Ca,L}}$ occurred with E2 treatment at physiological levels. This demonstrates E2 has the potential to create antagonist effects on the heart at physiological levels and interacts with other stimuli in heart muscle cells.

The sodium-calcium exchanger (NCX) is responsible for regulating some calcium and sodium movement into and out of the cell with reversible function (174). Its role in calcium removal depends on the species involved, but for the vast majority of animals this calcium removal mechanism is secondary to SERCA. Nonetheless, it has a physiological role and is impacted under conditions of stress. E2 regulates both NCX expression and activity in a sex-dependent manner, which may contribute to reported sex differences in $\text{Ca}^{2+}$ transients. Isolated cardiomyocytes from female rabbit hearts had greater NCX levels compared to those isolated from male hearts (24). Chronic E2 treatment upregulates NCX levels through a genomic mechanism in cardiomyocytes from females, but no effects were reported in cardiomyocytes from males (24). Withdrawal of estrogens by OVX increases NCX activity through a PKA-dependent mechanism without changing NCX levels (102). Although E2 upregulates NCX genomically, chronic E2 also decreases activity of NCX through a PKA-mediated pathway and may contribute to the sex differences seen in $\text{Ca}^{2+}$ transients.
Estrogen regulation of NCX activity and expression may act to relieve the negative effects on LTCC. Cardiomyocytes with upregulated NCX levels with chronic E2 treatment demonstrated increased susceptibility to early after depolarizations (24). This estrogen-dependent effect may explain, at least in part, why females tend to have lower levels of LTCC and are more prone to a prolonged QT interval (9).

RyR is responsible for the intracellular release of Ca^{2+} from the SR (25). Estrogens decrease RyR activity through a PKA-mediated pathway. Withdrawal of estrogens by OVX activated PKA and increased Ca^{2+} flux through RyR, which was subsequently reversed with estrogen replacement and PKA inhibition (47, 102). Similarly, in a separate study, OVX enhanced Ca^{2+} spark frequency and calcium transient amplitudes in mouse ventricular myocytes, possibly due to enhanced Ca^{2+} release by RyR (47). This evidence suggests estrogens have a role in decreasing RyR activity through a PKA-mediated pathway that may explain OVX enhanced Ca^{2+} sparks and sex differences in calcium stores.

Sex differences exist in the overall regulation of intracellular [Ca^{2+}] transients. A study in rat cardiomyocytes found that peak [Ca^{2+}] and the amplitude of the Ca^{2+} transient increased more in cardiomyocytes from males versus females. In addition, it took longer for cardiomyocytes from females to clear the Ca^{2+} with slowed times to peak shortening and 50% relaxation and a reduced extent of shortening (31). Bilateral OVX increases peak Ca^{2+} transients and contractions when compared to sham-operated animals. This further emphasizes the role of estrogens in regulating Ca^{2+} homeostasis in cardiomyocytes (30).

In addition to calcium regulation, estrogens regulate the activity and level of ion channels responsible for the heart’s relaxation. Females have higher susceptibility to torsade de pointes arrhythmias associated with prolonged baseline corrected QT intervals, and it is likely that
estrogens contribute to the associated sex differences in prevalence (126). Preclinical studies suggest E2 depresses K\(^+\) outward current that contributes to the repolarization phase during heart relaxation. K\(^+\) outward current changes according to fluctuating plasma E2 during a normal cycle in mice. Fast-transient outward K\(^+\) current (I\(\text{to, f}\)), ultrarapid delayed rectifier K\(^+\) current (I\(K_{\text{slow}}\)), and total K\(^+\) current were lower in female mice at the high E2 stage, estrus, compared to females in diestrus and males. These currents all declined with E2 treatment of OVX mice. Similarly, susceptibility to arrhythmias change throughout the menstrual cycle in women so that drug-induced corrected QT prolongation is more severe in the late follicular phase when E2 levels are high (177). High E2 levels are associated with longer action potential duration and corrected QT interval (186). A separate study found that E2 can acutely affect QT prolongation within only 10 min of E2 treatment, suggesting E2 acts through rapid nongenomic signalling (108). Lastly, ER\(\alpha\) and ER\(\beta\) knock out mice revealed that ER\(\alpha\) has a repressive role in regulating the K\(^+\) channel Kv3.2 and outward K\(^+\) current I\(\text{to}\) (60).

1.8 Myofilaments

Cardiac muscle cells are striated, branched, and connected to one another by intercalated disks containing gap junctions (191). Single cardiac muscle cells are ‘myocytes’, which largely consist of myofilament proteins organized to form sarcomeres, the contractile units of the heart (191). Sarcomeres join end-to-end to form myofibrils, which make up most of the cytoplasm of a myocyte (232).

Myofilaments are the central contractile units of the heart. Myofilaments are composed of interacting thick and thin filaments. Under normal physiological conditions an increase in free intracellular Ca\(^{2+}\) during excitation-contraction coupling allows Ca\(^{2+}\) to bind myofilaments to activate the contractile state of the cardiac cell. Calcium binds and is released from
myofilaments until calcium removal mechanisms reduce free calcium to levels that are below the activation threshold. The release of calcium from cardiac myofilaments is a passive process that can be promoted or inhibited by the post-translational modification of several myofilament proteins.

Myosin, the main component of the thick filament, uses chemical energy derived from ATP hydrolysis to produce mechanical work during its interactions with actin (127). Myosins have a conserved ~80 kDa catalytic domain, and an α-helical light chain-binding region consisting of one or more motifs (127). The myosin molecule is composed of two large polypeptide heavy chains and two pairs of light chains: the essential myosin light chain (MLC1) and the regulatory light chain (MLC2v) in ventricular myocardium (232). These combine with two globular heads of ‘heavy’ myosin, which are coupled to a long tail formed by the two intertwined heavy chains. The tails make up the backbone of the thick filaments and the two globular heads extend out to form cross-bridges with actin. Each globular head contains binding sites for actin and ATP, and an ATPase that hydrolyzes bound ATP (232).

The thin filament is composed of primarily actin, tropomyosin, and the troponin complex. Actin is a globular protein composed of a single polypeptide that polymerizes with other actin monomers to form two intertwined helical chains. These chains make up the backbone of the thin filament and each actin molecule contains a potential binding site for myosin (232). The approximate ratio of actin to troponin-tropomyosin is 7:1.

The intermediate filament protein desmin is involved in sarcomere structure and organization. Desmin surrounds Z-discs and links the sarcomere with the sarcolemma, cytoskeleton, and cytoplasmic organelles including the mitochondria and nucleus (137, 192). Hearts from desmin null studies are severely defective and display necrosis of the myocardium,
calcification, cardiomyocyte hypertrophy, and dilated cardiomyopathy, indicating the important role of desmin in cardiac function (136, 155). PKC-mediated phosphorylation can induce disassembly of desmin filaments, which may be involved in myofibrillar disarray (77, 80). A study demonstrated that desmin from failing hamster hearts contain more phosphorylation than that of normal control hearts (99).

Although the exact mechanisms by which desmin impacts heart function is not known, in general it acts as a biophysical linker between the subcellular compartments and facilitates mechanical signaling.

Cardiac myosin binding protein-C (MyBP-C) is a thick filament-associated protein that regulates the probability of cross-bridge interaction between myosin and actin. MyBP-C knockout models leads to cardiac hypertrophy (38). Dephosphorylated MyBP-C preferentially binds myosin to reduce its probability of binding actin. During increased physiological demands, PKA phosphorylates MyBP-C, which preferentially binds actin, to relieve the inhibition on myosin and promote cross-bridge interaction (141). MyBP-C phosphorylation state (141) affects the probability of cross-bridge interaction between myosin and actin according to its regulation of MyBP-C phosphorylation sites in MyBP-C models that have different contributions to force production. Ser-273, 282, and 302 in mice (58, 234). Overall, regulation of MyBP-C has roles in accelerating cross-bridge binding to actin (221), increasing Ca2+ sensitivity, and increasing the velocity of unloaded shortening (75).

The myosin light chains (MLC) regulate and contribute to the efficiency of cross-bridge cycling. MLC1, the essential myosin light chain, increases myofilament Ca2+ sensitivity by inducing a supramaximal increase in MgATPase activity at submaximal Ca2+ transients when it is phosphorylated (169). MLC2v phosphorylation by myosin light chain kinase (MLCK) can increase myosin lever arm stiffness and promote myosin head diffusion (194). This works to...
increase cardiac muscle force. MLC2v phosphorylation also promotes the movement of myosin heads closer to the actin filaments to slow down myosin cycling kinetics and prolong the cycle (193, 194). MLC2v phosphorylation is also a determinant of Ca$^{2+}$ sensitivity of myocardial force development (149).

Tropomyosin and the troponins are involved in the regulation of actin-myosin binding as determined by availability of intracellular Ca$^{2+}$ (205, 232). Tropomyosin is a rod-shaped molecule composed of two intertwined polypeptides arranged end-to-end along the actin thin filament. Its positioning on the thin filament physically inhibits the myosin-binding site on each actin molecule (205, 232). The troponin complex is composed of three subunits: troponin I (inhibitory), troponin T (tropomyosin), and troponin C (calcium-binding) (TnI, TnT, and TnC, respectively). TnT physically anchors the troponin complex to tropomyosin. TnC contains the calcium binding site involved in excitation-contraction coupling and relaxation. TnI physically inhibits myosin-binding directly through its N-terminal extension and indirectly through the positioning of tropomyosin (91, 205, 232).

After calcium binding, a structural change initiated in TnC but moving through the troponin complex removes the steric inhibition of actin-myosin interaction created by troponin-tropomyosin, allowing myosin to bind actin and undergo cross-bridge cycling (63, 91, 232). Cross-bridge cycling is powered by ATP cleavage, and ATP binding to the myosin head displaces myosin from actin. The cycle repeats in the presence of intracellular calcium until calcium detaches from TnC and troponin-tropomyosin inhibition of myosin binding returns (133, 205, 232).

Estrogens have well-recognized roles in the cardiovascular system and they are known to alter myocardial contractility through their effects on myofilament function. Rapid, nongenomic
activation of ERα depresses actomyosin MgATPase activity and decreases myofilament calcium sensitivity (106). Inhibition of p38 MAPK attenuated these effects, suggesting actions were mediated by the p38 MAPK pathway. TnI phosphorylation at S23/S24 was decreased, which is commonly associated with a decrease in myofilament calcium sensitivity (106). Similarly, withdrawal of estrogens by OVX increased myofibrillar Ca\(^{2+}\) sensitivity in isolated hearts, and this was attenuated by chronic estrogen replacement (228). A separate study found that myocardial contractility was preserved early after abrupt estrogen withdrawal by OVX in female rat heats but was reduced 60 days after OVX. The subsequent reduction was restored with estrogen replacement (151). Lastly, myocardial function can change with the estrous cycle. Ventricular myocytes isolated from female mice had larger contractions, larger Ca\(^{2+}\) transients, and lowest myofilament Ca\(^{2+}\) sensitivity during estrus compared with other stages (122).

Sex differences in contraction response may arise due to differences in the contractile machinery in cells. Female rat ventricles possessed higher levels of both α- and β-myosin heavy chain (MHC) mRNA and sarcomeric actin mRNA compared to males. The ratio of α- to β-MHC remained the same (182). β-MHC expression was increased in hearts of women and fertile female mice compared to age-matched males (153, 172). Deletion of β1- and β2-adrenergic receptors in mice, which were originally 40% lower in fertile females compared with males, abolished sex differences in β-MHC expression. In addition, sex differences in β-MHC expression disappeared after OVX or prepubertal mice, which suggests estrogen may be a possible regulator of β-MHC expression, and interacts with the same signalling pathways as β-adrenoreceptors (153).

Sex differences in myocardial contractile reserve arise during increased physiological stress. In trabeculae from cats, force produced by trabeculae from males was significantly higher
than that by females at increased stimulation frequencies (1.5 and 2.0 Hz) (158). Increases in preload associated with the Frank-Starling response resulted in smaller slopes in the force-frequency relationship in females compared to males. Also, SR calcium load increased with simulation frequency in trabeculae from males but not females (158). Yet, there was no difference in myofilament calcium sensitization in varying baths of extracellular calcium (158). Cardiac muscles from normal female hearts produce less force, take longer to reach peak force, and take a longer time to relax compared with age-matched males.

