NEW INSIGHTS INTO ESTROGEN/ESTROGEN RECEPTOR EFFECTS IN THE CARDIAC AND SKELETAL MUSCLE

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NEW INSIGHTS INTO ESTROGEN/ESTROGEN RECEPTOR EFFECTS IN THE CARDIAC AND SKELETAL MUSCLE

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EDITORIAL
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Editorial: New Insights into Estrogen/Estrogen Receptor Effects in the Cardiac and Skeletal Muscle

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Editorial on the Research Topic

New Insights into Estrogen/Estrogen Receptor Effects in the Cardiac and Skeletal Muscle

Decreased levels of the steroid hormone estrogen at menopause are associated with an increased incidence of cardiovascular disease and loss of skeletal muscle mass and strength. Consequently, it has been generally expected that estrogen may be a crucial protective factor against the development of cardiovascular disease and that it may be implicated in the regulation of skeletal muscle function in women. Estrogen signals through the classical nuclear estrogen receptors (ER) α and β, as well as the membrane G protein-coupled receptor GPR30 (also referred to as GPER), via the genomic or non-genomic pathway.

Studies reporting actions of estrogen in the heart show direct cardiac estrogenic effects (1, 2), which may differ significantly between the sexes (3–6). In this Research Topic, Ueda et al. provide an overview of ER signaling in the cardiovascular system, including cardiac myocytes and fibroblasts. Considerable effort has focused on non-nuclear ER signaling and non-genomic effects of estrogen. Accordingly, Puglisi et al. review non-genomic effects of estrogen on cell homeostasis and remodeling focusing on cardiac ischemia/reperfusion injury.

Mitochondrial bioenergetics are at the core of cardiac and skeletal muscle function. Ventura-Clapier et al. provide an overview of the effects of estrogen and its receptors in cardiac and skeletal muscle mitochondria. Mahmoodzadeh and Dworatzek take a closer look and review the regulation of cardiac mitochondrial function and Ca^{2+} ion channels by 17β-estradiol (E2) and its receptors, thereby affecting contractile function. The E2/ER axis also impacts skeletal muscle contractility and mitochondrial bioenergetics (7–11). Counts et al. report in their original research study that mitochondrial dysfunction was attenuated by the administration of E2 in a genetic mouse model of cachexia. In addition to regulating mitochondrial bioenergetics, estrogen is expected to confer protection against oxidative stress. In their original research article, Ogola et al. provide insight into how acute estrogen signaling via GPER provides cardiovascular protection in angiotensin II-induced hypertension characterized by increased oxidative stress.

The decline of estrogen at menopause is associated with changes in several cardiovascular risk factors, including the atherogenic lipid profile and calcification in cardiovascular structures. In this context, Zhang et al. provide a review of the effects of estrogen in basic biological pathways associated with vascular and cardiac valvular tissue calcification, as well as potential strategies of pharmacological therapy to reduce or slow these processes. Karvinen et al. investigated whether physical activity attenuates changes in the atherogenic lipid profile and cardiovascular risk in

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postmenopausal women. Their results suggest that physical activity may attenuate menopause-associated atherogenic changes of healthy middle-aged women to a certain extent. Interestingly, physical activity, in turn, is affected by estrogen (12, 13).

The E2/ER axis may also affect immune responses, thereby affecting the risk of infection and subsequent development of inflammatory heart disease, such as myocarditis, which may lead to cardiomyopathy and heart failure. In their original research study, Bruno et al. found that exposure to the endocrine disruptor bisphenol A led to altered ER expression in the heart, suggesting an increased risk of developing myocarditis after a viral infection in females.

Pronounced sex differences exist in the development and pathophysiology of cardiovascular diseases (14), such as pressure overload-induced left ventricular hypertrophy (15–20), as well as the response to therapy (21, 22). Estrogen is thought to play a major role in cardiovascular sex differences. Notably, it has been put forward that the decrease of estrogen at menopause may be a contributor to the development of heart failure with preserved ejection fraction (23), which mostly affects women (24). Considering the potential underlying pathomechanisms, Sickinghe et al. propose the hypothesis that the menopause-related estrogen decline contributes to myocardial microvascular dysfunction and they provide an overview of molecular targets of estrogen that might guide future research and treatment options. Along this line, Groban et al. outline the impact of GPER on diastolic function, left ventricular stiffness and aortic distensibility, among others.

Collectively, this Research Topic includes clinical- and pre-clinical studies in original research and state-of-the-art review articles focusing on the effects of estrogen on cardiovascular physiology and skeletal muscle biology and function. Detailed characterization of the regulation of (patho)physiology by estrogen and its receptors in cardiac and skeletal muscle, along with elucidation of the underlying mechanisms may lead to the identification of novel therapeutic targets, which may have a wide implication in the development of new and personalized therapies.

AUTHOR CONTRIBUTIONS

GK conceived the work and drafted the manuscript. DL revised critically the manuscript. DL and GK read and approved of the submitted manuscript.

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REVIEW
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The Role of 17β-Estradiol and Estrogen Receptors in Regulation of Ca^{2+} Channels and Mitochondrial Function in Cardiomyocytes

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Numerous epidemiological, clinical, and animal studies showed that cardiac function and manifestation of cardiovascular diseases (CVDs) are different between males and females. The underlying reasons for these sex differences are definitely multifactorial, but major evidence points to a causal role of the sex steroid hormone 17β-estradiol (E2) and its receptors (ER) in the physiology and pathophysiology of the heart. Interestingly, it has been shown that cardiac calcium (Ca^{2+}) ion channels and mitochondrial function are regulated in a sex-specific manner. Accurate mitochondrial function and Ca^{2+} signaling are of utmost importance for adequate heart function and crucial to maintaining the cardiovascular health. Due to the highly sensitive nature of these processes in the heart, this review article highlights the current knowledge regarding sex dimorphisms in the heart implicating the importance of E2 and ERs in the regulation of cardiac mitochondrial function and Ca^{2+} ion channels, thus the contractility. In particular, we provide an overview of *in-vitro* and *in-vivo* studies using either E2 deficiency; ER deficiency or selective ER activation, which suggest that E2 and ERs are strongly involved in these processes. In this context, this review also discusses the divergent E2-responses resulting from the activation of different ER subtypes in these processes. Detailed understanding of the E2 and ER-mediated molecular and cellular mechanisms in the heart under physiological and pathological conditions may help to design more specifically targeted drugs for the management of CVDs in men and women.

Keywords: estrogen, estrogen receptor, G-protein-coupled estrogen receptor, cardiomyocytes, sex difference, cardiac mitochondrial function, cardiac Ca^{2+} ion channel

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INTRODUCTION

Cardiovascular Diseases (CVDs) are one of the top age-associated chronic diseases with growing importance due to the dramatic increase in life expectancy (1) and are the leading cause of mortality in men and women worldwide (2). In the vast majority of CVDs, there are well described sex differences in the incidence, pathophysiology, and outcomes of diseases (3). As result of these observations, research over the last few decades has focused on the contribution of sex steroid hormones, specifically 17β-estradiol (E2), on the cardiovascular system and mechanistic pathways in the diseased heart.

Calcium (Ca^{2+}) is a key player in the regulation of myocardial contraction and the deregulation of Ca^{2+} signaling due to the alteration of Ca^{2+} ion channels function in cardiomyocytes is highly associated with the development of cardiac diseases, such as heart failure (4). Just like Ca^{2+}, mitochondria play an essential role in the regulation of energy metabolism of the heart, and defects of mitochondrial function also lead to the development and progression of cardiovascular diseases (5, 6). This review article provides an overview of the current knowledge regarding the sex differences in cardiac health and disease with the focus on the sexually dimorphic effects of E2 and estrogen receptors (ERs) in the regulation of cardiomyocyte's Ca^{2+} ion channels and mitochondrial function.

THE ROLE OF 17β-ESTRADIOL IN THE HEART

Epidemiological data suggest that premenopausal women are protected from the incidence of CVDs as well as from resulting morbidity and mortality compared with age-matched men, but that this protection is lost after menopause (7–9). This led to the generally accepted conclusion that the sex hormone E2 protects against CVDs in women (10). However, recent large-scale clinical trials revealed conflicting data about the effect of E2 on CVDs, which is still a matter of intense debate. For example, several observational studies such as the *Nurse's Health Study* showed that postmenopausal women with hormone replacement therapy (HRT) have a lower rate of CVDs and cardiac death, compared to women without HRT (11–14). In contrast, the *Women Health Initiative* (WHI) and the *Heart and Estrogen/Progestin Replacement Study* (HERS I and II) showed that HRT has no obvious beneficial effect on CVDs, and may actually increase the risk and events of CVDs in postmenopausal women (15–19). The reasons for this paradox remain unclear and many potential factors, such as the study design and subject characteristics, the form of applied E2 (which type of E2, combination of E2 with progestin), the dosage and pharmacokinetics of the HRT used, and the statistical power to address cardiac risk factors may contribute to the discrepant results and to the adverse outcome of HRT (20–22). In addition, another reason for the contradictory data could be the timing of HRT initiation. Recent studies such as the *Kronos Early Estrogen Prevention Study* (KEEPS) and the *Early vs. Late Intervention Trial with Estradiol* (ELITE) addressed the question of the so-called "timing hypothesis." They showed significant beneficial cardiovascular effects in women who initiated HRT in the early postmenopause vs. late menopause period (19, 23, 24), indicating the importance of the time point of HRT-application.

Modulatory effects of E2 on CVDs in men have also been reported (25, 26). In men with E2 deficiency due to a mutation in the cytochrome P450 aromatase gene (*Cyp19a1*), which catalyzes the aromatization of androgens to E2, or E2 resistance, caused by a point mutation in the ERα gene (*ESR1),* the following have been reported: increased total cholesterol level, the development of insulin resistance, impaired glucose tolerance, type 2 diabetes mellitus, and impaired vasodilatation (27–31). These data suggest that the physiological concentrations of E2 might reduce the risk of CVDs in men. Indeed, men with abnormally low (≤13 pg/mL) and abnormally high (≥37 pg/mL) E2-levels have been found to show the highest death rates from congestive heart failure (32). By contrast, individuals with levels of E2 in the range of 22–30 pg/mL had the least number of deaths over a 3-year period. However, the precise role of E2 in men in CVDs remains questionable (33).

ACTIONS OF 17β-ESTRADIOL AND ESTROGEN RECEPTORS

E2 belongs together with Estrone (E1) and Estriol (E3) to the group of sex steroids called Estrogens. Thereby, E2 is the predominant and most biologically active form (34). Estrogens have traditionally been associated with the female reproductive development and function, but it is now well-established that they also regulate male reproductive organs and play a physiological role in multiple organs in both sexes (26). In healthy premenopausal women, ovaries are the primary site of E2 production, and in men, E2 is produced in small amounts by the testes. E2 is also synthesized in a number of extragonadal tissues, through the conversion of testosterone by cytochrome p450 aromatase in both sexes, including bone, breast, adipose tissue, and the brain (35). There is increasing evidence that the aromatase is also expressed in the heart tissue and that E2 can also be produced locally in cardiac cells (36–39), suggesting that local cardiac E2 synthesis by aromatase plays a role in the E2-mediated effects on CVDs.

The physiological effects of E2 are predominantly mediated via estrogen receptor alpha (ERα) and beta (ERβ), which are members of the nuclear receptor superfamily (**Figure 1**) (40). Both receptors carry similar structural domains, however, they differ in their DNA- and ligand-binding regions, which are of crucial importance for their diverse transcriptional actions (41). E2-activated ERs can act as ligand-induced transcription factors inducing changes in transcription of E2 target genes, a process referred to as genomic actions. Here the binding of E2 to the ERs results in homo- or heterodimerization of ER and their translocation into the nucleus of cells. The E2/ER complex either binds to estrogen response elements (ERE) within the promoter of target genes or regulates gene transcription by interacting with other transcription factors, e.g., AP-1 and Sp1 (**Figure 1I**) (34, 42–44). Additionally, E2-bound ERs can also activate multiple signal transduction pathways, e.g., *mitogen-activated protein kinases ERK1/2* and *-p38* (ERK1/2-MAPK, p38-MAPK) as well as *phosphoinositide 3-kinase-serin/threonine-specific kinase* B (PI3K/AKT), which in turn phosphorylate ERs (45–47) or other promoter bound transcription factors that are involved in the regulation of E2-target gene expression (**Figure 1II**) (48–51). Moreover, through non-genomic actions, E2 rapidly mediates its effects by activation of ERs located in or adjacent to the plasma membrane, which in turn can activate different signal transduction cascades, such as PI3K/AKT and MAPK, leading for example to cytosolic eNOS activation (**Figure 1III**) (52, 53).

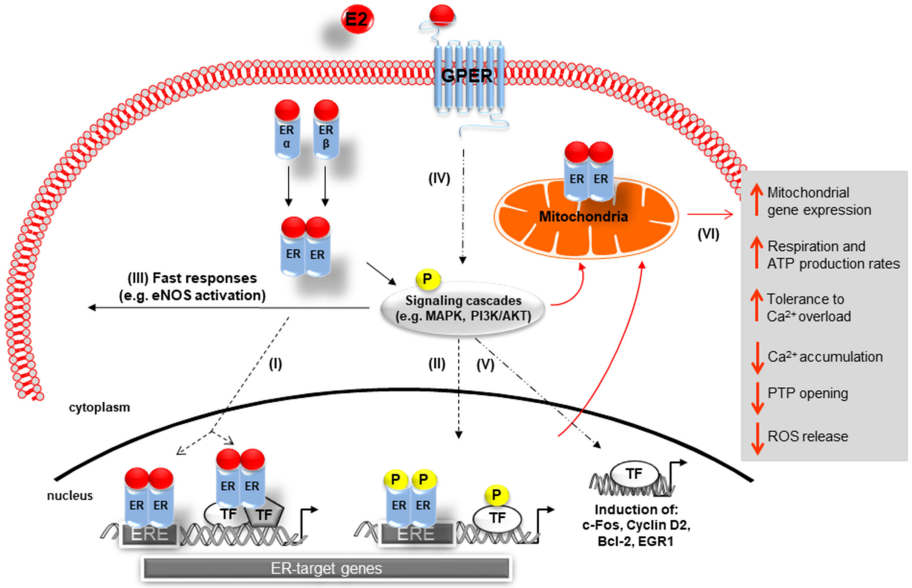

FIGURE 1 | Schematic representation of 17β-Estradiol induced estrogen receptor-alpha, -beta, and G-protein-coupled estrogen receptor signaling. Genomic pathway: **(I)** The E2/ER complex can bind to estrogen response elements (ERE) within the promoter of target genes or regulates gene transcription by interacting with other transcription factors (TF), e.g., AP-1 and Sp1. **(II)** In addition, E2/ER activate signaling transduction pathways, leading to phosphorylation of ER or other bound transcription factors modulating gene expression. In the non-genomic action: **(III)** E2-activated ER lead to rapid tissue responses via phosphorylation of cytosolic signaling cascades. **(IV)** GPER predominantly mediates rapid, non-genomic E2 signaling by the involvement of several kinases, ion channels, and second messengers. **(V)** GPER is also involved in gene expression regulation. **(VI)** E2 initiated cellular and mitochondrial ER/GPER genomic and non-genomic actions modulate mitochondrial respiration, ATP production, and ROS formation (indicated by red arrows). E2, 17β-estradiol; ER, estrogen receptor alpha and beta ERE, estrogen response element; TF, transcription factor; P, phosphorylation; GPER, G-protein-coupled estrogen receptor; Ca^{2+}, calcium, PTP, permeability transition pore; MAPK, mitogen-activated protein kinases; PI3K/AKT, phosphoinositide 3-kinase-serin/threonine-specific kinase B; eNOS: endothelial nitric oxide synthase.

ESTROGEN RECEPTORS IN THE HEART

Both ERs are localized in different cardiac cells such as cardiomyocytes, endothelial cells, smooth muscle cells, and cardiac fibroblasts in human hearts from both sexes (54, 55). Studies in rodents also showed that both ER are expressed in whole heart tissue from males and females (36, 39, 56–58). Recent observations from Pugach et al. showed that only ERα, but not ERβ, is expressed in left ventricular heart tissue from mice and isolated rat cardiomyocytes (59). However, there are several other studies that not only showed the expression of both ERs in cardiomyocytes of rodents but also their functional activity on genomic and non-genomic levels (36, 60–67).

Recent reports showed that E2 can signal through a third protein, the G-protein-coupled estrogen receptor (GPER), formerly known as GPR30, a membrane receptor with seven transmembrane spanning domains (68, 69). GPER is strongly expressed in both male and female human and rat cardiac tissue (70–73). Specifically, GPER is present in smooth muscle cells (74, 75), endothelial cells (76), cardiac fibroblasts (77), and cardiomyocytes (70). GPER has been implicated predominantly in the rapid, non-genomic E2 signaling by the involvement of several kinases, ion channels and second messengers in a wide variety of cell types (**Figure 1IV**) (69, 78–80). However, effects on gene expression, i.e., induction of c-fos, cyclin D2, Egr-1, and Bcl-2 expression, have also been described (81–85).

ASSOCIATION OF GENETIC ALTERATIONS AND POLYMORPHISMS OF THE ESTROGEN RECEPTOR GENES AND CARDIOVASCULAR DISEASE

Studies showed that mutations in the genes coding for ERα and ERβ are associated with differences in heart morphology, such as increased left ventricular mass and wall thickness (86, 87). Furthermore, single nucleotide polymorphisms (SNPs) in both ERα and ERβ have been shown to be associated with the susceptibility for CVDs. Most of the studies analyzing ERα focused on two SNPs: c.454-397T>C (rs2234693) and c.454-351A>G (rs9340799) located in the first intron of the ERα gene and 46 bp apart from each other (88). In fact, the ERα variant rs2234693 was linked to coronary heart disease among Finnish men (89), whereas a study of a Dutch cohort showed that ERα variants, rs2234693, and rs9340799, were associated with increased risk of myocardial infarction (MI) and ischemic heart disease (IHD) only in postmenopausal women, but not in men (90). In contrast, in a prospective study in men and women from the population based offspring cohort of the Framingham Heart Study showed that individuals of both sexes carrying the rs2234693 genotype have substantial increase in risk of MI (91). The authors confirmed their findings in men in a latter study, including 7,000 men in five cohorts from four countries (92).

In contrast, other studies found no association between these two SNPs or their haplotypes and MI or risk of CVD in either women or men (88, 93–95). Additionally, the absence of ERα in human vascular smooth muscle cells in premenopausal women (96) or the reduced ERα expression, due to methylation of the receptor with increasing age, is associated with the development of atherosclerosis in the cardiovascular system (97).

For ERβ, the SNP variant rs1271572 was associated with increased risk of MI in Spanish men (98), while Rexrode et al. identified this ERβ variant to be associated with increased risk of MI in women only (99). Additionally, this study showed the linkage of another ERβ variant, the rs1256049, with reduced risk of CVDs or MI in women (99).

The reasons for the inconsistency in data regarding the SNPs within the genes of ERα and ERβ could be due to the limited power within the studies, differences in methodology and study population (93). Despite the inconsistent findings, together these studies provide support for a relationship between ERα and ERβ polymorphisms and the risk of CVDs in men and women. The underlying mechanisms responsible for the phenotype associated with these genetic variants are not yet known. It is recognized that ER-SNPs can cause changes in E2-mediated downstream gene expression and signaling, which can alter the effects of E2 on the heart (100) and may be one possible explanation for the observed effects on the cardiovascular system. In contrast to ERα and ERβ, there are no studies so far regarding the association of polymorphisms within the GPER gene and cardiac risk in humans.

THE ROLE OF ESTROGEN RECEPTORS IN ANIMAL MODELS FOR HUMAN CARDIOVASCULAR DISEASES

The physiology of E2-actions through its multiple receptors is diverse and highly complex. The detailed understanding of their effects and underlying molecular mechanisms are essential for future therapeutic applications in humans. In order to clarify remaining questions regarding the functions of each individual receptor within the heart, different mouse models with a deficiency or overexpression of ERα, ERβ, and GPER have been generated (101, 102).

ERα

At the basal level, male and female whole body ERα-deficient (ERKO)-mice are obese and insulin resistant (103). They also exhibit altered cardiac substrate preference with a reduction in glucose uptake indicating that ERα is required to maintain glucose utilization in the mouse heart (104). However, ERKO-mice do not show any cardiac dysfunction under physiological conditions. Following cardiac injuries, such as ischemic-reperfusion (I/R) injury or induced chronic MI, male and female ERKO-mice show increased cardiomyocyte cell death, mitochondrial damage, marked coronary edema, decreased coronary flow rate, and poorer functional recovery of contractility (+dP/dt) and compliance (-dP/dt) in comparison to wild type (WT)-mice (105, 106). These data suggest a cardiac protective role of ERα in both sexes after I/R or MI. In contrast, following pressure overload induced myocardial hypertrophy by transverse aortic constriction, female ERKO-mice developed myocardial hypertrophy to an identical degree as that seen in WT females, indicating that ERα is not essential for the attenuation of pressure overload induced hypertrophy observed in females (107, 108).

Analysis of mice hearts carrying a cardiomyocyte-specific deletion of ERα (cs-ERKO) revealed variations in the expression of genes involved in metabolism, cell growth and differentiation, muscle architecture, and relaxation compared to WT-mice (109). Furthermore, under basal conditions hearts from male and female cs-ERKO-mice showed reduction of left ventricular mass accompanied by decreased left ventricle (LV) diameter compared with WT-mice. These data are in line with published findings in mice with cardiomyocyte specific ERα-overexpression (csERα-OE), showing that constitutive ERα-overexpression in cardiomyocytes resulted in higher left ventricular mass and increased ventricular volumes. In addition, greater cardiomyocyte length, augmented expression of hypertrophy-associated genes such as *nppa* and *nppb, but* no fibrosis development was observed (65). In agreement with these data, findings from ovariectomized (OVX) mice also emphasize an E2-dependent role of ERα on regulation of cardiomyocyte size and cardiac growth in healthy mice (110). Overall, these findings indicate that ERα restricted to the cardiomyocytes is associated with the growth in cardiac mass in both sexes.

Interestingly, the use of csERα-OE mice demonstrated that ERα provides cardioprotection in female mice by enhancing neovascularization and impairment of cardiac remodeling in response to cardiac ischemic injury (65). All together, these findings indicate that in the female sex, ERα in cardiomyocytes may have a therapeutic potential in the treatment of ischemic heart disease, leading to more efficient cardiac repair after cardiac injury.

ERβ

In contrast to ERKO-mice, male and female ERβ-deficient (BERKO)-mice show a mild metabolic phenotype characterized by increased cortical bone formation and loss of trabecular bone (111). In addition, ERβ deficiency protects against diet-induced insulin resistance and glucose intolerance (112). However, with increasing age, BERKO-mice show cardiac hypertrophy, hypertension, and pathology in other cell types as they age (113–115). Additionally, BERKO-mice develop severe cardiomyopathy with a disarray of cardiomyocytes, a disruption of intercalated discs, an increase in number and size of gap junctions, and alteration in nuclear structure (114).

Several studies in BERKO-mice demonstrate the relevant role of ERβ in male and female mice after cardiac injury. The lack of ERβ significantly decreased post-ischemic cardiac recovery and therefore myocardial function in female, but not male, mice (116). In OVX mice subjected to MI, E2-treatment did not reduce infarct size in female BERKO-mice, as observed in ERKO- and WT-mice (117). In line with these data, Pelzer et al. reported that OVX BERKO-mice subjected to chronic MI showed increased mortality rates and aggravated signs of heart failure (118). These

observations support the protective role of ERβ in response to I/R or MI in females. Following transverse aortic constriction, increase in left ventricular mass was not attenuated by E2-supplementation in OVX BERKO- as observed in WT- and ERKO-mice (108). Indeed, it has been shown that female BERKO-mice responded to transverse aortic constriction, as well as in the deoxycorticosterone acetate-salt mouse model, with a significantly higher increase in myocardial hypertrophy, marked increase in left ventricular diameters, increased cardiomyocyte size and apoptosis compared with female WT-mice (107, 119, 120). Fliegner et al. showed in male mice lacking ERβ significantly higher cardiomyocyte hypertrophy, increased myocyte apoptosis and faster progression toward heart failure (120). Thus, under pressure overload the loss of ERβ is detrimental for both males and females.

In a mouse model with a cardiomyocyte specific ERβ-overexpression (csERβ-OE), under basal conditions there were no observed differences in heart weight, morphology, and function in males and females (66). Interestingly, the overexpressed ERβ was located within the cytoplasm and nuclei of cardiomyocytes (66), while in csERα-OE mice the ERα protein was mainly located within the nuclei of cardiomyocytes (65). In response to MI, csERβ-OE exhibited improved survival in female and male mice compared to the WT counterparts (66). This was due to attenuated increase in heart weight and LV dilatation as well as improved systolic and diastolic function. In addition, both male and female csERβ-OE mice had a lower reduction of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) expression, suggesting less reduction in diastolic Ca^{2+}-reuptake into the sarcoplasmic reticulum post-MI. Most of these functional parameters were improved in both sexes by csERβ-OE; however, the effect on LV volumes and ejection fraction was more pronounced in males than females. This was possibly due to reduced cardiac remodeling with lower cardiac fibrosis and lower expression of fibrosis markers (collagen I and III, periostin and miR-21), which was observed particularly in male csERβ-OE hearts after MI.

GPER

There are several studies stating the phenotype of mice lacking GPER (101). The studies of GPER-KO-mice over the last decade revealed that GPER deficient mice show under basal conditions multiple physiological alterations, including obesity (75), insulin resistance, glucose intolerance, and increase in blood pressure (121). Interestingly, it has been reported that male, but not female, GPER-KO-mice show impaired cardiac function with enlarged LV and decreased +dP/dt and –dP/dt (122) or decreased ejection fraction and fractional shortening with increasing age (123). Under cardiac stress, one study reported in a mouse model of I/R that male WT-, ERKO-, and BERKO-mice respond to E2-treatment with an improved recovery and reduced infract size. However, the application of E2 to male GPER-KO-mice did not lead to observed cardioprotection after I/R (80).

A recent study in mice with a cardiomyocyte-specific GPER-KO (csGPER-KO) revealed under basal conditions adverse alterations in cardiac structure and impaired systolic and diastolic function in both sexes, in comparison to WT-mice, with more profound increases in LV dimensions, and wall-thinning among male KO-mice (124). Using DNA microarray analysis, the authors found differential expression profiles of genes affecting multiple transcriptional networks with marked differences in respect to sex and cardiomyocyte-specific GPER deletion. In detail, mitochondrial genes were enriched in cardiomyocytes from female GPER-KO- compared to female WT-mice, but not in male. In contrast, inflammatory response genes were enriched in GPER-KO- vs. WT-cardiomyocytes from male but not female mice (124, 125).

Although studies with transgenic ER mice failed to provide a clear consensus regarding the physiological and pathological roles of ERs, they suggest that each of the ER subtypes play a protective role in the heart.

THE ROLE OF 17β-ESTRADIOL AND ESTROGEN RECEPTORS IN REGULATION OF CA^{2+} CHANNELS AND CONTRACTILITY IN CARDIOMYOCYTES

Ca^{2+} is a critical regulator of myocardial function. Ca^{2+} regulates contraction, and deregulation of Ca^{2+} signaling has been associated with cardiac dysfunction and pathology such as arrhythmias and heart failure (4). In cardiomyocytes, Ca^{2+} levels are tightly regulated via the excitation-contraction (EC) coupling pathway (**Figure 2**). During action potential, in response to depolarization, Ca^{2+} crosses the sarcolemma and T-tubular membrane through the voltage gated L-type Ca^{2+} channels. This Ca^{2+} influx triggers the release of a larger quantity of Ca^{2+}, called Ca^{2+} sparks, from the sarcoplasmic reticulum (SR), through the opening of SR Ca^{2+} release channels, known as ryanodine receptors (RyRs, particularly RyR2). This process is termed Ca^{2+}-induced Ca^{2+} release. The combination of Ca^{2+} influx via the L-type Ca^{2+} channels and Ca^{2+} release from SR leads to the formation of cytosolic Ca^{2+} transients. The binding of cytosolic Ca^{2+} to the myofilaments then initiates cardiomyocyte contraction. Subsequent relaxation occurs by removal of Ca^{2+} from the cytosol mainly via the following mechanisms: (I) The SERCA2a re-uptakes the cytosolic Ca^{2+} back into the SR; the activity of this channel being modulated by its endogenous inhibitor phospholamban (PLN); (II) The Na^{+}/Ca^{2+} exchanger (NCX) extrudes the Ca^{2+} out of the cells; (III) The mitochondrial Ca^{2+} uniporter transports Ca^{2+} into the mitochondria (4, 126).

Numerous studies have documented sex differences in cardiac EC coupling (127–129). For example, at rest, women have longer QT intervals and higher left ventricular ejection fraction than men (130–132). Other studies showed that ventricular myocytes in the female human failing heart have significantly greater contractility and enhanced L-type Ca^{2+} current ($I_{Ca,L}$) compared to male patients (133–135). Studies in animal models also provide convincing evidence of sex differences in contractile function as observed in humans. It has been demonstrated that isolated cardiomyocytes from male rodents exhibit higher contraction than those from females (128, 136, 137). Furthermore, male rat cardiac myocyte and papillary muscle develop higher contractile force as well as significantly greater Ca^{2+} transient amplitude

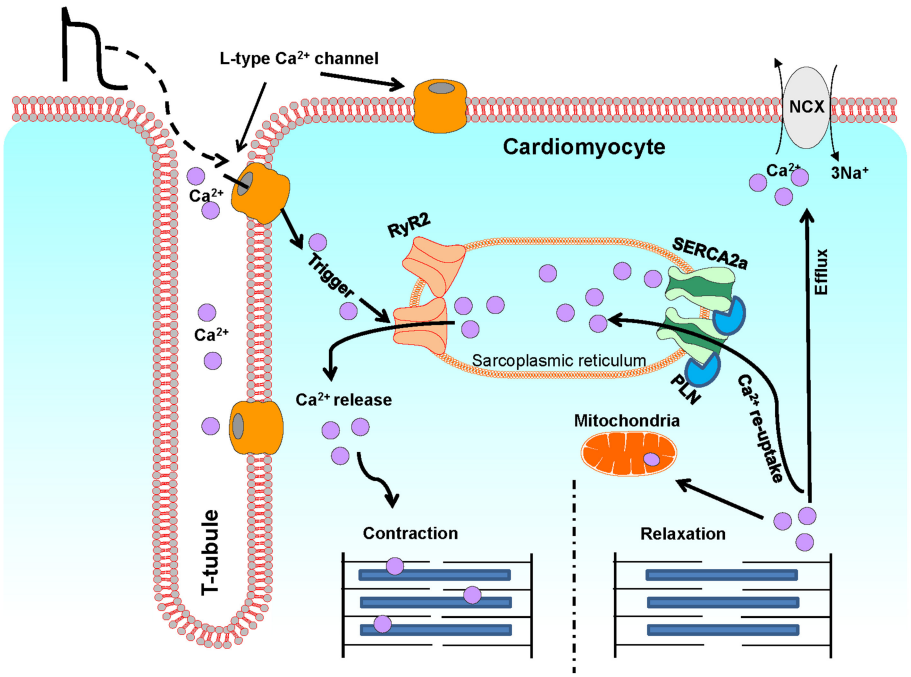

FIGURE 2 | A schematic illustration of Ca^{2+} fluxes during excitation-contraction in ventricular cardiomyocytes. This diagram depicts the most representative protein complexes and intercellular organelles involved in the cardiac excitation-contraction coupling. Ca^{2+}, calcium; SR, sarcoplasmic reticulum; M, Mitochondria; LTCC, L-type Ca^{2+} channel; RyR2, Ryanodine receptor 2; SERCA2a, Sarcoplasmic reticulum Ca^{2+}ATPase 2a; PLN, Phospholamban; NCX, Na+/Ca^{2+} exchanger.

than females (138–142). In studies, using paced cardiomyocytes at the rates of 0.5–1.0 Hz, cardiac relaxation rate was slower in cardiomyocytes from female rats compared to aged matched males (139, 143).

The expression and function of cardiac L-type Ca^{2+} channels, which have a direct impact on the functional changes in EC coupling pathway in the heart, also show significant sexual dimorphisms. In adult cardiomyocytes, the $Ca_v\alpha1C$ or $Ca_v1.2$ (cardiac voltage-gated L-type Ca^{2+} channel) is the most abundant cardiac L-type Ca^{2+} channel which triggering cardiac contraction by regulation of $I_{Ca,L}$ in cardiomyocytes (144–146). Therefore, it represents an important cellular site from which sex-based differences in myocardial intracellular Ca^{2+} handling and contractility may arise (138). Studies comparing the cardiac L-type Ca^{2+} Channel expression and $I_{Ca,L}$ that have included both female and male animals, are still limited and the existing data are controversial. It has been demonstrated that the levels of L-type Ca^{2+} channel expression increase or do not change at all in the ventricle of female rats and rabbits in comparison to males (147–149). Similarly, comparative studies using isolated cardiomyocytes from female and male rats, mice, guinea pigs, and dogs showed that compared to males, the $I_{Ca,L}$ density is either higher (147, 150–152) or lower in cells from females (153) or that there are no sex differences in $I_{Ca,L}$ density at all (137, 140, 141, 154, 155). Even with these discrepancies in the data, which might be due to variations in the experimental protocols, species, and used strains, sex differences in the regulation and expression of L-type Ca^{2+} channels are apparent, although the underlying signaling mechanisms implicated in these sex differences are poorly understood.

In recent years, several studies provided evidence that the distal part of the C-terminus of the α1C subunit (α1C-dCT) of $Ca_v1.2$ channel is proteolytically cleaved and shuttles between the plasma membrane and the nucleus of cardiomyocytes. It serves at the plasma membrane as an auto-inhibitor of $Ca_v1.2$ channel activity (156–159), and acts as transcription factor in the nucleus, regulating the expression of different genes, including $Ca_v1.2$ gene (CACNA1C) itself (160–163). Schroder et al. have provided evidence that the nuclear import of α1C-dCT in cardiomyocytes depresses $Ca_v1.2$ transcription, while nuclear export of α1C-dCT increases $Ca_v1.2$ channel activity consistent with a reduction of subsequent increase of $Ca_v1.2$ gene transcription rates (161). In a recent study, we observed a remarkable sex-disparity in nuclear shuttling of α1C-dCT in mouse cardiomyocytes (164). Here, the nuclear shuttling was significantly higher in isolated female cardiomyocytes compared to males. Furthermore, we found a significant decrease in nuclear shuttling of α1C-dCT in both female and male cardiomyocytes upon serum withdrawal. However, subsequent E2-treatment normalized the intracellular distribution of α1C-dCT only in male cardiomyocytes. This effect of E2 was reversed by the ER-antagonist ICI 182,780, indicating the involvement of ER in this signaling pathway. These findings provide a possible explanation for the cellular mechanisms responsible for sex differences in the regulation of L-type Ca^{2+} channel in the heart, revealing the role of E2/ER in this process.

In addition to the L-type calcium channel, sexual dimorphisms in the expression, and activity of other cardiac calcium channels have also been reported. For example, several studies found that the expression and/or current of NCX (I_{NCX}) are significantly higher in cardiomyocytes from female humans, rats, and rabbits compared to their male counterparts (135, 147–149, 165). Interestingly, Chen et al. showed that E2 administration increased NCX and I_{NCX} in female but not in male cardiomyocytes. These E2 effects appear to be mediated by a genomic mechanism involving the binding of E2 to its receptors, since these E2 effects were blunted by an ER antagonist (ICI 182,780) (165).

On the other hand, several studies have reported contradictory results on sex differences in the regulation of RyR2 expression and activity in the heart. It has been shown that the expression of RyR2 is higher in female rat cardiomyocytes compared to males (148, 149, 166), or that the expression does not differ in male and female rat and mice cardiomyocytes (155, 167). Bell et al. showed, however, that the regulation of RyR2 activity is different in male and female rat cardiomyocytes, with CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II)-mediated phosphorylation of RyR2 being lower in female cardiomyocytes than in male cardiomyocytes (167). This could be a possible explanation for the observed decrease in the gain of EC coupling (measured as SR Ca^{2+} release/Ca^{2+} current) in female rat and mice cardiomyocytes, which results from decreased size and duration of Ca^{2+} sparks by RyR2 (140, 155).

Collectively these findings suggest that the observed sex differences reflect, at least partly, the effects of E2 on myocardial Ca^{2+} handling, thus on contractility.

In this regard, studies with OVX rodents corroborate the effects of E2 on myocardial Ca^{2+} handling and contractility. Numerous studies with whole hearts or isolated cardiomyocytes from OVX mice, rats, rabbits, and pigs revealed that the E2 deficiency caused detrimental effects on both Ca^{2+} regulation and contractility of cardiomyocytes, such as enhanced Ca^{2}+ transients, increased Ca^{2}+ spark amplitudes, decreased myofilament Ca^{2+} sensitivity, and elevated contractions, in comparison to sham-operated controls (168–179). Remarkably, substitution of E2 effectively prevented the observed adverse effects (168, 169, 172, 174–179) and it could be shown that this is directly mediated via the ER by using the ER-antagonist ICI 182,780 (169).

In this context, several studies suggested that observed E2 effects are mediated by its receptors. Indeed, hearts of male ERKO-mice exhibit increased cardiac L-type Ca^{2+} channel expression and $I_{Ca,L}$ (180), as well as significantly higher Ca^{2+} accumulation compared to control hearts during I/R (106). In line with these data, a recent study demonstrated that both E2 pre-treatment and/or ERα activation of Tet-on/ERα H9c2 cardiomyoblast cells inhibited isoproterenol-induced cytosolic Ca^{2+} accumulation in these cells, and this protective effect of the E2/ERα was reversed by treatment with a specific inhibitor of ERα (181). These data indicate that E2/ERα signaling pathway is involved in the regulation of Ca^{2+} balance in cardiomyocytes, thereby preventing the harmful effects of Ca^{2+} overload in the pathophysiology of the heart. By contrast, another study using ERKO- and BERKO-mice could not show that the inhibition of $I_{Ca,L}$ and decrease in contraction depend on ERα or ERβ action (182). Moreover, it has been shown that in global GPER-KO mice, both left-ventricular contractility, and relaxation capacity were impaired only in males (122).

Furthermore, other studies have confirmed that the specific activation of different ER-isoforms affects cardiac contractility. Pelzer et al. showed that activation of ERα with the subtype-selective ERα agonist 16α-LE2 augments myocardial contractility to a measurable extent in OVX spontaneously hypertensive rats (183). Kulpa et al. showed that activation of ERα using the ERα agonist PPT (4,4',4"-(4-Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol) depressed actomyosin MgATPase activity and decreased myofilament Ca^{2+} sensitivity (184). Other studies have demonstrated the respective roles of ERβ and GPER activation in the regulation of SR Ca^{2+} handling proteins, such as SERCA2a and PLN, leading to improved contractility at the whole heart and single myocyte (66, 185).

These findings reveal that a solid understanding the roles of the various estrogen receptors in the regulation of cardiac contractility are needed in order to be able to find appropriate pharmacological agents that specifically target the receptors of interest.

THE ROLE OF 17β-ESTRADIOL AND ESTROGEN RECEPTORS IN CARDIAC MITOCHONDRIAL FUNCTION

Mitochondria are the main source of ATP and Reactive Oxygen Species (ROS) in the heart (186). It is considered that mitochondria play an essential role not only in regulation of cardiac contractility by providing ATP and by participating in Ca^{2+} homeostasis, but also they regulate cell death or apoptosis by ROS formation. Therefore, defects in mitochondrial structure and function are highly associated with CVDs (5, 186). E2 plays an important role in the supporting mitochondrial respiration, ATP production, and reducing ROS formation (**Figure 1VI**).

