ABSTRACT

Title of Dissertation: **CARDIAC AND VASCULAR FUNCTIONAL RESPONSES TO β2-ADRENERGIC RECEPTOR STIMULATION: EFFECTS OF SEX, AGE AND HEART FAILURE**

Yuan Liu, Doctor of Philosophy, 2024

Dissertation directed by: Assistant Professor, Sarah Kuzmiak-Glancy, Ph.D., Department of Kinesiology

Proper cardiovascular function is necessary to regulate the flow of blood to different parts of the body in response to demand. The ability of the heart to increase the amount of blood pumped and the precise control of blood flow to the skeletal muscle are of critical importance during movement – whether during exercise or while performing activities of daily living. This dissertation aims to assess the functional responses of the cardiac and vascular systems to β2-adrenergic receptor stimulation and identify factors influencing their responsiveness. Utilizing rat models of aging and heart failure, we investigated how sex, age, and heart failure impact the cardiac and vasculature responses to β-adrenergic receptor stimulation. In the first Aim, we explored the influence of the presence of estrogen on heart rate, coronary flow rate, and oxygen consumption rate when stimulated with a β2-adrenergic receptor agonist in perfused hearts from young and old, male and female rats. The presence of estrogen rescued the blunted heart rate response to β2-adrenergic receptor stimulation seen in young female compared to young male hearts. Old male and female hearts showed blunted heart rate responses compared to their young sex-matched controls; however, old males and females were similar in their responsiveness to β-adrenergic stimulation. In the second Aim, we evaluated the effects of a rat model of pressure-overload induced heart failure on cardiac responsiveness to β2-adrenergic stimulation in male and female hearts, again in the absence and presence of estrogen. Failing male and female hearts had similar heart rate responses to their sham counterparts. Comparing to the sham control female heart, heart failure female hearts show an impaired coronary flow rate increase in response to β-adrenergic stimulation with presence of estrogen, despite similar increases in heart rate. Aim 3 focused on measuring vascular responsiveness of an isolated muscular artery to β-adrenergic and estrogen receptor stimulation in young and middle-aged, male and female rats. Female rats demonstrated augmented vasodilation responses to β-adrenergic receptor stimulation compared to males, and estrogen enhances artery vasodilation response to β-adrenergic receptor stimulation in young female rats.

The primary goal was to investigate how the acute presence of estrogen affected cardiovascular regulation in young and old, male and female rats. Conducting experiments in young and old, male and female, heart failure and healthy rats uncovers how the acute presence of estrogen affects β-adrenergic receptor stimulation responsiveness in the ventricular myocardium and muscular artery vasculature. Our findings reveal sex differences in cardiac and vascular responses to β-adrenergic receptor stimulation, highlighting the influence of sex hormones, particularly estrogen in the regulation of the cardiovascular system. We propose these are due, at least in part, to the membrane estrogen receptor, GPR30, and its downstream signaling pathway. These insights contribute to a better understanding of estrogen's role in the acute regulation of cardiac and vascular function, informing future age and sex-specific treatments for cardiovascular diseases.

CARDIAC AND VASCULAR FUNCTIONAL RESPONSES TO β2-ADRENERGIC RECEPTOR STIMULATION: EFFECTS OF SEX, AGE AND HEART FAILURE

by

Yuan Liu

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2024

Advisory Committee:
  Assistant Professor Sarah Kuzmiak-Glancy Ph.D., Chair
  Associate Professor Sushant Ranadive, Ph.D.
  Associate Professor Steven J. Prior, Ph.D.
  Professor Matthew W. Kay, Ph.D.
  Associate Professor Byung-Eun Kim, Ph.D.

Dedication

I dedicate this dissertation to my parents, whose unwavering support and love have been the bedrock of my inspiration throughout my academic journey. Additionally, I extend heartfelt gratitude to my life partner, Dr. Yuan Yao, for not only believing in me but also for being my steadfast companion through all the highs and lows. Her support and guidance were instrumental in navigating the challenges of doctoral research, and this work stands as a testament to our shared journey.

Acknowledgements

I would like to express my deep appreciation and gratitude to those who have contributed to the completion of this dissertation. First and foremost, I am indebted to my dissertation advisor, Dr. Sarah Kuzmiak-Glancy, for her unwavering support, guidance, and scholarly insight. Her constructive feedback and mentorship have been invaluable throughout this research journey.

I extend my heartfelt thanks to the faculty members of the Department of Kinesiology at University of Maryland for their intellectual contributions and stimulating discussions. The collaborative environment fostered by this department has enriched my academic experience.

I am grateful to my research collaborators, Dr. Harry Zichen Li, Dr. Gabriel S. Pena, and Maria Canellas da Silva, for their collaboration, thoughtful insights, and shared commitment to advancing knowledge in our field.

Special appreciation goes to my life partner, Dr. Yuan Yao, whose encouragement, understanding, and sacrifices have been my pillars of strength. Your belief in me and unwavering support sustained me through the challenges of doctoral research.

I would also like to acknowledge my parents, Bingqin Liu and Qihai Liu, whose love, encouragement, and sacrifices laid the foundation for my academic pursuits. Your belief in my potential has been a driving force behind my achievements.

This dissertation is a collective effort, and I am deeply thankful to each individual and organization mentioned above for their contributions, whether big or small. Your support has been instrumental in the successful completion of this academic endeavor.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AC – Adenylyl cyclase

ACE – Angiotensin-converting enzyme

Ach – Acetylcholine

AKAP5 – A-Kinase Anchoring Protein 5

Ang II – Angiotensin II

ANS – Autonomic nervous system

ATPase – Adenosine triphosphatase

AV – Atrioventricular

β-ARs – β-adrenergic receptors

β1-ARs – β1-adrenergic receptors

β2-ARs – β2-adrenergic receptors

β3-ARs – β3-adrenergic receptors

βARK1 – β-adrenergic receptors kinase 1

cAMP – Cyclic adenosine monophosphate

CO – Cardiac output

COX-2 – Cyclooxygenase-2

CVD – Cardiovascular disease

EDCF – Endothelial-derived contracting factors

EDHF – Endothelial-derived hyperpolarizing factors

EDRF – Endothelial-derived relaxing factors

EDV – End-diastolic volume

EF – Ejection fraction

eNOS – Endothelial nitric oxide synthase

ERs – Estrogen receptors

ERα – Estrogen receptor α

ERβ – Estrogen receptor β

ET-1 – Endothelin-1

FAO – Fatty acid oxidation

G_i – Inhibitory unit of G protein

G_s – Stimulatory unit of G protein

GPR30 – G-protein-coupled receptor 30

HF – Heart failure

HR – Heart rate

IACUC – Institutional Animal Care and Use Committee

LV – Left ventricle

MAGUK – Membrane-associated guanylate kinases

MAPK – Mitogen-activated protein kinase

mRNA – Messenger ribonucleic acid

NO – Nitric oxide

OVX – Ovariectomized

PGH2 – Prostaglandin H2

PGI2 – Prostaglandin I2

PI3K – Phosphatidyl inositol 3 kinases

PKA – Protein kinase A

PNS – Parasympathetic nervous system

PO2 – Oxygen partial pressure

ROS – Reactive oxygen species

SA – Sinoatrial

SNS – Sympathetic nervous system

SV – Stroke volume

TAC – Transverse aortic constriction

TxA2 – Thromboxane A2

VEGF – Vascular endothelial growth factor

Chapter 1: Introduction

The cardiovascular system, which includes the heart, blood, and blood vessels, plays an essential role in transporting oxygen and nutrients throughout the body. The blood carries oxygen and nutrients and delivers them to the tissues to meet metabolic demands. The heart generates pressure to ensure continuous blood flow through the blood vessels. When the metabolic demand in the tissues increases, more oxygen and nutrients need to be delivered. Therefore, the heart needs to work harder to pump more blood to the tissues. As the heart works harder, it also needs more blood itself to provide oxygen and nutrients.

Certain types of cardiovascular diseases (CVD) are associated with the inability of heart to pump an adequate amount of blood to meet the body's demands during physical activity [1]. This contributes to the challenges individuals face to perform activities of daily living. [2]. This condition is known as exercise intolerance and is a critical aspect of heart failure (HF). Additionally, CVD present distinct risks for men and women, with women generally having a lower prevalence of CVD compared to age-matched men [3]. These elements contribute to the challenges individuals encounter in sustaining a high quality of life amid concerns related to cardiovascular health.

Cardiac output (CO) is the amount of blood pumped out of the heart per minute. CO is the product of the heart rate (HR) and the stroke volume (SV), which is the amount of blood pumped out of the heart per beat. CO plays an essential role in determining the delivery of oxygen and nutrients to the tissues. When the demand for oxygen and nutrients to a tissue increases, such as during exercise, HR and SV increase, resulting in an increased in CO to meet the demand. The skeletal muscle vasculature also plays a role in the regulation of blood flow to tissues as vasodilation and vasoconstriction work to increase and decrease blood flow, respectively, to different tissues. Together, the coordinated efforts of the heart and the vasculature work to increase the delivery blood to metabolically active tissues when there is an increase in demand.

1.1 Heart Rate

The sinoatrial (SA) node, located in the right atrium of the heart, generates an electrical signal to initiate cardiac contraction. In autorhythmic cells, the electrical activity is determined by the movements of Na^+, $K^{+,}$ and Ca^{2+}. During the spontaneous depolarization phase, the funny channels open to allow Na^+ to flow into the cell and K^+ to flow out. Once the membrane potential reaches the threshold, Ca^{2+} channels open, causing rapid Ca^{2+} flow into the cell to further depolarize it. Once the potential reaches its peak, K^+ channels open, allowing K^+ ions to move out, and the repolarization phase occurs. Therefore, iron channels, such as Ca^{2+} channels and K^+ channels, play essential roles in regulating the rate of the SA node [4].

The firing rate of the SA node's electrical signal determines the HR, making the SA node the pacemaker of the heart [4]. After being generated from the SA node, the electrical signal travels through the heart's electrical conduction system, including the atrioventricular (AV) node, and spreads throughout the ventricles, leading to ventricular contraction [4]. Several factors can modulate HR, such as the autonomic nervous system and catecholamines. The autonomic nervous system (ANS) contains two branches: sympathetic (SNS) and parasympathetic nervous system (PNS). HR is controlled by SNS and PNS activities, as both branches innervate the SA node. The firing rate of the SA node is around 100 action potentials per minute without innervation from the ANS. At rest, the PNS plays a dominant role in decreasing the action potential rate of the SA node to 60-80 per minute [5]. Activation of the PNS hyperpolarizes pacemaker cells, decreasing the rate of depolarization of the SA node, and decreases HR. During exercise, the increased HR is a result of the withdrawal of PNS and activation of the SNS [5].

Activation of the SNS increases heart rate by increasing both Na^+ and Ca^2 current into the autorhythmic cells, thereby increasing the rate of SA node depolarization [4]. During SNS activation, sympathetic neurons release norepinephrine, which binds to the β-adrenergic receptors, activating the cAMP secondary messenger system. cAMP augments the opening of funny channels and Ca^+ channels in autorhythmic cells to enhance both Na^+ and Ca^2 movement, which increases the spontaneous depolarization rate [4]. This results in an increase in the action potential frequency, increasing the HR. The enhanced SNS activity also stimulates the adrenal medulla to release epinephrine. The increased circulating level of epinephrine could also increase HR via binding β-adrenergic receptors. Conversely, the PNS releases acetylcholine (Ach) which slows HR. Ach slows the HR via its influence on Ca^+ and K^+ permeability. The K^+ permeability increases, leading to hyperpolarization, causing the pacemaker cells to be more negative [4]. Additionally, the Ca^+ permeability decreases, and the pacemaker has a slower depolarization. Together, HR is modulated through the PNS and SNS by altering the rate of action potential firing at the SA node.

1.2 Stroke Volume

One factor that modulates SV is the contraction force of the ventricles: when the ventricular contraction force increases, the SV increases. The amount of blood in the left ventricle and cardiac contractility (i.e. positive inotropy) both affect overall ventricular contractility. According to the Frank-Starling Law, which shows the relationship between the volume of blood and the force of contraction, a higher end-diastolic volume, increasing the length of muscle fiber, will lead to a higher contraction force [6]. The end-diastolic volume is largely determined by the venous return, which is influenced by three factors: the skeletal muscle pump, the respiratory pump, and sympathetic innervation [6]. The skeletal muscle pump refers to the squeezing of veins by the skeletal muscles during contraction, which pushes blood back to the heart. During the inspiration phase of respiration, the diaphragm moves down into the abdomen, enlarging the thoracic cavity and creating a subatmospheric pressure that draws more blood from veins in the abdomen into the vena cava. At the same time, higher pressure is placed on abdominal veins, compressing them. This combined effect creates the respiratory pump, which increases venous return. SNS stimulation can also affect the venous return. Skeletal muscle vasculature largely vasoconstricts in response to SNS innervation, resulting in more blood returning to the heart.

Nervous and endocrine systems both affect cardiac contractility. The catecholamines released from nervous and endocrine systems enhance cardiac contractility by binding to β-adrenergic receptors (β-ARs). β-ARs have three subtypes: β1, β2, and β3. β1-ARs are mainly found in the heart and are also located in the kidney and other tissue. β2-ARs have a more widespread distribution than β1-ARs, being expressed in the heart, lung, kidney, and blood vessels. β3-ARs are only found in the adipose tissues and heart [7]. All three β-ARs are expressed in the heart, with β1-ARs comprising 75-80% of total cardiac β-ARs, with β2-ARs comprise 20-25%. Only a small amount of β-ARs in the heart are β3-ARs [7]. Stimulation of β1-ARs leads to increased heart rate and enhanced cardiac contractility. Stimulation of β2-ARs has a generally weaker effect on heart rate and cardiac contractility than β1 but can also cause coronary artery vasodilation [7].

Stimulation of β-ARs affects cardiac contractility by playing a role in the regulation of cytosolic Ca^{2+} kinetics. Epinephrine and norepinephrine are two catecholamines that bind to and activate β-ARs on the myocardial cell membrane. Then the cAMP secondary messenger system is activated, resulting in the phosphorylation of both voltage-gated Ca^{2+} channels and phospholamban. Phosphorylation of the voltage-gated Ca^{2+} channels increase the channels' open time, allowing more Ca^{2+} to flow into the cells and increasing cytosolic Ca^{2+}. Phosphorylation of phospholamban also increases cytosolic Ca^{2+} by enhancing Ca^{2+}-ATPase on the sarcoplasmic reticulum (SR) [91]. The basic function of the SR is to take up and release Ca^{2+}, and the Ca^{2+}-ATPase is responsible for the reuptake of Ca^{2+} into the SR from the cell cytosol. The enhanced Ca^{2+}-ATPase activity concentrates Ca^{2+} in the SR, making more Ca^{2+} available to be released into the cytosol. Then, Ca^{2+} binds to actin filaments to allow the active sites to be exposed. The myosin head binds to the actin filament to form the crossbridge, which is the basic unit of muscular contraction. Therefore, a higher cytosolic Ca^{2+} level is predicted to activate more crossbridges and lead to a stronger contraction. After the power stroke generated by the crossbridge, the Ca^{2+} needs to be removed from the cytosol to relax the muscle fibers. The enhanced Ca^{2+}-ATPase uptake of Ca^{2+} from the cytosol shortens the removal time, increasing the speed of relaxation. Therefore, the increase in CO that occurs to an increase in demand is a function of both SNS and β-AR stimulation of HR and β-AR stimulation of the ventricular tissue, increasing cardiac contractility, and therefore, SV.

2. Responsiveness to β-Adrenergic Stimulation and β-ARs Expression in cardiac tissue

2.1 Effects of Age on β-ARs

Sex and age are important determinants of the cardiac response to β-adrenergic stimulation. Isoproterenol, a β-ARs agonist that activates all three receptor subtypes, shows decreased HR responses with age [8]. Old male rats have a blunted maximal HR and HR increase in response to isoproterenol compared to young rats. In men, the dose of isoproterenol required to increase resting HR by 25 beats per minute increases with age [9], indicating a decrease in the responsiveness of this pathway. One explanation for the lower β-ARs responsiveness could be the density of β-ARs, however experimental data on this are inconclusive. White et al. [10] reported that younger individuals had significantly higher β1-AR expression in the left ventricle compared to older individuals. Another investigation showed sex difference in aging effect on β1-AR expression. In women, β1-AR expression in heart decreases with age until menopause, where it stabilizes, but the correlation between age and β1-AR expression is not significant in men [11]. Moreover, another study that evaluated β-ARs expression in young and old male rat hearts found no difference [12]. Therefore, it remains unknown if altered β-ARs responsiveness is driven by altered β-ARs density, as this may differ between males and females, as well as between humans and animal models.

2.2 Effects of Sex on β-ARs

Male rat cardiac myocytes show a greater increase in contractility to non-selective β-ARs agonist stimulation than females, indicating stronger β-ARs signaling in male rats. Just like with age, differential β-AR responsiveness between male and female rat hearts may be explained, at least in part, by β-ARs expression [13]. According to Vizgirda et al. [13], male rat ventricular myocytes have twofold greater β-ARs expression compared to females. Peter et al. [14] also reported that male rat hearts express more β-ARs than female rats. These data support the functional studies demonstrating higher functional responsiveness to β-AR stimulation in male, compared to female, hearts. Interestingly, another study found no difference in ventricular β1-AR and β2-AR protein and mRNA levels between male and female rats [15]. Al-Gburi et al. [16] reported that β2-AR expression in blood vessels is similar in male and female rat hearts, but female rats have more β1-AR expression than male rats. Thus, sex differences in β-ARs expression exist, but ambiguity remains regarding the effect of sex on each subtype in the vasculature and the ventricular tissue.

There is copious evidence that estrogen affects the expression and responsiveness of β-ARs in the heart. Ovariectomy (removal of the ovaries) decreased β2-AR expression in female rat hearts, and four weeks of estrogen treatment restored it [17]. Another study reported that ovariectomy doubled ventricular β1-AR expression, which estrogen treatment brought back to the levels of sham-operated controls [18]. To evaluate the effect of estrogen on β-ARs responsiveness, Sherwood et al. [19] measured HR in response to the non-selective β-ARs agonist, isoproterenol, in age-matched pre- and post-menopausal women. Post-menopausal women have a blunted HR in response to the β-ARs agonist than pre-menopausal women even though they were the same age, suggesting that the chronic or acute presence of estrogen alters β-AR responsiveness.

2.3 Effects of Heart Failure on β-ARs

β-ARs responsiveness and expression are altered in HF [20]. The change in β-ARs responsiveness is partially due to changes in β-ARs expression, as downregulated β-ARs expression has been observed in HF patients. Additionally, β-ARs sensitivity may also contribute to the blunted responsiveness [21]. The remaining β-ARs may be desensitized due to increased circulating catecholamines [22]. The mechanisms of β-ARs desensitization in HF are likely related to the sympathetic nervous system and the release of catecholamines. HF is often associated with increased circulating catecholamines, which can lead to enhanced expression of β-ARs kinase (βARK1) [22]. βARK1 is a member of the G protein-coupled kinase family that is responsible for the desensitization and downregulation of β-ARs. Therefore, the failing heart may protect itself from chronic sympathetic stimulation by decreasing β-AR expression and responsiveness [22]. Another possible mechanism is the change in the expression ratio between β1-AR and β2-AR in HF. In a healthy heart, β1-AR expression is about four times higher than β2-AR [21]. In a failing heart, β1-AR expression decreases, and β2-AR expression increases or remains the same, resulting in a ratio that could reach 3:2 [21]. β1-AR only couples to the stimulatory unit of G protein, while β2-AR can couple to both the stimulatory (G_s) and inhibitory (G_i) units of G protein. The β2-AR/G_s pathway leads to cyclic adenosine monophosphate (cAMP) production and results in increased contractility, while the β2-AR/G_i pathway inhibits this effect of β2-AR/G_s activation [21]. Therefore, increased β2-AR expression may offset the enhanced contractility stimulated by the G_s pathway.

3. β-Adrenergic Regulation of Skeletal Muscle Vasculature

β-adrenergic receptor (β-AR) stimulation induces vasorelaxation in the skeletal muscle vasculature. This effect is abolished in β-AR knock-out mice [23]. Skeletal muscle vascular responses to β-AR stimulation appear to be sex- and age-dependent, with women showing greater responsiveness than men, and aging leading to blunted responses [24-26]. Human studies have shown that β2-AR agonists have sex-dependent effects on skeletal muscle vascular responses. In one study, forearm blood flow was measured after infusion of the β2-AR agonist albuterol in young men and women. Albuterol caused forearm vasodilation in both sexes, but women showed greater forearm blood flow to β2-AR stimulation than men [24]. Schutzer et al [26] reported that β2-AR-mediated vasodilation in rat aorta was significantly blunted in older rats compared to younger rats, indicating that the vasculature regulation is significantly influenced by aging. Estrogen may play an essential role in the sex difference in β-AR stimulation responses. A previous study of both pre- and post-menopausal women found that post-menopausal women had a blunted forearm blood flow increase after isoproterenol infusion compared to pre-menopausal women [27]. Ferrer et al. [28] reported that β-AR agonist-induced vasodilation in ovariectomized rat mesenteric arteries was significantly greater in estrogen-treated rats compared to untreated rats. Therefore, the acute or chronic presence of estrogen may enhance β2-AR responsiveness in the vasculature, as well as the ventricular tissue.

4. Role of Estrogen in Cardiovascular Health

Estrogen has a wide range of effects on the body, some of the most notable being vasodilation, inflammation reduction, and cholesterol levels maintenance. Estrogen is associated with increased angiogenesis, stimulation of vasodilation, and enhanced mitochondrial function, which play a protective role in cardiovascular health [29, 30]. Angiogenesis can be induced by estrogen stimulation of vascular endothelial growth factor (VEGF) gene expression [29, 30]. Estrogen regulates vascular function by modulating endothelial-dependent factors, including endothelial-derived relaxing factors (EDRF) and endothelial-derived contracting factors (EDCF) [31]. EDRF includes nitric oxide (NO), prostacyclin (PGI2), and endothelial-derived hyperpolarizing factors (EDHF), while EDCF includes endothelin-1 (ET-1), angiotensin II (Ang II), thromboxane A2 (TxA2), and prostaglandin H2 (PGH2). Estrogen enhances EDRF and attenuates EDCF activities and expressions, resulting in vasodilation of blood vessels.

Estrogen receptors (ERs) are crucial players in cardiovascular regulation, with three primary subtypes identified as estrogen receptor α (ERα), estrogen receptor β (ERβ), and G-protein-coupled receptor 30 (GPR30). These receptors are distributed between cellular compartments, with ERα and ERβ classified as both nucleus and membrane receptors, while GPR30 functions exclusively as a membrane receptor. Their presence in cardiovascular tissues, including ventricular myocytes, endothelial cells, and smooth muscle cells, underscores their significance in the cardiovascular homeostasis.

Estrogen, through its binding affinity with ERs, exerts its regulatory influence on gene expression and signaling molecules via both genomic and non-genomic pathways [32]. In the genomic pathway, estrogen binds to nuclear receptors ERα and ERβ, culminating in the activation of associated gene transcription. Notable among these genes are endothelial NO synthase (eNOS) and VEGF. Estrogen-mediated upregulation of eNOS gene expression enhances eNOS density, promoting increased nitric oxide (NO) production, thereby facilitating vasodilation [17, 33]. Additionally, VEGF, a key factor in angiogenesis, is subject to estrogen-induced modulation, as discussed above. Conversely, the non-genomic pathway involves the binding of estrogen to membrane receptors, including ERα and GPR30, leading to the activation of various signaling pathways, such as cAMP/protein kinase A (PKA), phosphatidylinositol 3 kinases (PI3K)/AKT, and MAPK [3, 32, 34]. These pathways collectively contribute to the orchestration of cellular responses, highlighting the estrogen impact on cardiovascular function.

5. Estrogen Effects on Vasculature

Estrogen has multiple effects on vascular function, including vasodilation, anti-inflammatory, antioxidant, and antiproliferative effects [35]. One mechanism by which estrogen affects vasodilation is by modulating endothelial-dependent factors, such as EDRF and EDCF. One of the primary effects of estrogen on EDRF is to promote the synthesis and release of nitric oxide (NO). Estrogen has both rapid and long-term effects on NO regulation. Rapidly, estrogen can activate eNOS to generate more NO, and chronically, estrogen can increase the expression of genes encoding eNOS to increase eNOS density via the genomic pathway [17, 36]. Estrogen activates eNOS through ERα and ERβ, and the PI3K/AKT/eNOS pathway is involved in both the acute increase in eNOS activity and the chronic enhancement of eNOS expression [37]. Additionally, the membrane receptor GPR30 also activates the PI3K/AKT/eNOS pathway. Cyclooxygenase-2 (COX-2) is an enzyme expressed in endothelial cells. When endothelial cells are stimulated by estrogen, COX-2 produces prostacyclin (PGI2), which can relax and widen blood vessels. Animal studies have shown that 18 hours of estrogen treatment enhances COX-2 expression and PGI2 production [38]. Ovariectomized (OVX) mice also show an increase in PGI2 production after long-term (7 days) estrogen exposure. Taken together, estrogen can cause vasodilation by increasing PGI2 production through both short- and long-term effects. Estrogen affects EDHF regulation in several ways [39]. For example, estrogen treatment restores EDHF-induced vasodilation in isolated small arteries from OVX animals, which indicates that the presence of estrogen can enhance EDHF-induced vasodilation [40]. Estrogen can also contribute to increased EDHF-mediated vascular responses to changes in vascular flow. Additionally, estrogen-induced acute vasodilation in coronary arteries also involves EDHF. Therefore, estrogen has been shown to play an essential role in EDHF-induced vasodilation in several arteries.

Estrogen inhibits the production of EDCFs, including endothelin-1 (ET-1) and angiotensin II (Ang II). ET-1 is a potent vasoconstrictor that can also stimulate cell proliferation and fibrosis. A study using human umbilical vein endothelial cells (HUVECs) found that 48 hours of estrogen treatment decreased ET-1 expression and release [41]. This indicates that estrogen blunts the vasoconstrictive effects of ET-1. There is a sex difference in sensitivity to ET-1 in coronary resistance vessels: male rats show more significant vasoconstriction in response to ET-1 than female rats [42]. However, as rats age, males tend to have decreased ET-1-induced vasoconstriction in coronary resistance vessels, while females have increased vasoconstriction in response to ET-1 [43]. This suggests that the enhanced vasoconstrictive effect of ET-1 in females may be related to decreased estrogen levels. Ang II is another potent vasoconstrictor that is regulated by estrogen. Estrogen reduces the activity of angiotensin-converting enzyme (ACE), a key enzyme in the conversion of Ang II. A study using a rat model found that OVX rats had higher ACE mRNA concentrations than OVX rats treated with estrogen [44]. This finding indicates that estrogen reduces ACE activity by inhibiting ACE mRNA synthesis, which in turn affects Ang II-induced vasoconstriction. Estrogen can also diminish the effects of Ang II on the sympathetic nervous system (SNS). Estrogen has a positive impact on parasympathetic nervous system (PNS) activity and a negative impact on SNS activity [45]. This offsets the positive effect of Ang II on the SNS. Overall, estrogen enhances vasodilation and attenuates vasoconstriction by increasing EDRF production and decreasing EDCF production.

6. Factors Affect Estrogen Receptors Expression

The expression of estrogen receptors (ERs) can be influenced by aging and HF. In women, serum estrogen levels decrease with age, but the effects of this decline on ER expression are not fully understood. Previous studies have shown that ERα expression decreases in aging female animals compared to younger animals [24, 46]. mRNA levels of ERα also show an age-related decline. There are conflicting findings regarding the effects of aging on ERβ expression. Koenig et al. [24] reported that ERβ expression was two-fold greater in old female mice than in young females, suggesting that aging has opposite effects on ERα and ERβ expression. Another study supports this finding, showing that ERβ mRNA levels also increased with age in mice [46]. However, Davezac et al. [46] found that ERβ expression in the aorta was slightly decreased in old females compared to young animals, similar to the effects of aging on ERα expression. There have been few studies on the effects of HF on ER expression. In one study that compared HF patients to a healthy control group, ERα mRNA and protein expression were nearly twofold higher in cardiomyocytes of HF patients [47]. Overall, more research is needed to understand the effects of aging and HF on ER expression, particularly ERβ and GPR30.