1.9 Inflammation

Inflammation is an important process in both injury and repair of the heart. Tumor necrosis factor α (TNFα) is a well-studied, potent proinflammatory cytokine involved in the pathogenesis of CVD, including congestive heart failure, septic shock, and myocarditis (222). TNFα acts on two receptors- TNFR1 (p55) and TNFR2 (p75) -which are both expressed in the adult heart under baseline conditions (222). Activation of TNFR1, but not TNFR2, led to a negative inotropic effect in isolated feline cardiac myocytes (222). Circulating levels of TNFα increases with heart failure progression as a mediator of myocardial dysfunction and adverse remodeling. TNFα is useful in evaluating risk assessment for heart failure patients with preserved and reduced ejection fraction where elevated TNFα levels are associated with a large decrease in HF patient survival (40). Treatment of the mouse heart with anti-TNFα adenoviral vector expression preserves myocardial diastolic function and reduced negative myocardial matrix remodeling (115).

TNFα levels change with levels of circulating estrogens. Hearts from OVX rats have increased expression levels of genes that code for inflammatory mediators, including TNFα. OVX also decreases expression of genes related to vasodilation, increased expression of
extracellular matrix genes and proapoptotic genes including caspase-3 and calpain (72). E2 replacement reversed many of these changes demonstrating a key role for estrogens (72). A small scale observational study of women investigated postmenopausal changes in serum cytokine levels with HRT and found an increase in TNFα and a decline in transforming growth factor β1 (TGFβ) in late postmenopausal women (90). HRT induced a significant increase in serum levels of macrophage colony-stimulating factor, but serum levels of other cytokines were not affected (90). These results suggest that estrogen changes following menopause may affect inflammatory markers in women and explain the increased risk for CVD.

Some of the protective effects of estrogens in I/R studies have been ascribed to the reduction in circulating cytokines. In rats, long-term estrogen treatment attenuated endotoxin-induced myocardial TNFα expression and ischemic injury, suggesting that the downregulation of TNFα provides a role in ischemic protection by estrogen (116). Chronic E2 treatment also attenuated the rise in TNFα levels in coronary effluent after I/R (235), improved post-I/R functional recovery, decreased LV apoptosis, and reduced myocardial necrosis (235).

In a large, nationwide, prospective study of women from the WHI the effects of HRT on inflammatory markers C-reactive protein (CRP) and IL-6 were investigated (163). CRP levels are largely stimulated through the IL-6 pathway, and TNFα is a known upstream cytokine inducer of IL-6 expression (22, 55). Women on HRT (E alone and E+P) were found to have two times higher CRP levels compared to non-users (111, 174). There was a positive relationship between plasma CRP levels and risk for CHD among both users and nonusers of HRT. Overall, CRP and IL-6 independently predict vascular events among apparently healthy postmenopausal women. HRT was shown to increase levels of CRP, but baseline levels of inflammatory markers were a better predictor of CVD risk than HRT use (163). A separate clinical study found elevated
serum IL-6, CRP, and spontaneous production of TNFα were associated with a 3-fold increased risk of HF, and this did not differ by sex (93). The same association between CRP and CHD has been confirmed in several clinical studies (33, 98, 105, 176, 224). In addition, IL-6 and CRP levels were elevated in women with traditional risk factors for CVD, including diabetes, hypertension, history of HRT, high BMI, and smoking. HRT remained associated with CRP even when adjusting for these factors (12). These studies demonstrate HRT influences IL-6 and CRP levels, which are both known markers for heart disease.

In comparison to IL-6, IL-1β is less well studied in association with CVD. However, it is well known that IL-1β stimulates CRP synthesis and that activation of CRP protein synthesis is associated with an increase in CHD risk (101). IL-1β stimulates CRP synthesis by inducing an NF-κB and C/EBPβ-dependent autocrine IL-6 loop (101). Both IL-1β and IL-6 are involved in the acute phase of CRP protein synthesis and they are considered equal inductors of CRP in cell line studies. Gaining a better understanding of how to modulate these cytokines through the use of estrogens may help suppress CRP expression for treatment of CVD.

Transforming growth factor β (TGFβ) is an important anti-inflammatory cytokine involved in CVD, particularly in cardiac remodeling, angiogenesis, and fibrosis. Higher levels of TGFβ is a good prognostic marker for hypertrophic cardiomyopathy and one of the most important predictors for heart failure (53). In response to increased afterload, such as aortic valve stenosis, the heart adapts with hypertrophy to preserve systolic function. However, chronic stress leads to excessive hypertrophy, reduced ejection fraction, elevated end-diastolic pressure, and increased ventricular stiffness (56). TGFβ regulates changes associated with permanent pressure overload, such as fibroblast growth, modulation of extracellular matrix metabolism, and stimulation of angiogenesis. In I/R studies, TGFβ plays a role in promoting angiogenesis,
attenuating myocardial necrosis, attenuating endothelial dysfunction, and neutrophil adherence to reduce injury (55). Overall, TGFβ is cardioprotective during an inflammation stimulating event.

Types of pathological hypertrophy differ between women and men. For example, compared with men, women develop more concentric hypertrophy with better maintained function in aortic stenosis, and women exhibit more diastolic dysfunction with small ventricles and thickened walls (170). Men mainly develop systolic dysfunction (214). As discussed previously, E2 has a well-known cardioprotective effect to reduce hypertrophy through the actions of ERβ and may help explain sex differences in hypertrophy. In a TAC OVX mouse model of pressure overload, E2 and an ERα agonist diminished the development of fibrosis, reduced TGFβ expression, and improved survival rate after TAC (230).

1.10 Apoptosis

Apoptotic death of cardiomyocytes contributes to the morbidity and mortality associated with heart disease (1). Apoptosis is involved in the development of reperfusion injury, end-stage heart failure, atherosclerosis, and pressure-overload hypertrophy (123). In contrast to necrosis, it is a highly regulated and controlled process that produces apoptotic bodies engulfed by phagocytic cells before spilling out and damaging surrounding cells (73). Apoptosis is a major form of early cell death in myocardial ischemia. Apoptosis causes cell death that is around 30-fold higher than necrosis in I/R injury (68).

Apoptotic cell death involves the activation of caspases. Caspases, previously known as IL-1β converting enzymes, are intracellular proteases that cleave substrate proteins behind aspartate residues. They have a highly conserved pentameric sequence and contain a cysteine residue in the catalytic center (73, 233). Caspases contain a prodomain and enzymatic region, where differences exist within the prodomain of each type. Caspases are produced as zymogens
that can be activated by autoactivation or cleavage by another caspase within the enzymatic domain. Caspases are grouped by both structure and function into upstream initiator caspases and downstream effector caspases (95). Initiator caspases, such as caspase-2, -8 and -9, possess a long prodomain along with a target interacting domain, and activate effector caspases (127). Effector caspases, such as caspase-3 and -7, contain short prodomains and depend on initiator caspases for activation (144). Effector caspases target a variety of proteins including nuclear proteins, signalling molecules, and cytoskeletal targets (73). Most of these proteins are cleaved by effector caspases-3 and -7 in the heart.

The activation of caspases takes place through two predominant mechanisms: the extrinsic death receptor pathway and the intrinsic mitochondrion-dependent pathway (73). The death receptor-mediated pathway begins with binding of a death ligand to a membrane-bound death receptor that contains a distinct cytoplasmic domain known as the “death domain.” Examples of death ligands include FasL, TNFα, Apo-3L, and TRAIL, and they bind to the death receptors Fas, TNFR1, DR3, DR4, and DR-5, respectively (131, 161, 211, 242). Interaction between the death ligands and receptors leads to the recruitment of intracellular adaptor proteins such as TNFR-associated death domain protein (TRADD) or Fas-associated death domain protein (FADD) to the cell membrane (26, 97). TNFα signalling overlaps with Fas signalling through the subsequent interaction of TRADD and FADD (73). FADD activates initiator caspase-8, which activates downstream effector caspases, including caspase-3 (206). In addition to TRADD, another protein that interacts with TNFR1 is receptor-interaction protein (RIP) (76). Initiator caspase-2 is recruited to RIP by RIP-associated ICH-1/Ced-3-homologous death domain protein (RAIDD) and then activates downstream effector caspases (39).
Human cardiomyocytes have the highest density of mitochondria of all mammalian cells (61). In the intrinsic mitochondrion-dependent pathway, the mitochondria initiates apoptosis in response to a stimulator such as oxidative stress or deoxyadenosine-5-triphosphate (dATP) (118). Mitochondria releases cytochrome c into the cytosol which complexes with apoptotic protein activating factor-1 (Apaf-1), caspase-9, and dATP or ATP to form the apoptosome (243). This leads to the autoactivation of initiator caspase-9 and subsequent activation of downstream effector caspases, including caspase-3 (203). In addition, mitochondria initiate apoptosis through the release of apoptosis-inducing factor (AIF) that translocates to the nucleus to cause DNA fragmentation independent of caspase activation (21).

There are several regulators of the apoptotic process. The B-cell lymphoma 2 gene (bcl-2) family of proteins regulate the intrinsic mitochondrion-dependent pathway and contain combinations of four conserved domains (73). Bcl-2 family members are categorized as either anti-apoptotic (bcl-2 and bcl-xL) or pro-apoptotic (bad, bak, and bax) (240). Expression of bcl-2, bcl-xL, and bax is reported in cardiomyocytes at physiological conditions. Bcl-2 expression is upregulated in hypertrophy and disease states, such as heart failure and MI (20, 82, 139). Overexpression of bcl-2 in cardiomyocytes prevents apoptotic cell death induced by p53, which shows that proteins required by bcl-2 for its antiapoptotic effects are expressed and functional in cardiomyocytes (96). Bcl-2-interacting protein (Bid) regulates the interaction between the extrinsic death receptor pathway and the intrinsic mitochondrial-dependent pathway. Cytosolic Bid is cleaved by activated caspase-8 to allow translocation to the mitochondria. Truncated Bid induces the clustering of mitochondria around the nuclei and the release of cytochrome c. Bcl-xL inhibits apoptotic changes by Bid (114).
Bcl-2 mediates its anti-apoptotic effects through four main mechanisms: a direct antioxidant effect; inhibition of the release of proapoptotic mitochondrial proteins; inhibition of proapoptotic proteins; and inhibition of the direct cytotoxic effects of proapoptotic regulators bax and bak (73). As an antioxidant, Bcl-2 protects the mitochondrion and makes it less likely to release proapoptotic factors. There is evidence that bcl-2 regulates protein translocation from the mitochondrial intermembrane space into the cytosol by decreasing the mitochondrial release of cytochrome c and AIF (213, 238).

Bax and bak lethality is suppressed by coexpression of bcl-2 and bcl-xL. In contrast, bax mediates its proapoptotic effects by interacting with and increasing the opening of the mitochondrial voltage-dependent anion channel. This promotes the loss in membrane potential and release of cytochrome c (215). The expression of bax is regulated by apoptosis inducing p53 (223).

Additional regulators of programmed cell death include inhibitor of apoptosis proteins (IAPs) that bind and inhibit activation of TNFR1 and TNFR2 (184). IAPs also directly block caspase function, mostly by directly binding (34, 185). In addition, the heart has been shown to have high levels of the protein ‘apoptosis repressor with caspase recruitment domain’ (ARC), which interacts with upstream caspases to block caspase-2 and -8, as well as cytochrome c release (44, 100).
Apoptotic death in cardiomyocytes is associated with myocardial infarction, I/R injury, and end-stage heart failure (45, 103, 239). Cardiomyocyte apoptosis is significantly different between men and women in failing hearts. Cardiomyocyte death is markedly lower in women than in men, with higher rates associated with early onset of heart failure (68).

Estrogens have a role in regulating cardiomyocyte apoptosis in cardioprotection, which occurs in a sex-specific manner. Both female sex and the expression of ERβ attenuated the development of apoptosis and fibrosis in a mouse model of MI. The loss of ERβ in knock out mice increased apoptosis by 100-fold in males and only 6-fold in females after TAC (52).

Evidence demonstrates the role of E2 in reducing cardiac death and regulating the PI3K/Akt and MAPK signalling pathways involved in apoptosis. The signalling protein p38β is known to be anti-apoptotic, whereas p38α is proapoptotic. During prolonged hypoxia and
subsequent reoxygenation (H/R), cardiomyocytes undergo p53-dependent apoptosis, after phosphorylation of p53 by p38α MAPK and p38β suppression. E2 significantly reduced cell death by inhibiting p38α-p53 signalling in apoptosis, mediated by both ERα and ERβ individually, and overall improved injury to H/R (117).