Sex differences in mitochondrial structure and function have been described. There is plenty of evidence that mitochondrial morphology and function differ between females and males in several organs and cell types. In the healthy mice hearts, although the female and male hearts displayed similar mitochondrial numbers, the proportion of large mitochondria ($\geq 1\ \mu m^2$) was significantly higher in female mice compared to males (56). Skeletal muscles from female rats show higher mitochondrial DNA and protein contents, as well as higher capacity of oxidative phosphorylation (OXPHOS) compared to male rats (187). Further, mitochondria in brain and liver from female mice exhibit higher antioxidant gene expression and lower oxidative damage under stress than in male animals (188). Additionally, several studies reported that the rate of ROS production is less in mitochondria from skeletal and cardiac muscle in female compared with aged matched male rats, particularly under stress conditions (187, 189, 190). Moreover, female rat hearts show altered posttranslational modification of several mitochondrial proteins under I/R in comparison to male hearts, including

aldehyde dehydrogenase-2 (ALDH2) (189), a protein that has been reported to be involved in cardioprotective processes (191). Whole genome expression profiling performed in hearts of old (78-week) male and female Fischer 344 rats showed that a majority of genes involved in oxidative phosphorylation had higher expression in females compared to male rats (192). These studies suggest that E2 plays a role in the regulation of mitochondrial function, which is supported by evidence from several studies in OVX animals.

In particular, a high throughput quantitative proteomic approach with isolated mitochondria from left ventricles of OVX rat relative to ovary-intact hearts revealed that about 50% of the identified proteins altered in OVX rat cardiac mitochondria are involved in mitochondrial ATP production (193). Indeed, the observed reduction of protein subunits of the electron transport chain complex I (NADH dehydrogenase), II (succinate dehydrogenase), III (cytochrome bc1 complex), IV (cytochrome c oxidase), and V (F0F1 ATP-synthase) in E2-deficint hearts was associated with reduced ATP production that may contribute to increased I/R injury and disease risk with E2 deficiency in aged female rats. Interestingly, in a mouse model of a human hypertrophic cardiomyopathy (cTnT-Q92), E2-supplementation of OVX mice significantly elevated myocardial ATP levels and mitochondrial respiratory function compared to untreated OVX mice, thereby improving diastolic heart function (194). In another model of cardiomyopathy, hearts from OVX rats showed higher Ca^{2+} accumulation in their mitochondria, lower mitochondrial respiratory function, severely structurally damaged mitochondria, and increased myocardial cell death after I/R injury in comparison to intact animals (195). Again, in this study, E2-treatment of the hearts from OVX animals attenuated cardiac damage by I/R, and thereby maintained the LV function. Furthermore, mitochondria from hearts of OVX rats showed higher expression of apoptotic markers compared to mitochondria of intact animals (196). However, chronic E2-treatment of these animals significantly attenuated mitochondria-dependent apoptotic pathways. These data directly show that alterations in mitochondrial function are a highly selective myocardial response to E2 deficiency, and that E2-mediated cardioprotection at the level of the mitochondria leads to improved cardiac function.

Indeed, several studies demonstrated that E2 through its ERs affects the cardiac mitochondria directly via regulation of mitochondrial gene/protein expression. It has been shown that ERα and ERβ are localized in the mitochondria of cardiac cells (62, 197–199). The presence of ERs in the mitochondria of cardiac cells suggests that they mediate the observed protective effects of E2, at least partly, by regulating mitochondrial structure and function in the heart. In line with the role of ERα and ERβ as transcription factors, distinct evidence supports the notion that mitochondrial DNA (mtDNA) could be one of the major targets for E2 acting via ER in cardiac cells. This is supported, for example, (1) by the presence of putative ERE on the mtDNA (200–202), (2) the E2-induced up-regulation of several mitochondrial-encoded genes, such as COXI and COXII (cytochrome c oxidase subunits I and II) (203, 204), and (3) the E2-induced expression of several nuclear-encoded mitochondrial genes, such as NRF-1 (nuclear respiratory factor 1), NRF-2 (nuclear respiratory factor 2), TFAM (mitochondrial transcription factor), PGC-1α (peroxisome proliferator-activated receptor gamma co-activator-1 alpha), and MEF2a (Myocyte enhancer factor 2A) (56, 202, 205, 206), whose proteins translocate into the mitochondria and thereby influence mitochondrial function. Additionally, it could be shown that in rat myocardium after severe hemorrhage the E2-induced increased expression of these genes was associated with an increase in COX IV (cytochrome c oxidase subunit IV), mtDNA-encoded COX I (cytochrome c oxidase subunit I), ATP synthase β-subunit, and mitochondrial ATP (207, 208). All these effects were abolished with the ER antagonist ICI 182,780, indicating an ER-specific effect.

The role of E2 and ER in the regulation of mitochondrial structure and function is established from studies with ER deficient mouse models. Microarray analysis using ERKO- and BERKO-mice showed that E2/ERβ pathways mediate down-regulation of mRNAs for nuclear-encoded subunits in each of the major complexes of the electron transport chain, whereas ERα is essential for most of the E2-mediated increase in gene expression including electron transport chain proteins and proteins involved in the anti-oxidative stress response (209). In a mouse model of exercise-induced physiological myocardial hypotrophy, we demonstrated that only female WT-mice showed an increase in the expression of key regulators of mitochondrial function e.g., NRF-1,−2, Mef2a, Atp5k (subunit E of mitochondrial F1F0-ATP synthase), and electron transport chain proteins (complexes I, III, and V) after running. Interestingly, ERβ deletion abolished the observed effects (56). Additionally, our study also showed that the activated ERβ significantly increased the expression of MEF2A, NRF-1, and−2 genes in a cardiomyocyte cell line (AC16 cells) (56). In line with these data, the expression of NRF-1 is diminished in BERKO hearts (209). On the other hand, Zhai et al. demonstrated that ERKO-mice hearts showed marked mitochondrial damages (fragmented and swollen mitochondria) and severe impairment of mitochondrial respiratory function compared to control hearts after I/R (106). To our knowledge a direct localization of GPER within the mitochondria has not been documented so far. However, analysis of DNA microarray data followed by Gene Set Enrichment Analysis (GSEA) from female and male cardiomyocytes of WT- and csGPER-KO-mice revealed that mitochondrial genes are enriched only in csGPER-KO females (124, 125), which provided direct evidence that the cardioprotective effects of GPER under physiological and pathological conditions in the female csGPER-KO-mice may be related to enhancements in mitochondrial function.

Several studies demonstrated that E2 also indirectly affects the cardiac mitochondria via regulation of ROS production. Elevated Ca^{2+} uptake by mitochondria results in the opening of the mitochondrial permeability transition pore (mPTP) and enhanced release of cytochrome c accompanied by dramatic increase in ROS formation, which leads to cell death via the induction of apoptosis pathways (210, 211). It has been shown that in comparison to male, mitochondria from female rat hearts accumulate Ca^{2+} more slowly (212), which might represent a mechanism that may underlie, at least partly, sex-related

differences accounting for females to suffer less injury with I/R. Indeed, several studies demonstrated that E2 administration can acutely attenuate the Ca^{2+} accumulation in mitochondria, inhibit Ca^{2+}-induced opening of mPTP in isolated heart mitochondria, prevent Ca^{2+}-induced release of cytochrome c from mitochondria, and inhibit ischemia-induced apoptosis in perfused heart (213–215). Interestingly, Feng et al. demonstrated that post-ischemic E2 administration to both male and OVX-female rats preserved mitochondrial structural integrity, which was associated with an increased tolerance to Ca^{2+} overload or augmented mitochondrial Ca^{2+} retention capacity (216) which reflects an inhibition of the mPTP opening in both male and OVX-female animals.

Here again, using ER deficient mice could be shown that these E2 effects are mediated by ERs. Male ERKO hearts subjected to I/R showed an accumulated Ca^{2+} deposition in their mitochondria which led to severe mitochondrial damage (fragmented and swollen mitochondria) in cardiomyocytes, and consequently to the depletion of ATP production (106). Using ERKO-, BERKO-, and ERα and ERβ double knockout (DERKO)-mice, Luo et al. found that both ER subtypes are necessary for E2-mediated cardioprotection during I/R in female hearts. Thereby, E2 and ER upregulate mitochondrial p38β-MAPK activity, with subsequent phosphorylation of the MnSOD (manganese superoxide dismutase), leading to enhanced SOD activity, thereby minimizing mitochondrial-derived ROS production and reduction of myocardial infarct size post I/R (217). By contrast, a systematic analysis of WT-, ERKO-, BERKO-, and GPER-KO-mice subjected to I/R showed that only GPER expression is essential for the acute action of E2 in cardioprotection against I/R injury in male mouse via a cascade involving PKC translocation, ERK1/2/GSK-3β (Glykogensynthase-Kinase 3β)- phosphorylation leading to the inhibition of the mPTP opening, resulting in reduction of harmful mitochondrial ROS generation (80). However, a pre-administration with G15, a specific GPER antagonist, reversed this estrogenic effect. This data indicate that GPER activation mediates E2-induced increase in mitochondrial Ca^{2+} retention capacity, and the GPER-mediated cardioprotective effect of post-ischaemic E2 is related to a decrease in mPTP sensitivity to Ca^{2+} overload, a process which is mediated via activation of the MEK/ERK/GSK-3β axis.

These data suggest that depending on the time period of E2-treatment, sex, and species different ERs can be activated by E2, which mediate the mitochondrial-dependent cardioprotective effect of E2 against I/R injury.

CONCLUSION

In the past, most clinical and animal studies did not include both sexes or differentiate between sexes in the data analysis. This might be the possible reason that our understanding of the molecular and cell-based mechanisms underlying sex-based differences in cardiovascular system are still incomplete so far. A more thorough understanding of underlying sex-dimorphic mechanisms in cardiac health and disease is required to effectively treat patients with CVDs. The presented data in this review support the concept that sex specific regulation of cardiac Ca^{2+} ion channels and mitochondrial function by E2 and ERs could be, at least partly, responsible for differences in cardiovascular disease incidence and outcomes. However, further attempts toward a more detailed understanding of E2 and ERs roles in the heart are needed to develop new drugs that target the beneficial effects on CVD in both sexes.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Estrogen Contributions to Microvascular Dysfunction Evolving to Heart Failure With Preserved Ejection Fraction

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Heart failure with preserved ejection fraction (HFpEF) is a syndrome involving microvascular dysfunction. No treatment is available yet and as the HFpEF patient group is expanding due to the aging population, more knowledge on dysfunction of the cardiac microvasculature is required. Endothelial dysfunction, impaired angiogenesis, (perivascular) fibrosis and the pruning of capillaries (rarefaction) may all contribute to microvascular dysfunction in the heart and other organs, e.g., the kidneys. The HFpEF patient group consists mainly of post-menopausal women and female sex itself is a risk factor for this syndrome. This may point toward a role of estrogen depletion after menopause in the development of HFpEF. Estrogens favor the ratio of vasodilating over vasoconstricting factors, which results in an overall lower blood pressure in women than in men. Furthermore, estrogens improve angiogenic capacity and attenuate (perivascular) fibrosis formation. Therefore, we hypothesize that the drop of estrogen levels after menopause contributes to myocardial microvascular dysfunction and renders post-menopausal women more vulnerable for heart diseases that involve the microvasculature. This review provides a detailed summary of molecular targets of estrogen, which might guide future research and treatment options.

Keywords: heart failure with preserved ejection fraction, microvascular dysfunction, sex differences, estrogens, endothelial dysfunction, impaired angiogenesis, (perivascular) fibrosis, capillary rarefaction

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INTRODUCTION

Cardiovascular disease (CVD) is the most common cause of death worldwide with 17.9 million deaths annually[1]. As the world adopts more westernized lifestyles, the incidence of CVD risk factors, like obesity and diabetes, increases. There are known sex differences in prevalence, incidence, risk factors, and prognosis of different CVDs (1). When corrected for age, in Europe, CVD is responsible for 49% of the deaths among women and 41% among men, causing death in over 4 million people every year (2). The incidence of CVDs is higher in men before the age of 50 than in pre-menopausal women. However, after menopause, CVD incidence in women rises and eventually exceeds CVD incidence in men (3). Furthermore, men more often die of ischemic heart disease, while women more often die from stroke and heart failure (HF) (3).

[1]http://www.who.int/en/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds).

Epidemiology and Comorbidities for HFpEF

Although HF was originally associated with a reduced left ventricular (LV) ejection fraction (HFrEF), it now seems that nearly half of the HF patients (45–50%) have a preserved ejection fraction (HFpEF) (4). In both sexes, the incidence of HFpEF, and HFrEF increases with age. However, HFpEF incidence is higher in women at any given age, especially in post-menopausal women. In contrast, HFrEF incidence is higher in men at any given age (4–6).

HFrEF develops following an ischemic event involving the bigger vessels of the heart, e.g., a myocardial infarction (MI), that leads to the reduced contraction of the ventricles (7). Comorbidities, like diabetes, obesity, and smoking are seen in both HFrEF, and HFpEF, but there are evident sex differences in the degree of association of some comorbidities for HF and underlying CVDs; hypertension, atrial fibrillation, female sex, and age have a stronger correlation with HFpEF[1]. Comorbidities and diseases present in HFpEF in combination with higher age (after menopause) and estrogen levels affect several pathophysiologic pathways, which will be illustrated in this review.

HFpEF is characterized by left ventricular diastolic dysfunction (LVDD) (8), and develops gradually suggested to involve the microvasculature of the heart (6, 9). In contrast to post-menopausal women, pre-menopausal women seem to be protected from HFpEF and other CVDs, which points toward the influence of estrogens in the development of microvascular dysfunction. Insights into the molecular basis of diseases affecting the microvasculature of the heart are lacking, but given that the prevalence of HFpEF is increasing, this information is crucial (4). Several pathological processes like endothelial dysfunction, impaired angiogenesis, (perivascular) fibrosis, and blood vessel rarefaction are estrogen-mediated and contribute to microvascular dysfunction. Expanding our knowledge on the role of estrogens on these pathophysiological processes may guide new treatment options for cardiac syndromes like HFpEF.

Abbreviations: AC, adenylate cyclase; ACS, acute coronary syndromes; AMPK, AMP activated protein kinase; Ang II, angiotensin-II; ANGPT, angiopoietin; AR, androgen receptor; ARE, androgen responsive element; AS, aortic stenosis; ATR, angiotensin-II receptor; BH_4, tetrahydrobioptin; CAD, coronary artery disease; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CHD, coronary heart disease; Co-R, co-receptor; COX, cyclooxogenase; CVD, cardiovascular disease; E2, 17β-estradiol; ECM, extracellular matrix; EDHF, endothelial-derived hyperpolarizing factor; EDV, end diastolic volume; eNOS, endothelial nitric oxide synthase; ER, estrogen receptor; ERE, estrogen response element; ET, endothelin-1 receptor; ET-1, endothelin-1; FGF, fibroblast growth factor; GPR30, G protein-coupled estrogen receptor 30; HF, heart failure; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; HT, hormone replacement therapy; I/R, ischemia/reperfusion; LVDD, left ventricular diastolic dysfunction; MAPK, mitogen-activated protein kinase; MI, myocardial infarction; NO, nitric oxide; NRF, nuclear respiratory factor; PDGF, platelet-derived growth factor; PGI_2, protacyclin; PI3K, phosphoinositide 3-kinase; PKG, protein kinase G; PPAR, peroxisome proliferator-activated receptor; RAAS, renin-angiotensin-aldosterone-system; RAMP3, receptor activity modifying protein 3; ROS, reactive oxygen species; SCAD, spontaneous coronary artery dissection; sGC, soluble guanylyl cyclase; SIRT, Sirtuin; TGF, transforming growth factor; TNF-α, tumor necrosis factor-α; TNFR, tumor necrosis factor receptor; TSP-1, thrombospondin-1; TXA_2, thromboxane A_2; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VSM, vascular smooth muscle.

This review will provide an in-depth summary of the impact of estrogens on pathophysiological processes in which the cardiac microvasculature is involved.

The Vascular Tone

The most important component of vascular function is the regulation of the vascular tone, which is coordinated by the ability of vascular smooth muscle (VSM) cells to react to changes in blood flow. The vascular tone is strictly regulated by vasoconstricting and vasodilating factors that can be systemically and locally derived and is coordinated by Ca^{2+} influx into the cells. If the vascular tone is chronically increased, hypertension develops, and as a consequence this contributes to the development of CVDs and cardiac remodeling (10, 11). Vascular tone can be regulated by the nervous system, which stimulates vasoconstriction through α-adrenergic receptors and vasodilatation through β-adrenergic receptors.

In the heart, the vascular tone is mostly controlled by energy demands of the myocardium, which are sensed in the microvasculature. Hypoxia activates the systemic renin-angiotensin (Ang II)-aldosterone-system (RAAS), in which Ang II induces VSM constriction. Sustained RAAS activation can lead to extensive collagen deposition (fibrosis) and stiffening of blood vessels, which culminates into an increased vascular tone (12). The vascular endothelium appears to be a key player in the regulation of the vascular tone: (sex) hormones and shear force trigger the endothelium to secrete factors that influence contractility of VSM cells (13).

Endothelial Dysfunction and Dysregulation of the Vascular Tone

HFpEF patients often present with dysregulation of the vascular tone involving a disturbed balance of secretion of vasoconstricting and -dilating factors by the endothelium of the microvasculature. Vasodilation almost always involves NO produced from L-arginine by three NO synthases of which endothelial NO synthase (eNOS) is the most important for vascular tone regulation (11). NO elevates cyclic GMP (cGMP) concentrations in VSM cells by stimulation of soluble guanylyl cyclase (sGC) activity. Elevated cGMP levels activate protein kinase G (PKG), which diminishes Ca^{2+} levels in the cell through multiple mechanisms, and consequently vasorelaxation is induced (14). Reduced availability and/or responsiveness to NO hinders the vasculature to accustom to constriction/dilatation in response to blood flow changes (15).

Vasodilating pathways can be modulated by estrogens, as depicted in **Figure 1**. In detail, 17β-estradiol (E2) can positively regulate eNOS activity and thereby NO production by binding to the estrogen receptors (ER)-α and -β that are both present in endothelial cells (represented in the mid-section of **Figure 1**) (16). Especially endothelial ER-α has cardioprotective properties. Although estrogen receptors are mostly present in the nucleus, where they act as transcription factors, a small pool of ERs has been reported to lay in the vicinity of the plasma membrane, where they stimulate eNOS activity involving protein kinases PI3K and Akt (right section of **Figure 1**) (17). ER-α activation prevents fatty streak formation by decreasing

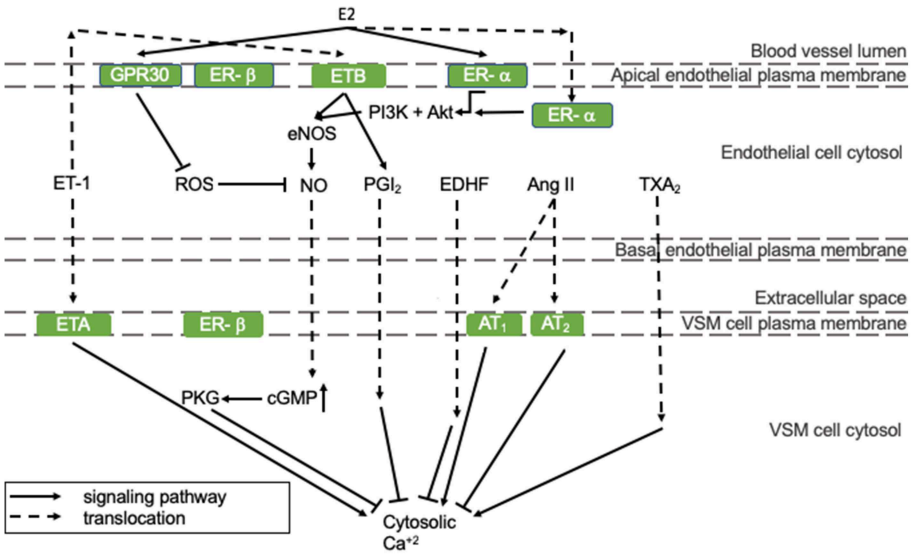

FIGURE 1 | The regulation of endothelial function. All presented pathways affect the cytosolic Ca^{2+} concentration in VSM cells by regulating endothelial function, which determines constriction/relaxation. Solid arrows indicate stimulation and lines with a flattened end indicate inhibition. Dashed lines indicate translocation of the molecule. Green squares are receptors. Ang II, angiotensin-II; AT, angiotensin-II receptor; cGMP, cyclic guanosine monophosphate; GPR30, G-protein coupled estrogen receptor 30; E2, 17β-estradiol; EDHF, endothelial-derived hyperpolarizing factor; eNOS, endothelial nitric oxide synthase; ER, estrogen receptor; ET, endothelin-1 receptor; ET-1, endothelin-1; MAPK/ERK, mitogen-associated protein kinase/extracellular signal-regulated kinases; NO, nitric oxide; PGI_2, prostacyclin; PI3K, phosphoinositide 3-kinase; PKG, protein kinase G; ROS, reactive oxygen species; TXA_2, thromboxane A_2.

lipoprotein deposition acting on local inflammation and immune regulation of the early atherosclerotic plaque. This effect is achieved independently of eNOS (18). As the HFpEF phenotype is associated with a proinflammatory state (19), this supports our hypothesis that E2 prevents HFpEF in pre-menopausal women. Alongside endothelial expression, ER-α and -β are also present in VSM cells, where they inhibit VSM cell proliferation in female, but not in male rats, through p38/MAPK signaling (20). In VSM cells, the expression of ER-α, and -β does not differ significantly between male and female rats (21). However, ER-β is up-regulated more in women following pressure overload, and this has been correlated with an inhibition of Ang II-induced hypertrophy in female mice (22, 23). In addition, it protects against reperfusion-induced arrhythmias and inflammation following ischemia in rodents (24).

Other vasodilating factors are prostacyclin (PGI_2), endothelial-derived hyperpolarizing factor (EDHF), and adenosine. E2 stimulates PGI2 production through ER-α and cyclooxygenase (COX)-1 signaling (25). EDHF can compensate for NO loss under pathological conditions and some studies indicate that EDHF-mediated vasodilatation is more prevalent in pre-menopausal women than in post-menopausal women and men, which is reviewed by Villar et al. (26). This might protect pre-menopausal women from impaired vasodilatation and the development of HFpEF. Adenosine receptor expression has been shown to be altered upon E2 administration in an *in vitro* model. However, the implication of this observation in vascular function has not been investigated yet (27).

Vasoconstricting factors are endothelin-1 (ET-1), Ang II, thromboxane A_2 (TXA_2), and reactive oxygen species (ROS) (11). The influence of E2 on Ang II generation and has not been reported. However, in a I/R mouse model, E2 influences the ratio of angiotensin II receptor (ANGTR)1/ANGTR2 in favor of the first, which was associated with protection against I/R injury (28). E2 does not influence TXA_2 production in an *in vitro* model (25). Although the effects of E2 on ET-1 expression and activity have not been reported, the expression pattern of ET-1 receptors in men renders them more vulnerable for ET-1 induced vasoconstriction compared to women (29).

ROS, such as superoxide, hydroxyl radical, lipid peroxyl radical, and alkoxyl radicals increase the influence of inflammation, diabetes, obesity, and age on endothelial function and thereby NO bioavailability (11). ROS levels are lower in women than men due to differences in phosphorylation patterns of mitochondrial proteins, e.g., aldehyde dehydrogenase (ALDH)2. In an ischemia/reperfusion (I/R) rat model, it was found that ALDH2, an enzyme that detoxifies ROS-generated aldehyde products, had an increased phosphorylation and activity in females compared to males. This was associated with less I/R injury in female mice (30).

E2 can effect ROS levels: E2 decreases oxidative stress by upregulating mitochondrial enzyme, e.g., manganese superoxide dismutase, levels and activity (31). Moreover, ROS formation can be inhibited by E2-G protein-coupled estrogen receptor 30 (GPR30) signaling, which is illustrated by the observation that GRP30 deficiency results in oxidative stress in ovariectomized rats (32). Furthermore, GPR30 signaling seems to be more prevalent in females than males, protecting females from oxidative stress (represented in the left section of **Figure 1**) (33). In a HFpEF mouse model, the GPR30 agonist G1 was able

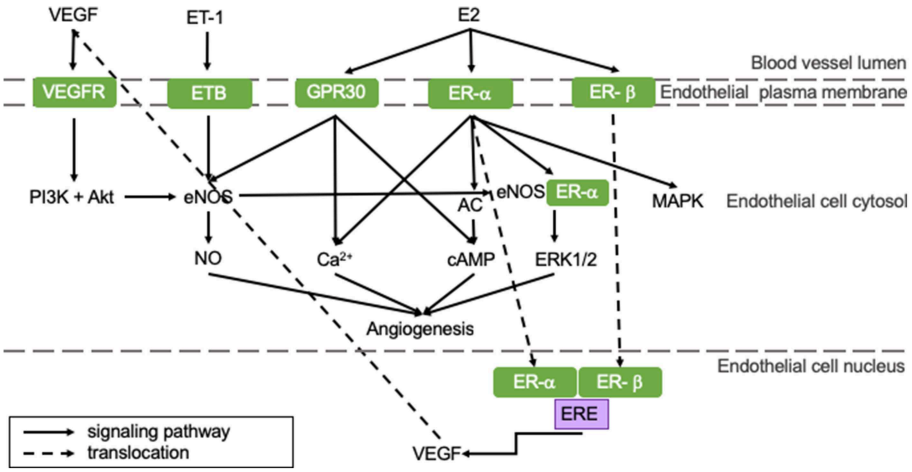

FIGURE 2 | E2 positively influences angiogenic pathways resulting in an increased angiogenic capacity of pre-menopausal women potentially protecting them from HFpEF. All represented pathways positively affect angiogenesis in the heart. Estrogens influence pro-angiogenetic processes, rendering women less susceptible for impaired angiogenesis. This potentially protects pre-menopausal women from HFpEF. AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; E2, 17β-estradiol; eNOS, endothelial nitric oxide synthase; ER, estrogen receptor; ERE, estrogen responsive element; ET, endothelin-1 receptor; ET-1, endothelin-1; GPR30, G protein-coupled estrogen receptor 30; MAPK, mitogen-activated protein kinase; NO, nitric oxide; PI3K, phosphoinositide 3-kinase; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

to abolish abnormal cardiac structure, fibrosis formation, and LVDD (34). This highlights the importance of GPR30 signaling in the pathophysiology of HFpEF. Furthermore, G1 might provide an interesting therapy option for HFpEF patients.

IMPAIRED ANGIOGENESIS

Angiogenesis is the process in which blood vessels sprout from pre-existing capillaries to meet the increased oxygen demands of developing or damaged tissue. It is essential for physiological processes, like growth, responses to sustained exercise, the estrous cycle, wound healing and aging, as well as for recovery from pathological processes like hypertrophy and ischemia (35). Angiogenesis is induced by a number of myocardium-derived factors, of which vascular endothelial growth factor (VEGF) is the most prominent. Other regulators include angiopoietin-1 and−2 (ANGPT-1 and−2), fibroblast growth factor (FGF), transforming growth factor (TGF), and platelet-derived growth factor (PDGF). These factors regulate angiogenesis through various signaling pathways on genomic and non-genomic levels and their role in myocardial angiogenesis has been extensively reviewed in the literature (36–39). The most important pathways that are influenced by E2 are shown in **Figure 2**.

E2 can stimulate angiogenesis: upon E2 binding, ER-α and -β translocate to the nucleus and bind the estrogen response element (ERE) of the *VEGF* gene, thereby upregulating transcription of VEGF and stimulation of angiogenesis (represented in the right section of **Figure 2**) (40). VEGF stimulates eNOS, which in turn activates tyrosine and PI3 kinases (left section of **Figure 2**). This regulates NO production and release by endothelial cells (41). Especially ER-α activation stimulates angiogenesis through both genomic and non-genomic processes (42). Non-genomic processes involve rapid changes in activities of adenylate cyclase (AC), mitogen-activated protein kinase (MAPK), PI3K and eNOS or in concentrations of cytoplasmic Ca^{2+}. GPR30-activation can induce angiogenesis via non-genomic processes, like Ca^{2+} influx, cyclic adenosine monophosphate (cAMP) synthesis or PI3K activation, which is presented in the right section of **Figure 2** (43). Furthermore, ER-α and eNOS assembly initiates ERK1/2 signaling, thereby promoting reendothelialization (44).

Pre-menopausal women have higher baseline levels of E2 compared to men and post-menopausal women. Therefore, they may have a better angiogenic capacity following MI (42). Furthermore, hypoxia can induce angiogenesis involving sirtuins (SIRT), proteins responsible for maintaining mitochondrial function and cellular metabolism. They have been shown to be key regulators in the coupling of hypoxia-induced angiogenesis and its levels decrease with age (45). SIRT3 transcription can be increased by E2-ER-β signaling in an *in vitro* model, thereby promoting hypoxia-induced angiogenesis (46). The importance of SIRT was indicated in a sirtuin-deleted mouse model, in which angiogenesis was impaired. This led to the development of LVDD (47). Taken all this together, we postulate that the reduced estrogen levels in post-menopausal women contribute to an impaired hypoxia-angiogenesis coupling evolving to LVDD and the development of HFpEF.

(PERIVASCULAR) FIBROSIS FORMATION

Perivascular fibrosis is the formation of fibrosis around blood vessels. While replacement fibrosis is more prevalent in HFrEF, HFpEF is associated with perivascular fibrosis formation in the microvasculature independent of epicardial stiffening (48–50).

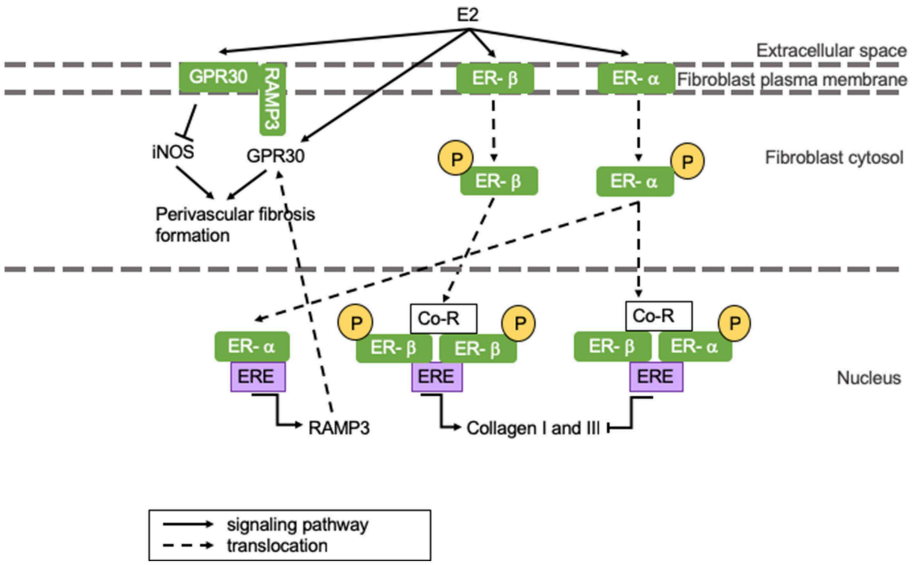

FIGURE 3 | The influence of E2 on (perivascular) fibrosis formation. (Perivascular) fibrosis formation is attenuated in women through E2-induced inhibition of collagen production. However, E2 receptor dimerization promotes fibrosis formation in men. GPR30 localization in men results in perivascular fibrosis formation, while GPR30 localization at the plasma membrane inhibits fibrosis formation in women. Yellow circles indicated with a "P" represent phosphorylated serine residues. ER-α is phosphorylated at Ser-118, while ER-β is phosphorylated at Ser108. Ang II, angiotensin II; AR, androgen receptor; ARE, androgen responsive element; ATR, angiotensin II receptor; Co-R, co-receptor; E2, 17β-estradiol; ER, estrogen receptor; ERE, ααestrogen responsive element; GPR30, G protein coupled receptor 30; iNOS, inducible nitric oxide synthase; RAMP3, receptor activity modifying protein 3; TGF-β, transforming growth factor-β.

Estrogens can influence fibrosis formation through several signaling pathways. A recent study shows that rat cardiac fibroblast ER-α activation by E2 leads to inhibition of collagen I and III production in females, while E2 binding to ER-β promotes collagen production in males (51). However, another study shows that increased levels of ER-β after MI protect from inflammation and fibrosis formation in female mice (52). These studies do not have to be conflictive seeing that ER-α and -β dimerization inhibits collagen deposition, and an increased ER-β concentration could amplify this (51). Interestingly, androgens can influence cardiac fibrosis formation by upregulation of TGF-β, which is known to induce extracellular matrix (ECM) deposition predisposing men to cardiac fibrosis (53). Sex differences in cardiac structural remodeling and fibrosis have been extensively reviewed and are beyond the scope of this review.

Perivascular fibrosis and cardiac hypertrophy can be reduced by E2-induced GPR30 activation, which results in the suppression of inducible NOS (iNOS) activity (represented in the left section of **Figure 3**) (34). iNOS activity is minimal under physiological conditions, but it is activated during infections and chronic inflammation, where it continuously produces NO (11). iNOS impairs vasoconstriction by activating sGC, but simultaneously reduces vasodilatation by limiting tetrahydrobiopterin (BH_4) availability for eNOS, thus inducing vascular dysfunction (54). When GPR30 is located at the plasma membrane, it appears to have cardioprotective effects, whilst GPR30 located at the cytosol is associated with perivascular fibrosis formation (55). GPR30 is held at the plasma membrane by receptor activity modifying protein 3 (RAMP3) (55). RAMP3 expression is regulated by E2, and as a consequence, women present with more GPR30 located at the plasma membrane than men (represented in the left section of **Figure 3**) (56). This is represented in **Figure 3**. We postulate that the drop of estrogen levels after menopause induces perivascular fibrosis formation involving above-mentioned mechanisms leading to HFpEF.

CAPILLARY RAREFACTION

Rarefaction is the decrease of capillary density, causing hypoxia in mice and rats (57). It is associated with hypertension (58, 59), hypertrophy (60), diabetes (61), and aging in multiple tissues in both men and women (62). Coronary microvascular rarefaction was shown to be a prominent cause of HFpEF in male and female patients (63, 64). However, mechanisms underlying coronary rarefaction are not fully understood, but are likely comparable to those in other circulatory organs, such as the kidneys. Inflammation, dysregulation of angiogenic molecules, and pericyte loss are phenomena underlying kidney rarefaction. E2 influences some of these pathologic processes and we postulate that these mechanisms could also play a role in coronary rarefaction contributing hypoxia in HFpEF, as will be described in the following section. However, capillary rarefaction may also be the result of cardiomyocyte death leading to a decreased oxygen need.

Lessons From Renal Rarefaction

Upon renal transplantation, capillary dilatation and rarefaction are strongly correlated with intracapillary inflammation (65). It is known that TNF-α mediated inflammation affects vascular

endothelial cells, which can result in apoptosis *in vitro* (66). Endothelial apoptosis leads to pruning of blood vessels in humans (67). TNF-α is a cytokine mediating the inflammatory response and promoting apoptosis by inhibition of Akt-mediated cell survival (68). E2 can inhibit TNF-α induced apoptosis by binding to its ER-β-receptor, which induces Akt phosphorylation and Notch1 expression, thereby promoting cell survival (66). Furthermore, women show lower LV expression of pro-inflammatory genes during pressure-overload than men (69). Consequently, pre-menopausal women might be protected from inflammation-induced apoptosis of vascular endothelial cells and resulting rarefaction.

Shear force is required for endothelial cell survival, through Akt phosphorylation (70). Furthermore, shear force stimulates the production of pro-angiogenic factors, such as NO and VEGF. When shear force is diminished, cell survival and pro-angiogenic signals are reduced and this results in vessel pruning (71). The role of VEGF dysregulation in kidney rarefaction is extensively reviewed (57). Whether this plays a role in coronary vascular endothelial cell apoptosis in a reaction to changes in shear force still has to be determined.

Role of Pericytes

Pericytes are perivascular cells that stabilize the vascular wall and maintain vascular quiescence and integrity. During kidney and lung fibrosis, TGF-β stimulates pericytes to detach from the vascular wall and differentiate into myofibroblasts, contributing to myocardial fibrosis (72, 73). The loss of pericytes in the perivascular region leads to destabilization of capillaries, capillary dysfunction and, ultimately, rarefaction. Although the role of pericytes in lung and kidney fibrosis has been established, the importance of pericytes in ventricular remodeling during HFpEF has not been fully established. There are implications that pericytes could play a role in coronary capillary rarefaction. Pericyte recruitment is regulated by the ratio of ANGPT-1 vs.-2, molecules that are secreted by mesenchymal and endothelial cells (74). Both molecules can bind the endothelial Tie-2 receptor. Binding of ANGPT-1 attracts pericytes and stimulates vessel assembly and maturation, whereas ANGPT-2 impairs pericyte recruitment. The latter was shown to induce vessel rarefaction in a tumor mouse model (74). Thrombospondin-1 (TSP-1) can disturb the ANGPT-1/-2 ratio in favor of ANGPT-2, thereby inducing capillary rarefaction in a diabetic mouse model (61). The influence of E2 on TSP-1 levels and responsiveness or ANGPT-1 and-2 stimulation have not been reported so far.

DISCUSSION

As established in this review, sex differences in the molecular mechanisms of endothelial dysfunction, impairment of angiogenesis, (perivascular) fibrosis formation, and capillary rarefaction are abundant and render men more vulnerable for CVDs at younger age than pre-menopausal women. However, after menopause, E2 levels drop and testosterone levels increase in women, which is associated with an elevated risk for CVDs, coronary heart disease (CHD) and HF (3, 75, 76). High E2 levels in post-menopausal women are associated with a lower risk of CHD (77). Post-menopausal women outnumber men in a ratio of 2:1 in CVDs, in which the microvasculature is affected, such as in HFpEF (78). Besides the effects of E2 on pathophysiologic processes described in this review, E2 affects other systemic and cellular processes. First of all, it is postulated that, in reaction to low E2 levels after menopause, RAAS is activated, which increases ROS and decreases NO. This results in an increase of collagen synthesis and LVDD, the main characteristic of HFpEF (6). Moreover, the effects of E2 on mitochondrial metabolism in LVDD have been described in the literature. In short, E2 maintains mitochondrial biogenesis and function through several signaling pathways, involving e.g., peroxisome proliferator-activated receptor (PPAR)α, nuclear respiratory factor (NRF)-1 and AMP-activated protein kinase (AMPK). Loss of E2 is associated with abnormal mitochondrial function, oxidative stress, and LVDD (79). Therefore, we hypothesize that due to the drop of E2 levels after menopause, the protective effects of E2 on the microvasculature involving the above-mentioned mechanisms are strongly diminished, which results in the incidence of HFpEF in the two decades following menopause. Also, non-hormone related causes can be appointed: in an acute I/R four core genotype mouse model, where you can investigate the sex chromosome effect independently of the sex hormone effect, the presence of a second X chromosome made mice more vulnerable for I/R damage. It was suggested that incomplete inactivation of the second X chromosome may result in escaping genes that are constitutively higher expressed in mice with two X chromosomes. This suggests that a second X chromosome by itself has a detrimental effect on the vasculature and the heart (80). If and how sex chromosomes interplay with sex hormones and affect HF subtypes is yet to be determined. Together, these studies suggest that the development of HFpEF is an interplay between several sex-related processes.

Future Perspectives and Treatment Options

Interestingly, E2 level-restoring therapies, like hormone therapy (HT), show conflicting effects. The Women's Health Initiative (WHI) hormone therapy trial was a large trial designed to investigate the effects of HT in women between 50 and 79 years of age. Although HT was beneficial for the management of menopausal symptoms in healthy women, the use of HT for the prevention of chronic disease was not supported. The risks of HT outweighed the benefits in all age groups (81). In this trial only one dose was used, therefore the smaller Kronos Early Estrogen Prevention Study (KEEPS) and the Early vs. Late Interventional Trial with Estradiol (ELITE) study sought to investigate the effects of different doses and forms of estradiol at several time points around menopause in women without CVD at entry. Although the ELITE study showed that estradiol could slow atherosclerosis progression in post-menopausal women within 6 years after menopause, the KEEPS study did not show beneficial effects on the vasculature. HT, however, did not seem to be harmful for cardiovascular health either (81, 82). Up to now, there is no data on the effects of HT on the development or progression of LVDD or HFpEF and it remains to be investigated if long-term menopausal HT could reduce the development of HFpEF.

