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Estrogen Therapy Rescues Advanced Heart Failure via Estrogen Receptor Beta

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular, Cellular & Integrative Physiology

by

Andrea Iorga

2015
ABSTRACT OF THE DISSERTATION

Estrogen Therapy Rescues Advanced Heart Failure via Estrogen Receptor Beta

By

Andrea Iorga
Doctor of Philosophy in Molecular, Cellular & Integrative Physiology
University of California, Los Angeles, 2012
Professor Mansoureh Eghbali, Chair

Cardiac hypertrophy, defined as an enlargement of the ventricles, is often triggered when the heart is subjected to hemodynamic stress from physiological stimuli such as pregnancy, or from pathological stimuli such as pressure overload-induced left ventricular hypertrophy or pulmonary hypertension-induced right ventricular hypertrophy. Physiological hypertrophy is beneficial and adaptive, while pathological hypertrophy is maladaptive and detrimental.

It is well documented that in pathological hypertrophy the proteasomal activity is greatly disrupted, but nothing was known about possible regulation of proteasome activity/expression in physiological hypertrophy. In chapter 2 I show that physiological hypertrophy is downregulated, with a concomitant decrease in protein ubiquitination and reactive oxygen production. These findings lead to the conclusion that during pregnancy, the levels of aberrant or misfolded proteins are decreased.
Secondly, estrogen treatment prior to the onset of pathological stimuli is known to attenuate the progression of the onset of ventricular hypertrophy, cardiac dysfunction and subsequent failure. However it was not known whether estrogen is also effective in rescuing heart failure since heart failure is not often diagnosed early and therapeutic intervention after the onset of hypertrophy and failure is necessary. In Chapters 3 and 4 I use two different models of hypertrophy and failure, the pressure overload-induced left ventricular hypertrophy and failure as well as the pulmonary hypertension-induced right ventricular hypertrophy and failure. I show that short-term estrogen therapy after the onset of cardiac dysfunction in both models rescues function via activation of the estrogen receptor β. This rescue action of estrogen is also associated with reversal of cardiac fibrosis and stimulation of angiogenesis, both of which are essential in nurturing the heart.
The dissertation of Andrea Iorga is approved.

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University of California, Los Angeles
2015
To my family, I am grateful for always believing in me and supporting me.
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Chapter 1:

Introduction
1.1. Cardiac Hypertrophy

Cardiac hypertrophy, defined as an enlargement of the ventricles, is often triggered when the heart is subjected to hemodynamic stress from volume or pressure overload\(^1\) and is an important compensatory response so the heart can maintain its pumping capacity. Sustained pressure overload often leads to concentric hypertrophy, which is characterized by increased wall thickness without a concomitant chamber enlargement. At this stage, the ventricle is capable of generating greater forces and higher pressures while the increased wall thickness maintains normal wall stress. Volume overload, on the other hand leads to eccentric hypertrophy characterized by a proportional enlargement of the chamber size and the wall thickness\(^2\). Heart hypertrophy can be physiological which is beneficial and adaptive or pathological which is maladaptive and detrimental (Figure 1).

1.2. Physiological Hypertrophy

Physiological hypertrophy occurs in response to normal exercise or pregnancy and is not associated with fibrosis, dysfunction, or increased morbidity and mortality. Physiological hypertrophy enables the heart to fulfill its function, and is often reversible without significant long-term detrimental effects on cardiac function\(^3-5\). In these aspects, pregnancy- and exercise-induced hypertrophies are similar. However, pregnancy is also accompanied by drastic hormonal changes. Both estrogen and testosterone steadily increase and reach their maximum levels at the end of pregnancy. Additionally, during pregnancy, unlike exercise, the force demand placed on the heart is continuous as opposed to sporadic. To this day, little is known about the molecular mechanisms that mediate pregnancy-induced hypertrophy, but recent reports have shown that its molecular signature is unique \(^6\) and differs from that induced by exercise\(^7,8\).
Figure 1. Pathological hypertrophy vs. pregnancy induced physiological hypertrophy. Top panels: Images of the whole heart in pathological hypertrophy and physiological hypertrophy in late pregnancy in mice; Middle panels: Hematoxylin-eosin staining of heart cross-sections; Bottom panels: Masson trichrome staining of heart cross sections, blue color indicates fibrosis. CTRL: control; HF: heart failure; NP: non-pregnant; LP: late pregnant. Note that the heart develops concentric hypertrophy in pressure overload and eccentric hypertrophy in physiological hypertrophy, in which no fibrosis is present.
1.3. Pathological hypertrophy and heart failure

Pathological heart hypertrophy often leads to heart failure if the stimulus persists. Heart failure (HF) is a clinical syndrome attributable to many factors. HF begins as a compensatory response to hypertrophic stimuli, followed by a decompensatory response which eventually results in failure. Heart failure is often preceded by heart hypertrophy, which is usually triggered by external stressors. While heart hypertrophy is presumed to be compensatory at the beginning it often progresses to chronic HF when insults persist.

1.3.1. Left ventricular hypertrophy and failure

Left ventricular (LV) hypertrophy initially develops due to increased force and stretch demand on the ventricle, and is initially compensatory. In its compensated state, the cardiac contractility and output is maintained. During the development of LV hypertrophy, the LV mass also increases representing a structural mechanism through which the LV adapts to reduce wall stress and maintain adequate stroke volume in response to pressure overload. If the hypertrophic stimulus is removed while the LV is in the compensated hypertrophy state, the cardiac remodeling is reversed back to normal functional state. However, if the hypertrophic stimulus persists, the LV eventually transitions to a non-reversible, decompensated state in which there is widespread adverse LV remodeling and eventual ventricular failure.

1.3.2. Right ventricular hypertrophy and failure

Right ventricular (RV) hypertrophy develops when the right ventricle works against increased pulmonary arterial pressures, and is initially a compensatory mechanism. Adaptation of the RV to this increased afterload can initially improve or maintain cardiac output. However, if the
afterload is not lowered, the increased right ventricular pressure leads to decreased RV perfusion and RV ischemia. The hypertrophied ventricle will progressively undergo changes that will impair RV filling and decrease stroke volume, increasing its dependence on a properly functioning right atrium. Furthermore, the increase in pressure results in increased wall stress and wall thickness, which further impedes myocardial perfusion. During the transition from compensated hypertrophy to decompensated hypertrophy and failure, the RV cannot sustain long-term pressure overload, resulting in an eventual decrease in the cardiac contractile force and RV dilation. At the current time, it is not clear what mechanism(s) mediated the transition from compensated RV hypertrophy to decompensated right heart failure.

1.4. Sex differences in cardiovascular disease

Heart failure is an increasing public health problem, with the majority of cases resulting from ischemic heart disease and hypertension. Myocardial infarction (MI) and chronic hypertension often lead to progressive LV remodeling that contributes to the development of heart failure, characterized by cardiomyocyte hypertrophy which in turn results in the activation of signaling pathways and regulation of gene expression.

Epidemiologic studies have previously suggested that females prior to menopause have reduced incidence of cardiovascular disease when compared to age-matched males. Coronary artery disease (CAD) as well as cardiovascular disease and myocardial infarction (MI) first present themselves in women approximately 10 years later than in men, and that the incidence and severity of cardiovascular disease increases post-menopause. The lower incidence of cardiovascular disease in women during reproductive age is believed to be related to estrogen. In the context of coronary artery disease which is the leading cause of death in western countries,
the protective role of estradiol (E2) has been highlighted in women. The incidence of coronary artery disease is higher in young women who had an oophorectomy\textsuperscript{14}. There has been evidence suggesting that pre-menopausal women withstand ischemia/reperfusion injury during open heart surgery better than males\textsuperscript{15}.

It has previously been shown that women with aortic stenosis develop a more concentric form of LV hypertrophy than men\textsuperscript{16}. In a study of patients with aortic stenosis before aortic valve replacement, echocardiography prior to replacement surgery revealed that males had significantly more maladaptive cardiac remodeling than women\textsuperscript{17}. Further transcriptome characterization also revealed that fibrosis and inflammation-related genes and pathways were upregulated in males but not in females, thus revealing distinct molecular processes between men and women in the overloaded left ventricle\textsuperscript{17}. Similarly to humans, female animals are also more protected against cardiovascular disease versus males\textsuperscript{18}.

The sex differences in cardiovascular disease between males and females can be attributed to sex hormones or to sex chromosome milieu.

\textbf{1.4.1. Role of sex hormones}

The lower incidence of cardiovascular disease in women during reproductive age is believed to be related to estrogen\textsuperscript{19}. In the context of coronary artery disease which is the leading cause of death in western countries, the protective role of estradiol (E2) has been highlighted in women. Intact female mice exhibit reduced ischemia/reperfusion injury compared with males. The infarct size in ovariectomized (OVX) female mice is larger than in gonadally intact mice, and E2 pre-treatment of OVX mice reduces the infarct size\textsuperscript{14}. Treatment of male rats with estrogen prior to ischemia also decreased infarct size, while estradiol administration improved contractile function.
in rat hearts after ischemia/reperfusion. In response to pressure overload it has been shown that hypertrophy is significantly more acute in males than in females, that ovariectomized mice develop a more robust left ventricular hypertrophy compared to intact female mice and estrogen pretreatment attenuates development of myocardial hypertrophy following transaortic constriction (TAC) in OVX mice. Patten et al. compared the effects of estrogen during MI and TAC, and found MI caused LV dilation and decreased fractional shortening (FS) that were unaltered by E2. TAC also caused LV dilation, reduced FS and increased LV mass, but in this model, E2 improved these parameters. In pathological heart hypertrophy beta myosin heavy chain (β-MHC) is known to be re-expressed. Atrial natriuretic peptide (ANP) has anti-hypertrophic properties and is constitutively expressed in the in the cardiac ventricles in response to stress induced by increased cardiac afterload or myocardial infarction. The study by Patten et al. further demonstrated that after MI, E2 led to increased cardiomyocyte cross-sectional area, ANP and β-MHC expression, while in the TAC model E2 decreased these factors. A more recent study by Costa et al. investigated the interplay of testosterone (T) addition to estrogen, as T has been added to hormone replacement therapy (HRT) to treat sexual dysfunction in postmenopausal women. Although estrogen has been associated with vascular protection, the group used conjugated equine estrogen (CEE, currently used in HRT), which is a combination of estrone, equilin and equilenin. Although estrone, which makes up circa 50% of CEE can be converted to estradiol, CEE does not contain any estradiol, the active estrogen found in women. The group found that CEE treatment increased endothelium-dependent relaxation, which was impaired in OVX rats, while this effect was lost in the CEE+T rats. Lastly, addition of T reversed the beneficial effects on reactive oxygen species (ROS). Therefore, the association of T with
CEE impairs the benefits of estrogen on OVX-associated endothelial dysfunction and ROS generation.

Sex differences in right ventricular hypertrophy (RVH) secondary to pulmonary hypertension (PH) have also been reported. Numerous experimental studies have demonstrated better outcome in female animals, exacerbation of the disease after ovariectomy and a strong protective effect of estrogen. These gender differences are attributed to the protective effects of estrogen in the females, as estrogen and its metabolites have been shown to prevent and rescue experimental RVH\textsuperscript{25-27}. On the other hand, some clinical studies have indirectly linked estrogen to increased risk of RVH, since the disease is more common in young women than men\textsuperscript{28}.

1.4.2. Role of sex chromosomes
Prior to the emergence of the four core genotype (FCG) model, the dominant theory of sexual differentiation was that all sex differences outside of the gonad were caused by gonadal secretions\textsuperscript{29}. The FCG mouse model was then developed to investigate the impact of sex chromosomes and gonadal type on phenotypes. In FCG mice, \( Sry \), the gene on the Y chromosome which causes testes formation and testosterone synthesis leading to male-typical development of many phenotypes, is deleted from the Y chromosome and a \( Sry \) transgene is instead inserted onto an autosome\textsuperscript{30}. Along with the typical XY male and XX female, deletion of \( Sry \) results in XY females with ovaries, and insertion of \( Sry \) in an autosome leads to XX males with testes. Thus using intact FCG mice can shed insight on the influence of sex steroids independent of sex chromosome content (the study of androgens in XX and XY gonadal males and of estrogens in XX and XY gonadal females). Conversely, depletion of gonadal steroids by gonadectomy of FCG mice unmask the effect of sex chromosomes. Using this mouse model, Li
et al. investigated the role of sex chromosomes in causing sex differences in myocardial I/R injury. The study found that after gonadectomy, the in vivo infarct size following left anterior descending (LAD) coronary artery ligation in XY mice was significantly smaller than XX mice regardless of gonadal type. Consistent with the in vivo data, ex vivo hearts of XY mice had a significantly smaller infarct size and better cardiac recovery in reperfusion. The mitochondrial calcium retention capacity, which is an indicator of mitochondrial health and overall cardiac health, was also significantly higher in XY compared to XX mice. Thus, this data suggests that the X chromosome number may contribute to lower cardioprotection regardless of sex chromosome complement.

1.5. Estrogen as therapy for heart failure in experimental models

Estrogen has been established to confer cardioprotection. Pretreatment with E2 has been previously shown to attenuate the development of HF due to pressure overload. E2 pre-treatment of OVX mice reduces the infarct size. Treatment of male rats with estrogen prior to ischemia also decreased infarct size, while estradiol administration has been shown to improve contractile function in rat hearts after ischemia/reperfusion. The mechanisms through which E2 is cardioprotective are comprehensive, as E2 exerts effects on the vasculature, cardiac fibrosis, angiogenesis and harmful reactive oxygen species.

1.5.1. Effect of estrogen on vasculature

E2 directly stimulates endothelial nitric oxide synthase (eNOS) activity and thus has a beneficial effect on vasculature. Many reports have shown that estrogen can cause arterial dilation of elastic and muscular arteries in-vivo via estrogen receptor (ER)-mediated activation of ERK and PI3K,
and stimulation of nitric oxide production\textsuperscript{33}. In addition, both ER\(\alpha\) and ER\(\beta\) receptors mediate estrogen’s vasodilative action\textsuperscript{34}. Isolated RNA from different tissues (kidney cortex, kidney medulla, lung and aorta) of OVX female rats treated with E2 for 21 days revealed a downregulation of angiotensin-converting enzyme (ACE) transcript levels\textsuperscript{35}. The downregulation of ACE and a consequent reduction in the circulating levels of the vasoconstrictor angiotensin II could be one of the mechanisms through which E2 confers its beneficial cardiovascular effects.

In vitro, similar NOS activation pathways have been established in isolated coronary, cerebral and mesenteric arteries\textsuperscript{36-38}. However, emerging studies show that the signaling mechanisms through which E2 exerts its powerful vasoactive action are still not well understood\textsuperscript{39}. White et al. found that E2 relaxed porcine coronary arteries in an endothelium-independent and dose-dependent fashion; however, when arteries were pretreated with agents to uncouple nitric oxide (NO) production from NO synthase (NOS), E2 elicited dose-dependent contractions of arteries. After E2-induced coronary contraction had reached its maximum levels, an inhibitor of superoxide (O\textsubscript{2}\textsuperscript{−}, Tempol) was able to reverse this response by reducing O\textsubscript{2}\textsuperscript{−}. Thus, they proposed that acute E2 administration of estrogen produces either coronary vasodilation via NO or vasoconstriction via O\textsubscript{2}\textsuperscript{−}. Another study by Sudhir et. al.\textsuperscript{40} also found that estrogen induced a significant increase in coronary cross-sectional area, flow velocity and volumetric blood flow in canine coronary arteries. The estrogen-induced vasodilation was not influenced by pretreatment with l-NAME (a constitutive inhibitor of NOS), indomethacin (an inhibitor of endothelial cell proliferation), ICI 182, 780 (a classic estrogen receptor antagonist), propranolol (a sympathic nonselective beta blocker used to treat arterial hypertension and tachycardia), glibenclamide (a vasorelaxant) and verapamil (another vasorelaxant used to treat arrhythmias). The investigators thus conclude that acute estrogen-induced dilation of coronary arteries is endothelium-
independent and is not mediated via the classic intracellular estrogen receptors, but presumably via through non-genomic mechanisms involving G protein coupled receptors at the plasma membrane\textsuperscript{40}.

Therefore, the direct action of E2 on the vessel wall could indirectly improve cardiac function, as E2 promotes vasodilatation by acting on the endothelial and smooth muscle cells\textsuperscript{41} which can in turn exert beneficial effects on cardiovascular function and improved cardiac hemodynamics.

\subsection*{1.5.2. Effects of estrogen on fibrosis}

Cardiac remodeling in heart failure is known to be associated with fibroblast proliferation, fibrosis, an accumulation of fibrillar collagens, and myocyte apoptosis\textsuperscript{42}. Increased expression of fibrillar collagens leads to stiffening of the cardiac muscle and myocytes electrical coupling, thus impeding both contraction and relaxation of the heart. Furthermore, increased levels of fibrosis cause a decrease in angiogenesis in the heart as well as an increase of oxygen diffusion distance, thus leading to hypoxia of myocytes\textsuperscript{43}.

The myocardial extracellular matrix (ECM) proteins, matrix metalloproteinases (MMPs), growth factors and cytokines are mainly produced by fibroblasts, all of which contribute to the myocardial structure maintenance as well as cardiac remodeling in diseased hearts\textsuperscript{44}. The normal ECM plays an important adaptive role in HF by preventing excessive dilatation when ventricular overload occurs. The ECM is mainly formed by type I and III collagens. An imbalance in the synthesis and/or inhibition in the expression of ECM proteins results in fibrosis. In the pressure overloaded ventricles however, cardiomyocyte hypertrophy is accompanied by increased collagen deposition between and around myocytes, resulting in interstitial and perivascular fibrosis\textsuperscript{45}. The ECM is enzymatically digested by MMPs, and an imbalance of collagen deposition
and the activity or expression of MMPs can lead to cardiac remodeling. In the normal heart, the majority of cells are fibroblasts, and external stressors cause fibroblasts to change their phenotype to myofibroblasts, which plays a pivotal role in inflammation and fibrosis.

The role of estrogen on cardiac fibroblasts has been elucidated by an early study. First of all, a study by Lee et al. first investigated the expression of the classic ERs in fibroblasts, and found that both ERα and ERβ were expressed, with the predominant receptor being ERβ expressed both in the cytosol and nucleus. The same study also found that treatment of fibroblasts with E2 increased DNA synthesis, which was prevented in the presence of tamoxifen, an anti-estrogen with high affinity for both estrogen receptors. This upregulation of DNA synthesis occurs through translocation of receptor protein and activation of MAPK. Thus, fibroblasts are a direct target of E2, which enhances the proliferative capacity of fibroblasts via ER and MAPK-dependent mechanisms. This data suggests that E2 acts on the fibroblasts to regulate ECM remodeling, thus impacting myocardial function. Interestingly, another early study by Dubey et al. found that E2 inhibited fetal calf serum-induced fibroblast proliferation (both DNA synthesis and cell number), as well as collagen synthesis. Furthermore, the estrogen metabolites 2-hydroxyestradiol and 2-methoxyestradiol were even more potent than E2 were more potent than E2 in inhibiting cell proliferation, DNA and collagen synthesis, and that these effects were enhanced in the presence of progesterone and 4-hydroxytamoxifen (a high-affinity ER ligand).

MMP-2 plays a pivotal role in cardiac remodeling. Its increased expression and activity occurs after aortic stenosis, MI, and left ventricular hypertrophy, and is believed to play adverse cardiac remodeling. Furthermore, its constitutive expression of MMP-2 causes systolic dysfunction and severe remodeling while its inhibition attenuates cardiac remodeling and improves survival after MI or pressure overload. More recently, Mahmoodzadeh et al. found that in adult rat...
and human fibroblasts, E2 significantly reduced MMP-2 gene expression via activation of the classic ERs. This effect was due to E2 phosphorylation of the transcription factor Elk-1 via the MAPK signaling pathway. Thus, this E2-induced repression of MMP-2 may contribute to sex differences in fibrotic processes.