ER activation can also act to reduce apoptosis through rapid PI3K/Akt signalling. In OVX female mice subjected to CAL, chronic E2 treatment significantly reduced cardiomyocyte apoptosis by decreasing caspase-3 activation and DNA fragmentation (154). E2-mediated reduced apoptosis was blocked by separate treatments of dominant negative-Akt overexpression, an ER antagonist, and PI3K inhibition; and E2 caused a rapid increase in Akt phosphorylation. This indicates E2 reduces apoptosis through a rapid ER-PI3K/Akt dependent pathway (154).

The MAPK and PI3K pathways may converge with E2 activation in the regulation of apoptosis. In rat cardiomyocytes E2 activates PI3K, which phosphorylates Akt and leads to an increase in p38β activity. p38β then suppressed ROS generation, which contributed to downstream inhibition of pro-apoptotic p38α (86). Overall, E2 drives a nongenomic cascade that reduces apoptosis in cardiac myocytes.

Another factor to consider in the effects of estrogens on apoptosis regulation are sex differences in the response to I/R injury. In both in vivo and isolated rat heart models of I/R, females had less injury and decreased ROS generation compared to males (110). Hearts from female rats also had a PI3K-dependent increase in the phosphorylation and activity of aldehyde dehydrogenase (ALDH) 2, an enzyme that detoxifies ROS-generated aldehyde adducts. Female rat hearts had increased phosphorylation of myocardial α-ketoglutarate dehydrogenase (α-KGDH), a major enzyme for ROS generation during I/R, that was protein kinase C (PKC) dependent (110). Previous reports also demonstrate that E2 regulates the PI3K pathway and
plays a role in mediating the sex differences in mitochondrial protein phosphorylation that reduces apoptosis (86).

The Timing Hypothesis suggests women who begin HRT during the perimenopausal period or shortly after the onset of menopause have better cardiovascular outcomes than women who initiate HRT several years postmenopause. Increasing levels of apoptosis and inflammation during an estrogen-deficient period following menopause may explain discrepant outcomes in differences of treatment timing. One study investigated the effects of E2 treatment timing post-OVX on inflammation and apoptosis (156). OVX rats were treated with E2 immediately after OVX or starting at 9 weeks post-OVX to represent different stages of HRT initiation. Hearts from OVX mice developed more apoptotic markers, including increased caspase-3, caspase-9, and calpain 2 expression levels; and increased levels of inflammatory cytokines, including TNFα and IL-6. While there was an increase in TNFα in late versus early E2 treatment, there were no differences in proapoptotic markers (156). Unfortunately, this OVX model is limited because the drastic withdrawal of E2 does not resemble the gradual transition into menopause typically experienced by most females.

1.11 Menopausal Model

As previously discussed, the efficacy of HRT on the outcome of CVD is much debated. One explanation that aims to resolve the discrepant findings between clinical trials, and between human and animal studies, is that the timing of HRT introduction may influence disease outcomes (183). However, traditional OVX animal models fail to recapitulate the key transitional stage of menopause (18). In order to understand how timing of HRT initiation may affect CVD outcomes, a more accurate animal model including the transition into menopause is better suited.
The 4-vinylcyclohexene diepoxide (VCD) mouse model can be used to better represent the gradual transition into menopause experienced by the majority of women. Daily VCD injections of laboratory animals over 15-20 days causes the gradual loss of ovarian small primary and primordial follicles by accelerating the natural process of atresia (120) to create a follicle-depleted, ovary-intact animal (18). VCD works by decreasing autophosphorylation of the type III tyrosine kinase receptor (KIT) expressed in oocytes and theca cells, inhibiting the PI3K pathway to promote cell apoptosis (130). After VCD has depleted all primordial follicles, there is a subsequent increase in estrous cycle length as the model transitions into a state analogous to the perimenopausal period in women (referred to as “perimenopause” throughout the remainder of this thesis). Estrogen levels fluctuate until they reach very low levels, unlike the abrupt depletion of ovarian estrogens in the OVX model. FSH and LH rise gradually and remain elevated after ovarian failure (132). The residual ovarian tissue continues to secrete androgens following ovarian failure and theca cells will continue to secrete androstenedione (132). These characteristics closely mimic the natural phase of perimenopause in humans and the transition into menopause (18). VCD dosing can be manipulated to alter the time span of the perimenopausal period (120).

The National Toxicology Program investigated potential toxic and carcinogenic effects of VCD in mice. To generate a menopause model, the dose is 980-fold less than that needed to induce any toxic or carcinogenic effects, which were limited to the reproductive tract. Histopathological evaluations on tissues 6 months after dosing showed no pathological effects in heart, lung, uterus, kidneys, adrenals, spleen, liver, brain, intestine, or pituitary tissues (18, 148).

The VCD model has been used to effectively study postmenopausal diabetes, metabolic syndrome, and hypertension (92, 162, 180), but no study has examined its physiological effects
on the heart during the transition to a menopausal state. VCD-induced ovarian failure provides a valuable approach to assess the transition into ovarian failure to better understand cardiac changes that occur during menopause in women.

1.12 Rationale and Hypothesis

Premenopausal women are protected from cardiovascular disease as compared with age-matched men, but their risk of heart disease increases following menopause (7, 8, 62, 207). Evidence from laboratory studies overwhelmingly concludes endogenous estrogens are cardioprotective, suggesting that the replacement of estrogens in postmenopausal women should decrease the incidence and severity of heart disease. Yet, large-scale clinical trials are inconsistent in finding cardioprotection with postmenopausal HRT (64, 78, 129). One untested theory that aims to explain discrepant clinical results is the timing hypothesis which posits that the age and stage of menopause at the time of HRT introduction affects cardiovascular outcomes. This theory suggests that the cardiovascular system evolves gradually with estrogen deficiency until a point at which HRT is no longer effective. Many animal studies have investigated E2 replacement in OVX models, but this model fails to include the gradual transition through a perimenopausal period, precluding perimenopausal studies and potentially producing a fundamentally different postmenopausal organ than would develop after a transitional phase. We hypothesize that the gradual loss of ovarian function induced with VCD injections leads to alterations in the myocardial response to ER activation. This study is the first to use the gradual ovarian failure model of VCD injection to investigate the transition into menopause and test the influence of timing on estrogen responsiveness.

Males have naturally lower levels of estrogens compared to females early in life. As females age estrogen levels decline to levels comparable to those of age-matched males. Despite
similarly low levels of circulating estrogens, the response to ER activation in males and females may not be the same, given the different developmental paths. Understanding sex-differences in response to ER activation is important for determining whether ER activation in males offers similar protection to that seen in females. Interestingly, clinical studies have already been done in this area: in human patients undergoing angioplasty the application of 17β-estradiol at reperfusion was shown to be protective in both males and females, although the vast majority of patients were ironically men (112, 113).

Estrogens initiate intracellular responses through binding to ERs in the classical genomic pathway, or through rapid, nongenomic pathways (104, 152, 157). ERα, ERβ, and GPER are expressed in adult cardiac myocytes and studies of their rapid activation have been explored (67, 106, 157, 164). ERα has been identified on the cardiomyocyte sarcolemma and acute agonist treatment specific to ERα has been associated with changes in myofilament activation (84, 106, 134). ERβ localization is less well studied, yet its effects on rapid MAPK signalling has been well demonstrated in its protection against LVH (5). Cardiac myofilaments make up the central contractile apparatus in the heart, and changes to their interaction with calcium alters their activity and subsequent force production of the whole heart. Despite research showing rapid, nongenomic effects that alter contractility, the investigation of non-genomic regulation of cardiac myofilament function by ER activation has been limited to a single study by our group (106), and no one has investigated sex differences or the changes that occur during a transition into a menopausal state. We hypothesize that ER activation rapidly alters cardiac myofilament function and that ovarian failure alters the myocardial milieu to affect ER regulation of cardiac contractility and myofilament function. We speculate that regulation by ER activation is
dependent on sex so that the regulation of cardiac myofilament function affects overall cardiac contractility.
2 MATERIALS AND METHODS

2.1 Animal Care

All animals were cared for in accordance with the guidelines of the Animal Care and use Committee of the University of Guelph and the Canadian Council on Animal Care.

2.2 Experimental VCD Groups

CD-1 female mice aged 78-105 days were obtained from Charles River Laboratories (St. Constant, QC). Mice were intraperitoneally injected daily with 160mg VCD/kg body weight or sesame oil vehicle at approximately 1:00pm for 15 days (18). Ovarian failure as determined by vaginal cytology was observed by day 120, as has been reported by others (120). VCD-treated mice were euthanized 60- and 120-days after the start of injections to investigate varying degrees of ovarian failure. Vehicle-injected and un-injected mice were raised for 120-days after the start of injections to represent reproductively intact groups. For comparisons between sexes, uninjected CD-1 male and female mice aged 78-105 days were used.

2.3 Vaginal Cytology

Reproductive status was evaluated according to estrous cycle regularity assessed by changes in vaginal cytology (28). Cells from the vaginal canal were collected at the same time daily (approximately 12:00 pm) by flushing with 20 μL of phosphate buffered saline (137mM NaCl, 2.7mM KCl, 13.1mM Na₃PO₄, 1.5mM KH₂PO₄, pH 7.2) and examined under a light microscope. To minimize differences in true reproductive potential and vaginal cycle regularity, female mice were housed in close proximity to males and bedding from male cages was distributed among female cages. Intact females maintained regular estrous cycles of ~4-6 days in length. Mice euthanized 60 days after the start of injections (60-day VCD) displayed irregular cycling, defined as less than 10 days in consecutive low-estrogen diestrus or irregular prolonged
periods of any estrous stage, and were used to model perimenopause. Mice euthanized 120 days after the start of injections (120-day VCD) halted cycling, defined as 10 or more consecutive days in diestrus, and were used to model the end of menopause.

2.4 Langendorff Isolated Heart Perfusion

Myocardial function and the cardiac response to acute ER activation was determined using a Langendorff isolated heart system (237). Hearts were harvested from CO₂ euthanized mice and placed in ice-cold Krebs-Henseleit solution with reduced CaCl₂ (0.2 mM CaCl₂). Excess tissue was dissected from the aorta and hearts were cannulated using a 22-g needle for retrograde perfusion. Hearts were perfused with oxygenated (95% O₂/5% CO₂) Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 24 mM NaHCO₃, 11.1 mM glucose, 1.8 mM CaCl₂, 1.2 mM KH₂PO₄, pH 7.4) maintained at 37°C and paced at constant heart rate (~425 bpm). A fluid-filled balloon attached to a pressure transducer (Ad Instruments, Powerlab 4/30) was passed through the left atrium into the left ventricle. The balloon was inflated to give a left ventricular end diastolic pressure (LVEDP) of less than 7 mmHg and left ventricular developed pressure (LVDP) of greater than 75 mmHg. Hearts were perfused for at least 15 min to ensure viability and stable function. Baseline function was taken as the average contractile values over the last 30 seconds of perfusion. Following baseline perfusion hearts were treated for 15 min with 100 nM PPT (ERα agonist), 1 nM diarylpropionitrile (DPN, ERβ agonist), or ethanol (EtOH, 0.1% and 0.01%) vehicle. Functional measurements were taken at 5, 10, and 15 min and were derived from 10 second data samples. After 15 min of treatment perfusion hearts were snap frozen in liquid nitrogen and stored at -80°C.
2.5 Myofilament Isolation

Cardiac myofilaments were isolated from whole heart samples according to our published protocol (167). Hearts were homogenized in ice-cold Standard Buffer (60 mM KCl, 30 mM Imidazole (pH 7.0), 2mM MgCl₂) containing protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.01 mM leupeptin, 0.2 mM benzamidine, pH 7.0) and phosphatase inhibitor cocktail (P0044 Sigma-Aldrich). Homogenates were centrifuged at 14,100g for 15 min at 4°C. The pellets were resuspended in ice-cold Skinning Buffer containing 1% Triton and gently agitated at 4°C for 45 min. The suspension was centrifuged at 1,100g for 15 min at 4°C and washed three times with ice-cold Standard Buffer. Protein concentration was determined in triplicate with the Bio-Rad Bradford Protein Assay (Bio-Rad Laboratories Ltd., Mississauga, ON).