Therapies that improve the vascular function of the heart might be promising. Angiogenic therapies have been under investigation during the last 20 years. Although preclinical results are promising, clinical trial results are unambiguous (83). Angiogenic gene therapy seems to be more effective in post-menopausal women than in men and younger women. But overall, angiogenic therapy alone does not improve vascularity significantly in a clinical setting (84). Better definition of patient sub-groups, improvement of targeted delivery and combination with other therapies might improve angiogenic therapy response. The information on sex differences in angiogenesis provided in this review might assist the development of such regenerative therapies, e.g., involving pericytes.

Revascularization therapy using pericytes has shown to be effective in the attenuation of cardiac remodeling. Transplantation of pericytes into the ischemic myocardium improves the capillary density in the heart with 45% compared to non-pericyte injected hearts in mice (85). Pericytes accomplish this effect by upregulating VEGF, PDGF-β, and TGF-β. The beneficial effects of TGF-β are questionable since other studies report that TGF-β induces pericyte differentiation and fibrosis (72, 73). Pericyte transplantation might be effective in the attenuation of cardiac remodeling evolving to HFpEF, but before clinical translation to HFpEF patients can be made more research should be conducted into molecular effects of pericyte transplantation in a HFpEF setting. Furthermore, the long-term efficiency of pericyte transplantation might be limited due to cell longevity and repeated injections could hinder the clinical application. The use of pericytes in regenerative medicine is relatively new and more research should be conducted into phenotyping and function should be conducted.

Inhibition of vascular decay by chemically promoting pericyte localization at the vascular wall could provide an efficient therapy against capillary rarefaction. This could also avoid trans-differentiation of pericytes into myofibroblasts decreasing (peri) vascular fibrosis. Especially in diseases like HFpEF, in which the smallest vessels in the heart are susceptible for decay, vascular stabilization poses an interesting research option. Vascular decay might also be interesting in the search for biomarkers for microvascular dysfunction, which could help detect vascular remodeling preceding CVDs in an early stage with low patient burden. Post-menopausal women suffering from HFpEF are underdiagnosed as efficient microvascular imaging techniques are expensive and time-consuming. The use of a microvascular decay biomarker might help the early detection of HFpEF patients.

CONCLUSION

Microvascular dysfunction is a prominent contributor to HFpEF in men and post-menopausal women. Treatment options for these diseases have not been successful so far. There are differences between the sexes and between pre- and post-menopausal women that render pre-menopausal women less vulnerable for microvascular dysfunction and subsequent CVDs. Considering differences in prevalence of the processes mentioned in this review, we hypothesize that capillary rarefaction might be more prevalent in post-menopausal women than in pre-menopausal women and men, thereby rendering them more vulnerable for CVDs associated with microvascular decay, e.g., HFpEF. On a molecular level, suggested therapeutic targets, including GPR30 and ETA. GPR30 agonists and ETA antagonists are promising for improving microvascular function and preventing decay, which might attenuate HFpEF (34, 86).

AUTHOR CONTRIBUTIONS

AS and EK conceived and designed the manuscript. AS drafted the manuscript with support of EK, who supervised the work. SK and HdR were involved in revising the manuscript critically for important intellectual content. All authors gave final approval of the manuscript to be published and agreed to be accountable for all aspects of the work.

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REVIEW
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Estrogens, Estrogen Receptors Effects on Cardiac and Skeletal Muscle Mitochondria

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Mitochondria are unique organelles present in almost all cell types. They are involved not only in the supply of energy to the host cell, but also in multiple biochemical and biological processes like calcium homeostasis, production, and regulation of reactive oxygen species (ROS), pH control, or cell death. The importance of mitochondria in cell biology and pathology is increasingly recognized. Being maternally inherited, mitochondria exhibit a tissue-specificity, because most of the mitochondrial proteins are encoded by the nuclear genome. This renders them exquisitely well-adapted to the physiology of the host cell. It is thus not surprising that mitochondria show a sexual dimorphism and that they are also prone to the influence of sex chromosomes and sex hormones. Estrogens affect mitochondria through multiple processes involving membrane and nuclear estrogen receptors (ERs) as well as more direct effects. Moreover, estrogen receptors have been identified within mitochondria. The effects of estrogens on mitochondria comprise protein content and specific activity of mitochondrial proteins, phospholipid content of membranes, oxidant and anti-oxidant capacities, oxidative phosphorylation, and calcium retention capacities. Herein we will briefly review the life cycle and functions of mitochondria, the importance of estrogen receptors and the effects of estrogens on heart and skeletal muscle mitochondria.

Keywords: estrogens, estrogen receptors, mitochondria, heart, skeletal muscle, sexual dimorphism

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CARDIAC AND SKELETAL MUSCLE MITOCHONDRIA

Mitochondria

Origin and Life Cycle

Mitochondria are small intracellular organelles of approximately one micron in size that are present in granular or filamentary form and in variable number from one cell type to another. Mitochondria derive from aerobic α-proteobacteria, which were engulfed by anaerobic archeobacteria giving rise to primitive eukaryotic cells a few billion years ago. The host cell provides the necessary substrates for the mitochondria that metabolize these substrates and provide ATP to the host cell through oxidative phosphorylations; in turn, mitochondria attenuate intracellular concentration of oxygen, which is a potentially toxic substance for cellular constituents.

Due to their ancient bacterial origin, mitochondria are surrounded by two membranes, an outer membrane, and an inner membrane exhibiting a particular lipid composition (rich in cardiolipin) and numerous invaginations or ridges, delimiting two spaces: the intermembrane space and the matrix space. The matrix of each mitochondrion contains several copies of a circular DNA (mtDNA) of 16 kb in mammals encoding

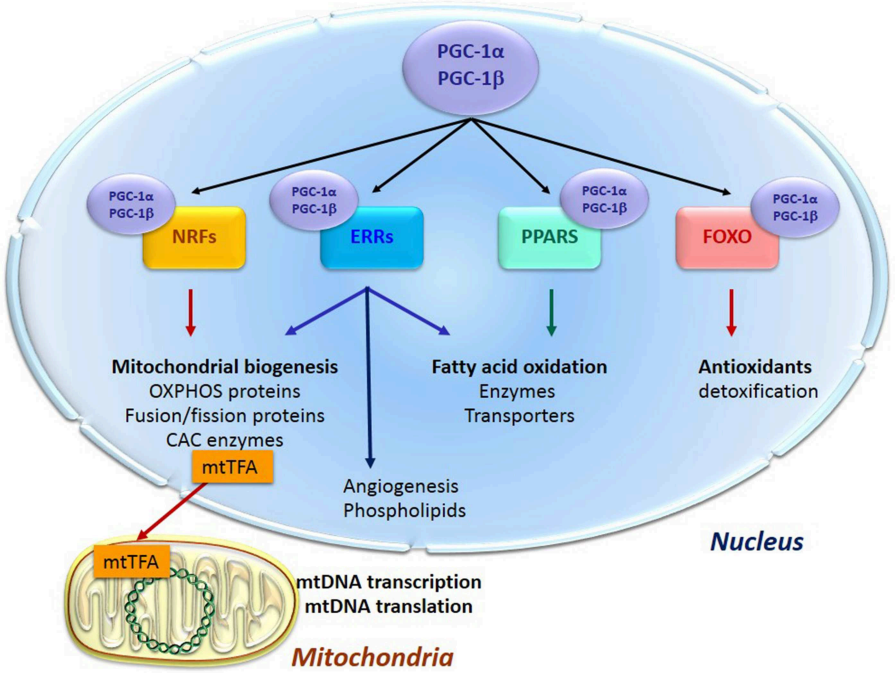

FIGURE 1 | Transcription cascade of mitochondrial biogenesis. Increased expression or activity of the master regulators of mitochondrial biogenesis "peroxisome proliferator activator receptor (PPAR) γ co-activators" (PGC-1s) α and β activate the expression of the nuclear respiratory factors 1 and 2 (NRFs) which induce the expression of the mitochondrial transcription factor A (mtTFA). The latter translocates to mitochondria, binds to mtDNA and activates its transcription and replication. PGC-1s also activate other transcription factors such as PPARα, mainly regulating lipid metabolism, estrogen-related receptors (ERRs) involved in substrate metabolism, energy transfer, angiogenesis and detoxification of reactive oxygen species, and the forkhead family of transcription factors (FOXOs) that activate antioxidant and detoxifying proteins.

some proteins as well as the RNAs required for the synthesis of these proteins. During evolution, mitochondrial DNA retained only 13 genes coding for subunits of the respiratory chain, two ribosomal RNA, and 22 transfer RNAs. Most of the mitochondrial genes have been transferred to the nucleus of the host cell. In mammals, the mitochondrial proteome is composed of more than a thousand of proteins (1). After fertilization, the mitochondria are of maternal inheritance. However, the fact that the vast majority of mitochondrial proteins are of nuclear origin underlies the tissue- and sex-specificity of mitochondria.

Energy supply and cell survival depend on the mitochondrial life cycle, which includes biogenesis, dynamics, and elimination of defective organelles by mitophagy. Mitochondrial proliferation consists of growing of preexisting organelles, through a process called mitochondrial biogenesis followed by mitochondrial division (fission). This biogenesis requires the coordination of both the nuclear and the mitochondrial genomes; it is mostly under the control of the nucleus through transcriptional cascades involving coactivators and specific transcription factors (**Figure 1**). Among the most important are (1) the family of the transcriptional coactivators "peroxisome proliferator activator receptor (PPAR) γ co-activators" (PGC-1s) α and β, the master regulators of mitochondrial biogenesis, (2) nuclear respiratory factors NRF1 and two involved in the nuclear control of mitochondrial biogenesis (2), (3) transcription factors such as PPARα, mainly regulating lipid metabolism, (4) estrogen-related receptors (ERRs) involved in substrate metabolism, energy transfer, angiogenesis, and detoxification of reactive oxygen species and (5) the mitochondrial transcription factor A (mtTFA) which once synthesized, migrates to the mitochondrial matrix where it activates the transcription and replication of mtDNA.

An important step in the mitochondrial life cycle is the fission of mother mitochondria into two daughter mitochondria, which is coordinated by specialized GTPases. In most cells, mitochondria are organized into a network that undergoes frequent fusion and fission events. This process is called mitochondrial dynamics. Fission is dependent on the DRP1 protein and its docking proteins, fission protein 1 (Fis1) and

Abbreviations: CK, creatine kinase; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; ERK1/2, extracellular signal-regulated kinase 1 and 2; ERR, estrogen-related receptors (ERRs); ETC, electron transport chain; Fis1, fission protein 1 (Fis1); GPER, G-protein coupled estrogen receptor; GSK3β, glycogen synthase kinase3β; JNK, c-Jun-NH2-terminal protein kinase; MERKO, muscle-specific ERα knock-out mice; Mff, mitochondrial fission factor; MFN, mitofusins; mtDNA, mitochondrial DNA; mTOR, mechanistic target of rapamycin; mtTFA, mitochondrial transcription factor A; NRF, nuclear respiratory factors; OPA1, optic atrophy 1; OVX, ovariectomy; OXPHOS, oxidative phosphorylations; p38 MAPK, mitogen-activated protein kinase; PARKIN, Parkinson juvenile disease protein 2; PCr, phosphocreatine; PGC-1, PPAR γ co-activator; PI3K, phosphoinositide 3-kinase; PINK1, PTEN-induced putative kinase 1; PKB, protein kinase B; PPAR, peroxisome proliferator activator receptor (PPAR) γ co-activators; PTP, permeability transition pore; ROS, reactive oxygen species; SERM, selective estrogen receptor modulator.

mitochondrial fission factor (MFF). Fusion is controlled by mitofusins (MFN1 and 2) for the outer membrane and optic atrophy 1 (OPA1) for the inner membrane. These fusion/fission processes are very slow in the adult cardiomyocyte under normal conditions (3).

Finally, dysfunctional mitochondria are eliminated through a specific mechanism of autophagy called mitophagy. Autophagy is the process by which cellular components such as macromolecules and organelles are recycled, allowing for the elimination of cellular waste and the reconstitution of nutrient stores. Mitophagy is the specific process for elimination of mitochondria and it is involved in quality control and mitochondrial turnover rate regulation. This process involves many proteins, among which the E3-ubiquitin ligase Parkinson juvenile disease protein 2 (PARKIN) and the PTEN-induced putative kinase 1 (PINK1) can be mentioned, allowing the identification of damaged mitochondria and their addressing to the autophagosome, the latter subsequently fusing with the lysosome to form autophagolysosomes.

Mitochondrial Functions and Regulation

Mitochondria are the main site of transformation of the energy of different substrates into chemical energy in the form of ATP. ATP consumption is extremely high in the heart where mitochondria provide more than 90% of the energy needed for contraction and cell pumps. The linear relationship between oxygen consumption and cardiac work shows that the mitochondria work in a "*pay as you go*" manner in this tissue. The heart can use a variety of substrates to regenerate ATP according to their availability, mainly fatty acids. While slow oxidative muscles also largely depend on oxidative metabolism for ATP production and uses fatty acids and glucose, fast skeletal muscles function in a "*work first pay later*" manner and preferably use glucose, glycogen and glycerol-3P. Lipid oxidation is responsible for 60 and 80% of totally consumed oxygen by soleus and cardiac muscle mitochondria, respectively, but for only a few percent by glycolytic gastrocnemius muscle mitochondria. On the opposite, glycerol-3-P utilization represents more than 80% of substrate used by gastrocnemius muscle mitochondria, while <10% of substrates used by cardiac mitochondria (4).

The common degradation product of lipids, amino acids and carbohydrates in mitochondria is acetyl-CoA, which enters the Krebs cycle in the mitochondrial matrix resulting in the production of reducing equivalents (NADH and $FADH_2$). These reducing equivalents are re-oxidized by the electron transport chain (ETC), also called respiratory chain, through the process of oxidative phosphorylations (OXPHOS). The respiratory chain is composed of four complexes forming a redox chain which results in the reduction of molecular oxygen to water and the creation of a proton gradient across the mitochondrial inner membrane. This electrochemical gradient of protons serves as a proton-motive force to rephosphorylate ATP from ADP by ATP synthase considered as complex V of ETC. Mitochondrial respiration is controlled by ADP according to a Michaelis/Menten relationship (5) and is modulated by calcium through the regulation of the activity of certain enzymes in the Krebs cycle and the respiratory chain (6). ATP is exchanged for ADP by the adenine nucleotide translocator anchored in the inner membrane. In muscle and cardiac cells, most of the ATP produced is immediately transformed in the inter-membrane space into phosphocreatine (PCr) by mitochondrial creatine kinase (CK) located on the outer surface of the inner membrane in the reaction:

$$\text{ATP} + \text{creatine} \Leftrightarrow \text{ADP} + \text{PCr} + \text{H}^+.$$

The newly formed PCr is channeled to the sites of energy utilization by the cytosolic CKs and allows the rephosphorylation of ADP locally produced by the ATPases (5, 7).

In addition to providing the greatest amount of energy to the host cell, mitochondria participate in other cellular functions such as ionic homeostasis among which calcium plays a pivotal role, production and regulation of reactive oxygen species (ROS), pH control and cell death. Besides, mitochondria are crucial for steroid hormone synthesis, thermogenesis, and lipid and carbohydrate utilization in a tissue-specific manner.

Mitochondria are the main site of ROS production in the cells and also the primary target of oxidative damages. ROS are mainly produced at the level of complex I and complex III of the respiratory chain. In physiological conditions, mitochondrial and cytosolic anti-oxidant defenses allow to control any excess of ROS production. Dysregulation of ROS production is involved in many pathological situations and in aging.

Another critical factor in mitochondrial function is the regulation of intracellular and intramitochondrial calcium. Although calcium is known to activate mitochondrial dehydrogenases, when present in excess, it uncouples mitochondria and decreases ATP production. High calcium collapses the mitochondrial membrane potential and induces the opening of the permeability transition pore (PTP), triggering the liberation of proapoptotic factors and leading to cell death (8).

Tissue Specificity of Mitochondria

Quantity, composition and function of mitochondria are adapted to the tissue in which they are embedded (9). It is generally believed that tissues with high and sustained ATP demand like striated muscles depend on oxidative phosphorylations ensured by the mitochondria. However, striking differences exist between oxidative cardiac and skeletal muscles on one hand and glycolytic skeletal muscles on the other hand. Cardiac muscle and postural skeletal muscles are strictly dependent on oxidative ATP production as they have to sustain long-term contractile activity. Oxidative muscles should have accurate adjustment of energy production to energy consumption. This is different from fast skeletal muscles whose contractile activity is rapid and short in duration and is mainly supported by ATP and PCr stores and glucose- or glycogen-driven ATP production.

In the myocardium, one of the most oxidative tissues of the body, mitochondria occupy 30–40% of the cell volume against 6–8% in the slow and 2–3% in the fast muscle fibers. The mitochondria in oxidative tissues like the heart and slow oxidative postural skeletal muscles are more numerous, located largely near the myofilaments, and able to use essentially fatty acids. They have a relatively low sensitivity to ADP and a significant amount of mitochondrial CK allowing them to

participate in intracellular energy transfer shuttles. They are adapted to the continuous supply of energy, necessary for muscular endurance work and cardiac function (10).

In fast skeletal muscles, mitochondria are less numerous, with a high sensitivity to cytosolic ADP (11) and are more dependent on glycolysis-derived substrates such as pyruvate and glycerol-3P (4). The energy required for the phasic activity of fast muscles is in fact essentially dependent on anaerobic glycolysis; energy metabolism reflects rapid and short-term use of large amounts of cytosolic ATP and PCr. Importantly, the CK system also exhibits important tissue specificity among striated muscles (9).

Sexual Dimorphism of Mitochondria

Although mitochondria are of maternal inheritance in both males and females, it should be kept in mind that, as discussed above, almost all mitochondrial proteins are encoded by the nucleus and thus under the influence of sex chromosomes, epigenetic marks and importantly, circulating sexual hormones. It is thus not surprising that in addition to a clear tissue specificity of mitochondria, a sexual dimorphism of mitochondria has been observed and starts to be studied in further details. Evidence is accumulating that this dimorphism takes part in the sex-specificity of important pathologies (12–14). Mechanistic pathways involved in sex differences in cardiovascular diseases has been recently reviewed in depth (15).

Cardiac Muscle

Gene expression profiling of mouse and human cardiac samples revealed sex and aged dependent expression patterns (16, 17). Among the differences, energy metabolism pathways are well-represented. However, it is generally accepted that cardiac oxidative capacity does not essentially differ between males and females (18–20) except for fatty acid utilization capacity that seems to be higher in young females, and for oxidative capacity that declines slower with age in older females (21, 22).

The most striking difference between male and female mitochondria lies in antioxidant defenses (12, 23). Female cardiac mitochondria seem to produce less ROS (18, 20) although this may be species or strain-dependent (19). Decreased ROS generation in female rat cardiac mitochondria has been attributed to posttranslational modifications of mitochondrial proteins involved in ROS handling (24).

Cardiac mitochondria of female rats have a greater calcium retention capacity than the male ones (25). Calcium kinetics also differ between male and female rat cardiac mitochondria (26). Mitochondria from females are more resistant to mitochondrial swelling at high calcium concentration (27). The lower Ca^{2+} uptake rates and the maintenance of mitochondrial membrane potential under conditions of high Ca^{2+} in female rat cardiac mitochondria have been attributed to modulation of the calcium uniporter (28).

Thus, sexual dimorphism of cardiac mitochondria involves a lower production of ROS, higher antioxidant defenses, and a better regulation of intra-mitochondrial calcium; all of which participate in the better resistance of female cardiomyocytes to ischemia/reperfusion injury, heart failure or cardiotoxicity.

Skeletal Muscle

It is well-established that female rodents have much higher endurance capacities than their male counterparts (29, 30). Interestingly woman skeletal muscle demonstrates an increased fatigue resistance compared to man muscle (31). Endurance capacity is highly dependent on skeletal muscle mitochondrial content and quality, as well as capacity to oxidize lipid substrates. Compared to males, females rely to a greater extent on fat oxidation during exercise (32) and skeletal muscles of women have higher intracellular lipid content than male muscles (33). Moreover, gastrocnemius muscle of female rats exhibits higher mitochondrial mass, oxidative-phosphorylative capacities, and mtDNA/nDNA compared to males (34, 35).

Different lean mass/body mass ratio in male and females, and the plasticity of skeletal muscle mitochondria in response to exercise training and exercise capacity (36), make comparison of skeletal muscle from men and women more difficult to interpret than for laboratory animals which are usually kept in standard low activity conditions. Recent studies have brought new knowledge on sex differences in human skeletal muscle mitochondria (37, 38). Whether these differences are due to differences in skeletal muscle composition in terms of oxidative vs. glycolytic fibers, remain to be determined. In young men and women of similar aerobic exercise capacity, a higher mitochondrial volume density and increased capacity for fatty acid and lactate oxidation were found in women skeletal muscle (39) thereby suggesting real differences between men and women mitochondrial properties.

Other aspects of sex differences in mitochondria include resistance to calcium overload, generation of radical oxygen species, and susceptibility to PTP opening and apoptosis. Only few data are available for skeletal muscle.

Thus, mitochondria of skeletal muscle also seem to exhibit a sexual dimorphism which could explain the higher facility of females to adapt to altered metabolic energy situations. However, data on skeletal muscle are so far sparse and undoubtedly, sexual dimorphism should be investigated more systematically in order to know whether skeletal muscle mitochondria contribute to different men and women muscle function and susceptibility to pathologies (13).

How much of these effects could be attributed to sex hormones and more specifically to estrogens and estrogen receptors will be discussed below.

ESTROGEN RECEPTORS AND MITOCHONDRIA

Estrogen effects are mainly mediated through estrogen receptors (ERs). ERs belong to the family of steroid hormone receptors themselves members of the family of ligand-activated transcription factors. Three estrogen-receptors have been described, estrogen-receptor alpha (ERα), estrogen receptor beta (ERβ), and the G-protein coupled estrogen receptor (GPER) with specific tissue and intracellular locations (**Figure 2**). These receptors are thought to be present in practically all cell types in

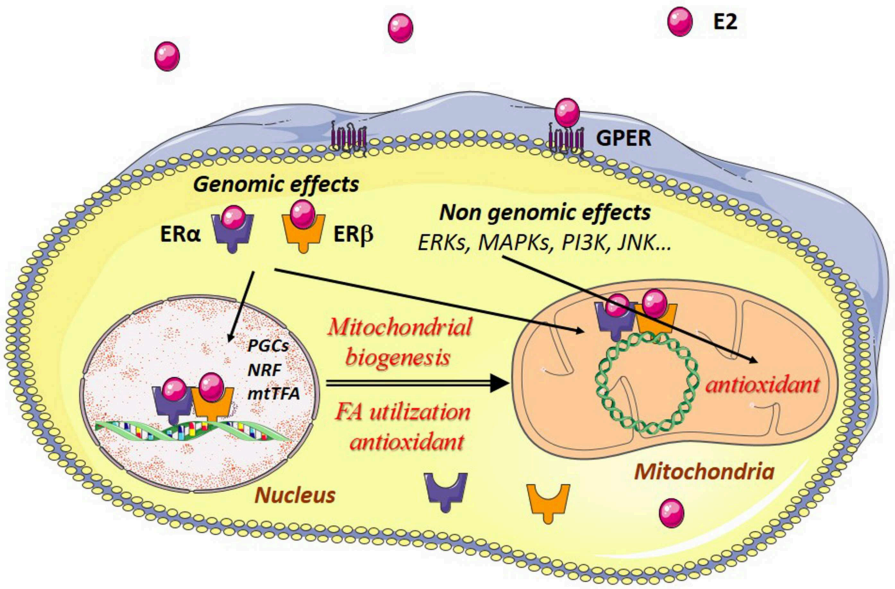

FIGURE 2 | Estrogen effects in muscle cells. Estradiol (E2) binds to its cytosolic receptors, estrogen-receptor α (ERα) and β (ERβ) inducing the translocation of the complex E2/estrogen receptors (ER) to the nucleus. Interaction of this complex with nuclear DNA results in the transcription of *Pgc-1α, Nrfs,* and other mitochondrial genes are involved in mitochondrial biogenesis, fatty acid utilization, and antioxidant defenses. Estradiol also interacts with ERs bound to mitochondrial DNA (mtDNA), thereby leading to transcription and replication of the mtDNA. E2 can also bind to G-protein-coupled estrogen receptors (GPER) at the plasma membrane, activating intracellular signaling pathways like extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38 mitogen-activated protein kinases (MAPKs), phosphoinositide 3-kinase (PI3K), c-Jun-NH2-terminal protein kinase (JNK), and others. This results in the transcription of genes encoding antioxidant enzymes, thus reinforcing in particular the antioxidant defenses of the mitochondria.

varying quantities. Their regulation and their location in striated muscles are still not well-understood.

Estrogens may have genomic and non-genomic effects. ERs may function as nuclear receptors and transcription factors in the nucleus and as signaling molecules in the plasma membrane. Estrogens regulate nuclear gene transcription by binding and activating the classical genomic estrogen receptors α and β (ERα and ERβ) and by activating plasma membrane-associated ERs, and GPER. The major circulating estrogen is 17β-estradiol (E2) which equally binds ERα and ERβ. The genomic effects involve dimerization of two ERs upon binding of E2 which then translocate to the nucleus and bind to estrogen response elements (ERE), thus regulating specific gene expression. Binding of E2/ERα complex to estrogen response elements (EREs) is regulated by phosphorylation independent of ligand binding. For detailed description of the genomic effects of E2/ER see (40).

Non-genomic effects involve rapid, within seconds or minutes, signaling actions through activation of membrane-associated ERs and GPER and subsequent activation of signaling pathways like extracellular signal-regulated kinase 1 and 2 (ERK1/2), increased phosphorylation of c-Jun-NH2-terminal protein kinase (JNK), phosphoinositide 3-kinase (PI3K), protein kinase B (PKB), glycogen synthase kinase3β (GSK3β), β-catenin, calcineurin, mechanistic target of rapamycin (mTOR), p38 mitogen-activated protein kinase (MAPK), and others (15, 41).

Both ERα and ERβ were found in male and female cardiomyocytes (42, 43). However, one study showed transcripts only for ERα in isolated cardiomyocytes from neonatal or adult rats suggesting that ERβ could be present in other cardiac cell types like fibroblasts or vascular cells (44). The presence of both ERs in cardiomyocytes is thus a matter of debate while skeletal muscles express both ERs with certainty (45, 46).

In addition to their presence in nuclei and plasma membrane, both ERα and ERβ have been detected in mitochondria of many cell types and species [for details see recent reviews (40, 47–49)]. However, ERβ seems to be the main estrogen receptor present in mitochondria as demonstrated by immunohistochemistry, immunocytochemistry, and immunoblots using a large panel of antibodies and mechanisms of import have been studied (see (50) for review).

Mitochondrial location of E2 receptors seems to be anyway tissue-specific. The presence of ERβ was detected in human heart mitochondrial proteins by Yang et al in 2004 (51) while not detected in liver cells by the same methods (MALDITOF) (52). ERβ was also identified in the skeletal muscle tissue and the C2C12 skeletal muscle cell line (53, 54). In this cell line, ERβ interacts with the chaperone HSP27 in the mitochondria and stabilizes it (55). However, additional experiments are needed to clearly identify the tissue specific expression and role of the different estrogen receptors in mitochondria.

ESTROGEN EFFECTS ON MITOCHONDRIA

The effects of estrogens on mitochondria are not restricted to their presence and role inside mitochondria. As discussed above, expression of mitochondrial proteins is mainly controlled by nuclear genome so that nucleus controls mitochondrial biogenesis. It is thus not surprising that the major effects of estrogen on mitochondria arise from their nuclear effects.

Estrogens exert a protective role on mitochondria by direct and indirect effects in a number of tissues [reviewed in (40, 48)]. Estrogen regulation of mitochondrial mass and function has been shown to participate in vascular, cardiac and neuronal protection (56–59). Estrogens appear to modulate various aspects of mitochondrial function including ATP production, ROS generation, antioxidant defenses, mitochondrial membrane potential, and calcium handling (50). Moreover, estrogens via ERs are involved in life cycle of mitochondria controlling mitochondrial biogenesis, mitochondrial quality control, and mitophagy. These effects may be mediated by both genomic and non-genomic effects.

Estrogens favor mitochondrial biogenesis in a tissue-specific manner by stimulating or inhibiting the expression of mitochondrial proteins from both nuclear and mitochondrial genomes (48, 60). Estrogens directly modulate the expression of mitochondrial protein genes by binding of ER/E2 to the ERE of metabolic genes in the nucleus. They increase the expression of the master regulator of energy metabolism and mitochondrial biogenesis PGC-1α and its downstream targets (61, 62). E2 also stimulates mitochondrial biogenesis by ER/E2 mediated activation of the transcription factors NRFs (56, 62, 63). NRF1 increases transcription of mitochondrial nuclear-encoded genes and of mitochondria-encoded genes through increased production of the transcription factor mtTFA (64). This is mediated by ERα and by the presence of an ERE in the promoter of the NRFs which can bind both ERα and ERβ in an estrogen-dependent manner (63).

ERs may also bind to mitochondrial DNA and are involved in the E2-induced expression of mtDNA and respiratory chain proteins, suggesting a role for these receptors in the regulation of mtDNA transcription and replication. The presence of ERs and their effects on both the nuclear and mitochondrial genomes would ensure their coordination in response to estrogens.

Whether ERα and ERβ exert similar or dissimilar roles in a tissue-specific manner is an additional level of complexity. Indeed, in addition to their subcellular localization, estrogen action depends on the expression and content of selective estrogen receptor modulators (SERMs). These SERMs induce various conformational changes in the ERs and differential recruitments of corepressors, coactivators, and transcription factors so that they can be either full or partial agonist or antagonists (47).

Thus, E2 is able to directly or indirectly modulate mitochondrial biogenesis and mitochondrial properties. The implication and role of ERα and/or ERβ may be tissue specific.

Estrogen Effects on Cardiac Mitochondria

Several studies have described the effects of ovariectomy on cardiac mitochondria. Quantitative proteomic analysis of cardiac mitochondria from ovariectomized (OVX) aged rats revealed a remarkable reduction in mitochondrial proteins involved in ETC, oxidative phosphorylation, lipid, and carbohydrate metabolism as well as oxidative stress and apoptosis (65). Ovariectomy decreases the activity of ETC complexes, increases mitochondrial membrane lipid peroxidation, and decreases calcium handling capacity (66). It decreases complex I-driven ATP synthesis and increases stress-induced ROS production of isolated cardiac mitochondria (58). Ovariectomy also downregulates cardiac mitochondrial biogenesis and function markers and increases oxidative stress through a possible GPER-mediated effect (67). These effects could be counteracted by E2 treatment. For example, E2 increases NRF-1, TFAM, PGC-1α, and mitochondrial protein levels in the heart of OVX mice (68). Mitochondrial alterations due to E2 deficiency may also be caused through miR-23a-mediated PGC-1α downregulation, which may subsequently be involved in the menopause-associated concentric cardiac hypertrophy (69).

On the other hand, physiological concentrations of estrogens do not affect mitochondrial respiratory functions in normal conditions but protect heart mitochondria from high calcium-induced release of cytochrome *c* (70). Activation of the membrane receptor GPER provides a positive effect after ischemia-reperfusion injury by inhibiting PTP opening, an effect mediated by the ERK pathway (71). E2 via ERβ mediates cardioprotection following trauma hemorrhage by activating the transcriptional cascade of mitochondrial biogenesis (62) and inhibiting the mitochondria-mediated apoptotic pathway (72). ERβ also appears to mediate the anti-apoptotic effects of estrogen in female adult heart following pressure overload (73). In cardiomyocytes exposed to ischemia/reperfusion, estrogens prevent cardiomyocyte apoptosis by inhibiting mitochondrial ROS formation (74). ERβ also mediates the increased expression of key regulators of cardiac mitochondrial function and respiratory chain proteins occurring in female mice following voluntary running (30). Thus, effects of E2 on mitochondria participate in the regulatory effects of estrogens. Finally, cardiomyocyte-specific deletion of ERα induces alterations in expression of some proteins involved in carbohydrate and lipid metabolism (75).

Whatsoever, estrogen actions in the heart seem to be more restricted to pathological states, during which estrogens mediate activation of mitochondrial biogenesis and cellular protection.

Estrogen Effects on Skeletal Muscle Mitochondria

Skeletal muscle possesses higher plasticity than cardiac muscle and exhibits considerable metabolic flexibility in response to hormonal stimulation, environmental factors, and exercise training. Skeletal muscles represent around 40% of body mass, and as a result they play a crucial role in insulin-sensitivity and glucose regulation.

Ovariectomy induces profound alteration of muscle biology and function. It decreases fatigue resistance, a factor known to depend on mitochondrial mass (76). Skeletal muscle of OVX mice shows lower use of fatty acid substrates, decreased PGC1α expression, reduced mitochondrial content, and increased compensatory extramitochondrial ATP synthesis during exercise,

most of which could be reverted by E2 treatment (77). Loss of E2 induces a rapid decline in skeletal muscle mitochondrial respiratory capacity, independently of mitochondrial content. This is likely mediated by changes in complex I function, accompanied by a ∼40% decrease in the reduced to oxidized glutathione ratio, effects that could also be reversed by E2 treatment (78).

Deletion of ERα in skeletal muscle has profound metabolic consequences. Muscle-specific ERα knock-out mice (MERKO) exhibit decreased muscle mass, obesity, and insulin resistance with muscle mitochondrial dysfunction, altered mitochondrial dynamics and diminished mitophagy (79). Knock-down of ERα in C2C12 myotubes reduces oxygen consumption rates and increased production of ROS thus promoting cellular oxidative stress (79). In parallel, the mammalian mitochondrial polymerase *Polg1* is also down-regulated following deletion of ERα, providing an additional mechanism for defective mitochondrial function in ERα deficiency. ERα also seems to affect mitochondrial quality control and dynamics because mitochondria of MERKO mouse muscle are elongated and hyperfused due to shift of the fusion/fission equilibrium toward fusion associated with a deficit in the fission protein DRP1 and increased levels of the fusion proteins OPA1 and MFN2 (79).

Conversely, estrogens are required to maintain muscle function because they have a positive effect on muscle mass, muscle regeneration after injury, and growth of satellite cells in young female mice [reviewed in (80)]. Estrogens regulate levels of enzymes associated with fatty acid metabolism explaining the higher capacity of woman skeletal muscles to utilize fatty acids (81, 82). In muscle cells, E2 plays also a major role in the inhibition of mitochondria-dependent apoptosis (83, 84). Physiological concentrations of E2 abrogate cytochrome *c* release and DNA damage induced by oxidative stress (47).

The beneficial effects of E2 derive from both genomic and non-genomic effects. In C2C12 muscle cells, E2 can interact with ERs located in the cell membrane and mitochondria to promote the activation of ERK, p38 MAPK, and the PI3K/Akt/p-Bad survival cascade thus abrogating the mitochondrial damage induced by hydrogen peroxide (83). E2 stimulates mitochondrial biogenesis in white gastrocnemius from OVX by increasing NRF1 transcript expression (85). Recently another mechanism by which E2 status broadly influences energy homeostasis has been described (78). It is shown that in skeletal muscle, E2 associates to the inner mitochondrial membrane, where it lowers membrane microviscosity, a mechanism that in turn promotes bioenergetic function, cellular redox balance, and insulin sensitivity thus offering a biophysical mechanism by which E2 influences energy homeostasis independently of ERα (78). This newly described mechanism of action of E2 on mitochondria may potentially be operative in other tissues; however, more studies are needed since this effect of E2 does not seem to be present in liver (86).

Importantly, postmenopausal women treated with hormone replacement therapy have substantially reduced relative risk of developing type 2 diabetes (87, 88). E2 exerts antidiabetogenic effects by protecting mitochondrial and cellular redox function in skeletal muscle of OVX mice (78). This insulin-sensitizing effect seems to be mediated by ERα. Estrogens induce insulin-sensitizing effects both by a reduction in the direct effects of ERα on insulin signaling as well as indirect effects of ERα on insulin action mediated by mitochondrial dysfunction (89). Estrogens thus appear to be an important target allowing to correct metabolic dysfunctions by pharmacological means (89, 90).

CONCLUSIONS AND PERSPECTIVES

Estrogens are important participants in metabolic regulation and mitochondrial function. Mitochondria are involved in a vast range of cellular processes, and their dysfunction is intrinsically associated with chronic diseases. Accumulating evidence suggests that many chronic diseases display gender specificity with females generally exhibiting beneficial metabolic profile. Among possible factors, sex-specificity of mitochondria plays a major role in the sexual dimorphism of chronic pathologies (12). Some of these effects could be mediated by estrogens via estrogen receptors. Whether present in nucleus, in mitochondria or bound to cell membrane these latters exert profound genomic and non-genomic effects on mitochondria. They affect among other things mitochondrial biogenesis, ROS production, calcium homeostasis, and mitochondrial pathway of apoptosis in a tissue-specific manner.

Female sex is known to be associated with a reduced risk of developing cardiovascular diseases when compared to males (15, 91), an effect mediated in large part by estrogen and estrogen receptors. It appears that although sex differences in cardiovascular pathologies and metabolic diseases are being recognized, considerations of this issue in myopathologies are yet underestimated (13). Certainly, knowing the importance of mitochondria in the genesis of myopathologies, the sexual dimorphism of skeletal muscle mitochondria could be a contributing factor (13). However, our understanding of sex differences is still incomplete and highlights the importance of developing mechanistic studies to delineate more precisely estrogen receptor expression, localization, and function in males and female heart and skeletal muscles. It is still distressing that, while we are entering the era of personalized medicine and personalized therapeutic strategies, gender differences are still poorly taken into account in the majority of the experimental and clinical researches.

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RV-C wrote the first version of the review. VV, AG, and JP participated actively in the writing of the final version.

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G Protein-Coupled Estrogen Receptor Protects From Angiotensin II-Induced Increases in Pulse Pressure and Oxidative Stress

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Our previous work showed that the G protein-coupled estrogen receptor (GPER) is protective in the vasculature and kidneys during angiotensin (Ang) II-dependent hypertension by inhibiting oxidative stress. The goal of the current study was to assess the impact of GPER deletion on sex differences in Ang II-induced hypertension and oxidative stress. Male and female wildtype and GPER knockout mice were implanted with radiotelemetry probes for measurement of baseline blood pressure before infusion of Ang II (700 ng/kg/min) for 2 weeks. Mean arterial pressure was increased to the same extent in all groups, but female wildtype mice were protected from Ang II-induced increases in pulse pressure, aortic wall thickness, and Nox4 mRNA. *In vitro* studies using vascular smooth muscle cells found that pre-treatment with the GPER agonist G-1 inhibited Ang II-induced ROS and NADP/NADPH. Ang II increased while G-1 decreased Nox4 mRNA and protein. The effects of Ang II were blocked by losartan and Nox4 siRNA, while the effects of G-1 were inhibited by adenylyl cyclase inhibition and mimicked by phosphodiesterase inhibition. We conclude that during conditions of elevated Ang II, GPER via the cAMP pathway suppresses Nox4 transcription to limit ROS production and prevent arterial stiffening. Taken together with our previous work, this study provides insight into how acute estrogen signaling via GPER provides cardiovascular protection during Ang II hypertension and potentially other diseases characterized by increased oxidative stress.