As ERβ is the predominant ER in fibroblasts, gender differences as well as the involvement of ERβ have been shown. WT male hearts exhibited a stronger induction of matrix-related genes vs. females, and ERβ knockout mice of both sexes exhibited a stronger induction as well as induction of proapoptotic genes.

Pedram et al found that ERβ activation prevents cardiac fibrosis. Angiotensin II (AngII) and endothelin-1 promote cardiac fibrotic deposition, and these factors directly promote the transition of fibroblasts to myofibroblasts. Both these agents stimulated the synthesis of TGFβ1, a potent inducer of cardiac fibrosis. Upregulation of TGFβ1 signaled through c-jun in the myofibroblast, which ultimately led to myofibroblast production of vimentin, fibronectin, and collagen I and III, which was inhibited by E2 as well as an ERβ agonist by signaling through cAMP and protein kinase A thus blocking the activation of c-jun kinase. Lastly, in OVX mice, AngII infusion induced cardiac fibrosis and hypertrophy, which was prevented by E2 administration in WT mice but not in ERβ knockout mice. Therefore, this study shows that fibroblasts play an important role in the development of cardiac hypertrophy and fibrosis, and that E2 and ERβ activation.

Lastly, E2 and ERβ activation has been shown to reverse the right ventricular adverse remodeling caused by pulmonary hypertension (PH) in rats. Chronic PH ultimately leads to right ventricular failure (RVF) which is characterized by exacerbated RV remodeling as well as upregulation of ECM-interacting cardiac fetal gene OPN. The study found that the transcript levels of two novel ECM-degrading disintegrin-metalloproteinases ADAM15 and ADAM17, as
well as OPN were elevated in the failing RV and E2 therapy after the onset of PH reversed these effects. Furthermore, an ERβ agonist was able to reverse ADAM15 and 17 transcript upregulation, RVF resulted in elevated Akt phosphorylation in the RV, while E2 reversed this phosphorylation\textsuperscript{58}. Thus, RV remodeling resulting from PH is associated with upregulation of ADAM15, ADAM17 and OPN, which were reversed by E2 likely through ERβ activation.

1.5.2. Effects of estrogen on angiogenesis

By definition, angiogenesis is the physiological process by which new blood vessels form from pre-existing vessels. Estrogen has previously been shown to be pro-angiogenic in various tissues \textsuperscript{59, 60}. Furthermore, increased cardiac angiogenesis has been shown to be a key event in maintaining LV function during adaptive hypertrophy\textsuperscript{61, 62}.

Several agents have been shown to induce angiogenesis. The fibroblast growth factor (FGF) family stimulates a number of processes such as cell differentiation, proliferation and matrix dissolution. From this family, the most potent angiogenesis growth factors are FGF-1 and FGF-2, which promote endothelial proliferation and organization into tubular structures \textsuperscript{63, 64}. The vascular endothelial growth factor (VEGF) has also been demonstrated to be a major contributor to angiogenesis by increasing the number of capillaries. VEGF is an important angiogenic factor and a critical determinant of capillary growth and density\textsuperscript{65}. VEGF binding to its receptor-2 (VEGFR-2) starts a signaling cascade which stimulates factors affecting eNOS and NO production, proliferation, migration of tube structures, and finally their differentiation into mature blood vessels\textsuperscript{66}. Furthermore, it is also believed that E2 induces the expression of vascular adhesion molecules\textsuperscript{67} and integrins\textsuperscript{68}, while inhibiting endothelial cell apoptosis\textsuperscript{69}. Other major contributors to angiogenesis are the matrix metalloproteinases (MMPs) which degrade the
proteins which keep the vessel wall solid, thus allowing endothelial cells to escape in the cell matrix and form new capillaries\textsuperscript{70}.

The majority of angiogenesis seen in mammals occurs during embryogenesis, which is downregulated in adult animals in nonpathological conditions\textsuperscript{71}. The exception to this occurs during the female menstrual cycle as well as embryonic implantation and development to support the developing embryo where adult angiogenesis is essential, thus suggesting that E2 is potent in stimulating angiogenesis\textsuperscript{72}. Indeed, E2 promotes new blood vessel formation both in vivo and in vitro\textsuperscript{73}, where endothelial cells plated on basement membrane matrix attach, migrate and form tubules with the lumen. Estrogen has also been postulated to be proangiogenic in ischemic tissues\textsuperscript{71}. Furthermore, in response to basic fibroblast growth factor, OVX mice displayed a 60-70\% reduction in vascularization versus intact female mice\textsuperscript{74}.

It has been shown that endothelial progenitor cells (EPCs) isolated from blood incorporate into foci of neovascularization in the adult\textsuperscript{75, 76}. As such, a study by Iwakura et al.\textsuperscript{77} investigated whether E2 can augment the incorporation of EPCs in the sites of cardiac ischemia vascularization, resulting in protection from ischemic injury. The group used OVX mice pretreated with placebo or E2 prior to MI induced by LAD, and found that E2 induced significant increases in circulating EPCs which led to increased cardiac capillary density. The study also found a greater preponderance of EPCs in the E2-treated animals at the ischemic sites\textsuperscript{77}. However, when using the same experimental protocol in eNOS-null mice, the effects of E2 were abolished, as was the cardiac functional recovery from acute myocardial ischemia. Furthermore, in eNOS-null mice, a decrease in MMP-9 was observed both in basal an E2 treated conditions, whereas MMP-9-null mice did not experience the mobilization of EPCs and the functional benefit conferred by E2\textsuperscript{77}. Therefore, E2 maintains the integrity of cardiac ischemic
tissue by increasing the mobilization and incorporation of EPCs by an eNOS-induced regulation of MMP-9. Later on, the same group investigated to role of ERα and ERβ in the E2-induced mobilization of EPCs which favorably affected neovascularization post cardiac ischemic injury. The study found that the E2-induced endothelial migration, tube formation and adhesion in EPCs derived from ERα knockout mice was severely, and moderately impaired in EPCs derived from ERβ knockout mice. When bone marrow was transplanted from either ERα or ERβ knockout mice into WT mice, it has been found that capillary density at the ischemic border zone was significantly reduced in both these models versus WT. ERα transcripts was expressed more abundantly than ERβ in EPCs, and VEGF expression was downregulated only in the ERα knockout EPCs both in vivo and in vitro when compared to WT. Thus both ERα and ERβ contribute to E2-induced EPC mobilization and preservation of cardiac function after MI, but ERα plays a more predominant role in this process. The fact that ERα can upregulate VEGF transcripts may hint to possible mechanisms through which E2 confers cardioprotection post ischemia.

1.5.3. The role of aromatase in the cardiovascular system

The enzyme aromatase catalyzes the final step of estrogen synthesis from testosterone. Aromatase is widely distributed in gonadal and extragonadal tissues such as bone, brain, adipose tissue and blood vessels, and plays important roles in their physiological functions through tissue-specific regulation of estrogen synthesis. As estrogen has been shown to have an atheroprotective effect against atherosclerotic progression in experimental animal models, aromatase plays a pivotal role in promoting vascular protection. Aromatase activity has been reported in rat arterial smooth muscle cells (SMCs) and in bovine coronary endothelial cells.
(ECs) in culture\textsuperscript{84, 85}. Harada et al.\textsuperscript{80} further proceeded to demonstrate aromatase activity in human arterial SMCs but not in ECs, which could potentially indicate an autocrine/paracrine estrogen activity. The authors speculate that estrogen produced in vascular SMCs acts to promote cardiac contractility, vasodilation and collagen synthesis, while also stimulating EC functions, such as NO production and angiogenesis\textsuperscript{80}. A later study by Georgi et al.\textsuperscript{86} demonstrated that the presence of aromatase and androgen receptors in human coronary vascular smooth muscle cells as well as rat cardiac fibroblasts, which indicates a the possibility that circulating T can act locally in androgenic or estrogenic fashion on the cardiovascular system.

It is well-known that the incidence of cardiovascular events in premenopausal women is significantly lower compared with that in men or postmenopausal women\textsuperscript{80}. However, the role of aromatase in the heart is not well understood. In postmenopausal women, aromatase plays an important role, as its peripheral activity becomes the primary source of E2 synthesis\textsuperscript{87}. Aromatase inhibitors, which are used clinically for treatment of breast cancer, carry the potential risk of increased cardiovascular events, partially due to the favorable effects of estrogen on vascular endothelial function\textsuperscript{88} and plasma lipids\textsuperscript{89}. Previous work from our group\textsuperscript{90} found that in female mice in proestrus (at which stage females have the highest surge of estrogen), the cardiac estrogen concentration was almost three times higher than in the circulating plasma. As the final cardiac estrogen concentrations would depend on the T and aromatase levels, we measured these two parameters. We found that females in estrus (in which stage the female heart is under the control of the preceding surge of estrogen from proestrus) have much lower levels of testosterone, while the aromatase protein levels were two fold higher versus males. Therefore, the higher aromatase expression in female hearts may underlie the high estrogen levels, thus resulting in cardioprotection. Estrogen in females has been conventionally considered to be
cardioprotective, but the role of aromatase in male cardioprotection was just recently established. Bell et al.\textsuperscript{91} studied whether upregulation of tissue aromatase expression could improve ischemic resilience in male hearts. The group used male mice in which aromatase was transgenically upregulated (AROM\textsuperscript{+}) and subjected them to ischemia/reperfusion studies. They found that in early reperfusion the ischemic contracture was attenuated in AROM\textsuperscript{+} male hearts. Female AROM\textsuperscript{+} hearts however, exhibited a diminished effect on ischemia/reperfusion function. Thus, these findings demonstrate a role of aromatase-induced modulation of cardiac function and protection against ischemic injury and maximize reperfusion recovery\textsuperscript{91}.

The ablation of aromatase has also been demonstrated to be associated with increased adiposity\textsuperscript{92} and progressive insulin resistance\textsuperscript{93, 94}, which are significant factors in the development of cardiovascular diseases such as atherosclerosis and diabetic cardiomyopathy. Paradoxically, two recent papers by Bell et al.\textsuperscript{95} investigated the role of aromatase deficiency by using aromatase knockout (ArKO) female mice, and found that in the context of ischemia/reperfusion injury, the recovery of left ventricular developed pressure as well calcium handling was substantially improved in ArKO versus wild-type (WT) hearts\textsuperscript{95}. Bell et. al.\textsuperscript{96} later demonstrated that in isolated cardiomyocytes from ArKO female mice, the basal calcium transient amplitude and extent of shortening were greater than in WT. Isolated cardiomyocytes from ArKO female mice exposed to a high calcium load also showed increased calcium transient and contractile amplitudes. The study concluded that the relative withdrawal of E2 in favor of T effects inotropic support via optimized calcium handling in response to stress, and aromatase inhibition may play a role in cardioprotection.
1.6. Estrogen receptors and cardioprotection

Estrogen binds to the traditional estrogen receptors ERα and ERβ, and can confer both genomic and nongenomic action. Intracellular ERα and ERβ act as transcription factors, and after binding estrogen they dimerize and enter to nucleus. In the nucleus, they bind to estrogen response elements, Ap1 and Sp1 sites on the promoter of E2-responsive genes, thus regulating their transcription. Estrogen can also bind to G-protein coupled receptors, such as the newly discovered GPER30, as well as to membrane-bound ERα and ERβ receptors which rapidly activates nuclear transcription factors via the MAPK pathway (Figure 2).

Estrogen receptors (ERs) can mediate cardioprotective processes such as angiogenesis and vasodilation, thus limiting cardiac remodeling and attenuating hypertrophy. Furthermore, a novel G-protein coupled receptor GPER has been found to be expressed in adult cardiomyocytes with a high affinity for E2. Activation of ERα, ERβ and GPER have been shown to have both nongenomic (acute) and genomic (chronic) effects.

As estrogen has been shown in previous experimental models to limit the extent of pressure-overload induced hypertrophy, a study by Donaldson et al. indeed corroborated these findings and determined that E2 pretreatment indeed reduced TAC-induced LV and myocytes hypertrophy and attenuated the deterioration of LV systolic function and contractility. The serine-threonine phosphatase calcineurin A (CnA) plays a critical role in the development of pathological hypertrophy. CnA targets the nuclear factor of activated T cell (NFAT) cellular localization and regulates its function by its phosphorylation state. Basally, NFAT is hyperphosphorylated and localized in the cytoplasm. Hypertrophic stimuli, CnA dephosphorylates NFAT which allows NFAT to localize in the nucleus, where, together with other transcriptional factors, activates the hypertrophic response. The study by Donaldson et al
Figure 2. Estrogen receptors and genomic and nongenomic action of estrogen. Estrogen binds to estrogen receptor alpha and/or beta, translocates to the nucleus in exerts its genomic action by binding to promoter regions of DNA via estrogen response elements or Sp1/Ap1. Nongenomically, membrane and cytosolic estrogen receptors activate transcription factors via the PI3K and MAPK signaling. E2, estrogen; ER, estrogen receptor; ERE, estrogen response element; GPCR, G-protein coupled receptor; PI3K, phosphoinositide 3-kinase; MAPK, mitogen activated protein kinase; AKT, protein kinase B.
found that the beneficial effects of E2 post pressure overload were associated with a reduced abundance of CnA and NFAT activation, while in vitro these effects were not observed in the presence of the broad estrogen receptor antagonist ICI 182, 780.

Estrogen’s protective role in the myocardial infarct model has also been previously elucidated. It was shown that female mice lacking the class II histone deacetylase (HDAC) genes -5 and -9 were protected against MI-induced cardiac dysfunction and LV remodeling, whereas males lacking these genes were hypersensitive to the pathological effects of MI. As HDAC-5 and -9 inhibit the expression of ER alpha, ablation of these genes in females allowed for ER alpha to confer protection by increasing angiogenesis in the infarcted region via upregulation of its target gene vascular endothelial growth factor (VEGF)\textsuperscript{103}.

A study by Zhai et al.\textsuperscript{104} highlighted the role of ERα in global myocardial ischemia and reperfusion by comparing male ERα knockout (ERKO) with wild-type mice. After ischemia, the ERKO hearts started beating later on during the reperfusion period, and had higher incidence of fibrillation and/or tachycardia, as well as a significantly lower coronary flow rate. ERKO hearts also displayed markedly increased interstitial edema and contraction bands, while having fewer viable myocytes versus wild-type controls. Transmission electron microscopy also revealed that ERKO hearts had swollen and fragmented mitochondria, loss of matrix and ruptured cristae\textsuperscript{104}. Therefore, this demonstrates that ERα plays a cardioprotective role in ischemia-reperfusion injury in males. Furthermore, in the in vivo rabbit model of I/R, acute pretreatment with E2 or the ERα agonist PPT, but not by the ERβ agonist DPN, significantly decreased infarct size, suggesting that ERα plays a significant role in the acute cardioprotective action of E2 \textsuperscript{99, 105}. Thus, acute activation of ERα seems to protect the heart from ischemic injury, while the data are conflicting regarding the role of ERα under conditions of chronic E2 exposure\textsuperscript{99}. 
Compared to ERα, which is primarily found in the sarcolemma, ERβ is primarily found in the nucleus, cytosol and mitochondria\textsuperscript{106} in adult murine hearts\textsuperscript{107}, thus suggesting a different mechanism of ERβ-mediated cardioprotection. Nonetheless, the cardioprotective role of ERβ has also been highlighted in previous studies. Female mice lacking the ERβ gene are less protected against myocardial I/R injury compared to WT female mice, and ERβ-knockout mice have abnormal vascular function and hypertension, increased mortality, and aggravated heart failure\textsuperscript{108}. Genetic deletion of ERβ results in hypertension in both female and male rats\textsuperscript{109}. Furthermore, both estrogen and ERβ have been shown to prevent hypertrophy in a variety of models of cardiac hypertrophy in mice\textsuperscript{19, 110}. Administration of an ERβ agonist to ovariectomized spontaneously hypertensive rats lowered peripheral artery resistance and systolic blood pressure, as well as attenuated hypertrophy\textsuperscript{111}. The possible pathways of ERβ-induced cardioprotection may be via increased gene expression involving fatty acid metabolism and nitric oxide (NO) production\textsuperscript{112}. ERβ, as well as E2, is also known to be anti-fibrotic, as it inhibits the transition of fibroblasts to myofibroblasts\textsuperscript{57}. ERβ is also shown to be responsible for the anti-hypertrophic and anti-fibrotic effects of E2\textsuperscript{21, 57, 109, 110}. Furthermore, long-term (2 weeks) pretreatment with the ERβ agonist DPN has been found to be cardioprotective in IR injury in ovariectomized female mice, and gene profiling in this experimental model showed long-term treatment with DPN resulted in the significant increase in cardioprotective genes, such as those encoding NO biosynthesis and antiapoptotic proteins\textsuperscript{113}. Another study by Lin et al.\textsuperscript{114} also showed that chronic E2 or DPN treatment leads to activation of protein S-nitrosylation and cardioprotection, which was blocked by NOS inhibition, which further supports that chronic E2 exposure protects the heart largely via ERβ activation and NO signaling\textsuperscript{114}. Activation of ERβ has also been shown to be beneficial in rescuing pulmonary hypertension-induced right ventricular heart failure in the
Recent work has demonstrated the existence of novel G protein coupled receptor 30, GPR30 (GPER) which has a high affinity for estrogen thus mediating its action\textsuperscript{115, 116}. GPER is an integral membrane protein, and seems to play an important role only in estrogen’s rapid nongenomic signaling\textsuperscript{117}. During rapid nongenomic estrogen signaling, estrogen activates adenylyl cyclase and mitogen-activated protein kinases and extracellular signal-regulated kinases Erk1 and Erk2, thus resulting in mobilization of intracellular calcium\textsuperscript{118}. It has previously been shown that during ischemia/ reperfusion, GPER activation results in cardioprotection\textsuperscript{119}. Bopassa et al.\textsuperscript{98} recently found that the mechanism through which GPER confers cardioprotection is via inhibition of the opening of the mitochondria permeability transition pore (mPTP) and activation of the Erk pathway. The mPTP appears to play a pivotal role in apoptosis following ischemia/ reperfusion\textsuperscript{120}. During ischemia, the mPTP remains closed and opens during the first few minutes of reperfusion due to oxidative stress, calcium overload and ATP depletion\textsuperscript{121}. Bopassa et al. investigated the role of GPER by using the highly specific GPER agonist G1 as well as G1 together with the Erk inhibitor PD-98059 (PD) to assess the cardiac function and infarct size post ischemia as well as the opening of the mPTP. The group has found that in the presence of G1, mouse hearts had better functional recovery as well as a smaller infarct size. Furthermore, the presence of G1 allowed for the mitochondria to uptake more calcium before the opening of the mPTP. All these effects were blocked by PD. Thus GPER confers cardioprotection by inhibiting the opening of the mPTP via activation of the Erk pathway\textsuperscript{98}.