7. β-ARs and ERs Pathways Interaction

G-protein-coupled receptor (GPCR) GPR30 couples to both stimulatory (G_s) and inhibitory (G_i) G protein subunits. G_s activation triggers the cAMP/PKA pathway, which can lead to increased cytosolic Ca^{2+} levels. GPR30 may also inhibit the adenylyl cyclase (AC) enzyme through the MAGUK/AKAP5 complex, which can inhibit the cAMP/PKA pathway. GPR30 activates the PI3K/AKT pathway via the G_i pathway, resulting in anti-apoptotic effects. However, GPR30 can also activate calmodulin/Ca^{2+}/calmodulin kinase II, which can lead to apoptosis. As GPCRs, both β-adrenergic receptors (β-ARs) and GPR30 activate the G_i and G_s pathways (Figure 1). β1-AR, β2-AR, and GPR30 can couple to G_s subunits, while β2-AR and GPR30 can couple to G_i subunits. Interestingly, cAMP levels in response to β-adrenergic stimulation are twice as high in male hearts as in female hearts [48]. If estrogen augments cAMP generation, this may enhance the response to β-adrenergic stimulation. Another study found that GPR30 expression in mouse hearts is greater than ER expression, suggesting that GPR30 may play an essential role in the acute response to estrogen activation [49].

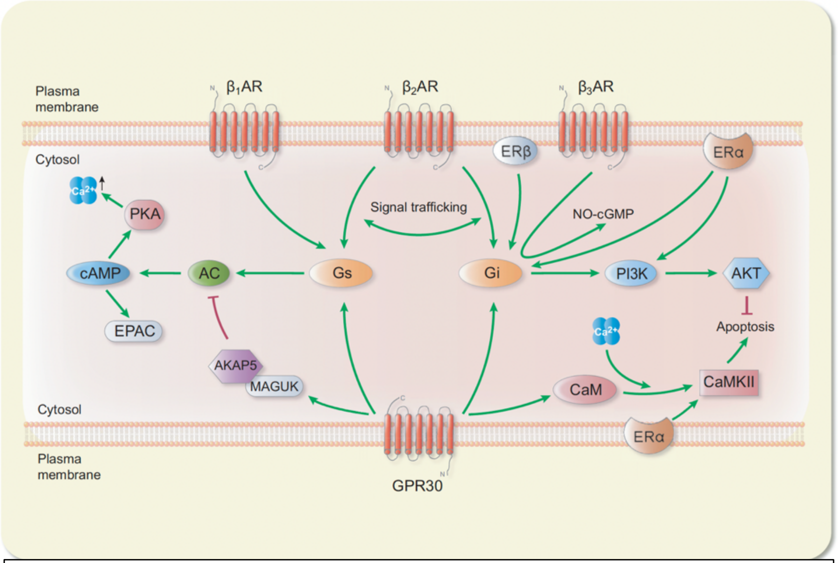


Figure 1. β-adrenergic receptors and estrogen receptors signaling pathways, taken from Machuki et al. [48].

8. Summary

The cardiovascular system is a vital component of the human body responsible for transporting oxygen and nutrients. Cardiac output, determined by HR and SV, is crucial for ensuring adequate blood flow to meet metabolic demands. β-ARs show significant influence in regulating cardiac function, includes increasing HR, enhancing cardiomyocyte contractility, and inducing coronary vasodilation. Sex, age, and HF are three factors that could affect β-ARs expression and responsiveness. Aging appears to result in a reduced β-AR response, either due to a decrease in β-AR density or a blunted responsiveness to stimulation. HF leads to alterations in both β-AR responsiveness and expression. The downregulation of β-AR expression, partly due to the increased circulating catecholamines in HF, contributes to the blunted responsiveness. Additionally, changes in the expression ratio between β1-AR and β2-AR can further impact the contractility of the heart. Moreover, research indicates that there is a sex difference in β-AR responsiveness, which is stronger in males compared to females, could be linked to the higher expression of β-ARs in males. Sex hormones, particularly estrogen, have been shown to positively affect β-ARs in the heart, increasing the expression and enhancing the responsiveness. In terms of skeletal muscle vasculature, β-ARs stimulation induces vasodilation, and this effect varies between sexes and ages. Studies have demonstrated that women exhibit greater responsiveness to β-ARs stimulation, while aging tends to diminish these responses. Taken together, estrogen has been found to influence β-ARs expression and responsiveness, especially in females, enhancing cardiac function and vasodilation. The interaction between estrogen and β-ARs is a complex and multifaceted process that involves multiple signaling pathways and has the potential to contribute to sex and age-related differences in cardiovascular responses. Understanding these interactions is essential for improving our knowledge of cardiovascular health and exploring potential therapeutic strategies. This dissertation will explore how the acute presence of estrogen affects cardiovascular responsiveness to β-ARs stimulation, and how age, biological sex, and heart failure modulate this response. Overall, this interaction between estrogen and β-ARs holds promise for uncovering new insights into the sex and age-related differences in cardiovascular health and offers potential avenues for interventions to optimize cardiovascular outcomes.

Chapter 2: Cardiac Functional Responses to β2-Adrenergic Receptor Stimulation: Effects of Sex and Age

Introduction

The cardiovascular system, which includes the heart, blood, and blood vessels, plays an essential role in transporting oxygen and nutrients throughout the body. Cardiovascular disease (CVD) represents a significant public health concern and that it has been observed that women exhibit a lower prevalence than age-matched men before menopause [50]. This pattern changes after menopause, as postmenopausal women experience a higher risk of CVD compared to age-matched men and premenopausal women [62]. Indeed, sex hormones, including estrogen, have been shown to play an essential role in cardiovascular protection. However, the precise mechanisms and the pathways underlying this shift remain unknown. Several studies concluded that the mechanism probably includes a combination of sex hormones, aging, and receptors in the regulation of the cardiovascular system [9. 10, 13, 15, 50, 62]. This underlines the clear association between several factors and CVD prevalence and highlights the need for a comprehensive understanding of the interactions among estrogen, aging, and receptors in managing cardiovascular health.

Estrogen modulates cardiovascular function by binding to estrogen receptors which are located within cardiovascular tissues. Estrogen receptors (ERs) have three subtypes: estrogen receptor α (ERα), estrogen receptor β (ERβ), and G-protein-coupled receptor 30 (GPR30). All three ER subtypes are expressed in cardiovascular tissues, including ventricular myocytes, endothelial cells, and smooth muscle cells [48, 49]. By binding to ERs, estrogen regulates gene expression and signaling molecules through both genomic and non-genomic pathways. Through the genomic pathway, estrogen binds to nuclear receptors ERα and ERβ to upregulate gene expression to increase the production of endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor (VEGF). The enhanced eNOS and VEGF expression will lead to vasodilation and angiogenesis [17, 32]. Through the non-genomic pathway, estrogen binds to membrane receptors (ERα and GPR30) to perform actions, such as rapidly activating nuclear transcription factors, which primarily cause the vasodilation [3]. In the cardiovascular system, when estrogen binds to ERs, it can have vasodilatory effects of coronary arteries. This vasodilation is crucial for maintaining proper blood flow to the heart, as well as the rest of the body, which is essential for overall cardiovascular function.

In addition to ERs, the β-ARs also play an important role in cardiovascular system regulation. β-ARs are G-protein-coupled receptors, and have three subtypes: β1, β2, and β3. Stimulation of β-ARs leads to increased heart rate, enhanced cardiac contractility and relaxed coronary smooth muscle [7]. Activation of β1-AR results in increased heart rate and improved cardiac contractility. In contrast, the stimulation of β2-AR typically exerts a milder impact on heart rate and cardiac contractility, although it can cause coronary artery vasodilation. Binding to the β-ARs in the sinoatrial (SA) node increases HR by activating the cAMP secondary messenger system. cAMP augments the opening of ion channels in autorhythmic cells to enhance both Na^+ and Ca^{2+} movement, which increases the spontaneous depolarization rate. As a result, the action potential frequency increases, shortening the duration of electrical activation and increasing HR. Stimulating the β-ARs in the cardiomyocytes results in increased myocardial contractility. This process is governed by the dynamics of cytosolic Ca^{2+} concentration. It involves an increase in cytosolic Ca^{2+} levels and an augmentation of Ca^{2+}-ATPase function, results in more activated muscle contraction units and a stronger contraction, it also leads to a shorter duration of muscular contraction. The activation of β2-AR in the smooth muscle of coronary vasculature induces a crucial physiological response known as vasodilation. This process involves the relaxation of the smooth muscle, resulting in the expansion of blood vessel diameters. The effects of β-ARs on cardiovascular system is mainly modulated by two catecholamines: epinephrine and norepinephrine. Through binding to β-ARs, the cAMP secondary messenger system is activated, leading to an increased heart rate, enhanced cardiac contractility, and vasodilation, depending on the specific β-AR receptor subtype and its location in the heart.

Age appears to be an important determinant of the cardiac response to β-adrenergic stimulation. Studies found that isoproterenol, a drug used as an agonist of all three β-AR subtypes, had reduced efficacy with age. Aging male rats show a blunted increase in HR in response to isoproterenol compared to young rats [8]. According to another study, as age increases, the dose of isoproterenol which increases resting HR by 25 beats per minute also increases in older men, which indicate that the sensitivity of β-ARs to agonist decreased as age increased [9]. The results indicated the age effects on decreased β-ARs responsiveness to stimulation in older individuals compared to younger ones.

In addition to aging, sex also affects β-ARs stimulation responsiveness. Men and women show different responses to age in β-ARs expression of heart [11]. In men, there is no significant association between β-ARs expression and age. However, β-ARs expression decreases as age increases in pre-menopausal women until menopause and stabilizes in post-menopausal [11]. Therefore, sex hormones, especially estrogen, may play an essential in β-ARs expression regulation. Abundant evidence supports the notion that estrogen plays a role in regulating β-ARs expression in the heart. In one study by Santos et al. [17], ovariectomy (removal of the ovaries) led to a decrease in β2 adrenergic receptor (β2-AR) expression in female rat hearts, and this reduction was reversed by four weeks of estrogen treatment. Another study by Chu et al. [18]

reported that ovariectomy doubled β1 adrenergic receptor (β1-AR) expression, but estrogen treatment brought it back to the levels observed in sham-operated controls. Taken together, estrogen has demonstrated a long-term effect in enhancing β-ARs expression.

In addition to its long-term effects, estrogen could also influences short-term regulation of β-ARs. β-ARs and GPR30 can play in acute cardiovascular regulation, as they both belong to G protein-coupled receptors and activate G_i and G_s pathways (Figure 1). β1-AR, β2-AR, and GPR30 can couple to G_s subunits, while β2-AR and GPR30 couple to G_i subunits. The interaction between β2-AR and GPR30 is complex and not fully understood. Cao et al. [53] reported that the estrogen enhanced the β2-AR-G_s pathway by increasing cAMP concentration and PKA phosphorylation in female rats, which indicates that estrogen may amplify signaling intermediates to promote the effects of β2-AR through GPR30. Additionally, there is evidence that these receptors might be interacting during pathological conditions as well. Both β2-AR and GPR30 are involved in the cardio protection against ischemic damage through PI3K/Akt pathway [54], which indicates the possible interaction between two receptors. Although there is evidence of potential cross-effects resulting from the activation of β2-AR and GPR30, it is currently unknown if these interactions are occuring within the cardiovascular system and the role of estrogen in β2-AR stimulation.

Therefore, the primary goal of this study was to evaluate the sex differences in cardiac functional responses to β2-AR stimulation in the absence and presence of estrogen in young and old, male and female rat hearts. We hypothesized that young female rats would have blunted cardiac responses to β2-AR stimulation compared to young male rats, and the presence of estrogen would augment the functional response to β2-AR stimulation in young female, but not male, hearts. Further, we hypothesized responsiveness of old male and female hearts be lower than young animals, but would not differ between the sexes, with no effect of the presence of estrogen in either group. A second goal of the study was to determine the expression of β2-AR and GPR30 in young and old, male and female rat hearts. We hypothesized that young male rat hearts will have higher β2-AR expression and lower GPR30 expression compared to young females, with old females will have both lower β2-AR and GPR30 expressions compared to young females, but old males only show lower β2-AR, not GPR30, expression compared to young males.

Methods

Animals

Male and female Hilltop SD rats (Rattus norvegicus, Hilltop Lab Animals, Inc.) were used for this study. All animal research procedures described below were approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC). Young rats were defined as less than thirty weeks old and old rats were more than eighty weeks old.

Langendorff perfused heart

After the rats are anesthetized with isoflurane (3% - 5%) and cessation of pain reflexes, the heart was excised, and the aorta was cannulated. The heart was Langendorff-perfused via the aorta at a consistent pressure of 62 mmHg with Krebs-Henseleit buffer, which is composed of NaCl 115 mM, KCl 16.5 mM, HEPES 25 mM, $CaCl_2$ 11 mM, $MgSO_4$ 7 mM, K_2HPO_4 1 mM, KH_2PO_4 1 mM, Glucose 5 mM, and Lactate 5 mM, pH 7.4 at 37°C and bubbled with 100% oxygen. The Krebs-Henseleit buffer provides oxygen and nutrients to make sure the heart could maintain ex vivo physiological function. With a consistent pressure of 62 mmHg above the aortic valve and no fluid in the left ventricle, the aortic valve remains closed and forces the buffer flow into the coronary arteries.

To evaluate the functional effects of estradiol, after 10 minutes of baseline perfusion, a 17-β-estradiol (E2) dose-response experiment was performed on young male and female rat hearts:

increasing concentrations of E2 (5, 10, 20, and 40 μM) were added every 5 minutes. HR and coronary flow rate (CFR) were continually monitored using PowerLab (ADIstruments, Sydney, Australia), while arterial and venous oxygen content were continually monitored by using NeoFox oxygen sensor kits (NEOFOX-KIT-PROBE, Ocean Insight, Orlando, FL). Oxygen consumption rate was calculated by using following equation: [(arterial oxygen concentration – venous oxygen concentration) * CFR * 760 * 1.3 * 10^-6 * 1000]. CFR and Oxygen consumption rate were normalized by the heart mass when comparing between groups. After looking at HR and CFR responses to increasing doses of estradiol, subsequent experiments utilized the 20 μM E2 dose, as this dose resulted in vasodilation in both male and female hearts, and increasing the dose to 40 μM resulted in a precipitous decline in HR in a subset of hearts.

To evaluate the cardiac response to β-adrenergic stimulation, a dose-response study using the β2-AR agonist, albuterol, was performed on young and old, male and female rat hearts. The hearts were perfused for 10 minutes of stabilization, then 0.1, 0.5, 1, and 5 μM of albuterol were added every 5 minutes, while HR, CFR, and arterial and venous oxygenation were monitored continuously. HR percentage change was calculated by using following equation: (heart rate at each drug dose – baseline heart rate) / baseline heart rate.

To evaluate the effects of E2 on the cardiac response to β-adrenergic stimulation, after 10 minutes stabilization, 20 μM E2 was added, then after 5 minutes, the albuterol dose-response described above was again performed, with doses addition again separated by 5 minutes. HR, CFR, and arterial and venous oxygen content were continually monitored. Heart rate percentage change was calculated by using following equation: (heart rate at each drug dose or heart rate at baseline – heart rate at 20 μM 17-β-estradiol) / heart rate at 20 μM 17-β-estradiol.

Western blot

β2, GPR30 and GAPDH protein expression were determined in homogenates made from the left ventricle and SA node. Following functional experiments, the left ventricle and SA node tissue samples were frozen for later analysis. Forty mg tissue was added to 400 µL 1% Triton buffer (Cat# J62289, Thermo Fisher Scientific, Waltham, MA) with 4 µL protease (Cat# 78430, Thermo Fisher Scientific, Waltham, MA) and 4 µL phosphatase inhibitors (Cat# 78428, Thermo Fisher Scientific, Waltham, MA) and homogenized by using handheld electric homogenizer. The samples were incubated on ice for 10 minutes and then centrifuged at 16,000 g for 10 minutes at 4°C. Protein concentration in prepared homogenates was determined using a BCA assay (Thermo Fisher Scientific, Waltham, MA)

Thirty-five micrograms of protein were loaded onto a 4-15% Mini-PROTEAN TGX Precast Protein Gel (Bio-Rad Laboratories, Hercules, CA) and electrophoresed at 110V for 60 minutes. The separated proteins were transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA) via wet transfer (transfer buffer - 20% methanol, 10% TBS, and 70% deionized water), 4°C, 70V for 90 minutes.

After transfer, the PVDF membrane was incubated for 5 min in TBST, then the membrane was blocked in 6% nonfat dry milk in TBST (10% TBS, 1% Tween-20, 89% deionized water) for 1 hour at room temperature. The membrane was then incubated with primary antibody against the β2-AR [anti-β2-AR rabbit monoclonal antibody (ab182136; Abcam), 1:1500 dilution], GPR30 [GPER1/GPR30 rabbit anti-Human polyclonal (C-Terminus) antibody (LS-A4272; Lifespan Biosciences), 1:2000 dilution] or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [Recombinant HRP Anti-GAPDH antibody (ab201822; Abcam), 1:2000 dilution] overnight at 4°C, followed by secondary antibody incubation (anti-Rabbit IgG, HRP-linked antibody #7074, 1:3000 dilution for β2 , 1:4000 dilution for GPR30 and 1:4000 dilution for GAPDH) for 2 hours at room temperature with 20-25 rpm shaker.

The signal was visualized using a ChemiDoc imaging system (Bio-Rad, CA). The β2, GPR30 and GAPDH were detected using ECL reagent (Cat# 32106, Thermo Fisher Scientific, Waltham, MA) after secondary antibody incubation. The total protein was detected after the protein was transferred from the protein gel to the PVDF membrane. Signal intensity was quantified using Bio-Rad Image Lab software and normalized to the total protein when analyzed left ventricle data. SA node β2 and GPR30 signal intensity were normalized to SA node GAPDH.

Statistical analysis

Statistical analyses were performed by using GraphPad Prism (version 10.0.0 for Windows, GraphPad Software, Boston, Massachusetts USA). A repeated measures two-way ANOVA was performed to evaluate the sex and E2 doses effects on HR, HR percentage change, oxygen consumption rate and venous oxygen partial pressure (PO2). A three-way ANOVA was used to examine the effects of sex, age and albuterol doses on the HR, HR percentage change, CFR, oxygen consumption rate and venous PO2 in responses to β2 stimulation. Following the three-way ANOVA, post hoc analyses were performed using Tukey's HSD test to explore differences between young and old rats, and between male and female rats. Independent t-tests were performed between young and old, male and female rats to determine the sex and age effects on protein expression. Independent t-tests were also performed to compare HR at baseline to HR after the administration of 20 μM E2 during the E2 + albuterol stimulation experiments (both absolute and relative HR). A $p < 0.05$ was deemed statistically significant.

Results

Rat characteristic data

Young and old, male and female rat characteristic data is showed in Table 1. Age was not significantly different between young male and young female, or between old male and old female rats; however, old rats were significantly older than young rats. Body mass was significantly higher in young male rats (450 ± 105 g) compared to young females (266 ± 40 g) ($p < 0.001$), as was heart mass: 1.58 ± 0.32 g in young males compared to 0.97 ± 0.19 g ($p < 0.001$) in young females. Similarly to young animals, in old animals, body mass and heart were also higher in old male compared to old female rats (688 ± 81 vs. 387 ± 47 and 2.09 ± 0.27 vs. 1.29 ± 0.15) ($p < 0.0001$).

Table 1. Characteristic data of young and old, male and female rats

		Age (weeks)	Rat Mass (g)	Heart Mass (g)	Heart/Rat Mass Ratio (g/kg)
		M *SD*	*M* *SD*	*M* *SD*	*M* *SD*
E2 Dose Response	YM (n=9)	20 ± 3	579 ± 38	1.81 ± 0.21	3.14 ± 0.38
	YF (n=7)	17 ± 4	245 ± 34***	1.02 ± 0.11***	4.22 ± 0.60*
Albuterol Dose Response	YM (n=11)	9 ± 2	450 ± 105	1.58 ± 0.32	3.80 ± 0.36
	YF (n=9)	7 ± 0	274 ± 35**	1.02 ± 0.16**	4.01 ± 0.44
	OM (n=6)	87 ± 1$^{\#\#\#}$	783 ± 106$^{\#\#\#}$	2.12 ± 0.24$^{\#}$	2.74 ± 0.41$^{\#\#}$
	OF (n=6)	95 ± 12$^{\#\#\#}$	409 ± 66$^{***\ \#\#}$	1.43 ± 0.25$^{**\ \#}$	3.61 ± 1.11
Albuterol Dose Response with E2	YM (n=14)	18 ± 6	510 ± 111	1.55 ± 0.27	3.11 ± 0.50
	YF (n=11)	18 ± 9	292 ± 50***	1.07 ± 0.14***	3.73 ± 0.59*
	OM (n=8)	96 ± 10$^{\#\#\#}$	688 ± 81$^{\#\#}$	2.09 ± 0.27$^{\#\#}$	3.06 ± 0.39
	OF (n=6)	88 ± 4$^{\#\#\#}$	387 ± 47$^{***\ \#}$	1.29 ± 0.15$^{***\ \#}$	3.36 ± 0.33

Data represented as mean (M) ± standard deviation (SD). Young male YM, young female YF, old male OM, old female OF, gram g, kilogram kg, 17-β-estradiol E2. Data were analyzed by using independent t-test. Comparing female to male (YF to YM and OF to OM), * $p \leq 0.01$, significantly different from age-matched males, ** $p < 0.001$, significantly different from age-matched males, *** $p < 0.0001$, significantly different from age-matched males. Comparing old to young (OM to YM and OF to YF), # $p \leq 0.01$, significantly different from young, ## $p < 0.001$, significantly different from young, ### $p < 0.0001$, significantly different from young.

E2 dose response in young male and female rat hearts

The relationship between E2 concentration and HR and CFR is illustrated in Figure 2. A two-way ANOVA was used to examine the effects of sex and E2 dose on HR, HR percentage change, CFR and oxygen consumption rate. Young females show significant higher HR compared to young males at baseline and all E2 doses: A significant main effect of sex on HR is found [sex effect (F(1, 68) = 55.39, $p < 0.0001$)] with post hoc analysis revealing differences between young male and young female rats at baseline and all E2 doses ($p < 0.05$) (Figure 2A). Additionally, the HR of both young males and young females decreases as E2 dose increases with a significant main effect of E2 dose on HR at 20 and 40 μM doses of E2, as indicated by the ANOVA [E2 dose effect (F(4, 68) = 5.132, $p = 0.0011$)]. For HR percentage change, young males and young females show similar decline as E2 dose increases with no significant main effect of sex by using two-way ANOVA. There is a significant main effect of E2 doses at 20 and 40 μM [E2 dose effect (F(4, 68) = 10.05, $p < 0.0001$)], indicating the significant effects of higher doses of E2 on negative HR percentage change (Figure 2B). Regarding CFR, the similar increases are observed in both groups and a significant main effect of E2 dose is found at 40 μM dose [E2 dose effect (F(4, 39) = 2.801, $p = 0.0389$)], but no significant main effect of sex is observed, indicating that 40 μM E2 leads to significant coronary vasodilation, with similar effects observed between young males and young females (Figure 2C). No significant main effect of sex or E2 dose on oxygen consumption rate, indicating E2 is not able to significantly affect oxygen consumption rate in either young males or young females (Figure 2D). Based on the presented results, the E2 dose of 20uM was determined as the optimal dose for the following experiments. Absolute CFR and oxygen consumption rate data for the 20 μM is presented in Table 2. Absolute CFR ($p < 0.01$) and oxygen consumption rate

($p < 0.05$) are significantly lower in young female rats at 20 µM E2 compared to young males (Table 2), due to their smaller size.

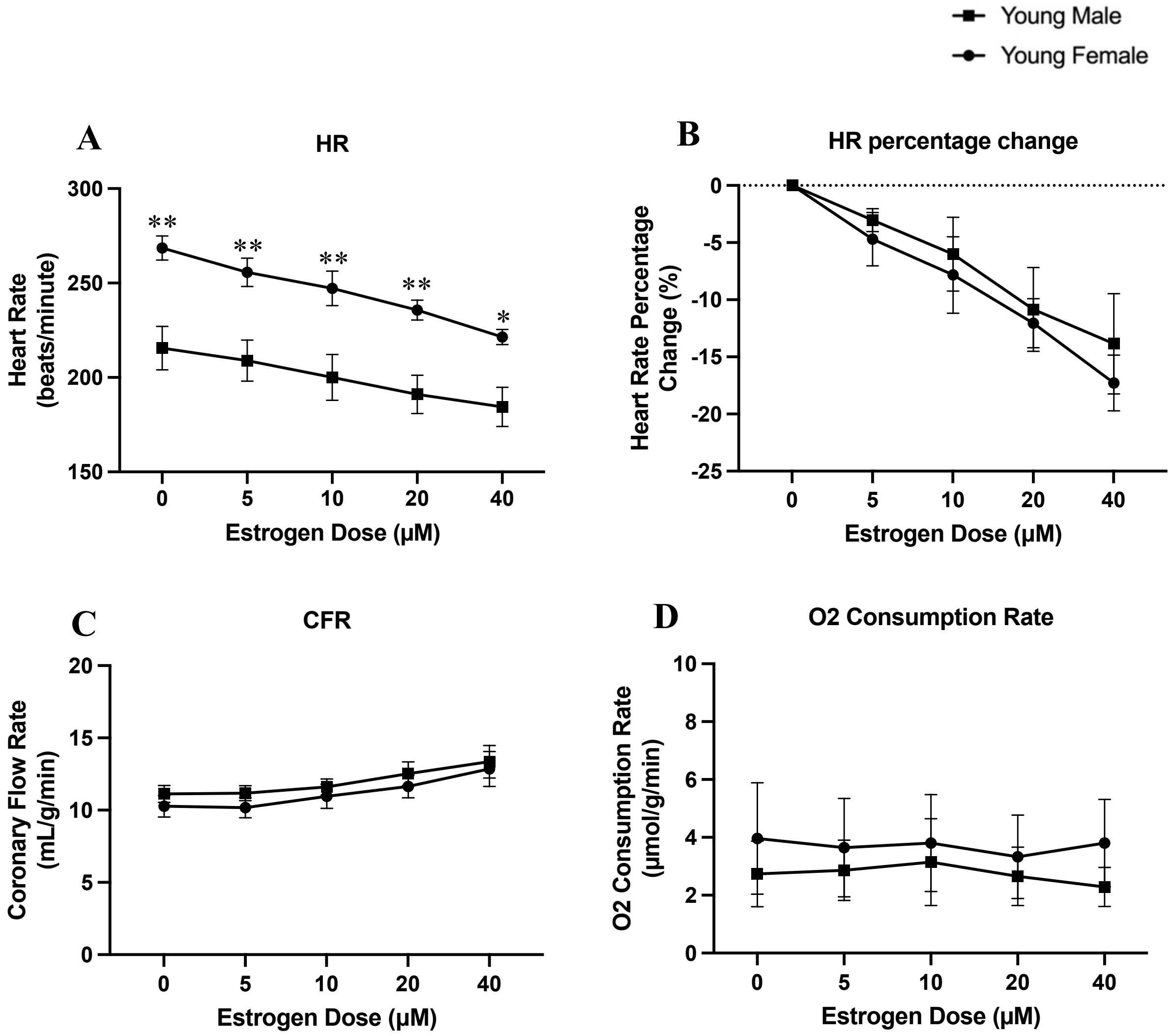


Figure 2. Young male and female rat function responses to increasing doses of E2. Data were analyzed by using repeated measures two-way ANOVA. Young female rats show significant higher HR compared to young males at every estrogen dose. There is no significant difference between young male and female rat in coronary flow rate normalized by heart weight, oxygen consumption rate or venous oxygen partial pressure. Heart rate HR, coronary flow rate CFR, milliliter/gram/minute mL/g/min, micromolar/gram/minute µmol/g/min. * $p \leq 0.01$, significantly different from young males, ** $p < 0.001$, significantly different from young males. Data are presented as mean ± standard error of mean (SEM).

Table 2. Absolute cardiac functional data at 20 µM E2 dose.

	Coronary Flow Rate (mL/min)	O_2 Consumption Rate (µmol/min)
	M *SEM*	*M* *SEM*
YM	19.0 ± 1.2	4.7 ± 1.7
YF	12.1 ± 0.6**	3.5 ± 1.5*

Data represented as mean (M) ± standard error of mean (SEM). Young male YM, young female YF, milliliter/minute mL/min, micromolar/minute µmol/min, oxygen O_2. Data were analyzed by using independent t-test. * $p \leq 0.05$, significantly different from male, ** $p \leq 0.01$, significantly different from male.

Albuterol dose response in young and old, male and female rat hearts in the absence and presence of E2

The role of sex and age on response to β2 stimulation was investigated by examining the effects of increasing albuterol concentration on HR, CFR, and oxygen consumption rate in young and old, male and female rat hearts. Next, in a separate group of young and old, male and female hearts, E2 (20 µM) was added prior to β2 stimulation, then, the effects of increasing albuterol concentration were investigated in the presence of estradiol.