Keywords: estrogen, G protein-coupled estrogen receptor, NADPH oxidase 4, oxidative stress, cell signaling/signal transduction

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INTRODUCTION

Premenopausal women are protected from cardiovascular disease compared with age-matched men, while aging narrows this sex difference (1). The G protein-coupled estrogen receptor (GPER), previously known as GPR30, mediates non-genomic signaling by estrogen and is expressed in vascular endothelial and smooth muscle cells (2–4). Numerous ligands bind to GPER such as estradiol (3), 2-methoxyestradiol (5), genistein (6), and the selective agonist G-1 (7). We previously showed that G-1 induces vasorelaxation by inducing nitric oxide release from endothelial cells and increasing cAMP signaling in smooth muscle cells (8). Pharmacological activation of GPER

ameliorates maladaptive tissue remodeling in the vasculature, heart, and kidneys of salt-sensitive mRen2 female rats (9–11), as well in doxorubicin-induced cardiotoxicity (12). Global GPER deletion does not impact reproductive function yet induces a variety of cardiometabolic deficits (13) including increased fat mass (14, 15), atherosclerosis (16), blood pressure, and glucose intolerance (17). Moreover, the first study to conditionally delete GPER shows that cardiomyocyte GPER is important for cardiac structure and function in both sexes (18). Interestingly, while GPER is expressed in the vasculature of both sexes (19, 20), the protective effects of GPER seem to be reversed in aging male mice, where global GPER deletion is protective against cardiac and vascular dysfunction (21, 22). Many of the cardiovascular effects of GPER are associated with changes in reactive oxygen species, suggesting an antioxidant role for this estrogen receptor.

Reactive oxygen species (ROS) are free radical and non-radical oxygen species including superoxide (O_2^-), hydrogen peroxide (H_2O_2), peroxynitrite (NO_3^-), and hydroxyl radical (HO•). Excessive ROS overwhelms the cellular antioxidant system, causes oxidative stress, and promotes atherosclerosis, hypertension, stroke, and pathophysiological vascular remodeling (23–26). NADPH oxidase (Nox) proteins mediate electron transfer through catalytic subunits and significantly contribute to the production of ROS including superoxide and H_2O_2 (27). The Nox family of enzymes consists of Nox1 to Nox5 and dual oxidases (Duox) Duox1 and Duox2 which play diverse roles in the cardiovascular system (28). Many of the deleterious effects of Ang II are attributed to the generation of ROS via the recruitment of Nox proteins, as well as accessory proteins, to form a complex at the membrane (29). More specifically, Ang II upregulates the expression of Nox4 in vascular smooth muscle cells (30), an effect that is reversed by increasing cAMP signaling (31). Increased Nox4 expression is also found in models of Ang II-dependent hypertension including the spontaneously hypertensive rat (31) and the mRen2 rodent model (30). Therefore, the regulation of Nox proteins may be critical in mediating the adverse effects of Ang II.

Since GPER decreases NADPH-generated superoxide in carotid and intracranial arteries (32), Nox proteins may play a role in its vascular antioxidant effects. *In vitro* application of a GPER antagonist upregulates Nox1 but not Nox2 or Nox4, while global GPER deletion is associated with lower expression of Nox1 in the aorta and heart of aging male mice (21). In contrast to the lack of changes in Nox4 in male mice, ovariectomy-induced upregulation of cardiac Nox4 is prevented by chronic administration of the GPER agonist G-1 (11), while cardiomyocyte-specific GPER deletion in female mice induces a 4-fold increase in Nox4 mRNA (33). Therefore, the objective of this study was to investigate sex differences in the impact of GPER on Ang II-induced hypertension, oxidative stress, and Nox expression. We hypothesized that female responses to Ang II would be lower than males, while global GPER deletion would attenuate the protective effects of female sex. Moreover, we hypothesized that the antioxidant effects of GPER would be associated with changes in Nox.

MATERIALS AND METHODS

Animals

All procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Tulane University Institutional Animal Care and Use Committee. The GPER knockout strain used in this study was derived from the original model created by homologous recombination (17, 34). Male and female wildtype and global GPER knockout mice were bred and maintained in the institutional vivarium. The presence or absence of GPER was verified using both genotyping and ddPCR as previously described (35). Mice had free access to food and water in a temperature-controlled room (65–75°F) with a 12 h light to dark cycle. Mice were anesthetized for implantation of radiotelemetry probes in the carotid artery. After recovery and recording of baseline cardiovascular parameters, osmotic minipumps (Alzet Model 1002) containing Ang II (Bachem) were implanted to infuse at a rate of 700 ng/kg/min for 2 weeks, a protocol previously shown to induce sex differences in Ang II-induced hypertension (36, 37). Mice were euthanized at 18–25 weeks of age using isoflurane, and mesenteric arteries were harvested for measurement of vascular reactivity as described below. Aortas were stripped of fat, washed in PBS, and stored in −80°C until use. Male and female Sprague Dawley rats were obtained at 3–6 months of age from Charles River for use in cell culture studies.

Vascular Reactivity

Mesenteric arteries were cleaned of surrounding connective tissue, cut into 2-mm ring segments, and mounted on two wires connected to an isometric force transducer (DMT 620 M, Ann Arbor, MI). Segments were bathed in Krebs buffer (118 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 11 mM glucose; pH 7.4) and mixed with 95% O_2 and 5% CO_2 at 37°C. Normalization and assessment of baseline vascular dynamics were done as previously described (35). Vascular contractility was assessed in response to increasing concentrations of angiotensin II (Ang II; 10^{-10} to 10^{-6} M) and prostaglandin F2α (PGF2α; 10^{-8} to 10^{-4} M). Vascular relaxation to increasing concentrations of sodium nitroprusside (SNP) or acetylcholine (10^{-10} to 10^{-5} M) were assessed in vessels pre-constricted with 10^{-5} M phenylephrine.

Cell Culture

The embryonic rat aortic smooth muscle (A7r5) cell line was obtained from ATCC (Cat# CRL-1444, RRID:CVCL_0137). Since these cells are of embryonic origin as assumed to be a mixture of both male and female cells, additional experiments utilized primary aortic smooth muscle cells isolated from the thoracic aorta of male and female Sprague Dawley rats (12–14 weeks of age). Cells were cultured for up to ten passages in Media 199 containing 10% FBS, 1% penicillin-streptomycin and 1% L-glutamine in 95% air, 5% CO_2 in 37°C incubator. Cells were grown to near confluence (80%-90%) then switched to phenol red-free Media 199 containing 0.5% charcoal-stripped serum, 1% penicillin-streptomycin, and 1% L-glutamine. Cells were treated in the presence or absence of GPER agonist G-1 (100 nM), GPER

antagonist G36 (10 μM) (38), adenylyl cyclase inhibitor SQ22536 (5 μM), and phosphodiesterase-4 inhibitor rolipram (5 μM) for 24 h before being exposed to Ang II (100 nM) for 4 h.

Immunoblotting

After treatment, cells were washed and collected in ice cold phosphate-buffered solution then lysed in RIPA buffer containing protease and phosphatase inhibitors. Protein was estimated using the Pierce™ BCA Protein Assay Kit, and 50 μg of protein was resolved in a 10% Sodium dodecyl sulfate gel by electrophoresis before being transferred to a 0.45 μm nitrocellulose membrane for 2 h. Membranes were blocked in 5% non-fat milk for 2 h and incubated overnight with anti-Nox4 (1:1,000; Abcam ab133303-16), an antibody whose protein specificity was validated in other studies (39) as well as in our hands using siRNA (**Figure 6D**). Blots were reprobed with anti-GAPDH (GeneTex gtx627408) or anti-β actin (Cell Signaling Technology 3700) as a loading control. Secondary antibodies against rabbit and mouse were used at a 1:1,000 dilution. Image Studio Lite Version 5.2 was used to analyze band intensity.

Histology

Aortas and hearts were fixed overnight in 10% PBS buffered formalin and followed by storage in 70% ethanol. Paraffin-embedded sections were stained with hematoxylin and eosin, and aortic wall thickness was measured by taking measurements along the aortic wall circumference perpendicular to the lumen at 10 points per sample. To assess for cardiac hypertrophy, the entire cross-section of the heart was imaged at 4× magnification with the Cytation 5 imaging reader (BioTek, Winooski, VT). Wall and lumen areas were measured to calculate the LV/lumen ratio.

RNA Extraction and Reverse Transcription-Quantitative PCR

Cells or tissues were subjected to RNA extraction using the Qiagen RNeasy mini kit (cat# 74106). The amount of RNA was estimated using a NanoDrop 3300 Fluorospectrometer

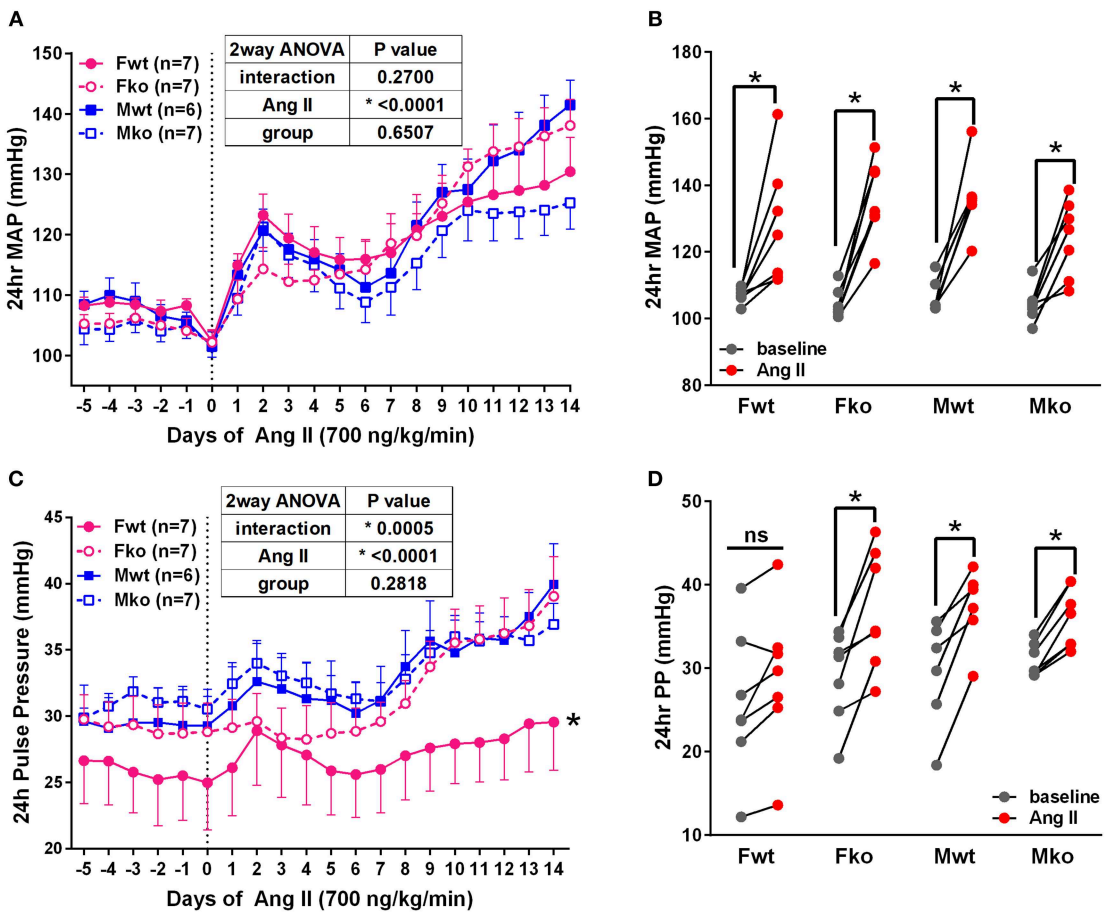

FIGURE 1 | GPER deletion increases pulse pressure in hypertensive female mice. **(A)** Radiotelemetry recordings show that MAP is not different at baseline or during 2-week Ang II infusion. **(B)** Grouped MAP pre and post Ang II shows a significant increase in all groups. 2-way repeated measures ANOVA, $p < 0.0001$ for Ang II, Sidak, *$p < 0.005$ for all groups. **(C)** Pulse pressure was significantly lower in Fwt mice. Tukey's test for main column effect, *$p < 0.001$ vs. all other groups. **(D)** Grouped pulse pressure data pre and post Ang II shows a significant increase in all groups except Fwt. 2-way repeated measures ANOVA, $p < 0.0001$ for Ang II, Sidak, *$p < 0.005$ vs. baseline.

(RRID:SCR_015804). For real-time polymerase chain reaction, a total of up to 500 ng RNA was used for PCR reaction. Specific rat primers for NOX4 (assay ID:qRnoCID0003969), NOX1 (assay ID:qRnoCID0004920), and GAPDH (assay ID:qRnoCID0057018) were obtained from Bio-Rad. For real-time PCR, iTaq™ Universal One-Step RT-qPCR Kit (cat# 172-5151) was used. The reaction mixture was set for 10 min at 50°C for cDNA synthesis, 5 min at 95°C for reverse transcription inactivation, and 10 s at 95°C for PCR cycling. Detection was done for 30 cycles followed by 30 s in 60°C and a melt curve analysis for 1 min at 95°C. The Bio-Rad® CFX96™ real-time PCR system was used to perform the assay in triplicate. To calculate the fold changes in mRNA expression, we normalized cycle threshold [C(t)] value of target genes to reference gene GAPDH using the $2^{-\Delta\Delta Ct}$ method.

Nox4 siRNA Transfection

Small interference RNA duplexes targeting Nox4 (rat) were obtained from Origene (cat# SR506919). Cells seeded in 35 mm dishes were grown to 60% confluence, and 20 μl of transfection reagent Lipofectamine® Plus™ was added to 180 μl Opti-MEM™ media for a final volume of 200 μl. One hundred nanomolar of siRNA was then added and mixed in a 1 ml tube and left to stand for 20 min at room temperature. The reaction mixture was added to each well to a final volume of 4 ml Opti-MEM™ media. Cells were grown for an additional 30 h after transfection before experiments were performed.

NADP/NADPH-Glo™ Assay

Cells treated in 24-well plates were washed with 37°C PBS and left to equilibrate at room temperature. NADP/NADPH-Glo™ detection reagent (cat#G9081, Promega) was added to each well and placed on a shaker for 1 h at room temperature. Luminescence was recorded using a Synergy™ HTX Multi-Mode Microplate Reader and normalized to protein in each well and expressed as relative luminescence units (RLU) per mg protein.

Catalase Colorimetric Assay Activity

Cells cultured in 12-well plates were washed twice in ice cold PBS and scraped followed by centrifugation at 250 × g for 10 min. The cell pellet was collected and re-suspended in 1X assay buffer and sonicated. Finally the lysate was centrifuged at 10,000 × g for 15 min and a portion of the supernatant was subjected to catalase colorimetric activity kit (ThermoFisher Scientific cat# EIACATC). Briefly, the generated standard curve and protein absorbance was read at 560 nm using Synergy™ HTX Multi-Mode Microplate Reader and normalized to protein in each well-estimated by BCA method. Final results are expressed as units (U) per mg protein.

Electron Spin Resonance Spectroscopy (ESR)

ESR was used to measure ROS in cells and isolated aortic tissues using the spin probe 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethyl-pyrrolidine (CMH) as previously described (40). Diethyldithiocarbamate (DETC; 2.5 μmol/l) and desferoxamine (DF, 25 μmol/l) were dissolved under nitrogen gas bubbling in ice-cold modified Krebs-Hepes (KH) buffer. Media containing drug treatments was removed before analysis, to avoid potential interference with the CMH signal. Cells or tissues were washed with calcium- and magnesium-free Dulbecco's phosphate-buffered saline (DPBS) and incubated with freshly prepared CMH (200 μmol/L) solution in KH buffer containing DETC and DF at 37°C for 60 min. Samples with buffer were transferred to 1 ml syringes, snap frozen in liquid nitrogen, and stored at −80°C until analysis. Samples were transferred to a finger Dewar vessel (Noxygen Science Transfer and Diagnostics, Germany) and analyzed using an EMX ESR Benchtop spectrometer (Bruker, Germany) with the following ESR settings: center field, 1.99 g; microwave power, 20 mW; modulation amplitude, 2 G; sweep time, 10 s; number of scans, 10; field sweep, 60 G. The amplitudes of the spectra were normalized using protein concentration and expressed as arbitrary units per mg protein.

Statistics

Statistical analysis was performed using GraphPad Prism 6.07 software (GraphPad Software). Outliers were identified using the ROUT method. For one factor analysis, the Shapiro-Wilk test was used to determine normality. Unpaired *t*-test was used to determine the difference between two groups. One-way ANOVA was used to determine differences between three or

TABLE 1 | Cardiovascular parameters.

	Fwt	Fko	Mwt	Mko	2-way ANOVA		
					Interaction	Ang II	Group
Baseline SBP	120 ± 2	118 ± 2	121 ± 2	119 ± 2	0.3337	* <0.0001	0.2670
Ang II SBP	142 ± 7	151 ± 6	156 ± 4	142 ± 5			
Baseline DBP	94 ± 2	89 ± 1	92 ± 2	88 ± 2	0.4870	* <0.0001	0.3790
Ang II DBP	114 ± 7	115 ± 4	118 ± 6	106 ± 4			
Baseline HR	596 ± 16	595 ± 10	550 ± 8	571 ± 12	0.4966	*0.0024	*0.0013 (Sidak, Mwt, $P = 0.04$)
Ang II HR	588 ± 13	580 ± 8	517 ± 11	551 ± 9			

Mean ± SEM and statistical analysis of systolic blood pressure (SBP, mmHg), diastolic blood pressure (DBP, mmHg), and heart rate (HR, beats-per-minute) obtained from 24 h telemetry recordings at baseline and after 2-week infusion of Ang II.

more groups, and if significant Tukey's multiple comparison test was performed. For data that was not normally distributed, Kruskal-Wallis with Dunn's multiple comparison was used. Two-way repeated measures ANOVA was used to analyze timeline and sex differences data, with no assumptions of sphericity, Geisser-Greenhouse corrections, and Tukey's test. For pre/post data, sphericity was assumed and multiple comparisons were made with Sidak's test. Comparisons where $P < 0.05$ were considered significant. All experiments were repeated at least once. Information on statistical tests used are also provided in graph legends.

RESULTS

GPER Deletion in Females Impacts Pulse Pressure but Not Mean Arterial Pressure

To determine the impact of genetic GPER deletion on cardiovascular parameters at baseline and during hypertension, male and female (M and F) wildtype and GPER knockout (wt and ko) mice were implanted with telemetry probes and exposed to Ang II for 2 weeks. As shown in **Figure 1**, no significant differences in MAP were found at baseline or in response to Ang II (**Figure 1A**). There was a trend for lower MAP in Mko

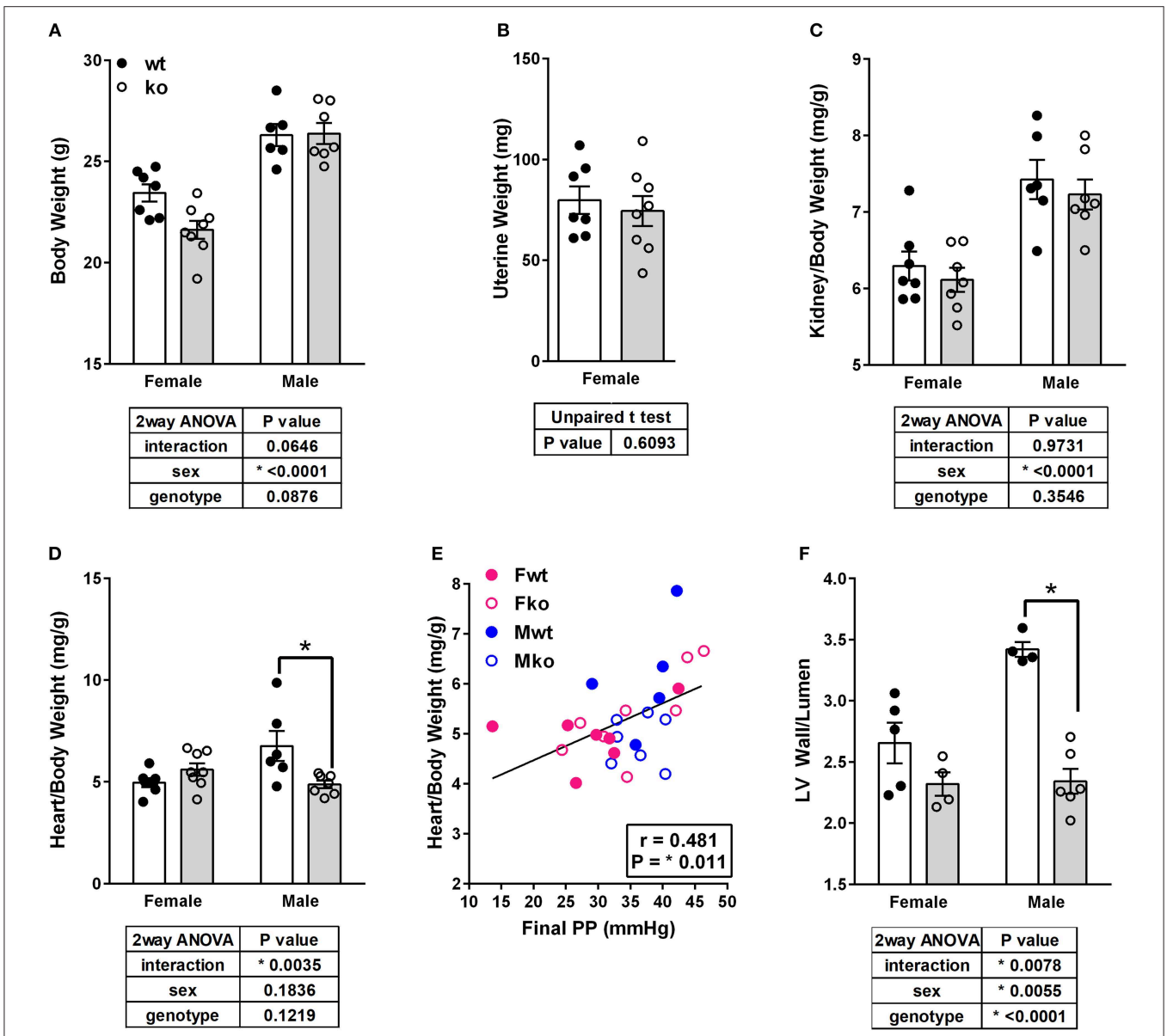

FIGURE 2 | GPER deletion impacts tissue weights and left ventricle wall thickness. **(A)** Body weight, **(B)** Uterine weight, **(C)** Kidney-to-body weight ratio, and **(D)** Heart-to-body weight ratio, all with respective statistical test results and Sidak's test if applicable. **(E)** A significant and positive correlation was found between final pulse pressure and heart-to-body weight ratio. **(F)** Left ventricular (LV) wall thickness-to-lumen ratio was higher in Ang II-infused Mwt versus Mko mice.

mice that did not reach statistical significance. Ang II induced a significant increase in blood pressure in all groups (**Figure 1B**). Analysis of systolic and diastolic pressures did not reveal any impact of genotype (**Table 1**). The ability of Ang II-induced hypertension to decrease heart rate was significant only in the Mwt group. Interestingly, Fwt mice had significantly lower pulse pressures than all other groups, while Fko pulse pressure was similar to Mwt and Mko mice (**Figure 1C**). In addition, pulse pressure was significantly increased by Ang II hypertension in all groups except Fwt mice (**Figure 1D**).

Impact of GPER Deletion on Wall Thickness, Nox Expression, and Vascular Reactivity

In Ang II-infused mice, body weight and kidney weight ratios were significantly higher in males but not impacted by genotype (**Figures 2A,C**). Uterine weights were not impacted by genotype (**Figure 2B**). A sex difference in cardiac weight ratios was observed in wt but not GPER ko mice (**Figure 2D**). Left ventricular wall-to-lumen ratio was impacted by GPER ko in male but not female mice (**Figure 2F**). A significant and positive correlation was found between final pulse pressure and heart-to-body weight ratio (**Figure 2E**). Assessment of aortic cross sections indicated a significant interaction between genotype and sex with increased wall thickness in Fko vs. Fwt with no impact in male mice (**Figure 3A**). To investigate the impact of Ang II on aortic Nox4 and Nox1, a separate cohort of mice was infused with Ang II for 2 weeks at the same dose or used as controls. Nox4 mRNA was significantly increased by Ang II treatment in all groups except Fwt mice (**Figure 3B**), while Nox1 mRNA was increased in female ko mice only (**Figure 3C**). Mesenteric arteries from female GPER ko and wt mice infused with Ang II were also assessed for vascular reactivity. Vessel contraction to increasing concentrations of PGF2α or Ang II was not significantly different between Fko and Fwt mice (**Figures 3D,E**). Similarly, relaxation to acetylcholine or SNP in pre-constricted vessels was also comparable between GPER ko and wt females (**Figures 3F,G**).

GPER Activation Prevented Ang II-Induced Increases in ROS and NADP/NADPH Ratio

To determine whether GPER impacts vascular Nox4 and oxidative stress, we designed *in vitro* experiments using A7r5 aortic smooth muscle cells (ASMC). Ang II-induced ROS was prevented by the GPER agonist G-1 (**Figure 4A**). Similarly, G-1 blocked Ang II-induced increases in NADP/NADPH ratio but did not alter levels when given alone (**Figure 4B**). Since A7r5 cells are embryonic and most likely contain a mixture of male and female cells, we assessed sex differences in the impact of GPER on oxidative stress. Surprisingly, primary isolated female ASMC had higher levels of ROS when assessed in estrogen-free conditions, but G-1 similarly attenuated the impact of Ang II in cells from both sexes (**Figure 4C**). To confirm involvement of

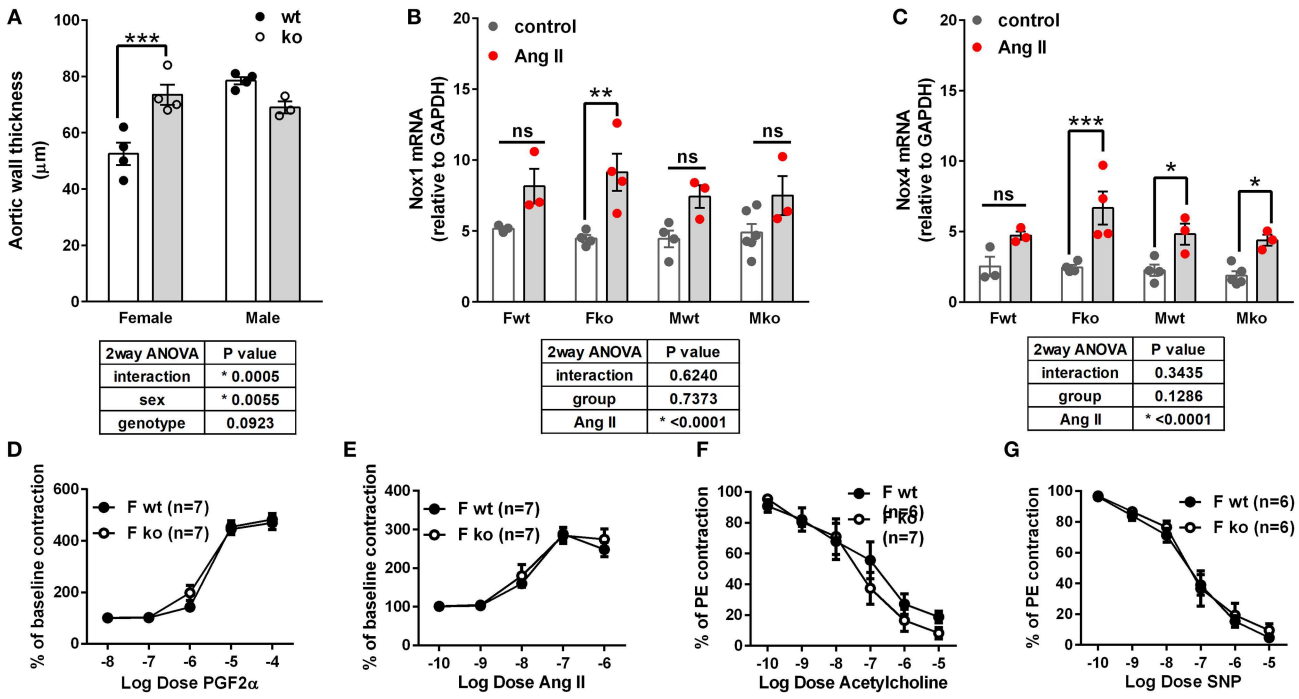

FIGURE 3 | Impact of GPER deletion on aortic wall thickness, Nox expression and vascular response. **(A)** Aortic wall thickness was increased in Ang II-infused Fko vs. Fwt mice, Sidak, $***p < 0.001$. **(B)** Nox1 mRNA was significantly increased by Ang II only in Fko mice, Sidak, $**p < 0.005$. **(C)** Nox4 mRNA was significantly increased by Ang II in all groups except Fwt, Sidak, $*p < 0.05$, $***p < 0.001$. Reactivity of Fwt and Fko mesenteric arteries after Ang II-infusion were not different when comparing **(D)** PGF2α-mediated contraction, **(E)** angiotensin (Ang) II-mediated contraction, **(F)** acetylcholine-induced vasorelaxation, and **(G)** sodium nitroprusside (SNP)-mediated relaxation.

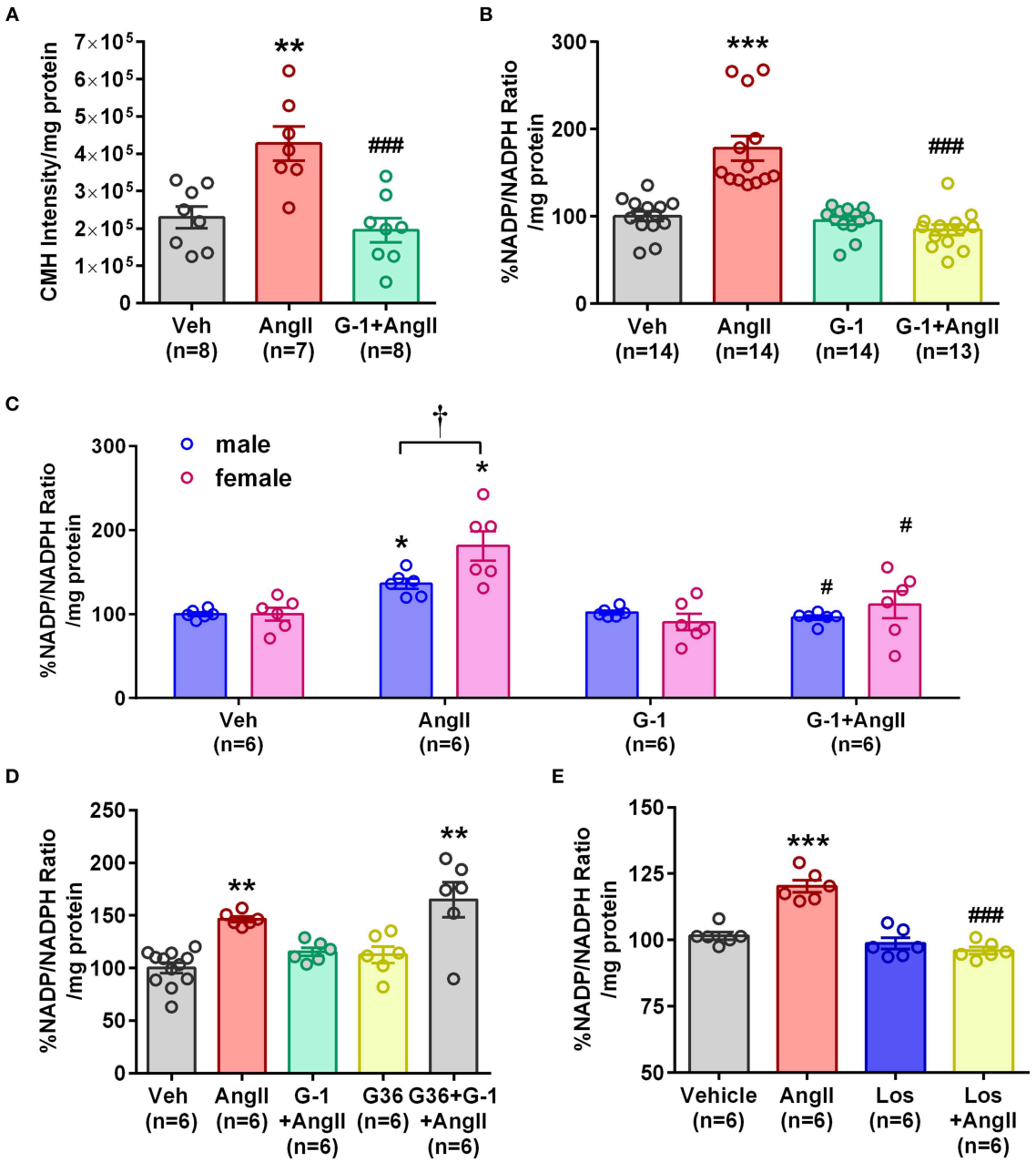

FIGURE 4 | GPER activation prevented Ang II-induced increases in ROS and NADP/NADPH ratio. **(A)** Stimulation of A7r5 cells with 100 nM Ang II for 4 h significantly increased ROS, which was prevented by pretreatment with the GPER agonist G-1 for 24 h. 1-way ANOVA, $p = 0.0004$, Tukey $^{**}p < 0.01$ vs. vehicle (Veh); $^{\#\#\#}p < 0.0001$ vs. Ang II. **(B)** Ang II-induced increases in the NADP/NADPH ratio were also mitigated by G-1. Kruskai-Wallis test, $p < 0.0001$, Dunn, $^{***}p < 0.001$ vs. Veh; $^{\#\#\#}p < 0.001$ vs. Ang II. **(C)** Ang II also increased NADP/NADPH ratio in primary male and female Sprague Dawley rat aortic smooth muscle cells, which was reversed by G-1. 2-way ANOVA with Geisser-Greenhouse correction, $^{*}p < 0.0323$ for interaction, Tukey, $^{*}p < 0.05$ vs. Veh; $^{\#}p < 0.05$ vs. Ang II; $^{\dagger}p < 0.01$ sex effect. **(D)** The GPER antagonist G36 blocked the ability of G-1 to inhibit Ang II effects on NADP/NADPH ratio. 1-way ANOVA, $p < 0.0001$, Tukey, $^{**}p < 0.01$ vs. Veh. **(E)** The increase in NADP/NADPH ratio induced by Ang II was completely prevented by losartan (Los). 1-way ANOVA, $p < 0.0001$, Tukey, $^{***}p < 0.001$ vs. vehicle; $^{\#\#\#}p < 0.001$ vs. Ang II.

GPER, cells were treated with Ang II and G-1 in the presence or absence of the GPER antagonist G36. The data consistently showed that Ang II increased while G-1 reversed NADP/NADPH ratio, but blocking GPER with G36 rendered G-1 ineffective in reversing the effect of Ang II (**Figure 4D**). To confirm the role of the Ang II type 1 receptor (AT1R) in mediating the effects of Ang II, we examined NADP/NADPH ratio in the presence or absence of the AT1R antagonist losartan (**Figure 4E**).

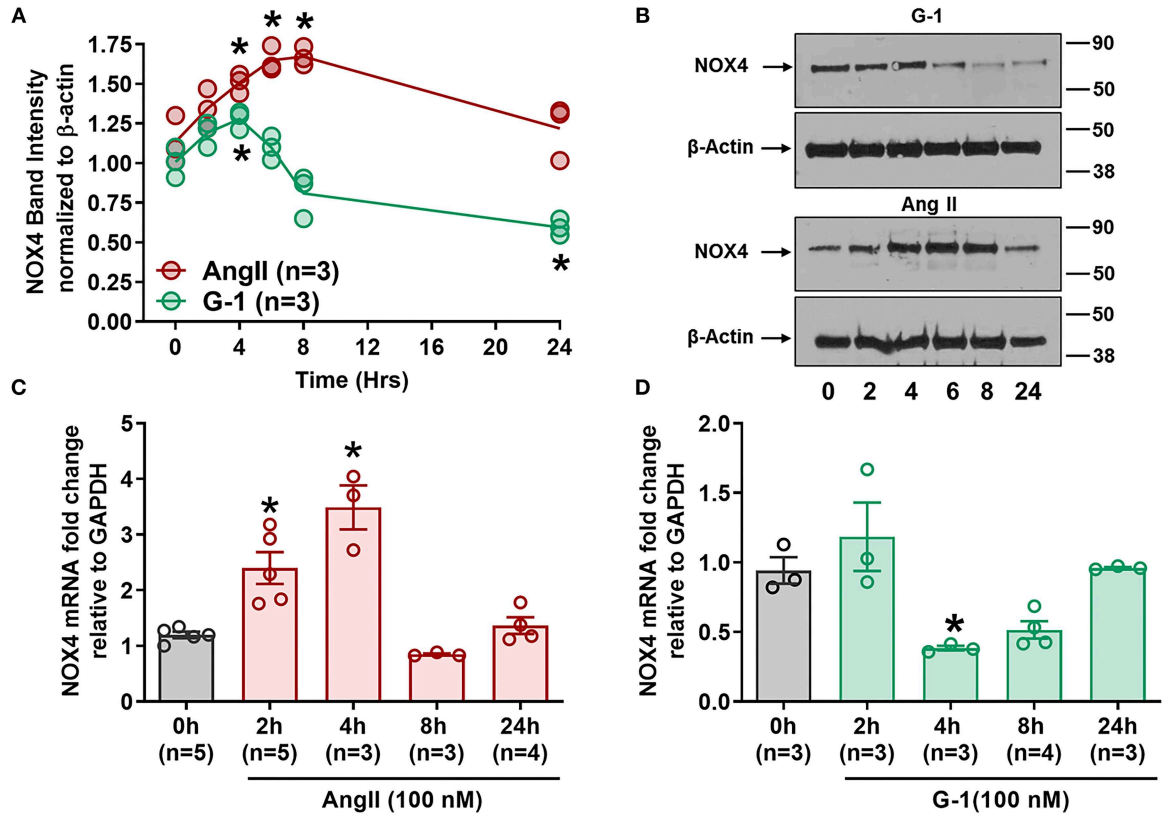

FIGURE 5 | GPER and Ang II regulate Nox4 protein and mRNA. **(A)** Ang II increased Nox4 protein at 4, 6, and 8 h while G-1 downregulated Nox4 protein at 24 h. 2-way ANOVA, $p < 0.001$ for interaction, Dunnett, $^*p < 0.05$ vs. 0 h. **(B)** Representative blots for **(A)**. **(C)** Ang II and **(D)** G-1 also impacted Nox4 mRNA. 1-way ANOVA, $p < 0.01$, Dunnett, $^*p < 0.05$ vs. 0 h.

Losartan completely blocked the Ang II-induced increase in NADP/NADPH ratio.

GPER and Ang II Regulate Nox4 Protein and mRNA

Since GPER attenuated Ang II-induced ROS and NADP/NADPH ratio, we next determined its impact on Nox4. Ang II upregulated Nox4 protein in A7r5 cells at 4, 6, and 8 h compared with baseline, while G-1 significantly decreased Nox4 protein expression at 24 h (**Figures 5A,B**). RT-qPCR showed that Nox4 mRNA levels were significantly increased by Ang II at 2 and 4 h (**Figure 5C**) but were decreased by G-1 at 4 h when compared with controls (**Figure 5D**). Nox4 mRNA was restored to control levels after 24 h of either G-1 or Ang II. These experiments indicated that Ang II and GPER regulate Nox4 in opposite directions at the transcriptional level.

GPER Activation Prevents Ang II-Induced Upregulation of Nox4 Protein

We next determined whether pretreatment with the GPER agonist prevented Ang II-induced increases in Nox4 mRNA and protein. G-1 pretreatment for 24 h did not alter Nox4 expression alone but prevented the upregulation induced by Ang II (**Figures 6A,B**). We used catalase activity to indirectly determine the amount of H_2O_2 produced when we blocked or activated GPER. Ang II significantly downregulated catalase activity, but this effect was ameliorated in the presence of G-1 (**Figure 6C**). The antagonist G36 eliminated the ability of G-1 to inhibit the effects of Ang II on Nox4 protein (**Figures 6D,E**) as well as catalase activity (**Figure 6F**).

siRNA Knockdown of Nox4 Reduced Ang II Effects

We utilized small interference (si) RNA for Nox4 to determine the role of this protein in Ang II-mediated oxidative stress, and validation of protein downregulation is shown in **Figure 7D**. Nox4 siRNA completely abrogated Ang II-induced ROS production (**Figure 7A**) and NADP/NADPH ratio (**Figure 7B**) compared with scrambled siRNA controls. Nox4 knockdown also prevented Ang II-induced Nox4 protein upregulation by 80% (**Figures 7C,E**), an effect similar to that seen with G-1 treatment.