To better understand the differences between the unique model of pregnancy-induced physiological hypertrophy versus pathological hypertrophy, I have elucidated several mechanisms through which these two types of hypertrophies are different in this dissertation. It
is well documented that in pathological hypertrophy the proteasomal activity is greatly disrupted, but nothing was known regarding the proteasome in pregnancy. In chapter 2, I show that indeed the proteasome is downregulated in pregnancy, which was also associated with decreased reactive oxygen species and protein ubiquitination thus indicating decreased levels of aberrant or misfolded proteins in the pregnant heart. In chapters 3 and 4 I sought to investigate possible therapeutic agents which would reverse the maladaptive cardiac remodeling and decreased function in both the pressure overload-induced left ventricular failure and the pulmonary hypertension-induced right ventricular failure. I found that short-term estrogen therapy restores cardiac function and reverses maladaptive cardiac remodeling both in the left and right ventricular models of hypertrophy and failure. I demonstrate that estrogen reverses fibrotic scarring and promotes angiogenesis, both of which are pivotal mechanisms for nurturing the heart back to health. Lastly, I show that estrogen exerts its beneficial effects on the heart by activating estrogen receptor beta in both the left and right ventricular models of heart failure.

1.7. Reference List


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Chapter 2:

Pregnancy Is Associated with Decreased Cardiac Proteasome Activity and Oxidative Stress in Mice
2.1. Abstract

During pregnancy, the heart develops physiological hypertrophy. Proteasomal degradation has been shown to be altered in various models of pathological cardiac hypertrophy. Since the molecular signature of pregnancy-induced heart hypertrophy differs significantly from that of pathological heart hypertrophy, we investigated whether the cardiac proteasomal proteolytic pathway is affected by pregnancy in mice. We measured the proteasome activity, expression of proteasome subunits, ubiquitination levels and reactive oxygen production in the hearts of four groups of female mice: i) non pregnant (NP) at diestrus stage, ii) late pregnant (LP), iii) one day post-partum (PP1) and iv) 7 days post-partum (PP7). The activities of the 26S proteasome subunits β1 (caspase-like), and β2 (trypsin-like) were significantly decreased in LP (β1: 83.26±1.96%; β2: 74.74 ± 1.7%, normalized to NP) whereas β5 (chymotrypsin-like) activity was not altered by pregnancy but significantly decreased 1 day post-partum. Interestingly, all three proteolytic activities of the proteasome were restored to normal levels 7 days post-partum. The decrease in proteasome activity in LP was not due to the surge of estrogen as estrogen treatment of ovariectomized mice did not alter the 26S proteasome activity. The transcript and protein levels of RPN2 and RPT4 (subunits of 19S) as well as β2 and α7 (subunits of 20S) were not significantly different among the four groups. High resolution confocal microscopy revealed that nuclear localization of both core (20S) and RPT4 in LP is increased ~2-fold and is fully reversed in PP7. Pregnancy was also associated with decreased production of reactive oxygen species and ubiquitinated protein levels, while the de-ubiquitination activity was not altered by pregnancy or parturition. These results
indicate that late pregnancy is associated with decreased ubiquitin-proteasome proteolytic activity and oxidative stress.
2.2. Introduction

Cardiac hypertrophy occurs in response to hemodynamic stress due to pressure overload. Cardiac hypertrophy is defined as an enlargement of the ventricles and cardiomyocytes, and can be adaptive or maladaptive. Sustained pressure overload leads to concentric hypertrophy, in which the ventricular wall thickens without a concomitant chamber enlargement. However, in response to normal exercise or pregnancy, physiological or eccentric hypertrophy develops. Eccentric hypertrophy which is characterized by an increase in cardiac pumping ability and muscle mass usually occurs due to volume overload-induced hypertrophy which is characterized by a proportional enlargement of the chamber size and the wall thickness. Eccentric hypertrophy is reversible without aberrant effects on cardiac function. In these aspects pregnancy- and exercise-induced hypertrophies are similar. However, the difference between pregnancy- and exercise-induced hypertrophies is that during pregnancy, acute changes in the mother’s hormonal environment occur, and the force demand placed on the woman’s heart is continuous as opposed to sporadic in exercise-induced hypertrophy.

The ubiquitin-proteasome system is the major pathway for protein degradation in the heart. Regulation of proteasome function can occur through the association of the core 20S proteasomal complex with different regulatory complexes such as 19S or 11S, which in turn affect proteasomal activity. Figure demonstrates the structure and function of the ubiquitin-proteasome system. Generally, covalent binding of ubiquitin moieties to the target protein dictates its degradation by the 26S proteasome. Following attachment of ubiquitin molecules to their target protein, the 19S regulatory subunits recognize the polyubiquitin tags and transfer the protein substrate to the inner pore of the 20S catalytic
Figure 1. Structure and function of the ubiquitin-proteasome system. Covalent binding of ubiquitin molecules to the target protein dictates its degradation by the 26S proteasome. Following attachment of ubiquitin molecules to their target protein, the 19S regulatory subunits recognize the polyubiquitin tags and transfer the protein substrate to the inner pore of the 20S catalytic core where the polypeptide is degraded. Image obtained from McNaught et al, *Nature Reviews Neuroscience* 2, 589-594(1).
core where the polypeptide is degraded\textsuperscript{13}. Dysfunctional ubiquitin-proteasome system in the heart leads to accumulation of abnormal, damaged or misfolded proteins\textsuperscript{6,14}.

Altered proteasome regulation has been reported in different types of cardiac hypertrophy and myopathy\textsuperscript{15,16}. However, the precise role and activity of the ubiquitin-proteasome system in physiological heart hypertrophy during pregnancy is not yet known. To investigate the role of the ubiquitin-proteasome system in the mouse heart during pregnancy, we measured proteasome activity, proteasome subunit expression and subcellular distribution, ubiquitination and de-ubiquitination levels, as well as reactive oxygen production in hearts from non pregnant (NP) mice in diestrus stage of the estrous cycle, late pregnant (LP), one day post-partum (PP1) and 7 days post-partum (PP7). We found that pregnancy is associated with decreased proteasomal activity, protein ubiquitination, and oxidative stress\textsuperscript{6}.

2.3. Methods

Animals and treatment

C57BL/6 3-4 months old female mice were used in non pregnant (NP, at diestrus stage), late pregnant (LP, day 20 of pregnancy), 1 day post-partum (PP1), 7 days post-partum (PP7) as well as ovariectomized (OVX). OVX mice were treated with a single subcutaneous 10-day continuous release 17β-estradiol (E2) pellet (0.012 mg/ pellet, Innovative Research of America, Sarasota, FL), or placebo pellets (containing 5 compounds: cholesterol, lactose, cellulose, phosphates and cerates) as vehicle for E2. This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health\textsuperscript{6}. 
Real Time PCR

Total RNA was isolated from hearts using Trizol (Invitrogen) and reverse transcribed with gene specific primers using the Omniscript RT kit (Qiagen). Controls were consisted of the reaction cocktail without reverse transcriptase and H2O instead of cDNA assessed by RT-qPCR. GAPDH transcript levels were used as an internal control6.

Western Blot

Whole heart cell lysates were prepared by homogenizing the hearts in: 50mM Tris (pH 7.5), 1mM EDTA, 5mM MgCl2, 150 mM NaCl, 1 mM DTT supplemented with Phosphatase and Protease Inhibitor cocktails (Roche). The samples were then centrifuged at 12,000 g for 10 min and the supernatants were collected. The protein concentrations were measured and 100 µg of protein was treated with SDS/DTT loading buffer prior to gel electrophoresis. The blots were probed with anti-RPN2, -RPT4 and -α7 (Enzo Life Sciences, 1:500) and with anti-mono- and polyubiquitinated conjugates (Enzo Life Sciences, clone FK2, 1:1000). Quantification of protein levels was achieved using Metamorph for protein levels of the proteasome subunits. For the quantification of ubiquitinated protein levels, protein lysates were subjected to Western Blotting procedure and immunolabeled with anti-mono- and polyubiquitinated antibodies. The fluorescence intensity of the entire lane was assessed in each group using ImageJ. The average fluorescence intensities were normalized to NP levels6.

Isolation of Cardiomyocytes

The hearts were quickly removed and perfused through the aorta with the following solutions: (i) Ca2+ -free Tyrode solution containing (in mM): 130 NaCl, 5.4 KCl, 1 MgCl2, 0.33 NaH2PO4, 10 HEPES, 5.5 glucose (pH adjusted to 7.35-7.37 with NaOH)
for 5 minutes, (ii) Ca\textsuperscript{2+} -free Tyrode solution containing 160.4 U/ml Collagenase Type II (Worthington) and 0.45 U/ml Protease Type XIV (Sigma) for ~15 min; and (iii) Krebs solution containing (in mM): 100 K-glutamate, 10 K-aspartate, 25 KCl, 10 KH\textsubscript{2}PO\textsubscript{4}, 2 MgSO\textsubscript{4}, 20 taurine, 5 creatine base, 0.5 EGTA, 5 HEPES, 20 glucose (pH adjusted to 7.2 with KOH) for 5 minutes. The solutions were oxygenated with 5% CO\textsubscript{2} and 95% O\textsubscript{2} prior to use and were maintained at 37 ±1 °C\textsuperscript{6}.

**Immunocytochemistry and imaging**

Freshly isolated cardiomyocytes were fixed in cold acetone for 10 min at -20°C prior to standard immunofluorescence staining with anti-core and anti-RPT4 (Enzo Life Sciences, 1:200) primary antibodies. Images were acquired at 0.0575 nm per pixel with a confocal microscope (Olympus Fluoview). For dihydroethidium (DHE, Invitrogen) staining, whole hearts were excised, washed thoroughly with ice-cold PBS and frozen in O.C.T. compound. Fresh 6µm sections were cut with a cryostat then incubated with 10 µM DHE in Krebs-HEPES buffer (containing in mM: 99 NaCl, 4.69 KCl, 25 NaHCO\textsubscript{3}, 1.03 KH\textsubscript{2}PO\textsubscript{4}, 5.6 D-Glucose, 20 Na-HEPES, 2.5 CaCl\textsubscript{2} and 1.2 MgSO\textsubscript{4}) for 1 hr and 15 min in the dark at room temperature. The sections were then visualized with a confocal microscope (Olympus Fluoview)\textsuperscript{6}.

**Proteasome activity assay**

Heart lysates were prepared by homogenizing the hearts in: 50mM Tris, 1mM EDTA, 5mM MgCl\textsubscript{2}, 150 mM NaCl, 1 mM DTT, pH 7.5. The samples were then centrifuged at 12,000 g for 10 min and the supernatants were collected. Proteasome activity of heart homogenates (20 µg/sample) was measured with fluorescent substrates of Z-LLE-AMC (β1), Boc-LSTR-AMC (β2) and Suc-LLVY-AMC (β5) as previously described\textsuperscript{17, 18}. The
proteasome activity was measured in the presence and absence of proteasome inhibitors (40µM Z-Pro-Nle-Asp-CHO for β1, 40µM epoxomicin for β2, and 20µM epoxomicin for β5). Assays were carried out in a total volume of 100 µl. The ATP-dependent 26S proteasome activities were measured in the presence of 50mM Tris, 1mM EDTA, 150mM NaCl, 10mM MgCl₂, 0.1mM ATP, pH 7.5. The ATP-independent 20S proteolytic activity for β5 was carried out in 25 mM HEPES (pH 7.5), 0.5 mM EDTA, and 0.03% SDS. The buffer composition was 25 mM HEPES (pH 7.5), 0.5 mM EDTA, 0.05% Nonidet P-40, and 0.001% SDS for β1 and β5 20S activity measurements. These buffers used for 20S proteasome activity were previously found to be optimal for proteasome activity in lysates from mouse heart tissue ¹⁹, ²⁰. Each assay was conducted in the absence and presence of a specific proteasome inhibitor ((40 µM Z-Pro-Nle-Asp-H for β1, 60 µM epoxomicin for β2 and 20 µM epoxomicin for β5) to determine proteasome-specific activity. Released AMC was measured using a Fluoroskan Ascent fluorometer (Thermo Electron, Walktham, MA) at an excitation wavelength of 390 nm and an emission wavelength of 460 nm⁶.

**De-ubiquitination assay**

De-ubiquitination activity was determined using 5µg of protein in 50mM Tris, 150 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, 2 mM DTT, pH 7.5. All assays were carried out in a final volume of 100 µl. The reaction was initiated by adding 400nM ubiquitin-AMC (Enzo Life Sciences, NY). Each assay was conducted in the absence and presence of a de-ubiquitination inhibitor (10 mM N-ethylmaleimide (NEM)) to determine de-ubiquitination-specific activity. Released AMC was measured using a Thermo
Fluoroskan Ascent fluorometer at an excitation wavelength of 390 nm and an emission wavelength of 460 nm.

**ELISA assay**

1 µg of protein lysate was bound overnight at 4°C on a 96 well-ELISA plate. 100 µl of FK1 (1:1000) detection antibody (which only recognizes polyubiquitinated proteins and not monoubiquitinated or free ubiquitin) was added for 1h, washed, and an anti-mouse HRP-conjugated secondary antibody was added for 1 hr. After the secondary antibody, 100 µl of Sureblue tetramethylbenzidine substrate (KPL Inc.) was added and incubated for 15 min. The reaction was stopped using 1M HCl and absorbance was measured at 450 nm.

**Statistics**

One-way ANOVA using SigmaStat 3.0 was used for statistical analysis. P values <0.05 were considered significant. Values are mean ± SEM.

**2.4. Results**

In late pregnancy the 26S proteasome activity is decreased

Physiological hypertrophy develops during pregnancy as a result of the natural volume overload (Figure 2). The ratio of the heart weight to body weight decreases in late pregnancy however, due to a significant increase in body weight at the end of pregnancy. One day post partum (PP1) the heart weight is reversed partially and fully one week post partum (PP7).

We measured the proteasome activity of total 26S ATP-dependent as well as the 20S ATP-independent proteasome to examine whether the proteasome function is altered in...
Figure 2. The increased in heart weight of late pregnant mice is reversed post-partum. Heart weights (HW, A), body weights (BW, B) and HW/BW in non-pregnant (NP, black bars, n=8), late pregnant (LP, white bars, n=11), one day post-partum (PP1, grey bars, n=6) and seven days post-partum (PP7, shaded bars, n=5) mice. Values are mean ± SEM, *p<0.05 and **p<0.001 vs. NP, #p<0.05 and ##p<0.001 vs. LP, ^p< 0.05 and ^^p<0.001 vs. PP1.
the heart in late pregnancy. The relative 26S caspase-like catalytic β1 subunit activity was decreased from 100 ± 6.82% in NP to 83.26 ± 1.96% in late pregnancy, and was restored fully one day post-partum (Figure 3). The trypsin-like β2 subunit activity was also decreased in late pregnancy from 100 ± 13.72% in NP to 74.74 ± 1.7% in LP, and was only fully restored in PP7 (111.56 ± 8.7%). However, the chymotrypsin-like β5 activity of the proteasome was significantly decreased 1 day post-partum (from 100 ± 4.7% in NP to 69.62 ± 4.5% in PP1). All of the 26S proteolytic activities were fully restored to normal levels 7 days after parturition (Figure 3). The proteasome activity of all three 20S subunits was however not altered in LP (Figure 3), while the activity of β1 and β2 subunits was significantly lower 7 days after parturition (from 100 ± 13.72% in NP to 64.69 ± 3.89% in PP7 and from 100 ± 13.35% in D to 55.41 ± 4.63% in PP7, respectively, Figure 3)

**Estrogen therapy does not regulate proteasome activity**

The levels of estrogen increase steadily during pregnancy and reach peak levels at the end of pregnancy. To understand whether decreased proteasome activity at the end of pregnancy was a result of the high estrogen milieu in pregnancy, ovariectomized (OVX) mice were treated with E2 or placebo. The ATP-dependent 26S proteasome activity was not affected by estrogen treatment, as there were no differences in the three catalytic subunits activities between E2 and placebo groups (Figure 4). Therefore, the rise of estrogen levels in pregnancy could not explain the decreased proteasome activity observed in late pregnancy.
Figure 3. Proteasome activity of 26S, but not 20S is reduced in late pregnancy. Activity of different proteasomal beta subunits of the 26S (A) and 20S (B) was measured after initiating the reaction with: Z-LLE-AMC (β1), Boc-LSTR-AMC (β2) and Suc-LLVY-AMC (β5) for non pregnant (NP, black bars), late pregnant (LP, white bars), one day post-partum (PP1, grey bars) and seven days post-partum (PP7, shaded bars). The fluorescence values in arbitrary units are represented as mean ± SEM *p<0.05 vs. NP (n=4 mice per group) and are normalized to NP levels. The raw proteasome activity values for the NP group are as follows (in nmol/min/mg protein): for the 26S ATP-dependent activities, β1 was 0.11 ± 0.01, β2 was 0.04 ± 0.01 and β5 was 0.16 ± 0.01, while for the 20S activity, β1 was 0.21 ± 0.03, β2 was 0.15 ± 0.02 and β5 was 0.12 ± 0.01.
Figure 4. Proteasome activity of the 26S is unaffected by estrogen treatment. Activity of the different proteasomal beta subunits of the 26S were measured after initiating the reaction with: Z-LLE-AMC (β₁), Boc-LSTR-AMC (β₂) and Suc-LLVY-AMC (β₅) in ovariectomized female mice treated with placebo (Placebo, black bars) or with 17-β estradiol for 10 days (E₂, white bars). The fluorescence values in arbitrary units are normalized to Placebo levels and represented as mean ± SEM (n= 4 mice per group)
Pregnancy does not alter the transcript and protein levels of proteasome subunits

To elucidate whether the observed decrease in the 26S proteasome proteolytic activity in pregnancy is due to decreased gene expression, we have performed Real-Time qPCR to quantify the transcript levels of 19S and 20S proteasome subunits. Because the 20S proteasome can also associate with an 11S regulator (PA28) which in turn can modulate 26S proteolytic activity, we also examined the transcript levels of the PA28α subunit of the 11S/PA28 regulator. We found that there were no significant differences in the transcript levels of these subunits neither with pregnancy nor up to one week after delivery (Figure 5). Western Blot also showed no significant differences in the expression levels of RPN2 and RPT4 (subunits of 19S, Figure 6A-D), in α7 (a subunit of 20S, Figure 6E-F), in PA28α (Figure 6G-H) nor in β5i (Figure 6I-J) with pregnancy or after parturition. For β5i, the two bands labeled with 26kDa and 30kDa, correspond to β5i and β5i containing its pro-peptide, respectively (Figure 6I). Both bands were considered for protein level quantification shown in Figure 5J. Quantification of either band independent of the other also showed no difference between groups (data not shown)⁶.