A three-way ANOVA was conducted to examine the effects of sex, age and albuterol dose on HR and HR percentage change in response to albuterol stimulation. As expected for a β2-AR agonist, albuterol causes HR increase in all groups. ANOVA results indicate that a significant main effect is observed for albuterol dose at 1 and 5 µM [albuterol dose effect ($F(4, 130) = 9.124$, $p < 0.001$)]. Although both male and female increase their HR in the presence of albuterol, the effects of albuterol is blunted in females compared to males [albuterol dose effect ($F(1, 130) = 7.451$, $p = 0.007$)]. Post hoc analysis reveals that young male and young female groups show significant differences at 0.5 µM (282 ± 18 beats/min in males vs. 249 ± 36 beats/min in females), 1 µM (288 ± 17 beats/min in males vs. 250 ± 43 beats/min in females), and 5 µM (300 ± 16 beats/min in males vs. 243 ± 53 beats/min in females) albuterol ($p < 0.05$) (Figure 3A). Similarly to sex, age was also found to have a significant impact on HR, showing significant differences between young and old animals at all albuterol doses. Comparing to young rats, old rats have relative lower HR in response to albuterol. Age has been found to be a significant main effect [age effect (1, 130) = 25.581, $p < 0.001$)], and the Post hoc analysis show young male and old male groups demonstrate significant differences at all albuterol doses ($p < 0.05$) (Figure 3A). These findings indicate that both sex and age are involved in the mechanism of β2-AR agonist-induced changes in HR, as a young female rats display a blunted response compared to young males, and in males, this response is also blunted in old compared to young (Figure 3A).

HR percentage change was analyzed to determine differences between groups that were not driven by differences in the initial HR. There are relationships between albuterol dose increase and HR percentage increase in all groups: the increased albuterol dose leads to the increase in HR percentage. The three-way ANOVA reveals that albuterol dose (at 0.5, 1, and 5 µM) [albuterol dose effect (F(4, 130) = 21.952, $p < 0.001$)] is a significant main effect of HR percentage change. Age also plays an important role in the HR respond to albuterol and a significant main effect is observed [age effect (F(1, 130) = 12.317, $p < 0.001$)]. When comparing young and old females during post hoc analysis, young ones showed a significant blunted response at 0.5 µM (7.41 ± 17.42 % in young vs. 20.36 ± 13.83 % in old), 1 µM (5.68 ± 19.01 % in young vs. 25.95 ± 18.77 % in old), and 5 µM (10.34 ± 17.10 % in young vs. 35.24 ± 20.93 % in old) albuterol ($p < 0.05$) (Figure 3C). Even though no significant main effect for sex is found, the further analysis indicates that young females (10.34 ± 17.10 %) demonstrate a significant lower HR percentage change at 5 µM albuterol compared to young males (22.69 ± 13.25 %) ($p < 0.05$) (Figure 3C).

To test the effects of the presences of E2 on responsiveness to β2-AR stimulation, 20 μM E2 was added to the perfusate prior to the administration of albuterol. HR (absolute and percent change) at baseline and after E2 administration were not significantly different, indicating, in these experiments, that 20 μM E2 alone does not result in a significant reduction in HR (Figure 3B&D).

We employed a three-way ANOVA to examine the simultaneous influence of sex, age and albuterol dose on HR and HR percentage change in response to albuterol with the presence of E2. All groups show increase in HR response to all albuterol doses with ANOVA results indicate that there is a significant main effect of albuterol dose at 0.5, 1 and 5 μM [albuterol dose effect (F(5, 204) = 26.874, $p < 0.001$)]. Similarly to the experiment with albuterol alone, old rats have blunted HR response compared to young rats. The three-way ANOVA suggests that age [age effect (F(1, 204) = 82.254, $p < 0.001$)] is a significant main effect. Post hoc analysis revealed HR was significantly different between young male and old male hearts at all albuterol doses, where old males show lower HR compared to young males ($p < 0.05$) (Figure 3B). Similarly, HR in old females is lower than young females at all albuterol doses except 5 μM ($p < 0.05$) (Figure 3B). Taken together, old rats show blunted HR in response to albuterol stimulation in the presence of E2.

Similar to absolute HR change, HR percentage change shows a positive relationship with albuterol in the presence of E2 dose, and old rats have blunted changes compared to young rats. There are significant main effects for age [age effect (F(1, 231) = 5.487, $p = 0.02$)] and albuterol dose at 0.5, 1, and 5 μM [albuterol dose effect (F(5, 231) = 43.974, $p < 0.001$)]. Further analysis using Post hoc demonstrates significant differences between groups. Old males exhibit significantly lower in HR percentage (25.24 ± 9.62 %) change compared to old females (44.2 ± 24.27 %) at the 5 μM albuterol dose ($p < 0.05$) (Figure 3D). Moreover, comparing to old females, young females demonstrate significant lower HR percentage change at 1 μM (14.15 ± 12.38 % in young vs. 25.45 ± 14.83 % in old) and 5 μM (18.20 ± 18.33 % in young vs. 44.2 ± 24.27 % in old) albuterol doses ($p < 0.05$) (Figure 3D).

In order to directly compare the effects of albuterol alone to E2 + albuterol within a given group (e.g. old females), we conducted a two-way ANOVA examining the influence of the presence of E2 on the HR response to albuterol (Figure 4). In young males, albuterol shows similar effects on HR increase in the absence and presence of E2, with a significant main effect of albuterol dose at 0.5, 1, and 5 μM [albuterol dose effect ($F(4, 103) = 21.83$, $p < 0.0001$)]. However, there is a significant main effect of E2 [E2 dose effect ($F(1, 85) = 3.994$, $p = 0.0489$)] on HR in young female, indicates that the presence of E2 affects albuterol-induced HR increase. Post hoc tests uncover notable variations among the groups and show significant differences between the absence and presence of E2 in HR responses to albuterol in young female rats at 5 μM albuterol doses ($p < 0.05$) (Figure 4B). In old rats, there is no significant difference in HR response to albuterol stimulation between absence and presence of E2. For old male rats, there are significant main effects of albuterol dose at 0.5, 1, and 5 μM [albuterol dose effect ($F(4, 60) = 14.24$, $p < 0.0001$)], and for old females, significant main effects are observed at 1 and 5 μM [albuterol dose effect ($F(4, 50) = 7.9$, $p < 0.0001$)]. Therefore, the presence of E2 specifically influences the heart rate response to albuterol stimulation solely in young female rats, without exerting a significant effect in young males, old males, or old females (Figure 4).

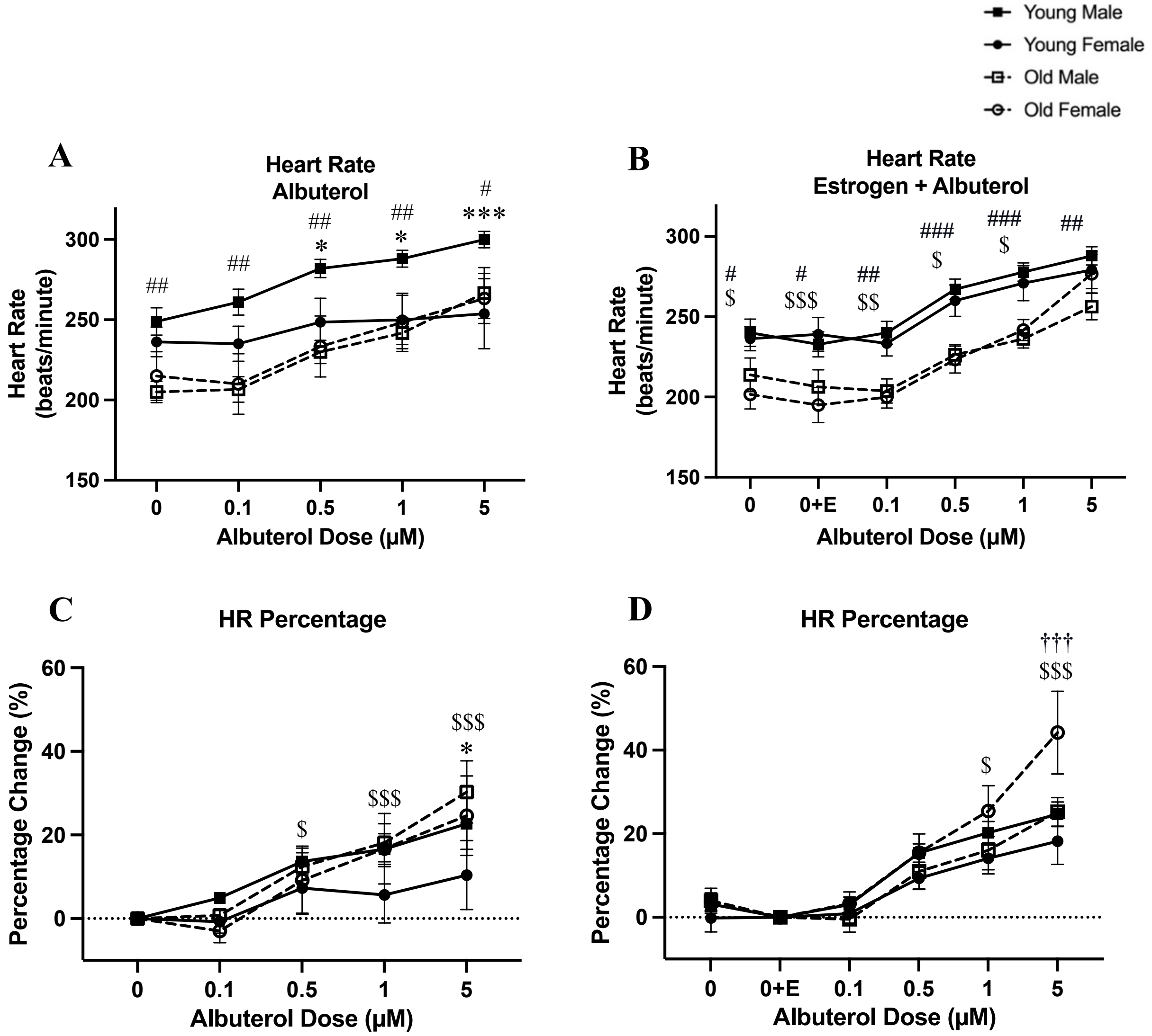


Figure 3. Heart rate response to albuterol is blunted in young female compared to young male hearts, but is rescued with the acute presence of E2. Old female and old male rat heart rate responses to albuterol did not differ from each other, whether E2 was absent or present. Old female rats show significant higher percentage change in heart rate compared to young females and old males. Young and old, male and female rats heart rate responses to increasing albuterol concentration in the absence (A&C) and presence of E2 (B&D). Both absolute heart rate (A&B) and percent change in heart rate were determined (C, D). Data were analyzed by using three-way ANOVA. * $p < 0.05$, *** $p < 0.001$, significantly different between young male and young female rats, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, significantly different between young male and old male rats, $ $p < 0.05$, $$ $p < 0.01$, $$$ $p < 0.001$, significantly different between young female and old female rats, † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ significantly different between old male and old female rats. Data are presented as mean ± standard error of mean (SEM).

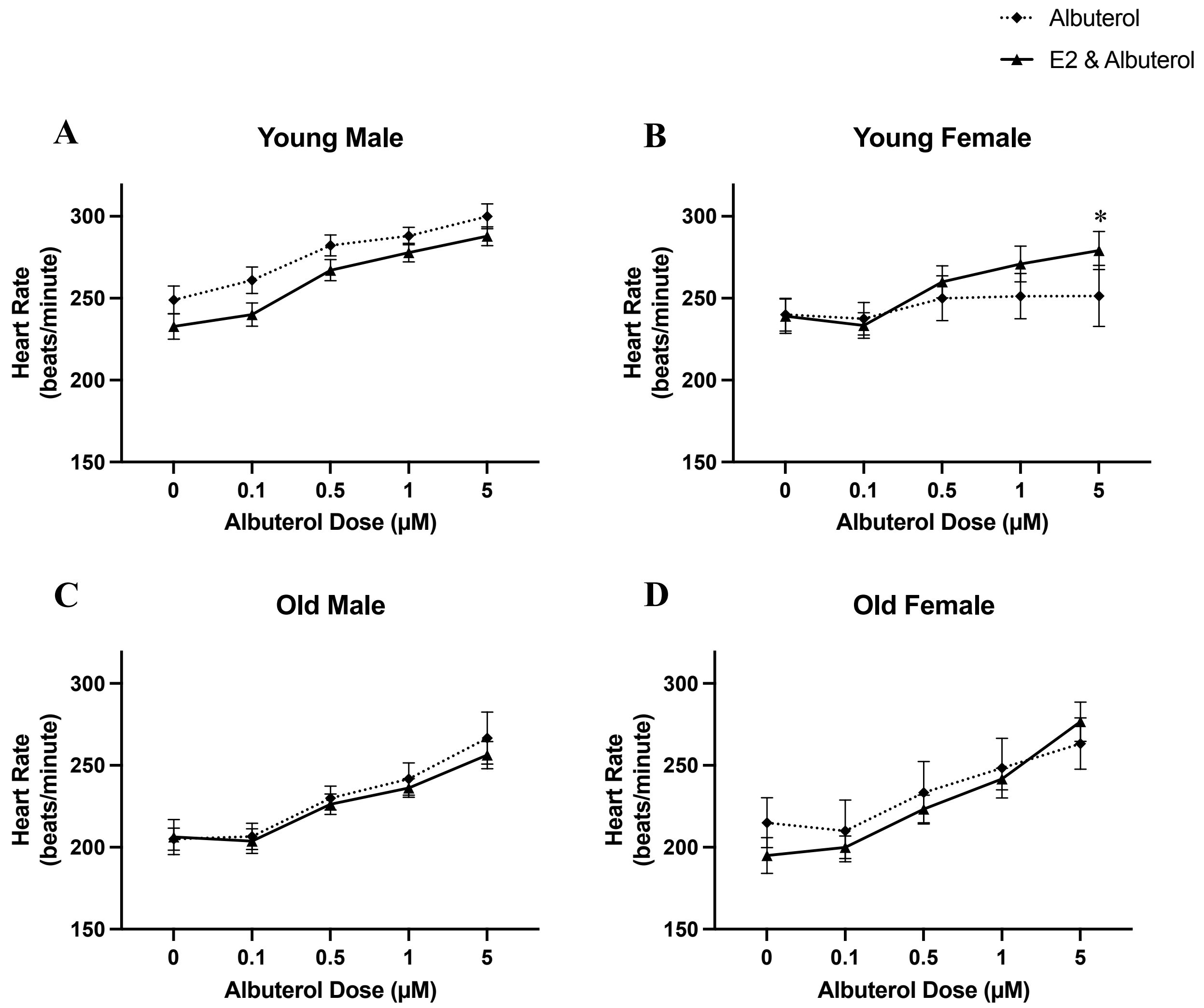
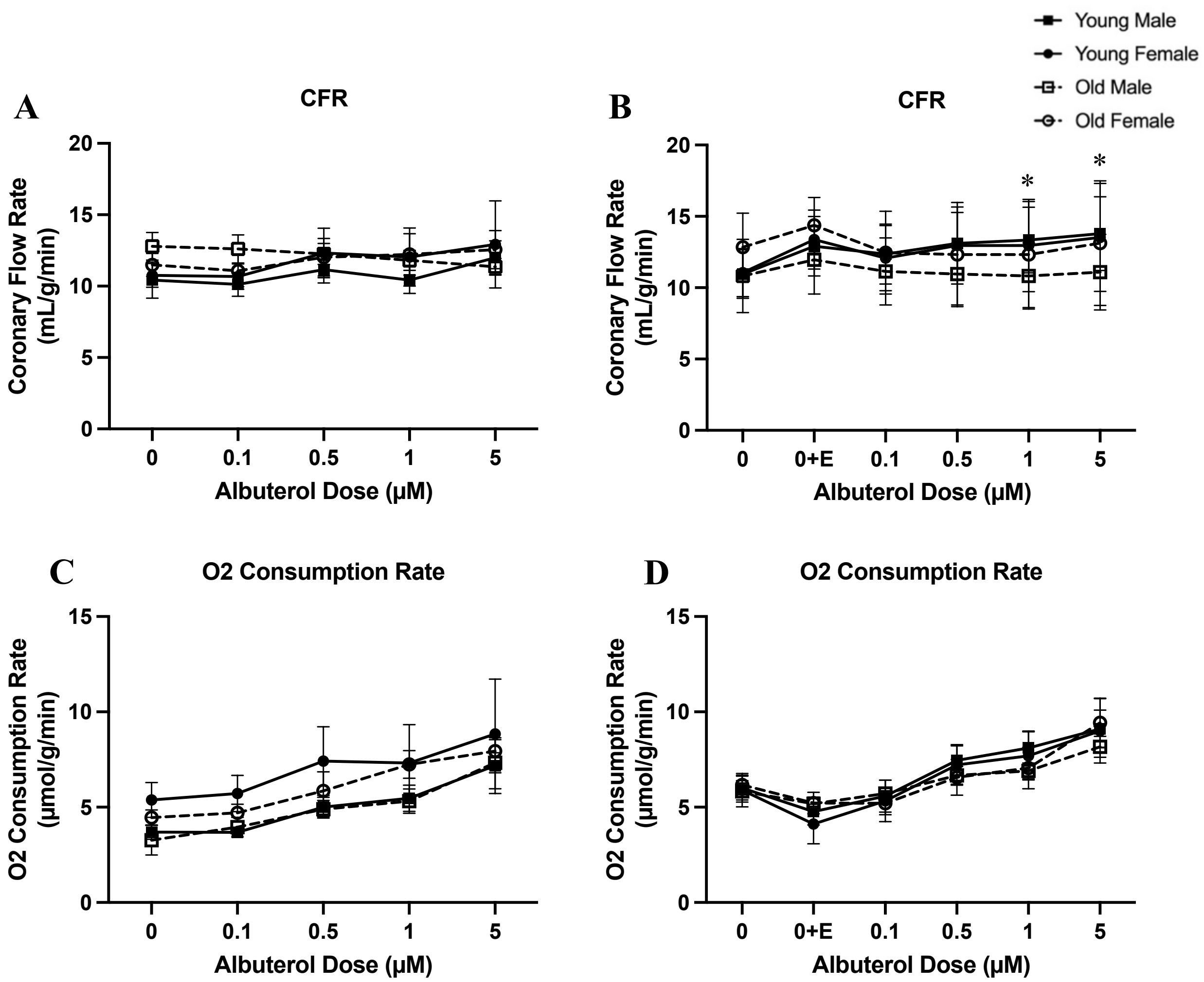


Figure 4. Heart rate response to albuterol in the absence and presence of E2. Young and old, male and female rats heart rate responses to increasing albuterol concentration in the absence and presence of E2. Utilizing a repeated measures two-way ANOVA revealed that the presence or absence of E2 only result in a significant difference in the heart rate response to 0.5 μM albuterol in young male rats. 17-β-estradiol E2. * $p < 0.05$, significantly different in heart rate responses to albuterol between absence and presence of E2. Data are presented as mean ± standard error of mean (SEM).

A three-way ANOVA is conducted to examine the main effects of sex, age and albuterol dose on CFR, oxygen consumption rate and venous PO2 in the absence and presence of E2. For CFR, in the absence of E2, there is no significant difference between any groups (Figure 5A). In the presence of E2, the independent t-test shows no significant difference between CFR at baseline and E2 dose, which suggests that E2 does not affect CFR response.

Old males have a significant lower CFR compared to old females [sex effect (F(1, 204) = 4.983, $p = 0.027$)]. Using Tukey's Honestly Significant Difference test in post hoc analysis, young male and old male groups demonstrate significant differences at 1 μM (13.34 ± 2.31 mL/g/min in young vs. 10.81 ± 2.50 mL/g/min in old) and 5 μM (13.79 ± 2.59 mL/g/min in young vs. 11.07 ± 2.86 mL/g/min in old) albuterol doses ($p < 0.05$) (Figure 5B).

The independent t-test indicates no significant difference in oxygen consumption rate and venous PO2 between baseline and E2 dose. For oxygen consumption rate, there are slightly increases in all groups in response to albuterol in the absence and presence of E2. Significant main effects are observed for albuterol at 1 and 5 μM (F(1, 100) = 8.29, $p < 0.001$) without E2 (Figure 5C) and at 5 μM (F(1, 150) = 11.29, $p < 0.001$) with E2(Figure 5D) . Regardless of age, female rats have significant higher oxygen consumption rate compared to males [sex effect (F(1, 100) = 10.57, $p = 0.002$)] (Figure 5C). However, in the presence of E2, this sex difference disappears (Figure 5D). For venous PO2, the increased albuterol dose leads to the decline in PO2 with or without the presence of E2 (Figure 5E&F). The ANOVA results support the findings: there are significant main effects for albuterol dose at 1 and 5 μM (F(4, 105) = 12.77, $p < 0.001$) without E2 and at 5 μM albuterol dose (F(5, 148) = 13.896, $p < 0.001$) with E2. Old males demonstrate significant higher venous PO2 compared to old females in the absence of E2 [sex effect (F(1, 105) = 10.487, $p = 0.002$)]. Post hoc analysis employing Tukey's Honestly Significant Difference tests show old male and old female groups demonstrate significant differences at 1 μM albuterol doses ($p < 0.05$), which is 242.98 ± 103.09 mmHg in old males vs. 318.06 ± 91.33 mmHg in old females.


Young Male
Young Female
Old Male
Old Female
A
CFR
Coronary Flow Rate (mL/g/min)
20
15
10
5
0
0
0.1
0.5
1
5
Albuterol Dose (μM)
B
CFR
Coronary Flow Rate (mL/g/min)
20
15
10
5
0
0
0+E
0.1
0.5
1
5
*
*
Albuterol Dose (μM)
C
O2 Consumption Rate
O2 Consumption Rate (μmol/g/min)
15
10
5
0
0
0.1
0.5
1
5
Albuterol Dose (μM)
D
O2 Consumption Rate
O2 Consumption Rate (μmol/g/min)
15
10
5
0
0
0+E
0.1
0.5
1
5
Albuterol Dose (μM)

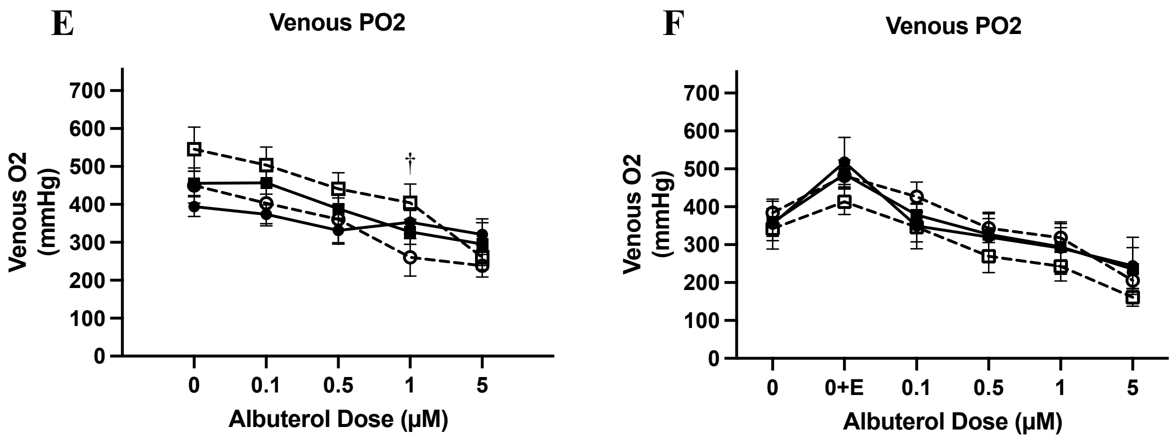


Figure 5. Young and old, male and female rats coronary flow rate and O2 consumption responses to difference albuterol doses in the absence and presence of E2. There is no significant difference between young and old, male and female rat coronary flow rate normalized by heart weight (A&B). Oxygen consumption rate is no significant difference between young and old, male and female rat coronary flow rate normalized by heart weight (C&D). There is no significant different in venous oxygen partial pressure between any groups. Venous oxygen partial pressure was similar in all groups (E&F). Data were analyzed by using three-way ANOVA. * $p < 0.05$, significantly different between young male and young female rats, † $p < 0.05$, significantly different between old male and old female rats. 17-β-estradiol E2, milliliter/gram/minute mL/g/min, micromolar/gram/minute µmol/g/min, oxygen partial pressure PO2, milliliter mercury mmHg. Data are presented as mean ± standard error of mean (SEM).

Table 3. Absolute cardiac functional data at highest albuterol dose in absence and presence of E2.

		Coronary Flow Rate (mL/min)	O2 Consumption Rate (µmol/min)
		M SEM	*M SEM*
Albuterol Dose Response	YM	17.0 ± 1.1	10.2 ± 1.5
	YF	9.7 ± 1.6**	6.2 ± 1.6
	OM	$23.9 \pm 0.9^{\#\#\#}$	$15.5 \pm 1.2^{\#}$
	OF	$17.6 \pm 1.8^{*}$ $^{\#\#}$	$10.7 \pm 0.4^{*}$ $^{\#}$
Albuterol Dose Response with E2	YM	21.0 ± 1.0	13.3 ± 1.2
	YF	14.1 ± 1.0***	9.8 ± 1.8
	OM	22.7 ± 1.4	$16.8 \pm 0.7^{\#}$
	OF	16.6 ± 1.8*	11.9 ± 1.3**

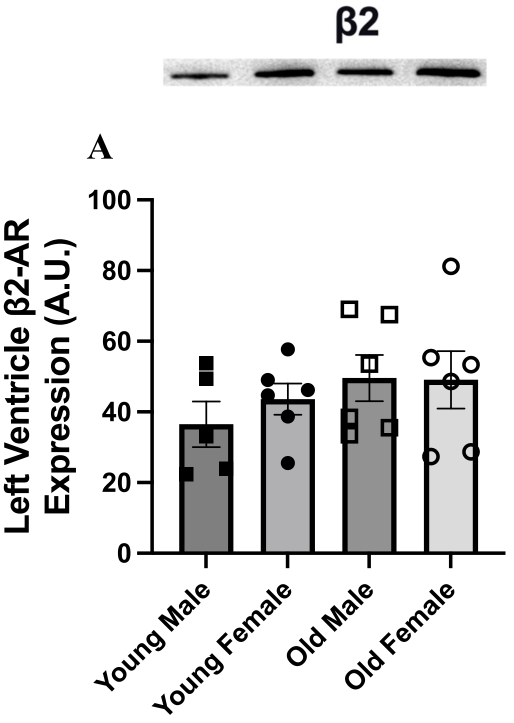
Data represented as mean (M) ± standard error of mean (SEM). Young male YM, young female YF, old male OM, old female OF, 17-β-estradiol E2, milliliter/minute mL/min, micromolar/minute µmol/min, oxygen O2. Data were analyzed by using independent t-test. Comparing female to male (YF to YM and OF to OM), * $p \leq 0.05$, significantly different from male, ** $p < 0.01$, significantly different from male, *** $p < 0.001$, significantly different from male. Comparing old to young

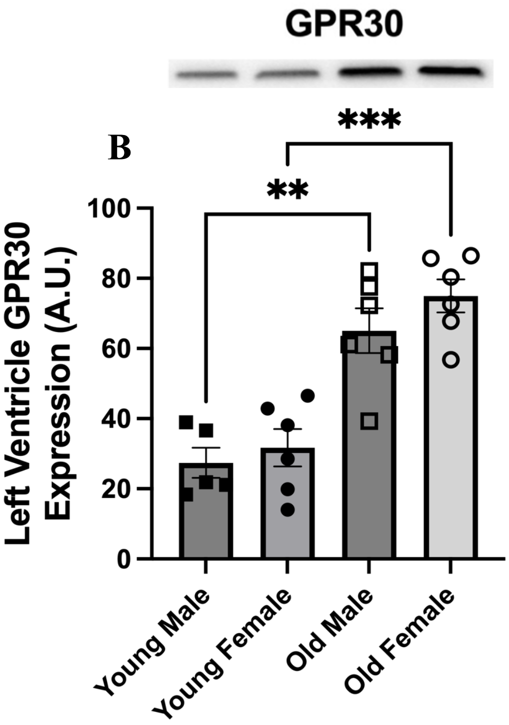
(OM to YM and OF to YF), # $p \leq 0.05$, significantly different from young, ## $p < 0.01$, significantly different from young, ### $p < 0.001$, significantly different from young.