2.6 Actomyosin MgATPase Assay

Isolated cardiac myofilaments (50 μg) were incubated in reaction buffers containing variable free Ca²⁺ concentrations using combinations of Activating (pCa 4.0) (23.5 mM KCl, 5 mM MgCl₂, 3.2 mM ATP, 2 mM EGTA, 20 mM imidazole, and 2.2 mM CaCl₂, 0.1 mM phenylmethylsulfonyl fluoride, 0.01 mM leupeptin, 0.2 mM benzamidine, pH 7.0) and Relaxing (pCa 9.0) buffers (26 mM KCl, 5.1 mM MgCl₂, 3.2 mM ATP, 2mM EGTA, 20 mM imidazole, 4.9 μM CaCl₂). Buffers were supplemented with protease and protein phosphatase inhibitors as described earlier. Myofilaments were incubated in reaction buffers for 10 min at 32°C and reactions quenched with ice-cold 10% trichloroacetic acid. Actomyosin MgATPase activity was determined using a modified Carter assay (236). Inorganic phosphate produced from ATP consumption was measured following the addition of an equal volume of 0.5% FeSO₄ and 0.5% ammonium molybdate in 0.5 M H₂SO₄ and after 10 minutes reading the absorbance at 630 nm.
on a spectrophotometer. Actomyosin MgATPase activity was plotted against free calcium and curves fit using the Hill equation (32).

2.7 Myofilament Protein Phosphorylation

Myofilament proteins (10 μg) were separated using 12% bis-acrylamide gels and SDS-PAGE (237). Gels were fixed in 50% methanol-10% acetic acid at room temperature overnight and washed twice with ddH₂O. Proteins were stained with ProQ Diamond phosphoprotein gel stain (Invitrogen) according to the manufacturer’s instructions, and gels were destained with 5% sodium acetate-20% acetonitrile. Imaging was done using a Bio-Rad Imaging MP at 532-560 nm excitation and 580nm emission. Protein loading was assessed after phosphoprotein imaging by Coomassie staining (0.2% coomassie R-250, 20% methanol, 5% glacial acetic acid). Gels were destained with 20% methanol-5% glacial acetic acid. Densitometric analysis was done using ImageJ software (Scion Inc) using total protein as the loading control.

2.8 Immunoblotting

Homogenized heart samples (100 μg) were separated using SDS-PAGE with 12% bis-acrylamide gels to detect ER protein levels or 15% bis-acrylamide gels to detect inflammatory cytokines and apoptotic markers. Proteins were transferred to nitrocellulose membranes, blocked for one hour in Tris-buffered saline (TBS; 0.01M Tris-base, 0.15M NaCl) with 5% milk, and probed with primary antibodies overnight at 4°C. Antibodies for ERα (sc-542 Santa Cruz Biotechnology rabbit) and ERβ (GTX70174 GeneTex mouse) were diluted 1:1000 in Tris-buffered saline with tween (TBS-T) (0.5mM tween) with 1% milk. Antibodies for interleukin-6 (IL-6; M-19 Santa Cruz Biotechnology goat), tumor necrosis factor α (TNFα; N19 Santa Cruz Biotechnology goat), interleukin-1β (IL-1β; H-153 Santa Cruz Biotechnology goat) and
transforming growth factor β (TGFβ; V Santa Cruz Biotechnology rabbit) were diluted 1:1000 in TBS-T with 1% milk. Antibodies for caspase-3 (MAB4703 Millipore mouse), bax (MABC1176 Millipore mouse), and bcl-2 (MABC573 Millipore rat) were diluted 1:1000 in TBS-T with 1% milk. Secondary antibodies conjugated to horseradish peroxidase were anti-goat (1:5000, A5420 Sigma Aldrich); anti-rabbit (1:5000, A0545 Sigma Aldrich), anti-mouse (1:1000; A2304 Sigma Aldrich), or anti-rat (1:10,000, A10549 Invitrogen). Protein loading was assessed by stripping membranes with 0.1 M glycine (pH 2.9) and re-probing with α-actinin antibody (1:2000, Mab 1682 Millipore mouse). The secondary antibody used for re-probing was anti-mouse (1:1000; A2304 Sigma Aldrich). Protein bands were detected using Western Lightning ECL Pro (PerkinElmer, Inc.), imaged using Bio-Rad Imaging MP, and analyzed using ImageJ.

2.9 Statistical Analysis

All data are shown as mean ± SEM. Perfusion and protein phosphorylation statistical analyses were performed using two-way ANOVA. Once significance was established, one-way ANOVA was used within treatment groups. Immunoblotting statistical analyses was carried out using one-way ANOVA. Post-hoc Fisher’s Least Significant Difference test was used for perfusion, protein phosphorylation, and immunoblotting analysis. Post-hoc Tukey’s test was used for Myofilament Mg\(^{2+}\)ATPase analysis. Homogeneity of variance was evaluated using the Levene’s test. Normality was evaluated using the Shapiro-Wilk test. In cases where the normality assumption was invalid, a logarithmic transformation was applied to satisfy the Shapiro-Wilk test. p < 0.05 was considered statistically significant.
3 RESULTS

3.1 Heart Function

Baseline Heart Function.

Left ventricular developed pressure (LVDP) and the rate of contraction, dP/dt\textsubscript{max}, were measured to assess contractility or systolic function. The rate of relaxation, dP/dt\textsubscript{min}, was measured to assess diastolic function. Baseline values were not different between experimental groups (Tables 1 and 2).

Effects of acute ER activation on left ventricular function in females.

Hearts from ovary intact mice were perfused for 15 min with estrogen receptor-\(\alpha\) (PPT) or -\(\beta\) (DPN) agonists. Both PPT and DPN treatment rapidly reduced all measures of LV function at 5 min, and all measures remained below intact control values at 10 and 15 min of treatment (Figure 1). At 5 min, PPT reduced LVDP by 8.2 ± 1.6%, dP/dt\textsubscript{max} by 8.5 ± 1.7%, and dP/dt\textsubscript{min} by 13.3 ± 2.2%. At 5 min, DPN reduced LVDP by 5.6 ± 1.2%, dP/dt\textsubscript{max} by 5.8 ± 1.4%, and dP/dt\textsubscript{min} by 10.4 ± 2.1%. This is the first study to show rapid effects of acute ER-specific activation on myocardial function.

VCD-induced ovarian failure alters ER-dependent regulation of LV function in females.

Hearts from mice 60 days after VCD treatment had an attenuated response to ER activation. PPT and DPN treatment both tended to reduce contractility at 5 min of treatment, but this did not reach statistical significance (\(p = 0.104\)) (Figure 2). PPT significantly reduced dP/dt\textsubscript{max} only at 10 min by 6.05 ± 1.3%, and no other measured parameter was significantly impacted by treatment (Figure 2). At 5 min, DPN transiently decreased dP/dt\textsubscript{max} by 9.8 ± 1.8%,
and \( \frac{dP}{dt_{\text{max}}} \) remained below intact control values at 10 min but not 15 min of treatment. \( \frac{dP}{dt_{\text{min}}} \) was not impacted with ER activation (Figure 2).

In hearts from mice 120 days after VCD treatment, the depressive effects of PPT were largely restored (Figure 3). All measured parameters declined at 5 min of PPT treatment and remained significantly below intact values with the exception of \( \frac{dP}{dt_{\text{max}}} \) at 10 min (\( p = 0.054 \)). At 5 min, PPT reduced LVDP by 8.6 \( \pm \) 1.2\%, \( \frac{dP}{dt_{\text{max}}} \) by 9.1 \( \pm \) 1.9\%, and \( \frac{dP}{dt_{\text{min}}} \) by 14.5 \( \pm \) 2.5\%. Hearts from 120-day VCD mice had no significant response to DPN (Figure 3). These results are the first to show that the myocardial response to ER activation is affected in a non-linear manner by VCD-induced ovarian failure.

**Myocardial functional response in females is dependent on both ER-subtype and VCD treatment.**

There is an interaction effect of ER agonist and VCD treatment on contractility as measured by LVDP starting at 5 min (Figures 1-3). This relationship remains significant at 10 and 15 min of agonist treatment. The response to \( \frac{dP}{dt_{\text{max}}} \) and \( \frac{dP}{dt_{\text{min}}} \) tended to depend on both agonist and VCD treatment but this trend did not reach statistical significance (\( p = 0.24 \) and 0.52 at 15 min).

**Effects of acute ER activation on left ventricular function in males.**

Hearts from male mice were perfused for 15 min with PPT and DPN. PPT significantly reduced all measures of heart function at 10 min of treatment, and these measures remained significantly reduced compared to male control values at 15 min (Figure 4). At 10 min, PPT reduced LVDP by 10.9 \( \pm \) 2.2\%, \( \frac{dP}{dt_{\text{max}}} \) by 11.1 \( \pm \) 2.0\%, and \( \frac{dP}{dt_{\text{min}}} \) by 18.9 \( \pm \) 2.4\%. DPN significantly reduced all measures of heart function by 15 min of treatment, and \( \frac{dP}{dt_{\text{max}}} \) became significantly reduced at 10 min of treatment compared to male control values (Figure 4). DPN
significantly reduced LVDP by $12.2 \pm 1.8\%$ and $dP/dt_{\text{min}}$ by $19.9 \pm 3.7\%$ at 15 min of treatment; and $dP/dt_{\text{max}}$ by $11.1 \pm 2.0\%$ at 10 min of treatment. Males responded to ER agonist treatment in a similar manner as females, and there was no interaction between sex and agonist on any LV functional measures.
### Baseline values for Ovarian Failure Groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PPT</th>
<th>DPN</th>
<th>Control</th>
<th>PPT</th>
<th>DPN</th>
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<th>DPN</th>
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<td>10</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Heart Rate (bpm)</td>
<td>427.1±1.7</td>
<td>423.8±1.2</td>
<td>426.3±2.3</td>
<td>425.3±0.4</td>
<td>425.5±0.3</td>
<td>425.2±0.3</td>
<td>428.1±4.5</td>
<td>429.29±2.6</td>
<td>427.9±4.0</td>
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<td>LVEDP (mmHg)</td>
<td>2.3±0.4</td>
<td>3.3±0.6</td>
<td>3.0±0.4</td>
<td>3.7±0.4</td>
<td>3.6±0.6</td>
<td>3.9±0.6</td>
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<td>3.3±0.5</td>
<td>2.9±0.4</td>
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<tr>
<td>LVDP (mmHg)</td>
<td>88.0±2.9</td>
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<td>87.0±2.9</td>
<td>83.7±1.8</td>
<td>87.0±2.1</td>
<td>88.2±5.0</td>
<td>89.8±2.3</td>
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<tr>
<td>Coronary Flow (mL/min)</td>
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<td>1.9±0.1</td>
<td>2.0±0.1</td>
<td>1.9±0.2</td>
<td>1.8±0.3</td>
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<td>1.8±0.1</td>
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</tbody>
</table>

Table 1. Cardiac functional baseline values of Langendorff perfused reproductively intact and VCD-injected mouse hearts. No significant differences in pre-treatment heart rate, LVEDP, LVDP, and coronary flow. All data excluding sample size are presented as means ± SEM.

### Baseline values for Sex Difference Groups

<table>
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<th></th>
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<th>DPN</th>
<th>Control</th>
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<td>11</td>
<td>10</td>
<td>7</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Heart Rate (bpm)</td>
<td>427.1±1.7</td>
<td>423.8±1.2</td>
<td>426.3±2.3</td>
<td>425.8±0.3</td>
<td>424.5±0.6</td>
<td>424.9±0.1</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>2.3±0.4</td>
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<td>3.0±0.4</td>
<td>4.3±0.4</td>
<td>4.0±0.4</td>
<td>3.8±0.7</td>
</tr>
<tr>
<td>LVDP (mmHg)</td>
<td>88.0±2.9</td>
<td>88.2±2.4</td>
<td>87.0±2.9</td>
<td>89.3±3.1</td>
<td>83.7±3.2</td>
<td>88.2±4.9</td>
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<tr>
<td>Coronary Flow (mL/min)</td>
<td>1.9±0.1</td>
<td>1.9±0.1</td>
<td>2.0±0.1</td>
<td>2.3±0.3</td>
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</tr>
</tbody>
</table>

Table 2. Cardiac functional baseline values of Langendorff perfused reproductively intact female and male mouse hearts. No significant differences in pre-treatment heart rate, LVEDP, LVDP, and coronary flow. All data excluding sample size are presented as means ± SEM.
Figure 1. Left ventricular functional response to acute ER activation in hearts from ovary intact mice. 

A. Perfusion protocol. Hearts were perfused for 15 min for equilibration, with baseline values derived from the final 30 seconds of perfusion. All hearts were subjected to 15 min treatment with 100 nM (4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT, ERα agonist) or 1 nM diarylpropionitril (DPN, ERβ agonist). 

B. At 5, 10, and 15 min of PPT and DPN treatment, LVDP was significantly reduced compared to the vehicle-treated control group. 

C. Similarly, at 5, 10, and 15 min of PPT and DPN treatment, dP/dt_max was significantly reduced compared to the control group. 