Nuclear labeling of 20S core and 19S RPT4 is increased during pregnancy in cardiomyocytes

We have performed confocal microscopy to examine whether there are any changes in the subcellular distribution of proteasomal subunits in cardiomyocytes in pregnancy. Fig 7 shows representative images of high resolution confocal microscopy of cardiomyocytes co-labeled with anti-core (an antibody which recognizes six 20S subunits: α5/7, β1, β5, β5i, and β7) and anti-RPT4 (a subunit of 19S) antibodies. The vast majority of the stained proteins resided in the t-tubules and the nuclear labeling was very weak in cells from non
Figure 5. Transcript levels of proteasome 19S and 20S subunits, as well as the regulatory subunit PA28α, are not modified in late pregnancy. Relative transcript expression of the cardiac proteasome measured by Real-Time PCR in non pregnant (NP, black bars), in late pregnancy (LP, white bars), 1 day post-partum (PP1, grey bars) and 7 days post-partum (PP7, shaded bars) for RPN2 and RPT4, which are subunits of 19S (A-B), β2 and α7, which are subunits of 20S (C-D) and the proteasome regulatory subunit PA28α (E). GAPDH was used as the internal reference gene (data not shown). Values are mean ± SEM as normalized to NP (n= 3-5 per group).
Figure 6. Protein levels of α7, RPT4, RPN2, PA28α and β5i are unaffected by pregnancy. Immunoblotting of whole heart lysates (100 µg) from non pregnant (NP, black bars), late pregnant (LP, white bars), 1 day post-partum (PP1, grey bars) and 7 days post-partum (PP7, shaded bars) with anti-RPN2 (A-B), -RPT4 (C-D), -α7 (E-F), -PA28α (G-H) and -β5i (I-J) antibodies. In (I), the upper 30kDa band is the β5i containing the pro-peptide. The bar graphs represent the quantification of fluorescent signal intensity normalized to Vinculin. For β5i both bands were taken into consideration in the quantification of protein levels. Vinculin was used as the loading control (n=4 per group). Values are mean ± SEM in arbitrary units.
pregnant animals (Figure 7A). In late pregnancy however, there is increased nuclear localization of both core and RPT4, which was reversed one week after parturition. Quantification of nuclear labeling revealed approximately a 2-fold upregulation with pregnancy (from 1 ± 0.04 to 1.97 ± 0.20 for core and from 1 ± 0.04 to 1.73 ± 0.16 for RPT4). The increased nuclear labeling of both proteins remained one day post partum (1.95 ± 0.17 for core and 1.73 ± 0.06 for RPT4) and was only reversed back to NP levels seven days after delivery (to 0.88 ± 0.06 for core and 1.15 ± 0.07 for RPT4, Figure 7B).6

**Pregnancy is associated with decreased production of reactive oxygen species**

The nuclear proteasome selectively degrades oxidatively-damaged histones in the nuclei of mammalian cells 22. Therefore we performed dihydroethidium (DHE) staining of cardiac cross-sections to assess reactive oxygen species production. We observed decreased levels of reactive oxygen species in late pregnancy, which remain low up to one week after parturition (Figure 8). The average DHE staining intensity normalized to non-pregnant levels is reduced about 5-fold and partially recovers only seven days post-partum (0.198 ± 0.010 in LP, 0.213 ± 0004 in PP1, and 0.405 ± 0.030 in PP7, normalized to NP).6

**Decreased levels of ubiquitinated proteins are associated with pregnancy**

To further understand the mechanism for the observed decrease of 26S proteasome activity in late pregnancy, we quantified polyubiquitinated proteins using two different methods in the heart. Western Blot against mono- and polyubiquitinated proteins revealed that ubiquitinated protein levels are significantly decreased in late pregnancy from 1 ± 0.05 in NP to 0.6 ± 0.06 in LP, and they remain low 7 days post-partum (0.65 ± 0.04, in arbitrary units normalized to NP, Figure 9A-C). These results were further confirmed by
Figure 7. Increased nuclear labeling of Core Subunits and RPT4 in late pregnancy was reversed one week postpartum. A. Representative single confocal sections of cardiomyocytes dissociated from non pregnant (NP), late pregnant (LP), one day post-partum (PP1) and seven days post-partum (PP7) are co-immunostained with anti-core (green) and -RPT4 (red) antibodies. The nuclear overlay of Core and RPT4 are also shown at higher resolution. These results are representative of the labeling pattern observed in myocytes from 3 different animals in each group. B. Quantification of nuclear fluorescence labeling in the four groups mentioned above for core (green bars) and RPT4 (red bars) from at least 20-25 cells per group (n=3 mice/group). Only the nucleus in the confocal plane of focus was taken into account. ** denotes $p < 0.001$ vs. NP, # $p < 0.05$ vs. LP and ^ $p < 0.05$ vs. PP1.
Figure 8. Superoxide production is decreased with pregnancy and remains low seven days after parturition. A. Representative dihydroethidium (DHE) staining of transverse heart sections in non pregnant (NP), late pregnant (LP), 1 day post-partum (PP1) and 7 days post-partum (PP7). Red staining indicates the presence of reactive oxygen species (ROS). B. Quantification of the DHE staining for detection of ROS in non pregnant (NP, black bar), late pregnant (LP, white bar), 1 day post-partum (PP1, grey bar) and 7 days post-partum (PP7, shaded bar). Values are mean ± SEM as normalized to NP (n=3 per group), and ** denotes p< 0.001 vs. NP, # p< 0.05 vs. LP and ^ p <0.05 vs. PP1.
Figure 9. Pregnancy is associated with decreased polyubiquitinated protein levels, but not de-ubiquitination levels. A. Representative Western Blot of polyubiquitinated proteins (using the FK2 antibody) in whole heart lysates (100 µg) from non pregnant (NP), late pregnant (LP), 1 day post-partum (PP1) and 7 days post-partum (PP7). B. PonceauS was used as the loading control (n=4 per group). C. Quantification of the polyubiquitinated proteins by Western Blot in non-pregnant (NP, black bar), late pregnant (LP, white bar), 1 day post-partum (PP1, grey bar) and 7 days post-partum (PP7, shaded bar). D. Polyubiquitination levels in NP, LP, PP1 and PP7 as determined by ELISA (using the FK1 antibody). E. De-ubiquitination activity levels in NP, LP, PP1 and PP7. Values are mean ± SEM and are normalized to NP, n=4 per group and * denotes p <0.05 vs. NP.
ELISA, as the relative amount was significantly downregulated from 100 ± 10.41% in NP to 66.68 ± 3.69% in LP (Figure 9D). We have also observed that there are no significant changes in protein de-ubiquitination in pregnancy and parturition (Figure 9E). Therefore, the observed decrease in the levels of ubiquitinated proteins is not due to an increase in de-ubiquitination activity.

2.5. Discussion

Previous characterization of cardiac physiological hypertrophy during pregnancy led us to speculate that the late pregnant heart is “a better functioning heart.” During pregnancy, contractile efficiency and capacity is enhanced in response to increased force and stretch demand. Here we show for the first time that the activity of the total 26S ATP-dependent proteasome, polyubiquitinated protein levels as well as the production of reactive oxygen species are reduced at the end of pregnancy. These findings further support our previous suggestion that a late pregnant heart is a better functioning heart.

Cardiac hypertrophy and proteasome activity

The heart is the only organ in the body that is constantly bearing a heavy workload and a high metabolic rate, which is why it is essential that cardiac cells maintain a tightly controlled and very efficient system for removal of misfolded or damaged proteins. During cardiac hypertrophy, the increased protein synthesis in cardiomyocytes could potentially result in an increase of misfolded or aberrant proteins. For cardiac maintenance, an increase in 26S proteasomal degradation could result in the clearance of these aberrantly folded proteins. Alternatively, an increase in protein degradation by the proteasome could lead to tissue atrophy. Previous work describing proteasomal activity
during cardiac dysfunction and hypertrophy remains controversial. Decreased proteasome activities have been shown during the progression of cardiac dysfunction $^{25}$, while many other studies report increased proteasome activity in compensated heart hypertrophy induced by trans-aortic constriction (TAC) both in mouse and canine models $^{26,27}$. However, increased proteasomal activity has been suggested to be required for the development of compensated heart hypertrophy $^{28,29}$. Although during pregnancy the heart also develops compensated hypertrophy, the proteasome activity in this unique model of hypertrophy is not increased. In fact, the activity of the 26S proteasome is decreased in the late pregnant heart, which was not reflected by any changes in the inducible $\beta$5i subunit or in the PA28$\alpha$ subunit of the 11S/PA28 regulator.

**Cardiac hypertrophy and the expression of proteasome subunits**

Some controversy regarding the expression of proteasome subunits at the mRNA and protein levels exists. Most reports show an increase in 26S proteasome expression in different models of cardiomyopathy and hypertrophy $^{30}$, possibly due to a compensatory mechanism in response to impaired proteasome activity. Increased expression of the representative subunits of 19S (RPN2 and RPT11) and 20S (\(\alpha_6\)) have also been reported in the subendocardium of the canine model of left ventricular hypertrophy $^{31}$, while transcript levels of representative 20S subunits have been shown to be decreased in failing hearts $^{32}$ thus suggesting possible post-translational modifications. Here we did not observe any significant differences in the transcript or protein levels of \(\alpha_7\), RPN2 and RPT4 as well as in the regulatory subunit PA28$\alpha$ with pregnancy (Figures 5, 6), further suggesting that pregnancy-induced hypertrophy has an unique molecular signature unlike all other hypertrophy models.
Decreased levels of ubiquitinated proteins are associated with pregnancy

Before a protein is targeted for degradation, it must first initially be covalently tagged with ubiquitin molecules by the E2-E3 ligase complex. These molecules are then recognized by the 19S regulatory particle of the 26S proteasome complex in an ATP-dependent binding. Thus, protein ubiquitination is a pivotal mechanism for targeting a peptide to be degraded by the proteasome’s proteolytic pathway, and ubiquitination levels are also important for the proteasome activity (Figure 1). Here, we observe decreased levels of ubiquitinated proteins in pregnancy (Figure 9). Two independent methods revealed that the level of ubiquitination in the heart was decreased in late pregnancy. However, unlike pregnancy, immunocytochemical experiments previously revealed markedly increased expression levels of ubiquitin in patients with decompensated cardiomyopathy. Increased ubiquitination levels have also been reported in experimental models of pressure overload-induced left ventricular hypertrophy in murine and canine hearts. Lower proteasome activity may conserve energy as less ATP would be needed for protein unfolding by the 19S complex and this may be beneficial to the heart. Changes in protein ubiquitination can occur from changes in proteasome activity, changes in de-ubiquitination activity or changes in the ubiquitin-conjugating activity system. Lower proteasome activities are unlikely to cause lower polyubiquitination levels since the proteasome readily degrades polyubiquinated proteins. Our findings confirmed this speculation, as the deubiquination activity was not significant affected by pregnancy. These results suggest that the ubiquitin-conjugating activity system may be lowered by pregnancy. It is possible that since the proteasome
has potentially less substrates to degrade is responsible for the reduced activity of the proteasome, since the proteasome expression levels were unchanged in pregnancy.

**The 26S proteasome and reactive oxygen species**

The proteasomal system has previously been shown to be the major proteolytic system involved in the removal of oxidized proteins, with the 26S proteasome being the most sensitive to oxidative stress. Although the 26S proteasome generally functions as part of the ubiquitin-proteasome pathway, it can also degrade unfolded or damaged proteins (Figure 1), including “aged” or denatured proteins, or proteins that have been oxidatively damaged without initial ubiquitin tagging. We observed that the levels of reactive oxygen species are decreased in pregnant rats and remain low one week after delivery (Figure 8), which may account for the decreased polyubiquitination levels in the late pregnant hearts.

**Hormones and proteasome activity**

It is also possible that estrogen may affect the proteasome as interferon-induced oxidative stress has been shown to be associated with decreased proteasome amount and increased polyubiquitination. Hormones can affect the ubiquitin-mediated control of protein degradation, as glucocorticoids have previously been shown to cause catabolic protein breakdown. In skeletal muscle, degradation of cell proteins is of major physiological importance, and the size of a muscle cell is tightly regulated by the overall rate of proteolysis, a process precisely regulated by hormones and cytokines. The level of estrogen drastically increases at the end of pregnancy, but it is not clear whether estrogen treatment could regulate proteasome activity in the heart. Here we report for the first time that estrogen did not have any effect on the three proteolytic activities of the mouse.
cardiac 26S proteasome. Thus, the changes in proteasome activity occurring in pregnancy cannot be attributed to the surge of estrogen.

Taken together, our results suggest that the ubiquitination, proteasome proteolytic pathway and the production of reactive oxygen species are affected by pregnancy. Late pregnancy is associated with a decrease in the polyubiquitination levels, which could be explained at least in part by reduced reactive oxygen species production.

2.6. Reference List


(12) Gomes AV, Zong C, Ping P. Protein degradation by the 26S proteasome system in the normal and stressed myocardium. Antioxid Redox Signal 2006 September;8(9-10):1677-91.


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Chapter 3:

Estrogen Rescues Pre-existing Severe Pulmonary Hypertension in Rats
3.1. Abstract

**Rationale:** Pulmonary hypertension (PH) is characterized by progressive increase in pulmonary artery pressure leading to right-ventricular (RV) hypertrophy, RV-failure and death. Current treatments only temporarily reduce severity of the disease and an ideal therapy is still lacking.

**Objectives:** Estrogen pre-treatment has been shown to attenuate development of RV hypertrophy. Since PH and RV hypertrophy is not often diagnosed early, we examined if estrogen can rescue pre-existing RV hypertrophy before it transitions to RV failure.

**Methods:** RV hypertrophy secondary to PH was induced in male rats with monocrotaline (60mg/kg). At day 21 in the RV hypertrophy state, rats were either treated with 17-β estradiol (E2, 42.5μg/kg/day), the estrogen receptor beta agonist DPN (850μg/kg/day), or the estrogen receptor alpha agonist PPT (850μg/kg/day) for 10 days or left untreated to develop RV failure. Serial echocardiography, cardiac catheterization, immunohistochemistry and immunocytochemistry were performed.

**Main results and measurements:** Estrogen therapy prevented progression of PH and RV hypertrophy to RV failure, and restored lung and RV structure and function. This restoration was maintained even after removal of estrogen at day 30, resulting in 100% survival at day 42. E2 treatment restored the loss of blood vessels in the lungs and RV. In the presence of angiogenesis inhibitor, TNP-470(30mg/kg) or estrogen receptor β antagonist (PHTPP, 850μg/kg/day), estrogen failed to rescue PH. Estrogen receptor β selective agonist (DPN) was as effective as estrogen in rescuing PH and RV hypertrophy.

**Conclusions** Estrogen rescues pre-existing severe RV hypertrophy secondary to PH in rats by restoring lung and RV structure and function that is maintained even after removal
of estrogen. Estrogen-induced rescue of PH and RV hypertrophy is associated with stimulation of cardiopulmonary neoangiogenesis and suppression fibrosis, mainly via activation of estrogen receptor. Furthermore, estrogen-rescue is likely mediated through estrogen receptor β.
3.2. Introduction

Pulmonary hypertension (PH) is a chronic lung disease characterized by progressive increase in pulmonary artery (PA) pressure and pulmonary vascular remodelling leading to right ventricular (RV) hypertrophy and RV failure (RVF). End-stage RVF has long been regarded as a terminal state of pathological cardiopulmonary remodelling, characterized by chamber dilation and fibrotic scarring, which currently still unresponsive to available therapies. Advanced PH is most often treated with aggressive non-pharmacological therapies such as lung transplantation, but this approach can only be taken for a fraction of patients. Cell and gene therapies have shown great potential for treatment of PH in animal models\textsuperscript{1,2} and humans\textsuperscript{3} in the past decade. Therefore, effective pharmacological therapy for treatment of advanced PH patients is still needed, as would be much more practical and much more cost-effective. Several agents have been identified to attenuate the development of PH when the therapy is started before the initiating stimuli\textsuperscript{4-6}. Unfortunately, up to now, there is no known effective pharmacological therapy to reverse advanced PH\textsuperscript{7}.

Although the incidence of PH remains higher in female patients\textsuperscript{8}, in various animal models females have been shown to be protected against PH\textsuperscript{9-11}. This phenomenon has been termed the ‘estrogen paradox’ of PH. Ovariectomy exacerbates PH and pre-treatment with E2 and its metabolites attenuates the progression of different animal models of PH, thus this gender difference in susceptibility to experimental PH has been suggested to be in part due to the action of estrogen (E2)\textsuperscript{4,10,12,13}.

As PH is not always diagnosed early, we hypothesized that E2 may also be able to reverse pre-existing severe PH and examined the molecular mechanisms involved in the
rescue of pre-existing PH by E2 therapy. We used the well-established model of monocrotaline (MCT)-induced PH in rats, which has been previously well established\textsuperscript{14}.\textsuperscript{15}. We found that a 10-day E2-treatment after the onset of PH significantly reversed MCT-induced pre-existing PH by restoring both lung and heart structure and function. E2 therapy resulted in 100% survival even after withdrawal of E2 for 12 days. E2 restored angiogenesis both in RV and lung, which could be an important mechanism of E2 action in rescuing PH. Furthermore, this rescue action of E2 is likely mediated through an estrogen receptor $\beta$ (ER$\beta$)--dependent mechanism\textsuperscript{16}.

3.3. Methods

Animals and treatments

Male and female Sprague-Dawley rats (350-400 g) were used. To induce PH, rats were treated with a single subcutaneous injection of MCT (60 mg/kg, Sigma). This model has been extensively used by many investigators and has been shown to be reproducible\textsuperscript{17},\textsuperscript{18}. From day 21 to day 30, the animals were randomly assigned to receive E2 via subcutaneous continuous-release pellets (42.5 $\mu$g/kg/day, Innovative Research of America) or to remain untreated. Placebo pellets (containing 5 compounds: cholesterol, lactose, cellulose, phosphates and cerates) had no effects as the disease progression. In some experiments animals were treated with E2 pellets together with subcutaneous administration of the angiogenesis inhibitor TNP-470 (30 mg/kg, Sigma) once every other day for 10 days\textsuperscript{19}. A weight loss of more than 10% per day for 2 consecutive days and arterial oxygen tension of less than 80% were the criteria required to sacrifice the
animals and counted as a ‘loss’ in survival measurements. Protocols received institutional review and committee approval\textsuperscript{20}.

**Cardiac and pulmonary hemodynamics**

B-mode, M-mode and pulmonary pulsed wave Doppler echocardiography was performed using a VisualSonics Vevo 2100 equipped with a 30-MHz linear transducer. During the course of the experiment, serial echocardiography was performed to accurately monitor the stage of the disease by measuring cardiac and pulmonary hemodynamic parameters and RV structure. RV pressure was calculated from pulsed wave Doppler echocardiography of pulmonary artery flow using Mahan’s regression equation: MPAP = 79 – 0.45×PAAT\textsuperscript{21} (MPAP is mean pulmonary artery pressure and PAAT is the pulmonary artery acceleration time). The RV pressure was also measured terminally by direct catheterization (1.4F Millar SPR-671) connected to a pressure transducer (Power Lab, ADInstruments) into the RV. The values of RV pressure measured by both methods were very similar. The RV ejection fraction, RV free wall thickness and RV cavity dimensions were quantified using M-mode\textsuperscript{22}.

**Gross histologic evaluation**

The RV wall, the left ventricular wall and the interventricular septum were dissected and weighed, and the ratio of the right ventricle to left ventricle plus septum weight [RV/(LV+IVS)] was calculated as an index of RV hypertrophy. Wet lung weight was determined by weighing the lung tissue\textsuperscript{23}.

**Immunohistochemistry**

Whole hearts and lungs were fixed in 4% paraformaldehyde and transversal 6-7 μm sections were obtained with a cryostat. Tissue sections were stained with standard
hematoxylin-eosin and Masson trichrome staining, as well as with anti-smooth muscle
actin, -von Willebrand Factor antibodies and TO PRO (for nucleic identification) for
immunofluorescence. The images were acquired using a light microscope (Nikon) or with a
laser-scanning confocal microscope (Nikon)24.

**Histological Analyses**

Pulmonary arteriolar medial wall thickness was determined in lung sections using \( \alpha \)-
smooth muscle actin staining of pulmonary arterioles. The wall thickness of arterioles
was quantified using ImageJ software by measuring the maximum thickness of arteriolar
walls. The values are normalized to control and shown as % increase in wall thickness
compared to control, where control is 100%.

Pulmonary angiogenesis was quantified by counting the number of small blood vessels
(up to 50\( \mu \)m) stained with von Willebrand Factor in high power fields.

Percent tissue fibrosis in lung sections was determined using Masson trichrome stain for
collagen. A grid containing 100 squares was used to assess the presence. Results are
expressed as the percentage occupied by fibrosis to the total area examined25.