Impact of cAMP Signaling

We next investigated the role of GPER-mediated cAMP production in the protective effects of G-1 on NADPH oxidase activity and Nox4 protein expression. A7r5 cells were treated with Ang II in the presence or absence of the GPER agonist

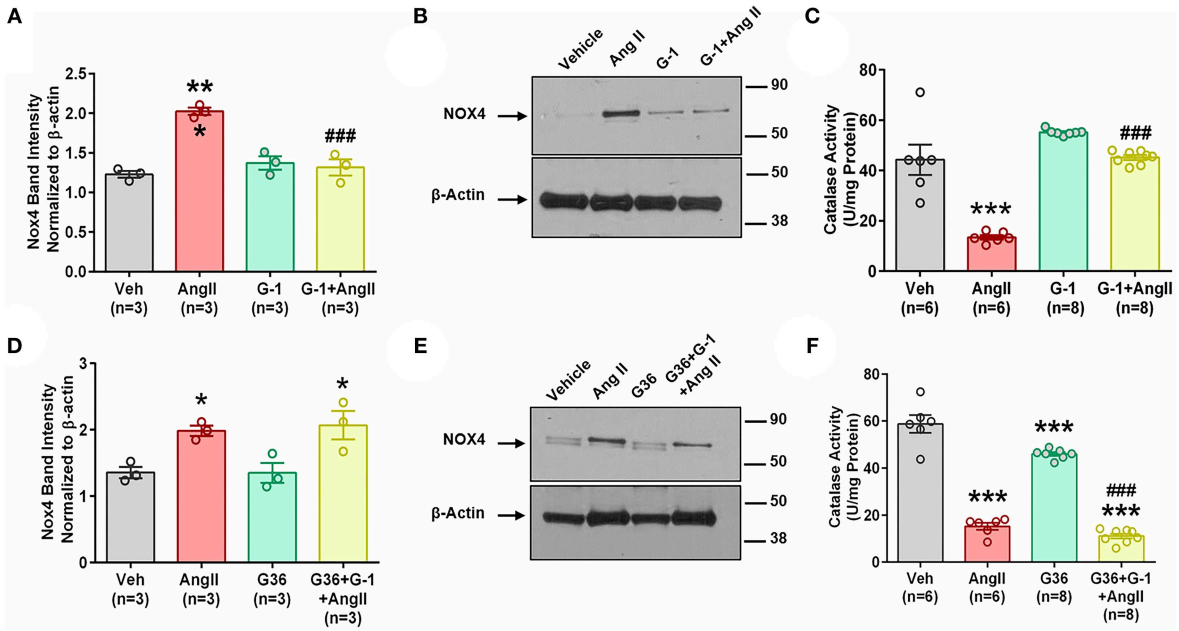

FIGURE 6 | GPER activation prevents Ang II-induced upregulation of Nox4 protein. **(A)** Ang II-induced Nox4 protein is reversed by G-1. 1-way ANOVA, $p = 0.0002$, Tukey, $^{***}p < 0.001$ vs. vehicle (Veh); $^{\#\#\#}p < 0.001$ vs. Ang II. **(B)** Representative blot for **(A)**. **(C)** Ang II-induced decreased catalase activity while G-1 prevented the decrease. 1-way ANOVA, $p < 0.0001$, Tukey, $^{***}p < 0.001$ vs. vehicle; $^{\#\#\#}p < 0.001$ vs. Ang II. **(D)** The GPER antagonist G36 blocked the ability of G-1 to inhibit Ang II effects on Nox4 protein. 1-way ANOVA, $p = 0.0106$, Tukey, $^{*}p < 0.05$ vs. Veh. **(E)** Representative immunoblot for **(C)**. **(F)** G36 blocked the effect of G-1 on Ang II-induced decreased catalase activity. 1-way ANOVA, $p < 0.0001$, Tukey, $^{***}p < 0.001$ vs. Veh; $^{\#\#\#}p < 0.001$ vs. G36.

G-1, the adenylyl cyclase inhibitor SQ22536 (SQ), or the phosphodiesterase (PDE) 4 inhibitor rolipram, which increases intracellular cAMP levels by preventing its breakdown. G-1 again prevented Ang II-induced increases in NADP/NADPH ratio, but SQ blocked this effect (**Figure 8A**). Similarly, when adenylyl cyclase was inhibited, G-1 was unable to prevent the effect of Ang II on Nox4 protein (**Figures 8B,C**) and catalase activity (**Figure 8D**). Rolipram mimicked the effects of GPER activation by blocking Ang II-induced NADP/NADPH activity (**Figure 8E**), Nox4 upregulation (**Figures 8F,G**), and decreased catalase activity (**Figure 8H**).

DISCUSSION

The novel finding from the current study is that despite similar blood pressures, GPER deletion in female mice significantly increased pulse pressure and exacerbated the upregulation of aortic NADPH oxidases in response to Ang II. In parallel, *in vitro* activation of GPER attenuated oxidative stress via cAMP-mediated regulation of Nox4. We demonstrated that Nox4 plays a major role in Ang II-induced ROS production in cultured VSMC. Furthermore, we showed that GPER opposed the effects of Ang II by downregulating Nox4 at the transcriptional level and restoring catalase activity. These findings have important implications since randomized clinical trials fail to significantly inhibit oxidative stress using currently available antioxidants such as vitamin E (41–43), while preclinical studies with Nox1 and 4 inhibitors (44, 45) are promising. Since oxidative stress is detrimental to cardiovascular tissues, GPER may provide a novel target for inhibition of vascular ROS.

In contrast to previous studies, we did not find a sex difference in Ang II-induced hypertension as found in other studies using the same or similar protocol (36, 37). A study using a higher dose of Ang II also failed to detect sex differences in telemetry blood pressure recordings (46). Since the mice used in this study were developed from a 129/Sv strain and backcrossed to the C57bl/6, some genetic aspects of the 129/Sv strain may have carried over (34, 47), since mice of this strain have two renin genes and higher blood pressures even after two generations of backcrossing with C57bl/6 mice (48, 49). Four GPER knockout mice strains have been created using slightly different methods (13), and in this study the model created by homologous recombination was utilized (17, 34). The results from these different models is varied, for example previous data from this same strain utilized in the current study shows increased body weight in both male and female knockout mice at 10 months of age (15). In contrast, data from a different GPER knockout strain shows lower body weight in Fko at 19 weeks of age, and also finds increased mean arterial pressure in female knockout mice at 9 months of age but not at 6 months, the latter of which is comparable to the ages of the mice in our study (17). We found no difference in body weight or blood pressure before or after Ang II infusion, suggesting that age and strain are important factors in observing these phenotypes.

Since sex did not influence Ang II hypertension and the hypothesis was that GPER deletion would remove sex differences, we were not surprised to find similar MAP in wt and ko mice.

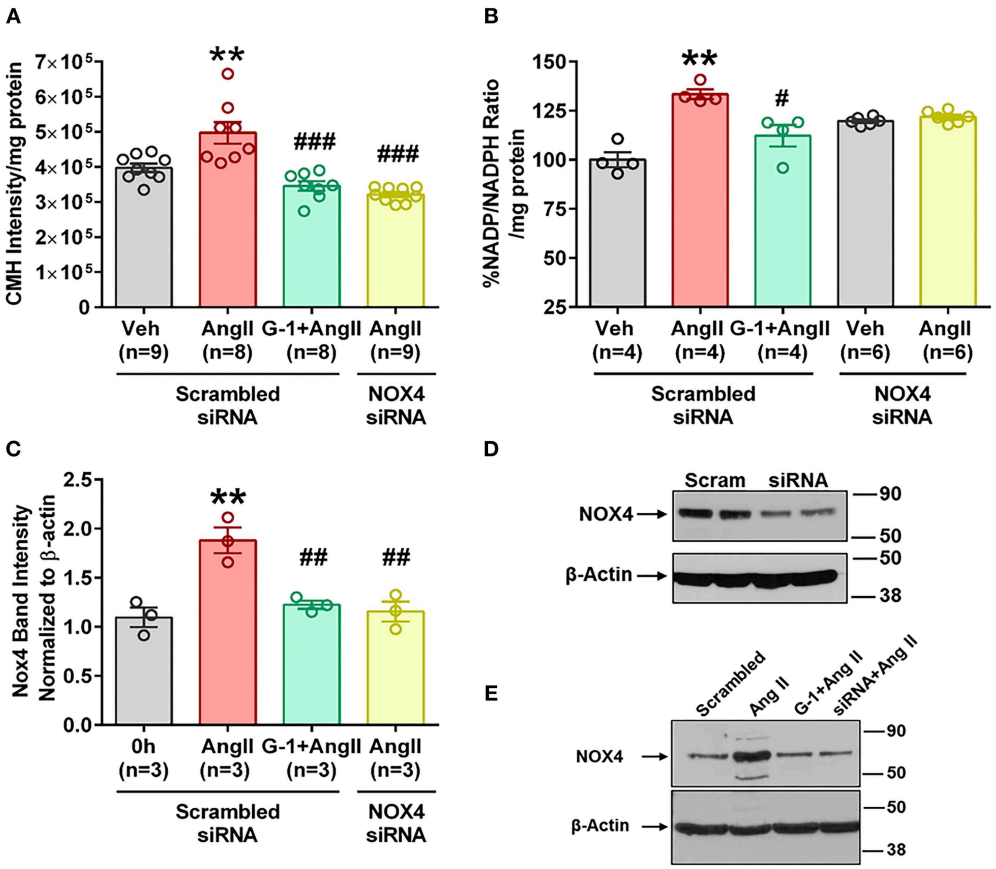

FIGURE 7 | siRNA knockdown of Nox4 reduced Ang II effects. **(A)** Nox4 siRNA significantly reduced Ang II-induced ROS. 1-way ANOVA, $p < 0.0001$, Holm-Sidak, $^{**}p < 0.01$ vs. Veh; $^{\#\#\#}p < 0.0001$ vs. Ang II. **(B)** Nox4 siRNA significantly reduced Ang II-induced NADP/NADPH ratio. Kruskai-Wallis test, $p = 0.0012$, Dunn $^{**}p < 0.01$ vs. Veh; $^{\#}p < 0.05$ vs. Ang II. **(C)** Ang II upregulated Nox4 protein in the presence of scrambled (Scr) siRNA but not with Nox4 siRNA. 1-way ANOVA, $p = 0.0017$, Tukey, $^{*}p < 0.05$ vs. Veh; $^{\#\#}p < 0.01$ vs. Ang II. **(D)** Validation of Nox4 antibody and siRNA. **(E)** Representative blot for **(C)**.

However, we found a significant impact of GPER deletion when analyzing pulse pressure, an indicator of arterial stiffening, which was increased in Fko mice to a level comparable to male wt and ko mice. These data support our previous study in salt-loaded mRen2 rats where despite similar levels of hypertension, aortic wall thickness was significantly reduced by chronic treatment with the GPER agonist G-1 (9). The increased stiffness observed may precede changes in pressure, considering that arterial stiffening is observed before increases in blood pressure in aging humans as well as a mouse model of high fat diet-induced hypertension (50–52). While arterial stiffening increases afterload, cardiac hypertrophy was not different in Fwt vs. Fko mice in the current study. However, the significant positive correlation with pulse pressure indicates that arterial stiffening is associated with increased cardiac remodeling, but longer Ang II infusion may be required to observe differences between groups. The current study indicates that in female mice, GPER provides protection from Ang II-induced vascular remodeling and pulse pressure increases, but not hypertension.

Using ESR spectroscopy, the best method for detecting and analyzing ROS in biological samples (40), we confirmed that GPER activation promotes antioxidant defenses in the vasculature. This result is consistent with studies showing that estrogen attenuates oxidative stress in VSMC (53) and endothelial cells (6). Previous work from our laboratory indicates that GPER attenuates vascular oxidative stress and remodeling (9), decreases cardiac ROS (11), and reduces renal oxidative damage in rats fed a high salt diet (10). In addition, deficiency of GPER in cardiomyocytes of female mice is associated with increased cardiac oxidative stress (33). Mechanisms for the antioxidant effect of GPER in other cell types include a reduction in mitochondrial permeability transition pore opening in cardiac cells (54), regulation of several antioxidant genes including glutathione peroxidase and thioredoxin-interacting protein in skeletal muscle (33), and reduced H_2O_2 peroxidation in the liver (6). Recent evidence also suggests that GPER induces superoxide dismutase during methotrexate-induced kidney damage (55). In the rat heart, ovariectomy increases Nox4 expression and oxidative stress and is reversed by administration of the GPER agonist G1 (11). Notably, deletion of GPER in cardiomyocytes increases Nox4 and oxidative stress in female mice (33), suggesting a detrimental role for this enzyme in the heart (56).

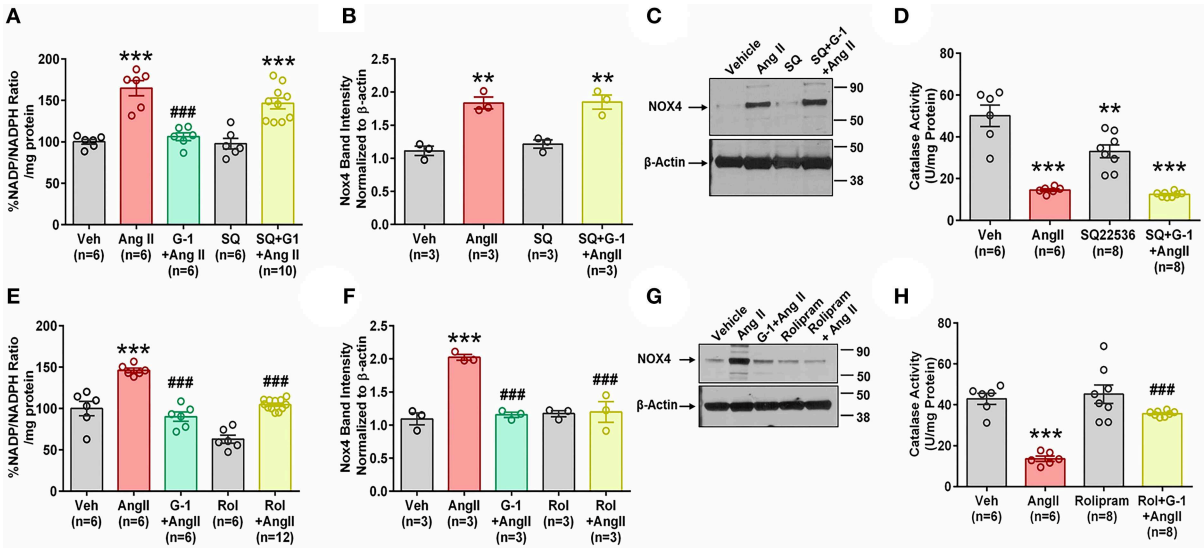

FIGURE 8 | Impact of cAMP signaling. The adenylyl cyclase inhibitor SQ22536 (SQ) blocked the ability of GPER to oppose the impact of Ang II on **(A)** NADP/NADPH ratio. 1-way ANOVA, $p < 0.0001$, Tukey, $^{***}p < 0.001$ vs. Veh, $^{\#\#\#}p < 0.001$ vs. Ang II. **(B)** Nox4 protein. 1-way ANOVA, $p = 0.0003$, Holm-Sidak, $^{**}p < 0.01$ vs. Veh. **(C)** Representative blot for **(B)**. **(D)** SQ also blocks G-1's ability to reverse Ang II-induced decreased catalase activity. 1-way ANOVA, $p < 0.0001$, Tukey, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Veh. Increasing cAMP with the phosphodiesterase inhibitor rolipram (Rol) mimics the impact of G-1 on **(E)** NADP/NADPH ratio. 1-way ANOVA, $p < 0.0001$, Tukey, $^{***}p < 0.001$ vs. Veh, $^{\#\#\#}p < 0.001$ vs. Ang II. **(F)** Nox4 protein. 1-way ANOVA, $p < 0.0001$, Tukey, $^{\#\#\#}p < 0.001$ vs. Veh, $^{\#\#\#}p < 0.001$ vs. Ang II. **(G)** Representative blot for **(E)**. **(H)** Ang II-induced decreased catalase activity is restored by Roland G-1. 1-way ANOVA, $p < 0.0001$, Tukey, $^{***}p < 0.001$ vs. Veh; $^{\#\#\#}p < 0.001$ vs. Ang II.

These studies indicate a connection between GPER, oxidative stress, and Nox4 in multiple cardiovascular tissues.

Our experiments using GPER ko and wt mice indicate that females with intact GPER signaling were protected from Ang II-induced increases in aortic Nox1 and Nox4. While the ability of Ang II to upregulate Nox1/2 and negatively impact cardiovascular health is well-established, the role of Nox4 is still debatable. Studies show both up and downregulation of Nox4 in response to Ang II (30, 57–60). Functionally, Nox4 induces VSMC hypertrophy, oxidation of lipids, and inactivation of nitric oxide (23, 29), but is required for VSMC differentiation (61) and protects endothelial cells during hypoxia (62). This conflicting data is also observed in Nox4 knockout mice, where Ang II-hypertension is not impacted but aortic wall thickness is *increased* (63). Nox4-induced endothelium derived hyperpolarizing factor mediates a decrease in blood pressure, suggesting a vasculoprotective role (64). While Nox4 promotes nitric oxide production during shear stress in endothelial cells, during aging Nox4 uncouples endothelial nitric oxide synthase and induces oxidative stress (65). Importantly for the increased pulse pressure and aortic remodeling observed in the current study, Nox4 is upregulated in aortic smooth muscle during aging and contributes to mitochondrial ROS and vascular stiffening (66). The same group recently showed that overexpression of mitochondrial Nox4 increases aortic smooth muscle cell stiffness and pulse wave velocity (67). Similarly, pharmacological inhibition of Nox4 using GKT137831 attenuates hypoxia-induced pulmonary artery remodeling (68), and a Nox4 dominant negative mutation protects atherosclerotic mice from increases in pulse wave velocity (69). Since GPER antagonism reduces aortic ROS in aged male mice and was associated with downregulation of Nox1 but not Nox4 (21), the role of Nox4 in the vasculature may be altered during the aging process.

Another factor may be the relative amounts of superoxide vs. H_2O_2 that are produced by the Nox4 enzyme, which may depend on cell type. Nox4 produces superoxide in neurons and rat aortic smooth muscle cells (31, 70), but H_2O_2 in endothelial cells (71). The protective vs. detrimental impact of H_2O_2 may also differ by cell type. In a tamoxifen-inducible endothelial Nox4 knockout mouse model, H_2O_2 produced by Nox4 increases angiogenesis after femoral artery ligation injury demonstrating a protective role (63). However, smooth muscle cell overexpression of catalase, which quenches H_2O_2, protects from Ang II-induced aortic remodeling (23). Our data indicates that in cultured vascular smooth muscle cells, catalase activity is reduced in the presence of Ang II and reversed by the GPER agonist G-1. Ang II most likely downregulates catalase activity by increasing superoxide which reacts with superoxide dismutase to form H_2O_2 (72).

Since most studies are performed only in male mice, sex differences in Nox4 expression or function may underlie its beneficial vs. detrimental vascular effects. In males, Nox4 is highly expressed in basilar cerebral arteries (73), while females express high Nox4 in mesenteric (74) and porcine coronary arteries (75), suggesting sex and functional differences. Sexual dimorphisms may also become important when considering GPER, since we found a trend for lower MAP and cardiac protection in Mko mice in the current study. Similarly, GPER

antagonism in aging male mice confers protection from oxidative stress by decreasing vascular Nox1 with no impact on Nox4 (21). Nox1 and 4 may interact in the regulation of ROS since non-specific Nox1/4 inhibitors GKT136901 or GKT137831 attenuate oxidative stress (44, 45, 68). Our study indicates that in female mice infused with Ang II, Nox4 plays a detrimental role in vascular smooth muscle cell remodeling, while intact signaling by GPER confers protection.

Surprisingly, activation of a G protein-coupled receptor known for its role in acute estrogen signaling had a significant impact on Nox4 mRNA within 4 h. Other GPCRs such as endothelin-1 and thrombin receptors also regulate NADPH oxidases (76). Our study also demonstrated that cAMP activation by GPER is necessary to regulate both NADP/NADPH ratio and Nox4 expression. Activation of GPER by the agonist G-1 activates adenylyl cyclase, leading to accumulation of cAMP (77), and work from our lab and others show that this GPER signaling cascade induces vasorelaxation (8, 78). Downstream phosphorylation of protein kinase A activates cAMP response element binding protein (CREB), a transcription factor that regulates several genes including Nox1 and Nox5 (79, 80). Activation of the cAMP-CREB pathway attenuates VSMC migration (81), while reductions in cAMP increase NADP oxidation to promote ROS in aortic smooth muscle cells (31, 82). Our results connecting GPER-induced cAMP increases with Nox4 regulation and NADP/NADPH ratio indicate that this signaling pathway plays an important role in attenuating Ang II-induced oxidative stress and remodeling. Interestingly, Ang II-induced downregulation of catalase activity can also be reversed by G-1 and rolipram, suggesting a distinct pathway involving the stimulation of adenylyl cyclase and accumulation of cAMP for protection against Ang II-induced ROS.

In addition to the current findings, activation of GPER attenuates oxidative damage to pancreatic beta cells in diabetes (47) and protects neurons from oxidative stress in the brain (83). Since oxidative stress is involved in many disease processes and GPER is ubiquitously distributed in mammals, therapeutic targeting of this estrogen receptor may provide benefits. While menopause is associated with an increase in cardiovascular disease, vascular stiffening, and increased ROS production (84), GPER may have the capacity to selectively decrease oxidative stress without activating nuclear estrogenic signaling. Therefore, inclusion of GPER as a therapeutic target may alleviate deleterious effects in both cardiovascular and metabolic diseases.

DATA AVAILABILITY

The datasets generated during the current study are available in the Harvard Dataverse repository: https://dataverse.harvard.edu/dataset.xhtml?persistentId=doi:10.7910/DVN/Z9FEPX.

ETHICS STATEMENT

All procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Tulane University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

BO, KM, PK, and SL contributed conception and design of the study. BO, VS, JD, KG, MZ, and GC performed the experiments. BO and SL performed the statistical analysis. BO wrote the first draft of the manuscript. SL wrote sections of the manuscript. All authors contributed to manuscript revisions and approved the submitted version.

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Menopausal Status and Physical Activity Are Independently Associated With Cardiovascular Risk Factors of Healthy Middle-Aged Women: Cross-Sectional and Longitudinal Evidence

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Cardiovascular disease (CVD) is the primary cause of mortality in women in developed countries. CVD risk rises with age, yet for women there is a rapid increase in CVD risk that occurs after the onset of menopause. This observation suggests the presence of factors in the middle-aged women that accelerate the progression of CVD independent of chronological aging. Leisure time physical activity (LTPA) is a well-established protective factor against CVD. However, its role in attenuating atherogenic lipid profile changes and CVD risk in post-menopausal women has not been well-established. The present study is part of the Estrogenic Regulation of Muscle Apoptosis (ERMA) study, a population-based cohort study in which middle-aged Caucasian women (47–55) were classified into pre-menopausal, peri-menopausal, and post-menopausal groups based on follicle stimulating hormone levels and bleeding patterns. Comprehensive questionnaires, laboratory visits, anthropometric measurements, and physical activity monitoring by accelerometers were used to characterize the menopausal groups and serum lipid profiles were analyzed to quantify CV (cardiovascular) risk factors. Based on our findings, LTPA may attenuate menopause-associated atherogenic changes in the serum CV risk factors of healthy middle-aged women. However, LTPA does not seem to entirely offset the lipid profile changes associated with the menopausal transition.

Keywords: menopause, cardiovascular disease, physical activity, cholesterol, HDL, LDL, triglycerides, fasting blood glucose

INTRODUCTION

Cardiovascular disease (CVD) is the primary cause of mortality in women in developed countries (1). It has long been noted that CVD seems to be rare among women younger than 45 years of age, but by age 70, women experience CVD at the same rate as their male counterparts (2). Furthermore, studies in pre-menopausal women show that hysterectomy is associated with higherrisk of CVD and stroke (3–5) and increments in blood lipids and lipoprotein cholesterol levels (6–8).

In addition, menopause associates with unfavorable changes in body composition, leading to increased fat tissue mass, and decreased lean body mass (9) as well as unfavorable changes in the blood lipid profile [i.e., increased serum total cholesterol, low-density lipoprotein cholesterol (LDL-C) and triglyceride levels and decreased high-density lipoprotein cholesterol (HDL-C) levels] (2). Therefore, menopause has been seen as one risk factor for developing metabolic syndrome and CVD, which may occur even independent of chronological aging (10–12).

Yet, some studies have reported an inconsistent association of menopause with the serum cardiovascular (CV) risk factors, including total cholesterol, LDL-C, HDL-C, triglycerides, and fasting blood glucose. To our knowledge at least nine cross-sectional (7, 13–20) and four longitudinal (21–24) studies are currently available with measurements of all or most of the above-mentioned clinically measured CV risk factors. Total cholesterol and LDL-C were found to be higher in post-menopausal women compared to pre-menopausal women by all the listed cross-sectional studies except one (16) in which difference in the LDL-C level was not significant. The results were much more variable for HDL-C, which two studies found to be lower (13, 17) and two higher (7, 20) in post-menopausal women compared to pre-menopausal, while five studies (14–16, 18, 19) reported no significant difference between menopausal groups. Of the discussed cross-sectional studies, one did not measure triglycerides (16) and five did not find significant group differences (7, 15, 18–20), while three (13, 14, 17) found higher levels for post-menopausal compared to pre-menopausal women. Fasting blood glucose was measured only in three of the studies (15, 17, 20), of which only Cho et al. reported significantly higher levels in post-menopausal women, while others did not find significant group differences. The longitudinal studies over the menopausal transition have also provided partially contradicting results. Jensen et al., Do et al., and Matthews et al. reported upregulation of total cholesterol, LDL-C and triglycerides over the menopausal transition while Abdulnour et al. did not find significant change for any of them. HDL-C was reported to be downregulated by Jensen et al. and Do et al. and upregulated by Matthews et al. and Abdulnour et al. Fasting glucose was measured only in studies by Matthews et al. and Abdulnour et al., which both found it to be upregulated over the menopausal transition. However, studies by Do et al. and Matthews et al. made specific remarks that not all of the results were associated specifically with the menopausal transition, but more likely with aging *per se*. Therefore, it is still controversial whether menopausal status or chronological aging has a more prominent association with serum CV risk factors.

The variability in the results obtained by the previous studies may partially be due to differences in the mean ages of the menopausal groups and in the determination of menopausal status, which in most cases relies on self-reports regarding menstrual cycle. Only six (13, 18, 20, 21, 23, 24) out of the 13 studies used additional hormone measurements to determine menopausal status. Furthermore, there may be confounding factors which have not been taken similarly into account in all studies. For example, only seven of the studies (13, 17, 18, 20, 22–24) attempted to measure potential differences in the physical activity (PA) levels of the study participants, and, of the seven, only Abdulnour et al. (24) used accelerometer first to measure physical activity and then to control differences in PA in their analysis. Therefore, large scale studies with precise determination of the menopausal status and PA are warranted to overcome the variability of the results and to eventually conclude if the menopausal transition has an effect on CV risk factors independent of aging.

The present study is a part of large-scale population-based cohort study, Estrogenic Regulation of Muscle Apoptosis (ERMA) (25), that comprised of healthy women aged 47–55 years and included both cross-sectional (*base-ERMA*) and longitudinal (*core-ERMA*) study designs. The current study had three major objectives. First, it sought to determine whether there are significant differences in serum CV risk factors between groups of middle-aged women classified as pre-menopausal, peri-menopausal, and post-menopausal according to follicle stimulating hormone (FSH) levels and bleeding patterns. Second, it examined the changes in serum CV risk factors over the menopausal transition using a longitudinal study design. Third, it evaluated whether any of the serum CV risk factor differences between study participants could be independently explained by menopausal status and leisure time physical activity (LTPA).

RESULTS

The current study is part of the ERMA study described previously (25). In brief, the ERMA study is a population-based cohort study comprising of women aged 47–55 years living in the city of Jyväskylä and neighboring municipalities, in Finland. The data presented here includes both cross-sectional (*base-ERMA*, $n = 886$) and longitudinal (*core-ERMA*, $n = 193$) study designs (**Figure 1**). Initially 1,393 participants completed the menopausal group assignments and health screen questionnaire, but between the first lab visit and the following physiological and psychological assessments, 507 participants were excluded or discontinued the study. Furthermore, participants taking prescription lipid-lowering medications (23 participants) were excluded from the final analysis. Finally, 886 women remained in the cross-sectional *base-ERMA* study setup and were used to explore the associations of menopausal status and LTPA with CV risk factors in a population-based, large-scale study design. The peri-menopausal women of the *base-ERMA* study entered into the longitudinal *core-ERMA* study. Of them, 193 went through the natural menopausal transition during the 16 ± 8 month follow-up period. The progression of the menopausal transition was evaluated based on menstrual bleeding diaries and hormone assessments taken in 3–6 month intervals. Therefore, the *core-ERMA* design enabled close follow-up of the menopausal transition from peri-menopause to early post-menopause, minimizing confounding factors.

Base-ERMA: Socio-Demographic and Life-Style Characteristics of the Participants

Socio-demographic and life-style factors including LTPA for each *base-ERMA* group are shown in **Table 1**. As expected, the mean age in the peri-menopausal group was significantly higher

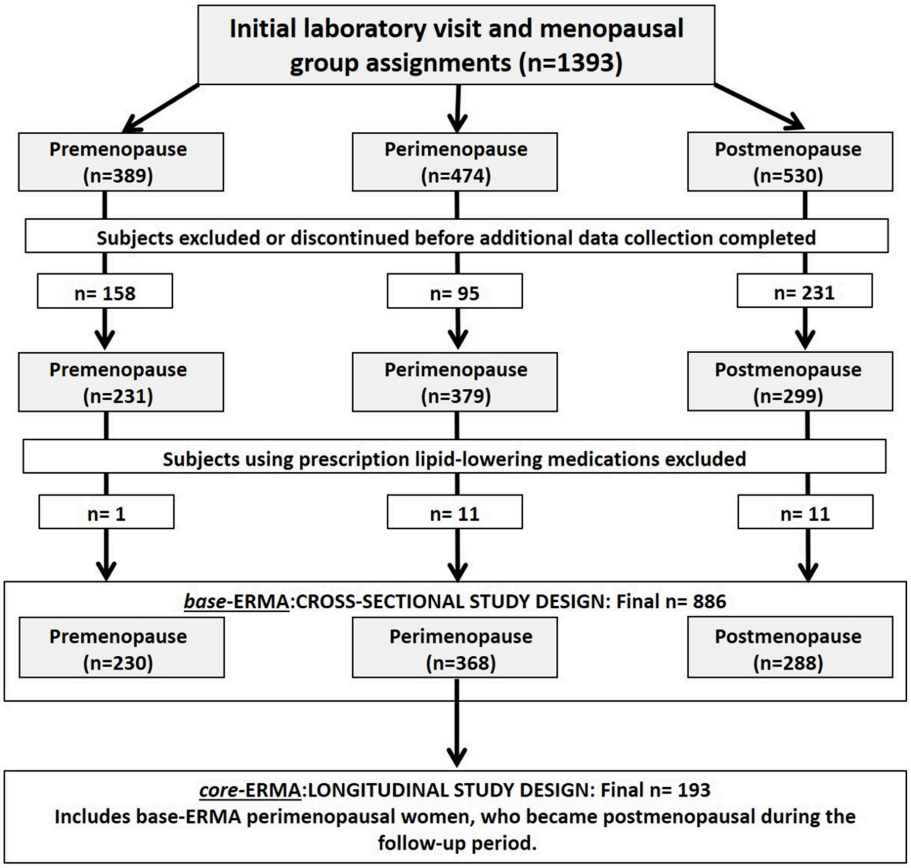

FIGURE 1 | Flow chart of the final subject group determination for the cross-sectional (*base-ERMA*) and longitudinal (*core-ERMA*) study designs.

than in the pre-menopausal group ($p < 0.001$), while the post-menopausal participants were significantly older than those in both the pre- and peri-menopausal groups ($p < 0.001$). The highest level of education achieved differed between the groups ($p = 0.019$), with pre- and peri-menopausal women appearing to achieve tertiary (master's or doctoral) levels of education at higher rates, while post-menopausal women were more likely to have achieved a secondary education level. The pre-menopausal group had higher total and lean body mass than the post-menopausal group ($p < 0.050$). Meanwhile, the post-menopausal group had significantly lower body mass index (BMI) and lean body mass than the peri-menopausal group ($p < 0.050$) but did not differ in percent body fat. The post-menopausal women had significantly higher leisure time vigorous PA in accelerometer-based activity measures compared with pre- and peri-menopausal women ($p < 0.050$), yet the groups did not differ in their PA total counts, self-reported LTPA or cardiorespiratory fitness assessed by 6-min walking test.

Base-ERMA: Gynecologic and Other Medical Factors

Data related to gynecologic and health factors as well as diseases and medication for each *base-ERMA* group are shown in **Table 2**. The pre-menopausal group had significantly lower FSH and higher estradiol levels than either the peri- or post-menopausal groups, and the post-menopausal FSH concentration was higher and estradiol level lower than the peri-menopausal group ($p < 0.001$). The groups differed significantly in the use of hormonal forms of contraceptives, with the pre-menopausal group appearing to use at higher rates than the other groups ($p < 0.001$). The groups did not differ in disease incidence or medications used. Fasting blood glucose concentrations were lower in the post-menopausal group than in both the pre-menopausal and peri-menopausal groups ($p < 0.05$).

Core-ERMA: Characteristics of the Participants

The participant group in the *core-ERMA* study design was comprised of a subset of subjects from the *base-ERMA* design's peri-menopausal group (**Figure 1**). Of the 368 women, 193 went through the menopausal transition from peri-menopause to early post-menopause in the course of the study thus enabling longitudinal data (**Table 3**). The characteristics from the *core-ERMA* study closely followed the differences identified in the *base-ERMA* (peri-menopausal vs. post-menopausal group). The FSH level increased and estradiol level decreased after

TABLE 1 | Characteristics of the study participants of the cross-sectional study design (*base-ERMA*).

	Pre-menopause *n* = 230	Peri-menopause *n* = 368	Post-menopause *n* = 288	*p*-value
Age [years]	50.64 (1.62)	51.19 (1.91)‡	52.54 (1.91)‡¶	**<0.001**
Age Range [years]	47-54	47-55	48-55	
Education [*n* (%)]			†*	**0.019**
Primary	1 (0.4)	8 (2.2)	7 (2.4)	
Secondary	126 (54.8)	198 (53.8)	181 (62.8)	
Tertiary	103 (44.8)	162 (44.0)	100 (34.7)	
Cigarette Smoking				0.837
Never Smoked	153 (66.5)	247 (67.1)	198 (68.8)	
Quit smoking	63 (27.4)	90 (24.5)	71 (24.7)	
Current Smoker	14 (6.1)	31 (8.4)	19 (6.6)	
Alcoholic drinks/wk	3.45 (3.17)	4.02 (3.67)	4.02 (4.18)	0.283
Percent Body Fat [%]	29.58 (7.12)	31.02 (7.77)	30.46 (7.32)	0.068
Body mass [kg][¤]	70.05 (10.13)	70.37 (11.08)	68.41 (10.99)†*	**0.050**
Lean Body Mass [kg]	45.99 (4.49)	45.23 (5.18)†	44.25 (4.66)‡*	**<0.001**
Body Mass Index [kg/m^2]	25.37 (3.29)	25.75 (3.85)	25.01 (3.74)*	**0.029**
Normal (<24.9)	113 (49.1)	180 (49.0)	162 (56.3)	
Overweight (25.0-29.9)	95 (41.3)	127 (34.6)	91 (31.6)	
Obese (>30.0)	22 (9.6)	60 (16.3)	35 (12.2)	
Self-reported leisure time PA [MET hours/day]	3.77 (3.94)	3.68 (3.80)	3.72 (3.42)	0.825
Accelerometer measured leisure time PA[§]				
Sedentary [min/day]	364.58 (45.89)	367.14 (52.44)	361.90 (50.66)	0.394
Light [min/day]	197.37 (40.83)	195.09 (43.59)	198.18 (43.44)	0.681
Moderate [min/day]	34.03 (16.52)	33.91 (17.96)	34.86 (17.79)	0.766
Vigorous [min/day]	4.03 (7.40)	3.86 (6.55)	5.05 (7.82)†*	**0.036**
Total counts	4.3×10^5 (1.2×10^5)	4.3×10^5 (1.4×10^5)	4.4×10^5 (1.3×10^5)	0.171
6-min walking distance [m][£]	674.76 (58.71)	666.44 (64.32)	669.60 (58.17)	0.281

Values are mean (standard deviation) for continuous variables and n (percentage) for categorical variables. †*p < 0.05 and p < 0.001 compared with pre-menopausal women. p < 0.05 and* ¶*p < 0.001 compared with peri-menopausal women.* §*Missing values = 126,* £*missing values = 76, and* ¤*missing values = 2. Statistically significant findings (p < 0.05) are marked with bold in all of the tables.*

menopause compared to the peri-menopausal values ($p < 0.010$). Body mass and body fat percentage increased and lean body mass decreased during the menopausal transition ($p < 0.010$). The prevalence of CVD or type 2 diabetes did not increase, yet hypertension became more common accompanied by an increase in the use of RAAS-acting agents ($p < 0.050$). Nevertheless, the group mean values of both systolic and diastolic blood pressure slightly decreased over the menopausal transition ($p = 0.035$ and $p < 0.001$, respectively). Of the 193 participants, 12 began to use HT during the follow-up period, yet this did not significantly affect the results (*data not shown*). There was no change in self-reported or accelerometer-measured LTPA.

Base- and *core-ERMA*: Association of Menopausal Status With Serum CV Risk Factors

The serum CV risk factors are presented in **Figure 2**. In the *base-ERMA* population serum total cholesterol concentrations were significantly higher in the post-menopausal group than either of the other groups ($p < 0.001$). Total cholesterol levels were also higher in the peri-menopausal group compared to the pre-menopausal group ($p < 0.050$). Serum LDL-C and HDL-C concentrations followed similar trends, with higher concentrations in the post-menopausal group compared to the pre- and peri-menopausal subjects ($p < 0.001$). A similar trend was seen in the *core-ERMA* population, as serum total cholesterol, LDL-C, HDL-C, and triglyceride levels increased already at early post-menopause compared to the previous peri-menopausal values ($p < 0.010$). In the *core-ERMA* population thetriglyceride level also increased during the menopausal transition ($p < 0.001$).

Table 4 displays output from regression models of the *base-ERMA* population constructed with menopausal status as an independent predictor of serum CV risk factors. In addition, the *p*-values from the Generalized Estimating Equations (GEE) model comparing peri- and early post-menopausal levels of the studied parameters from the *core-ERMA* population are included in the table. The direction of the changes is the same in the *base*-ERMA and *core*-ERMA populations (*β coefficients for GEE models not shown*). In *base-ERMA,* menopausal status was positively associated with all three cholesterol measures in the univariate model, models that had been adjusted only for self-reported LTPA, and fully adjusted (adjusted for

TABLE 2 | Gynecologic and health factors of the study participants of the cross-sectional study design (*base-ERMA*).