D. Likewise, at 5, 10, and 15 min of PPT and DPN treatment, dP/dt_min was significantly reduced compared to the control group. N = 11 intact Control and intact PPT. N = 10 intact DPN. * p < 0.05 vs. intact control.
Figure 2. Left ventricular functional response to acute ER activation in hearts from mice 60 days post-VCD treatment. Hearts were harvested 60 days after VCD injections. Hearts were perfused for 15 min for equilibration, with baseline values derived from the final 30 seconds of perfusion. All hearts were subjected to 15 min treatment with 100 nM (4,4’,4”-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT, ERα agonist) or 1 nM diarylpropionitril (DPN, ERβ agonist). A. LVDP was not significantly different with PPT and DPN treatment compared to the vehicle-treated control group at all time points. B. dP/dt\textsubscript{max} in hearts from 60-day VCD mice was significantly reduced at 10 min of PPT treatment and at 5 and 10 min with DPN treatment compared to the vehicle-treated control group. C. dP/dt\textsubscript{min} was not significantly different with PPT and DPN treatment compared to the control group. N = 7 60d VCD control. N = 6 60d VCD PPT and 60d VCD DPN. * p<0.05 vs. 60d VCD control.
Figure 3. Left ventricular functional response to acute ER activation in hearts from mice 120 days post-VCD treatment. Hearts were harvested 120 days after VCD injections. Hearts were perfused for 15 min for equilibration, with baseline values derived from the final 30 seconds of perfusion. All hearts were subjected to 15 min treatment with 100 nM (4,4’,4”-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT, ERα agonist) or 1 nM diarylpropionitril (DPN, ERβ agonist). A. At 5, 10, and 15 min, LVDP was significantly reduced with PPT treatment compared to the vehicle-treated control group. All measures of functional response did not change with DPN treatment at all timepoints. B. At 5 and 15 min, dP/dt_{max} was significantly reduced compared to the control group. C. At 5, 10, and 15 min, dP/dT_{min} was reduced with PPT-treatment compared to the control group. N = 7 120d VCD control and 120d VCD DPN. N = 5 120d VCD PPT. * p<0.05 vs. 120d VCD control.
Figure 4. Sex differences in functional response to acute ER activation in male mouse hearts. Hearts were perfused for 15 min for equilibration, with baseline values derived from the final 30 seconds of perfusion. All hearts were subjected to 15 min treatment with 100 nM (4,4',4"-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT, ERα agonist) or 1 nM diarylpropionitril (DPN, ERβ agonist). A. At 10 and 15 min of PPT treatment, and 15 min of DPN treatment, LVDP was significantly reduced compared to the male vehicle-treated control group. B. At 10 and 15 min of PPT and DPN treatment, dP/dt_max was significantly reduced in males compared to the male vehicle-treated control group. C. At 10 and 15 min of PPT treatment, and 15 min of DPN treatment, dP/dt_min was significantly reduced in males compared to their controls. LVDP, dP/dt_max, and dP/dt_min responses to ER agonists were independent of sex. N = 7 male control. N = 6 male PPT. N = 5 male DPN. *p<0.05 vs. male control.
3.2 Myofilament Function

Actomyosin MgATPase activity was measured using myofilaments isolated from the left ventricles of female mice following Langendorff perfusion with PPT, DPN, or ethanol (control) at varying days after VCD or sesame oil injections. Sex comparisons were evaluated using hearts from intact females and males perfused with PPT, DPN, or ethanol.

**Myofilament function changes with ovarian failure.**

Ovarian failure increased maximal actomyosin MgATPase activity and decreased myofilament calcium sensitivity. ATPase activity at saturating levels of free calcium was significantly greater in 60-day VCD control samples compared to intact controls (Figure 5A). The trend continued in 120-day VCD controls but did not reach statistical significance. Normalized curves demonstrated a right-ward shift in the Ca$^{2+}$-ATPase curve that reached significance in 120-day VCD controls (Figure 5). This was illustrated by a significant increase in the Ca$^{2+}$ concentration defined by mid-point ATPase activity (EC$_{50}$) from 0.737 ± 0.083 µM in intact controls to 0.749 ± 0.071 µM in 60-day VCD controls and 0.833 ± 0.045 µM in 120-day VCD controls (Figure 5B). Co-operativity as determined by the Hill coefficient was not different between any of the groups studied.

**Acute ER-specific activation changes myofilament function.**

Acute ER-specific activation in intact samples led to increases in maximal actomyosin MgATPase activities and Ca$^{2+}$ sensitivities compared to controls (Figure 5C). ATPase activity at saturating levels of free calcium was significantly increased after PPT treatment and DPN treatment. Myofilaments decreased their level of sensitivity to Ca$^{2+}$ in response to ER$\beta$-activation evident by an increase in EC$_{50}$ from 0.737 ± 0.083 µM in intact controls to 0.842 ±
0.026 μM with DPN treatment. There was no change in Ca\(^{2+}\) sensitivity with PPT treatment (Figure 5D).

**Myofilament functional response to acute ER activation changes with ovarian failure.**

ER\(\alpha\) activation by PPT treatment resulted in changes to actomyosin Ca\(^{2+}\) sensitivity dependent on degree of ovarian failure. While there was no significant change in Ca\(^{2+}\) sensitivity in intact samples after PPT treatment, the same treatment in 60-day VCD samples resulted in a right-ward shift in the Ca\(^{2+}\)-ATPase curve and decrease in Ca\(^{2+}\) sensitivity, measured by changes in EC\(_{50}\) from 0.749 ± 0.071 μM in control-treated to 0.844 ± 0.032 μM in PPT-treated 60-day VCD samples (Figure 6A and 6B). Interestingly, PPT treatment in 120-day VCD samples resulted in a left-ward shift in the ATPase-Ca\(^{2+}\) curve and increase in Ca\(^{2+}\) sensitivity, as measured by changes in EC\(_{50}\) from 0.833 ± 0.045 μM in controls to 0.701 ± 0.067 μM in PPT-treated 120-day VCD samples (Figure 6C and 6D).

Generally similar to functional data, the response to ER\(\beta\) activation by DPN treatment was diminished with ovarian failure (Figure 6). While there was a significant decline in ATPase Ca\(^{2+}\) sensitivity and rise in maximal ATPase activity in intact samples with DPN treatment compared with intact controls, there were no differences in ATPase Ca\(^{2+}\) sensitivities and maximal activities among DPN-treated 60-day samples and 120-day samples compared to their respective controls (Figure 6).

**Sex differences in myofilament functional regulation with ER-specific subtype activation.**

While there was an increase in maximal actomyosin ATPase activity in intact females with PPT treatment, there were no detectable changes in males (Figure 5 and 7). ER\(\beta\) activation resulted in similar myofilament functional effects in males as females. DPN treatment in males
tended to increase maximum ATPase activity and decrease Ca$^{2+}$ sensitivity, measured by an increase in EC$_{50}$ from 0.726 ± 0.097 µM to 0.839 ± 0.022 µM (Figure 7).
Figure 5. Ovarian failure and acute ER-specific activation changes cardiac myofilament actomyosin MgATPase activity. Hearts were treated with ER agonists or vehicle. Isolated myofilaments were subjected to modified carter assay (236). A. Mice were treated with VCD and hearts were harvested 60- and 120-days after injections. Maximal actomyosin MgATPase activity exhibited at saturating concentrations of calcium was significantly greater in 60d VCD control hearts than intact hearts. B. Ovarian failure in 120d VCD controls led to a decrease in actomyosin MgATPase calcium sensitivity demonstrated by a right-ward shift in the Ca$^{2+}$-ATPase curve and increase in EC$_{50}$. C. In cardiac myofilaments from intact mice, maximal actomyosin ATPase activity was significantly greater with acute ER$\alpha$- and ER$\beta$- activation after 15 min of PPT and DPN treatment. D. PPT treatment in intact samples led to a decrease in actomyosin MgATPase calcium sensitivity demonstrated by a right-ward shift in the Ca$^{2+}$-ATPase curve and increase in EC$_{50}$. Data points are means ± SEM. N = 6 intact control. N = 5 60d VCD control, 120d VCD control, and intact DPN. N = 4 intact PPT. *p < 0.05 60d VCD vs. intact control (A). *p < 0.05 vs. intact control (B and D). *p < 0.05 intact PPT and intact DPN vs. intact Control (C).
Figure 6. Cardiac myofilament actomyosin MgATPase activity after acute ER-specific activation depends on ovarian failure. Mice were treated with VCD and hearts were harvested 60- and 120-days after injections. Hearts were treated with ER agonists or vehicle. Isolated myofilaments were subjected to modified carter assay (236). A. There were no detectable changes in maximal actomyosin MgATPase activity in 60d VCD samples with PPT and DPN treatment. B. Actomyosin MgATPase calcium sensitivity decreased with PPT treatment in 60d VCD samples demonstrated by a right-ward shift in the Ca^{2+}-ATPase curve and increase in EC_{50} compared to controls. C. There were no detectable changes in maximal actomyosin MgATPase activity in 60d VCD samples with PPT and DPN treatment. D. PPT treatment after ovarian failure in 120d VCD samples led to an increase in actomyosin MgATPase calcium sensitivity demonstrated by a left-ward shift in the Ca^{2+}-ATPase curve and decrease in EC_{50}. Data points are means ± SEM. N = 5 60d VCD Control, 60d VCD DPN, and 120d VCD control. N = 7 60d VCD PPT. n = 6 120d VCD PPT and 120d VCD DPN. *p < 0.05 vs. control.
Figure 7. Sex differences in cardiac myofilament actomyosin MgATPase activity after acute ERα- and ERβ- activation. Hearts were treated with ER agonists or vehicle. Isolated myofilaments were subjected to modified carter assay (236). A. Maximal actomyosin MgATPase activity tended to increase in male samples after acute DPN treatment. B. Actomyosin MgATPase calcium sensitivity decrease with DPN treatment in 120d VCD samples demonstrated by a right-ward shift in the Ca²⁺-ATPase curve and increase in EC₅₀ compared to vehicle-treated controls. Data points are means ± SEM. N = 4 male control, male PPT, and male DPN. *p < 0.05 vs. control.
3.3 Myofilament Protein Phosphorylation

Ovarian failure generally reduces myofilament protein phosphorylation.

Myofilament protein phosphorylation can mediate both acute and chronic changes in myofilament function. When comparing control-treated hearts across degrees of ovarian failure, there were significant reductions in total phosphorylation levels of several key myofilament proteins. With the exception of MLC-1, all measured proteins in the VCD treatment group had decreased levels of total phosphorylation (Figure 8). In hearts from mice 60-days post-VCD treatment, MyBP-C levels were reduced by 25 ± 10%; desmin phosphorylation was reduced by 35 ± 10%; TnT phosphorylation was reduced by 29 ± 8%; Tpm phosphorylation was reduced by 26 ± 8%; TnI phosphorylation was reduced by 29 ± 7%; and MLC-2 phosphorylation was reduced by 35 ± 11% compared to the intact control group. In hearts from mice 120-days post-VCD treatment, MyBP-C levels were reduced by 21 ± 12%, desmin phosphorylation was reduced by 40 ± 7%, TnT phosphorylation was reduced by 34 ± 8%, Tpm phosphorylation was reduced by 22 ± 9%, TnI phosphorylation was reduced by 30 ± 7%, and MLC-2 phosphorylation was reduced by 39 ± 9% compared to the intact control group (Figure 8).

Effect of acute ER activation on myofilament protein phosphorylation during ovarian failure in females.

While there are no detectable phosphorylation changes in intact PPT- and DPN-treated hearts compared to intact controls (Figure 9), there are significant differences following treatment in ovarian failure mice. In 60-day VCD hearts, DPN increased desmin phosphorylation by 30 ± 10%, compared to controls (Figure 10). PPT decreased MLC-1 phosphorylation by 15 ± 5% compared to controls (Figure 10). In 120-day VCD hearts, PPT increased MLC-2
phosphorylation by $26 \pm 6\%$ compared to controls (Figure 11). There were no detectable changes in TnT, TnI, Tpm, and MyBP-C phosphorylation with ER activation.