**Statistical Analysis**

One-way ANOVA was used to compare between groups and within the same group at
different time points using SPSS13.0 and subjected to *post-hoc* tests (Bonferroni
correction) which allowed for multiple comparisons. P-values <0.05 were considered
statistically significant. Values are expressed as mean ± SEM26.
3.4. Results

Estrogen reverses PH by improving cardiac and pulmonary structure and function

Rats were injected with a single dose of monocrotaline (MCT, 60 mg/kg, Figure 1A) to induce PH as has been extensively used by many investigators. By day 21, rats already developed severe PH (PH group), as peak RV systolic pressure (RVSP) had increased from 31.1±0.9 in control (CTRL) to 67.7±1.1 mmHg in PH group (Figure 1C). The severity of PH at day 21 was further confirmed by increased RV hypertrophy (RV/(LV+IVS) from 0.23±0.02 in CTRL to 0.64±0.05 in PH Figure 1D); 3) pulmonary arteriolar medial hypertrophy (Figure 2A) and 4) appearance of a mid-systolic notch on Doppler echocardiography of PA flow (Figure 2B). These data clearly agrees with previous studies using this time point as an initiating point for therapy of advanced PH and demonstrate the development of malignant PH by day 21. Therefore, we started E2-therapy at day 21 in one group of rats by subcutaneously implanting E2 pellets for 10 days from day 21 to 30 (E2 group), whereas another group was left untreated to develop RV failure (RVF group) by day 30. E2 therapy gradually improved RVSP, as these parameters had returned to almost CTRL levels within 10 days of E2 treatment. In untreated animals, however, the RVSP significantly increased even further to 72.0±1.4 mmHg (Figure 1C). However, the RV hypertrophy index (Figure 1D) did not increase after 21 days any further. E2 therapy was also associated with the regression of RV hypertrophy (RV/(LV+IVS)=0.34±0.01g vs. 0.64±0.05 at day 21, Figure 1D). Moreover, pulmonary arteriolar medial hypertrophy was completely reversed (Figure 2A).
Figure 1. E2 rescues severe PH. A. Experimental protocol, male rats were injected with MCT or PBS at day 0. The thick horizontal lines represent the length of each experimental group. At day 21, animals were either sacrificed (PH group), or left untreated to develop RVF (RVF group). Both E2 and E2-W groups received E2 only from day 21 to day 30. All of the rats in E2-group were sacrificed at day 30, whereas the rats in E2-W group were kept for another 12 days until day 42 after E2 withdrawal at day 30. B. Survival plot. C. RVP measured directly by inserting a catheter into the RV right before sacrifice and D. RV hypertrophy index in CTRL (black bar), PH (red), RVF (purple), E2 (dark blue) and E2-W (light blue). *P<0.05 vs. CTRL, **P<0.001 vs. CTRL; †P<0.001 vs. PH; ††P<0.001 vs. PH; ^^P<0.001 vs. RVF and #P<0.05 vs. E2 (n=6-8 rats per group)(1).
The improvements in lung and RV structure and function mediated by E2 led to 100% survival, as all E2-treated animals were alive at day 30, whereas in untreated rats, the mortality started at day 24, sharply increased to 45% by day 28 and to 75% by day 30 (Figure 1B)\(^{32}\).

**Estrogen rescue of PH persists after estrogen withdrawal**

To investigate whether the improved structural and functional changes achieved by E2 therapy were maintained once E2 was no longer available, some E2-treated rats were kept for another 12 days until day 42 after E2 withdrawal at day 30 (E2-W group). Surprisingly, these rats improved even further and were fully restored to normal levels as in CTRL by day 42 (Figures 1C, D; 2A, B). All rats with PH treated with E2 for 10 days survived to at least 42 days after MCT injection whereas none survived after day 32 in the untreated PH group (Figure 1B). Hence, these data confirmed that E2-rescue of PH persisted even after discontinuation of E2-therapy, suggesting that short-term E2-therapy was sufficient to maintain and further improve pulmonary function and structure\(^{33}\).

**Estrogen reverses pulmonary fibrosis and inflammation**

To further investigate the action of estrogen in the lungs, we assessed pulmonary fibrosis and inflammation. In the PH group, lung fibrosis had increased considerably (16.6±1.65% in PH vs. 2.6±0.35% in CTRL), and E2 mediated a complete reversal of this fibrosis (2.93±0.15%) (Figure 3A,B). Pulmonary arteriolar medial hypertrophy in the lungs of the PH group was significantly increased in PH (281±14% in PH vs. 100±7% in CTRL) and was fully reversed by E2 (131±7%) (Figure 3C, D)\(^{34}\).
Figure 2. Reversal of cardiopulmonary structure and function by E2. A. Hematoxylin-Eosin staining for lung arterioles and heart cross-sections in male rats. B. Echocardiographic images of M-mode (upper panels) showing RV (EDD, end diastolic diameter), LV and IVS and Pulse-wave Doppler (lower panels) in male rats. Yellow arrows show mid-systolic notch present in PH and RVF only(1).
Figure 3. Reversal of lung inflammation and remodeling by E2. A. Masson trichrome staining of lung sections in CTRL, PH and E2, blue color indicates fibrosis. B. quantification of lung fibrosis showing % lung fibrosis in CTRL, PH and E2 groups. C. Immunofluorescence labeling of pulmonary arterioles stained for smooth muscle actin (green) and C. Immunoperoxidase labeling of pulmonary arterioles stained with anti-smooth muscle actin antibody (brown) together with haematoxylin stained nuclei (blue). J. Bar graph for quantification of pulmonary arteriolar medial wall thickness in CTRL, PH and E2 groups. *P<0.05 vs. CTRL, **P<0.001 vs. CTRL, †P<0.05 vs. PH, ††P<0.001 vs. PH (n=4 animals per group)(1).
Estrogen stimulates pulmonary and cardiac angiogenesis

Stimulation of pulmonary neoangiogenesis has been suggested as a potential therapeutic strategy for treatment of PH\(^{35}\). We examined whether stimulation of both cardiac and pulmonary neoangiogenesis by E2 participated in E2-induced rescue of PH. Pulmonary vessel density was significantly reduced in PH (5.5±0.5 vessels per high power field (HPF) in PH vs. 16.5±0.5 in CTRL, Figure 4A,B). E2 reversed the loss of blood vessels associated with PH in lung (14.3±1.4) (Figure 4B). E2 was also able to reverse the loss of pulmonary microvasculature in females very similarly to males. In females, pulmonary vessel density was significantly reduced by ~2.5-fold in PH (7±0.4 vessels per HPF in PH vs. 16±0.5 in CTRL, Figure 5). E2 reversed the loss of blood vessels associated with PH in lung (14.8±1.13 in E2). Loss of RV microvessels has been previously reported to occur in PH patients\(^{36}\). Thus, we examined whether E2 could also restore the loss of RV microvessels and found that RV capillary density was significantly reduced in PH (0.65±0.06 microvessels per cardiomyocyte in PH vs. 0.95±0.07 in CTRL, Figure 6A-D), while E2 significantly enhanced capillary density even beyond healthy CTRL (1.54±0.12 in E2 vs. 0.95±0.07 in CTRL) (Figure 6D).

To further confirm the direct role of angiogenesis in E2-induced rescue of PH, animals were treated with E2 alone or E2 together with the angiogenesis inhibitor TNP-470 (TNP, Figure 6A) at day 21. In the presence of TNP, E2 failed to rescue PH as RVSP (59.4±1.8 mmHg) and RV hypertrophy index (0.68±0.09) had not significant improvement after 10 days of E2+TNP therapy (Figure 7B, C). These data strongly support the vital role of angiogenesis in the rescue action of E2\(^{37}\).
Figure 4. Stimulation of pulmonary neoangiogenesis by E2. A. Single confocal images of lung sections of male rats immunostained for von Willebrand Factor (green, upper panel), overlay of von Willebrand Factor and Nuclei (stained red with TO PRO, middle panel), and at higher magnification of the respective fields (lower panel). B. Quantification of vessels/high power field (HPF) in CTRL (black bar), PH (red) and E2 (blue). **P<0.001 vs. CTRL; ††P<0.001 vs. PH (n=4 animals per group)(1).
Figure 5. E2 therapy restores PH-induced loss of blood vessels in the lungs of female rats. A. Single confocal images of lung sections of female rats immunostained for von Willebrand Factor (green, upper panel), overlay of von Willebrand Factor and Nuclei (stained red with TO PRO, middle panel) and at higher display magnification of the respective fields (lower panel). B. Quantification of vessels/high power field in CTRL (black bar), PH (red) and E2 (blue). **P<0.001 vs. CTRL; ††P<0.001 vs. PH (n=3 animals per group)(1).
Figure 6. Stimulation of cardiac neoangiogenesis by E2. A. Single confocal images of RV sections of male rats immunostained for CD31 (green, C), overlay of CD31 and WGA (red, B) and at higher display magnification (C). D. Quantification of microvessels/cardiomyocyte in CTRL (black bar), PH (red) and E2 (blue). *P<0.05 vs. CTRL, **P<0.001 vs. CTRL; ††P<0.001 vs. PH (n=4 animals per group)(1).
Figure 7. E2 fails to rescue PH in the presence of an angiogenesis inhibitor. A. Experimental protocol. B. Terminal catheterization RVSP. C. RV hypertrophy index RV/(LV+IVS) for CTRL (black bar), PH (shaded gray), E2 (gray) and E2+TNP (black), at day 30 except for PH group which was at day 21. *P<0.05 vs. CTRL, **P<0.001 vs. CTRL; †P<0.05 vs. PH, ††P<0.001 vs. PH; †††P<0.05 vs. E2, ††††P<0.001 vs. E2 (n=3-8 animals per group)(1).
**Estrogen receptor β mediates estrogen-induced rescue of PH**

To investigate whether E2 receptors (ERs) are involved in the rescue action of E2, rats were treated with a selective agonist of ERα, PPT, or a selective agonist of ERβ, DPN at day 21 after MCT injection for 10 days (Figure 8A). DPN was as effective as E2 in rescuing PH (RVSP=34±1mmHg, RV/(LV+IVS)= 0.30±0.01, RVEF=64±4% and lung weight=1.5±0.4g), whereas PPT was not as effective as E2 (RVSP=56±1mmHg, RV/(LV+IVS)=0.60±0.01, RVEF=40±4% and lung weight=2.0±0.1g). PPT was only able to reduce RVSP significantly compared to PH, but the RVEF, lung weight and RV hypertrophy index were not different from PH (Figure 8B-E). To further confirm the role of ERβ in the rescue action of E2, we treated PH rats with E2 in the presence of the ERβ-specific antagonist PHTPP for 10 days. E2 could not rescue PH in the presence of PHTPP, as the beneficial effects of E2 were all abolished (RVSP=60±4mmHg, RV/(LV+IVS)= 0.58±0.05, RVEF=42±1.7% and lung weight=1.93±0.01g) (Figure 8B-E). These results suggest that the rescue of PH by E2 is most likely mediated via the ERβ ^38_.

Thus we propose that E2 therapy after the onset of PH decreases lung fibrosis, while stimulating angiogenesis and restoring right ventricular and pulmonary function to levels similar to CTRL (Figure 9).

### 3.5. Discussion

The present study demonstrates that E2 therapy can rescue agent for advanced pre-existing PH. E2 reversed PH by improving adverse cardiac and pulmonary remodelling,
structure and function leading to 100% survival even after withdrawal of E2. Our approach of starting E2 treatment after the onset of PH has major advantages over

Figure 8. Selective ERβ agonist DPN is as effective as E2 in rescuing severe PH and E2 fails to rescue PH in the presence of the ERβ antagonist PHTPP. A. Experimental design. B-E. RVSP (B), RVEF (C), lung weight (D) and RV hypertrophy index (E) at day 30 except for PH which was at day 21 after MCT injection, for CTRL (white), PH (black), E2 (gray), DPN (ERβ agonist, vertical stripes), PPT (ERα agonist, shaded gray) and E2+PHTPP (ERβ antagonist, horizontal stripes) in male rats. *P<0.05 vs. CTRL, **P<0.001 vs. CTRL; ††P<0.001 vs. PH; #P<0.05 vs. E2, ##P<0.001 vs. E2; $P<0.05 vs. DPN, $$$P<0.001 vs. DPN (n=3-8 animals per group)(1).
Figure 9. Proposed mechanisms underlying E2-induced rescue of PH. Injection of MCT induced PH in healthy rats after 21 days, which led to RVF if left untreated. E2 therapy starting at the PH stage not only prevented the transition to RVF, but also normalized the RV pressure and RVEF. Stimulation of cardiopulmonary angiogenesis together with suppression of lung inflammation and fibrosis by E2 are the key mechanisms in rescuing the lung and the heart(1).
previous studies\textsuperscript{4, 12} which used E2 therapy before the initiation of the disease. As PH is not often diagnosed early, our approach is more practical for patients who already have severe PH as this disease is not often diagnosed early. Furthermore, our data strongly suggests that E2-induced rescue is mainly mediated by ER$\beta$ since the selective ER$\beta$ agonist DPN efficiently rescued PH to levels similar to E2, and in the presence of the ER$\beta$ antagonist PHTPP, E2 could not exert it cardiopulmonary beneficial action (Figure 8). Stimulation of lung and RV angiogenesis by E2 is the other key mechanism involved in E2-induced rescue of PH (Figures 4-6).

However, it is not clear whether the beneficial action of E2 acts directly on the heart, thus improving lung function, or whether E2 acts directly on the vessel wall of pulmonary arteries and arterioles as it has previously been described\textsuperscript{39} to improve lung function. As E2 was shown to attenuate LV hypertrophy of ventricular myocytes\textsuperscript{40}, we speculate that in addition to the action of E2 on the lung, E2 also directly acts on the RV to restore function (Figure 1C) and reverse RV hypertrophy (Figure 1D).

**E2 therapy reverses pulmonary fibrosis and stimulates cardiac angiogenesis**

Recently it has been shown that there is a gender difference in the development of pulmonary fibrosis, as females are better protected than males\textsuperscript{41}. In the present study, we demonstrate that E2 significantly reduces pulmonary fibrosis.

Impaired pulmonary angiogenesis and loss of existing small blood vessels contribute to the increased pulmonary pressures and progression of PH. In the RV, loss of myocardial microvessels together with increased oxygen demand in PH patients with normal coronary arteries have been shown to result in RV ischemia\textsuperscript{42}. This has been proposed to be due to insufficient upregulation of angiogenic factors\textsuperscript{43}. Here we found that PH was
associated with loss of small blood vessels both in lungs and in RV, and that E2 therapy reversed the loss of vessels associated with PH in the lungs in both male and female rats. Furthermore, E2 stimulated the growth of new capillaries beyond the levels of healthy CTRL. During the development of left ventricular hypertrophy, increased angiogenesis has been shown to be a key event in maintaining cardiac function. Therefore, increased cardiac and pulmonary angiogenesis by E2 therapy may underlie the decrease in the severity of PH thus leading to improved cardiopulmonary structure and function. The fact that E2 failed to rescue PH in the presence of the potent angiogenesis inhibitor TNP strongly supports our findings that stimulation of angiogenesis in both lung and RV by E2 is one of the key mechanisms in E2-induced rescue of PH.

The rescue action of estrogen is likely mediated through ERβ

E2 exerts its biological effects mainly via ERα and ERβ. Both receptor subtypes are present in lungs and heart. ERβ has been shown to protect the heart against pressure overload-induced LV failure and ischemia/reperfusion injury, as well as the lungs in the trauma-hemorrhage model. ERβ-knockout mice demonstrated abnormal vascular function and hypertension, increased mortality, and aggravation of heart failure. The data presented in this study strongly supports our hypothesis that the rescue action of E2 is mainly mediated through ERβ. We show that a selective ERβ agonist is able to rescue PH as efficiently as E2 and that E2 fails to rescue PH in the presence of an ERβ-selective antagonist. The estrogen receptor-mediated action of E2 has recently been reported in the hypoxia-induced PH model. Pretreatment with E2 attenuates hypoxia-induced PH through an estrogen receptor-dependent mechanism, as the preventive effect of E2 is abolished in the presence of the broad ERα and ERβ antagonist ICI. Our findings are in
agreement with many other previous studies demonstrating that the estrogen-mediated protection against vascular injury, hypertension, cardiac remodeling and apoptosis are mediated through ERβ54-56.

**The estrogen paradox in pulmonary hypertension**

The prevalence of idiopathic pulmonary hypertension is more profound in young women, which in contrary to rodent studies 57. Female rats with PH induced by either MCT or chronic hypoxia exhibit less severe PH than their male counterparts58-60. This gender difference in susceptibility to PH has been suggested to be partly due to the protective action of E2, as E2 replacement attenuates the progression of PH in ovariectomized animals61 as well as in male rats 4. In addition to E2, estradiol metabolites such as 2-methoxyestradiol (2-ME)10, the natural soybean derived phytoestrogen genistein62, and the selective estrogen receptor modulator raloxifene63 have all been shown to slow down the progression of MCT- or chronic hypoxia-induced PH in rodents.

Lahm et al.64 speculated that the discrepancy between the prevalence of PH in young women vs. animal models can be due to a defect in the estrogen receptors, estrogen receptor signalling, estrogen metabolism or genetic susceptibility in certain female individuals could make them more prone to this disease65. Future clinical studies are needed to investigate the possible correlations between ERα and ERβ pulmonary expression, plasma estrogen levels, and the severity of PH in these female individuals.

The fact that postmenopausal women exhibit an increased risk of developing PH 66 and that hormone replacement therapy may prevent the development of pulmonary hypertension in these patients further support the beneficial role of E2 in PH patients67.
Other sex steroids and PH

Progesterone has also been shown to prevent the progression of experimental PH similarly to E2\(^{68}\). Although testosterone has been shown to be a pulmonary vasodilator in isolated pulmonary arteries\(^{69-71}\), male rats develop more severe PH in different experimental models\(^{10, 11, 72}\). Dehydroepiandrosterone (DHEA) can be metabolized to estrogens and androgens. The plasma levels of estradiol and testosterone have been shown to be higher in DHEA-treated than in DHEA-untreated animals\(^{73}\). As testosterone does not seem to be beneficial in experimental PH, we speculate that the beneficial action of DHEA in experimental PH is due to the conversion of DHEA to estrogen.

ER\(\beta\)-selective ligands as novel therapeutic agents for PH patients

Our findings raise the intriguing concept that E2 therapy may be of significant benefit for patients with severe PH. ER\(\beta\) has been shown to have anti-proliferative activity, while ER\(\alpha\) promotes epithelial proliferation and has pro-estrogenic effects in the breasts and uterus\(^{74, 75}\), we propose that selective ER\(\beta\) ligands could provide a novel treatment modality. Activation of ER\(\beta\) in PH patients as opposed to E2 therapy would minimize estrogenic side effects, a concept which most definitely warrants further investigation.

3.6. Reference List


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Chapter 4:

Rescue of Pressure Overload-Induced Advanced Heart Failure by Estrogen Therapy
4.1. Abstract

**Background-** The heart has all machinery to biosynthesize estrogen from testosterone by aromatase. However, the cardiac and plasma levels of estrogen, testosterone or aromatase and their role in heart failure (HF) has not been investigated. Although estrogen pretreatment attenuates the development of heart hypertrophy, it is not known whether estrogen could also rescue pre-existing HF.

**Objectives-** Here we hypothesized that local heart estrogen concentration is reduced in HF and examined whether exogenous estrogen therapy *after* the onset of advanced HF induced by pressure overload can rescue HF.

**Methods-** Severe HF was induced by transaortic constriction. Mice in HF were treated with estrogen or with estrogen receptor alpha (ERα) or –beta (ERβ) agonists for 10 days. Cardiac structure and function, angiogenesis, fibrosis and inflammation were assessed. Estrogen and testosterone levels were measured both in plasma and heart tissue using enzyme immunoassays.