β2, GPR30 and GAPDH protein expression

β2 and GPR30 protein expression was quantified in samples collected from the left ventricle and SA node. Both young male and young female rats exhibited similar β2-AR expression levels in both the left ventricle and SA node, as illustrated in Figure 6 and Figure 7. Additionally, there were no significant differences observed in β2-AR expression between old male and old female rat left ventricles. Further, no significant differences were found comparing young rats to old rats. Regarding GPR30 expression, there were no differences in protein expression between young male and young female rats or between old male and old female rats in the left ventricle. However, it is worth noting that old male rats exhibit a significantly higher GPR30 expression compared to young male rats ($p = 0.0049$), and old female rats demonstrate a higher expression compared to young female rats ($p = 0.0063$). In the SA node, no significant differences in GPR30 expression were observed between young male and young female rats.

GAPDH expression levels were assessed in samples obtained from both the left ventricle (Figure 6C) and the SA node (Figure 7C). In the left ventricle, no significant differences were observed between young male and young female rats, as well as between old male and old female rats. Notably, there was a significantly higher GAPDH expression in old male rats compared to young males ($p = 0.0153$), and in old female rats compared to young females ($p = 0.0008$). There were no differences in GAPDH expression between young male and female rats in the SA node (Figure 7).


β2
A
Left Ventricle β2-AR Expression (A.U.)
0
20
40
60
80
100
Young Male
Young Female
Old Male
Old Female


GPR30
B
***
**
Left Ventricle GPR30 Expression (A.U.)
0
20
40
60
80
100
Young Male
Young Female
Old Male
Old Female

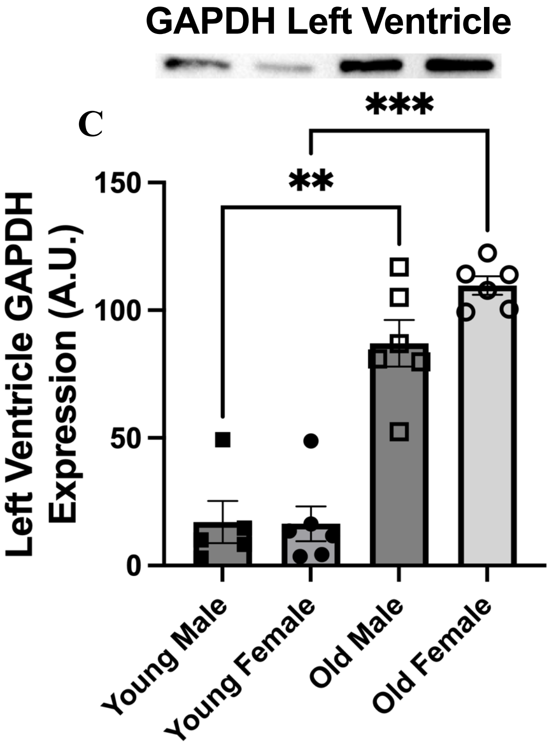
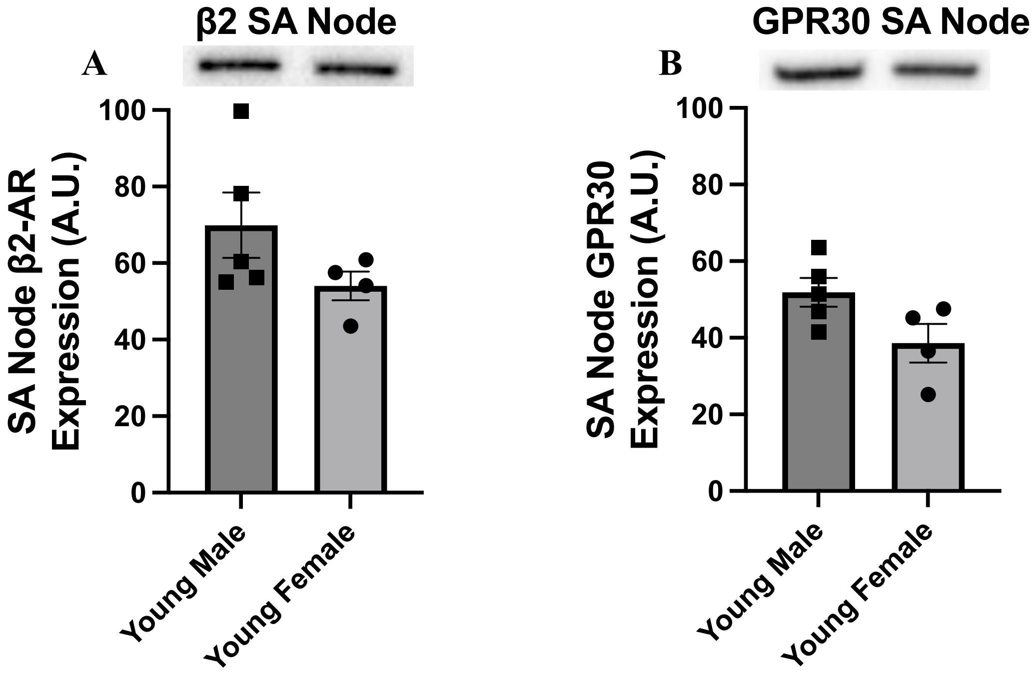

GAPDH Left Ventricle
C
***
**
Left Ventricle GAPDH Expression (A.U.)
0
50
100
150
Young Male
Young Female
Old Male
Old Female

Figure 6. β2, GPR30 and GAPDH expression in young and old, male and female rat left ventricle. By using independent t-test, there is no significant difference between young and old, male and female rat left ventricle in β2 expression (A). Old male and female show significant higher GPR30 expression comparing to young male ($p \leq 0.001$) and female ($p \leq 0.0001$), respectively (B). Old male and female show significant higher GAPDH expression comparing to young male ($p < 0.001$) and female ($p < 0.0001$), respectively (C). There is no significant difference between young male and young female, or old male and old female rat left ventricle in GPR30 (B) or GAPDH expression (C). ** $p < 0.001$, significantly different from male, *** $p < 0.0001$, significantly different from male. Data are presented as mean ± standard error of mean (SEM).



C

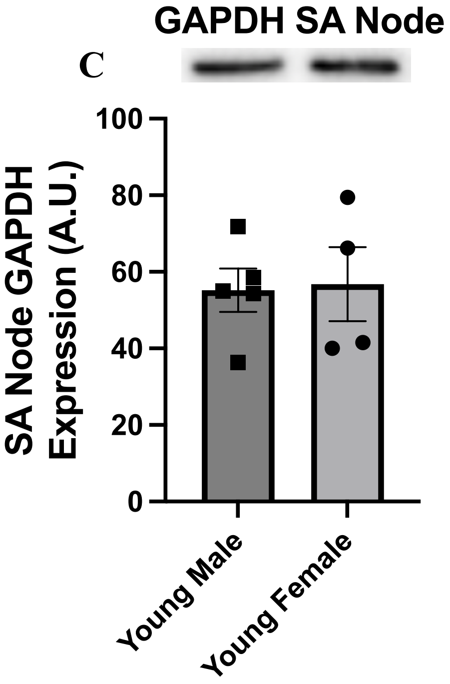


Figure 7. β2, GPR30 and GAPDH expression in young and old, male and female rat SA node. There is no significant difference between young male and female rat SA node in β2, GPR 30 and GAPDH expression (A, B&C) by using independent t-test. Data are presented as mean ± standard error of mean (SEM).

Discussion

The main outcome of this study revealed that young female rats exhibit a diminished HR response to β2-AR stimulation in comparison to their young male counterparts, and the acute presence of E2 eliminates this observed sex-based difference in young rats. This indicates that E2 may play a key role in young female rats HR response to β2-AR stimulation. This is the first study, to our knowledge to reveal that in the intact heart there is a sex difference in HR responses to β2-AR stimulation that is modulated by the acute presence of E2. Intriguingly, the sex difference in HR response to β2-AR stimulation was not observed in old male and old female rats, and the presence of E2 did not affect the responses in the old animals. The findings contribute to our understanding of the interactions between sex hormones and β2-AR stimulation, providing insights into the mechanisms by which estrogen regulates cardiac function. Furthermore, we found the expression of the E2 membrane receptor GPR30 is significantly higher in old compared to young rat ventricular tissue, which contributes to our understanding of the aging effect on heart GPR30 expression. Taken together, this data indicates that the different functional response between young and old, male and female hearts to β2-AR stimulation is not driven by different expression of the β2-AR and GPR30, but somewhere downstream in the signaling pathway likely contribute to this difference.

Young male and female rats show different HR responses

As we hypothesized, young female rat hearts exhibit a blunted HR response to β2-AR stimulation compared to young male hearts, consistent with the previous research [55], which reported that blunted HR response to non-selective β-ARs agonist, isoproterenol, stimulation in female rabbit hearts compared to male rabbits [55]. This sex difference in β2-AR responsiveness can be attributed to differences in heart β2-AR protein expression. According to findings by Peter et al. [14], female rat hearts exhibit lower β2-AR mRNA level compared to male rats, which may lead to a lower β2-AR density in female rats. The low β2-AR density could contribute to the attenuated HR responsiveness to the β2-AR stimulation in female rats. However, we did not see a difference in β2-AR density between male and female in the SA node. Therefore, it is unlikely that SA node β2-AR density explains the sex differences in HR responses we observed in young rats. The attenuated HR response observed in young female rat hearts may related to other factors influencing signaling pathway of β2-AR.

β2-AR regulates HR response through adenylate cyclase/ cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling pathway. The secondary messenger system cAMP plays an essential role related to β2-AR stimulation, which enhances depolarization rate of SA node cells by activating funny channels. Basal cAMP levels were lower in female rat cardiomyocyte compared to male rat [56], indicating there might be sex-based differences in this signaling pathway. Moreover, cAMP production was higher in response to non-selective β-ARs agonist, isoproterenol, stimulation in male rat ventricular myocytes compared to female rats [13]. Thus, even though there was no difference in β2-AR expression in the SA node, if stimulation results in attenuated cAMP levels in females, this may explain the functional difference between males and females. Therefore, the effects on HR might be influenced by cAMP levels and its role in β2-AR response, rather than by receptor density itself.

E2 effects on HR in response to β-AR stimulation

In the E2 dose response experiments, both young male and young female rats HR only showed a decrease at the higher concentration of E2. As a member of G protein-coupled receptor family, GPR30 could bind to both the stimulatory (G_s) and inhibitory (G_i) units of G protein [48]. Following E2 binds to GPR30, GPR30/G_s signaling pathway is activated, leading to an increase in HR. Simultaneously, the GPR30/G_i signaling pathway counteracts the effects of the GPR30/G_s pathway. Thus, the decrease HR may be due to the dominate effect of GPR30/G_i signaling pathway in response to GPR30 stimulation. In the albuterol dose response with the presence of E2, an increased HR is observed in both young male and young female rats. Since β2-AR and GPR30 share the G-protein coupled signaling pathway and β2-AR could also bind to both G_s and G_i, one possible mechanism could be that β2-AR/ G_s signaling pathway plays a domination role in HR response, overpowering the effect of GPR30/G_i.

Previous research demonstrates that E2 influences β-ARs expression: in summary, ovariectomy leads to reduced β-ARs expression in female rats, a phenomenon that can be reversed with E2 treatment [17, 18]. However, the acute effects of E2 on β-ARs stimulation remain largely uninvestigated. Because females have demonstrated lower basal cAMP levels as well as blunted increases in response to non-selective β-ARs stimulation, it is likely the intracellular signaling cascade is different between the sexes. As mentioned above the β2-AR and GPR30 share the G-protein coupled signaling pathway, which results in the production of cAMP. Therefore, the augmented β2-AR responsiveness in young females in the presence of E2 may be due to the stimulation of the GPR-30 pathway. However, no significant effect of E2 on HR in response to β2-AR stimulation was found in old rats. This may because of the age-related reduction of GPR30 responsiveness in the heart.

Age effects on β-ARs stimulation

We found no sex difference in cardiac responses to β2-AR stimulation in old male and female rats, regardless of the presence of E2. Furthermore, old rats exhibited a blunted HR response to β2-AR stimulation compared to young rats, consistent with previous studies that demonstrated blunted β-ARs responsiveness to non-selective β-ARs agonist in old male rats compared to young males [8]. Previous research also supports these findings, as older individuals require a higher dose of non-selective β-ARs agonist to increase resting HR by 25 beats compared to young individuals [9]. One possible mechanism for this age-related difference is the decrease in β2-AR expression with age. β2-AR expression in old rat hearts was significant lower compared to young rats [51]. However, we found no differences in β2-AR expression in the left ventricle between young and old rat hearts. Therefore, the blunted HR response to β2-AR stimulation in old rats may related to other factors, such as cAMP level. Aging is associated with the decreased adenylyl cyclase level [58], which catalyzes ATP to generate cAMP. The decreased cAMP leads to the blunted β2-AR responsiveness in older animals [58]. β2-AR could couple to both the G_s and

G_i units of G protein. The β2-AR/G_s pathway leads to cAMP production and results in greater contractility, whereas the β2-AR/G_i pathway inhibits this effect of β2-AR/G_s activation [21, 59]. Age-related G_i upregulation has been shown in human [60] and animal [61] research. The enhanced G_i activity may also contribute to the decreased β2-AR responsiveness.

Young and old rats show different GPR30 and GAPDH expression

As a novel estrogen receptor, several studies suggest that GPR30 plays a role in cardiac function regulation [77, 94, 95]. Filice et al. [95] reported that G-1, a specific GPR30 agonist, elevated HR in Langendorff-perfused male rat hearts. Another study supports this, showing that G-1-induced cardiac function improvement in a heart failure model. G-1 has also been shown to increased HR in the failing hearts of the ovariectomized female rats [94]. However, in our study, both young male and female rats demonstrated decreased HR in response to E2 stimulation. The difference in our study and previous research is that we use the non-selective ER agonist E2, encompassing ERα, ERβ, and GPR30, rather than GPR30 specific agonist G-1. Thus, E2 may bind to ERα and ERβ, potentially counteracting the effect of GPR30 on HR. However, Filice et al. [95] demonstrated that both ERα and ERβ agonists could individually increase HR. Dose variation may also contribute to the observed HR decrease, given that the lowest E2 dose in our study is 5 μM, whereas the highest dose in Filice's research is 0.01 μM. A possible mechanism is that higher E2 doses may act through ERα and ERβ, contributing to the decline in HR.

Most studies investigate the aging effect on the estrogen receptor expressions in heart have primarily focus on ERα and ERβ. Previous studies have shown that ERα expression decreases in aging female animals compared to young individuals [24, 46], and mRNA levels of ERα also decline with age. Controversial findings exist regarding ERβ expression when comparing young to old animals, with some studies reporting an increase in ERβ expression with age [24] and others finding no significant change [46]. However, few studies have been conducted to evaluate the effect of age on GPR30 expression. Previous research showed opposite aging effect on GPR30 expression. Lindsey et al. [62] found that GPR30 expression in mesenteric arteries form aging female rats was significant lower compared to the young females. Moreover, another study that evaluate GPR 30 expression in the vascular smooth muscle cell of rats showed the similar results: old rats had significant lower GPR30 expression than the young rats [63]. In our study, we observed a significant increase in ventricular GPR30 expression as age increased in both male and female rat hearts, but no significant change in HR response to E2 was observed in old rats compared to their young counterparts. These findings indicate that either the responsiveness of GPR30 is impaired in old rats compared to young rats, or the GPR30-induced HR increase is offset by the activation of ERα and ERβ. A potential mechanism of the elevated GPR30 expression in old rats could be the uncoupling of GPR30 from the G protein in old rat hearts and to maintain the function of GPR30, an overexpression phenomenon is likely to occur.

In our study, a noteworthy observation is the significantly higher GAPDH expression in the left ventricle of old rats compared to young rats. GAPDH, a key enzyme in the glycolysis metabolic pathway, plays a crucial role in breaking down glucose to generate ATP. Although the heart primarily relies on fatty acids as its main energy source, it is also capable of utilizing glucose, lactate, and ketone bodies for energy [61]. Our findings reveal that, with advancing age, there is a significant increase in GAPDH expression, suggesting an augmented reliance on glycolysis as a crucial energy source for the heart. This age-associated shift towards enhanced glycolytic activity aligns with previous research findings. Kates et al. [64] reported an age-related alteration in myocardial substrate metabolism in healthy humans, characterized by a decreased contribution from fatty acids and an increased reliance on glucose. Even though they did not specifically measure GAPDH expression, the heightened glycolytic activity they observed could potentially be linked to increased GAPDH expression. Another study, focusing on glucose transporters, reported an age-related rise in Glut-4 content, indicative of heightened glucose utilization by the heart [65]. These collective findings support the notion that, with aging, the heart undergoes metabolic adaptations, emphasizing the importance of glycolysis and reflected in increased GAPDH expression.

Limitations

We measured the cardiac functional responses to β2-AR stimulation in young and old, male and female rat hearts. One of the limitations of this study is that we did not directly measure ventricular contractility, which is a critical parameter representing the effects of β2-AR stimulation and could have provided additional insights when considered alongside ventricular β2-AR and GPR30 expression to evaluate sex differences. Another limitation of our study is the exclusion of β1-AR from consideration. β1-AR is a crucial factor influencing cardiac function and shares signaling pathways with GPR30. Incorporating β1-AR into our analysis could provide a more comprehensive understanding of our findings. Third, we did not compare SA node β2-AR and GPR30 expression between young rats and old rats, which could have provided valuable insights, especially considering the age-related differences in heart rate. This comparison could have contributed to a better understanding of the underlying mechanisms. Finally, the estradiol dose-response experiments were only performed in young rats, and it is possible the estradiol dose that elicited optimal CFR and HR effects differed between young and old animals.

Conclusions

In conclusion, our study revealed that young female rat hearts exhibit a blunted HR response to β2-AR stimulation compared to their male counterparts, and the presence of E2 eliminates this sex difference. Interestingly, the observed sex difference in HR response could not be attributed to variations in β2-AR expression in the SA node, suggesting that other factors, such as differences in cAMP levels, may contribute. Additionally, our study presents novel findings regarding the increased expression of GPR30 and GAPDH in the left ventricle of rat hearts with advancing age, marking a notable contribution to the existing knowledge. As this is the first documentation of such age-related changes, future investigations should delve into exploring ventricular contractility and cAMP levels in both the SA node and left ventricle of young and old, male and female rat hearts. These investigations will provide further insights into the mechanisms underlying cardiac β-ARs responsiveness and aging-related alterations.

Chapter 3: Cardiac Functional Responses to β2-Adrenergic Receptor Stimulation: Effects of Heart Failure

Introduction

Heart failure (HF) has been a major public health threat worldwide. Over 60 million people worldwide are living with HF, and approximately 1% to 2% of the adult population in developed countries has HF [66]. HF is a complex clinical syndrome characterized by the inability of the heart to pump blood effectively to meet the body's metabolic demands. The pathology of HF involves structural and functional changes in the heart that compromise its ability to maintain adequate cardiac function. β-adrenergic receptors (β-ARs) are G-protein-coupled receptors which play an essential role in the regulation of cardiac function. Overall, stimulation of β-ARs increases heart rate, cardiomyocyte contractility, and conduction velocity. Two catecholamines, epinephrine and norepinephrine, could bind to and activate β-ARs on the myocardial cell membrane to affect cardiac contractility via cAMP secondary messenger system. Thus, β-ARs may play essential role in regulation of cardiac function in HF patients.

β-ARs dysfunction in HF heart includes downregulation and desensitization [20, 59]. β-ARs expression is decreased in the HF heart compared to the healthy heart. Bristow et al. [67] reported that in the HF heart, the density of total β-ARs was approximately 50% lower in the ventricular chambers compared to healthy heart. Another study supports this downregulation, demonstrate a significant reduction in β1-AR numbers in the left ventricle of failing human hearts compared to non-failing hearts. Additionally, a decrease in β2-adrenergic receptor (β2-AR) expression was observed, although the difference was not statistically significant. [68]. Furthermore, the remaining β-ARs become less effective in HF. This diminished effectiveness is attributed to the desensitization of the remaining β-ARs, a process influenced by increased circulating catecholamines and hyperactive sympathetic nervous system (SNS) [22], both commonly associated with HF. The increased circulation of catecholamines and enhanced SNS activity result in the upregulation of G protein-coupled receptor kinase 2 (GRK2) in cardiomyocytes [38, 69, 70]. GRK2 function involves the recognition and phosphorylation of agonist-activated G protein-coupled receptors, such as β-ARs, serving as a mechanism to terminate signaling. Therefore, the elevated GRK2 level in HF heart leads to enhanced phosphorylation of β-ARs, resulting in the uncoupling β-ARs for G proteins such as G_s and G_i [68]. This process ultimately leads to the desensitization of β-ARs. Taken together, β-ARs play essential roles in the cardiac dysfunction observed in HF.

Pre-menopausal women have a lower prevalence of HF compared to age-matched men and post-menopausal women. Therefore, sex hormones may play an important role in regulating cardiac function. Estrogen has been shown as the protector of cardiac health through improvement of angiogenesis, stimulation of vasodilation, and enhancement of mitochondrial function [3]. Estrogen regulates vascular function by modulating endothelial-dependent factors, including endothelial-derived relaxing factors (EDRF) and endothelial-derived contracting factors (EDCF) [31]. By increasing EDRF and inhibiting EDCF generate and release, estrogen causes vasodilation in blood vessels. There are three estrogen receptors (ERs): estrogen receptor α (ERα), estrogen receptor β (ERβ), and G-protein-coupled receptor 30 (GPR30). ERα and ERβ are categorized as nucleus and membrane receptors, while GPR30 is a membrane receptor. There were few studies focused on the effects of HF on ERs expression. According to a previous study that compared HF patients to a healthy control group, the ERα mRNA and protein expression were almost twofold increase in cardiomyocytes of HF patients [47]. Taken together, more studies related to the effects of HF on ERs need to be done, especially on ERβ and GPR30. Therefore, the purpose of this study was to evaluate the cardiac function in response to β2-AR stimulation and the role of estrogen in the HF and healthy rats, and β2-AR and GPR30 expression in HF rat hearts. We hypothesized that 1) HF rats will exhibit impaired cardiac responses to β2-AR stimulation compared to healthy rats, and the presence of estrogen will restore the impaired cardiac responses in HF rats, 2) HF rats will show lower β2-AR expression compared to healthy rats, but only female HF and healthy rats show the difference in GPR30 expression, which will be higher in healthy rats compared to HF.

Methods

Animals

Male and female Hilltop SD rats (Rattus norvegicus, Hilltop Lab Animals, Inc.) were used for this study. All animal research procedures described below have been approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC). TAC and SHAM rats were purchased at 3 weeks and underwent surgery at 4 weeks. For TAC rats, the specific surgery was performed on both male and female rats to induce HF, while SHAM rats were performed a similar surgery as TAC, but the aorta arch was not constricted and were not induce HF.

Transverse aortic constriction surgery

At 4 weeks of age, male and female rats underwent TAC or SHAM surgery. The surgical instruments and operating area were cleaned, and sterilized, and 4-week-old male and female Sprague-Dawley rats were anesthetized with isoflurane (3% - 5%) until the absence of pain reflexes. Isoflurane administration continued via nose cone during the whole surgery. Immediately after anesthetization, buprenorphine (0.05 - 0.1 mg/kg) was administered via intraperitoneal injection. Briefly, the rat was placed supine, and the surgical area was shaved and disinfected. An incision was made at the anterior cervical area, and a silk suture was passed around the aortic arch between the right innominate and left common carotid artery. For TAC procedure, the suture was tied to the size of a 20-gauge needle to induce constriction of the aorta arch. For SHAM procedure, the suture was passed around aorta arch, but no constriction was applied, serving as a surgical control. The surgical area was closed by stitching after the procedure, as described previously [90].

Ultrasound imaging

The Vevo 3100 Preclinical Imaging System (Fujifilm VisualSonics, Toronto, Canada) was used to get ultrasound images from TAC and SHAM rats. The rats were prepared for imaging following the guidelines and positioned on the imaging platform. The heart rate (HR), breathing rate, and body temperature were collected via the four paw pads and the temperature probe. The chest area was shaved and the pre-warmed ultrasound gel was placed on the chest of rats. A transducer (MX250, Scan Depth 30 mm, Vevo 3100, FujiFilm VisualSonics, Toronto, ON, Canada) was used to obtain M-mode recording from the Parasternal Short Axis view of the heart and aorta arch. The ultrasound images we collected were analyzed by using Vevo LAB analysis software (Fujifilm VisualSonics, Toronto, Canada). The aortic arch diameter was assessed to determine the success of the TAC surgery. Ejection fraction left ventricular and septal wall thickness were assessed to determine the presence of HF in the rats.

Langendorff perfused heart

All TAC and SHAM rats were sacrificed at 44 weeks (40 weeks after surgery). After being anesthetized with isoflurane (3% - 5%) and cessation of pain reflexes, the heart was excised, and the aorta was cannulated. The heart was Langendorff-perfused via the aorta at a consistent pressure of 62 mmHg with Krebs-Henseleit buffer, which is composed of NaCl 115 mM, KCl 16.5 mM, HEPES 25 mM, $CaCl_2$ 11 mM, $MgSO_4$ 7 mM, K_2HPO_4 1 mM, KH_2PO_4 1 mM, Glucose 5 mM, and Lactate 5 mM, pH 7.4 at 37°C and bubbled with 100% oxygen. With a consistent pressure of 62 mmHg above the aortic valve and no fluid in the left ventricle, the aortic valve remains closed and forces the buffer flow into the coronary arteries. The Krebs-Henseleit buffer provides oxygen and nutrients to make sure the heart could maintain ex vivo physiological function.

To evaluate the cardiac response to β-adrenergic stimulation, a dose-response experiment was perfused using the β2-AR agonist, albuterol, on hearts from male and female, TAC and SHAM rats. The hearts were perfused for 10 minutes of stabilization, then 0.1, 0.5, 1, and 5 μM of albuterol were added every 5 minutes, while HR and coronary flow rate (CFR) were continually monitored using PowerLab (ADIstruments, Sydney, Australia), and arterial and venous oxygen content were continually monitored by using NeoFox oxygen sensor kits (NEOFOX-KIT-PROBE, Ocean Insight, Orlando, FL). Oxygen consumption rate was calculated by using following equation: [(arterial oxygen concentration – venous oxygen concentration) * CFR * 760 * 1.3 * 10^-6 * 1000]. CFR and Oxygen consumption rate were normalized by the heart mass. HR percentage change was calculated by using following equation: (HR at each drug dose – baseline HR) / baseline HR.

To evaluate the effects of estrogen on the cardiac response to β-adrenergic stimulation, after 10-mintue stabilization, 20 μM 17-β-estradiol (E2) was added, then after 5 minutes, the albuterol dose-response described above was again performed, with doses addition again separated by 5 minutes. HR, CFR, and arterial and venous oxygen content were continually monitored. HR percentage change was calculated by using following equation: (HR at each drug dose or HR at baseline – HR at 20 μM E2) / HR at 20 μM E2.

Western blot

β2, GPR30 and GAPDH protein expression were determined in homogenates made from the left ventricle. Following functional experiments, the left ventricle tissue samples were frozen for later analysis. 40 mg tissue was added to 400 µL 1% Triton buffer (Cat# J62289, Thermo Fisher Scientific, Waltham, MA) with 4 µL protease (Cat# 78430, Thermo Fisher Scientific, Waltham, MA) and 4 µL phosphatase inhibitors (Cat# 78428, Thermo Fisher Scientific, Waltham, MA) and homogenized by using handheld electric homogenizer. The samples were incubated on ice for 10 minutes and then centrifuged at 16,000 g for 10 minutes at 4°C. Protein concentration in prepared homogenates was determined using a BCA assay (Thermo Fisher Scientific, Waltham, MA)

Thirty-five micrograms of protein were loaded onto a 4-15% Mini-PROTEAN TGX Precast Protein Gel (Bio-Rad Laboratories, Hercules, CA) and electrophoresed at 110V for 60 minutes. The separated proteins were transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA) via wet transfer (transfer buffer - 20% methanol, 10% TBS, and 70% deionized water), 4°C, 70V for 90 minutes.

After transfer, the PVDF membrane was incubated for 5 min in TBST, then the membrane was blocked in 6% nonfat dry milk in TBST (10% TBS, 1% Tween-20, 89% deionized water) for 1 hour at room temperature. The membrane was then incubated with primary antibody against the β2-AR [anti-β2-AR rabbit monoclonal antibody (ab182136; Abcam), 1:1500 dilution], GPR30 [GPER1/GPR30 rabbit anti-Human polyclonal (C-Terminus) antibody (LS-A4272; Lifespan Biosciences), 1:2000 dilution] or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [Recombinant HRP Anti-GAPDH antibody (ab201822; Abcam), 1:2000 dilution] overnight at 4°C, followed by secondary antibody incubation (anti-Rabbit IgG, HRP-linked antibody #7074, 1:3000 dilution for β2 , 1:4000 dilution for GPR30 and 1:4000 dilution for GAPDH) for 2 hours at room temperature with 20-25 rpm shaker.