	Pre-menopause *n* = 230	Peri-menopause *n* = 368	Post-menopause *n* = 288	*p*-value
Follicle stimulating hormone [IU/L]	7.91 (3.51)	31.81 (21.02)‡	83.00 (29.84)‡¶	**<0.001**
Estradiol [nmol/L]	0.621 (0.659)	0.329 (0.262)‡	0.142 (0.964‡¶	**<0.001**
Hysterectomy	22 (9.6)	19 (5.2)	22 (7.6)	0.333
Contraceptive use [*n* (%)]				**<0.001**
No use	88 (38.3)	198 (53.8)	158 (54.9)	
Former	37 (16.1)	58 (15.8)	56 (19.4)	
Current	105 (45.7)	112 (30.4)	74 (25.7)	
LDL:HDL ratio	1.90 (0.77)	1.91 (0.77)	2.00 (1.01)	0.668
Fasting blood glucose [mmol/L]	5.20 (0.47)	5.23 (0.58)	5.13 (0.64)†*	**0.016**
Systolic BP [mm Hg]	130.42 (16.85)	131.45 (18.66)	130.66 (16.77)	0.900
Diastolic BP [mm Hg]	83.31 (9.48)	83.95 (10.12)	83.55 (9.69)	0.585
Leptin [ng/ml]§	16.40 (12.67)	16.24 (10.48)	15.88 (11.66)	0.565
Disease [*n* (%)]				
CVD	3 (1.3)	1 (0.3)	1 (0.3)	0.218
Hypertension	25 (10.9)	55 (14.9)	36 (12.5)	0.334
Type 2 diabetes	2 (0.9)	4 (1.1)	2 (0.7)	0.869
Medications [*n* (%)]				
RAAS-acting agents	17 (7.4)	46 (12.5)	27 (9.4)	0.115
β-blockers	16 (7.0)	24 (6.5)	23 (8.0)	0.765
Calcium-channel blockers	5 (2.2)	9 (2.4)	8 (2.8)	0.907
Drugs used in diabetes	0 (0.0)	3 (0.8)	2 (0.7)	0.406

*Values are mean (standard deviation) for continuous variables and n (percentage) for categorical variables. †$p < 0.05$ and ‡$p < 0.001$ compared with pre-menopausal women. *$p < 0.05$ and ¶$p < 0.001$ compared with peri-menopausal women. §Missing values = 7. Statistically significant findings ($p < 0.05$) are marked with bold in all of the tables.*

age, education, smoking, alcohol consumption, and body fat percentage) models ($p < 0.001$). The same analysis using accelerometer-measured LTPA as an adjustor is presented in the **Supplementary Table 1**. The longitudinal analysis using the core-ERMA population verified that the natural menopausal transition is the contributing factor. Also, in the adjusted models of *base-ERMA*, menopausal status was associated with triglyceride and fasting blood glucose levels ($p < 0.001$). In the *core-ERMA* population, the menopausal transition was positively associated with triglyceride levels ($p < 0.001$) but not with blood glucose levels. Leptin levels were measured only from the *base-ERMA* population and were not associated with menopausal status.

Base- and *core-ERMA*: Association of LTPA With Serum CV Risk Factors

Table 5 displays output from regression models of the *base-ERMA* population and the *p*-values from the GEE model with self-reported LTPA (MET-h/day) as an independent predictor of serum CV risk factors. The direction of the changes is the same in the *base*-ERMA and *core*-ERMA populations (*β coefficients for GEE models not shown*). In *base-ERMA,* self-reported LTPA was positively associated with HDL-C and negatively associated with all of the other measured serum CV risk markers in univariate and fully adjusted models with or without percentage of body fat (adjusted for age, education, smoking, and alcohol consumption, $p < 0.050$) except for total cholesterol, which was negatively associated with self-reported LTPA in a fully adjusted model only when percent body fat was not included as an adjustor ($p < 0.050$). In *base-ERMA*, we were also able to investigate the associations between PA and serum CV risk factors by using accelerometer measured LTPA as a variable. The self-reported LTPA correlated positively both with accelerometer measured LTPA and total PA ($\beta = 0.353$, $p < 0.001$, and $\beta = 0.270$, $p < 0.001$; respectively). When regression models were built using accelerometer measured LTPA (total counts), which was available from 760 participants from *base-ERMA* population, the results obtained by using self-reported LTPA were verified for HDL-C, triglycerides, and leptin, but, possibly due to lower number of participants leading to reduced statistical power, not for other investigated CV risk factors. Accelerometer measured LTPA was positively associated with HDL-C and negatively associated with triglycerides and leptin levels ($p < 0.05$, **Supplementary Tables 1**, **2**), and the same result was acquired when regression models were built using accelerometer measured total PA, which also included work time PA *(data not shown)*.

In the longitudinal *core-ERMA* population, very similar results than in the cross-sectional *base-ERMA* population were obtained. Self-reported LTPA was positively associated with HDL-C, and negatively with triglyceride and fasting blood glucose levels in univariate and fully-adjusted models with and without percentage body fat ($p < 0.050$). Self-reported LTPA was also negatively associated with LDL-C levels in univariate and fully-adjusted models without body fat percentage in the *core-ERMA* population ($p < 0.050$), yet LTPA was not associated with total cholesterol levels. Leptin levels were measured only from the *base-ERMA* population.

TABLE 3 | Characteristics of the participants of the longitudinal study design (*core-ERMA, n* = 193).

	Peri-menopause (baseline)	Early post-menopause (follow-up)	*p*-value
Follicle stimulating hormone [IU/L]	36.82 (21.10)	66.49 (34.73)	**<0.001**
Estradiol [nmol/L]	0.334 (0.245)	0.263 (0.265)	**<0.001**
LDL:HDL ratio	1.84 (0.76)	1.81 (0.73)	0.263
Fasting blood glucose [mmol/L]	5.20 (0.54)	5.17 (0.60)	0.569
Systolic BP [mm Hg][§]	131.59 (19.56)	129.71 (18.55)	**0.035**
Diastolic BP [mm Hg][§]	83.62 (10.31)	81.49 (10.05)	**<0.001**
Percent Body Fat [%]	30.79 (8.27)	31.84 (7.89)	**<0.001**
Body mass [kg][†]	69.66 (11.23)	70.36 (11.48)	**0.001**
Lean Body Mass [kg]	44.91 (5.37)	44.57 (5.23)	**0.002**
Disease [*n* (%)][§]			
CVD	0 (0.00)	0 (0.00)	1.00
Hypertension	25 (13.20)	28 (14.80)	**<0.001**
Type 2 diabetes	2 (1.10)	2 (1.10)	1.00
Medications [*n* (%)]			
RAAS-acting agents	19 (9.80)	24 (12.40)	**0.025**
β-blockers	16 (8.30)	19 (9.80)	0.083
Calcium-channel blockers	4 (2.10)	6 (3.10)	0.157
HT-usage (*n*)	0 (0.0)	12 (6.20)	0.317
Self-reported leisure time PA [MET hours/day][¤]	3.66 (3.84)	3.43 (3.77)	0.151
Accelerometer measured leisure time PA (total counts)[#]	4.18×10^5 (1.28×10^5)	4.11×10^5 (1.27×10^5)	0.459

Values are mean (standard deviation) for continuous variables and n (percentage) for categorical variables. [§]*Missing values = 2,* [†]*missing values = 3,* [¤]*missing values = 4,* [#]*missing values = 44. Statistically significant findings (p < 0.05) are marked with bold in all of the tables.*

DISCUSSION

The present study observed the menopause-associated changes in the serum lipid profile and the role of LTPA in decreasing the levels of these CV risk factors in healthy middle-aged women both with cross-sectional (*base-ERMA*) and longitudinal (*core-ERMA*) study designs. Our study showed that menopausal status was independently associated with higher serum total cholesterol, LDL-C and HDL-C concentrations, and an increasing trend was observed in each of these parameters with advancing menopause status while adjusting for age and LTPA both in *base-ERMA* and *core-ERMA* study designs. Our results highlight that the menopausal transition, not aging, drives the increase in total cholesterol and LDL-C levels, as well as an increase in the less-reported HDL-C level. In addition, we found that self-reported LTPA was associated with higher serum HDL-C and lower serum LDL-C levels as well as lower triglyceride and fasting blood glucose levels, independent of menopausal status in both of our study designs.

Menopausal Status Is Associated With Serum CV Risk Factors Over Chronological Age

An accumulating body of literature shows that menopause is associated with unfavorable changes in lipid metabolism leading to an increased likelihood of developing metabolic syndrome and CVD (9–11, 23, 26). These changes can partially be opposed by estrogen-containing hormone therapy (HT). The mechanistic pathways of how estrogen and its receptors affect CVD have been studied extensively, yet mostly in pathophysiological conditions with animal models (27). In women, when started at post-menopause, orally administered estrogen reduces LDL-C levels (28, 29). The observed reduction in LDL-C seems to be a result of accelerated conversion of hepatic cholesterol to bile acids and increased expression of LDL receptors on cell surfaces leading to improved clearance of LDL from plasma (30, 31). Orally administered estrogen also increases HDL-C levels in post-menopausal women, which is due to decreased hepatic lipase activity and increased production of apolipoprotein A, the main protein component of HDL particles (29). However, there is a variety of HDL subclasses with different functions. Thus far, subparticle HDL_2 is considered the most active in reverse cholesterol transport making it the key candidate in decreasing CV risk (32). Furthermore, in female mice, estrogen signaling through hepatocyte ERα was shown to regulate reverse cholesterol transport (33). The deletion of hepatocyte ERα both decreased the capacity of HDL to export cholesterol and increased serum total cholesterol level as well as HDL particle size. Yet we lack the information regarding which specific HDL particles increase with HT and only a few studies have investigated if menopause negatively affects functionality of HDL particles (34, 35).

Nevertheless, the existing literature has reported somewhat inconsistent associations of menopause with triglyceride and HDL-C levels. In contrast to our findings, two large European cross-sectional studies observed no significant differences in triglyceride or HDL-C levels after adjustment for age (14, 15), highlighting the role of chronological age on these particular serum CV risk factors. To further support this observation, a longitudinal American study found the increment in total

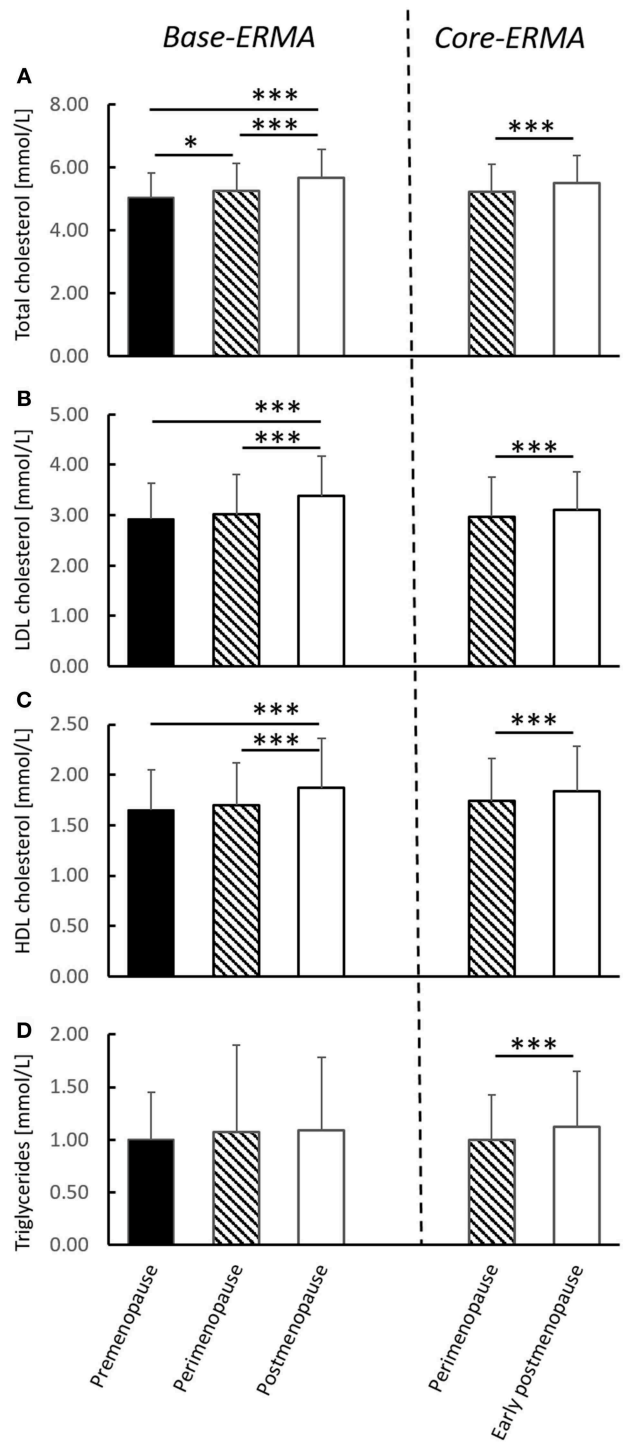

FIGURE 2 | Serum CV risk factors from the cross-sectional (*base-ERMA*) and longitudinal (*core-ERMA*) study designs. Total cholesterol **(A)**, LDL-C **(B)**, HDL-C **(C)**, and triglyceride levels **(D)** from the pre-, peri- and post-menopausal groups in the *base-ERMA* design and from peri-menopause (baseline) and early post-menopause (follow-up) from the *core-ERMA* population. Recommended levels: Total cholesterol <5.2 mmol/L (<200 mg/dl), LDL <2.6 mmol/L (<100 mg/dl), HDL >1.5 mmol/L (60 mg/dl), and triglycerides <1.7 mmol/L (<150 mg/dl) (Mayo clinic, USA). Figures show average + SD, $^{*}p < 0.050$, $^{***}p < 0.001$.

cholesterol and LDL-C to follow the curve of FSH consistent with menopause-induced changes, while the increment in triglycerides and HDL-C were more gradual and followed the chronologic aging curve (23). In the present study, total cholesterol, LDL-C and HDL-C increased in both study designs and triglycerides in *core-ERMA* during the menopausal transition. The *Core-ERMA* study was designed to measure women as soon as possible after they became post-menopausal (verified by repeatedly high FSH levels), and thus was able to already capture changes in the measured biomarkers with this rather short (16 ± 8 months) follow up period only expanding to early post-menopause, strongly suggesting the underlying cause of changes in CV risk factors to be menopausal hormonal changes rather than aging. In previous studies, the age range of the subjects has been large (from 18 to 70-year-old women) and/or the determination of menopausal status has relied mostly on retrospective self-reports. In our present study, the age range of the subjects is very narrow (47–55 years) and menopausal status was defined both by using the bleeding diaries and blood hormonal level measurements, making our study design more robust in studying the effects of aging and menopausal status on CV risk factors. Our *core-ERMA* is the first longitudinal study to report that menopausal transition associates not only with higher LDL-C and total cholesterol levels, but also with higher triglycerides and HDL-C, independent of chronological aging, while controlling differences in physical activity.

Some other studies have found HDL-C levels to increase acutely before menopause and decrease after menopause (11, 23, 36). In our *base-ERMA* study, HDL-C was progressively higher across the stages of menopause from pre- to post-menopause as well as increased during the menopausal transition from peri-menopause to early post-menopause in *core-ERMA* (**Figure 2**). In line with our results, increased HDL-C levels with menopause have been previously reported (36–38), although this has been generally less highlighted compared to the menopausal increment in LDL-C. Traditionally, higher HDL-C levels have been considered to be atheroprotective. Therefore, it is not straightforward to understand why increased HDL-C accompanies the otherwise clearly negative menopausal changes in the lipid profile. There have been several attempts to explain this unexpected change, such as relatively high physical activity levels (38), yet all explanations point to the fact that low HDL-C levels do not seem to be the main factor causing increased CVD risk or metabolic syndrome in post-menopausal women (37). In our study, the results of the regression and GEE models did not change after adjusting for LTPA, i.e., the changes in lipid profile were not due to decrease in LTPA level. Woodard et al. (36) speculated that the higher HDL-C at post-menopause may be explained by functional and compositional changes in HDL particles (36, 39). More specifically, among women, the profile of the HDL and LDL particle sizes seems to shift around menopause or over the menopausal transition toward more atherogenic lipoprotein subtypes (13, 26, 36). Indeed, it seems that the atheroprotective effect of HDL may be weaker in women after menopause (36, 40). Therefore, in the future, measuring lipoprotein particles and separately analyzing lipoprotein subclasses with simultaneous analysis of

TABLE 4 | Univariate and multivariate linear regression models with menopausal status as independent predictor of serum CV risk factors (*base-ERMA*).

	Total Cholesterol [mmol/l]					**LDL Cholesterol [mmol/l]**					**HDL Cholesterol [mmol/l]**				
	β	***p*-value**	**R^2**	***p*-value**	***p*-value**	**β**	***p*-value**	**R^2**	***p*-value**	***p*-value**	**β**	***p*-value**	**R^2**	***p*-value**	***p*-value**
	Menopausal status		**Full model**		**GEE-model***	**Menopausal status**		**Full model**		**GEE-model***	**Menopausal status**		**Full model**		**GEE-model***
Univariate model	0.282	**<0.001**	0.080	**<0.001**	**<0.001**	0.242	**<0.001**	0.058	**<0.001**	**0.001**	0.200	**<0.001**	0.040	**<0.001**	**<0.001**
LTPA-adjusted model	0.282	**<0.001**	0.088	**<0.001**	**<0.001**	0.242	**<0.001**	0.077	**<0.001**	**0.001**	0.200	**<0.001**	0.065	**<0.001**	**<0.001**
Fully adjusted model[§]	0.238	**<0.001**	0.115	**<0.001**	**<0.001**	0.185	**<0.001**	0.118	**<0.001**	**0.001**	0.181	**<0.001**	0.148	**<0.001**	**<0.001**

	Triglycerides [mmol/l]					**Fasting blood glucose [mmol/l]**					**Leptin [ng/ml]**			
	β	***p*-value**	**R^2**	***p*-value**	***p*-value**	**β**	***p*-value**	**R^2**	***p*-value**	***p*-value**	**β**	***p*-value**	**R^2**	***p*-value**
	Menopausal status		**Full model**		**GEE-model***	**Menopausal status**		**Full model**		**GEE-model***	**Menopausal status**		**Full model**	
Univariate model	0.043	0.204	0.002	0.204	**<0.001**	−0.062	0.066	0.004	0.066	0.383	−0.018	0.588	0.000	0.588
LTPA-adjusted model	0.042	0.202	0.032	**<0.001**	**<0.001**	−0.062	0.061	0.031	**<0.001**	0.383	−0.019	0.551	0.074	**<0.001**
Fully adjusted model[§]	0.036	0.309	0.093	**<0.001**	**<0.001**	−0.076	**0.031**	0.076	**<0.001**	0.383	−0.035	0.177	0.519	**<0.001**

**GEE model of serum CV risk factors (core-ERMA). β, standardized regression coefficient; R^2, Coefficient of determination, statistically significant coefficients are highlighted. [§]Model is adjusted for age, education level, smoking status, alcohol consumption, and percent body fat. Statistically significant findings ($p < 0.05$) are marked with bold in all of the tables.*

TABLE 5 | Univariate and multivariate linear regression models with self-reported LTPA (MET-h/day) as independent predictor of serum CV risk factors (*base-ERMA*).

	Total Cholesterol [mmol/l]					**LDL Cholesterol [mmol/l]**					**HDL Cholesterol [mmol/l]**				
	β	***p*-value**	**R^2**	***p*-value**	***p*-value**	**β**	***p*-value**	**R^2**	***p*-value**	***p*-value**	**β**	***p*-value**	**R^2**	***p*-value**	***p*-value**
	LTPA		**Full model**		**GEE-model***	**LTPA**		**Full model**		**GEE-model***	**LTPA**		**Full model**		**GEE-model***
Univariate model	−0.093	**0.005**	0.009	**0.005**	0.475	−0.137	**<0.001**	0.018	**<0.001**	**0.013**	0.157	**<0.001**	0.025	**<0.001**	**<0.001**
Fully adjusted model[£]	−0.048	0.157	0.115	**<0.001**	0.804	−0.072	**0.033**	0.118	**<0.001**	0.363	0.086	**0.009**	0.148	**<0.001**	**0.004**
Fully adjusted model without percent body fat[$]	−0.076	**0.019**	0.107	**<0.001**	0.622	−0.119	**<0.001**	0.095	**<0.001**	**0.038**	0.165	**<0.001**	0.084	**<0.001**	**<0.001**

	Triglycerides [mmol/l]					**Fasting blood glucose [mmol/l]**					**Leptin [ng/ml]**			
	β	***p*-value**	**R^2**	***p*-value**	***p*-value**	**β**	***p*-value**	**R^2**	***p*-value**	***p*-value**	**β**	***p*-value**	**R^2**	***p*-value**
	LTPA		**Full model**		**GEE-model***	**LTPA**		**Full model**		**GEE-model***	**LTPA**		**Full model**	
Univariate model	−0.173	**<0.001**	0.030	**<0.001**	**<0.001**	−0.166	**<0.001**	0.027	**<0.001**	**<0.001**	−0.272	**<0.001**	0.074	**<0.001**
Fully adjusted model[£]	−0.098	**0.004**	0.093	**<0.001**	**0.002**	−0.098	**0.005**	0.076	**<0.001**	**0.001**	−0.061	**0.013**	0.519	**<0.001**
Fully adjusted model without percent body fat[$]	−0.166	**<0.001**	0.045	**<0.001**	**<0.001**	−0.160	**<0.001**	0.037	**<0.001**	**<0.001**	−0.269	**<0.001**	0.075	**<0.001**

**GEE model of serum CV risk factors (core-ERMA). β, standardized regression coefficient; R^2, Coefficient of determination, statistically significant coefficients are highlighted. [£]Model is adjusted for age, education level, smoking status, alcohol consumption, menopausal status, and percent body fat. [$]Model is adjusted for age, education level, smoking status, alcohol consumption and menopausal status. Statistically significant findings ($p < 0.05$) are marked with bold in all of the tables.*

blood hormone levels may provide additional insight into the association between lipids and CVD risk factors at menopause.

LTPA May Attenuate Menopause-Associated Unfavorable Changes in Serum CV Risk Factors

More recently, studies have concentrated on how PA affects the detrimental changes associated with menopause. The current literature has rather controversial results regarding the effect of exercise on blood lipid profiles of menopausal women. Some controlled intervention trials show an improvement on blood lipid levels after either resistance or aerobic exercise training in post-menopausal women (41–44). Yet in all these intervention studies, exercise was combined with either estrogen-containing hormone replacement therapy (HT) or weight-reducing diets, or the study subjects were dyslipidemic at the start of the intervention. Two more recent studies (20, 45) with healthy subjects showed a positive effect of exercise on the blood lipid profile. Behall et al. (45) compared the type of exercise to effects on plasma lipids in overweight, pre- and post-menopausal women, and found that the type of exercise (resistance or aerobic) was more important to post-menopausal than pre-menopausal women, aerobic exercise being more efficient on lowering serum total cholesterol level (45). Mandrup et al. (20) found that 3 month high-intensity training improved several CVD factors in healthy, non-obese post-menopausal women, including lower diastolic blood pressure, total cholesterol, and LDL-C (20). Nonetheless, some intervention studies have shown no improvement in blood lipid levels after exercise training (46–49), even with training interventions similar to those associated with positive effects in other studies. In all, exercise appears to result in improvements in the blood lipid profile of overweight or dyslipidemic post-menopausal subjects, yet results are less consistent with subjects that are healthy at baseline.

In the present study designs, self-reported LTPA level was associated with higher HDL-C and lower LDL-C levels in fully adjusted models that did not include percent body fat (**Table 5**). In the *base-ERMA* population, LTPA was no longer associated with total cholesterol when percent body fat was included in the models, although it still was associated with lower LDL-C and higher HDL-C. Excess body weight has been associated with higher serum total cholesterol, LDL-C and triglyceride levels, indicating that high body weight, or high body fat percentage, is linked to dyslipidemia (50–52). This association most likely causes the difference in the predictive power of LTPA with and without body fat percentage in cholesterol levels in our study setup. Correlation analysis showed that increasing PA levels were associated with decreased total and LDL-C concentrations and increased HDL-C. Self-reported LTPA was also a highly significant predictor of triglycerides, fasting blood glucose, and leptin (**Table 5**). Moreover, we found no interaction between LTPA and menopausal status in the *base-ERMA* population serum CV risk factor regression models, suggesting that LTPA associates similarly among middle-aged women regardless of their menopausal status. This is important comprehensive evidence on several different serum CV risk factors indicating that PA beneficially affects the CVD risk of healthy middle-aged women. In particular, PA has the potential to combat the progressive increment in LDL-C levels with advancing menopause status, which, in combination with its association with other biomarkers of CVD risk, may further alleviate disease risk.

Even though high LTPA evidently has health benefits, our results revealed that a similar LTPA level than at pre-menopause was not sufficient to prevent the unfavorable lipid profile changes associated with the menopausal transition. This observation is in line with previous literature, where exercise intervention was either accompanied with HT and/or diet (42, 43), or exercise was done at high-intensity level (20) to gain healthier blood lipid profiles in post-menopausal women. Hence, to obtain a clinically relevant effect on serum CV risk factors, a greater LTPA dose and/or simultaneous changes in diet may be necessary in healthy, post-menopausal women.

Study Strengths and Limitations

This study has several important strengths to note. It is part of a large, comprehensive cohort study designed to characterize the changes that occur in women at the time of menopause using previously validated research methods. It relied on a combination of bleeding diaries and serum FSH levels, rather than on self-report alone to accurately classify women into the proper menopausal groups. Participants were subjected to extensive questionnaires, physical measurements, and lab testing to effectively identify the variables exerting an effect on the subjects' CVD risk. In addition, we were able to obtain data from longitudinal study design from the same participants as in the cross-sectional study design, further strengthening our findings. The discontinuation rate was low, and the participant number was larger than in many studies that have previously attempted to address related issues.

The current study also has some limitations. Although our study provides valuable information about changing CV risk profiles associated with different menopausal status and degree of PA, its clinical relevance is somewhat limited by the lack of prospective, patient-centered outcomes, such as CV event incidence, CV event mortality, and all-cause mortality.

We collected retrospective survey data on the occurrence of myocardial infarction and stroke. This historical data was helpful to assess the baseline cardiovascular health of the participants. However, if accelerated atherosclerosis is indeed the primary mechanism by which menopause increases CVD risk, it is unlikely that the adverse event incidence would change quickly enough to be captured in this study. Currently we are performing a follow-up study from the ERMA study population, termed EsmiRs (www.jyu.fi/esmirs/en). EsmiRs will provide 4-year follow-up since the baseline of the ERMA study allowing us to investigate if the serum lipid profile continues to worsen in this population. However, even longer follow-ups are needed to define how detrimental the currently revealed early signs of worsening lipid profiles due to menopause will clinically turn out to be.

This study was designed to intensively characterize the differences seen due to menopause among women aged 47–55 years and during the menopausal transition from peri-menopause to early post-menopause and identify differences and early changes in CV risk factors that may help to explain the well-documented increases in CVD in post-menopausal women later in their lives. In the future, more sophisticated metabolomics analyses may further increase the understanding of the relation of LTPA and menopausal transition to CV risk factors (53).

In the present study, we chose to use self-reported LTPA in the association analyses to retain higher sample sizes and to utilize an estimation of LTPA activity representing a longer period than captured by the usual 7-day use of the accelerometer. Using accelerometer-measured LTPA decreases the sample size in cross-sectional study design by 126 subjects, which greatly decreases the power of the analysis, as shown in **Supplementary Tables 1**, **2**. The self-reported questionnaire data focused on capturing the average physical activity level over an extended period of time, which is what will influence the cardiovascular risk profile. The accelerometers, by contrast, offered only a 7-day snapshot of the physical activity. However, it is known that self-reports are prone to over-estimation of PA. Therefore, we also used accelerometers to measure LTPA and did not find significant differences between group means of total activity counts. Self-reported LTPA correlated significantly both with accelerometer measured LTPA and total PA. Also, the group difference we observed in accelerometer measured vigorous LTPA (highest level in post-menopausal women) would not favor the worse cholesterol profile observed in the post-menopausal group. Therefore, it is unlikely that a possible reporting bias in self-reported LTPA would lead to overestimation of associations observed between menopausal status or LTPA and serum CV risk factors.

Finally, the population studied here was highly homogenous, as every woman enrolled identified herself as Caucasian/white. Therefore, it is unknown the extent to which these results are generalizable to women of other ethnic groups or those living in developing countries with less robust health systems.

CONCLUSIONS

In conclusion, this large-scale cohort study of middle-aged women complemented by longitudinal investigations of women over menopause found that the menopausal transition is associated with increases in serum levels of total cholesterol, LDL-C, and HDL-C that are independent of age, percent body fat and LTPA. The level of LTPA, in turn, is an independent predictor of the variation in LDL-C and HDL-C concentrations between the participants and higher PA level is associated with cardioprotective effects such as lower LDL-C and higher HDL-C, as well as lower levels of triglycerides, fasting blood glucose, and leptin. These results suggest that the menopausal transition is a stronger determinant of serum CV risk factors than chronological age. We observed that LTPA may attenuate menopause-associated atherogenic changes of healthy middle-aged women, yet it does not seem to entirely compensate for the alterations in the serum lipid profile. Hence, to obtain a clinically relevant effect on serum CV risk factors, a greater LTPA dose and/or or simultaneous dietary intervention may be necessary in healthy, post-menopausal women.

MATERIALS AND METHODS

Study Population

The ERMA study is a population-based cohort study comprising of Caucasian women aged 47–55 years living in the city of Jyväskylä (Finland) and neighboring municipalities (**Figure 1**). The collection of the cross-sectional *base-ERMA* data proceeded in three phases (pre-questionnaire, group assignments and health screen questionnaire, and laboratory visit with psychological and physiological measures), as detailed in Kovanen et al. (25). Of the total cohort, 82% was approached by a postal inquiry and invited to take part in the study and to return the pre-questionnaire enabling screening for exclusion. Of the initial 1,393 participants who completed the group assignments and health screen questionnaire, 389 were pre-menopausal, 474 peri-menopausal, and 530 post-menopausal. The response rate for the postal inquiry was 45%. Those willing to participate and fulfilling the inclusion criteria were asked to fill out a menstrual diary for at least 12 weeks before the first laboratory visit for initial health screening and blood sampling. Participants who currently used, had used during the past 3 months estrogen-containing contraceptives or other estrogen-containing medications, used lipid lowering medication, had had bilateral oophorectomy, were pregnant or lactating, had polycystic ovary syndrome or other conditions affecting ovarian function, had a BMI $>$ 35 kg/m^2 (based on self-reported height and weight), or had any musculoskeletal disorder seriously affecting everyday PA were excluded. Additionally, participants having conditions or use of medications affecting daily mental or physical function or systemic hormone or inflammatory status were excluded (25). Between the initial health screening questionnaire and the following physiological and psychological assessments, 471 participants were excluded and 36 discontinued the study. Finally, participants without available data for all variables used in the regression models, due to missing values or study discontinuation (4 participants), as well as those taking prescription lipid-lowering medications (23 participants) were excluded from the final analysis of the current study. After all exclusions and dropouts, 886 women remained in the cross-sectional *base-ERMA* study setup: 230 pre-menopausal, 368 peri-menopausal, and 288 post-menopausal. The *base-ERMA* population was recruited to explore the associations of menopausal status and LTPA on serum CV risk factors in a population based, large-scale study design.

Of the 368 peri-menopausal women in the *base-ERMA* study, 193 went through the natural menopausal transition during the 16 $\pm$ 8 month follow-up period, forming the longitudinal *core-ERMA* study design (**Figure 1**). The *core-ERMA* design was reconstructed from *base-ERMA* to enable the examination of a smaller cohort of the exact same individuals from peri-menopause to early post-menopause, minimizing confounding factors. The same exclusion criteria as for the *base-ERMA* study were applied for the *core-ERMA* study, with the exception that participants who had begun using hormone-replacement therapy ($n = 12$) were not excluded from the analysis.

Ethics Statement

The ERMA study was approved by the Ethics Committee of the Central Finland Health Care District (KSSHP Dnro 8U/2014). All study participants gave written informed consent. The study protocol followed good clinical and scientific practice and the Declaration of Helsinki.

Blood Sampling and Serum CV Risk Factor Profiling

Fasting (12 h) blood samples were taken from the antecubital vein in supine position between 7:00 and 10:00 a.m. and during the first 5 days of the menstrual cycle, when the cycle was predictable. For serum separation, whole blood was left to clot for 30 min at room temperature and centrifuged at 2,200 × g before aliquoting and storing the sera at −80°C. Serum FSH and estradiol levels were determined using IMMULITE® 2000 XPi (Siemens Healthcare Diagnostics, UK) according to manufacturer's instructions. Blood glucose, total cholesterol, LDL-C, HDL-C and triglycerides were measured using KONELAB 20 XTi analyzer (Thermo Fischer Scientific, Finland) according to manufacturer's instructions. Leptin was measured using Human Leptin ELISA-kit (RD191001100, BioVendor, Czech Republic) according to manufacturer's instructions.

Determination of Menopausal Status

The determination of menopausal status as pre-menopausal, peri-menopausal, or post-menopausal is based on the menstrual diary and measured FSH level as has been presented in detail earlier (25). The main procedure follows the Stages of Reproductive Aging Workshop (STRAW)+10 guidelines, which define different menopausal states as follows; pre-menopausal women have regular menstrual cycle with variable FSH and estradiol, peri-menopausal women have irregular menstrual cycle with elevated FSH and lowered estradiol and post-menopausal women have at least 12 months of amenorrhea with high (> 30 IU/L) FSH and low estradiol (54). However, due to study feasibility reasons, participants were asked to keep bleeding diaries for a minimum of 3 months before blood was drawn for hormone assessment, which may have led some peri-menopausal women to be erroneously categorized as post-menopausal. By the time of the second laboratory visit, women categorized as naturally post-menopausal had provided diaries showing an average of 150 ± 71 days without menstrual bleeding and FSH levels 83 ± 30 IU/L.

Another potential source for misclassification is the unreliability of the bleeding pattern. Therefore, for participants whose menstrual bleeding pattern was not completely natural, i.e., they had undergone hysterectomy or were using progesterone-containing contraceptives, the group assignment relied on the FSH assessment, but with more stringent cut-off values (given in parentheses) than those used for participants with natural menstrual bleeding. A participant was categorized as pre-menopausal, if she had a regular bleeding pattern and FSH < 17 IU/L (15 IU/L), as peri-menopausal, if she had irregular or no bleeding and FSH 17–30 IU/L (15–39 IU/L) and as post-menopausal, if she had no menstrual bleeding and FSH > 30 IU/L (>39 IU/L). A few women who had very high FSH levels (>130 IU/L), but had still occasional bleeding days, were also categorized as post-menopausal.

The *core-ERMA* participants, who at baseline were determined to be peri-menopausal, revisited our laboratory in 3–6 months intervals and their hormone levels were measured. Before determining women had reached early post-menopausal status, the FSH levels needed to be high in two consecutive measurements.

Socio-Demographic and Life-Style Factors and Health-Related Variables

Education, smoking, and use of alcohol were assessed by standard questionnaires. Education was classified as primary, secondary or tertiary based on the highest education level reported by the participant. Participants were categorized based on their smoking habits as never, quitter, or current smoker. According to reported use of alcohol, participants' mean consumption per week was calculated. Information regarding diseases, use of medication, and gynecological issues was self-reported using standard, pre-structured questionnaires. The presence of chronic conditions and use of prescribed medication were confirmed via nurse's interview according to a pre-structured questionnaire and current prescriptions. The Anatomical Therapeutic Chemical (ATC) Classification System by the World Health Organization (WHO) was used for drug classification, with A10: drugs used in diabetes, C07: β-blockers, C08: calcium channel blockers, and C09: agents acting on the renin–angiotensin (RAAS) system (https://www.whocc.no/atc_ddd_index). Only those drug classes known to influence the serum CV risk factors used in cardiovascular risk assessments are reported here.

Anthropometrics, Body Composition, Physical Fitness, and Physical Activity

Anthropometrics and body composition were measured between 7:00 and 10:00 a.m. after overnight fasting and physical fitness after light breakfast in the same day as reported in Kovanen et al. (25). Briefly, body weight was measured with a beam scale and height by a stadiometer with the participant wearing only undergarments and BMI was calculated as weight (kg)/height squared (m^2). Body fat percentage and lean body mass were assessed with a multifrequency bioelectrical impedance analyzer (InBody^{TM} 720; Biospace, Seoul, Korea). Physical fitness was assessed by 6-min walking test (55). The test was performed on a 20-m indoor track, and participants were instructed to complete as many laps as possible within 6 min to assess submaximal exercise tolerance and aerobic capacity.

For PA assessments, both self-reports and accelerometer measures were used. The PA questionnaire is described previously (56). Briefly, the metabolic equivalent (MET) of PA was calculated as a product of intensity, duration and frequency from 3 questions of LTPA. The following MET values were used: 4 (for PA intensity corresponding to walking), 6 (vigorous walking to jogging), 10 (jogging), and 13 (running). The total LTPA was expressed as MET-h/day. In addition PA was measured with 7-day use of GT3X+ or wGT3X+ ActiGraph

accelerometer (Pensacola, Florida, USA) as reported previously (57). Participants were instructed to wear the monitors on their right hip for 7 consecutive days during their waking hours except while bathing or doing other water-based activities. They were also provided with a diary and instructed to record their wake-up time, working hours, and periods, when the monitor was removed for longer than 30 min. Raw acceleration data were collected at 60 Hz, filtered and converted into 60-s epoch counts. A customized Excel-based program was used for further data analysis. Mean time spent at different PA intensities i.e., light, moderate, and vigorous was calculated for each participant using tri-axial vector magnitude cut-points of 450, 2,690, and 6,166 cpm, respectively (57, 58). The cut-point of upper vector magnitude limit was 25,000 cpm. LTPA was separated from whole day PA based on the notes of working hours in the activity diaries. The PA data were then normalized to 10-h wake time for LTPA and 16-h wake time for total PA (59). For association analyses, the self-reported LTPA was used. This enabled using the whole dataset ($n = 886$) with higher statistical power, as accelerometer measured PA was available only from 760 subjects. Furthermore, the self-reported questionnaire data estimated the average physical activity level over an extended period of time instead of the accelerometer measured, 7-day measurements of the PA.

Statistics

Data are presented as mean and standard deviation (SD) for continuous variables and as frequency (n) and percentage (%) for categorical variables. The normality of the variables was assessed using the Shapiro-Wilks test. As only a few variables met the normal distribution criteria, differences between the menopausal groups were investigated using the Kruskal-Wallis and Mann-Whitney tests. To estimate the independent association of predictor variables with the serum lipid variables in the *base-ERMA* design, univariate, and multivariate linear regression models were constructed. Before building up the multivariate regression models, homoscedasticity was tested, the autocorrelation was tested with Durbin-Watson test, and multicollinearity was inspected with variance inflation factor to fit into the recommended range. Correlations between self-reported and accelerometer-measured LTPA and total PA were investigated using Spearman's correlation. The change in characteristics of peri-menopausal women, who went through the menopausal transition during follow-up time (the *core-ERMA* design), were examined using Wilcoxon test. To estimate the independent association of predictor variables with the serum CV risk factors in the *core-ERMA* design, the Generalized Estimating Equations (GEE) model was used. In the GEE model, the baseline measurement was used for age, education, smoking status, alcohol consumption, fat percentage and self-reported LTPA. Data analysis was carried out using IBM SPSS Statistics software version 24 (Chicago, IL, US), and the level of significance was set at $p < 0.05$.

Sensitivity Analyses

Separate sensitivity analyses were performed for the *base-ERMA* population, with the participants using progesterone-based contraceptives in the past 3 months or having had hysterectomies excluded from the analysis in order to evaluate if unreliability issues in the menstrual bleeding patterns or the complete lack of bleeding had influenced our primary analyses. The results of the sensitivity analyses did not markedly differ from our primary analysis (*data not shown*). Separate sensitivity analyses were also performed for the *core-ERMA* population excluding the participants using HT ($n = 12$), and results did not significantly differ from our primary analysis (*data not shown*).