**Results-** In male mice local heart estrogen and testosterone concentrations, as well as cardiac aromatase transcripts were significantly reduced in HF. Interestingly, plasma estrogen remained unchanged, whereas plasma testosterone was increased in HF. Estrogen therapy of HF mice not only increased cardiac estrogen by 3 fold, it also restored cardiac testosterone levels and aromatase transcripts. E2 therapy also rescued pre-existing HF by restoring cardiac function to ~55% both in male and female mice. Stimulation of cardiac angiogenesis by estrogen is one of the key mechanisms, as in the presence of an angiogenesis inhibitor, estrogen failed to rescue HF. Furthermore, estrogen therapy also reversed HF-induced fibrosis and inflammation. Estrogen rescue is mediated potentially through ERβ since the ERβ agonist DPN rescued HF by stimulating cardiac angiogenesis and reversing fibrosis.
Conclusions- E2 rescues pre-existing HF by restoring cardiac testosterone and aromatase transcripts, stimulating cardiac angiogenesis, suppressing fibrosis, inflammation and activation of ERβ pathway.
4.2. Introduction

Heart failure (HF) is often preceded by heart hypertrophy, which is usually triggered by external stressors. While heart hypertrophy is presumed to be compensatory at the beginning, it often progresses to chronic HF when insults persist. Advanced chronic HF is most often treated with aggressive non-pharmacological therapies such as heart transplantation or implantable left ventricular assist devices. Effective pharmacological therapies for treatment of HF patients would possess major advantages due to their non-invasive and more cost-effective nature. Several agents such as a phosphodiesterase 5A inhibitor as well as the Gβγ small molecule inhibitor gallein have been shown to reverse cardiac hypertrophy and even halt the progression of failure. However, effective pharmacological intervention which can rescue HF at an advanced stage is still necessary.

The heart has all the machinery for biosynthesis of estrogen from testosterone by the help of aromatase. However, it is not known whether local heart E2 biosynthesis is disrupted in HF. In addition, estrogen pretreatment has been shown to attenuate the development of heart hypertrophy, but it is not known if E2 could rescue advanced HF. Here we found that HF induced by pressure overload is associated with lower local heart, but not plasma estrogen concentrations. We found that both cardiac aromatase transcripts and testosterone levels were significantly downregulated in HF. We hypothesized that local heart estrogen concentration is reduced in HF possibly due to downregulation of cardiac aromatase and testosterone, and therefore exogenous estrogen therapy after the onset of advanced HF may rescue pre-existing HF. We now show that short term estrogen therapy starting after the onset of advanced HF restores ejection fraction from ~30% to ~55% and cardiac hemodynamics. We demonstrate that beneficial effects of estrogen are mediated via ERβ activation and seem to result from the
interplay of various factors, including stimulation of angiogenesis and suppression of fibrosis and inflammation.

4.3. Methods

Animals - Wild-type male and female CD-1, wild-type male C57BL/6, and Aromatase knockout (ARO-KO) mice 3-4 months old were used. All protocols received institutional review and committee approval from the Division of Laboratory Animal Medicine at UCLA, and all procedures have conformed to NIH guidelines.

Experimental protocol- Healthy mice with an ejection fraction (EF) of ~60% were randomly subjected to sham or TAC surgery as described previously. Commercially available aromatase knockout (ARO-KO, Jackson Laboratories) mice were also used without surgical intervention. Serial echocardiography was performed to monitor the heart structure and function throughout the course of the experiment. Signs of compensated heart hypertrophy (CHH) were evident two to three weeks after the TAC surgery. Decompensated HF was achieved 8-10 weeks after the TAC surgery with an EF of ~30%. Once TAC mice reached HF, mice were randomly assigned to be left untreated (HF group), were or to one of the following treatment groups for 10 days: E2 via a subcutaneous 10-day continuous release pellet of 0.012 mg E2 (Innovative Research of America, E2-RES group), the angiogenesis inhibitor TNP-470 (30mg/kg, Sigma, once every other day) alone, or TNP-470 together with E2 pellets (E2+TNP). Placebo pellets (containing 5 compounds: cholesterol, lactose, cellulose, phosphates and cerates) were used for 10 days as vehicle for E2, had no effect on the heart structure or function. For both TAC surgery as well as euthanasia, a dose of ketamine/xylazine of 80mg/10mg/1000g was used intraperitoneally. For the Serial echocardiography was performed on the ARO-KO mice to monitor the heart structure and function, and then they were implanted with the same E2 pellet as previously described without
surgical intervention. Throughout the echocardiography as well as for pellet implantation, 1.5 - 2% isofluorane was used as anesthetic.

**Plasma and heart estrogen measurements** - For the estradiol EIA (Cayman chemical) in plasma, the manufacturer's protocol was performed according to the instructions. For tissue measurements, hearts were first frozen and powdered with a mortar and pestle, then homogenized with a polytron and subsequently sonicated. For both E2 and T measurements, the lysates were then centrifuged (12,000 rpm for 10 min), the supernatants collected and subjected to EIA. The estradiol kit was then tested both for plasma and tissue homogenates. There was no interference of proteins, and measurements gave accurate results when compared to known sample concentrations (male samples spiked with estrogen). Thus, the samples were measured according to manufacturer’s protocol. For T measurements however, initial measurements in plasma did not yield accurate results, thus the kit was optimized. A female plasma sample was spiked with known levels of testosterone and serially diluted up to 1:64 and subjected to EIA. Corrected values accounting for the dilution factor and spike recovery were calculated, and for each serial dilution the percent agreement values as well as spike recovery were determined. It was found that the dilutional linearity was within acceptable range (80-120%), and that 1:16 sample dilutions did not yield any protein interference of the T measurements. This dilution factor also yielded concentrations within the detectable range of the standard curve. Thus, for T measurements all plasma and heart lysates were diluted 1:16 and then subjected to EIA.

**Cardiac hemodynamics** - Serial B-Mode and M-Mode echocardiography was performed using a VisualSonics Vevo 2100 equipped with a 30-MHz linear transducer to accurately monitor the stage of the disease by measuring cardiac hemodynamic parameters and assessing heart structure. The LV ejection fraction, LV wall thickness and LV cavity dimensions were quantified using
M-mode. The left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP) and heart rate (HR) were recorded directly by inserting a catheter (1.4F Millar SPR-671) connected to a pressure transducer (Power Lab, ADInstruments) into the LV right before sacrifice. The left ventricular developed pressure (LVDP) and rate pressure product (RPP) were calculated as LVDP = LVSP – LVEDP, RPP = HR*LVDP. The maximum rate of the LV pressure rise (dP/dt\text{max}) and decline (-dP/dt\text{min}) were directly calculated from the recordings.

**Real-Time qPCR** - For RT-qPCR quantification, hearts were excised and rinsed in ice-cold PBS. The heart weight was measured, the atrias were then removed and the ventricles were snap-frozen in liquid nitrogen and stored at -80°C. For RNA extraction, hearts were powdered with a mortar and pestle on dry ice, suspended in 2 ml Trizol (Invitrogen) and homogenized with a polytron (Kinematica). RNA quality was assessed via gel electrophoresis. For cDNA synthesis, RNA concentration was assessed using a spectrophotometer (Beckman) and 2 µg of RNA with purity (A260/A280) of 1.7–2.0 was reverse transcribed using the Omniscript RT kit (Qiagen) according to manufacturer’s protocol in a final reaction volume of 20 µl. The real-time qPCR was performed using IQ SYBR Green supermix (BioRad) according to manufacturer’s protocol in a final volume of 25µl using a BioRad CFX RT-qPCR machine. For all assays, there were at least 3 samples/group assayed in duplicate. Threshold cycle (Cq value) was determined using CFX Manager, and the Cq value of the gene of interest was normalized to the Cq value of its own internal control gene (GAPDH). Controls consisted of: the reaction cocktail without reverse transcriptase and H2O instead of cDNA tested by RT-qPCR.

**RT-qPCR primer validation** - Prior to the RT-qPCR experiments, each primer set was validated to ensure that it yields a single sharp peak in the RT-qPCR melting curve. The controls for each primer set consisted of the reaction cocktail without reverse transcriptase and H2O instead of
cDNA tested simultaneously by RT-qPCR. All the RT-qPCR products, including the negative controls, were then subjected to gel electrophoresis to ensure amplification of a single product of the expected molecular size and that the negative controls yield no product.

Immunochemistry and imaging- Whole hearts were fixed in 4% paraformaldehyde and transversal 6-7 μm sections were obtained with a cryostat. Tissue sections were used for standard eosin/hematoxylin and Masson’s Trichrome (Sigma) staining according to manufacturer’s protocol and images were acquired with a light microscope (Nikon). For immunofluorescent confocal microscopy, sections were labeled with anti-PECAM1 (04-1074, Millipore, 1:200 dilution) and wheat germ agglutinin (WGA, Invitrogen, 1:500 dilution) and images were acquired with a confocal scanning microscope (Nikon).

Statistical Analysis- One-way ANOVA with Holm-Sidak post hoc tests were used to compare between groups using SPSS SigmaStat. P values less than 0.05 were considered statistically significant. Values are expressed as mean ± SEM.

4.4. Results

Local heart estrogen and T concentrations and cardiac aromatase expression levels are significantly lower in HF male mice

Figure 1A and B demonstrate that the local estrogen and testosterone concentrations in the heart tissue were significantly lower in HF compared to CTRL (7.97±2.08 pg/ml vs. 17.86±3.78 pg/ml in CTRL for estrogen, and 136.26±11.23 vs. 356.05±15.88 pg/ml in CTRL for testosterone). Interestingly, plasma estrogen concentrations remained unchanged in HF (26.29±6.10 pg/ml in HF and 27.43±4.27 pg/ml in CTRL), while testosterone levels were increased (3.41±0.65 ng/ml
Figure 1. Local heart E2 and T concentrations, as well as cardiac aromatase transcripts are reduced in HF. (A) Estrogen concentration in plasma and in heart, n = 6-13 mice/group. (B, C) Testosterone concentration in heart (n = 6 mice/group) and in plasma (n = 6-13 mice/group) and (D) Relative transcript expression of cardiac aromatase normalized to CTRL. GAPDH was used as an internal control (n = 4-5 hearts/group). *P<0.05 vs. CTRL; **P<0.001 vs. CTRL; ^P<0.05 vs. HF and ^^P<0.001 vs. HF.
in HF vs. 1.94±0.38 in CTRL, Figure 1A,C). Additionally, cardiac aromatase transcript levels were also ~5 fold lower in HF compared to CTRL (0.19±0.04 in HF, normalized to CTRL, Figure 1D). Estrogen therapy increased cardiac and plasma E2 concentration to a similar levels (67.12±8.60 pg/ml and 66.48±9.65, respectively), and restored aromatase transcripts to CTRL (0.95±0.08, normalized to CTRL, Figure 1D). Lastly, we found that E2 therapy partially restored cardiac and plasma testosterone levels to 237.89±46.56 pg/ml and 1.23±0.11 ng/ml, respectively (Figure 1B,C).

To understand the contribution of gonadal T in HF, we assessed the plasma and heart E2 and T levels in ORC male mice in CTRL and HF. As expected, these mice had very low T levels in the plasma, which were unaffected in HF (45.48±7.58 in CTRL and 47.08±11.38 pg/ml in HF, Figure 2). The levels of T were also very low in the heart and remained unchanged between CTRL and HF (46.78±4.34 in CTRL and 49.79±2.60 pg/ml in HF). Furthermore, both plasma and heart [E2] were also lower, and remained unchanged in HF (12.13±2.44 in CTRL and 10.19±1.74 pg/ml in HF in plasma, and 13.31±1.54 in CTRL and 12.22±1.69 pg/ml in HF in the heart, Figure 2B).

To further investigate the role of cardiac aromatase in the heart function, we assessed the heart function of Aro-KO mice at the baseline in the absence of any cardiac stress. Aro-KO male mice had significantly depressed ejection fractions compared to wild-type mice (47.46±1.81% vs. 64±1.05% in WT, Figure 3) at the baseline at 4 months of age. These data highlight the important role of aromatase and local heart hormonal milieu on cardiac function.

**Estrogen therapy rescues pre-existing advanced HF induced by pressure overload by restoring heart function and structure as in compensated state both in male and female mice**
Figure 2. Local heart E2 and T concentrations in orchidectomized mice remain unchanged in heart failure. (A) Testosterone and (B) estrogen concentrations in plasma and in hearts of orchidectomized CTRL and HF male mice (n=5 mice/group).
Figure 3. Aromatase knockout mice have lower cardiac function than wild type mice at baseline. (A) Examples of M-mode images of the parasternal short axis view by echocardiography from wild type and aromatase knockout (Aro-KO) male mice at the baseline (B). Ejection fraction in WT (circles, n=10) and in Aro-KO mice (diamonds, n=6). The open shapes represent individual measurements and filled shapes the averages.
We explored whether exogenous treatment of estrogen could *rescue* pre-existing advanced HF. Fig 4A illustrates a typical example of an M-mode echocardiogram from the same male mouse at the baseline before the TAC operation (CTRL), in compensated heart hypertrophy (CHH), and in advanced HF before and after E2 treatment. E2 therapy restored the ejection fraction (EF) of HF mice in a surprisingly efficient manner from 33.2±1.1% in HF to 53.1±1.3 % within 10 days of E2 treatment (Figure 4A,B) as in compensated hypertrophy state. Placebo pellets had no effect on cardiac structure and function. E2 therapy also reversed the changes that occurred in LV structure (LV posterior wall and intraventricular septum dimensions) from CHH to HF (Figure 4C, Table-1, Figure 5A,B). E2 reversed LV structural morphology to levels similar to CHH as reflected in the heart weight to body weight ratio (HW/BW, mg/g: 5.39±0.14 CTRL; 8.55±0.21 CHH; 10.15±0.3 HF and 7.88±0.44 in E2-RES, Figure 5C) as well as in the myocytes cross section diameter (CSD, normalized to CTRL: 1.67±+0.02 CHH; 1.7±0.07 HF; 1.39±0.02 E2-RES, Figure 5D). In pathological heart hypertrophy beta myosin heavy chain ($\beta$-MHC) is known to be re-expressed. E2 therapy reversed HF-induced rise of $\beta$-MHC to similar levels as in CHH (Figure 5E).

As in male mice, E2 was also able to rescue HF in female mice as in CHH (55.35±2.78% in E2, 31.66±0.15 in HF and 52.33±0.9 in CHH vs. 65.53±1.24% in CTRL, Figure 6A,B). Similarly to male mice, HW/BW ratio of HF mice was also decreased by E2 therapy (7.38±0.62 in HF to 5.14±0.21 in E2 therapy vs. 4.33±0.09 in CTRL). To ensure that our findings are not limited only to CD1 strain, we also explored if E2 can rescue HF in C57BL/6 mice, the most common strain used in pressure overload studies. We found that E2 is able to efficiently restore cardiac function in C57BL/6 mice similarly to CD-1 mice to an EF of 56.24±2.40% (Figure 7).

Since Aro-KO mice had depressed cardiac function even at the baseline, we did not perform
Figure 4. Estrogen therapy rescues HF by restoring heart function and structure similarly to the compensated state prior to the transition to heart failure in male mice. (A) Examples of M-mode images of the parasternal short axis view by echocardiography from the same mouse before TAC, after TAC surgery in CHH, in HF and after treatment with E2. (B) Averaged EF%. (C) Left ventricle posterior wall thickness (LVPW) at the end of diastole (filled bars) and systole. Values and the number of animals are given in Table-1. **P<0.001 vs. CTRL; ††P<0.001 vs. CHH and ^^P<0.001 vs. HF.
Figure 5. E2-induced rescue of HF is associated with a decreased heart weight to body weight ratio, cardiomyocyte diameter and \( \beta \)-MHC fetal gene transcript expression. (A) Images of the whole heart as well as (B) hematoxylin and eosin staining of cross sections and longitudinal sections of hearts in CTRL, HF and E2-RESC (C) The heart weight to body weight ratio (HW/BW, n=6-9 animals per group), (D) Cardiomyocyte cross sectional diameter (CSD, 20-30 cells per animal, normalized to CTRL) and (E) Relative transcript expression of \( \beta \)-MHC normalized to CTRL GAPDH was used as an internal control and its transcripts were similar in all conditions. \( ^{**}P<0.001 \) vs. CTRL; \( ^{†}P<0.05 \) and \( ^{‡‡}P<0.001 \) vs. CHH, \( ^{^^}P<0.001 \) and \( ^{^}P<0.05 \) vs. HF (n=3-4 animals per group).
Figure 6. Estrogen therapy rescues HF in female mice. (A) Examples of M-mode images of the parasternal short axis view by echocardiography at baseline before TAC, after TAC surgery in HF and after treatment with E2. (B) Averaged EF%. (C) Left ventricle posterior wall thickness (LVPW) at the end of diastole (filled bars) and systole (open bars). *P<0.05 vs. CTRL; **P<0.001 vs. CTRL; †P<0.05 vs. CHH; ††P<0.001 vs. CHH and ^^P<0.001 vs. HF (n= 6-9 animals/group).
Figure 7. Estrogen therapy rescues HF by restoring heart function and structure in C57BL/6 male mice (A) Examples of M-mode images of the parasternal short axis view by echocardiography before TAC, after TAC surgery in HF and after treatment with E2. (B) Averaged EF %. *P<0.05 vs. CTRL; **P<0.001 vs. CTRL and ^^P<0.001 vs. HF (n=6 animals/group).
TAC surgery in these mice and examined whether exogenous E2 therapy could improve the heart function of Aro-KO mice in the absence of pressure overload. We found that estrogen therapy for 10 days was able to improve the ejection fractions of Aro-KO mice to some extent to 54.58±2.29% from 47.46±1.81% in the absence of TAC (Figure 8).

Therefore, exogenous E2 therapy rescues advanced HF in both male and female CD-1 as well as in male C57BL/6 mice by restoring cardiac function. Furthermore, E2 significantly improves the impaired cardiac contractility at baseline of ARO-KO mice.

**Stimulation of cardiac angiogenesis by E2 therapy is a key mechanism in E2-induced rescue of HF.**

Reduced cardiac angiogenesis in HF has been reported. Thus, we examined if stimulation of cardiac angiogenesis by E2 could be one of the mechanisms participating in the E2-induced rescue of HF. The transcript levels of two angiogenic markers, vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1a (HIF1a) were both significantly upregulated in CHH (~2 fold) but were downregulated (~5 fold) in HF male mice. E2 treatment was not only able to reverse the downregulation of VEGF and HIF1a transcript levels as observed in HF, but also upregulated both transcripts ~3 fold higher than in healthy controls (3.24±0.1 in E2 vs. 0.26±0.06 in HF, Figure 9A). Quantification of capillary density also revealed that E2 therapy significantly enhanced capillary density by ~ 4 fold compared to the HF group (2.83±0.14 in E2 vs. 0.66±0.07 in HF, normalized to CTRL, Figure 9B, C). To further confirm the role of angiogenesis in the E2-induced rescue of HF, HF males were treated with the angiogenesis inhibitor TNP-470 alone or E2 together with TNP-470 (TNP group, Figure 9D). All the HF animals treated with TNP-470 alone died shortly after commencing treatment. E2 failed to rescue HF in the presence of TNP-470 as EF (29.3±2.1%) was not significantly improved by 10 days of E2 therapy (Figure 9D).
Figure 8. E2 therapy restores myocardial contractile deficiency of Aro-KO mice. Ejection fraction in WT (circles, n=10) and in Aro-KO mice (diamonds, n=6) before and after 10 days E2 therapy (squares). The open shapes are individual measurements and filled shapes are the averages. Note that the TAC surgery was not performed on Aro-KO mice. *P<0.05 vs. baseline measurements.
Figure 9. Stimulation of cardiac angiogenesis by E2. (A) Relative transcript expression of HIF1a, VEGF and GAPDH normalized to CTRL. GAPDH was used as an internal control. (B) Single confocal images of LV sections immunostained for CD-31 (green, left panels), the overlay of CD31 and WGA (red, middle panels) and at higher display magnification of the white squares (right panels). (C) Quantification of capillary density as microvessels per cardiomyocyte. (D) Averaged EF%. **P<0.001 vs. CTRL; †P<0.05 vs. CHH; ††P<0.001 vs. CHH, ^^P<0.001 vs. HF and §§ P<0.001 vs. E2 (n=3-4 animals per group).
also prevented E2 from stimulating the capillary density (0.53±0.07 in E2+TNP vs. 2.83±0.14 in E2).