**Sex differences in myofilament protein phosphorylation.**

There were no detectable myofilament phosphorylation differences between control-treated male and reproductively intact female hearts (Figure 12). Similar to intact female hearts, there were no detectable changes in myofilament protein phosphorylation levels in male hearts with PPT and DPN treatment compared to male controls (Figure 13).
**Figure 8.** Cardiac myofilament protein phosphorylation is altered with ovarian failure. Mice were treated with VCD and hearts were harvested 60- and 120-days after injections. Hearts were treated with ER agonists or vehicle. Isolated myofilaments were separated by SDS-PAGE. Phosphorylation was determined with ProQ Diamond staining. Total protein load was determined by Coomassie staining. **A.** Protein phosphorylation levels of cardiac myofilament proteins. **B.** Total protein load for normalization. **C.** Total myofilament protein phosphorylation is reduced with ovarian failure. $n = 9$ Intact, $n = 6$ 60d VCD, $n = 5$ 120d VCD. *p < 0.05 vs intact control. MyBP-C = myosin binding protein C. TnT = troponin T. Tpm = tropomyosin. TnI = troponin I. MLC-1 = myosin light chain 1. MLC-2 = myosin light chain 2. Ctrl = control. PS = Peppermint Stick molecular weight standard.
Figure 9. Cardiac myofilament protein phosphorylation in reproductively intact hearts with acute ER activation. Hearts were treated with ER agonists or vehicle. Isolated myofilaments were separated by SDS-PAGE. Phosphorylation was determined with ProQ Diamond staining. Total protein load was determined by Coomassie staining. A. Protein phosphorylation levels of cardiac myofilament proteins. B. Total protein load for normalization. C. No detectable changes in total protein phosphorylation. n = 4 all groups. p > 0.05. MyBP-C = myosin binding protein C. TnT = troponin T. Tpm = tropomyosin. TnI = troponin I. MLC-1 = myosin light chain 1. MLC-2 = myosin light chain 2. Ctrl = control. PS = Peppermint Stick molecular weight standard.
Figure 10. Cardiac myofilament protein phosphorylation in 60-day VCD hearts with acute ER activation. Mice were treated with VCD and hearts were harvested 60- and 120-days after injections. Hearts were treated with ER agonists or vehicle. Isolated myofilaments were separated by SDS-PAGE. Phosphorylation was determined with ProQ Diamond staining. Total protein load was determined by Coomassie staining. **A.** Protein phosphorylation levels of cardiac myofilament proteins. **B.** Total protein load for normalization. **C.** Acute DPN treatment significantly increased desmin phosphorylation compared to controls. Acute PPT treatment significantly reduced MLC-1 phosphorylation compared to controls and DPN treatment. n = 6 60d VCD Ctrl, n = 5 60d VCD PPT, n = 6 60d VCD DPN. *p < 0.05 vs 60d VCD control. MyBP-C = myosin binding protein C. TnT = troponin T. Tpm = tropomyosin. TnI = troponin I. MLC-1 = myosin light chain 1. MLC-2 = myosin light chain 2. Ctrl = control. PS = Peppermint Stick molecular weight standard.
Figure 11. Cardiac myofilament protein phosphorylation in 120-day VCD hearts with acute ER activation. Mice were treated with VCD and hearts were harvested 60- and 120-days after injections. Hearts were treated with ER agonists or vehicle. Isolated myofilaments were separated by SDS-PAGE. Phosphorylation was determined with ProQ Diamond staining. Total protein load was determined by Coomassie staining. A. Protein phosphorylation levels of cardiac myofilament proteins. B. Total protein load for normalization. C. Acute PPT treatment significantly increased MLC-1 phosphorylation compared to controls. n = 5 120d VCD Ctrl, n = 7 120d VCD PPT, n = 8 120d VCD DPN. *p < 0.05 vs 120d VCD control. MyBP-C = myosin binding protein C. TnT = troponin T. Tpm = tropomyosin. TnI = troponin I. MLC-1 = myosin light chain 1. MLC-2 = myosin light chain 2. Ctrl = control. PS = Peppermint Stick molecular weight standard.
Figure 12. Cardiac myofilament protein phosphorylation in male and reproductively intact female untreated controls. Hearts were treated with ER agonists or vehicle. Isolated myofilaments were separated by SDS-PAGE. Phosphorylation was determined with ProQ Diamond staining. Total protein load was determined by Coomassie staining. A. Protein phosphorylation levels of cardiac myofilament proteins. B. Total protein load for normalization. C. No detectable difference between myofilament phosphorylation levels at baseline. n = 5 female Ctrl, n = 7 male Ctrl. p > 0.05. MyBP-C = myosin binding protein C. TnT = troponin T. Tpm = tropomyosin. TnI = troponin I. MLC-1 = myosin light chain 1. MLC-2 = myosin light chain 2. Ctrl = control. PS = Peppermint Stick molecular weight standard.
Figure 13. Cardiac myofilament protein phosphorylation in male hearts with acute ER activation. Hearts were treated with ER agonists or vehicle. Isolated myofilaments were separated by SDS-PAGE. Phosphorylation was determined with ProQ Diamond staining. Total protein load was determined by Coomassie staining. A. Protein phosphorylation levels of cardiac myofilament proteins. B. Total protein load for normalization. C. No detectable difference between myofilament phosphorylation levels in males with PPT and DPN treatment compared to controls. n = 7 male ctrl, n = 4 male PPT, n = 4 male DPN. p > 0.05. MyBP-C = myosin binding protein C. TnT = troponin T. Tpm = tropomyosin. Tnl = troponin I. MLC-1 = myosin light chain 1. MLC-2 = myosin light chain 2. Ctrl = control. PS = Peppermint Stick molecular weight standard.
3.4 Immunoblotting

ER levels are unchanged by ovarian failure and sex.

Differences in the myocardial response to estrogen receptor agonists may be the result of variations in cardiac expression of estrogen receptors, either between the sexes or during perimenopause. Myocardial ER\(\alpha\) and ER\(\beta\) levels were quantified using immunoblotting. Protein expression levels were normalized to \(\alpha\)-actinin. ER levels remained consistent across all groups of ovarian failure (Figure 14). There were no detectable sex differences in ER\(\alpha\) and ER\(\beta\) levels (Figure 15).

VCD-induced ovarian failure alters inflammatory profile and inconclusively predisposes the heart to disease.

Increasing levels of inflammatory markers are associated with heart disease and can change with estrogen exposure (55, 116, 202). Myocardial levels of inflammatory cytokines were measured by immunoblotting in ovarian intact controls and hearts from mice at varying stages of ovarian failure. Cytokine expression levels were normalized to \(\alpha\)-actinin to control for protein load. Logarithmic transformation was applied to TNF\(\alpha\) and TGF\(\beta\) data to satisfy Shapiro-Wilk’s test for normality. Immunoblotting demonstrated no change in TNF\(\alpha\) levels in hearts following VCD treatment compared to intact controls (Figure 16). IL-6 levels significantly increased by 77 ± 21% in 60-day VCD hearts compared to intact hearts. In 120-day VCD hearts, there tended to be greater IL-6 levels compared to intact controls, but this did not reach significance (\(p = 0.089\)) (Figure 16). IL-1\(\beta\) levels were significantly increased by 52 ± 7% in 60-day VCD hearts and remained significantly greater in 120-day VCD hearts by 43 ± 5%, compared to intact hearts (Figure 16). TGF\(\beta\) levels demonstrated a significant increase in 60-day VCD hearts by 93 ± 40% compared to controls; however, there was no significant change in
120-day VCD hearts compared to controls (Figure 16). Overall, there was a tendency towards higher levels of pro-inflammatory cytokines with VCD treatment.

**Ovarian failure promotes an apoptotic state by the upregulation of markers.**

The process of apoptotic death in the heart is associated with the development and severity of heart disease, including MI and heart failure (45, 103, 239). Understanding how ovarian failure changes baseline levels of apoptotic markers can provide insight for susceptibility to heart disease. Logarithmic transformation was applied to bcl-2 and bax data to satisfy Shapiro-Wilk’s test for normality. Immunoblotting demonstrated a significant rise in pro-apoptotic full-caspase levels by 78 ± 9.4% in 60-day VCD hearts and 57 ± 22% in 120-day VCD hearts compared to intact controls. Active caspase-3 significantly increased by 3.0-fold in 60-day VCD hearts and 4.9-fold in 120-day VCD hearts compared to intact controls (Figure 17). Consistent with these results, pro-apoptotic bax significantly increased by 3.7-fold in 60-day VCD hearts and 3.2-fold in 120-day VCD hearts compared to intact controls (Figure 18). In addition, anti-apoptotic bcl-2 levels tended to decline but did not reach significance (p=0.06) (Figure 18).
Figure 14. ERα and ERβ expression are consistent across ovarian failure. Mice were treated with VCD and hearts were harvested 60- and 120-days after injections. Samples were homogenized and isolated by SDS-PAGE. ERα and ERβ were detected by immunoblotting. A. ERα and ERβ levels and α-actinin loading control. B. There are no significant differences across ERα and ERβ levels of intact female, 60-day VCD female, and 120-day VCD female hearts. n = 5 Intact, 60d VCD, n = 4 120d VCD. p > 0.05. ER = estrogen receptor.
Figure 15. ERα and ERβ expression levels are consistent between the sexes. Heart samples were homogenized and isolated by SDS-PAGE. ERα and ERβ were detected by immunoblotting. A. ERα and ERβ levels and α-actinin loading control. B. There are no significant differences in ERα and ERβ levels of intact female and male mouse hearts. n = 5 all groups. Normalization to α-actinin and female. p > 0.05. ER = estrogen receptor.
Inflammatory cytokines are altered with ovarian failure. Mice were treated with VCD and hearts were harvested 60- and 120-days after injections. Samples were homogenized and isolated by SDS-PAGE. Inflammatory cytokines were detected by immunoblotting. A. Inflammatory marker levels and α-actinin loading control. B. TNFα levels were significantly reduced in 120-day VCD hearts compared to intact controls. IL-6 levels tended to increase in 60-day VCD hearts compared to controls and 120-day VCD hearts. IL-1β levels were increased in both 60- and 120-day VCD hearts compared to controls. TGFβ levels were increased in 60-day VCD hearts compared to controls. n = 6 all groups. Log transformations for TNFα and TGFβ data were applied before analysis to satisfy normality assumption. *p < 0.05 vs intact control. TNFα = tumour necrosis factor α. IL = interleukin factor. TGFβ = transforming growth factor β.
Figure 17. Apoptotic markers are altered with VCD treatment. Mice were treated with VCD and hearts were harvested 60- and 120-days after injections. Samples were homogenized and isolated by SDS-PAGE. Apoptotic markers were detected by immunoblotting. A. Apoptotic bands and α-actinin loading control. B. Active caspase-3 was significantly increased with ovarian failure 60- and 120-days after VCD treatment. n = 6 all groups. Normalization to α-actinin. *p < 0.05 vs intact control.
Figure 18. Apoptotic markers are altered with VCD treatment. Mice were treated with VCD and hearts were harvested 60- and 120-days after injections. Samples were homogenized and isolated by SDS-PAGE. Apoptotic markers were detected by immunoblotting. A. Apoptotic bands and α-actinin loading control. B. Preliminary results show an increased trend in bax expression levels with VCD treatment. C. Decreased trend in bcl-2 expression levels with VCD treatment. n = 6 all groups. Normalization to α-actinin. Log transformation was applied to all data before analysis to satisfy normality assumption. *p < 0.05 vs intact control.
4 DISCUSSION

Estrogen regulation of the cardiovascular system is thought to be cardioprotective, but this has much been debated. The mechanisms by which estrogen mediates its myocardial effects are not yet fully understood, and the complexity of estrogen signalling may contribute to the discrepant results of studies on the cardiovascular outcomes of HRT. Estrogens regulate through both genomic and nongenomic mechanisms and function by activating various ER subtypes in cardiomyocytes and other cells of the cardiovascular system. Understanding the effects and mechanisms of action specific to each ER isoform in the heart is important to fully understand how estrogens regulate myocardial function and mediate their potentially protective effects. This is the first study to show that ER regulation in the heart is dependent on the specific ER involved and is influenced profoundly by the stage of ovarian failure. We present the new observation that nongenomic ER-specific activation changes heart function in a non-linear manner with the stage of ovarian failure. We show that while myofilaments are altered by ER activation their changes are not sufficient to explain the myocardial effects. Changes in myocardial cytokines and markers of apoptosis at the end of a menopausal phase in an experimental animal model suggest an increased susceptibility to myocardial stressors that explains the decline in cardioprotection seen in post-menopausal women. Together this study indicates that myocardial changes early in menopause subtly remodel the heart and its ability to respond to estrogens, but these non-pathological changes may mediate significant effects and mitigate the beneficial effects of HRT. This study is one of the first to test and provide supporting evidence for the Timing Hypothesis of HRT.
4.1 The VCD-induced ovarian failure model enables the investigation of perimenopause.