DATA AVAILABILITY

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

MJ and SK wrote the manuscript and did the statistical analysis of the studied parameters. MH helped drafting the manuscript and ran the regression analysis. TT is responsible for the accelerometer data. PA for the gynecological data. UK for the medical examinations. SS, VK, and EL planned the ERMA study and provided financial support. EL and UK supervised drafting the manuscript and interpretation of the data regarding the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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BPA Alters Estrogen Receptor Expression in the Heart After Viral Infection Activating Cardiac Mast Cells and T Cells Leading to Perimyocarditis and Fibrosis

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Myocarditis is an inflammatory heart disease that leads to dilated cardiomyopathy (DCM) and heart failure. Sex hormones play an important role in the development of myocarditis with testosterone driving disease in males and estrogen being cardioprotective in females. The human population is widely exposed to the endocrine disruptor bisphenol A (BPA) from plastics such as water bottles, plastic food containers, copy paper, and receipts. Several clinical and numerous animal studies have found an association between elevated BPA levels and cardiovascular disease. A recent report found elevated levels of BPA in the serum of patients with DCM compared to healthy controls. In this study we examined whether exposure to BPA for 2 weeks prior to viral infection and leading up to myocarditis at day 10 altered inflammation in female BALB/c mice housed in standard plastic cages/water bottles with soy-free food and bedding. We found that a human relevant dose of BPA (25 μg/L) in drinking water, with an estimated exposure of 5 μg BPA/kg BW, significantly increased myocarditis and pericarditis compared to control water without altering viral genome levels in the heart. BPA exposure activated ERα and ERβ in the spleen 24 h after infection and phosphorylated ERα and ERβ during myocarditis, but decreased ERα and increased ERβ mRNA in the heart as measured by qRT-PCR. Exposure to BPA significantly increased $CD4^+$ T cells, IFNγ, IL-17A, TLR4, caspase-1, and IL-1β in the heart. BPA exposure also increased cardiac fibrosis compared to controls. Mast cells, which are associated with cardiac remodeling, were found to increase in number and degranulation, particularly along the pericardium. Interestingly, plastic caging/water bottle exposure alone led to increased mast cell

numbers, pericardial degranulation and fibrosis in female BALB/c mice compared to animals housed in glass cages/water bottles with soy-free food and bedding. These data suggest that BPA exposure may increase the risk of developing myocarditis after a viral infection in women.

Keywords: myocarditis, endocrine disruptor, bisphenol A, estrogen receptor, mast cells

INTRODUCTION

Myocarditis is an inflammatory heart disease that can lead to dilated cardiomyopathy (DCM) and heart failure and is most often caused by viral infections such as coxsackievirus B3 (CVB3) (1–3). We recently reported that cardiac inflammation during viral myocarditis in mice is increased by testosterone and reduced by 17β-estradiol, and the sex ratio in patients with myocarditis in the study was 3.5:1 male to female (4). Additionally, men with myocarditis are more likely to develop cardiac fibrosis than women and progress to DCM and heart failure (5, 6).

Estrogen receptors (ERs) are located on/in immune cells, cardiomyocytes, endothelial cells, and cardiac fibroblasts (7–9). ERα is believed to mediate most of the cardioprotective effects of estrogen in women and female mice (7). ERα has been found to protect against CVB3 myocarditis by increasing disease in ERα knockout mice while infected male mice treated with the ERα agonist propylpyrazoletriol were protected (10, 11). ERβ protects male and female mice from hypertrophy induced using transverse aortic constriction (TAC) by decreasing genes associated with mitochondrial damage, especially in males (12). However, ERβ has also been found to increase collagen synthesis from cardiac fibroblasts in male and female mice, while ERα decreases collagen synthesis (13–15). In CVB3 myocarditis, ERβ signaling was found to promote myocarditis in male or female mice treated with the ERβ agonist diarylpropionitrile (10, 11). Little is known about the effect of estrogen-related receptor (ERR)γ signaling on/in immune cells or on cardiac physiology or disease (16). Since CVB3 myocarditis is influenced by sex hormones and ERs, endocrine disruptors could play a role in the development and severity of disease.

People of all ages have detectable levels of bisphenol A (BPA) or its metabolized products in their body fluids (17, 18). BPA, an endocrine disruptor, is known to be found in items such as plastic water bottles, plastic food containers, the lining of cans, on thermal receipts, and photocopy paper (19–22). BPA has been found in nearly all patients when assessed in urine or blood, but the actual effect on heath and disease is largely unknown (18).

There have been studies assessing the pharmacokinetics and metabolism of BPA in humans and mice including following oral exposure. The half-life of BPA is 6 h and it is primarily metabolized to the glucuronide form prior to excretion (23). BPA has other metabolites that make up a smaller portion of its metabolism (23). BPA is metabolized to BPA glucuronide by UDP-glucuronosyltransferase and by sulfotransferase to BPA sulfate to a lesser degree (24). BPA is excreted in both the feces and urine with unconjugated BPA primarily excreted in feces whereas metabolized BPA is more predominantly excreted in the urine (23). Studies have also determined that the exposure route of BPA influences its pharmacokinetics and the clinical relevance of animal studies. Oral exposure of BPA has been found to more closely match levels found in humans compared to subcutaneous injection or bolus gavage routes (25). Recent studies have concluded that the pharmacokinetics of oral exposure to BPA is more similar between mice and humans than originally thought (26). Serum levels of BPA in mice vary greatly depending on the exposure route, with an oral exposure route being the most clinically relevant (27).

BPA can act as an estrogen agonist when it binds ERs including ERα, ERβ, and ERRγ but BPA has also been found to act on the androgen receptor (AR) (28, 29). BPA can bind to the AR where it acts in an anti-androgenic manner, but the AR is not able to bind to the androgen response element when BPA is bound instead of androgen (30). BPA preferentially binds ERβ over ERα but the metabolized forms of BPA, such as BPA glucuronide, do not bind the ERs (28). Epidemiological and animal data indicate that increased exposure to BPA worsens cardiovascular diseases including hypertension (31–33), atherosclerosis (34–36), myocardial infarct (37), arrhythmias (13, 14), and DCM (38). To our knowledge, no one has examined the role of BPA on myocarditis. In this study we examined whether BPA exposure in drinking water could alter CVB3 myocarditis in adult female BALB/c mice housed in plastic cages. Different doses of BPA were dissolved in drinking water for 2 weeks prior to intraperitoneal (ip) injection with CVB3 and self-tissue to induce autoimmune myocarditis and exposure continued until harvest at day 10 post-infection (pi). In order to determine the possible effect of exposure of mice to BPA or other plastics/ chemicals leached from the plastic cages, we compared the effect of plastic vs. glass cages/housing on myocarditis in separate experiments. Our autoimmune model of CVB3 myocarditis has the advantage of being a highly translatable animal model where the disease time-course, pathology, severity, sex differences, biomarkers and progression to DCM closely match patients (4, 39), providing a good animal model to test whether BPA is able to alter cardiac inflammation and the severity of disease.

MATERIALS AND METHODS

Animal Care Ethics Statement

Mice were used in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Mice were maintained under pathogen-free conditions in the animal facility at the Johns Hopkins School of Medicine and at Mayo Clinic Florida, and

approval obtained from the Animal Care and Use Committee at Johns Hopkins University and Mayo Clinic Florida for all procedures. Mice were sacrificed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

CVB3-Induced Autoimmune Myocarditis Model

Female BALB/c (stock #651) 5 week old adult mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were maintained under pathogen-free conditions in the animal facility at the Johns Hopkins School of Medicine or the Mayo Clinic Florida animal facility. The number of mice used in individual experiments are listed in the text or figure legends. At 8 weeks of age mice were inoculated intraperitoneally (ip) with 10^3 plaque forming units (PFU) of heart-passaged stock of CVB3 that contained cardiac self-tissue on day 0 and acute myocarditis examined at day 10 pi, as previously described (40). CVB3 (Nancy strain) was originally obtained from the American Type Culture Collection (ATCC; Manassas, VA) and grown in Vero cells (ATCC), as previously described (40).

Bisphenol A and Housing

Mice arrived from Jackson Labs at 5 weeks of age and were placed in plastic cages/plastic water bottles for BPA exposure experiments or glass cages/glass water bottles for control experiments. All experiments used bedding (Envigo-Tekland, 7990.BG) and food (Envigo-Tekland, 2020X) from Envigo (Minneapolis, MN) that were free of soy and phytoestrogens to exclude other naturally occurring estrogenic compounds.

Varying doses of BPA were given to 6 week old mice dissolved in drinking water for 2 weeks prior to inoculation ip with CVB3 containing cardiac self-tissue. BPA exposure was continued from day 0 of viral infection until harvest at day 10 pi. At harvest heart tissue was divided in half and ½ heart used for histology, qRT-PCR, ELISA or western blot. Separate experiments were conducted in order to obtain all endpoints. Estimated intake of BPA for mice in drinking water was based on Jenkins et al. (41) who found that a 20 g mouse drinks an estimated 4 mL of water a day (**Table 1**). Jenkins et al. and Cagen et al. previously reported that BPA at these doses in water is stable for 1 week (41, 42). For this reason, as well as to provide the mice with fresh water, BPA water was replaced each week of the experiment.

TABLE 1 | Bisphenol A (BPA) doses[a].

Treatment (μg BPA/L)	Estimated intake (μg BPA/kg BW)	Human exposure level
0	0	Control
2.5	0.5	Human relevant exposure
25	5	High human relevant exposure
250	50	EPA reference dose

[a](41).

The doses of BPA that were administered were 2.5, 25, and 250 μg BPA/L in drinking water, which is equivalent to an estimated intake of 0.5, 5, and 50 μg BPA/kg body weight (BW), respectively, based on predicted daily exposure levels in the human population [(41); **Table 1**]. At the time of the development of this project Jenkins et al. was the only study available that assessed the effect of BPA in a mouse model using oral exposure with a clinically relevant dose of BPA in the drinking water. The EPA reference dose was calculated using a safety factor of 1,000x the lowest observable adverse effect level (LOAEL) (43). The EPA reference dose is defined as an estimate of the daily exposure to a susceptible individual without an appreciable risk of deleterious effects during a lifetime.

Histology

Mouse hearts were cut longitudinally and fixed in 10% phosphate-buffered formalin and embedded in paraffin for histological analysis. Five micrometer sections were stained with hematoxylin and eosin (H&E) to detect inflammation, Masson's trichrome and picrosirius red to detect collagen or toluidine blue to detect mast cell granules. Myocarditis, pericarditis and fibrosis were assessed as the percentage of the heart with inflammation or fibrosis compared to the overall size of the heart section using a microscope eyepiece grid, as previously (44). Sections were scored by at least two individuals blinded to the treatment group.

Quantitative Real-Time PCR

At harvest spleen or half heart was collected and stored at −80°C for RNA isolation. Spleens and hearts were homogenized and lysed using a Tissuelyser (Qiagen) with 7 mm stainless steel beads in RTL buffer with 0.5% DX buffer to reduce foam (Hilden, Germany). The homogenate was placed in an automated RNA isolation and purification instrument, QIAcube, with reagents for RNase Easy Mini Kit with a DNase step for spleen and cells or RNase Easy Fibrous Mini Kit including a DNase and proteinase K step for heart tissue (Qiagen). Spleen RNA was eluted into 30 μL and heart RNA into 60 μL RNase free water (Qiagen). RNA quantification was determined in μg/μL using NanoDrop (Thermo Scientific, Waltham, MA).

Cell Isolation

Cardiac tissue was dissociated using enzyme buffer mixtures from the Miltenyi Biotec Mouse and Rat Heart Dissociation kit (#130-098-373) in gentleMACS C tubes (#130-098-237; 130-096-334) according to manufacturer's instructions. CD45 cells were isolated from the heart using the Miltenyi Biotec "LS" bead capture system (CD45 Ly-5 MicroBeads rat IgG2b, clone 30F11.1). We confirmed that the CD45 population had a purity of 94% while the cardiac/cardiomyocyte fraction was 97% pure using qRT-PCR for the markers CD45 and Myl2, respectively.

qRT-PCR

Total messenger RNA (mRNA) from mouse spleens, hearts or heart cell isolates was assessed by quantitative real time (qRT)-PCR using Assay-on-Demand primers and probe sets and the ABI 7000 Taqman System from Applied Biosystems (Foster City, CA) after RNA was

converted to cDNA using High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems), as previously described (44). Data are shown as relative gene expression (RGE) normalized to the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (Hprt).

Measurement of CVB3 Genome Levels by qRT-PCR

Probe sets to detect CVB3 in the heart were developed by Antoniak et al. and obtained from Integrated DNA Technologies (Coralville, IA) (45). Probe sets: CVB3 forward, 5′-CCCTGAAT GCGGCTAATCC-3′; CVB3 reverse, 5′-ATTGTCACCATAAG CAGCCA-3′; CVB3 probe, 5′-FAM-TGCAGCGGAACCG-TA MRA-3′.

ELISA

Mouse hearts were homogenized at 10% w/v in 2% minimal essential medium for use in ELISAs (R&D Systems, Minneapolis, MN), as previously described (46).

Western Blot

Heart tissues were dissected, snap frozen on dry ice and stored at −80°C. Tissues were lysed by homogenizing using mechanical cell disperser with RIPA buffer (Santa Cruz Biotechnology) and protease/phosphate inhibitor cocktail (BioRad) to obtain proteins from membrane, cytoplasm and nucleus. Extracted proteins were separated on a Criterion XT precast bis-tris 4–12% gel (BioRad) then transferred onto nitrocellulose membrane (BioRad). Samples were probed with primary antibodies (Abcam) ERα (mouse IgG_1 monoclonal, H226, 1:200), phospho-ERα (rat polyclonal IgG, Serine 118, 1:200), ERβ (mouse monoclonal IgG_{2a}, 1531 1:200), phospho-ERβ (rat polyclonal IgG, Serine 87, 1:200), and normalized to Hprt (goat polyclonal IgG, N-15, 1:200). The molecular weight of the proteins of interest were: Hprt−23 kDa, ERα/pERα-66 kDa, and ERβ/pERβ-56 kDa. The phosphorylation of the ER does not add additional molecular weight when assessing via western blot. Species-specific secondary antibodies were conjugated to horseradish peroxidase (HRP). Protein concentrations were normalized to the housekeeping gene (Hprt) and experimental groups were compared using densitometric analyses (Image J).

Statistical Analysis

Normally distributed data comparing two groups were analyzed using a 1- or 2-tailed Student's *t*-test. The Mann-Whitney rank sum test was used to evaluate non-parametric data. When comparing more than two groups 1-way or 2-way ANOVA was performed followed by multiple comparison analysis (Dunnett's multiple comparisons test or Tukey's multiple comparison, respectively) with each group compared to the corresponding control group. Data are expressed as mean ± SEM. A value of $p < 0.05$ was considered significant.

RESULTS

Disease Development in Response to BPA

BPA Exposure in Drinking Water Increases Viral Myocarditis in Female BALB/c Mice Housed in Plastic Cages

To assess the effect of BPA exposure on CVB3-induced myocarditis, female adult BALB/c mice housed in traditional plastic cages were fed varying doses of BPA in drinking water *ad libitum* for 2 weeks prior to ip infection with CVB3 and heart tissues on day 0 until harvest at day 10 pi during myocarditis. Mice were provided soy-free bedding and food, because soy contains genistein which is estrogenic. We found that a high human relevant dose (5 μg BPA/kg BW) ($p = 0.006$) and EPA reference dose (50 μg BPA/kg BW) ($p = 0.04$) of BPA increased myocarditis compared to control water without BPA (0 BPA) using 2-tailed Student's *t*-test and one-way ANOVA (all doses $p = 0.01$) (**Figure 1A**). However, after adjusting for multiple comparisons only the 5 μg/kg BW dose of BPA was significantly different from the control water ($p < 0.01$).

Laboratory animals are traditionally housed in polycarbonate cages with polysulfone water bottles. Both of these plastics contain BPA which can leach from the plastic, especially when heated as occurs when cages are routinely autoclaved for sterilization (47). As cages age, more plastic can leach due to degradation of the surface (47). In order to determine whether BPA that may have leached from the plastic cages and plastic water bottles used in our experiments affected myocarditis, in separate experiments we compared glass cages and water bottles (no added BPA) to plastic cages and water bottles (possible BPA or other plastics/chemicals released due to leaching). We used soy-free bedding and food to remove the effect of these estrogenic compounds. The water bottles in glass and plastic cages contained normal water (i.e., did not contain additional BPA) throughout the experiment. Female BALB/c mice were infected with CVB3 ip on day 0 and myocarditis assessed at day 10 pi. We found that there was no significant difference between the level of myocardial inflammation (i.e., myocarditis) that developed in female BALB/c mice housed in plastic compared to glass cages ($p = 0.50$) (**Figure 1B**). Thus, BPA or other chemicals that leached from plastic cages and/or plastic water bottles was not able to increase myocarditis, indicating that BPA added to the drinking water was responsible for the increase in myocarditis over controls.

BPA Exposure in Drinking Water Does Not Alter Viral Levels in the Heart of Female BALB/c Mice Housed in Plastic Cages

We examined whether BPA exposure in drinking water altered CVB3 levels in the heart during myocarditis at day 10 pi in female mice housed in plastic cages. We found that BPA exposure did not significantly alter viral genome levels in the heart by qRT-PCR for any dose of BPA (0 BPA vs. 0.5 μg BPA/kg BW, $p = 0.29$; vs. 5 μg BPA/kg BW, $p = 0.13$; or vs. 50 μg BPA/kg BW, $p = 0.41$). There was also no difference in viral genome levels in female BALB/c mice with myocarditis that were housed in plastic vs. glass cages without addition of BPA to drinking water ($p =$

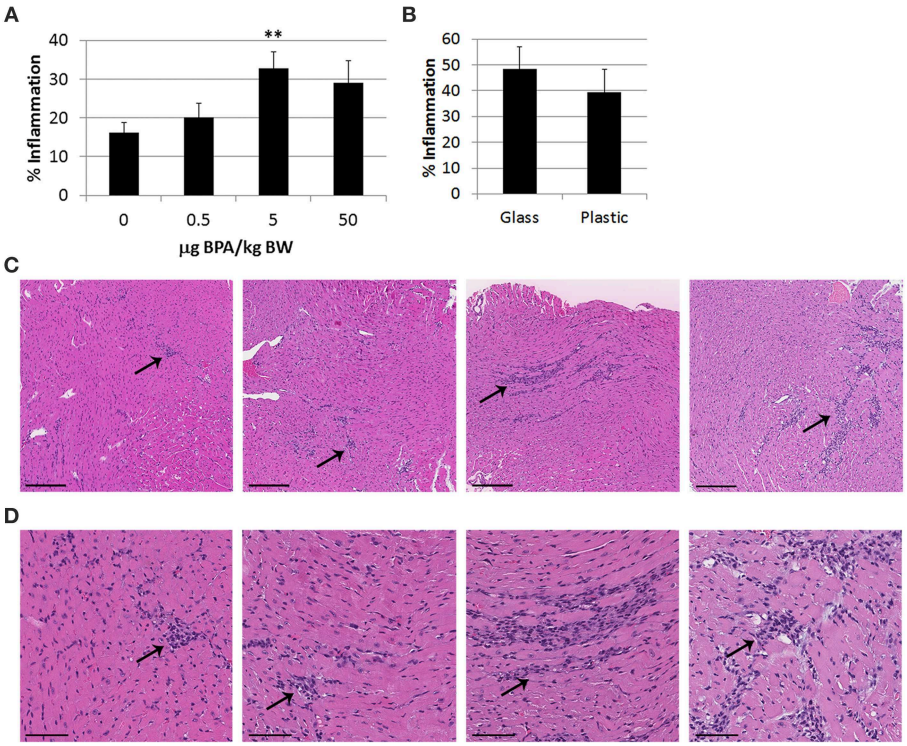

FIGURE 1 | BPA exposure in drinking water increases viral myocarditis in female BALB/c mice housed in plastic cages. **(A)** Female BALB/c mice housed in plastic cages with no soy in food or bedding were given 0, 0.5, 5, and 50 µg BPA/kg BW BPA in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 on day 0 and exposure continued until harvest for myocarditis at day 10 pi. Myocarditis was assessed as % inflammation in the heart compared to the overall size of the heart section by histology using an eyepiece grid. Data show the mean ±SEM (0 µg BPA/kg $n = 10$, 0.5 µg BPA/kg $n = 9$, 5 µg BPA/kg $n = 9$, 50 µg BPA/kg $n = 10$). One-way ANOVA found a significant difference between all groups ($p = 0.01$) and 0 vs. 5 µg BPA/kg BW (**$p < 0.01$). **(B)** Female BALB/c mice were housed in glass or plastic cages with glass or plastic water bottles, respectively, and no soy in food or bedding for 2 weeks prior to ip infection with CVB3 to induce myocarditis. The drinking water did not contain BPA. Mice were injected ip with 10^3 PFU CVB3 ip on day 0 and hearts were harvested at day 10 pi during acute myocarditis. Data show the mean ± SEM (glass $n = 10$, plastic $n = 10$). Two-tailed Student's *t*-test found no significant difference between groups. **(C,D)** Representative H&E photos depict myocarditis at 0, 0.5, 5, and 50 µg BPA/kg BW at **(C)** magnification x100, scale bar 200 µm or **(D)** magnification x300, scale bar 70 µm, arrows indicate inflammatory foci.

0.18). Thus, BPA exposure in drinking water did not alter viral replication, and increased myocarditis with BPA exposure was not due to increased viral replication.

BPA Exposure in Drinking Water Increases Pericarditis in Female BALB/c Mice Housed in Plastic Cages

Pericarditis is common in patients with myocarditis and is observed in our animal model (48). We found that BPA exposure in drinking water of female BALB/c mice housed in plastic cages significantly increased pericarditis at day 10 pi (1-way ANOVA, $p = 0.002$) (**Figure 2A**). Controlling for multiple comparisons revealed that the 5 µg and 50 µg/kg BW doses of BPA significantly increased pericarditis at day 10 pi compared to control water ($p < 0.05$ and $p < 0.001$, respectively). When we examined whether BPA leached from plastic cages or water bottles could alter pericarditis compared to glass cages and water bottles we found that there was no significant difference between glass and plastic caging ($p = 0.18$) (**Figure 2B**). Normal pericardium consists of one layer of pericardial cells on the outer surface of the heart as depicted in **Figure 2C**. A representative photo of pericarditis that was promoted by exposure to BPA in drinking water is depicted in **Figure 2D**.

BPA Exposure in Drinking Water Increases Cardiac $CD4^+$ T and Mast Cells in Mice Housed in Plastic Cages, Plastic Cages Alone Increase Mast Cells

Because myocarditis was significantly increased by the high human relevant dose of BPA (i.e., 5 µg BPA/kg BW) (**Figure 1**), all future analyses compared the 0 BPA to the 5 µg BPA/kg BW (5 BPA) groups.

In female BALB/c mice exposure to 5 µg BPA/kg BW significantly increased cardiac $CD4^+$ T cells based on expression of CD4 levels ($p = 0.03$) and mast cells based on cKit/CD117 expression ($p = 0.004$) vs. 0 BPA controls (**Table 2**). We also examined expression of CD45, GR1, CD11b, F4/80, CD3, CD8, B220, and Foxp3, but found no significant difference between 0 BPA and 5 µg BPA/kg BW for any of the other immune cell markers (**Table 2**). An increase in $CD4^+$ T cells and mast cells during myocarditis following

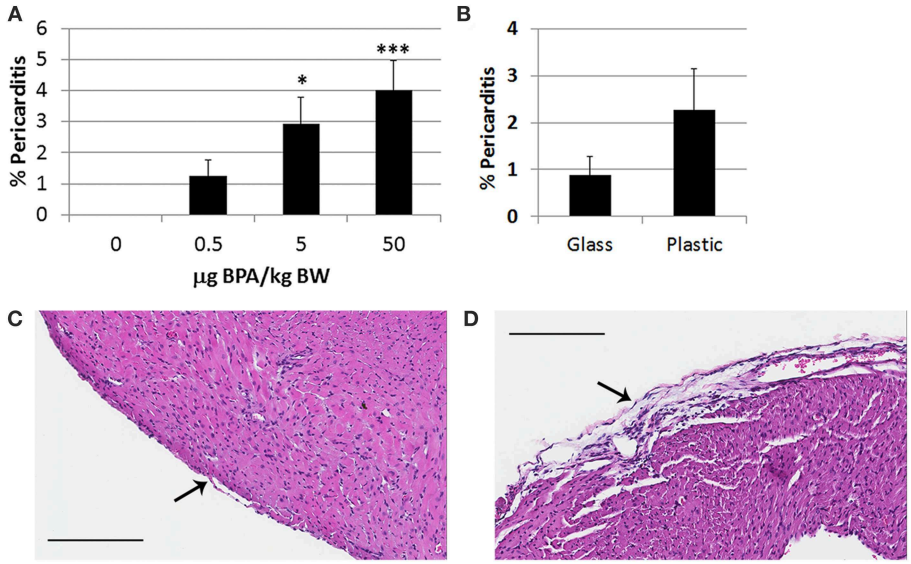

FIGURE 2 | BPA exposure in drinking water increases pericarditis in female BALB/c mice housed in plastic cages. Female BALB/c mice housed in plastic cages with no soy in food or bedding were given increasing doses of BPA in drinking water for 2 weeks and injected with 10^3 PFU CVB3 ip on day 0 and pericarditis examined at day 10 pi during acute myocarditis. BPA exposure continued from day 0 to 10 pi. Pericarditis was assessed as the % pericardial inflammation in the heart with H&E compared to the overall size of the heart section by histology using an eyepiece grid. **(A)** Data show the mean ± SEM (0 µg BPA/kg $n = 10$, 0.5 µg BPA/kg $n = 9$, 5 µg BPA/kg $n = 9$, 50 µg BPA/kg $n = 10$). One-way ANOVA found a significant difference existed between groups ($p = 0.002$). After controlling for multiple comparisons, the 5 and 50 µg BPA/kg BW groups were significantly different than 0 BPA control water (*$p < 0.05$ and ***$p < 0.001$, respectively). **(B)** Female BALB/c mice were housed in glass or plastic cages with glass or plastic water bottles and no soy in food or bedding for 2 weeks prior to ip infection with CVB3 to induce myocarditis. The drinking water did not contain BPA. Data show the mean ± SEM (glass $n = 10$, plastic $n = 10$). Two-tailed Student's *t*-test found no significant difference between groups. Representative photos depict **(C)** 0 BPA and **(D)** 50 µg BPA/kg BW, bar 200 µm, magnification 100x, arrows point to outer pericardial layer of the heart.

TABLE 2 | Effect of BPA exposure in drinking water in mice housed in plastic cages on expression of immune cell markers in the heart during myocarditis using qRT-PCR.

Cell marker	Description	0 BPA[a,b]	5 BPA	*P*-value
CD45	Total immune cells	4.3 ± 1.1	6.2 ± 1.8	0.43
GR1	Neutrophils	7.5 ± 0.7	7.4 ± 2.2	0.35
CD11b	Mac, neu, MC, DC	4.7 ± 0.8	3.1 ± 0.6	0.09
F4/80	Macrophages	4.7 ± 0.6	4.1 ± 0.7	0.47
CD3	All T cells	20.3 ± 8.3	4.4 ± 1.4	0.43
CD4	$CD4^+$ T cells	2.8 ± 0.4	4.9 ± 1.1	0.03
CD8	$CD8^+$ CTL cells	108.9 ± 58.6	6.3 ± 2.0	0.75
Foxp3	Regulatory T cells	20.3 ± 8.3	4.4 ± 1.4	0.43
B220	B cells	10.5 ± 3.9	5.4 ± 1.1	0.58
cKit	MC	76.6 ± 6.1	131.7 ± 20.5	0.004

[a]*CLT, cytolytic T cells; DC, dendritic cells; Mac, macrophages; MC, mast cells; Neu, neutrophils.*

[b]*0 BPA, control water without BPA; 5 BPA, high human relevant dose (5 µg/kg BW).*

TABLE 3 | Effect of plastic vs. glass housing on expression of immune cell markers in the heart during myocarditis using qRT-PCR.

Cell marker	Description	Glass	Plastic	*P*-value
CD45	Total immune cells	8.72 ± 1.8	8.2 ± 2.2	0.87
CD11b	Mac[a], neu, MC, DC	6.2 ± 1.0	5.5 ± 1.3	0.68
F4/80	Macrophages	6.8 ± 1.0	6.3 ± 1.4	0.77
CD3	All T cells	6.9 ± 1.6	6.3 ± 1.3	0.73
CD4	$CD4^+$ T cells	10.1 ± 1.9	9.1 ± 2.5	0.77
CD8	$CD8^+$ CTL cells	7.7 ± 1.1	7.1 ± 2.4	0.81
Foxp3	Regulatory T cells	2,073 ± 1,191	1,990 ± 627	0.95
CD19	B cells	5.9 ± 1.4	4.6 ± 1.5	0.53
cKit	Mast cells	1.9 ± 0.2	3.1 ± 0.4	0.04

[a]*CLT, cytolytic T cells; DC, dendritic cells; Mac, macrophages; MC, mast cells; Neu, neutrophils.*

BPA exposure is consistent with the increased myocardial and pericardial inflammation observed with histology for the 5 µg BPA/kg BW dose (**Figures 1**, **2**). Interestingly, cardiac mast cells were increased by qRT-PCR based on cKit expression in plastic vs. glass cages in mice with CVB3 myocarditis (**Table 3**). No other immune cell population was significantly altered by exposure to plastic compared to glass cages (**Table 3**).

BPA Exposure in Drinking Water Increases Cardiac Mast Cell Numbers and Pericardial Mast Cell Degranulation in Mice Housed in Plastic Cages

Previously, BPA exposure in culture was found to increase mast cell number and activation (49); however, the effect of

BPA on cardiac mast cells during myocarditis was unknown. The effect of 5 μg BPA/kg BW exposure on cardiac mast cells during myocarditis at day 10 pi was assessed using qRT-PCR and toluidine blue staining of heart histology sections, which detects mast cell granules. We found that BPA exposure significantly increased the expression of the mast cell marker cKit by qRT-PCR in the heart (0 BPA 76.6 ± 6.1 vs. 5 BPA 131.7 ± 20.5, $p = 0.004$) (**Figure 3A**).We found that BPA exposure significantly increased the total number of mast cells in the heart by histology ($p = 0.007$) compared to 0 BPA control (**Figure 3B**). A representative photo of mast cells that are not degranulating are shown in **Figure 3C** and degranulating mast cells in **Figure 3D**.

By 2-way ANOVA we found that during myocarditis more mast cells were not degranulating compared to those that were degranulating ($p < 0.0001$) (**Figure 3E**), and BPA exposure in drinking water increased mast cell degranulation in the heart overall (2-way ANOVA, $p = 0.0006$) (**Figure 3E**). However, 5 μg BPA/kg BW did not increase the number of mast cells that were not degranulating, but significantly increased the number of degranulating mast cells during myocarditis ($p < 0.05$) compared to the 0 BPA group (**Figure 3E**).

Next we examined the number of degranulating vs. non-degranulating mast cells in different locations of the heart (i.e., myocardial, vascular, pericardial). We found that more myocardial mast cells were not degranulating than degranulating ($p < 0.0001$) (**Figure 3F**), but note the few number of mast cells found at this location. BPA did not increase the number of myocardial mast cells in the heart overall ($p = 0.71$) (**Figure 3F**). Two-way ANOVA and a Student's *t*-test ($p = 0.09$) comparison of 0 BPA to 5 BPA did not find a significant difference in mast cell degranulation in the myocardium. Similar to myocardial and overall mast cell numbers, there was no increase in the number of vessel-associated mast cells that were not degranulating vs. degranulating following BPA exposure ($p = 0.11$) (**Figure 3G**). There was an increase in vessel-associated mast cells that were degranulating after BPA exposure using Student's *t*-test ($p = 0.005$), but this difference was no longer significant by 2-way ANOVA (**Figure 3G**). Similar to other locations, more pericardial mast cells were not degranulating than degranulating (2-way ANOVA, $p < 0.0002$) (**Figure 3H**). In contrast to other locations, however, BPA exposure significantly increased degranulation of pericardial mast cells overall (2-way ANOVA, $p = 0.0004$) and in the degranulating subgroup ($p < 0.01$) compared to the 0 BPA controls (**Figure 3H**). Note that the largest numbers of mast cells in the heart during myocarditis are located along the pericardium.

We found other markers of mast cell activation were also increased in the heart during myocarditis by 5 BPA compared to control by qRT-PCR including IgE receptor-γ (0 BPA 1.7 ± 0.1 vs. 5 BPA 2.1 ± 0.0.3, $p = 0.03$) and the anaphylaxis receptor C3aR1 (0 BPA 2.3 ± 0.2 vs. 5 BPA 3.1 ± 0.3, $p = 0.04$).

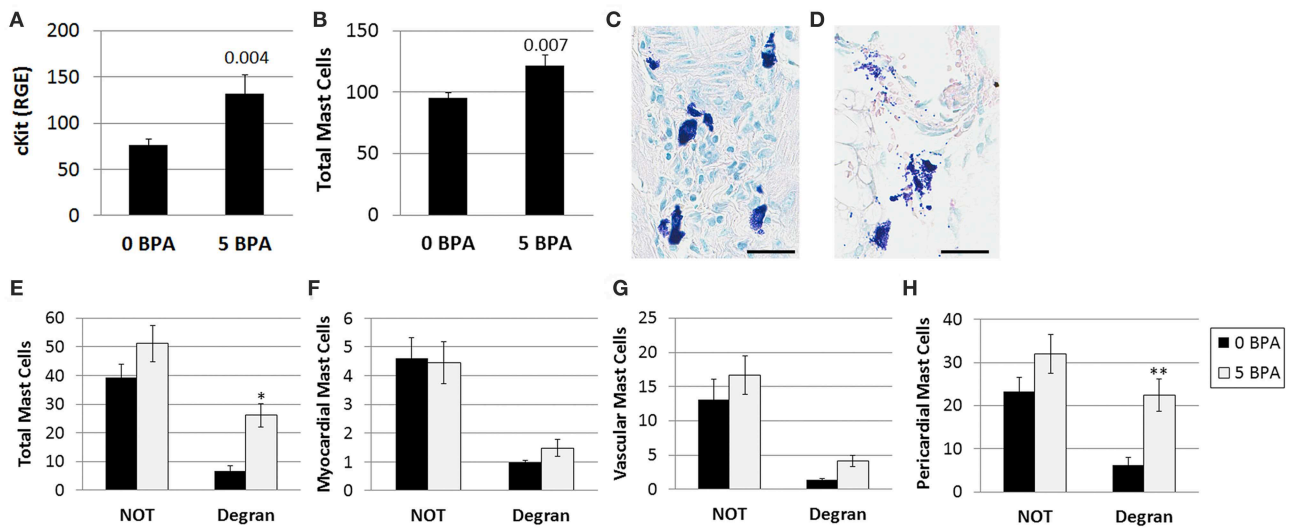

FIGURE 3 | BPA increases the number of mast cells and degranulation of pericardial mast cells during viral myocarditis. Female BALB/c mice were given 0 or 5 μg/kg BW BPA (0 BPA vs. 5 BPA) in drinking water for 2 weeks and injected with 10^3 PFU CVB3 ip on day 0. BPA exposure continued from day 0 to 10 pi and hearts were harvested at day 10 pi during acute myocarditis. **(A)** Relative gene expression (RGE) of mast cells (cKit/ CD117) vs. the housekeeping gene Hprt was analyzed in whole hearts by qRT-PCR at day 10 pi comparing 0 to 5 μg BPA/kg BW groups. (0 BPA $n = 10$, 5 BPA $n = 8$). Toluidine blue was used to detect mast cell granules by histology and the total numbers of mast cells in the heart section were normalized to the size of the heart using an eyepiece grid (50). **(B)** Data show the mean ± SEM using the Mann-Whitney rank test (0 BPA $n = 20$, 5 BPA $n = 20$). A representative photo of **(C)** non-degranulating (NOT) and **(D)** degranulating (Degran) mast cells. Mast cell granules stain dark purple, magnification 400x, scale bar 30 μm. **(E)** Data show the mean ± SEM of 10 mice/group. Two-way ANOVA found significant results for degranulation ($p < 0.0001$) and BPA ($p = 0.0006$). *$p < 0.05$ indicates *ad-hoc* analysis comparing 0 vs. 5 BPA for degranulating cells (Degran). **(F,G)** Two-way ANOVA found significant results for degranulation $p = 0.0001$, but not for BPA for **(F)** myocardial and **(G)** vascular MCs, $n = 10$/group. **(H)** In contrast, 2-way ANOVA found significant results for degranulation ($p < 0.0002$) and BPA ($p = 0.0004$) for pericardial mast cells. After Tukey's multiple comparison test, 5 BPA was found to significantly increase degranulation in pericardial mast cells compared to control water (**$p < 0.01$).

Plastic Cages Increase Cardiac Mast Cell Numbers and Pericardial Mast Cell Degranulation

We found mast cell levels were significantly increased in the heart of female mice with myocarditis that were housed in plastic cages compared to those housed in glass cages based on the mast cell marker cKit (glass 2.0 ± 0.2 vs. plastic 3.1 ± 1.4, $p = 0.04$) (**Figure 4A**). Housing mice in plastic cages compared to glass cages did not alter the total number of mast cells assessed histologically by toluidine blue staining ($p = 0.79$) (**Figure 4B**). However, by 2-way ANOVA we found that during myocarditis more mast cells were not degranulating compared to those that were degranulating ($p < 0.0001$) for mice housed in glass cages, whereas a similar number of mast cells were degranulating vs. not degranulating for mice housed in plastic cages ($p = 0.74$) (**Figure 4C**). So comparing glass to plastic, overall more mast cells were degranulating in the hearts of mice housed in plastic cages than in glass cages ($p = 0.006$) (**Figure 4C**).

Examining the number of degranulating vs. non-degranulating mast cells in different locations of the heart (i.e., myocardial, vascular, pericardial), we found that more mast cells located in the myocardium were not degranulating compared to those that were degranulating by 2-way ANOVA ($p < 0.0008$) for mice housed in glass cages, whereas a similar number of mast cells were degranulating vs. not degranulating for mice housed in plastic cages ($p = 0.94$) (**Figure 4D**). Additionally, there was no significant difference in the number of myocardial mast cells that were degranulating in glass vs. plastic cages ($p = 0.21$). Similarly, most mast cells associated with vessels were not degranulating in the heart of mice housed in glass cages ($p = 0.0006$), and a similar number of vascular mast cells were degranulating vs. not degranulating in plastic cages ($p = 0.78$) (**Figure 4E**). There was no significant difference in the number of vessel-associated mast cells that were degranulating vs. not degranulating comparing glass to plastic cages ($p = 0.33$). Similar to myocardial and vascular mast cells, mast cells along the pericardium were mainly not degranulating if mice were housed in glass cages ($p = 0.0001$) (**Figure 4F**). In contrast to mast cells in the other locations, more pericardial mast cells were degranulating than not degranulating for mice housed in plastic cages ($p = 0.02$), and more pericardial mast cells were degranulating when mice were housed in plastic compared to glass cages ($p = 0.0002$) (**Figure 4F**). Thus, the elevated cKit levels detected by PCR from mice housed in plastic cages corroborate the increased number of pericardial degranulating mast cells detected in the heart using histology. Additionally, BPA, other plastics and/or other chemicals that are leaching from plastic cages/plastic water bottles are causing the increase in number and pericardial degranulation of mast cells observed in the experiments where mice are exposed to BPA in drinking water (**Figure 3**).