Similar to males, E2 was also able to stimulate the loss of capillary density in HF female mice ~3 fold higher than control levels (2.21±0.09 in E2 vs. 0.62±0.06 in HF, normalized to CTRL; Figure 10A, B).

Next, we investigated whether E2 has any effect on myocardial neoangiogenesis in the absence of cardiac insult. Mice without TAC were treated with E2 or placebo for 10 days. Quantification of the myocardial capillary density revealed that even in the absence of TAC, E2 therapy significantly stimulates cardiac neovascularization by about 30% (1±0.07 in CTRL to 1.29±0.08 with estrogen treatment, Figure 11).

**Estrogen reverses HF-induced myocardial fibrosis and inflammation**

Extensive myocardial fibrosis has been identified in both severe HF patients as well as in different experimental models of HF\(^\text{11}\). Masson’s Trichrome staining revealed that the interstitial and perivascular fibrosis observed in HF was absent in E2 treated male mice (Figure 12A). Mice in HF had 45.5±2.8% fibrous tissue in cardiac sections vs. 1.2±0.4% in CTRL, while E2 therapy significantly decreased fibrotic scarring to levels lower than CHH (5.3±1.0 in E2 vs. 12.6±2.6 in CHH, Figure 12A). RT-PCR also revealed that the transcript levels of several pro-fibrotic markers were significantly upregulated in HF (from 1.0±0.2 to 1.8±0.1 for collagen I; 1.0±0.2 to 1.5±0.1 for collagen III; 1.0±0.9 to 4.3±0.6 for TGF-β1; 1.0±0.5 to 3.6±0.2 for Fibrosin I (FBRS) and 1.0±0.3 to 2.9±0.3 for Lysil Oxidase (LOX)), while E2 treatment fully restored these transcripts to levels comparable to healthy CTRL group (Figure 12B). Estrogen had similar effects in reducing fibrosis in females as in males (Figure 13).
Figure 10. Stimulation of cardiac neoangiogenesis by E2 in female HF mice. (A) Single confocal images of LV sections immunostained for CD-31 (green, top panels), the overlay of CD31 and WGA (red, middle panels) and at higher display magnification of the white squares (bottom panels). (B) Quantification of capillary density as microvessels per cardiomyocyte and (C) heart weight to body weight (HW/BW) ratio in CTRL (black bars), HF (red bars), and after E2 treatment (purple bars). *P<0.05 vs. CTRL; **P<0.001 vs. CTRL; ^P<0.05 vs. CTRL and ^^P<0.001 vs. HF (n=3-6 animals per group).
Figure 11. Stimulation of cardiac neoangiogenesis by E2 in the absence of TAC. (A) Single confocal images of LV sections immunostained for CD31 (green, left panels), the overlay of CD31 and WGA (red, middle panels) and at higher display magnification of the white squares (right panels). (B) Quantification of capillary density as microvessels per cardiomyocyte. *P<0.05 vs. CTRL.
Figure 12. E2-induced rescue of HF is associated with suppression of fibrosis (A) Representative Masson’s Trichrome staining of LV sections (interstitial, top panels; perivascular, lower panels) and total fibrosis quantification. B) Relative transcript expression of pro-fibrotic genes normalized to CTRL. GAPDH was used as an internal control. *P<0.05 vs. CTRL; **P<0.001 vs. CTRL, ††P<0.001 vs. CHH and ^P<0.05 vs. HF (n=3-5 hearts/group).
Figure 13. E2-induced rescue of HF is associated with suppression of fibrosis in female mice. Representative Masson’s Trichrome staining of LV sections (interstitial, top panels; perivascular, lower panels) in CTRL, HF and E2-RES; blue color indicates fibrosis (n=3-6 animals/group).
E2 also reversed the transcript levels of the proinflammatory markers IL-1β and IL-6, which were upregulated in HF male mice from 1.0±0.2 to 1.9±0.1 and 1.0±0.3 to 5.3±0.8, respectively. The anti-inflammatory interleukin IL-10 was downregulated in HF from 1.0±0.2 to 0.5±0.03 and was partially restored with E2 to 0.7±0.1 (Figure 14).

**Estrogen exerts its cardioprotective effects mainly via the estrogen receptor beta**

Most of biological actions of E2 are mediated through ERα or ERβ, and both of these receptors are present in the heart\(^{12}\). EF of HF male mice treated with the ERβ agonist DPN significantly improved (from 33.2±1.1% in HF to 45.3±2.1%), while there was no improvement in the EF of HF mice treated with the ERα agonist PPT (31.1±2.3%, Figure 15). To further investigate the involvement of ERβ in the rescue action of E2, HF mice were treated with E2 in the presence of the ERβ antagonist PHTPP. E2 failed to rescue HF in the presence of PHTPP, as there was no significant improvement in their EF at the end of the 10 day treatment (32.5±5.2%, Figure 15B). ERα and ERβ transcript and protein levels remain unchanged in HF (Figure 16). Interestingly, E2 treatment resulted only in increased expression of ERβ (Figure 16C). Next, we examined the role of ERβ in the stimulation of angiogenesis. We found that the capillary density of HF mice treated with the ERβ agonist DPN was restored to CTRL levels (0.98±0.09 in DPN vs. 0.66±0.07 in HF and 1±0.08 in CTRL, Figure 17), while the ERα agonist PPT could not stimulate cardiac angiogenesis in HF male mice (0.62±0.05 in PPT vs. 0.66±0.07 in HF, Figure 17).

We then examined the role of ERβ in reversing fibrosis observed in HF. We found that DPN therapy was also able to reverse both interstitial and perivascular fibrosis, while the ERα-specific agonist PPT was not very effective (Figure 18A). To investigate the mechanism through which DPN can exert its beneficial actions on reversing fibrosis, we assessed the transcript levels of the fibrotic markers Collagen I and TGF-β1 \textit{in vivo}. Real-time PCR revealed that DPN treatment of
Figure 14. E2 therapy reverses HF-associated myocardial inflammation. Relative transcript expression of pro-inflammatory markers IL-1β and IL6 and anti-inflammatory IL-10 in CTRL (black bar), HF (red bar) and E2-RES (purple bar) normalized to CTRL. GAPDH was used as an internal control and its transcripts were similar in all conditions. *P<0.05 vs. CTRL; ^P<0.05 vs. HF and n=3-4 animals/group.
Figure 15. An ERβ, but not ERα agonist improves EF. (A) Examples of M-mode images of the parasternal short axis view by echocardiography 10 days after treatment with an ERα agonist (PPT, left panels, n=6) or an ERβ agonist (DPN, center panels, n=7) and E2 together with the ERβ-specific antagonist PHTPP (right panels, n=3). (B) Averaged EF as a function of time. §P<0.05 vs. E2; §§P<0.001 vs. E2 and $$P<0.001 vs. DPN.
Figure 16. E2 therapy upregulates ERβ protein levels. (A) Relative mRNA transcript levels normalized to CTRL. GAPDH was used as an internal control. Representative Western Blot of whole heart lysates for (B) the 45 kDa and 66 kDa isoforms ERα, and (C) ERβ. Bar graphs represent the quantification of fluorescent signal normalized to the corresponding Vinculin and to CTRL. Vinculin was used as a loading control. *P<0.05 vs. CTRL and ^P<0.05 vs. HF.
Figure 17. ERβ agonist, but not ERα agonist, restores cardiac angiogenesis in HF mice. (A) Single confocal images of LV sections immunostained for CD31 (green, top panels), the overlay of CD31 and WGA (red, middle panels) and at higher display magnification of the white squares (bottom panels). (B) Quantification of capillary density as microvessels per cardiomyocyte. *P<0.05 vs. CTRL; **P<0.001 vs. CTRL; †P<0.05 vs. HF; ††P<0.001 vs. HF; and ^^P<0.001 vs. E2 (n=3-5 animals per group).
HF mice was able to fully restore both transcripts to levels similar to CTRL, while PPT had a partial restorative effect (Figure 18B, C).

Consistent with our in-vivo findings, E2 and DPN treatments were able to reverse AngII induced the transcript upregulation of Collagen I and TGF-β1 in a co-culture of neonatal rat fibroblasts and ventricular myocytes, while PPT was only partially effective in reducing the levels of TGF-β1 but had no effect on Collagen I expression (Figure 18D, E).

In summary, the rescue action of E2 is mainly mediated through ERβ since the ERβ agonist was able to restore cardiac contractility, stimulate cardiac angiogenesis and reverse cardiac fibrosis to a great extent, whereas ERα agonist had no significant effect on any of the above mentioned parameters.

**E2 therapy restores cardiac hemodynamic parameters of HF male mice**

Direct catheterization revealed that LV developed pressure (LVDP) increased by ~50% in CHH compared to CTRL (97.8±2.6 vs. 150.3±2.3 mmHg) and was reduced in HF (100±5.2). Consistent with E2 treatment-regulated improvement of mechanical performance of the heart, LVDP increased to 131±4.6 mmHg near CHH values (Table-1). E2 also improved the rate pressure product (RPP) from 43284±4527 mmHg*beats/min in HF to 65455±5533 mmHg*beats/min, Figure 19A). The contractile and relaxation defects induced by HF were also corrected by E2 therapy, as the maximum rate of LV pressure rise (dP/dt$_{max}$) and decline (dP/dt$_{min}$) were restored with E2 treatment similar to healthy hearts in spite of the sustained presence of the TAC stress stimulus (Figure 19B, Table-1). Furthermore, DPN treatment of HF mice was also able to significantly improve the RPP to (38701±3284.73 in HF to 62260.7±5963.43 (p<0.001), while PPT treatment had no effect on LV mechanical performance.
Figure 18. The ERβ agonist DPN reverses interstitial and perivascular cardiac fibrosis in HF mice. (A) Representative Masson’s Trichrome staining of LV sections (interstitial, top panels; perivascular, lower panels) in HF mice treated with the ERα agonist PPT, or the ERβ agonist DPN for 10 days; blue color indicates fibrosis. (B, C) Relative transcript expression of *in-vivo* collagen I and TGF-β1 normalized to CTRL. (n=3-4 animals/group). (D, E) Relative transcript expression of *in-vitro* collagen I and TGF-β1 in CTRL (black), angiotensin-II alone (Ang II, blue) or together with E2 (purple), DPN (green) or PPT (red) as normalized to CTRL (n=3-5 individual treatments/group). GAPDH was used as an internal control and its transcripts were similar in all conditions. *P<0.05 vs. CTRL; **P<0.001 vs. CTRL; ^P<0.001 versus HF, ^P<0.05 vs. HF $P<0.05$ vs. Ang II $^{SS}$P<0.001 vs. DPN and $^SP<0.05$ vs. DPN.
Figure 19. E2 therapy restores systolic function and corrects contraction and relaxation defects induced by HF. (A) RPP and (B) dP/dt_max (filled bars) and dP/dt_min (open bars). The values and the number of animals are given in the Table-1. N=8 animals for the PPT and DPN groups. *P<0.05 vs. CTRL; **P<0.001 vs. CTRL; †P<0.05 vs. CHH; ††P<0.001 vs. CHH; ^P<0.05 vs. HF; ^^P<0.001 vs. HF; P<0.05 vs. E2; ††P<0.001 vs. E2; ¶P<0.05 vs. E2+TNP and #P<0.05 vs. PPT (n=6-10 animals per group). (C) Proposed mechanism underlying E2 rescue of pressure overload-induced HF. Pressure overload initially induces compensated heart hypertrophy, which eventually transitions into HF when the pressure is sustained. E2 therapy started after the onset of HF rescues pre-existing severe HF by restoring heart function fully to compensated heart hypertrophy mainly through stimulation of angiogenesis and suppression of fibrosis and inflammation. E2 also restores cardiac E2 and T concentrations as well as aromatase levels.
as the RPP was not significantly different from their corresponding values in HF (Figure 19A). The relaxation and contraction defects of HF mice (dP/dt\text{max} and dP/dt\text{min}) were also significantly improved by DPN treatment compared to HF (dP/dt\text{max} from 4871.4±511.9 mmHg/s to 6405.7±461.2, and dP/dt\text{min} from 4429.2±280.1 mmHg/s to 5828.6±322.2), while PPT had no significant effect on these parameters. As expected, RPP and dP/dt could not be restored by E2 in the presence of the angiogenesis inhibitor TNP-470 (Figure 19A, B).

4.5. Discussion

Here we show for the first time that local heart E2 and T are reduced significantly in advanced HF. Furthermore, cardiac aromatase transcripts were also downregulated in HF. Surprisingly plasma T was increased ~2 fold in HF whereas plasma E2 remained unchanged. Despite the presence of sustained pressure overload, pharmacological intervention using E2 therapy restored ejection fraction of TAC-induced HF in male and female mice from ~30% to ~55%. E2 therapy also restored heart E2, T as well as aromatase transcript levels. In the plasma, E2 therapy also restored T levels. Starting E2 treatment after the establishment of severe HF has major clinical advantages over previous studies that used E2 therapy prior to the initiation of the disease\textsuperscript{5}, as our approach (i) is more practical for patients who already have severe HF, as the disease is not often diagnosed early, and (ii) the therapy requires shorter duration (10 days vs. 28 or 56 days) and a lower dose of E2 (0.0012 mg E2/day versus 0.003 mg/day\textsuperscript{5}), thereby minimizing E2 side effects. Regarding the possible mechanisms involved in E2-induced rescue of HF, our data strongly support that the rescue action of E2 is mediated through ER\textbeta since 1) selective estrogen receptor beta agonist, but not alpha agonist, is able to rescue pre-existing HF; and 2) E2 fails to rescue HF in the presence of an ER\textbeta selective antagonist. E2-induced rescue of HF is strongly
correlated with stimulation of LV angiogenesis, as the angiogenesis inhibitor TNP-470 prevented E2 action. In addition, the beneficial effects of E2 in HF also associated with reversal of myocardial fibrosis and reduced inflammation (Figure 19C).

**Regulation of cardiac E2, T and aromatase in HF**

Here we show that while cardiac E2 levels are reduced in HF, plasma E2 remains the same (Figure 1). Surprisingly, despite having much higher levels of T in the plasma, cardiac T levels were low in HF, possibly due to reduced T uptake by the failing heart. Interestingly, aromatase transcripts are also reduced in HF, therefore reduced cardiac E2 could be due to a reduction in both T and aromatase levels, as well as a lower uptake of plasma E2 by cardiac tissue. Most importantly, E2 therapy restores cardiac and plasma T levels, as well as cardiac aromatase transcripts. The normalization of cardiac T and aromatase transcripts by E2 therapy could be result of improved heart function leading to better T uptake. In addition, E2 therapy could directly normalize aromatase transcripts by binding to a half ERE site, or indirectly through transcription factors as our analysis of 5 kb promoter region of aromatase gene shows several half ERE as well as Sp1 and Ap1 elements. In ORC mice the levels of plasma T and E2 were very low as expected and remained unchanged in HF (Figure 2). Furthermore, the cardiac levels of E2 and T, which were much lower than in normal hearts, remained unaffected by HF. These data support the view that plasma levels of these hormones are important for their local cardiac concentration possibly by an alteration in their uptake and/or local biosynthesis in HF. Significantly lower levels (~50 fold) of plasma T in ORC animals compared to intact males could greatly reduce the cardiac uptake of T and local E2 concentrations.

The role of aromatase in the heart is not well understood. In postmenopausal women, aromatase plays an important role, as its peripheral activity becomes the primary source of E2 synthesis.\(^\text{13}\)
The ablation of aromatase has also been demonstrated to be associated with increased adiposity\textsuperscript{14} and progressive insulin resistance\textsuperscript{15, 16}, which play significant roles in the development of cardiovascular diseases such as atherosclerosis and diabetic cardiomyopathy. Here we show for the first time that aromatase KO mice, in the absence of any stress stimulus (pressure overload), have significantly lower EF than age-matched wild type mice, which further highlights the role of aromatase in cardiac function. Therefore, restoring aromatase in the heart by E2 therapy could be an important process in preserving the heart function.

The higher plasma T levels in HF could be due to increased stress associated with the progression of HF. Consistent with our results, previous reports have shown that stress provokes a rise in plasma T\textsuperscript{17, 18}. T has recently emerged as a new therapeutic approach for treatment of chronic HF, as 25\% of men with chronic heart failure suffer from testosterone deficiency\textsuperscript{19}. Supplementation of heart failure therapy with testosterone has been examined in small clinical trials, where T improved the exercise capacity and muscle strength of heart failure patients\textsuperscript{20}. However, T therapy had no effect on the LV EF\textsuperscript{21}. Consistent with this study, our findings also suggest that the beneficial effects of E2 on the failing heart could be at least in part due to restoration of T in HF.

**Stimulation of cardiac angiogenesis by E2**

Increased cardiac angiogenesis has been shown to be a key event in maintaining LV function during adaptive hypertrophy induced by pressure overload, however the imbalance between cardiac growth and neoangiogenesis eventually leads to the transition from CHF to HF\textsuperscript{10}. VEGF is an important angiogenic factor and a critical determinant of capillary growth and density\textsuperscript{22}. We have shown that E2 strongly stimulated VEGF expression and growth of blood vessels in the LV of HF mice (Figure 9). The fact that E2 failed to rescue HF in the presence of the angiogenesis
inhibitor TNP (Figure 9D) strongly supports the critical role of angiogenesis in the rescue action of E2. In the absence of TAC stimulus, E2 was also able to induce cardiac angiogenesis by about 30%. This is not surprising as estrogen has been shown to be pro-angiogenic in various tissues and organs such as the uterus, breast, brain and limbs. However, the 30% increase in angiogenesis in healthy mice is not as dramatic as the 300% observed in HF mice treated with E2 (Figure 11). Since the existing angiogenesis meets the demands of the heart under basal conditions (in the absence of TAC), there is only a marginal increase in angiogenesis by E2. In HF, despite the larger LV size and obvious need for more blood vessels to support the heart muscle, angiogenesis is drastically decreased. Therefore the 300% increase in neoangiogenesis by estrogen therapy is a vital process in the protection against heart from failure.

**Reverse remodeling of cardiac fibrosis by E2 therapy**

End-stage heart failure has long been regarded as a terminal state of cardiac pathological remodeling, consisting of fibrosis and extracellular matrix (ECM) remodeling that are almost impossible to reverse by any currently available therapy. E2 treatment has been demonstrated to mitigate adverse ECM remodeling in LV hypertrophy and failure by decreasing collagen deposition and metalloproteinase expression. Here we show that E2-induced rescue of HF is associated with reversal of LV fibrosis by reversing increased expression of the pro-fibrotic genes collagen I, III, TGF-β1, LOX and FBRS (Figure 12). Although attenuation of fibrotic deposition in the heart with tetrahydrobiopterin (a NOS coupler), Tempol (a broad antioxidant), as well as FTY-720 (an immunomodulator for treating multiple sclerosis) in the TAC model has been demonstrated, our study reports the reversal of extensive fibrosis at a late stage of HF with E2 therapy. Our data also support the view that E2 therapy reverses LV failure by inhibiting fibrosis, and adverse ECM remodeling likely through an ERβ-mediated mechanism. This is
possible as E2 can prevent cardiac fibrosis by inhibiting the transition of fibroblasts to myofibroblasts through an ERβ-mediated mechanism as shown in another model of fibrosis\textsuperscript{29}.