In females, menopause is associated with an increase in risk for several diseases such as CVD, osteoporosis, diabetes, the metabolic syndrome, and ovarian cancer (18). The VCD mouse model of menopause results in gradual ovarian failure in a number of species from rodents to primates. VCD drives ovarian failure by progressively depleting ovarian follicles in ovary-intact animals and better represents the typical human progression through perimenopause than surgical ovariectomy (120). Despite the loss of estrogen-producing follicles, the residual ovarian tissue continues to secrete androgens (132). In addition, hormone production is gradually decreased to create a profile that better models the progressive nature of perimenopause. Our study uses the VCD-induced ovarian failure model to investigate changes in heart and myofilament function during the transition into menopause (18). Previous studies have used the VCD model of menopause to investigate the impact of menopause on the progression of diabetic kidney disease, the metabolic syndrome, and hypertension. A previous report found increased severity of diabetic kidney disease and upregulation of associated genes in VCD-treated diabetic female mice compared with cycling controls (35). In addition, VCD-treated female mice were more likely than cycling female mice to gain weight on a high fat diet, and promoted an increase in cholesterol and free fatty acids (180). Interestingly, angiotensin II-induced hypertension in the VCD mouse model of menopause was shown to be prevented by E2 replacement during perimenopause, but not in menopausal mice (162). The promising findings reported in the current and previous studies using the VCD mouse model provide evidence for its usefulness in evaluating the transition into menopause.
4.2 Functional changes during rapid ERα- and ERβ- activation depend on ovarian failure.

No studies have explored the acute effects of ER-specific activation on whole heart function nor have any compared sex differences in the responses. We have investigated the rapid functional effects of ERα- and ERβ- activation, and their dependence on sex and ovarian failure. Rapid ER activation results in a contractile decline that is generally diminished with ovarian failure. These results are consistent with previous reports that generally show a decline in cardiac function after acute E2 treatment (41, 168). E2, which activates ERα and ERβ with equal affinity (6), reduced left ventricular pressure in the isolated rabbit heart with concentrations starting from $10^{-6}$ M (168). The ER-dependent decrease in contractility is independent of sex. Consistent with our results on sex comparisons between ERα- and ERβ- activation, earlier studies found the negative inotropic effect of E2 was independent of sex (168). In addition, acute E2 treatment at $10^{-6}$ M concentration on isolated male rat hearts resulted in a decrease of heart rate, also demonstrating a negative chronotropic effect (41). These reports are consistent with our demonstrated functional decline after acute ERα- and ERβ- activation in hearts from intact mice. However, none of these studies looked at the effects mediated specifically by ER subtypes. Functional responses to ER subtypes diverge with ovarian failure so that the ERα response returns by the end of menopause, but the ERβ response remains attenuated 120 days after VCD treatment. Our work is the first to show timing-dependent changes in ER-responsiveness during the transition to a post-menopausal state.

No studies have evaluated the functional responses to acute ER-specific activation in the heart, and this has never been studied in models of ovarian failure or OVX. However, rapid ER-specific activation has been well studied in the vasculature and there is evidence of its dependence on long-term estrogen withdrawal. Previous reports of acute estrogen treatment in
the vasculature generally report a vasodilatory effect. Short-term E2 treatment in isolated porcine coronary arteries resulted in decreased vasoconstriction that was maintained in the presence of a protein synthesis inhibitor, suggesting non-genomic mechanisms (218). Vasculature relaxation by estrogens are subtype-specific and have been shown to rapidly act through ER\(\alpha\) and not ER\(\beta\) (14). Interestingly, consistent with the demonstrated dependence of ER activation on heart function with ovarian failure, ER-specific activation in the vasculature has shown to be dependent on estrogen withdrawal by OVX (14). Acute treatment with the ER\(\alpha\)-specific agonist PPT, but not the ER\(\beta\) agonist DPN, resulted in relaxation of isolated rat aorta (14). In isolated aorta from rats with estrogen withdrawal by OVX, neither E2 nor PPT induced relaxation. After chronic E2 replacement in OVX rats, the relaxation response by PPT was restored but remained diminished by DPN. In addition to our study, these studies provide additional evidence for a rapid functional response to ER activation that depends on both subtype and long-term estrogen deprivation (14). The combination of acute functional effects on both the heart and vasculature that depend on estrogen deprivation may contribute to changes seen in cardiovascular disease risk after the onset of menopause. Furthermore, the ER type-specific effects on the cardiovascular system raise the idea of a more directed HRT model in which ER-specific agonists may be used in place of broad-spectrum estrogens.

Both calcium handling and myofilament function drive the contractile force of cardiac muscle. During a normal contraction cycle in a cardiac myocyte, voltage-gated LTCC set the influx of Ca\(^{2+}\) into the cell (13, 43). The rise in intracellular Ca\(^{2+}\) activates the release of additional Ca\(^{2+}\) from SR intracellular stores through RyR. Ca\(^{2+}\) subsequently binds myofilaments to form cross-bridges and activate the contractile system (205). Ca\(^{2+}\) removal is driven largely by SERCA with some assistance from NCX (209). Estrogens acutely reduce myocardial
contractility, a functional effect that can be mediated, at least in part, by decreased Ca\(^{2+}\) transients (85). Calcium entry into cardiomyocytes are reduced by rapid nongenomic effects of E2 (19, 135, 200). Acute E2 treatment reversibly inhibited Ca\(^{2+}\) current through LTCC by 15-20% in cardiac myocytes from humans, guinea pigs, and rats, independent of sex and species (135). Consistent with these cellular experiments, our results show a contractile decline with acute ER\(\alpha\)- and ER\(\beta\)- activation that is independent of sex in intact mice. In addition, in isolated human ventricular myocardium, E2 had negative inotropic effects and antagonistic effects on LTCC current within 5 min of treatment (200). However, a separate study using rat ventricular myocardium contradicted most studies by showing an increase in calcium uptake within 5 min of E2 exposure at physiological concentrations (10\(^{-10}\) M) (19). While the evidence available is contradictory, these studies consistently show rapid ER activation plays a role in Ca\(^{2+}\) regulation inside the cardiac myocytes, which can have a major impact on function.

Other reports have identified roles for estrogens in reducing RyR and NCX activity, which also impacts the cardiomyocyte Ca\(^{2+}\) transient. OVX increases Ca\(^{2+}\) flux through both RyR and NCX, which was reversed by chronic E2 replacement and inhibition of PKA (102). While E2 treatment was provided over 6 weeks in this study, there is potential for these changes to result from nongenomic regulation through PKA. Overall, chronic E2 treatment attenuated increased intracellular Ca\(^{2+}\) transient after OVX by the increased release of Ca\(^{2+}\) by RyR and removal of Ca\(^{2+}\) by NCX responsible for the greater contractility and relaxation after OVX. These mechanisms may contribute to reported sex differences in the Ca\(^{2+}\) transient and functional changes after VCD treatment (31, 102).

Our results show a decrease in heart contractile function with both acute ER\(\alpha\)- and ER\(\beta\)-activation in hearts from intact female mice. These results are consistent with the majority of
studies that report E2 treatment results in rapid reductions in LTCC current. Similarly, E2 decreases RyR activity and NCX activity potentially through nongenomic mechanisms (102). These changes contribute to reduced myofilament activation and is consistent with our results of decreased heart contractility after ERα- and ERβ- activation (135, 200, 205). Our results show heart function in response to ER activation changes with ovarian failure. Since evidence has shown estrogens have a role in cardiomyocyte Ca^{2+} regulation, future studies can explore how these mechanisms may change with ovarian failure and result in functional changes that characterize the post-menopausal state.

4.3 Ovarian failure and ER activation regulate myofilament function and protein phosphorylation.

Altering the Ca^{2+} sensitivity of the myofilaments is one of the basic mechanisms that determine the force of cardiac contraction at the subcellular level. The data presented in this thesis is the first to investigate myofilament changes with ER-specific activation after gradual ovarian failure. 120-days after VCD treatment represents the end of menopause and was found to be associated with a decrease in Ca^{2+} sensitivity, as measured by a right-ward shift in Ca^{2+}-actomyosin MgATPase curve. Maximum myofilament ATPase activity increased with ovarian failure starting at 60-days after VCD treatment. ERα and ERβ activation led to rapid changes in myofilament function that were affected by ovarian failure. Interestingly, trends with myofilament changes following ER activation tended to follow changes in whole heart function. While there were no functional changes in myofilaments from untreated controls across VCD groups, the response with ERβ activation was present in intact myofilaments and later diminished with ovarian failure, similar to pressure observations. With acute ERα activation, myofilament function changed in a non-linear manner such that the 60-day VCD group diverged
from both intact and 120-day VCD groups. Further studies can investigate the molecular mechanisms that contribute to changes in ER response.

Few studies have investigated the effects of estrogen on cardiac myofilaments. A study performed in collaboration with our group determined that myofilament calcium sensitivity in adult female mice fluctuate with estrous cycle (122). Other studies in female rats using OVX as a model of menopause have shown that OVX results in the suppression of maximum myofilament ATPase activity and an increase in myofilament calcium sensitivity in cardiac myofibrils (188, 228). In both cases, these changes were reversed with long-term supplementation of E2. A previous study performed by our group investigated acute ERα-specific activation which led to a significant decrease in ATPase Ca\(^{2+}\) sensitivity in as early as 5 min of PPT treatment (106). These studies suggest that E2 may decrease myofilament Ca\(^{2+}\) sensitivity at least in part by acute activation of ERα. The results in this thesis are novel because we are the first to investigate myofilament function during the transition into ovarian failure and its regulation by ER activation. Our data show a decrease in myofilament ATPase sensitivity to Ca\(^{2+}\) with VCD treatment. While this is inconsistent with studies that report myofilament hypersensitivity to Ca\(^{2+}\) after OVX, OVX studies represent a post-menopausal model because of its long-term withdrawal of estrogens. The lack of a transitional phase with OVX may produce fundamentally different hearts than those that result following gradual ovarian failure. Our results are novel because we are the first to treat hearts with ER-specific treatment and measure how ovarian failure itself impacts ER response and myofilament function. Results show that the manner in which myofilaments are regulated by acute ER activation depend on the stage of ovarian failure.

Myofilament protein phosphorylation states are associated with changes in actomyosin myofilament function. Dephosphorylated MyBP-C preferentially binds myosin to reduce its
probability of binding actin (221). Phosphorylation of desmin, involved in sarcomere organization, is generally related to changes in myofibrillar structure and diastolic dysfunction (77, 195). Phosphorylation of cardiac TnT at Thr206 is associated with decreased actomyosin ATPase activity and myofilament calcium sensitivity whereas the other phosphorylation sites within TnT appear to be silent (212). Decreased Tpm phosphorylation at S382 has been shown to influence calcium regulation by increasing SERCA levels and PLB phosphorylation in response to stress (190). Decreased TnI phosphorylation at S23/S24 decreases myofilament calcium sensitivity (106). Phosphorylation of the ventricular myosin regulatory light chains increases Ca$^{2+}$ sensitivity in cardiac muscles (149). This study is the first to investigate changes in total myofilament protein phosphorylation with acute ER-specific activation and its dependency on ovarian failure. Our data show that myofilament phosphorylation is generally reduced with ovarian failure. These mechanisms are generally consistent with a decrease in actomyosin Ca$^{2+}$ sensitivity, also observed with ovarian failure. However, the large number of changes and a technical inability to investigate the numerous phosphorylatable residues did not allow us to determine which phosphorylation changes drive the functional alterations. Although these phosphorylation changes do not manifest in changes in overall heart function, they show that the heart is altered at the molecular level, and that may help explain why different responses are seen after ER activation (i.e. different myofilament backgrounds). In addition, acute ER$\alpha$- and ER$\beta$-activation led to changes in myofilament phosphorylation. These changes were dependent on ovarian failure but were independent of sex. Changes in the myofilament phosphorylation profile may contribute to the non-linear differences observed in myofilament function with ER activation. A detailed investigation of the site-specific changes in myofilament protein phosphorylation is warranted based on the complex alterations reported here.
4.4 ER expression remains consistent between the sexes and after ovarian failure.