The signal was visualized using a ChemiDoc imaging system (Bio-Rad, CA). The β2, GPR30 and GAPDH were detected using ECL reagent (Cat# 32106, Thermo Fisher Scientific, Waltham, MA) after secondary antibody incubation. The total protein was detected after the protein was transferred from the protein gel to the PVDF membrane, and expression of individual proteins was normalized to total protein.

Statistical Analysis

Statistical analyses were performed by using GraphPad Prism (version 10.0.0 for Windows, GraphPad Software, Boston, Massachusetts USA). A three-way ANOVA was used to examine the effects of sex, HF and albuterol doses on the HR, HR percentage change, CFR, oxygen consumption rate and venous oxygen partial pressure (PO2) in responses to β2 stimulation. Following the three-way ANOVA, post hoc analyses were performed using Tukey's Honestly Significant Difference test to explore differences between TAC and SHAM rats, and between male and female rats. Independent t-tests were performed between TAC and SHAM, male and female rats to determine the sex and age effects on protein expression. Independent t-tests were also performed to compare baseline and E2 dose in HR and percentage change in HR responses to albuterol stimulation. A $p < 0.05$ was deemed statistically significant.

Results

Rat characteristic data

TAC and SHAM, male and female rat characteristic data are presented in Table 4, while ventricular wall thickness data is provided in Table 5. Age was not significantly different between TAC and SHAM groups for both male and female rats in the albuterol dose response experiment. However, in the albuterol dose response with E2, SHAM males are older ($p < 0.05$) and heavier ($p < 0.01$) than TAC males. Furthermore, SHAM males demonstrate a significantly greater heart mass and septal wall thickness compared to TAC males ($p < 0.05$); however, this is likely due to their greater mass, as heart mass normalized to body mass was not different between TAC and

SHAM males. No significant differences are observed in other parameters between TAC and SHAM rats. When comparing male and female rats, males exhibit significantly higher body mass and heart mass, along with greater ventricular wall thickness.

Table 4. Characteristic data of TAC and SHAM, male and female rats

		Age (weeks)	Rat Mass (g)	Heart Mass (g)	Heart/Rat Mass Ratio (g/kg)
		M *SD*	*M* *SD*	*M* *SD*	*M* *SD*
Albuterol Dose Response	TM (n=6)	46 ± 1	597 ± 40	1.80 ± 0.24	3.02 ± 0.45
	SM (n=9)	45 ± 1	622 ± 37	1.83 ± 0.14	2.94 ± 0.24
	TF (n=7)	45 ± 1	310 ± 30$^{\#\#\#}$	1.09 ± 0.09$^{\#\#\#}$	3.52 ± 0.26$^{\#}$
	SF (n=7)	45 ± 2	318 ± 20$^{\#\#\#}$	1.10 ± 0.12$^{\#\#\#}$	3.49 ± 0.44$^{\#}$
Albuterol Dose Response with E2	TM (n=6)	54 ± 23	618 ± 52	2.01 ± 0.54	3.24 ± 0.82
	SM (n=6)	81 ± 11*	773 ± 79**	2.10 ± 0.19	2.73 ± 0.21
	TF (n=5)	71 ± 18	387 ± 84$^{\#\#\#}$	1.37 ± 0.30$^{\#}$	3.57 ± 0.68
	SF (n=6)	67 ± 18	378 ± 61$^{\#\#\#}$	1.29 ± 0.22$^{\#\#\#}$	3.42 ± 0.17$^{\#\#}$

Data represented as mean (M) ± standard deviation (SD). TAC male TM, SHAM male SM, TAC female TF, SHAM female SF, gram g, kilogram, kg, 17-β-estradiol E2. Data were analyzed by using independent t-test. Comparing SHAM to TAC, * $p < 0.05$, significantly different from TAC, ** $p < 0.01$, significantly different from TAC. Comparing females to males (TF to TM and SF to SM), # $p < 0.05$, significantly different from male, ## $p < 0.01$, significantly different from male, ### $p < 0.001$, significantly different from male.

Table 5. Ventricle wall thickness of TAC and SHAM, male and female rats

		Left Ventricle (mm)	Septal Wall (mm)
		M *SD*	*M* *SD*
Albuterol Dose Response	TM (n=6)	4.54 ± 0.37	3.38 ± 0.74
	SM (n=9)	4.23 ± 0.28	3.30 ± 0.25
	TF (n=7)	3.51 ± 0.46$^{\#\#\#}$	2.63 ± 0.36$^{\#}$
	SF (n=7)	3.46 ± 0.48$^{\#\#}$	2.81 ± 0.39$^{\#}$
Albuterol Dose Response with E2	TM (n=6)	4.24 ± 0.62	2.97 ± 0.41
	SM (n=6)	4.71 ± 0.27	3.82 ± 0.48*
	TF (n=5)	4.09 ± 0.49	3.11 ± 0.50
	SF (n=6)	4.03 ± 0.55$^{\#}$	3.00 ± 0.48$^{\#}$

Data represented as mean (M) ± standard deviation (SD). TAC male TM, SHAM male SM, TAC female TF, SHAM female SF, 17-β-estradiol E2. Data were analyzed by using independent t-test.

Comparing SHAM to TAC (SM to TM and SF to TF), * $p < 0.05$, significantly different from TAC. Comparing females to males (TF to TM and SF to SM), # $p < 0.05$, significantly different from male, ## $p < 0.01$, significantly different from male, ### $p < 0.001$, significantly different from male.

Echocardiogram results

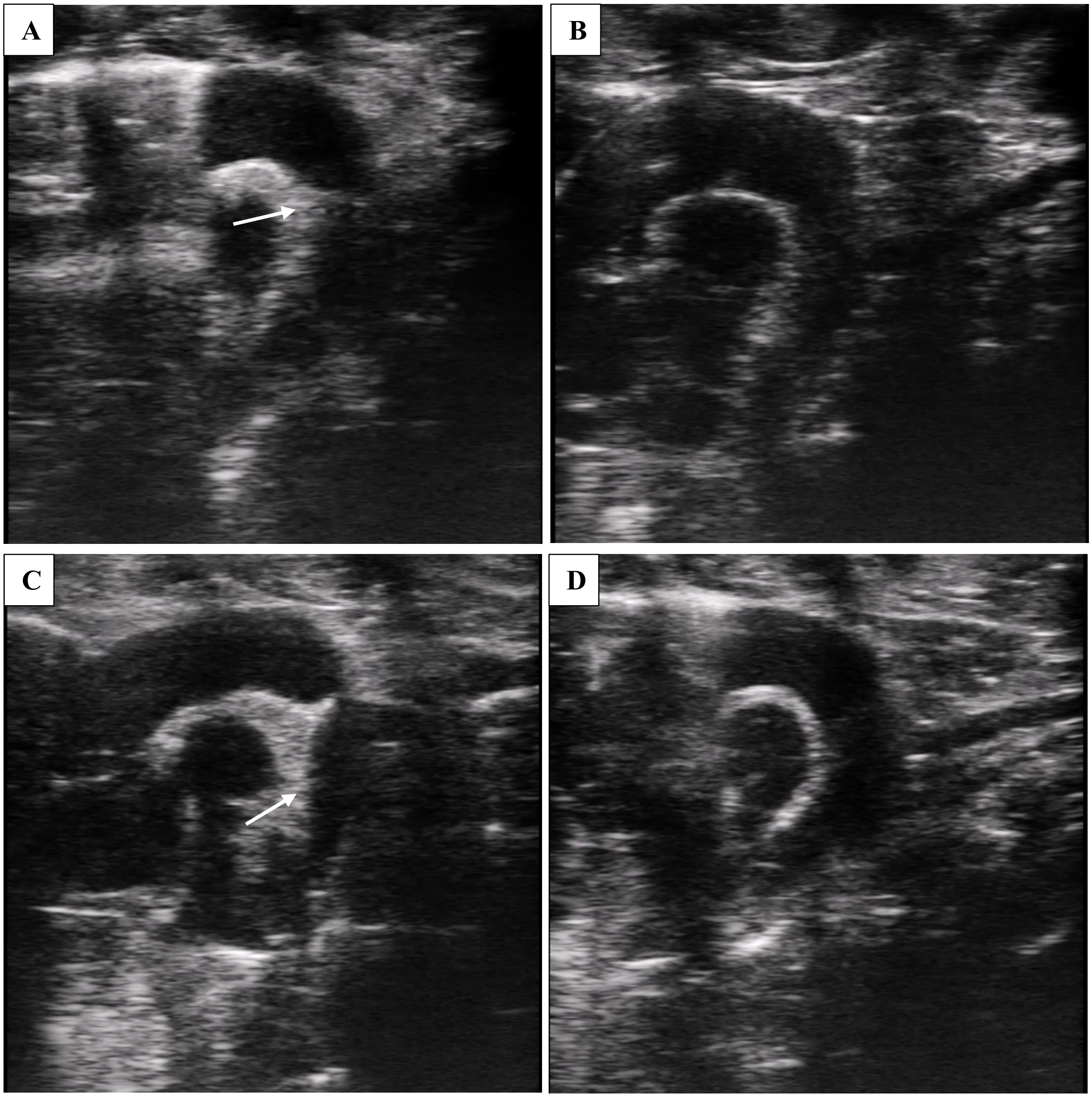


Figure 8. TAC and SHAM, male and female rat aorta arch echocardiogram. TAC male (A) and TAC female (C) rats showed a constriction on aorta arch. There was no constriction on aorta arch of SHAM male (B) and SHAM female (D).

HR response to albuterol in TAC and SHAM, male and female rat hearts in the absence and presence of E2

To assess the impact of sex and HF on the response to β2 stimulation, albuterol dose-response curves are generated for male and female rat hearts in both TAC and SHAM groups. Subsequently, albuterol dose-response curves are generated once more with the presence of E2 across all groups.

A three-way ANOVA is conducted to examine the main effects of sex, HF and albuterol dose on HR and HR percentage change in response to albuterol stimulation. The results reveal a significant elevation in HR and HR percentage change in response to albuterol across all groups, as evidenced by F(4, 125) = 19.27 and F(4, 125) = 29.349, respectively, with p-values below 0.001 (Figure 9A&C). TAC rats exhibit a comparatively greater response in HR percentage change than their SHAM counterparts [HF effect (F(1, 125) = 6.864, p = 0.01)]. Further analysis using Tukey's Honestly Significant Difference test reveals TAC males (35.2 ± 8.5 %) exhibit significant greater HR percentage change 5 μM albuterol doses ($p < 0.05$) compared to TAC females (22.0 ± 5.6 %) (Figure 9C). Even though there is sex difference between TAC male and female rats in the response to albuterol, overall, TAC and SHAM, male and female rat hearts exhibit similar HR responses to the administered albuterol doses.

In order to evaluate the effects of the presences of E2 on responsiveness to β2-AR stimulation, 20 μM E2 was added to the perfusate prior to the administration of albuterol. The results indicate that there are no statistically significant differences in HR, both in absolute terms and percentage change, between the baseline and after the administration of E2. This suggests that the presence of 20 μM E2 alone does not lead to a significant reduction in HR, as illustrated in Figure 9B&D.

To assess the effects of E2 on HR and HR percentage change in response to albuterol stimulation, a three-way ANOVA was conducted. In the presence of E2, albuterol increases both HR [albuterol dose effect (F(5, 114) = 18.306, $p < 0.001$)] and HR percentage change [albuterol dose effect (F(5, 114) = 30.684, $p < 0.001$)] across all groups (Figure 9B&D). By using Post hoc analysis, TAC males show significant greater HR percentage change at 1 and 5 μM albuterol doses ($p < 0.05$) compared to their SHAM male counterparts (Figure 9D). These findings indicate that TAC and SHAM, male and female rat hearts have similar HR responses to albuterol and HR displays a consistent increase with escalating albuterol concentrations in all groups in the presence of E2.

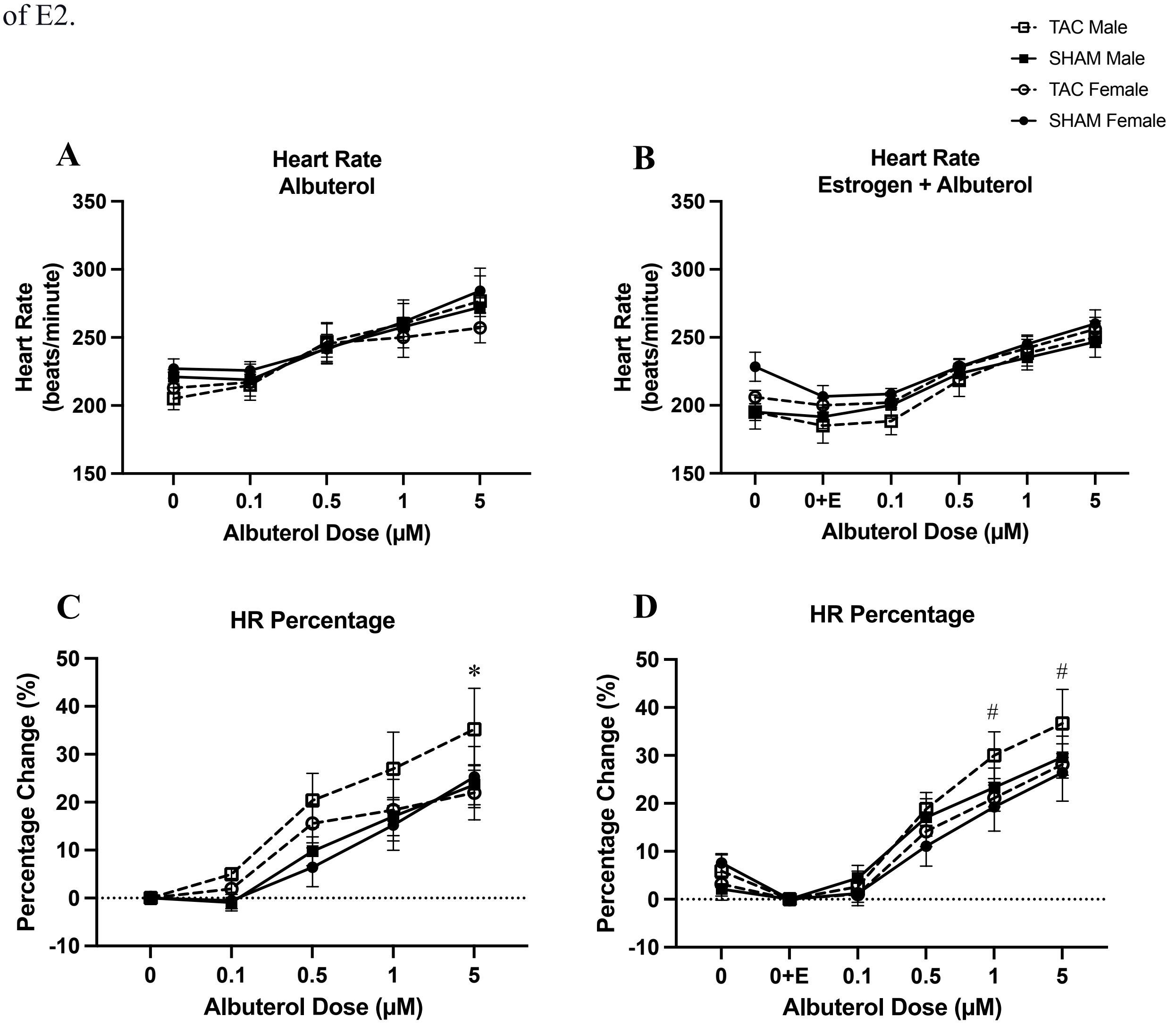


Figure 9. TAC and SHAM, male and female rats heart rate responses to difference albuterol doses in the absence and presence of estrogen. TAC and SHAM, male and female rats show similar heart rate response to β2 stimulation in the absence (A) and presence (B) of estrogen. Heart rate percentage change also showed in the figure (C&D) and no significant different is found between any groups. Data were analyzed by using three-way ANOVA. * $p < 0.05$, significantly different between TAC male and TAC female rats. # $p < 0.05$, significantly different between TAC male and SHAM male rats. Heart rate HR. Data are presented as mean ± standard error of mean (SEM).

CFR, O2 consumption and venous PO2 response to albuterol in TAC and SHAM, male and female rat hearts in the absence and presence of E2

A three-way ANOVA is conducted to examine the main effects of sex, age and albuterol dose on CFR, oxygen consumption rate and venous PO2 in the absence and presence of E2. To evaluate the effects of the presences of E2 on responsiveness to β2-AR stimulation, 20 μM E2 was added to the perfusate prior to the administration of albuterol. The results reveal no statistically significant differences in CFR, oxygen consumption rate and venous PO2, both in absolute terms and percentage change, between the baseline and after the administration of E2. This suggests that the presence of 20 μM E2 alone does not lead to a significant reduction in CFR, oxygen consumption rate and venous PO2 (Figure 10B&D&F).

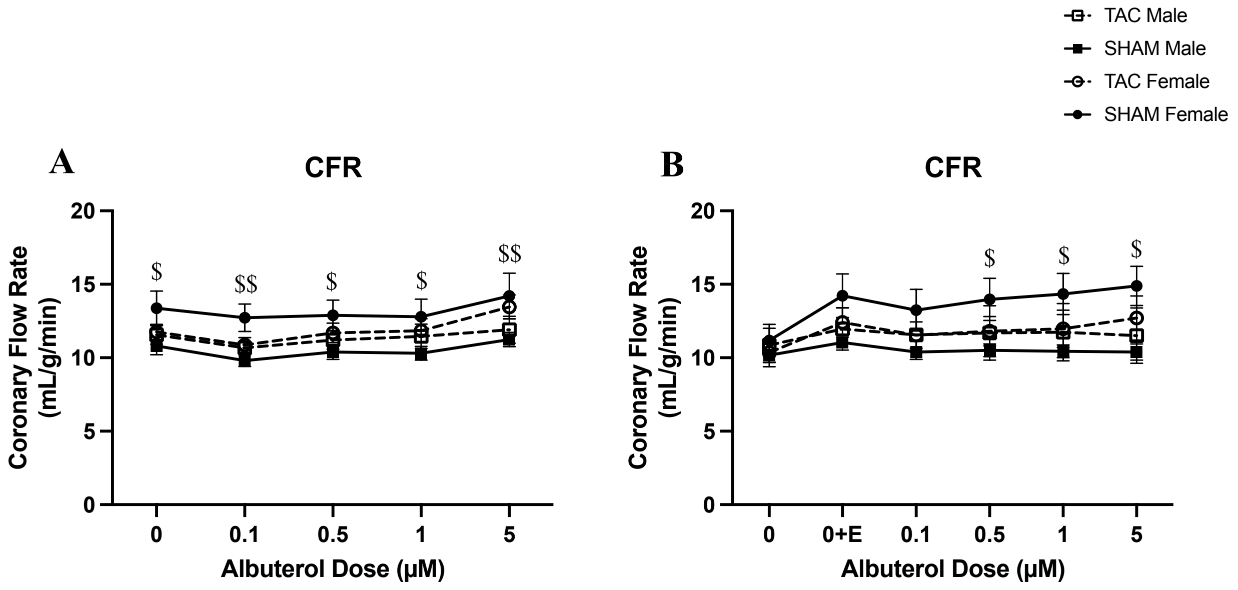
In the absence of E2, CFR analysis reveals a significant higher in SHAM females compared to SHAM males [sex effect ($F(1, 125) = 20.965$, $p < 0.001$)], with notable interaction effects between sex and HF ($F(1, 125) = 8.867$, $p = 0.003$). Subsequent post hoc analysis using Tukey's Honestly Significant Difference test identifies group differences, particularly in the sex variable, where SHAM females exhibit significant higher CFR compared to SHAM males at all albuterol doses ($p < 0.05$) (Figure 10A). In the presence of E2, a significant main effect is observed for sex ($F(1, 114) = 10.884$, $p = 0.001$), accompanied by significant interaction effects for sex and HF (1, 114) = 7.9, $p = 0.006$). Post hoc analysis using Tukey's Honestly Significant Difference test reveals group differences, with SHAM females demonstrate significant higher CFR compared to SHAM males all albuterol doses except 0.1 µM ($p < 0.05$) (Figure 10B). These results indicates that SHAM male and SHAM female rats have different CFR in response to albuterol stimulation in the absence and presence of E2, while TAC males and females demonstrate similar CFR responses regardless of the presence of E2.

Albuterol increases oxygen consumption rate in all groups in the absence (F(4, 120) = 27.097, $p < 0.001$) and presence (F51, 114) = 12.125, $p < 0.001$) of E2. Additionally, the three-way ANOVA analysis highlights significant main effects for sex, indicating that females exhibit a higher oxygen consumption rate compared to males, irrespective of HF and the presence of E2. Post hoc analysis, which concerning sex, indicates that SHAM females have significant higher oxygen consumption rate compared to SHAM males at all albuterol doses in the absence ($p < 0.05$) and presence ($p < 0.05$) of E2 (Figure 10C&D). In the presence of E2, SHAM females exhibit significant higher oxygen consumption rate compared to TAC females at 1 µM (12.8 ± 1.5 mmol/g/min in SHAM vs. 10.0 ± 1.0 mmol/g/min in TAC) and 5 µM (15.3 ± 1.6 mmol/g/min in SHAM vs. 11.3 ± 1.1 mmol/g/min in TAC) albuterol doses ($p < 0.05$) (Figure 10D). The results suggest that, both in the absence and presence of E2, SHAM females exhibit a significantly higher oxygen consumption rate compared to SHAM males. Additionally, when compared to TAC female rats, SHAM females demonstrate a significantly higher oxygen consumption rate specifically in the presence of E2.

In the absence of E2, male rats exhibit significantly higher values for venous PO2 compared to females [sex effect (F(1, 120) = 9.768, p = 0.002)], and there is a notable decrease in venous PO2 with increased albuterol doses [albuterol dose effect (F(4, 120) = 28.572, p < 0.001)] according to three-way ANOVA analysis. Additionally, significant interaction effects are observed for sex and HF (1, 120) = 19.208, p < 0.001). Further analysis was applied to evaluate the sex and

HF effects by using Tukey's Honestly Significant Difference test. For sex, comparing to SHAM females, SHAM males demonstrate significant higher values of venous PO2 at all albuterol doses except 5 µM ($p < 0.05$) (Figure 10E). Regarding HF, at 0.5 µM albuterol dose, TAC males (310.2 ± 24.5 mmHg) show significant lower venous PO2 compared to SHAM males (414.5 ± 30.8 mmHg) ($p < 0.05$) (Figure 10E). In the presence of E2, male rats also show significantly higher values for venous PO2 compared to females, and all groups demonstrate significant decreased venous PO2 in response to albuterol. There are significant main effects for sex (F(1, 114) = 5.896, $p = 0.017$) and albuterol dose (F(5, 114) = 11.072, $p < 0.001$).

In summary, the collective findings indicate that both TAC and SHAM rats, regardless of sex, exhibit comparable responses in CFR, oxygen consumption rate, and venous PO2 to albuterol, both in the absence and presence of E2. Furthermore, any observed differences between SHAM males and SHAM females may be associated with the notable age difference, with SHAM males being significantly older compared to their female counterparts.



C **D**

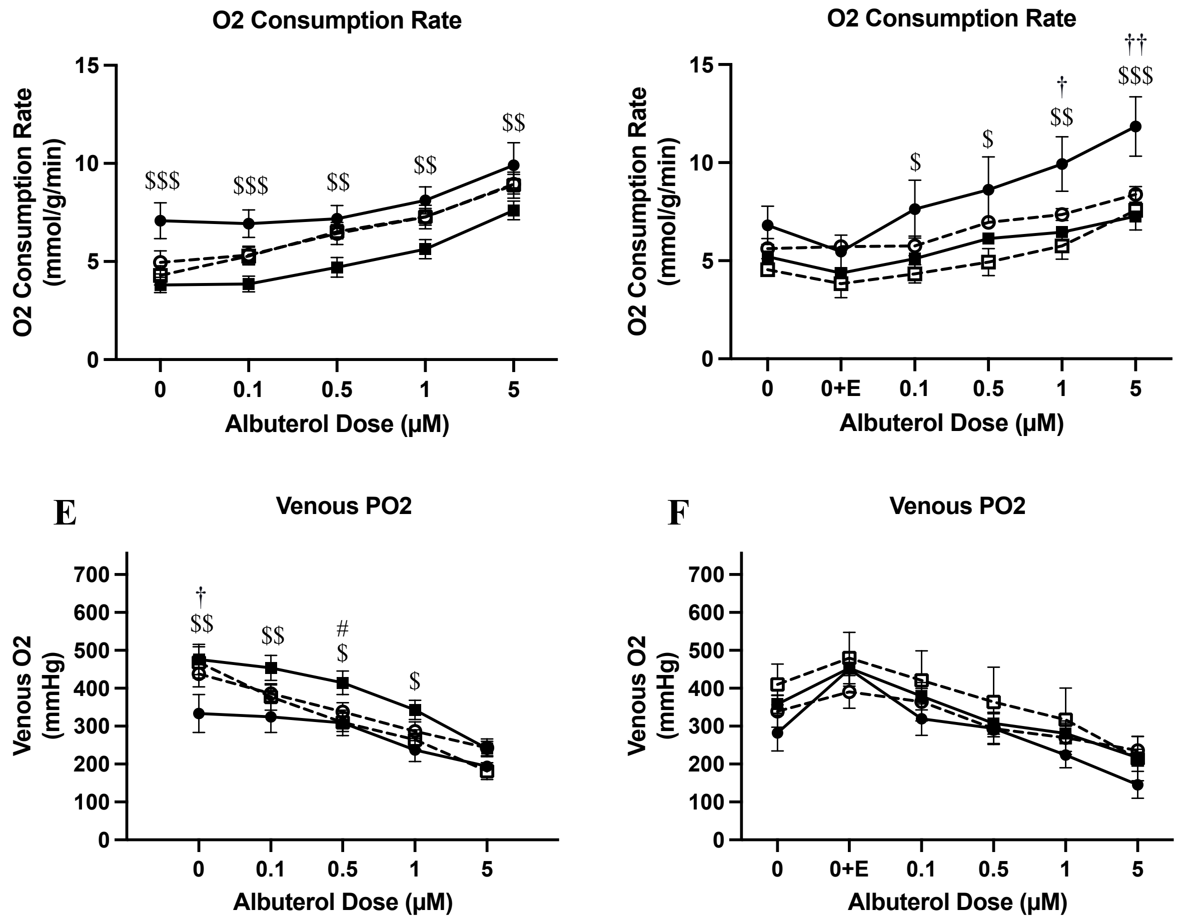


Figure 10. TAC and SHAM, male and female rats coronary flow rate and O2 consumption responses to difference albuterol doses in the absence and presence of estrogen. There is no significant difference between TAC and SHAM, male and female rat heart weight normalized coronary flow rate (CFR) and oxygen consumption rate to β2 stimulation (A&C). With the presence of estrogen, SHAM females show significant higher CFR ($p = 0.004$) and oxygen consumption rate ($p = 0.002$) compared to SHAM males (B&D). Comparing to TAC females, SHAM females also show significant higher oxygen consumption rate ($p = 0.016$) with the presence of estrogen. There is no significant different in venous oxygen partial pressure between any groups. Data were analyzed by using three-way ANOVA. # $p < 0.05$, significantly different between TAC male and SHAM male rats. † $p < 0.05$, †† $p < 0.01$, significantly different between TAC female and SHAM female rats. \$ $p < 0.05$, \$\$ $p < 0.01$, \$\$\$ $p < 0.001$, significantly different between SHAM male and SHAM female rats. milliliter/gram/minute mL/g/min, millimolar/gram/minute mmol/g/min, oxygen partial pressure PO2, milliliter mercury mmHg. Data are presented as mean ± standard error of mean (SEM).

Table 6 presents the absolute data for CFR and oxygen consumption rate in response to the maximal albuterol dose (5 μM). In both the absence and presence of E2, there is no significant difference between TAC males and SHAM males or TAC females and SHAM females in either

CFR or oxygen consumption rate following albuterol administration. Nevertheless, in the absence of E2, TAC females exhibit a significantly lower CFR ($p = 0.001$) and oxygen consumption rate ($p = 0.003$) in comparison to TAC males. Concurrently, SHAM females demonstrate significantly reduced CFR ($p = 0.011$) and oxygen consumption rate ($p = 0.041$) relative to SHAM males in response to albuterol under similar E2-deprived conditions. No statistically significant differences are observed between male and female rats in response to albuterol in the presence of E2.

Table 6. Absolute cardiac functional data at the highest albuterol dose in absence and presence of E2.