**Receptor mediated effects of E2 in the rescue of HF**

Estrogen mainly acts through classical ER\(\alpha\) and/or ER\(\beta\) and both receptors are present in the heart\textsuperscript{30}. The beneficial role of ER\(\beta\) in cardioprotection has been highlighted in the recent years\textsuperscript{31, 32}. Female mice lacking the ER\(\beta\) gene are less protected against myocardial I/R injury compared to intact female mice. Furthermore, ER\(\beta\)-knockout mice have abnormal vascular function and hypertension, increased mortality, and aggravated heart failure\textsuperscript{33}. ER\(\beta\) is also shown to be responsible for the anti-hypertrophic and anti-fibrotic effects of E2\textsuperscript{5, 34}. Various studies have shown that E2 can differentially regulate estrogen receptors depending on the tissues and cell types\textsuperscript{35-37}. Here we report that E2 could induce the expression of ER\(\beta\), but not ER\(\alpha\) in HF suggesting that ER\(\beta\) induction may be involved in E2 mediated rescue. Our data using specific ER\(\alpha\) and ER\(\beta\) agonists clearly demonstrated that ER\(\beta\) activation is responsible for the salutary effects of E2 in the rescue of HF (Figure 15). Furthermore, the fact that E2 failed to rescue HF in the presence of a specific ER\(\beta\) antagonist further confirmed the role of ER\(\beta\) in E2-induced rescue of pressure overload-mediated HF. Here we also show that anti-fibrotic action of estrogen in the failing heart is mediated through ER\(\beta\), as treatment with the ER\(\beta\) agonist DPN decreased fibrosis, as well as effectively restored the levels of the fibrotic markers Collagen I and TGF-\(\beta\)\textsubscript{1} to levels similar to control in vivo. However, the ER\(\alpha\) agonist PPT had no effect in the reversal of fibrosis in the heart but was able to decrease the transcript levels of Collagen I and TGF-\(\beta\)\textsubscript{1}. In vitro DPN fully restored transcript levels of these two markers to CTRL levels, while PPT had no effect at all. This difference could also be due to the fact that in the working myocardium, we observe the contribution and effect of other cell types and not just fibroblasts.
Regarding angiogenesis stimulation, although ERβ activation was able to normalize angiogenesis in HF mice to levels similar to CTRL, ERβ activation was not potent enough as E2 to stimulate angiogenesis 3 fold above control levels, which could partially account for the lower EF observed in HF mice treated with DPN compared to E2 (from ~45% in DPN vs ~55% in E2). E2 is also more effective in rescuing HF than DPN possibly due to its anti-inflammatory and vasodilatory properties\textsuperscript{38}. Furthermore, it is possible that protective action of E2 is mediated through the newly identified estrogen receptor GPR30 \textsuperscript{39}. All these factors could contribute to a better cardiac functional recovery after E2 therapy when compared to DPN.

**The beneficial action of E2 on the heart**

Our data show that E2 therapy restores systolic function and corrects contraction and relaxation defects associated with HF in spite of the sustained presence of the stimulus (Figure 19A, B). These hemodynamic data, together with E2-induced stimulation of cardiac angiogenesis (Figures 9, 10) and reversal of LV fibrosis (Figure 12) support the view that the improvement of LV function is mediated via the action of E2 on the heart. However, the direct action of E2 on the vessel wall could not be excluded, as E2 promotes vasodilation by acting on the endothelial and smooth muscle cells\textsuperscript{40}. E2 directly stimulates endothelial nitric oxide synthase (eNOS) activity and thus has a beneficial effect on vasculature. Many reports have shown that estrogen can cause arterial dilation of elastic and muscular arteries \textit{in-vivo} via estrogen receptor (ER)-mediated activation of ERK and PI3K, and stimulation of nitric oxide production \textsuperscript{41}. Furthermore, estrogen has also been shown to promote dilation of canine coronary arteries in an endothelium-independent manner not mediated by the classic intracellular estrogen receptors \textsuperscript{42}, thus unloading of the heart may thus be an important explanatory factor for the rescue effect of E2 therapy. In addition, both ERα and ERβ receptors mediate estrogen’s vasodilatory action\textsuperscript{43}.
Therefore, it is possible that vasodilation caused by estrogen on the vessel wall has an additional contribution to the observed E2-induced rescue of HF and results in beneficial effects on cardiovascular function and improved cardiac hemodynamics.

Here we show that E2 rescues advanced HF induced by pressure overload. The beneficial action of estrogen is not limited to only pressure overload induced by TAC. Estrogen has been shown to be protective in rescuing other models of HF such as ischemia/reperfusion injury \(^44\), volume overload \(^45\), as well as right ventricular failure secondary to pulmonary hypertension\(^46\).

**Controversial role of E2 therapy for cardiovascular disease in women**

Although estrogen has been shown to be protective in several animal models of heart disease \(^5\), \(^44\), \(^47\), two large scale clinical trials, Women’s Health Initiative (WHI) and Heart and Estrogen/progestin Replacement Study (HERS), failed to show that hormone replacement therapy (HRT) can reduce heart disease in postmenopausal women\(^{48, 49}\). Recent reviews discuss possible reasons why HRT was not successful, as there was a wide range in subjects’ age (50-80 years old) and in the time of administration of HRT after menopause and women’s life style was not accounted. Furthermore, in HRT estrogen does not seem to be detrimental, since medroxyprogesterone acetate (the form of progesterone administered in HRT) significantly attenuated the beneficial effects of E2 on coronary artery plaque in monkeys\(^{50}\). The ongoing Kronos Early Estrogen Prevention Study (KEEPS) however is designed to determine the effect of different route of estradiol administration with or without progestin on atherosclerosis and coronary calcification in women which are receiving HRT within 3 years of menopause\(^{51}\). KEEPS is thus expected to shed valuable light regarding protocol, route and timing of HRT for cardiovascular disease prevention in women\(^{52}\).

Here we show that E2-induced rescue of HF is associated with the reversal of adverse LV
remodeling. The beneficial effects of E2 treatment in HF seem to result from an interplay of different factors among which restoration of cardiac aromatase and T levels, stimulation of cardiac neoangiogenesis and reversal of fibrosis are key mechanisms.

Even though E2 may have unwanted long-term side effects, our findings with relatively short term E2 treatment raise the exciting possibility to expand the application of E2 or targeted ERβ activators for the treatment of chronic heart failure within a relatively safe time frame, a concept which most definitely warrants further investigation. As both E2 and the ERβ agonist DPN reverse left ventricular dysfunction associated with a number of cardiovascular diseases, our findings have broad clinical implications.
Table 4.1. E2 therapy restores cardiac function

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<td>53.6±1.04**</td>
<td>33.2±1.13**††</td>
<td>53.1±1.3**††</td>
</tr>
<tr>
<td><strong>LV %FS</strong></td>
<td>33±0.53</td>
<td>27.6±0.696**</td>
<td>15.7±0.58**††</td>
<td>27.3±0.84**††</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>10 mice</th>
<th>5 mice</th>
<th>7 mice</th>
<th>7 mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVSP</td>
<td>104.7±3.9</td>
<td>168.5±3.5**</td>
<td>95.6±5.5†</td>
<td>135±4.6††††</td>
</tr>
<tr>
<td>LVEDP</td>
<td>6.9±3.8</td>
<td>18.2±2.6</td>
<td>9.5±4.9</td>
<td>4±2††</td>
</tr>
<tr>
<td>LVDP</td>
<td>97.8±2.66</td>
<td>150.3±2.3**</td>
<td>86.2±2.7†††</td>
<td>131±4.6††††</td>
</tr>
<tr>
<td><strong>RPP</strong></td>
<td>60868.6±2412.8</td>
<td>79454.9±8241.3</td>
<td>36251.9±3531.7</td>
<td>65455±5533.2††</td>
</tr>
<tr>
<td>(dP/dt)_{Max}</td>
<td>11036.6±495.2</td>
<td>13974.7±1172.6</td>
<td>4871.4±511.9 †† ††</td>
<td>8541.2±638.5 ††††</td>
</tr>
<tr>
<td>(dP/dt)_{Min}</td>
<td>8390.9±469.3</td>
<td>11048.5±935.57</td>
<td>4429.2±280.1 †† † †</td>
<td>7862.6±754.9 † ††</td>
</tr>
<tr>
<td><strong>HR</strong></td>
<td>622.8±19</td>
<td>530±56.5</td>
<td>421.7±40.4**</td>
<td>499.2±35.1</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. IVS indicates intra-ventricular septum; LV PW, left ventricular posterior wall; LV %EF, left ventricular ejection fraction; LV %FS, left ventricular fractional shortening; LVDP, left ventricular developed pressure; RPP, rate pressure product; (dP/dt)_{Max},
maximum rate of LV pressure rise and \((dP/dt)_{\text{Min}}\), maximum rate of LV pressure decline. Intraventricular septum (IVS), LV posterior wall (LVPW), LV chamber dimensions and LV mass were obtained from M-mode images of parasternal short axis view similar to those shown in Figure 2A. The number of animals is given in each section. *\(P<0.05\) vs. CTRL; **\(P<0.001\) vs. CTRL; †\(P<0.05\) vs. CHH; ††\(P<0.001\) vs. CHH; ^\(P<0.05\) vs. HF and ^^\(P<0.001\) vs. HF.
4.6. Reference List


Chapter 5:

Conclusions and Future Directions
In this dissertation, I first characterized the ubiquitin-proteasome system in the unique pregnancy-induced physiological hypertrophy, and then I proceeded to examine the therapeutic role of estrogen in pre-existing left ventricular heart failure induced by pressure overload as well as right ventricular failure secondary to pulmonary hypertension. Here I show that physiological hypertrophy is downregulated, with a concomitant decrease in protein ubiquitination and reactive oxygen production. To examine the therapeutic role of estrogen, I used short-duration estrogen therapy after the onset of heart failure and determined that just 10 days of estrogen therapy within physiological levels restored cardiac function. Advanced heart failure has been shown to be associated with extensive fibrosis, which has been long considered to be irreversible. Furthermore, the loss of capillary density has been identified to be a key event in the transition from cardiac hypertrophy and failure. In this dissertation I have shown that estrogen therapy can indeed reverse fibrotic scarring as well as promote angiogenesis and restore cardiac function both in the left ventricular model of heart failure as well as in the right ventricular model of hypertrophy and failure due to pulmonary hypertension.

In this dissertation I also demonstrate that estrogen exerts its beneficial action on cardiovascular function via activation estrogen receptor beta in both right and left ventricular failure models. I also demonstrate that in the pressure overload-induced left ventricular failure model, a selective estrogen receptor beta agonist was able to reverse fibrosis as well as restore cardiac angiogenesis, two processes which are vital in nurturing the heart.

Furthermore, in this dissertation I measure for the first time local estrogen and testosterone concentrations in the heart tissue. I show that i) while cardiac estrogen levels are reduced in heart failure, plasma estrogen levels remain the same, ii) in spite of having much higher levels of testosterone in the plasma in heart failure, cardiac testosterone were low, and iii) cardiac
aromatase levels are decreased in heart failure. I also show that estrogen therapy is associated with restoration of estrogen and testosterone concentrations, as well as aromatase expression in the heart.

5.1. Cardiovascular implications of estrogen replacement therapy in women

The results presented in this dissertation clearly demonstrate that estrogen therapy is very effective in rescuing cardiac function in both models of left right ventricular heart failure in mice and rats. However, two large scale clinical trials Women’s Health Initiative (WHI) and Heart and Estrogen/progestin Replacement Study (HERS), failed to support the ability of hormone replacement therapy (HRT) in postmenopausal women in reducing the risk of ischemic heart disease. The recent reviews discuss as to why HRT was deemed not to be protective since there was a wide range in age (50-80) years old, time of administration after menopause, dosage, the hormonal content of therapy, route of administration, as well as women’s life style were not accounted for. The failure of HRT therapy could also be attributed to the presence of progesterone and not estrogen, since medroxyprogesterone acetate (MPA, the form of progesterone administered in HRT) significantly attenuated the beneficial effects of E2 on coronary artery plaque in monkeys.

Two large scale randomized, double-blind, placebo-controlled clinical trials Women’s Health Initiative (WHI) and Heart and Estrogen/progestin Replacement Study (HERS), in which daily use of conjugated equine estrogens (CEE, 0.625 mg/day) plus medroxyprogesterone acetate (MPA, 2.5 mg/day) was administered to postmenopausal women with coronary disease (50-79 years old) and concluded that overall, there were no significant differences between the groups neither in the primary (WHI and HERS) nor in any of the secondary cardiovascular outcomes.
(HERS). However, both WHI and HERS failed to take into consideration the women’s lifestyle in evaluating the effect of HRT. The Copenhagen HRT examined the association between self-reported lifestyle factors, health condition and the use of HRT in over 14,000 Danish nurses, and concluded that the usage of HRT was not necessarily associated with a particularly healthy lifestyle of better overall health\(^8\). The study advises to specifically consider lifestyle factors when evaluating the effect of HRT. Moreover, it is not clear whether the hormone dosage and combination employed in by WHI and HERS is appropriate for preventing or reducing CVD risk. CEE alone at a lower dose (0.3 mg/day) was able to decrease major coronary events in women \(^9\), and decreased coronary artery atherosclerosis in monkeys\(^{10}\). Progestins however downregulate estrogen receptors and have direct progestin receptor-mediated effects which oppose estrogenic action\(^{11}\), and MPA may do this to a greater extent. The ongoing Kronos Early Estrogen Prevention Study (KEEPS) however is designed to determine the effect of different route of estradiol administration (CEE or transdermal E2) with or without progestin on atherosclerosis and coronary calcification in women which are receiving HRT within 3 years of menopause\(^{12}\). KEEPS is thus expected to shed valuable light regarding protocol, route and timing of HRT for CVD prevention in women\(^{13}\).

5.2. Estrogen treatment of male patients

The results of my study show that estrogen therapy is very effective in rescuing heart failure in male mice as in females. Estrogen therapy has been previously used in men and none of these studies reported any complications resulting from estrogen therapy, including in the cardiovascular system. Several decades ago, Carani et al. demonstrated the efficacy of transdermal estradiol (TE) in closing the epiphyses in a man with an inactivating mutation of the
aromatase gene\textsuperscript{14}. Other studies have also shown that estrogen supplementation to male patients promotes an increase in bone mass as well as bone maturation\textsuperscript{14-16} as seen in women\textsuperscript{2, 3}. Albeit the estrogens used differ in composition between the studies, the dose of estrogens administered was much higher and for much longer duration than used in our studies. For example, Bilzeikian et al. used 0.3 mg/day conjugated estrogens, which was then increased gradually to 0.75 mg/day for at least one year\textsuperscript{15}, while Hermann et al. used estradiol at a dose 0.83 µg/kg/wk for 3 months, and then reduced it to 0.42 µg/kg/wk \textsuperscript{16}. The dose of estrogen used in our studies in male (and female) mice and rats equates to roughly 0.03 µg/kg/day for a short duration. This short-term and low dose treatment with estrogen conferred significant beneficial effects on the cardiovascular system, a finding which warrants further investigation.

5.3. Study limitations and future directions

The studies presented in this dissertation indicate that estrogen exerts its beneficial effects on the cardiovascular system (both in the pressure overloaded left ventricle as well as the right ventricular hypertrophy and failure secondary to pulmonary hypertension) mainly via estrogen receptor beta activation. selective estrogen receptor beta agonist was able to rescue the heart failure in both models, whereas estrogen receptor alpha agonist had no effect in my study. The agonists have much higher selectivity for their particular subreceptor, but they can still bind to the other estrogen receptors, possibly even GPER\textsuperscript{30}. Also, these agonists might have other unknown physiological effects. As I show that estrogen rescues both right and left ventricular dysfunction by estrogen receptor beta activation, further knockout studies are required. Indeed, if estrogen has the ability to recover cardiac function in estrogen receptor alpha knockout, but not
beta knockout animals, agonists of estrogen receptor beta for treatment of heart failure would eliminate the risk for unwanted estrogen effects.

Although I show remarkable rescue by estrogen in the pressure overloaded failing left ventricle, I did not assess the long-term effects after estrogen cessation. All animals presented in this study have sacrificed immediately after 10 days of estrogen treatment. Therefore, further experiments are required to examine whether the beneficial actions of estrogen on the heart are maintained after estrogen withdrawal. Animals should be followed long-term after estrogen withdrawal and their cardiovascular function should be serially monitored by echocardiography to understand whether estrogen exerts its beneficial effects long-term, or whether there is a time point at which cardiovascular function in estrogen-rescued animals starts to decline. It would also be of interest to know whether decreased fibrosis and normalized angiogenesis persist long-term, or in case there is a time point in which cardiovascular function starts declining, whether these two beneficial actions of estrogen are diminished.

Although we show that the effects of estrogen in reversing right ventricular dysfunction persist 12 days after estrogen withdrawal, the long-term effects of this therapy on the right ventricle also remain to be elucidated. Although the data presented in this dissertation indicates that 12 days after estrogen cessation the right ventricular systolic pressures are significantly reduced versus immediately after estrogen treatment, it is also of interest to know for how long these effects persist. Therefore, estrogen withdrawal animals should also be monitored long-term and their right ventricular function and pulmonary arterial pressures should be serially determined via echocardiography. Similarly to left ventricular rescue, fibrosis and angiogenesis should be assessed long term or at the point in which estrogen-induced rescue begins to decline.
Here I show that estrogen reverses cardiac fibrosis both in left and right ventricular failure, which may seem surprising as fibrosis has been long considered to be permanent and irreversible scarring. Therefore, it is very important to unravel the molecular mechanisms as how estrogen is reversing cardiac fibrosis. MicroRNAs are short noncoding RNAs that either inhibit transcription or regulate translation of genes, and most recently they have emerged as key players in mammalian biology. Several reports have implicated microRNAs in the regulation of fibrosis in different tissues \(^{17, 18}\). Thus, RNA samples from both left and right ventricular failure and estrogen rescued animals should be sent for microarray analysis to investigate whether heart failure regulates microRNAs implicated in fibrosis, and whether estrogen therapy is able to restore the levels of these microRNAs to control animals. These findings can lead to a more definitive mechanism for estrogen induced rescue of the two models of ventricular failure discussed in this dissertation.

### 5.4. Concluding remarks

In summary, during the course of my dissertation project I have acquired a wealth of knowledge regarding the physiological and pathological cardiac adaptations both in the left and right ventricle. This has allowed me to gain novel insights into the molecular mechanisms occurring during cardiac hypertrophy, as well as to determine potential therapeutic targets for heart failure. Considering that cardiovascular disease is still the number one cause of morbidity and mortality, I hope that these findings can be applied towards improving cardiac function for this lethal disease. Even though estrogen may have unwanted long-term side effects, our findings with relatively short term estrogen treatment raise the exciting possibility to expand the application of estrogen or targeted estrogen receptor activators for the treatment of chronic heart failure within
a relatively safe time frame, a concept which most definitely warrants further investigation. As both estrogen and an estrogen receptor beta agonist reverse left and right ventricular dysfunction associated with a number of cardiovascular diseases, these findings have broad clinical implications and certainly warrant further investigation in larger animal models which have more similarities to human hearts.

5.5. Reference List