ERα and ERβ differentially regulate whole heart and myofilament function. Their upregulation has been associated with the prevalence of heart disease, such as DCM, and may represent a protective compensatory process (124). Differences in their expression levels could explain alterations in the myocardial responses to ER agonists observed with VCD treatment, however we found no changes in expression levels. Expression and localization patterns of ERα and ERβ have been well studied. Previous studies have investigated ER transcript levels using qRT-PCR. ERα transcript expression in mouse LV myocardium did not differ between males and females, nor with ageing (166). The same results are observed in sex differences of expression in human studies (124). ERα transcript levels did not change in the hearts of female rats after OVX, nor with OVX and E2 replacement (138). These studies on transcript levels are consistent with studies measuring ER protein level expression. A study compared sex differences and the impact of OVX on protein expression and distribution of ERα and ERβ in the CD1 mouse heart using subcellular fractionation and immunoblotting (119). ERα was predominantly found in the sarcolemma, whereas ERβ was predominantly located in the nucleus and cytosol. ERα expression was greater in the ventricles compared to the atria, whereas ERβ expression was similar in both chambers. The overall distribution and abundance of ERα and ERβ protein levels did not differ between sexes (119). At 6-weeks post-OVX there was an upregulation of ERα expression, but not ERβ. No change in ERα and ERβ levels were observed at 1- or 3- weeks post-OVX (119). Consistent with previous reports, the results in this study found no changes in protein levels of ERα and ERβ between the sexes and across VCD groups. While a previous report found an upregulation of ERα 6-weeks post-OVX, this models the post-menopause period of long-term estrogen deprivation by ovarian failure.
4.5 Ovarian failure alters the regulation of cardiovascular disease

The Timing Hypothesis suggests women who begin HRT during the perimenopausal period or shortly after the onset of menopause have better cardiovascular outcomes than women who initiate HRT several years postmenopause (183). Increasing levels of apoptosis and inflammation during an estrogen-deficient period may explain discrepant outcomes in differences of treatment timing. Inflammatory cytokines and apoptotic markers are both measures for risk, severity, and prognosis of heart disease. Inflammatory cytokines have shown to be useful in evaluating risk assessment for heart failure and they function in injury and recovery regulation (40, 53). Apoptosis is a major contributor in the development of cardiac injury, resulting in the development of reperfusion injury, heart failure, and hypertrophy (123). In preclinical studies, E2 has a generally protective role in reducing injury by regulating markers of inflammation and apoptosis (156). OVX resulted in an increase in caspase-3, caspase-9, and calpain 2 apoptotic marker expression levels, as well as TNFα and IL-6 inflammatory markers. Chronic E2 replacement treated early after OVX attenuated the upregulation of these markers (156).

The results in this study investigated the role of ovarian failure in regulating markers of disease and sought to determine how early these markers change. Our results indicate that VCD-induced ovarian failure promotes a pro-inflammatory and pro-apoptotic profile in the absence of disease stressors as early as peri-menopause. TNFα, IL-6 and IL-1β are pro-inflammatory cytokines associated with increased risk and severity of heart disease. IL-6 and IL-1β both significantly increased with ovarian failure, and TNFα was not changed. IL-6 and CRP, a protein target of expression by both IL-6 and IL-1β, both independently predict vascular events among
apparently healthy postmenopausal women (163). Interestingly, TGFβ increased in a non-linear manner with ovarian failure so that it was significantly greater in 60-day VCD hearts compared to intact hearts, but then returned to levels similar to intact hearts 120 days after VCD treatment. TGFβ is an important anti-inflammatory cytokine involved in cardiac remodeling and angiogenesis (55). Higher levels of TGFβ is a good prognostic marker for CVD, such as hypertrophic cardiomyopathy and heart failure (53). TGFβ may be upregulated in 60-day VCD hearts as a compensatory mechanism that is lost before complete ovarian failure in the 120-day VCD group. This data presents evidence that suggests VCD-induced ovarian failure promotes a pro-inflammatory state within the heart early in the peri-menopausal phase which may contribute to an increased risk for CVD by the onset of menopause.

In addition to an inflammatory profile, this study investigated the role of ovarian failure on apoptotic markers. The process of apoptosis in cardiac myocytes is mediated largely through the activation of caspases (203). Once activated, effector caspases target a variety of cellular proteins for cleavage, including nuclear proteins, signalling molecules, and cytoskeletal targets, driving the death of the cell (73). Caspases are activated through a receptor- or mitochondrion-dependent pathway. Bax regulates the release of pro-apoptotic signalling molecules from mitochondria and an increase in bax levels promotes caspase activation and apoptosis (215). Bcl-2 functions antagonistically to bax and inhibits the mitochondria from releasing pro-apoptotic factors (215). The results in this study show that VCD-induced ovarian failure promote an apoptotic state in which pro-apoptotic activated caspase-3, full caspase-3, and bax expression levels were upregulated. In addition, pro-apoptotic bax significantly increased and anti-apoptotic marker bcl-2 tended to decline but did not reach significance. This evidence shows that VCD-
induced ovarian failure promotes an apoptotic state at least in part by promoting the activation of caspases through the mitochondrion-dependent pathway.

5 LIMITATIONS

5.1 Limitations of study design

This study used the VCD-induced ovarian failure mouse model to produce a gradual transition into a menopause state. While there are significant benefits in using this model compared to the OVX model, there are limitations to be considered. The risk for CVD rises in women with the onset of menopause, which occurs for most by late adulthood (7, 8). Most women who are going through menopause are impacted by physiological changes due to both ovarian failure and aging. Aging independently poses the largest risk factor for CVD (147). This study evaluates the impact of ovarian failure on heart function independent of aging. The application of VCD treatment in young female mice to induce ovarian failure can be considered a model of premature ovarian insufficiency (POI), a condition of early menopause in females. This design allows us to investigate mechanisms and changes specific to ovarian failure without the complications of ageing, which is helpful in developing a targeted treatment that affects estrogen loss. Moreover, women who experience POI have a higher risk of CHD, CVD mortality, and overall morality compared to age-matched premenopausal women (142, 178).

In addition, menopause and ovarian failure involve the loss of many hormones, including progesterone. VCD-induced ovarian failure creates a representative hormonal profile model of human menopause and has shown to deplete progesterone levels (171). In combined HRT, progesterone is replaced in addition to estrogen to prevent endometrial hypertrophy and cancer, unless a low dose of estrogen is used (64). This study is limited because it has isolated the effects
of specific estrogen receptor activation associated with E only therapy, which may not be
generalizable to the effects of combined (E+P) therapy.

ER expression levels were measured to investigate their possible contributions to
observed diminished responses. Homogenized LV samples were isolated using SDS-PAGE and
protein bands were quantified with immunoblotting. While this provides data on total ER protein
expression, this does not provide evidence for ER subcellular localization. Hearts were treated
acutely with ERα- and ERβ- agonists, which bring about their rapid effects by activation of ERs
on the sarcolemma. Subcellular fractionation can be used to evaluate relative quantities of ERs in
different cellular locations. However, we were limited by sample collection, due to the time- and
labour-intensive protocols involved in VCD-treatment and subsequent heart perfusion to
generate samples. In addition, a previous study reported the protein expression and subcellular
distributions of ERα and ERβ is not different among the sexes and does not change in females 3
weeks after OVX, so we would not expect any changes in distribution with VCD treatment
(119).

In order to evaluate whether ovarian failure impacts susceptibility to CVD, protein
expression levels of inflammatory cytokines and apoptotic markers involved in the risk and
development of heart disease were measured using immunoblotting. Although these signalling
molecules are involved in the regulation of these injury processes, these measurements do not
indicate whether or not the heart is in a pathological state or whether the changes are sufficient to
increase risk. Further studies can be performed to measure relative levels of apoptosis and to
evaluate disease susceptibility.
5.2 Technical Limitations

Phosphorylation of the cardiac myofilaments are well established mechanisms that modulate cardiac contractility. This study measured total protein phosphorylation levels of the myofilaments. However, specific phosphorylation sites can contribute singly or in combination to influence cardiac function. Currently, only two methods are available to measure site-specific myofilament phosphorylation: mass spectrometry, which is costly, and immunoblotting using phospho-specific antibodies, which is limited by their availability and accuracy (219). The results in this study provide support for more detailed investigations of the site-specific changes where complex alterations in total phosphorylation were reported.

In addition, the actomyosin ATPase assay is a limited measure of myofilament function, as it does not measure myofilament force generation. In order to correlate myofilament functioning with force generation, either on the myofilament or cellular level, force studies using isolated cardiac myocytes or skinned myofibrils would need to be conducted.

6 FUTURE DIRECTIONS

Based on the work conducted in this study, there are several future directions that can be taken. First, further investigations can be performed to address study limitations. For example, ER subcellular localization levels can be investigated using immunohistochemistry. This would be helpful in determining whether ER distribution within the cell changes with VCD-induced ovarian failure. If relative levels of ERs at the sarcolemma are decreased with ovarian failure, this may provide insight into a mechanism that explains a diminished ER functional response. However, previous studies using the OVX mouse model fail to show any changes with ER distribution early after OVX, so changes in distribution patterns are not expected.
Changes in disease susceptibility associated with ovarian failure can be further evaluated by performing measurements on extent of programmed cell death. These can be evaluated using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay for detecting DNA fragmentation generated during apoptosis (109). In addition, Pfu-labelling can be used to detect blunt end DNA fragmentation generated during necrosis (68). However, these investigations would be more appropriate in studies with the application of a cardiac stressor, such as ischemia, to induce more detectable amounts of injury.

Site-specific phosphorylation can have a significant impact on protein function. In this study, total protein phosphorylation of the myofilaments was evaluated after ovarian failure and ER agonist treatment. This provides evidence for changes in overall protein phosphorylation, but this does not specify which phosphorylation sites are being regulated. Further biochemical studies can be done to evaluate changes in phosphorylation sites of the myofilament proteins that are known to have a significant impact on myofilament function. For example, there are phospho-specific antibodies that target phosphorylated S23/24 on TnI, which is associated with a decrease in myofilament Ca$^{2+}$ sensitivity (106).

Myofilament function was assessed using the modified carter assay by measuring changes in inorganic phosphate as a result of the reaction (236). Changes in myofilament function can be further investigated by conducting actomyosin MgATPase assays with simultaneous force measurements. For example, force-frequency relationship (FFR) experiments using myocardium can be performed to measure physical force generation in varying concentrations of extracellular Ca$^{2+}$ to evaluate calcium sensitivity (158). These studies can provide an understanding of myofilament function in vivo.
Acute ER activation resulted in altered heart and myofilament function. Previous reports from our group indicate that ERα has a role in rapid p38 MAPK signalling known to affect myofilament function (106). In addition, contractile proteins are targets of several protein kinases, including PKA and PKC (204). Further studies can investigate the relationships between acute ER activation and the cAMP-dependent PKA pathway and MAPK cascades. This can provide a greater understanding of rapid ER regulation in the heart and create opportunities for control by treatment.

Future studies can be done with certain parameters added to gain our understanding of ER regulation and ovarian failure. The third G protein-coupled estrogen receptor, GPR30, is localized to the plasma membrane known to activate multiple signalling pathways upon activation (50, 173). Separate studies using acute GPR30 agonist treatment can investigate its functional response and impact on myofilament regulation during ovarian failure. Additional studies can be performed to investigate the acute response to progesterone exposure during ovarian failure. Since certain hormone therapies are a combination of estrogen and progesterone, it would be useful in evaluating the regulation of both steroid hormones. Previous reports indicate acute progesterone treatment attenuates cardiac contraction and modifies myofilament calcium sensitivity (48). In addition to changes in treatment, an additional VCD group aged 150 days or more can be established to model the postmenopausal period. Harvesting the heart more days after VCD injections will expose the heart to ovarian failure for a longer period of time. Also, this study can be repeated in aged mice that model late adulthood to evaluate the impact of both ovarian failure and aging on heart function and ER regulation.

Preclinical studies provide evidence for the protective effects of estrogens in reducing cardiac injury after MI (15, 16, 70). It would be interesting to investigate whether the protection
of estrogen is limited to earlier stages of menopause. I/R protocol can be applied to Langendorff isolated heart perfusion experiments and hearts can be pretreated with ER agonists to evaluate ER-specific effects. Hearts from mice of varying VCD-induced ovarian failure can be compared to determine if there is a timepoint at which the heart becomes resistant to the protective effects of estrogen. Myofilament function and disease progression can be further evaluated.

7 CONCLUSIONS

Overall our results show that the way the heart responds to acute ERα- and ERβ-activation depends on ovarian failure and changes in function are regulated in a non-linear manner. The functional response to ER activation leads to decreased function which is attenuated with ovarian failure and is subtype-specific. Both ovarian failure and ER activation lead to changes in the regulation of myofilament function and protein phosphorylation that contribute to force of contraction. Ovarian failure promotes a pro-inflammatory and pro-apoptotic state represented by altered disease markers in the heart. Together these findings suggest that the timing of onset of menopause can have an impact on response to estrogen supplementation and overall CVD outcomes.
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