		Coronary Flow Rate (mL/min)	O2 Consumption Rate (mmol/min)
		M *SEM*	*M* *SEM*
Albuterol Dose Response	TM	21.2 ± 1.4	15.9 ± 1.3
	SM	20.6 ± 1.2	14.0 ± 1.1
	TF	14.6 ± 1.0$^{\#\#\#}$	9.8 ± 0.8$^{\#\#}$
	SF	15.4 ± 1.3$^{\#\#}$	10.7 ± 0.9$^{\#}$
Albuterol Dose Response with E2	TM	21.1 ± 1.9	14.3 ± 1.0
	SM	21.7 ± 0.8	15.2 ± 0.4
	TF	16.9 ± 1.6	11.3 ± 1.1
	SF	18.8 ± 1.0	15.3 ± 1.6

Data represented as mean (M) ± standard error of mean (SEM). TAC male TM, SHAM male SM, TAC female TF, SHAM female SF, 17-β-estradiol E2, milliliter/minute mL/min, millimolar/minute mmol/ min. Data are analyzed by using independent t-test. Comparing SHAM to TAC (SM to TM and SF to TF), no significant difference is found. Comparing female to male (TF to TM and SF to SM), # $p < 0.05$, significantly different from male, ## $p < 0.01$, significantly different from male, ### $p < 0.001$, significantly different from male.

β2 and GPR30 protein expression

The evaluation of β2 and GPR30 protein expression in left ventricle samples obtained from TAC and SHAM rats, both male and female, reveals no significant differences in the expression levels of β2 and GPR30 proteins across all experimental groups (Figure 11).

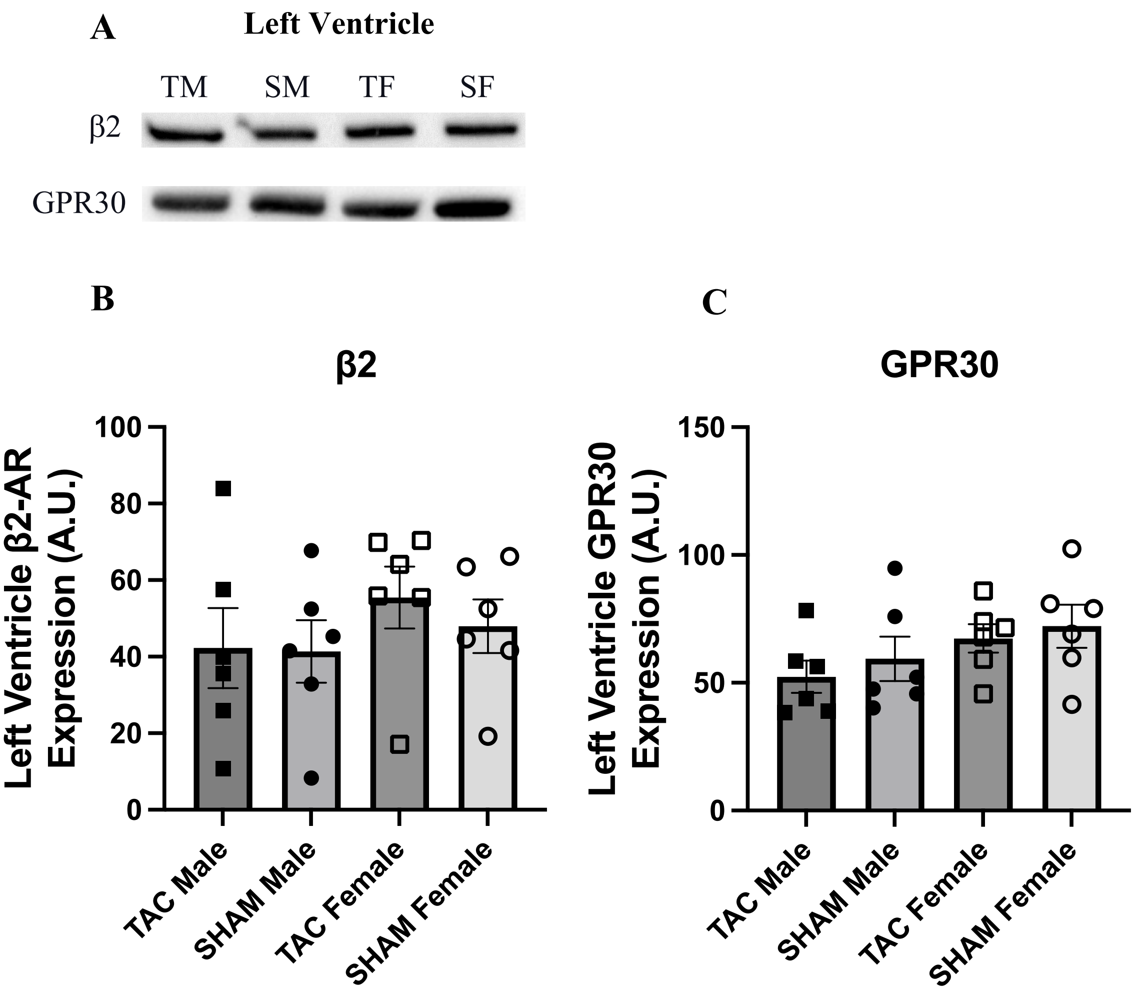


Figure 11. TAC and SHAM, male and female rats left ventricle β2-AR and GPR30 protein expression. Data were analyzed by using independent t-test. There is no significant difference between TAC and SHAM, male and female rat in neither β2-AR (B) nor GPR30 (C) expression. Data are presented as mean ± standard error of mean (SEM).

Discussion

The primary goal of our study was to investigate differences in cardiac responses to β2-AR stimulation between TAC and SHAM, male and female rats. Additionally, we explored the role of estrogen (E2) on β2-AR responses. First, our study found modest changes in ventricular hypertrophy and function as a result of 40-weeks of TAC in male and female rates. Further, there are were minor variations in parameters of cardiac function in response to β2-AR stimulation among TAC and SHAM male and female rats. Moreover, the presence of E2 did not alter the responsiveness of male and female rat hearts to β2-AR stimulation between the absence and presence of E2. Overall, the results indicate that TAC and SHAM, male and female rats, exhibit comparable patterns in the cardiac responses to β2-AR stimulation, regardless of the presence of E2.

HR responses to β-ARs stimulation in TAC and SHAM rats

Despite albuterol inducing a notable increase in HR across all groups, no distinction in the response to β2-AR stimulation was evident between HF rat and healthy rat hearts. This observation contrasts with prior research indicating a reduction in β-ARs expression and desensitization in failing hearts, characterized by decreased β1-AR mRNA and protein levels and uncoupling of β2-AR from signaling pathways [1, 71, 72]. Consistent with these findings, our study revealed no significant alteration in the mRNA and protein levels of β2-AR [68, 72]. Furthermore, our investigation aligns with previous research, as there was no significant difference in left ventricle β2-AR expression between TAC and SHAM rats. These results highlight that the β2-AR expression pattern remains stable even in the TAC rats.

Given the uncoupling of cardiac β2-AR in HF, the expectation would be a diminished HR response to β2-AR stimulation in HF hearts compared to their healthy counterparts. This anticipation aligns with a previous study by Chang et al. [73], who observed a significantly lower beating rate in the sinoatrial (SA) node from HF rabbit hearts compared to controls, implying a potentially blunted HR response to β-ARs stimulation in HF. Verkerk et al. [74] further supported this notion, demonstrating a decrease in firing rate in single SA node cells due to HF. Contrary to these expectations, our findings diverged as the HF heart did not exhibit a blunted HR response compared to the control heart. This difference may be attributed to the dual, opposing signaling pathways of β2-AR stimulation. Specifically, β2-AR stimulation has been reported to engage both stimulatory (G_s) and inhibitory (G_i) units of the G protein [1, 21]. The β2-AR/G_s pathway fosters cyclic adenosine monophosphate (cAMP) production, enhancing the firing rate of the SA node. Conversely, the β2-AR/G_i pathway mitigates the effects of β2-AR/G_s activation, exerting an inhibitory influence [21]. In the context of HF, the altered β1/β2-AR ratio and upregulation of G_i [72] suggest an enhanced role for inhibitory G protein units in the HF heart's response to β2-AR stimulation. Consequently, the stimulation of β2-AR may not manifest a discernible difference in increased HR between TAC and SHAM rats. These results highlight the interaction of signaling pathways, emphasizing the need for more research to understand the complexities of HR responses to β2-AR stimulation in the context of HF.

E2 effects on HF heart

One of E2 effects on vascular tone maintenance is the preservation of vasodilation. Specifically, E2 is implicated in binding to membrane estrogen receptors, eliciting the release of NO through a non-genomic pathway [3]. NO, a crucial molecule in the regulation of vascular function, exerts its influence by inducing vasodilation and modulating blood pressure [75]. This effect suggests a potential involvement of membrane estrogen receptors, particularly GPR30, in the HF treatment. Recent research has underscored the critical role of GPR30 in HF and posited it as a prospective treatment target for HF patients [76-78]. Zhang et al. [76] demonstrated that chronic GPR30 activation treatment restored cardiac function in male mice and reversed the progression of HF. Another study reported the attenuation of HF in female rats through chronic GPR30 activation treatment [77]. One major effect of GPR30 activation is the enhancement of cardiac contractility. Filice et al. [95] reported that G-1, a specific GPR30 agonist, enhanced left ventricle contractility in Langendorff perfused male rat hearts. Another study supports this G-1-

induced cardiac function improvement. In the heart failure heart model, G-1 also increased the left ventricle contractility in ovariectomized (removal of ovaries) female rats [94]. Furthermore, G-1 treatment also improve the myocardial cells contraction in the failing hearts of ovariectomized female rats [94]. The mechanisms underlying the therapeutic effects of GPR30 activation encompassed improvements in β2-AR expression and activity, enhanced Ca^{2+} regulation, and augmented ion channel activity [76, 79]. These findings not only corroborate the vasodilatory effect of E2 observed in our study but also emphasize the potential of GPR30 as a therapeutic target in the context of HF, offering avenues for further exploration and potential clinical interventions.

Nonetheless, our investigation did not reveal any discernible difference in HR, CFR or oxygen consumption rate between TAC and SHAM rats. In our study, TAC rats only have mild HF based on the ventricular parameters from ultrasound data. The divergence from prior studies might be attributed to the deliberate focus of our experimental design on the short-term effects of E2. E2 exerts its influence through binding to both nuclear receptors (ERα and ERβ) and membrane receptors (ERα, ERβ, and GPR30). Through nuclear receptors, E2 facilitates chronic genomic regulation, regulating gene expression and the activity of signaling molecules [3]. Conversely, acute non-genomic regulation involves E2 binding to membrane receptors to promptly modulate signaling molecules. In our study, we emphasize the acute and immediate impact of E2 on β2-AR stimulation and no significant differences were observed between TAC and SHAM. One possible reason is that the TAC rats in this study may only exhibit mild HF. The short-term effects of E2 on β2-AR stimulation could result in similar HR, CFR or oxygen consumption rate in mild HF TAC rats compared to SHAM rats. While our study did not reveal disparities in HR, CFR or oxygen consumption rate between TAC and SHAM rats the interaction between estrogen genomic and non-genomic regulatory mechanisms invites further exploration to elucidate the E2 acute impact on HR, CFR and oxygen consumption rate.

Limitations

There are some limitations in this study. First, for the albuterol dose response experiment in the presence of E2, the SHAM male rats are significantly older than TAC males. Thus, age may also affect the results beyond the HF. Second, even though ultrasound images show there is constriction on the aorta arch and the ventricular wall thickness is relative higher in TAC compared to SHAM rats, the TAC rats in this study may either exhibit only mild HF or early HF, and this may explain the lack of differences between TAC and SHAM rats. Third, we did not measure the contractility of left ventricle in HF rat hearts. HF mainly affects the cardiomyocyte contractility and the contraction force generated by left ventricle could help us with a better understanding of cardiac responses to β2-AR stimulation.

Conclusions

In conclusion, our study found similar responsiveness of male and female rat hearts to β2-AR stimulation in both TAC and SHAM groups. Despite albuterol-induced HR increases, no discernible differences were observed between TAC and SHAM rat hearts, challenging previous expectations. These findings suggest that the TAC model utilized in our study may not fully represent the heart failure phenotypes. Additionally, it's noteworthy that in certain experiments, SHAM male rats were notably older compared to other groups, introducing the potential influence of aging on our findings.

Chapter 4: Muscular Artery Vascular Functional Responses to β2-Adrenergic Receptor Stimulation in Male and Female Rats

Introduction

Muscular arteries are able to regulate blood flow to meet the varying demands of different tissues during various physiological conditions. β-adrenergic receptors (β-ARs) are a subtype of adrenergic receptors that play a crucial role in regulating muscular artery function. In muscular arteries, β-ARs are primarily found on smooth muscle cells in the arterial walls. When activated, a cascade of intracellular events is initiated, which mediate smooth muscle tone and endothelial function regulation. The primary effect of β-ARs on muscular arteries is vasodilation. Two major β-ARs are involved in the regulation of muscular artery function: the β1-adrenergic receptor (β1-AR) and the β2-adrenergic receptor (β2-AR). The predominance of β2-AR in the smooth muscle cells of muscular arteries initiates vasodilation through multiple signaling pathways, and the contribution of β1-ARs to these vasodilatory effects is relatively minor.

Vasodilation, which can be initiated by β2-AR stimulation, is coordinated through three primary signaling pathways: eNOS, cAMP/PKA, and calcium-dependent potassium channels [81]. When β2-AR are activated, a series of enzymatic events lead to the activation of eNOS, releasing NO and causing vasodilation. Simultaneously, the cAMP/PKA pathway, also activated by β2-AR, aids in lowering intracellular calcium concentration in vascular smooth muscle cells, resulting in vasodilation. Additionally, β2-AR activation prompts the opening of K^+ channels, leading to increased extracellular K^+ concentration, reduced membrane potential, and hyperpolarization. As a result, voltage-dependent calcium channels close, initiating relaxation in vascular smooth muscle. Previous research indicates that β-AR induced vasodilation disappears in multiple blood vessels of β1-AR/β2-AR knockout mice [23], indicating the essential role of β-ARs in muscular artery vasodilation. Albuterol, the β2-AR agonist, increases forearm blood flow in both young men and women, but young women show a greater increase in forearm blood flow than young men [82]. This finding indicates that young women are more sensitive to β2-AR stimulation compared to young men.

Estrogen may play an essential role in mediating the sex differences of β2-AR stimulation of arterial vasodilatory responses in muscle. Post-menopausal women have a blunted forearm blood flow increase after adding the same dose of isoproterenol, a non-selective β-ARs agonist, compared to pre-menopausal women [27]. Thus, the difference in forearm vasodilation between pre- and post-menopausal women may be related to different estrogen levels. Kneale et al. [82] investigated the sexual dimorphism in the response of the forearm vasculature to stimulation of β2-AR and found that, in comparison to young men, young women exhibited a more pronounced forearm vasodilation. This observation underscores that the presence of estrogen contributes to an augmented responsiveness to β2-AR stimulation in the context of vascular regulation. Taken together, muscular artery vasculature β2-AR vasodilation may be affected by estrogen.

A plausible mechanism to explain estrogen’s role in muscular artery vasculature β2-AR vasodilation is estrogen’s potential modulation of β-ARs expression. Studies have demonstrated that estrogen regulates β1-AR expression in the ovariectomized (removal of ovaries) female rat aorta by elevating the mRNA levels of β1-AR [89], suggesting a potential upregulation of β1-AR expression by estrogen. For β2-AR, previous research also report that estrogen upregulates the expression in heart. Wu et al. [92] and Liu et al. [93] both found that female ovariectomized rats downregulate β2-AR expression in ventricular myocytes, an effect that was rescued with estrogen treatment.

Beyond modulating vasculature vasodilation through affecting β-ARs expression, estrogen could also modulate vasculature through the regulation of endothelial-derived relaxing factors (EDRF) and endothelial-derived contracting factors (EDCF) [31]. Estrogen enhances vasodilation and attenuates vasoconstriction by modulating endothelial-dependent factors, thus facilitating blood vessel vasodilation [17, 37]. These dual mechanisms highlight the role of estrogen in influencing vascular function, encompassing both receptor-level regulation and modulation of endothelial factors. Estrogen modulates vasculature function by binding to estrogen receptors (ERs) on the vascular endothelial and smooth muscle cells. ERs can be broadly categorized into two main types based on their subcellular localization: nuclear ERs and membrane ERs. Estrogen receptor α (ERα) and Estrogen receptor β (ERβ) are primarily localized in the cell nucleus, while GPR30 is membrane-bound receptor. Membrane receptor, GPR30, can mediate acute, non-genomic responses, while nuclear receptors, ERα and ERβ, contribute to chronic, genomic responses that involve changes in gene expression.

In addition to estrogen, β2-AR stimulation *in situ* leads to vasodilation in the vascular beds. The underlying mechanism is that as G protein-coupled receptors, β2-AR and GPR30 share similar signaling pathways, including the PI3K/Akt/eNOS pathway which leads to vasodilation. The observed sex differences in vasodilation may be attributed to the variation in β2-AR density, estrogen effects on β2-AR stimulation and signaling pathways interaction between β2-AR and GPR30. Despite these insights, the direct vasoactive effects of β2-adrenergic stimulation and the role of estrogen in the muscular arteries are still not fully understood. Therefore, the goal of this study was to evaluate the sex differences in muscular artery vasculature responses to β2-AR stimulation in the absence and presence of estrogen in male and female rats. We hypothesized that the femoral artery in young female rats exhibits a heightened vasodilation response to β2-AR stimulation in comparison to young male rats, and estrogen may enhance this vasodilation response specifically in young female rats. Furthermore, we proposed that with advancing age, the estrogen-augmented vasodilation response observed in young female rats diminishes or ceases.

Methods

Animals

Male and female Hilltop SD rats (Rattus norvegicus, Hilltop Lab Animals, Inc.) were used for this study. All animal research procedures described below have been approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC). Young rats (4 males, 5 females) were defined as less than twelve weeks old, middle-aged rats (5 males, 5 females) were around forty-two weeks old.

Femoral artery perfusion

The rats were anesthetized with isoflurane (3% - 5%) until pain reflexes ceased. The heart was excised via bilateral thoracotomy. Utilizing microsurgical scissors, the skin was removed from the left and right hindlimbs to expose approximate 6 mm section of femoral arteries. The femoral artery and associated nerve and vein were gently dissected and separated from the musculature to minimize damage to the artery. The femoral artery was carefully dissected from the nerve and vein using microsurgical scissors, with care taken to avoid damage to the artery. Each femoral artery was dissected into two ~3 mm long segments, resulting in 4 femoral artery segments from one rat to perform experiments.

The femoral artery was placed in ice-cold physiological saline solution (PSS) and continuously bubbled with 100% oxygen. PSS buffer is composed of NaCl 141.9 mM, KCl 4.7 mM, HEPES 10 mM, CaCl2 2.8 mM, MgSO4 1.7 mM, KH2PO4 1.2 mM, Glucose 5 mM, and EDTA 0.5 mM, pH of 7.4. After 15 minutes of equilibration in the ice-cold PSS, the femoral artery was cannulated with custom-made cannula, a modified 27-gauge needle (outside diameter 0.413 mm) at both ends and was put in the vessel chamber (Single Vessel Chamber CH-1, Living System Instrumentation, Fairfax, VT) maintained at 37°C, with the PSS continuously bubbled with 100% oxygen. Two syringes, filled with PSS and positioned at an elevated level, were connected to respective cannulas. This setup allowed for the perfusion of the femoral artery with PSS, maintaining a consistent pressure of 90 mmHg throughout the duration of the experiment.

After the stabilization of basal tone, PSS buffer was removed and high potassium PSS (KPSS) buffer, which is composed of NaCl 95.8 mM, KCl 38 mM, HEPES 10 mM, CaCl2 2.8 mM, MgSO4 1.7 mM, KH2PO4 1.2 mM, Glucose 5 mM, and EDTA 0.5 mM, pH 7.4, was added to the vessel chamber to fully depolarize vascular smooth muscle cells to evaluate the vascular function. Then the chamber was rinsed with PSS three times every ten minutes and PSS was added again in the chamber after the rinse. After 10 minutes of stabilization, phenylephrine, a vasoconstrictor, was added in the chamber to achieve the maximum vasoconstriction. All vessels underwent this treatment. Subsequently, separate vessel segments received one of the following interventions: 1) 17-β-estradiol (E2) dose-response, 2) albuterol dose-response, or 3) albuterol dose-response in the presence of E2, to assess muscular artery vascular function.

Femoral artery vasodilation experiments

To assess the effects of E2 on femoral artery vasodilation, a dose-response experiment was performed on young male and female rat femoral artery in which increasing concentrations of 17-β-estradiol was added to the bath surrounding the cannulated artery every 5 minutes following an initial 10-minute stabilization, resulting in final E2 concentrations of 20, 40, 100, 200 and 500 μM. To evaluate the vasculature response to β adrenergic stimulation, albuterol dose-response was performed on young male and female rat femoral artery. The femoral artery was perfused for 10 minutes of stabilization, then, increasing albuterol concentrations were added to the bath surrounding the vessel every 5 minutes resulting in final albuterol concentrations of 0.1, 0.5, 1, and 5 μM. To evaluate if the presence of E2 affected the femoral artery response to β-ARs stimulation, after 10 minutes stabilization, first, 20 μM 17-β-estradiol was to the bath surrounding the vessel. After 5 minutes, albuterol was again added as described above, resulting in final albuterol concentrations of 0.1, 0.5, 1, 5 μM.

The lumen diameter of the femoral artery was continually monitored using the open-source pressure myography software, VasoTracker. The femoral artery lumen diameter percentage change was calculated by the following equation: (lumen diameter at each drug dose – lumen diameter at baseline) / lumen diameter at baseline * 100%. In dose response experiments (E2 and albuterol), the baseline lumen diameter was determined as the diameter after adding phenylephrine. However, in experiments investigating the effect of E2 on albuterol, the baseline lumen diameter was identified as the diameter after adding 20 μM E2, not after the addition of phenylephrine.

Statistical Analysis

Statistical analyses were performed by using GraphPad Prism (version 10.0.0 for Windows, GraphPad Software, Boston, Massachusetts USA). A three-way ANOVA was used to examine the effects of sex, age and drug doses on the femoral lumen diameter percentage change in responses to drug stimulation. Following the three-way ANOVA, post hoc analyses were performed using Tukey's HSD test to explore differences between young and middle-aged rats, and between male and female rats. A $p < 0.05$ was deemed statistically significant.

Results

Rat characteristic data

Characteristic data for male and female rats is presented in Table 7. In all experimental groups, male rats displayed significantly higher body mass and heart mass compared to their female counterparts ($p < 0.05$). Furthermore, middle-aged rats, irrespective of sex, demonstrated significantly elevated body mass and heart mass across all experimental groups ($p < 0.05$).

Table 7. Characteristic data of male and female rats.

		Age (weeks)	Rat Mass (g)	Heart Mass (g)	Heart/Rat Mass Ratio (g/kg)
		M *SD*	*M* *SD*	*M* *SD*	*M* *SD*
E2 Dose Response	YM (n=3)	9 ± 2	332 ± 54	1.01 ± 0.07	3.08 ± 0.30
	YF (n=4)	10 ± 2	$211 \pm 31^{*}$	$0.70 \pm 0.05^{**}$	3.33 ± 0.26
	MM (n=2)	$43 \pm 2^{\#\#}$	$586 \pm 6^{\#}$	1.42 ± 0.35	2.41 ± 0.57
	MF (n=2)	$43 \pm 1^{\#\#\#}$	327 ± 44	1.12 ± 0.24	3.51 ± 1.21
Albuterol Dose Response	YM (n=4)	10 ± 2	354 ± 62	1.07 ± 0.13	3.06 ± 0.25
	YF (n=4)	11 ± 2	$210 \pm 32^{*}$	$0.71 \pm 0.05^{**}$	3.39 ± 0.32
	MM (n=4)	$42 \pm 1^{\#\#\#}$	$615 \pm 18^{\#\#}$	$2.01 \pm 0.41^{\#}$	3.26 ± 0.57
	MF (n=2)	$42 \pm 0^{\#\#\#}$	$342 \pm 23^{**\ \#}$	$1.06 \pm 0.16^{*}$	3.12 ± 0.66
Albuterol Dose Response with E2	YM (n=3)	10 ± 2	378 ± 49	1.11 ± 0.13	2.95 ± 0.14
	YF (n=4)	11 ± 2	$210 \pm 32^{*}$	$0.71 \pm 0.05^{*}$	3.39 ± 0.32
	MM (n=3)	$43 \pm 1^{\#\#\#}$	$600 \pm 9^{\#}$	$1.75 \pm 0.16^{\#\#}$	2.91 ± 0.25
	MF (n=4)	$42 \pm 1^{\#\#\#}$	$365 \pm 43^{**\ \#\#}$	$1.17 \pm 0.12^{**\ \#\#}$	3.27 ± 0.70

Data represented as mean (M) ± standard deviation (SD). Young male YM, young female YF, middle-aged male MM, middle-aged female MF, gram g, kilogram kg, 17-β-estradiol E2. Data were analyzed by using independent t-test. Comparing female to male (YF to YM, MF to MM), * $p < 0.05$, significantly different from male, ** $p < 0.01$, significantly different from male, *** $p < 0.001$, significantly different from male. Comparing middle-aged to young (MM to YM, MF to YF), # $p < 0.05$, significantly different from young, ## $p < 0.01$, significantly different from young, ### $p < 0.001$, significantly different from young.

Muscular artery vasculature responses to β2-AR and E2 stimulation

A three-way ANOVA was conducted to examine the main effects of sex, age and E2 dose on femoral artery lumen diameter percentage change. Post hoc analysis employing Tukey's HSD test was used to identify significant group differences. E2 led to significant femoral artery vasodilation in all groups with a significant main effect for E2 dose (F(5, 42) = 45.285, $p < 0.001$). There were no significant differences between males and females at lower E2 doses. By using Post hoc analysis, young male rats show significant greater vasodilation compared to young females at 40 µM (22.53 ± 13.01 % in males vs. 2.87 ± 2.11 % in females, $p = 0.024$) and 100 µM (32.87 ± 10.75 % in males vs. 8.65 ± 5.20 % in females, $p = 0.006$) E2 doses (Figure 12A). However, no significant difference was observed between young males and young females at higher E2 doses (200 and 500 µM). In the middle-aged groups, there were no significant difference in femoral vasodilation response to E2, except at the 500 µM dose, where middle-aged females (73.48 ± 14.92%) exhibited greater vasodilation compared to males (48.99 ± 0.23%) ($p = 0.033$) (Figure 12A).

In the albuterol dose response experiments, albuterol-induced femoral artery vasodilation was observed in all groups. Three-way ANOVA results support these findings, as a significant main effect was observed for albuterol dose at 1 and 5 µM [albuterol dose effect (F(4, 45) = 7.175, $p < 0.001$)] in femoral artery lumen diameter percentage change (Figure 12B). Young male and young female rats exhibited similar vasodilation in response to albuterol, as do the middle-aged male and middle-aged female rats. However, the young rats displayed greater vasodilation compared to their middle-aged counterparts [age effect (F(1, 45) = 13.58, $p < 0.001$)] and Post hoc analysis using Tukey's HSD showed young male and middle-aged male groups were significantly different at 5 µM albuterol dose (32.28 ± 9.55 % in young vs. 10.88 ± 10.88 % in middle-aged, $p = 0.018$), while young female and middle-aged female groups also demonstrated significant differences at 5 µM albuterol dose (31.70 ± 10.74 % in young vs. 16.76 ± 5.31 % in middle-aged, $p = 0.045$) (Figure 12B). These findings indicate that albuterol elicited vasodilation in the femoral arteries across both young and middle-aged males and females. Notably, middle-aged rats exhibited blunted vasodilation effects at the highest albuterol dose compared to young rats.

In the presence of E2, albuterol also led to vasodilation in young and middle-aged, male and female rat femoral artery with a significant main effect for albuterol dose (F(5, 60) = 24.047, $p < 0.001$) in femoral artery lumen diameter percentage change by using ANOVA. Young rats also exhibited greater vasodilation compared to their middle-aged counterparts [age effect (F(1, 60) = 49.747, $p < 0.001$)], as confirmed by post hoc analysis. Specifically, for males, significant differences were observed between young male and middle-aged male groups at the 1 μM albuterol dose ($p < 0.05$). Similarly, among females, significant differences were noted between young female and middle-aged female groups at the 0.1, 0.5, 1, and 5 μM albuterol doses ($p < 0.05$). Middle-aged males and middle-aged females displayed similar vasodilation responses to albuterol in the presence of E2. However, young females exhibited greater vasodilation compared to young males [sex effect (F(1, 60) = 4.774, $p = 0.033$)] by using three-way ANOVA, and post hoc analysis indicated young male and young female groups had significant differences at 5 μM albuterol dose (20.58± 6.25 % in males vs. 45.88 ± 6.63 % in females, $p < 0.001$). These findings indicated that young females exhibited a more pronounced albuterol-induced vasodilation of the femoral artery compared to middle-aged females.

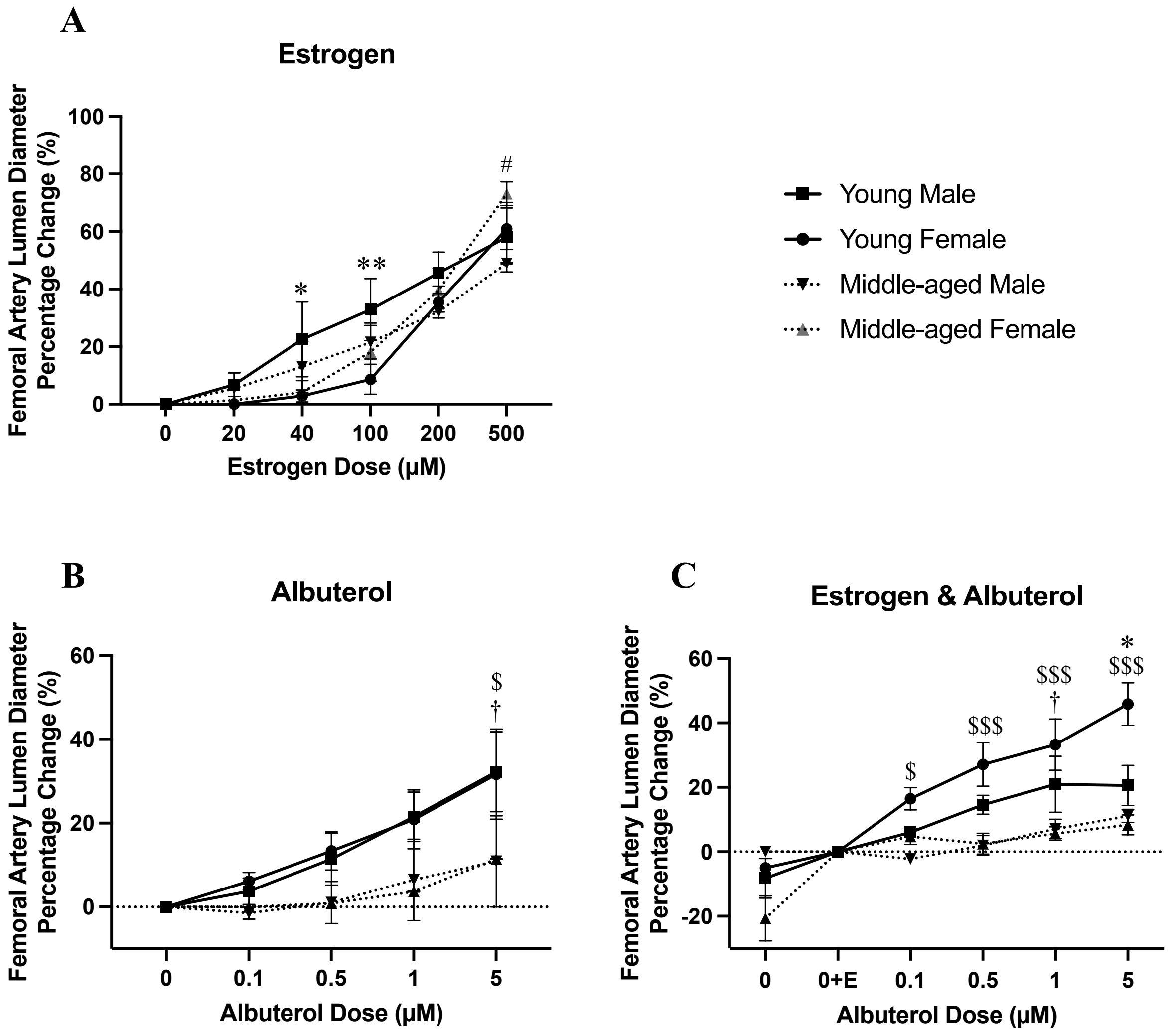


Figure 12. Male and female rat femoral artery responses to difference estrogen doses, albuterol doses in the absence and presence of estrogen. Estrogen caused vasodilation in all groups. Young and middle-aged female rats show significant greater vasodilation response to estrogen stimulation compared to young and middle-aged males, respectively (A). Young and middle-aged, male and female rats show similar femoral artery vasodilation responded to albuterol stimulation (B). With the presence of estrogen, young female rats show significant greater vasodilation compared to young males (C). Data were analyzed by using three-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significantly different between young male and young female rats, # $p < 0.05$ significantly different between middle-aged male and middle-aged female rats, † $p < 0.05$ significantly different between young male and middle-aged male rats , $\$\ p < 0.05$, $\$\$\$\ p < 0.001$ significantly different between young female and middle-aged

Table 8. Simple linear regression of albuterol and E2 dose response.

	Femoral Artery	F	df	R^2	*p*-value
E2 Dose Response	YM (n = 3)	12.43	1, 13	.489	0.0037**
	YF (n = 4)	97.98	1, 18	.845	< 0.0001****

	MM (n=2)	37.39	1, 8	.824	0.0003***
	MF (n=2)	11.13	1, 8	.582	0.0103*
	OM (n=2)	92.79	1, 8	.921	< 0.0001****
	OF (n=2)	11.74	1, 8	.595	0.009**
Albuterol Dose Response	YM (n=4)	8.372	1, 14	.374	0.0118*
	YF (n=4)	6.634	1, 14	.322	0.0220*
	MM (n=3)	1.448	1, 10	.127	0.257
	MF (n=2)	181.4	1, 6	.968	< 0.0001****
Albuterol Dose Response with E2	YM (n=3)	1.618	1, 10	.139	0.2321
	YF (n=4)	8.920	1, 14	.389	0.0098**
	MM (n=3)	9.807	1, 10	.495	0.0107*
	MF (n=4)	1.877	1, 14	.118	0.192

Data represented as mean (M) ± standard deviation (SD). Young male YM, young female YF, middle-aged male MM, middle-aged female MF, old male OM, old female OF, 17-β-estradiol E2. Data are analyzed by using simple linear regression. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Femoral lumen diameter responds to E2 and albuterol

The data for femoral artery lumen diameter is provided in Table 9. When comparing young male and female rats, young females exhibited a significantly lower femoral lumen diameter at multiple doses (baseline, 20, 40, and 100 μM), although no significant differences were observed between middle-aged male and female rats. When comparing young and middle-aged rats, there was no significant difference in femoral lumen diameter response to E2, regardless of sex. In the context of the albuterol dose response, young females displayed a significantly lower femoral lumen diameter than young males at various doses (baseline, 0.1, and 1 μM), while middle-aged females exhibited a significantly lower femoral lumen diameter compared to middle-aged males at all doses except the highest one. In the presence of E2, there were no significant differences between the femoral lumen diameter of young male and female rats. However, middle-aged female rats demonstrated a significantly lower femoral artery lumen diameter in response to several albuterol doses (baseline, 1, and 5 µM) compared to middle-aged males.

Table 9. Femoral artery lumen diameter.

	Lumen Diameter (mm)					
	baseline	20 µM E2	40 µM E2	100 µM E2	200 µM E2	500 µM E2
YM	0.77 ± 0.02	0.79 ± 0.02	0.85 ± 0.02	0.88 ± 0.01	0.93 ± 0.01	0.98 ± 0.02
YF	0.65 ± 0.02*	0.65 ± 0.02**	0.66 ± 0.03**	0.67 ± 0.03**	0.75 ± 0.06	0.80 ± 0.06
MM	0.70 ± 0.07	0.73 ± 0.05	0.76 ± 0.03	0.80 ± 0.04	0.85 ± 0.06	0.93 ± 0.06
MF	0.64 ± 0.02	0.68 ± 0.04	0.73 ± 0.03	0.80 ± 0.01	0.85 ± 0.02	0.88 ± 0.02
	baseline		0.1 µM A	0.5 µM A	1 µM A	5 µM A
YM	0.77 ± 0.05		0.78 ± 0.05	0.80 ± 0.05	0.83 ± 0.05	0.86 ± 0.05
YF	0.61 ± 0.03*		0.63 ± 0.03*	0.65 ± 0.04	0.67 ± 0.04*	0.69 ± 0.05
MM	0.81 ± 0.01		0.80 ± 0.02	0.81 ± 0.03	0.83 ± 0.05	0.85 ± 0.05
MF	0.62 ± 0.04*		0.62 ± 0.04*	0.63 ± 0.04*	0.65 ± 0.04*	0.67 ± 0.03
	baseline	20 µM E2	0.1 µM A	0.5 µM A	1 µM A	5 µM A
YM	0.72 ± 0.09	0.75 ± 0.09	0.77 ± 0.09	0.80 ± 0.08	0.82 ± 0.09	0.82 ± 0.08
YF	0.60 ± 0.04	0.66 ± 0.07	0.70 ± 0.07	0.72 ± 0.07	0.74 ± 0.07	0.77 ± 0.06
MM	0.80 ± 0.01	0.80 ± 0.01	0.79 ± 0.01	0.81 ± 0.02	0.83 ± 0.01	0.84 ± 0.01
MF	0.66 ± 0.03*	0.71 ± 0.04	0.71 ± 0.03	0.70 ± 0.04	0.71 ± 0.03*	0.72 ± 0.03*

Data represented as mean (M) ± standard error of mean (SEM). Young male YM, young female YF, middle-aged male MM, middle-aged female MF, old male OM, old female OF, 17-β-estradiol E2, albuterol A. Data was analyzed by using independent t-test. Comparing middle-aged to young (MM to YM, MF to YF), no significant difference is found. Comparing female to male (YF to YM, MF to MM), * $p < 0.05$ comparing to age-matched male, ** $p < 0.01$ comparing to age-matched male.

Discussion

The primary aim of our study was to assess the vascular function of the femoral artery in both young and middle-aged male and female rats. Additionally, we investigated the individual effects of estrogen and albuterol, as well as their potential interaction effects on femoral artery.

The primary finding of this study was that young and middle-aged female rats exhibited a greater femoral artery vasodilation in response to E2 stimulation when compared to their male counterparts. Despite the fact both young and middle-aged male and female rat femoral arteries demonstrated similar vasodilatory responses to β2-AR stimulation, when 20 μM E2 was added prior to albuterol, young female rat femoral arteries demonstrated augmented vasodilation compared to middle-aged females. This difference not observed between young and middle-aged male rats. These findings highlight the acute effect of E2 on young female rats in vascular responsiveness and potential impact on age-related changes on β2-AR stimulation.

E2 induces vasodilation in both male and female rat femoral artery

E2 caused vasodilation in situ in the femoral artery of both male and female rats at all ages. This finding aligns with previous studies demonstrating the acute vasodilatory effect of estrogen in various blood vessels. Sudhir et al. [83] reported that male and female dog coronary arteries showed a significant increase in cross-sectional area and flow velocity in response to acute presence of E2. In another study, Traupe et al [84] indicated that estrogen induced acute relaxation in epicardial coronary arteries of male and female pigs. In both young and middle-aged groups, female rats show more significant femoral artery vasodilation compared to male rats. Our finding aligns with the previous research. Kawano et al. [85] demonstrated that a short-term estrogen supplementation augmented endothelium-dependent brachial artery vasodilation in women but not in men. This sex difference may be explained by the estrogen-induced NO synthase and release, and the available number of ERs are initial lower in men compared to women [85].

Given the study's focus on the short-term effect of E2, the membrane estrogen receptor GPR30, in addition to ERα and ERβ, plays a crucial role in vasodilation. According to Lindsey et al. [86], estrogen and the GPR30 agonist G-1 caused similar vasodilation in aortic rings and mesenteric vessels, but the GPR30 antagonist significantly attenuated vasodilation effects in both blood vessels. Sudhir et al. [83] indicates that the ERα antagonist did not significantly inhibit estrogen-induced vasodilation, suggest other ERs, like GPR30, may play a predominant role in vasodilation. Therefore, more research is needed to evaluate the role of GPR30 in estrogen-induced muscular artery vasodilation.

β2-AR stimulation leads to vasodilation in muscular artery

Consistent with previous studies, we found that β2-AR stimulation leads to muscular artery vasodilation in both male and female rats. Kneale et al. [82] reported that the β2-AR agonist albuterol led to acute forearm vasodilation in both men and women, indicating that β2-AR plays an essential role in muscular artery vasodilation. However, the age effect on femoral vascular response to β2 stimulation was not found in this study, young and middle-aged rats had similar vasodilation responsiveness. This finding is not consistent with previous studies that showed the age effect on vascular function resulted in impaired vasodilation. [25, 26]. This may be because β1-AR, not β2-AR, plays the more predominant role in artery vasodilation. Chruscinski et al. [23] measured the maximal relaxation of femoral artery using β-AR knock-out mice, finding that β1-AR knock-out mice showed slightly vasodilation response to β-ARs agonists, while β2-AR knock-out mice showed similar vasodilation as the control group. This finding suggests that β1-AR may play a more predominant role as the vasodilator receptor than β2-AR in isolated mouse femoral artery. In contrast, Schutzer et al. [26] reported the opposite results. They found that the selective β1-AR antagonist only slightly reduces the maximal vasodilation induced by non-selective β-ARs agonist in male rat aorta artery. The controversial findings about the predominant β-ARs may be related to the different species and arteries used in research settings, and more studies are needed to evaluate the roles of β1-AR and β2-AR in muscular artery vascular function.

β2-AR and GPR30 signaling pathway interaction

We also found that, in the acute presence of 20 µM E2, femoral artery from young female rats showed greater vasodilatory response to β2-AR stimulation when compared to middle-aged females. This may be due to the vasodilatory effect of E2. However, there was no difference between the absence and presence of E2 when comparing young and middle-aged male rats in response to acute β2-AR stimulation. Therefore, E2 may enhance the acute β2 stimulation effect only on young female femoral artery vasodilation. This result can be explained by the fact that estrogen membrane receptor GPR30 and β2-AR share similar non-genomic acute signaling pathways leading to vasodilation [48]. Both β2-AR and GPR30 are members of G protein-coupled receptors and could activate the cAMP/PKA pathway via the stimulatory unit of the G protein (G_s), which leads to the relaxation of vascular smooth muscle. Thus, the acute presence of E2 enhanced the vasodilation effect of β2-AR stimulation in the female rat femoral artery. Previous research supports our findings. Limberg et al. [87] measured the non-selective β-AR agonist induced vasodilation in forearm between young women currently using oral contraceptives (placebo phase, normal estrogen level) and those not using oral contraceptives (early follicular phase, low estrogen level). The results showed that non-selective β-AR agonist induced vasodilation in forearm is greater in oral contraceptives using group compared to not using oral contraceptives group, indicates that the estrogen enhanced acute vascular response to β-AR stimulation.

Estrogen concentration effect on β-ARs stimulation

In the current investigation, we scrutinized the impact of E2 on acute β-ARs stimulation. Our results revealed that the acute presence of E2 significantly augmented vasodilation induced by β2-AR agonist stimulation in the femoral artery of young female rats. However, this enhanced effect of E2 was not evident in middle-aged female rats, potentially attributed to the decline in estrogen levels associated with aging [24, 46]. Collectively, our findings suggest a role for E2 in regulating vasculature responsiveness during β-AR stimulation. Supporting our observations, a prior study [88] reported reduced exercise performance during the early follicular phase, characterized by the lowest estrogen levels in the menstrual cycle. This reduction in exercise performance during low estrogen concentration phases may be linked to the influence of estrogen on β-AR stimulation. During exercise, heightened sympathetic nervous system activity releases catecholamines, stimulating β-ARs and resulting in increased heart rate, enhanced cardiomyocyte contractility, and blood vessel vasodilation. Given that low estrogen concentration is known to impact exercise performance, it is plausible that this effect operates through the modulation of β-AR stimulation. Consequently, our findings provide insights that may elucidate the observed reduction in exercise performance during the early follicular phase characterized by low estrogen concentrations. Moreover, they suggest that the acute presence of estrogen may exert an influence on the responsiveness to β-AR stimulation, shedding light on the complex interplay between hormonal fluctuations and cardiovascular responses during physiological challenges such as exercise.

Limitations

There were several limitations in this study. Firstly, we did not measure the effect of different E2 doses on the femoral artery's response to β2-AR stimulation. Both male and female rats did not exhibit a significant response to the relatively low E2 concentration (20 μM) we used for the experiment. A higher E2 concentration may eliminate the sex difference in the vasodilation effect of albuterol. Secondly, we only include β2-AR into consideration. According to the previous, whether β1-AR or β2-AR plays a more dominate role in muscular artery vasodilation is still not fully illustrated. Thus, measuring femoral artery respond to β1-AR stimulation may help us with a better understanding of muscular artery vasculature function.

Conclusions

E2-induced femoral artery vasodilation is blunted in male rats compared to female ones, indicating sex differences in E2 responsiveness. Both young and middle-aged male and female rat femoral arteries responded similarly to β2-AR agonist-induced vasodilation. Whether β1-AR or β2-AR plays a more dominate role in muscular artery vasodilation needs more investigation. The acute presence of E2 enhanced the vasodilation effect of β2 stimulation in young females compared to middle-aged females. These results emphasize the acute effect of E2 on muscular artery vasculature function and the interaction between the β2-AR and GPR30 signaling pathways was evident. However, this effect was not observed between young and middle-aged males, possibly due to sex-related E2 responsiveness. These findings underscore the acute effects of E2 on β2-AR stimulation, sex-related vasculature responsiveness, and age-related vascular changes, providing valuable insights for further exploration.

Chapter 5: Summary of Findings

This dissertation aimed to investigate the impact of sex, age, and heart failure on cardiac responses to β2-AR stimulation, as well as the sexual dimorphism in femoral artery responses to β2-AR stimulation using a rat model. The first study involved assessing cardiac function responses to β2-AR stimulation in young and old, male and female rats, examining parameters such as heart rate, coronary flow rate, and oxygen consumption rate. In the second study, rats underwent TAC surgery to induce increased afterload and heart failure, allowing for the evaluation of the impact of heart failure on cardiac function in male and female rats. Additionally, the first set of studies included measurements of β2-AR and estrogen receptor GPR 30 expressions in the left ventricle and SA node. Beyond cardiac function, the third study focused on assessing skeletal muscle vasculature responses to β-ARs stimulation in young male and female rats by employing the pressure myography method on the femoral artery. This comprehensive approach contributes to a deeper understanding of both cardiac and skeletal muscle vascular responses to β-ARs stimulation in various physiological conditions.

Our investigation into cardiac responses to β2-AR stimulation delineated sex-based differences and the regulatory influence of estrogen in both young and old rats. In young female rats, a diminished HR response compared to males was observed. Expression of β2-AR in the left ventricle and SA node showed no sex difference in young rats. The secondary messenger system, cAMP, likely contributes to the blunted HR responses in young females, characterized by lower basal cAMP levels in female rat cardiomyocytes. However, the presence of estrogen eliminated this sex difference, underscoring its pivotal role in β2 stimulation responses. The shared signaling pathway involving β2-AR and GPR30, mediated by cAMP, suggests a mechanism through which estrogen restores HR in female rat hearts. Additionally, GPR30 expression significantly upregulated in the left ventricle of aged rat hearts prompts questions about its implications for aging cardiac tissue and its role in modulating cardiac responses.

In contrast, no sex difference was found in old male and female rats, regardless of estrogen presence, with aging associated with a blunted HR response to β2-AR stimulation, consistent with previous studies. Proposed mechanisms for reduced β-ARs responsiveness in older animals included age-related decreases in β2-AR and GPR30 expression and increased G_i activity. Notably, GPR30 expression exhibited significant upregulation in the left ventricle of aged rat hearts, prompting further questions about its role in aging cardiac tissue. The study also highlighted an age-associated increase in GAPDH expression, suggesting an enhanced reliance on glycolysis as a crucial energy source for the aging heart.

To evaluate the heart failure effects on cardiac function to β2-AR stimulation, TAC rat model was utilized. The study found a consistent response to β2-AR stimulation and a comparable expression profile of β2-AR and GPR30 receptors in the left ventricle across TAC and SHAM, male and female rat groups. Despite albuterol inducing a increase in HR across all groups, no discernible distinction in the response to β2-AR stimulation was observed between hearts with HF and healthy hearts. This contradicts previous research suggesting a reduction in β-ARs expression and desensitization in failing hearts. The study also explored the potential inhibitory influence of the β2-AR/G_i pathway in the context of HF, suggesting that altered signaling pathways may contribute to the observed HR responses. There is no discernible difference in cardiac function between TAC and SHAM rats, indicates that the TAC model utilized in this study may not represent the HF phenotypes.

This study utilized a pressure myography model to examine the femoral artery vascular function, aiming to enhance our comprehension of sex differences in response to β2-AR stimulation and the role of estrogen. Consistent with prior research, estrogen demonstrated a vasodilatory effect, indicating its acute impact on various blood vessels. We discovered that young and middle-aged male rats exhibited reduced femoral artery vasodilation in response to estrogen stimulation compared to their female counterparts. The study emphasized the acute presence of estrogen and its crucial role in vasodilation. Confirming the vasodilatory effect of β2-AR stimulation in both young male and female rats, the study highlighted the unclear predominant role of β1-AR or β2-AR in arteries, necessitating further investigation. Furthermore, the interaction between β2-AR and the GPR30 signaling pathway was explored. In the presence of estrogen, young female rat femoral arteries exhibited increased vasodilation in response to acute β2-AR stimulation compared to middle-aged females, suggesting an enhancement of the acute vasodilatory effect of β2 stimulation by estrogen. This result is attributed to shared non-genomic signaling pathways between estrogen's membrane receptor GPR30 and β2-AR, activating the cAMP/PKA pathway. Supporting evidence from prior research indicated greater β-ARs agonist-induced vasodilation in women using oral contraceptives with normal estrogen levels compared to those with low estrogen levels, emphasizing estrogen's role in augmenting vascular responses to β-ARs stimulation. Importantly, this sex-specific effect was not observed in middle-aged rats, highlighting the acute impact of estrogen on young females and its potential influence on age-related changes in β2-AR stimulation. Furthermore, the study explored the concentration effect of estrogen on acute β-ARs stimulation, revealing that the enhanced vasodilatory effect of estrogen diminished in young male rats. This diminishing effect is attributed to sex-related difference in estrogen levels. In summary, these findings underscore the interplay between estrogen, β2-AR stimulation, and age-related vascular changes, underscoring the imperative for further research to elucidate the specific roles of estrogen receptors and hormonal influences on acute vascular responses.

Future Directions

Building upon the insights gained from this dissertation, future research directions should address several key avenues to deepen our understanding of cardiac responses to β-ARs stimulation and the interaction with sex, age, and heart failure. Firstly, extending the investigation into cardiac responses to β2-AR stimulation, a more granular exploration of the cAMP signaling pathway is warranted, particularly in the SA node. The observed blunted HR response suggests a nuanced role of cAMP in mediating sex-related variations, necessitating detailed mechanistic studies. Additionally, further exploration of GPR30 upregulation in the left ventricle of aged rat hearts is essential. Understanding its implications for aging cardiac tissue and its role in modulating cardiac responses could unveil potential therapeutic targets for age-related cardiovascular issues.

In the context of heart failure, despite a consistent response to β2-AR stimulation and comparable receptor expression profiles, the unexpected lack of distinction in HR responses between HF and healthy hearts prompts further investigation into altered signaling pathways. Exploring the β2-AR/G_i pathway in heart failure heart SA node could elucidate the underlying mechanisms contributing to the observed responses. Moreover, given the therapeutic potential of estrogen-induced coronary vasodilation, a more comprehensive examination of GPR30 role and downstream signaling pathways in HF conditions would be valuable. This could pave the way for targeted interventions aimed at leveraging estrogen's beneficial effects in mitigating heart failure.

Expanding the focus to femoral artery vascular function, the unresolved question of the predominant role between β1-AR and β2-AR in arteries should be addressed through systematic studies. Moreover, considering the potential role of the GPR30 receptor in mediating estrogen-induced vasodilation, exploring the downstream signaling pathways in detail could provide valuable insights for therapeutic interventions targeting vascular health. This study exclusively examines the acute impact of estrogen on cardiac and skeletal muscle vascular responses to β-ARs stimulation. Consequently, there is a compelling need for subsequent research endeavors aimed at assessing the chronic effects of estrogen on β-ARs stimulation in both the cardiac and vascular systems. Such investigations would provide a more comprehensive understanding of the prolonged influences of estrogen on adrenergic responsiveness, offering valuable insights into the sustained physiological implications and potential implications for therapeutic interventions.

In conclusion, future research endeavors should delve deeper into the molecular mechanisms governing cardiac and vascular responses to β-ARs stimulation, with a particular emphasis on elucidating sex-specific nuances, unraveling the complexities of heart failure conditions, and addressing unresolved questions regarding the roles of estrogen receptors. These endeavors hold promise for advancing our understanding of cardiovascular physiology and guiding the development of targeted therapeutic interventions in the realm of cardiac health and disease.

Bibliography

1. Brum, P. C., Rolim, N. P. L., Bacurau, A. V. N., & Medeiros, A. (2006). Neurohumoral activation in heart failure: the role of adrenergic receptors. *Anais Da Academia Brasileira de Ciências*, *78*(3), 485–503. https://doi.org/10.1590/s0001-37652006000300009
2. Piepoli, Massimo F., et al. “Exercise Intolerance in Chronic Heart Failure: Mechanisms and Therapies. Part I.” European Journal of Cardiovascular Prevention & Rehabilitation, vol. 17, no. 6, Dec. 2010, pp. 637–642, https://doi.org/10.1097/hjr.0b013e3283361dc5. Accessed 14 Jan. 2024.
3. Iorga, A., Cunningham, C. M., Moazeni, S., Ruffenach, G., Umar, S., & Eghbali, M. (2017). The protective role of estrogen and estrogen receptors in cardiovascular disease and the controversial use of estrogen therapy. *Biology of Sex Differences*, *8*(1). https://doi.org/10.1186/s13293-017-0152-8
4. MacDonald, Eilidh A., et al. “Neurohumoral Control of Sinoatrial Node Activity and Heart Rate: Insight from Experimental Models and Findings from Humans.” Frontiers in Physiology, vol. 11, 3 Mar. 2020, https://doi.org/10.3389/fphys.2020.00170. Accessed 14 Jan. 2024.
5. Carter, James B., et al. “Effect of Endurance Exercise on Autonomic Control of Heart Rate.” Sports Medicine, vol. 33, no. 1, 2003, pp. 33–46, https://doi.org/10.2165/00007256-200333010-00003. Accessed 14 Jan. 2024.
6. Sequeira, Vasco, and Jolanda van der Velden. “Historical Perspective on Heart Function: The Frank–Starling Law.” *Biophysical Reviews*, vol. 7, no. 4, 19 Nov. 2015, pp. 421–447, https://doi.org/10.1007/s12551-015-0184-4. Accessed 14 Jan. 2024.
7. Ali, D. C., Naveed, M., Gordon, A., Majeed, F., Saeed, M., Ogbuke, M. I., Atif, M., Zubair, H. M., & Changxing, L. (2019). β-Adrenergic receptor, an essential target in cardiovascular diseases. *Heart Failure Reviews*, *25*(2), 343–354. https://doi.org/10.1007/s10741-019-09825-x
8. Abrass, I. B., Davis, J. L., & Scarpace, P. J. (1982). Isoproterenol Responsiveness and Myocardial -Adrenergic Receptors in Young and Old Rats. *Journal of Gerontology*, *37*(2), 156–160. https://doi.org/10.1093/geronj/37.2.156
9. Vestal, R. E., Wood, A. J., & Shand, D. G. (1979). Reduced beta-adrenoceptor sensitivity in the elderly. *Clinical Pharmacology and Therapeutics*, *26*(2), 181–186. https://doi.org/10.1002/cpt1979262181
10. White, M., Roden, R., Minobe, W., Khan, M. F., Larrabee, P., Wollmering, M., Port, J. D., Anderson, F., Campbell, D., & Feldman, A. M. (1994). Age-related changes in beta-adrenergic neuroeffector systems in the human heart. *Circulation*, *90*(3), 1225–1238. https://doi.org/10.1161/01.cir.90.3.1225
11. Lindenfeld, JoAnn, et al. “Sex-Related Differences in Age-Associated Downregulation of Human Ventricular Myocardial β1-Adrenergic Receptors.” The Journal of Heart and Lung Transplantation, vol. 35, no. 3, 1 Mar. 2016, pp. 352–361, www.sciencedirect.com/science/article/abs/pii/S1053249815014825, https://doi.org/10.1016/j.healun.2015.10.021. Accessed 8 Jan. 2024.
12. Kusumoto, F., Lurie, K. G., Dutton, J. P., Capili, H., & Schwartz, J. B. (1994). Effects of aging on AV nodal and ventricular beta-adrenergic receptors in the Fischer 344 rat. *American Journal of Physiology-Heart and Circulatory Physiology*, *266*(4), H1408–H1415. https://doi.org/10.1152/ajpheart.1994.266.4.h1408

13. Vizgirda, V. M., Wahler, G. M., Sondgeroth, K. L., Ziolo, M. T., & Schwertz, D. W. (2002). Mechanisms of sex differences in rat cardiac myocyte response to β-adrenergic stimulation. *American Journal of Physiology-Heart and Circulatory Physiology*, *282*(1), H256–H263. https://doi.org/10.1152/ajpheart.2002.282.1.h256
14. Peter, A. K., Walker, C. J., Ceccato, T., Trexler, C. L., Ozeroff, C. D., Lugo, K. R., Perry, A. R., Anseth, K. S., & Leinwand, L. A. (2021). Cardiac Fibroblasts Mediate a Sexually Dimorphic Fibrotic Response to β-Adrenergic Stimulation. *Journal of the American Heart Association*, *10*(11). https://doi.org/10.1161/jaha.120.018876
15. CHU, S., SUTHERLAND, K., BECK, J., KOWALSKI, J., GOLDSPINK, P., & SCHWERTZ, D. (2005). Sex differences in expression of calcium-handling proteins and beta-adrenergic receptors in rat heart ventricle. *Life Sciences*, *76*(23), 2735–2749. https://doi.org/10.1016/j.lfs.2004.12.013
16. Al-Gburi, S., Deussen, A., Zatschler, B., Weber, S., Künzel, S., El-Armouche, A., Lorenz, K., Cybularz, M., Morawietz, H., & Kopaliani, I. (2017). Sex-difference in expression and function of beta-adrenoceptors in macrovessels: role of the endothelium. Basic Research in Cardiology, 112(3). https://doi.org/10.1007/s00395-017-0617-2
17. Lyrio, R., Juliana Trindade Lima, Wender Nascimento Rouver, & Margareth Ribeiro Moysés. (2016). Deficiency of sex hormones does not affect 17-ß-estradiol-induced coronary vasodilation in the isolated rat heart. *Brazilian Journal of Medical and Biological Research*, *49*(5). https://doi.org/10.1590/1414-431x20165058
18. Chu, S. H., Goldspink, P., Kowalski, J., Beck, J., & Schwertz, D. W. (2006). Effect of estrogen on calcium-handling proteins, β-adrenergic receptors, and function in rat heart. *Life Sciences*, *79*(13), 1257–1267. https://doi.org/10.1016/j.lfs.2006.03.037
19. Sherwood, A., Park, S. B., Hughes, J. W., Blumenthal, J. A., Hinderliter, A., Trivedi, R., & McFetridge-Durdle, J. (2010). Cardiovascular hemodynamics during stress in premenopausal versus postmenopausal women. *Menopause*, *17*(2), 403–409. https://doi.org/10.1097/gme.0b013e3181b9b061
20. Lohse, M. J., Engelhardt, S., & Eschenhagen, T. (2003). What Is the Role of β-Adrenergic Signaling in Heart Failure? *Circulation Research*, *93*(10), 896–906. https://doi.org/10.1161/01.res.0000102042.83024.ca
21. Najafi, A., Sequeira, V., Kuster, D. W. D., & van der Velden, J. (2016). β-adrenergic receptor signalling and its functional consequences in the diseased heart. *European Journal of Clinical Investigation*, *46*(4), 362–374. https://doi.org/10.1111/eci.12598
22. White, D. C., Hata, J. A., Shah, A. S., Glower, D. D., Lefkowitz, R. J., & Koch, W. J. (2000). Preservation of myocardial β-adrenergic receptor signaling delays the development of heart failure after myocardial infarction. *Proceedings of the National Academy of Sciences*, *97*(10), 5428–5433. https://doi.org/10.1073/pnas.090091197
23. Chruscinski, A., Brede, M. E., Meinel, L., Lohse, M. J., Kobilka, B. K., & Hein, L. (2001). Differential Distribution of β-Adrenergic Receptor Subtypes in Blood Vessels of Knockout Mice Lacking β_1- or β_2-Adrenergic Receptors. *Molecular Pharmacology*, *60*(5), 955–962. https://doi.org/10.1124/mol.60.5.955
24. Koenig, A., Buskiewicz, I., & Huber, S. A. (2017). Age-Associated Changes in Estrogen Receptor Ratios Correlate with Increased Female Susceptibility to Coxsackievirus B3-Induced Myocarditis. *Frontiers in Immunology*, *8*. https://doi.org/10.3389/fimmu.2017.01585

25. Schutzer, W. E., & Mader, S. L. (2003). Age-related changes in vascular adrenergic signaling: clinical and mechanistic implications. *Ageing Research Reviews*, *2*(2), 169–190. https://doi.org/10.1016/s1568-1637(02)00063-6
26. Schutzer, W. E., Xue, H., Reed, J. F., & Mader, S. L. (2006). Effect of Age on Vascular ss2-Adrenergic Receptor Desensitization Is Not Mediated by the Receptor Coupling to G i Proteins. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *61*(9), 899–906. https://doi.org/10.1093/gerona/61.9.899
27. Harvey, R. E., Barnes, J. N., Charkoudian, N., Curry, T. B., Eisenach, J. H., Hart, E. C., & Joyner, M. J. (2014). Forearm vasodilator responses to a *β* -adrenergic receptor agonist in premenopausal and postmenopausal women. *Physiological Reports*, *2*(6), e12032. https://doi.org/10.14814/phy2.12032
28. Ferrer, M., Meyer, M., & Osol, G. (1996). Estrogen Replacement Increases β-Adrenoceptor-Mediated Relaxation of Rat Mesenteric Arteries. *Journal of Vascular Research*, *33*(2), 124–131. https://doi.org/10.1159/000159140
29. Barnabas, O., Wang, H., & Gao, X.-M. (2013). Role of estrogen in angiogenesis in cardiovascular diseases. *Journal of Geriatric Cardiology: JGC*, *10*(4), 377–382. https://doi.org/10.3969/j.issn.1671-5411.2013.04.008
30. Losordo, D. W., & Isner, J. M. (2001). Estrogen and Angiogenesis. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *21*(1), 6–12. https://doi.org/10.1161/01.atv.21.1.6
31. Rubanyi, G. M., Johns, A., & Kauser, K. (2002). Effect of estrogen on endothelial function and angiogenesis. *Vascular Pharmacology*, *38*(2), 89–98. https://doi.org/10.1016/s0306-3623(02)00131-3
32. Marino, M., Galluzzo, P., & Ascenzi, P. (2006). Estrogen Signaling Multiple Pathways to Impact Gene Transcription. *Current Genomics*, *7*(8), 497–508. https://doi.org/10.2174/138920206779315737
33. Batenburg, W. W., Jansen, P. M., van den Bogaerdt, A. J., & J. Danser, A. H. (2012). Angiotensin II-aldosterone interaction in human coronary microarteries involves GPR30, EGFR, and endothelial NO synthase. *Cardiovascular Research*, *94*(1), 136–143. https://doi.org/10.1093/cvr/cvs016
34. Randeva. (2010). G-protein coupled estrogen receptor 1 expression in rat and human heart: Protective role during ischaemic stress. *International Journal of Molecular Medicine*, *26*(2). https://doi.org/10.3892/ijmm_00000452
35. Mallikarjuna, X., Pabbidi, R., Kuppusamy, M., Didion, S., Sanapureddy, P., Reed, J., Sontakke, S., & Pabbidi, M. (2018). Signaling Mechanisms 17␤-estradiol. *Am J Physiol Heart Circ Physiol*, *315*, 1499–1518.
36. Ndzie Noah, M. L., Adzika, G. K., Mprah, R., Adekunle, A. O., Adu-Amankwaah, J., & Sun, H. (2021). Sex–Gender Disparities in Cardiovascular Diseases: The Effects of Estrogen on eNOS, Lipid Profile, and NFATs During Catecholamine Stress. *Frontiers in Cardiovascular Medicine*, *8*. https://doi.org/10.3389/fcvm.2021.639946
37. Simoncini, T., Mannella, P., Fornari, L., Caruso, A., Varone, G., & Genazzani, A. R. (2004). Genomic and non-genomic effects of estrogens on endothelial cells. *Steroids*, *69*(8-9), 537–542. https://doi.org/10.1016/j.steroids.2004.05.009
38. SHAH, B. (2005). Estrogen stimulation of COX-2-derived PGI2 confers atheroprotection. *Trends in Endocrinology and Metabolism*, *16*(5), 199–201. https://doi.org/10.1016/j.tem.2005.05.008

39. Luksha, L., Agewall, S., & Kublickiene, K. (2009). Endothelium-derived hyperpolarizing factor in vascular physiology and cardiovascular disease. *Atherosclerosis*, *202*(2), 330–344. https://doi.org/10.1016/j.atherosclerosis.2008.06.008
40. Gokina, N. I., Burger, N. Z., Osol, G., & Kuzina, O. Y. (2009). Estrogen replacement enhances EDHF-mediated vasodilation of mesenteric and uterine resistance arteries: role of endothelial cell Ca $^{2+}$. *The FASEB Journal*, *23*(S1). https://doi.org/10.1096/fasebj.23.1_supplement.965.10
41. Bilsel, A. (2000). 17β-Estradiol modulates endothelin-1 expression and release in human endothelial cells. *Cardiovascular Research*, *46*(3), 579–584. https://doi.org/10.1016/s0008-6363(00)00046-8
42. Kingma jr, J. G., & Laher, I. (2021). Effect of endothelin on sex-dependent regulation of tone in coronary resistance vessels. *Biochemical and Biophysical Research Communications*, *540*, 56–60. https://doi.org/10.1016/j.bbrc.2020.12.103
43. LeBlanc, A. J., Chen, B., Dougherty, P. J., Reyes, R. A., Shipley, R. D., Korzick, D. H., & Muller-Delp, J. M. (2013). Divergent Effects of Aging and Sex on Vasoconstriction to Endothelin in Coronary Arterioles. *Microcirculation*, *20*(5), 365–376. https://doi.org/10.1111/micc.12028
44. Gallagher, P. E., Li, P., Lenhart, J. R., Chappell, M. C., & Brosnihan, K. B. (1999). Estrogen Regulation of Angiotensin-Converting Enzyme mRNA. *Hypertension*, *33*(1), 323–328. https://doi.org/10.1161/01.hyp.33.1.323
45. Xue, B., Pamidimukkala, J., Lubahn, D. B., & Hay, M. (2007). Estrogen receptor-α mediates estrogen protection from angiotensin II-induced hypertension in conscious female mice. *American Journal of Physiology-Heart and Circulatory Physiology*, *292*(4), H1770–H1776. https://doi.org/10.1152/ajpheart.01011.2005
46. Davezac, M., Buscato, M., Zahreddine, R., Lacolley, P., Henrion, D., Lenfant, F., Arnal, J.-F., & Fontaine, C. (2021). Estrogen Receptor and Vascular Aging. *Frontiers in Aging*, *2*. https://doi.org/10.3389/fragi.2021.727380
47. Shokoufeh Mahmoodzadeh, Eder, S., Nordmeyer, J., Ehler, E., Huber, O., Martus, P., Weiske, J., Reinhard Pregla, Hetzer, R., & Regitz-Zagrosek, V. (2006). Estrogen receptor alpha up-regulation and redistribution in human heart failure. *The FASEB Journal*, *20*(7), 926–934. https://doi.org/10.1096/fj.05-5148com
48. Machuki, J. O., Zhang, H. Y., Harding, S. E., & Sun, H. (2017). Molecular pathways of oestrogen receptors and β-adrenergic receptors in cardiac cells: Recognition of their similarities, interactions and therapeutic value. *Acta Physiologica*, *222*(2). https://doi.org/10.1111/apha.12978
49. Kabir, M. E., Singh, H., Lu, R., Olde, B., Leeb-Lundberg, L. M. F., & Bopassa, J. C. (2015). G Protein-Coupled Estrogen Receptor 1 Mediates Acute Estrogen-Induced Cardioprotection via MEK/ERK/GSK-3β Pathway after Ischemia/Reperfusion. *PLOS ONE*, *10*(9), e0135988. https://doi.org/10.1371/journal.pone.0135988
50. *National Health Interview Survey (NHIS) - Health, United States*. (2022, August 8). Www.cdc.gov. https://www.cdc.gov/nchs/hus/sources-definitions/nhis.htm
51. Xiao, Rui, et al. "Age-Associated Reductions in Cardiac Beta1- and Beta2-Adrenergic Responses without Changes in Inhibitory G Proteins or Receptor Kinases." Journal of Clinical Investigation, vol. 101, no. 6, 15 Mar. 1998, pp. 1273–1282, https://doi.org/10.1172/jci1335

52. Cerbai, E., Guerra, L., Varani, K., Barbieri, M., Borea, P. A., & Mugelli, A. (1995). β-Adrenoceptor subtypes in young and old rat ventricular myocytes: a combined patch-clamp and binding study. *British Journal of Pharmacology*, *116*(2), 1835–1842. https://doi.org/10.1111/j.1476-5381.1995.tb16671.x
53. Cao, X., Zhou, C., Chong, J., Fu, L., Zhang, L., Sun, D., Hou, H., Zhang, Y., Li, D., & Sun, H. (2015). Estrogen resisted stress-induced cardiomyopathy through increasing the activity of β2AR–Gαs signal pathway in female rats. *International Journal of Cardiology*, *187*, 377–386. https://doi.org/10.1016/j.ijcard.2015.02.113
54. Xu, C., Àiyīng Liú, Sun, H., Sun, Y., Wang, G., Gao, L., Hao, Y., & Yan, C. (2010). β2-Adrenoceptor confers cardioprotection against hypoxia in isolated ventricular myocytes and the effects depend on estrogenic environment. *Journal of Receptors and Signal Transduction*, *30*(4), 255–261. https://doi.org/10.3109/10799893.2010.488242
55. Hoeker, G. S., Hood, A. R., Katra, R. P., Poelzing, S., & Pogwizd, S. M. (2014). Sex Differences in β-Adrenergic Responsiveness of Action Potentials and Intracellular Calcium Handling in Isolated Rabbit Hearts. *PLoS ONE*, *9*(10), e111411. https://doi.org/10.1371/journal.pone.0111411
56. Parks, R. J., Ray, G., Bienvenu, L. A., Rose, R. A., & Howlett, S. E. (2014). Sex differences in SR Ca2+ release in murine ventricular myocytes are regulated by the cAMP/PKA pathway. *Journal of Molecular and Cellular Cardiology*, *75*, 162–173. https://doi.org/10.1016/j.yjmcc.2014.07.006
57. Cerbai, E., et al. “β-Adrenoceptor Subtypes in Young and Old Rat Ventricular Myocytes: A Combined Patch-Clamp and Binding Study.” British Journal of Pharmacology, vol. 116, no. 2, Sept. 1995, pp. 1835–1842, https://doi.org/10.1111/j.1476-5381.1995.tb16671.x. Accessed 9 Jan. 2024.
58. Tang, T., Hammond, H. K., Firth, A., Yang, Y., Gao, M. H., Yuan, J. X.-J. ., & Lai, N. C. (2011). Adenylyl Cyclase 6 Improves Calcium Uptake and Left Ventricular Function in Aged Hearts. *Journal of the American College of Cardiology*, *57*(18), 1846–1855. https://doi.org/10.1016/j.jacc.2010.11.052
59. Lucia, C. de, Eguchi, A., & Koch, W. J. (2018). New Insights in Cardiac β-Adrenergic Signaling During Heart Failure and Aging. *Frontiers in Pharmacology*, *9*. https://doi.org/10.3389/fphar.2018.00904
60. Brodde, O.-E. ., Zerkowski, H.-R. ., Schranz, D., Broede-Sitz, A., Michel-Reher, M., Schäfer-Beisenbusch, E., Piotrowski, J. A., & Oelert, H. (1995). Age-Dependent Changes in the β-Adrenoceptor—G-Protein(s)—Adenylyl Cyclase System in Human Right Atrium. *Journal of Cardiovascular Pharmacology*, *26*(1), 20. https://journals.lww.com/cardiovascularpharm/Abstract/1995/07000/Age_Dependent_Changes_in_the.4.aspx
61. Böhm, M., Dorner, H., Patrik Htun, Lensche, H., Platt, D., & Erdmann, E. (1993). Effects of exercise on myocardial adenylate cyclase and Gi alpha expression in senescence. *American Journal of Physiology-Heart and Circulatory Physiology*, *264*(3), H805–H814. https://doi.org/10.1152/ajpheart.1993.264.3.h805
62. Lindsey, S. H., Ariel, Silva, M. S., & Chappell, M. C. (2013). Reduced vasorelaxation to estradiol and G-1 in aged female and adult male rats is associated with GPR30 downregulation. *American Journal of Physiology-Endocrinology and Metabolism*, *305*(1), E113–E118. https://doi.org/10.1152/ajpendo.00649.2012

63. Li, X., Huang, F., Yin, J., Li, K., Li, Y., Qi, H., Fang, L., Yuan, C., Liu, W., & Wang, M. (2016). GPR30 decreases with vascular aging and promotes vascular smooth muscle cells maintaining differentiated phenotype and suppressing migration via activation of ERK1/2. *OncoTargets and Therapy*, 3415. https://doi.org/10.2147/ott.s104972
64. Kates, A. M., Herrero, P., Dence, C., Soto, P., Srinivasan, M., Delano, D. G., Ehsani, A., & Gropler, R. J. (2003). Impact of aging on substrate metabolism by the human heart. *Journal of the American College of Cardiology*, *41*(2), 293–299. https://doi.org/10.1016/s0735-1097(02)02714-6
65. Martineau, L. C., Chadan, S. G., & Parkhouse, W. S. (1999). Age-associated alterations in cardiac and skeletal muscle glucose transporters, insulin and IGF-1 receptors, and PI3-kinase protein contents in the C57BL/6 mouse. *Mechanisms of Ageing and Development*, *106*(3), 217–232. https://doi.org/10.1016/S0047-6374(98)00106-7
66. Groenewegen, A., Rutten, F. H., Mosterd, A., & Hoes, A. W. (2020). Epidemiology of heart failure. *European Journal of Heart Failure*, *22*(8), 1342–1356. https://doi.org/10.1002/ejhf.1858
67. Bristow, M. R., Ginsburg, R., Umans, V., Fowler, M., Minobe, W., Rasmussen, R., Zera, P., Menlove, R., Shah, P., & Jamieson, S. (1986). Beta 1- and beta 2-adrenergic-receptor subpopulations in nonfailing and failing human ventricular myocardium: coupling of both receptor subtypes to muscle contraction and selective beta 1-receptor down-regulation in heart failure. *Circulation Research*, *59*(3), 297–309. https://doi.org/10.1161/01.res.59.3.297
68. Ungerer, M., Böhm, M., Elce, J. S., Erdmann, E., & Lohse, M. J. (1993). Altered expression of beta-adrenergic receptor kinase and beta 1-adrenergic receptors in the failing human heart. *Circulation*, *87*(2), 454–463. https://doi.org/10.1161/01.cir.87.2.454
69. Sato, P. Y., Chuprun, J. K., Schwartz, M., & Koch, W. J. (2015). The Evolving Impact of G Protein-Coupled Receptor Kinases in Cardiac Health and Disease. *Physiological Reviews*, *95*(2), 377–404. https://doi.org/10.1152/physrev.00015.2014
70. Rengo, G., Perrone-Filardi, P., Femminella, G. D., Liccardo, D., Zincarelli, C., de Lucia, C., Pagano, G., Marsico, F., Lymperopoulos, A., & Leosco, D. (2012). Targeting the β-Adrenergic Receptor System Through G-Protein–Coupled Receptor Kinase 2: A New Paradigm for Therapy and Prognostic Evaluation in Heart Failure. *Circulation: Heart Failure*, *5*(3), 385–391. https://doi.org/10.1161/circheartfailure.112.966895
71. Lefkowitz, R. J., Rockman, H. A., & Koch, W. J. (2000). Catecholamines, Cardiac β-Adrenergic Receptors, and Heart Failure. *Circulation*, *101*(14), 1634–1637. https://doi.org/10.1161/01.cir.101.14.1634
72. Brodde, O.-E., & Michel, M. (1999). *Adrenergic and Muscarinic Receptors in the Human Heart*.
73. Chang, S.-L., Chuang, H.-L., Chen, Y.-C., Kao, Y.-H., Lin, Y.-K., Yeh, Y.-H., Chen, S.-A., & Chen, Y.-J. (2016). Heart failure modulates electropharmacological characteristics of sinoatrial nodes. *Experimental and Therapeutic Medicine*, *13*(2), 771–779. https://doi.org/10.3892/etm.2016.4015
74. Verkerk, A. O., Wilders, R., Coronel, R., Ravesloot, J. H., & Verheijck, E. E. (2003). Ionic Remodeling of Sinoatrial Node Cells by Heart Failure. *Circulation*, *108*(6), 760–766. https://doi.org/10.1161/01.cir.0000083719.51661.b9
75. Forstermann, U., & Sessa, W. C. (2011). Nitric oxide synthases: regulation and function. *European Heart Journal*, *33*(7), 829–837. https://doi.org/10.1093/eurheartj/ehr304

76. Zhang, X., Li, T., Cheng, H.-J., Wang, H., Ferrario, C. M., Groban, L., & Cheng, C. P. (2021). Chronic GPR30 agonist therapy causes restoration of normal cardiac functional performance in a male mouse model of progressive heart failure: Insights into cellular mechanisms. *Life Sciences*, *285*, 119955. https://doi.org/10.1016/j.lfs.2021.119955
77. Kang, S., Liu, Y., Sun, D., Zhou, C., Liu, A., Xu, C., Hao, Y., Li, D., Yan, C., & Sun, H. (2012). Chronic Activation of the G Protein-Coupled Receptor 30 with Agonist G-1 Attenuates Heart Failure. *PLoS ONE*, *7*(10), e48185. https://doi.org/10.1371/journal.pone.0048185
78. Aryan, L., Younessi, D., Zargari, M., Banerjee, S., Agopian, J., Rahman, S., Borna, R., Ruffenach, G., Umar, S., & Eghbali, M. (2020). The Role of Estrogen Receptors in Cardiovascular Disease. *International Journal of Molecular Sciences*, *21*(12), 4314. https://doi.org/10.3390/ijms21124314
79. Groban, L., Tran, Q.-K., Ferrario, C. M., Sun, X., Cheng, C. P., Kitzman, D. W., Wang, H., & Lindsey, S. H. (2020). Female Heart Health: Is GPER the Missing Link? *Frontiers in Endocrinology*, *10*. https://doi.org/10.3389/fendo.2019.00919
80. Ma, Y., Qiao, X., Falone, A. E., Reslan, O. M., Sheppard, S. J., & Khalil, R. A. (2010). Gender-Specific Reduction in Contraction is Associated with Increased Estrogen Receptor Expression in Single Vascular Smooth Muscle Cells of Female Rat. *Cellular Physiology and Biochemistry*, *26*(3), 457–470. https://doi.org/10.1159/000320569
81. Silva, A., & Zanesco, A. (2010). *Physical exercise, ß-adrenergic receptors, and vascular response Exercício físico, receptores ß-adrenérgicos e resposta vascular*.
82. Kneale, B. J., Chowienczyk, P. J., Brett, S. E., Coltart, D. John., & Ritter, J. M. (2000). Gender differences in sensitivity to adrenergic agonists of forearm resistance vasculature. *Journal of the American College of Cardiology*, *36*(4), 1233–1238. https://doi.org/10.1016/s0735-1097(00)00849-4
83. Sudhir, K., Chou, T. M., Mullen, W. L., Hausmann, D., Collins, P., Yock, P. G., & Chatterjee, K. (1995). Mechanisms of estrogen-induced vasodilation: In vivo studies in canine coronary conductance and resistance arteries. *Journal of the American College of Cardiology*, *26*(3), 807–814. https://doi.org/10.1016/0735-1097(95)00248-3
84. Traupe, T., Stettler, C. D., Li, H., Haas, E., Bhattacharya, I., Minotti, R., & Barton, M. (2007). Distinct Roles of Estrogen Receptors α and β Mediating Acute Vasodilation of Epicardial Coronary Arteries. *Hypertension*, *49*(6), 1364–1370. https://doi.org/10.1161/hypertensionaha.106.081554
85. Kawano, H., Motoyama, T., Kugiyama, K., Hirashima, O., Ohgushi, M., Fujii, H., Ogawa, H., & Yasue, H. (1997). Gender Difference in Improvement of Endothelium-Dependent Vasodilation After Estrogen Supplementation. *Journal of the American College of Cardiology*, *30*(4), 914–919. https://doi.org/10.1016/s0735-1097(97)00234-9
86. Lindsey, S. H., Carver, K. A., Prossnitz, E. R., & Chappell, M. C. (2011). Vasodilation in Response to the GPR30 Agonist G-1 is Not Different From Estradiol in the mRen2.Lewis Female Rat. *Journal of Cardiovascular Pharmacology*, *57*(5), 598–603. https://doi.org/10.1097/fjc.0b013e3182135f1c
87. Limberg, J. K., Peltonen, G. L., Johansson, R. E., Harrell, J. W., Kellawan, J. M., Eldridge, M. W., Sebranek, J. J., Walker, B. J., & Schrage, W. G. (2016). Greater Beta-Adrenergic Receptor Mediated Vasodilation in Women Using Oral Contraceptives. *Frontiers in Physiology*, *7*. https://doi.org/10.3389/fphys.2016.00215

88. Mcnulty, K., Kirsty, Elliott-Sale, J., Dolan, Paul, Swinton, A., Ansdell, Goodall, Thomas, K., Hicks, M., & Elliott, K. (2020). *The Effects of Menstrual Cycle Phase on Exercise Performance in Eumenorrheic Women: A Systematic Review and Meta-Analysis*. *50*, 1813–1827.
89. Riedel, K., Deussen, A. J., Tolkmitt, J., Weber, S., Schlinkert, P., Zatschler, B., Friebel, C., Müller, B., El-Armouche, A., Morawietz, H., Matschke, K., & Kopaliani, I. (2019). Estrogen determines sex differences in adrenergic vessel tone by regulation of endothelial β-adrenoceptor expression. *American Journal of Physiology-Heart and Circulatory Physiology*, *317*(2), H243–H254. https://doi.org/10.1152/ajpheart.00456.2018
90. Garrott, K., Dyavanapalli, J., Cauley, E., Dwyer, M. K., Kuzmiak-Glancy, S., Wang, X., Mendelowitz, D., & Kay, M. W. (2017). Chronic activation of hypothalamic oxytocin neurons improves cardiac function during left ventricular hypertrophy-induced heart failure. *Cardiovascular Research*, *113*(11), 1318–1328. https://doi.org/10.1093/cvr/cvx084
91. Marks, A. R. (2013). Calcium cycling proteins and heart failure: mechanisms and therapeutics. *Journal of Clinical Investigation*, *123*(1), 46–52. https://doi.org/10.1172/jci62834
92. Wu, Qin, et al. “Oestrogen Changed Cardiomyocyte Contraction and β-Adrenoceptor Expression in Rat Hearts Subjected to Ischaemia–Reperfusion.” *Experimental Physiology*, vol. 93, no. 9, 14 Aug. 2008, pp. 1034–1043, https://doi.org/10.1113/expphysiol.2007.041939.
93. Liu, Aiying, et al. “Testosterone Enhances Estradiol’s Cardioprotection in Ovariectomized Rats.” *Journal of Endocrinology*, vol. 212, no. 1, 30 Sept. 2011, pp. 61–69, https://doi.org/10.1530/joe-11-0181.
94. Wang, Hao, et al. “Cardiomyocyte-Specific Deletion of the G Protein-Coupled Estrogen Receptor (GPER) Leads to Left Ventricular Dysfunction and Adverse Remodeling: A Sex-Specific Gene Profiling Analysis.” Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, vol. 1863, no. 8, Aug. 2017, pp. 1870–1882, https://doi.org/10.1016/j.bbadis.2016.10.003.
95. Filice, E, et al. “A New Membrane G Protein-Coupled Receptor (GPR30) Is Involved in the Cardiac Effects of 17beta-Estradiol in the Male Rat.” PubMed, vol. 60, no. 4, 1 Dec. 2009, pp. 3–10.