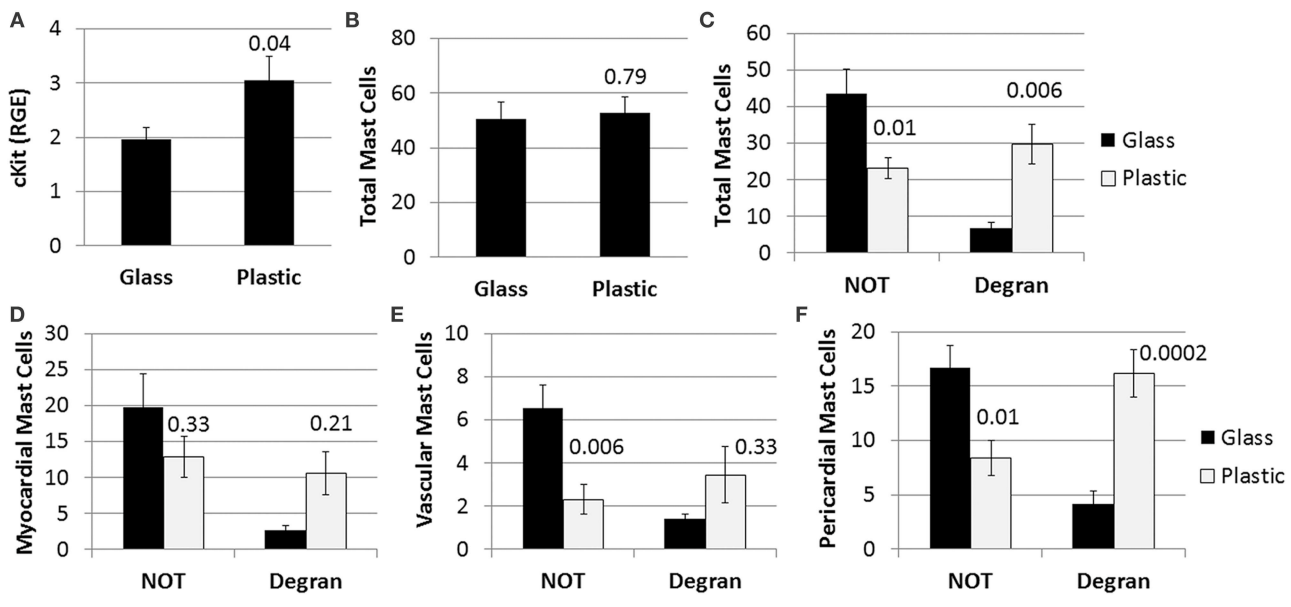

FIGURE 4 | Plastic cages/water bottles, without the addition of BPA in water, increase mast cell numbers and pericardial degranulation in BALB/c females. Female BALB/c mice were housed in glass or plastic cages with glass or plastic water bottles and no soy in food or bedding for 3 weeks prior to ip infection with CVB3 to induce myocarditis. The drinking water did not contain BPA. Mice were injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi during acute myocarditis. **(A)** Relative gene expression (RGE) of mast cells (cKit/ CD117) vs. the housekeeping gene Hprt was analyzed in whole hearts by qRT-PCR at day 10 pi comparing mice in glass vs. plastic cages (glass $n = 10$, plastic $n = 10$). **(B–F)** Toluidine blue was used to detect mast cell granules by histology and the total numbers of MCs in the heart section were normalized to the size of the heart using an eyepiece grid [(50); glass $n = 10$, plastic $n = 10$]. **(A,B)** Data show the mean ± SEM using the Mann-Whitney rank test of total mast cells (NOT and Degran). **(C–F)** Two-way ANOVA found significant results for degranulation ($p < 0.0001$) and BPA ($p = 0.0006$). *P*-values depict *ad-hoc* analyses comparing glass to plastic by Tukey's multiple comparison.

BPA Effect on ERs in the Spleen and Heart

ERα, ERβ, and AR Are More Highly Expressed on Cardiac Tissue Than Immune Cells During Viral Myocarditis in Mice Housed in Plastic Cages

To determine whether immune cells (i.e., $CD45^+$ cells) or cardiac tissue (i.e., cardiomyocytes, fibroblasts, endothelial cells) expressed more ERs or AR, we isolated these two cell populations from the heart during myocarditis in female BALB/c mice that were housed in plastic cages without soy in their food or bedding and no BPA added to their drinking water. We found that cardiac tissue had significantly higher expression of ERα (CD45 2.7 ± 0.4 vs. cardiac 17.9 ± 5.0, $p = 1.3 \times 10^{-5}$), ERβ (CD45 0.0 vs. cardiac 2.8 ± 1.2, $p = 0.007$), and AR (CD45 3.0 ± 0.6 vs. cardiac 42.2 ± 7.4, $p = 1.3 \times 10^{-5}$) compared to immune cells normalized to Hprt using qRT-PCR (data not shown). There was no significant difference between the two populations for ERRγ expression after normalization (CD45 20.8 ± 7.0 vs. cardiac 19.8 ± 8.9, $p = 0.76$).

BPA Exposure in Drinking Water Increases ERα and ERβ Expression at 24 h pi in the Spleen in Mice Housed in Plastic Cages

To assess whether BPA exposure altered ER expression on/in splenic immune cells during the innate immune response to CVB3 infection, we exposed mice for 2 weeks prior to ip injection with CVB3 on day 0 to 5 μg BPA/kg BW (5 BPA) in drinking water and examined ERα, ERβ, ERRγ, and AR expression by qRT-PCR in the spleen at 24 h pi compared to 0 BPA (we continued BPA exposure until harvest). We found that BPA exposure significantly increased ERα (0 BPA 4.5 ± 0.5 vs. 5 BPA 21.6 ± 9.4, $p = 0.04$) and ERβ (0 BPA 2.2 ± 0.4 vs. 5 BPA 30.0 ± 8.5, $p = 0.001$) expression in the spleen, but had no significant effect on ERRγ (0 BPA 1.3 ± 0.1 vs. 5 BPA 1.4 ± 0.1, $p = 0.19$) or the AR (0 BPA 1.3 ± 0.1 vs. 5 BPA 1.3 ± 0.1, $p = 0.84$) compared to 0 BPA (**Figure 5A**, **Table 4**).

BPA Exposure in Drinking Water Decreases ERα and Increases ERβ Expression in the Heart During Viral Myocarditis in Mice Housed in Plastic Cages

BPA binds to ERα and ERβ at a 10,000-fold lower affinity than natural estrogen, but binds ERRγ with a higher affinity (16). Therefore, exposure to BPA has the potential to alter myocarditis. BPA has been found to be able to bind to the AR directly (30), and one study found that BPA can act as both an agonist and antagonist for the AR (51). In this study, we found that exposure to 5 μg BPA/kg BW decreased the mRNA expression of ERα in the heart during CVB3 myocarditis ($p = 0.02$) and increased the expression of ERβ ($p = 0.04$), but had no significant effect on ERRγ ($p = 0.38$) or the androgen receptor (AR) ($p = 0.91$) (**Figure 5B**, **Table 4**). Based on our finding that BPA exposure increased acute myocarditis in female BALB/c mice (**Figure 1**), these data suggest that ERα signaling reduces while ERβ signaling increases our model of autoimmune CVB3

TABLE 4 | Effect of BPA exposure in drinking water in mouse housed in plastic cages on expression of steroid hormones in spleen and heart using qRT-PCR.

Cell marker	Description	0 BPA[a,b]	5 BPA	*P*-value
24 hr spleen				
ERα	Estrogen receptor alpha	4.5 ± 0.5	22 ± 9.4	0.04
ERβ	Estrogen receptor beta	2.2 ± 0.4	30 ± 8.5	0.001
ERRγ	Estrogen related receptor gamma	1.3 ± 0.1	1.4 ± 0.1	0.19
AR	Androgen receptor	1.3 ± 0.1	1.3 ± 0.1	0.84
d10 heart				
ERα	Estrogen receptor alpha	3.7 ± 0.5	2.3 ± 0.3	0.02
ERβ	Estrogen receptor beta	46 ± 7.4	70 ± 10	0.04
ERRγ	Estrogen related receptor gamma	1.0 ± 0.1	1.1 ± 0.1	0.38
AR	Androgen receptor	3.4 ± 0.5	3.5 ± 0.8	0.91

[a] *AR, androgen receptor; d, day; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; ERRγ, estrogen related receptor gamma; hr, hour.*
[b] *0 BPA, control water without BPA; 5 BPA, high human relevant dose (5 μg/kg BW).*

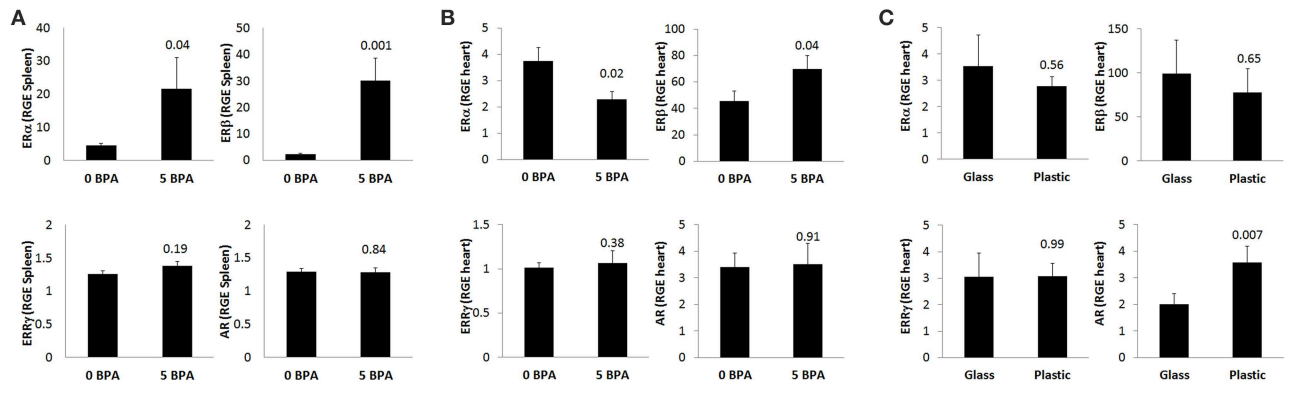

FIGURE 5 | BPA exposure in drinking water increases splenic ERα/ERβ, decreases cardiac ERα but increases ERβ during myocarditis, while plastic cages increase cardiac AR during myocarditis. Relative gene expression (RGE) of ERα, ERβ, ERRγ, and AR vs. the housekeeping gene Hprt were analyzed in **(A)** spleen at 24 h (0 BPA $n = 10$, 5 BPA $n = 10$), **(B)** whole hearts at day 10 pi by qRT-PCR comparing 0–5 μg BPA/kg BW (0 BPA $n = 10$, 5 BPA $n = 8$) or **(C)** whole hearts at day 10 pi by qRT-PCR comparing glass vs. plastic caging (glass $n = 10$, plastic $n = 10$). Data show the mean ± SEM using a two-tailed Student's *t* or Mann-Whitney rank test.

myocarditis- confirming the role for these receptors that have been shown previously by Huber et al. using a different model of CVB3 myocarditis (10). These findings also suggest that BPA mediates its effect by altering ERα and ERβ expression levels in the heart and/or on cardiac inflammation.

BPA Exposure in Drinking Water Activates Cardiac ERα and ERβ During Viral Myocarditis in Mice Housed in Plastic Cages

Activation of ERs requires phosphorylation (52). We examined whether BPA altered activation of ERα and/or ERβ by determining protein levels of the phosphorylated form of the receptors by western blot. We found that BPA exposure in drinking water did not significantly alter total protein levels of ERα (**Figures 6A,B**) or ERβ (**Figures 6C,D**) in the heart during myocarditis, but significantly increased phosphorylated-ERα (p-ERα) (**Figures 6A,B**) and phosphorylated-ERβ (p-ERβ) (**Figures 6C,D**) in mice exposed to 5 μg BPA/kg BW. Thus, BPA exposure activated cardiac ERα and ERβ in the heart during myocarditis.

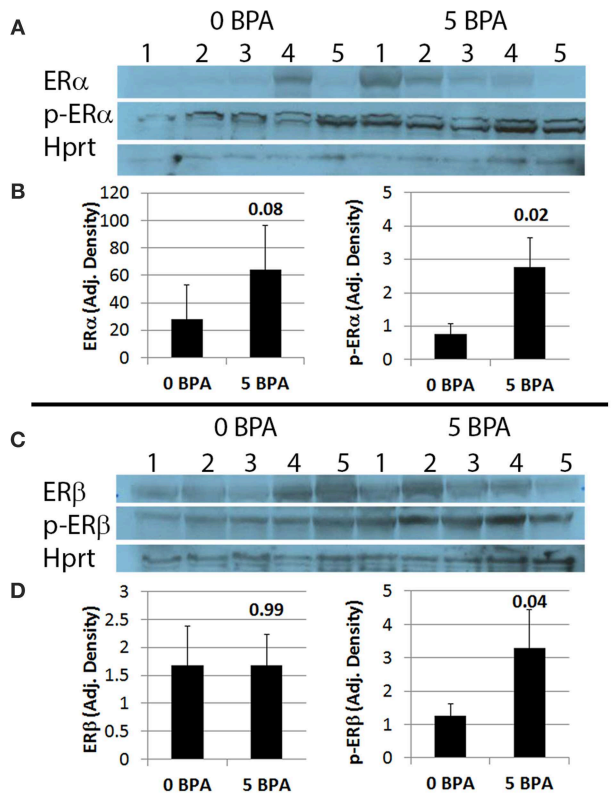

FIGURE 6 | BPA exposure in drinking water significantly increases phosphorylation of ERα (p-ERα) and ERβ (p-ERβ) in the heart during myocarditis. **(A)** Western blot images for ERα, p-ERα and Hprt. One to five represent individual samples from hearts with myocarditis. **(B)** Quantitation of western blot data for ERα (left) and p-ERα (right) from hearts adjusted to Hprt levels. Data show the mean ± SEM using a two-tailed Mann-Whitney rank test with 5 mice/group. **(C)** Western blot images from hearts for ERβ, p-ERβ, and Hprt. One to five represent individual samples. **(D)** Quantitation of western blot data for ERβ (left) and p-ERβ (right) adjusted to Hprt levels. Data show the mean ± SEM using a one-tailed Mann-Whitney rank test with 5 mice/group.

Plastic Cages Increase AR Expression in the Heart During Myocarditis

Next we determined ER and AR expression in the heart using qRT-PCR of female mice with myocarditis housed in glass vs. plastic cages with food and bedding that did not contain soy. We found that plastic cages significantly increased AR ($p = 0.007$) expression in females during acute myocarditis compared to glass cages, but did not significantly alter ER expression in the heart (**Figure 5C**, **Table 5**).

BPA Effect on Inflammatory Mediators

BPA Exposure in Drinking Water Significantly Increases Cardiac IFNγ and IL-17A in Mice Housed in Plastic Cages

BPA exposure has been shown to increase both Th1 and Th2 immune responses in various inflammatory animal models, like the OVA model of asthma for example (53). Because $CD4^+$ T cells were increased by BPA exposure in drinking water (**Table 2**), we examined whether IFNγ, IL-4, and/or IL-17A cytokine levels were altered in the heart during acute CVB3 myocarditis following BPA exposure. Changes in these cytokines are often used to indicate Th1, Th2, and/or Th17 responses, respectively (46). We found that IFNγ (0 BPA 39.2 ± 4.5 vs. 5 BPA 91.4 ± 14.3, $p = 5 \times 10^{-5}$) (**Figure 7A**) and IL-17A (0 BPA 57.8 ± 9.2 vs. 5 BPA 94.0 ± 15.5, $p = 0.03$) (**Figure 7C**) were significantly increased in the heart by exposure to 5 μg BPA/kg BW during myocarditis, but IL-4 levels were not significantly altered (0 BPA 282.5 ± 85.0 vs. 5 BPA 385.9 ± 72.1, $p = 0.98$) (**Figure 7B**).

BPA Exposure in Drinking Water Increases TLR4, Caspase-1, and IL-1β in the Heart During Myocarditis in Mice Housed in Plastic Cages

We previously demonstrated that components of IL-1R-mediated signaling (i.e., TLR4, caspase-1, IL-1β, and IL-18) are upregulated in the heart during viral myocarditis in male mice and that testosterone elevates this pathway on mast cells and macrophages during the innate and adaptive immune response to CVB3 infection in our model (44, 46). In this study we found that 5 μg BPA/kg BW exposure significantly increased

TABLE 5 | Effect of plastic vs. glass caging on expression of steroid hormones in the heart using qRT-PCR.

Cell marker	Description[a]	Glass	Plastic	*P*-value
d10 heart				
ERα	Estrogen receptor alpha	3.5 ± 1.2	2.8 ± 0.4	0.56
ERβ	Estrogen receptor beta	98 ± 38	77 ± 27	0.65
ERRγ	Estrogen related receptor gamma	3.1 ± 0.9	3.1 ± 0.5	0.99
AR	Androgen receptor	2.0 ± 0.4	3.6 ± 0.6	0.007

[a] *d, day; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta.*

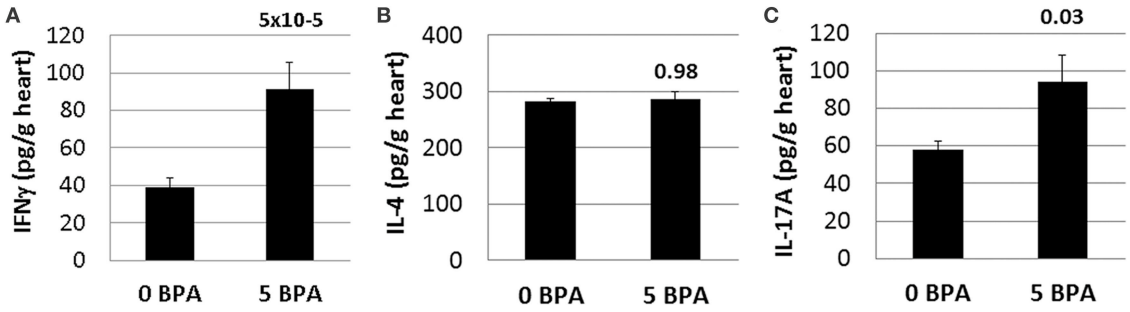

FIGURE 7 | Cardiac IFNγ and IL-17A levels significantly increased by BPA exposure in drinking water. Hearts were harvested at day 10 pi and homogenized and the supernatant used to measure **(A)** IFNγ, **(B)** IL-4, and **(C)** IL-17A levels in the heart by ELISA. Data show the mean ± SEM using a one-tailed (IL-17A) or 2-tailed Student's *t* (IFNγ and IL-4) or Mann-Whitney rank test with 10 mice/group.

expression of this pathway in the heart during acute CVB3 myocarditis in female BALB/c mice. We found that TLR4 (0 BPA 3.1 ± 0.5 vs. 5 BPA 5.7 ± 1.3, $p = 0.01$), caspase-1 (0 BPA 4.2 ± 0.9 vs. 5 BPA 9.8 ± 1.3, $p = 0.001$), and IL-1β (0 BPA 178.7 ± 97.3 vs. 5 BPA 577.9 ± 116.3, $p = 0.009$) levels were significantly increased by BPA exposure in drinking water compared to control water (**Figures 8A–C**). In contrast, BPA that may have leached from plastic cages and water bottles did not significantly alter TLR4 (glass 3.6 ± 0.6 vs. plastic 5.0 ± 0.9, $p = 0.20$), caspase-1 (glass 3.3 ± 0.7 vs. plastic 4.3 ± 0.7, $p = 0.32$) or IL-1R2 (glass 1.9 ± 0.2 vs. plastic 2.4 ± 0.4, $p = 0.18$) in the heart (**Figures 8D–F**) indicating that BPA exposure in drinking water led to elevated TLR4 signaling rather than plastic caging alone.

BPA Exposure in Drinking Water Increases Pericardial, Myocardial, and Vascular Fibrosis

Fibrosis is not present in the heart of male or female mice during acute CVB3 myocarditis at day 10 pi, and only begins to appear during chronic myocarditis around day 35 pi (54). We found that exposure to 5 or 50 μg BPA/kg BW in drinking water in mice housed in plastic cages significantly increased fibrosis in the heart of females with myocarditis ($p = 0.007$ and $p = 0.01$, respectively) using 2-tailed Student's *t*-test and 1-way ANOVA (all doses $p = 0.04$) (**Figure 9A**). BPA that may have leached from plastic cages and water bottles not only increased the number of mast cells in the heart (**Figure 4A**), but also significantly increased fibrosis in the heart compared to mice housed in glass cages (glass 9.8 ± 0.8 vs. plastic 13.8 ± 1.2, $p = 0.01$) (**Figure 9B**). We showed previously that mast cell degranulation is associated with increased fibrosis during myocarditis (50).

Next we determined expression of collagen genes in the heart during myocarditis and found that BPA exposure in drinking water significantly increased expression of collagen I (*Col1a1*) ($p = 0.005$) (**Figure 9C**) and collagen III (*Col3a1*) ($p = 0.02$) (**Figure 9D**) compared to control water. We also assessed the effect of housing on collagen gene expression in the heart and found that plastic caging had no effect on the production of collagen I ($p = 0.28$) (**Figure 9E**) or collagen III ($p = 0.67$) (**Figure 9F**) during myocarditis. Mice exposed to BPA in drinking water also had greater pericardial (**Figure 10B**), myocardial (**Figures 10C,F**), and perivascular fibrosis (**Figures 10E,H**) compared to mice that had no BPA added to their water based on Masson's trichrome stain which uses three stains to identify muscle, collagen and fibrin (**Figures 10A–E**) and picosirius red which detects collagen [(55); **Figures 10F–H**].

DISCUSSION

In this study we found that BPA exposure at a high human relevant dose (25 μg BPA/L water or 5 μg BPA/kg BW) administered to adult female BALB/c mice significantly increased CVB3 myocarditis (**Figure 1A**) and pericarditis (**Figure 2A**) at day 10 pi compared to control water. The increase in myocardial and pericardial inflammation was not due to elevated levels of virus in the heart and, importantly, was not due to BPA that leached from the plastic cages or water bottles (**Figures 1B**, **2B**). BPA exposure in water significantly increased ERβ expression in the heart (**Figures 5B**, **6C,D**), which has been found to promote CVB3 myocarditis in male and female mice (10, 11), but was not significantly altered by plastic caging alone (**Figure 5C**). BPA also increased T cell numbers in the heart and proinflammatory and profibrotic cytokines (i.e., IFNγ, IL-17A, IL-1β) and signaling pathways (i.e., TLR4/IL-1R) that were not increased by BPA leaching from plastic cages and water bottles alone (**Figures 7**, **8**). However, BPA (or other plastics/chemicals) leaching from plastic cages and water bottles was found to activate cardiac mast cells especially along the pericardium (**Figures 3**, **4**) and to increase cardiac fibrosis (**Figures 9**, **10**).

These findings have broad implications. First, the cages used for these experiments are the traditional cages used by many research facilities and investigator's experiments may be influenced by BPA leaching from cages and water bottles leading to mast cell activation. Our studies suggest that animal models that use viral infections to induce disease may be especially vulnerable to alterations caused by mast cell activation, although investigators studying the effects of allergy may also be affected by BPA leaching from caging materials. Second, women exposed

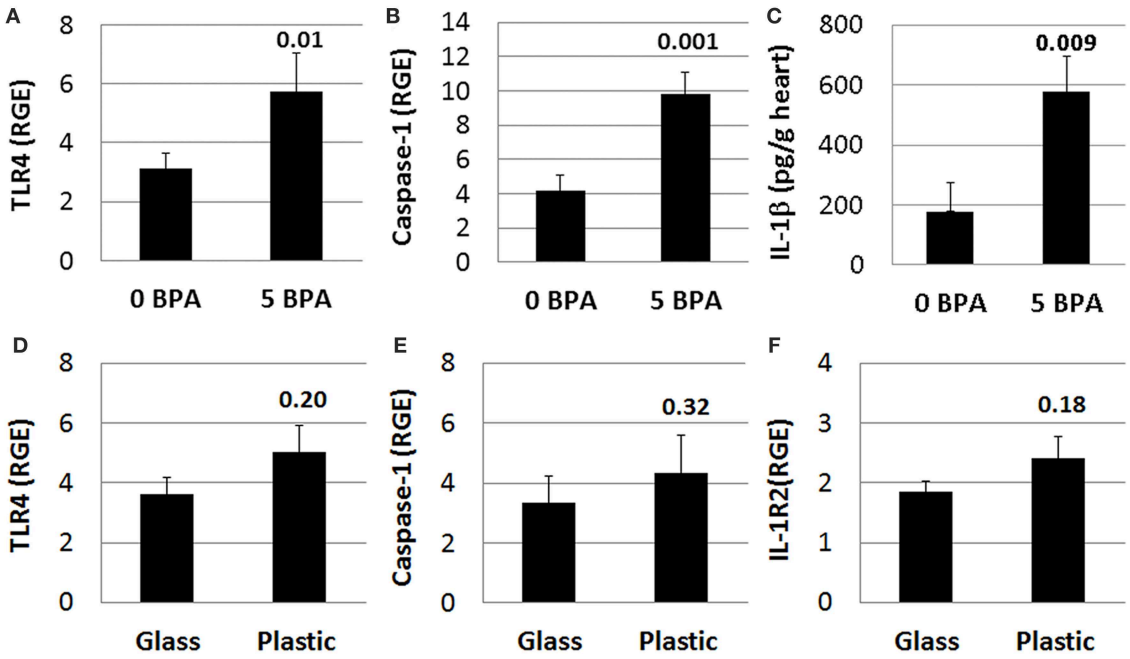

FIGURE 8 | BPA exposure in drinking water activates TLR4 pathway during myocarditis. **(A–C)** BPA exposure in drinking water, **(D–F)** plastic vs. glass caging (no BPA added to water). Relative gene expression (RGE) of genes vs. the housekeeping control Hprt for **(A)** TLR4 (0 BPA $n = 10$, 5 BPA $n = 7$) and **(B)** caspase-1 (0 BPA $n = 10$, 5 BPA $n = 9$) were examined in whole hearts by qRT-PCR at day 10 pi comparing 0–5 μg BPA/kg BW. **(C)** IL-1β protein levels were determined using ELISA from homogenized whole heart supernatants comparing 0 to 5 μg BPA/kg BW (0 BPA $n = 8$, 5 BPA $n = 10$). RGE of genes vs. the housekeeping control Hprt for **(D)** TLR4 (glass $n = 10$, plastic $n = 10$), **(E)** caspase-1 (glass $n = 10$, plastic $n = 10$), and **(F)** IL-1R2 (glass $n = 10$, plastic $n = 10$) were examined in whole hearts by qRT-PCR at day 10 pi comparing glass to plastic. Data show the mean ± SEM using a two-tailed Student's *t* or Mann-Whitney rank test.

to a high dose of BPA such as workers in factories that use plastic products as part of the manufacturing or packaging process, sales clerks handling receipts throughout the day, clerical staff that photocopy frequently, and/or certain furniture manufactures that use BPA resins in the production process, for example, may be at a higher risk of developing myocarditis following CVB infection. Individuals may not need a high lifetime exposure to BPA but an elevated exposure just prior to viral infection could be enough to increase their risk. The high use of plastics, especially associated with food and drink products, has made BPA and other plastic endocrine disruptors a ubiquitous exposure. Additionally, the discovery that BPA at higher doses in drinking water or leaching from plastic cages could increase fibrosis is important because female mice and women are highly protected from remodeling and fibrosis that leads to DCM and heart failure but we found in this study that remodeling and fibrosis are promoted by this endocrine disrupting chemical.

In our study, we administered BPA dissolved in drinking water based on Jenkins et al. (41) and found that a high human relevant exposure significantly increased myocarditis in our mouse model (**Figure 1A**). Animal studies researching the effect of BPA use many different exposure routes such as ip, gavage and by applying oil-based BPA to the chow of mice (56). We chose water as the exposure route as it has been found to be the most clinically relevant exposure route (25) and is a continuous exposure compared to using a high dose one-time injection/ bolus. People are exposed to BPA in many different ways including orally (i.e., drinking water from water bottles or eating food from aluminum cans coated with BPA) and by contact (by touching shiny receipts, fresh epoxy resin or through medical devices such as implants or tubing) (19–22).

In our BPA exposure experiments we housed all mice, both those exposed to BPA water and those receiving control water, in the same Allentown plastic standard mouse cages with plastic water bottles. The housing and water bottles are both made of plastics that contain BPA and other bisphenols that could leach from the caging (56–58). Studies have found that the age of cages can have a dose effect on the amount of BPA that is leached, with new cages releasing no more BPA than BPA-free or glass cages while older cages leach significantly more BPA (47, 56–58). The cages that we used for these experiments were not new and were all the same age. This means that the 0 BPA group is likely not truly zero because the mice were housed in older plastic cages. However, the potential BPA exposure from plastic cages was the same for all groups and our experiments comparing glass to plastic cages revealed the effect of the plastic cages and water bottles on our experimental endpoints.

At the time we started these studies we chose BPA doses based on Jenkins et al. who found that the 5 BPA dose was equivalent to a high human relevant exposure [(41); **Table 1**]. This dose of BPA is thought to be equal to exposures experienced

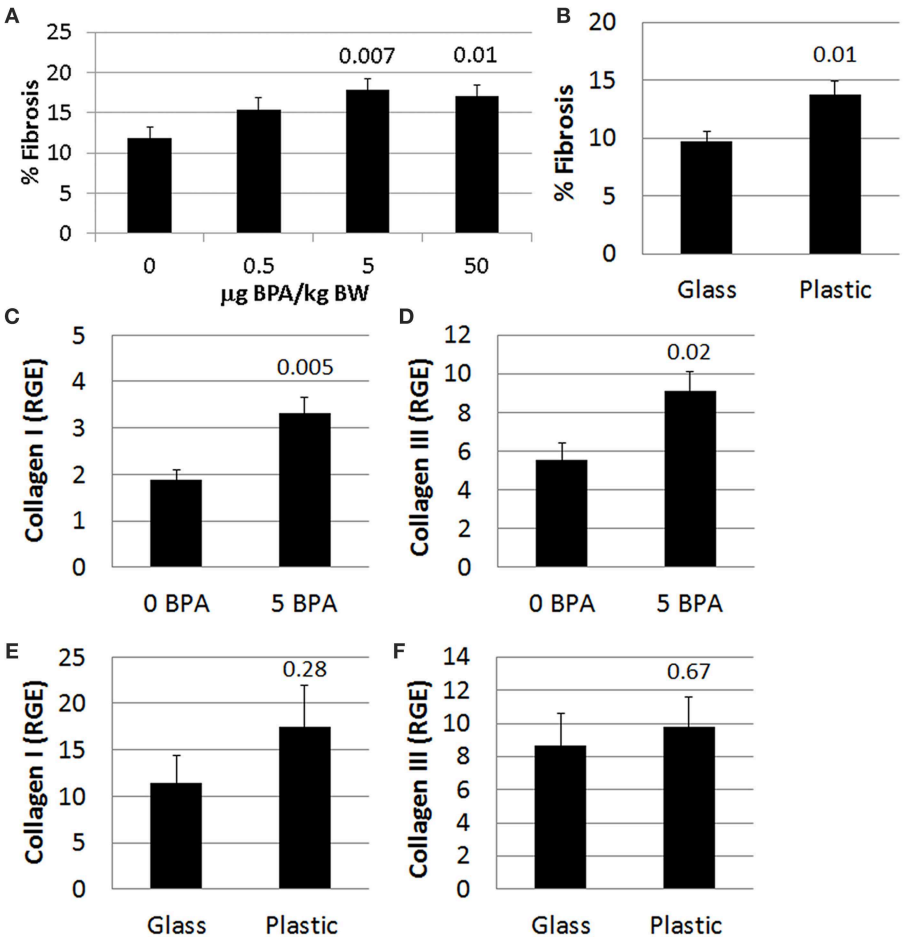

FIGURE 9 | BPA exposure increased fibrosis. Female BALB/c mice were given 0, 0.5, 5, and 50 µg BPA/kg BW in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 on day 0 and exposure continued until harvest for myocarditis at day 10 pi. Fibrosis in the heart was assessed using Masson's trichrome blue to detect collagen deposition, which stains bright blue. **(A,B)** Quantification of cardiac fibrosis was calculated histologically as % collagen staining blue in the heart normalized to the overall size of the heart section using an eyepiece grid. Data show the mean ± SEM. **(A)** comparing BPA exposed mice to mice given no BPA in water (0 µg BPA/kg $n = 10$, 0.5 µg BPA/kg $n = 9$, 5 µg BPA/kg $n = 9$, 50 µg BPA/kg $n = 10$). One-way ANOVA found a significant difference existed between groups ($p = 0.04$). After controlling for multiple comparisons (Dunnett's multiple comparisons), the 5 and 50 µg BPA/kg BW groups were significantly different compared to control water (Glass $n = 10$, Plastic $n = 10$). **(B)** Fibrosis assessed comparing glass vs. plastic caging without BPA exposure in water bottles. Relative gene expression (RGE) of **(C,E)** collagen I (*Col1a1*) and **(D,F)** collagen III (*Col3a1*) vs. the housekeeping gene Hprt were analyzed in whole hearts by qRT-PCR at day 10 pi. **(C,D)** Compare 0–5 µg BPA/kg BW groups (0 BPA $n = 9$, 5 BPA $n = 9$). **(E,F)** Compare glass vs. plastic cages (glass $n = 10$, plastic $n = 10$).

by workers who are constantly exposed to BPA through their occupation as well as through personal exposures from food cans, water bottles, heating leftover food in plastic containers, etc. Occupations thought to have heavy exposure to BPA include cashiers (coated receipts), craftsman industries such as furniture design (epoxy resin), and canning companies (lining of cans), for example (19–22). Our data found that the highest dose of BPA (50 BPA), the EPA reference dose, did not significantly increase myocarditis (**Figure 1A**). Although most exogenous drugs/radiation/chemotherapy are thought to have linear dose-response curves, that is not the case for hormones (59, 60). The dose-response curve for hormones is a bell shaped curve that is termed hormesis because low and high concentrations of the hormone have a detrimental effect on health while a middle or moderate level is optimal for health (61). This principle may also apply to endocrine disrupting hormones such as BPA, where low and high levels of BPA have no detrimental effect on health but a middle or moderate exposure promotes disease (62).

Since BPA is known to act via ERs our data show that activating and/or altering the expression level of these receptors in/on immune or cardiac cells can increase the severity of autoimmune CVB3 myocarditis and promote pericarditis. Altering ER expression and/or ERα to ERβ ratios may also shift the type of immune response (i.e., Th2 to Th1/Th17A) following BPA exposure promoting a more cardio-damaging phenotype that increases inflammation, fibrosis and promotes progression to DCM. In this study we found that exposure to BPA in drinking water elevated proinflammatory cytokines/ receptors in

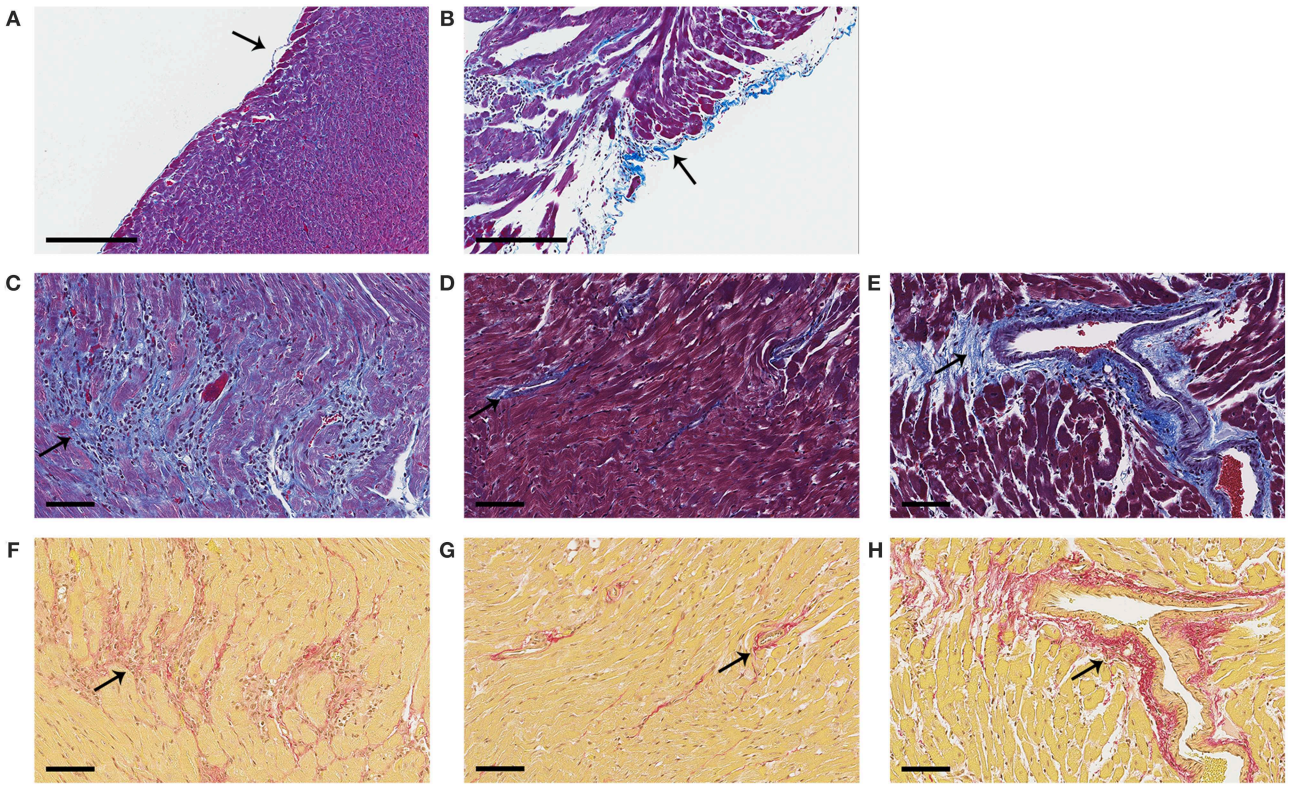

FIGURE 10 | Representative photos of pericardial, myocardial and perivascular fibrosis. Female BALB/c mice were given 0, 0.5, 5, and 50 μg BPA/kg BW BPA in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and exposure continued until harvest for myocarditis at day 10 pi. **(A–E)** Fibrosis was assessed using Masson's trichrome blue to detect collagen and fibrin deposition, which stains bright blue. **(F–H)** Picosirius red was used to assess collagen deposition, which stains red. **(A)** Normal pericardial layer; a single layer of pericardial cells lift from the myocardium and stain blue with Masson's trichrome. **(B)** Severe pericarditis with collagen (i.e., fibrosis) that stains bright blue. **(C)** Myocardial fibrosis staining bright blue or **(F)** red. **(D,G)** Normal myocardium with small amount of collagen staining mainly around vessels. **(E,H)** Perivascular fibrosis. Photos depict **(A,D,G)** 0 BPA, **(B,C,E,F,H)** 5 μg BPA/kg BW. **(A**, **B)** Magnification 100x, scale bar 200 μm. **(C–H)** Magnification 300x, scale bar 70 μm. Arrows point to staining for collagen.

the heart of female mice during CVB3 myocarditis that would typically be elevated in male mice or men with mycarditis such as TLR4, caspase-1, IL-1β, IL-17A, and IFNγ (44, 46, 63). Other investigators have reported that BPA exposure causes a shift from a Th2- (associated with IL-4) to a Th1- (associated with IFNγ) type immune response (64–66) which is similar to our findings. Thus, the immune response following BPA exposure resembles the cardiac inflammation characteristic of male mice with myocarditis suggesting that exposure to BPA could increase the risk of women developing myocarditis who may normally be protected by estrogen.

BPA exposure to myeloid immune cells from a mouse model of lupus or human peripheral blood cells was found to activate the TLR4 pathway causing the release of mature IL-1β and IL-18 (65). BPA treatment in cell culture of a monocyte-like cell line derived from a leukemia patient and human peripheral blood macrophages have been found to release the proinflammatory cytokines TNFα and IL-6 and to decrease the anti-inflammatory/ regulatory cytokines IL-10 and TGFβ (67). Lui et al. showed that this effect on cytokines by BPA was mediated through ERα and ERβ (67). Nowak et al. reviews many studies examining the effects of endocrine disrupting chemicals including BPA on immune cell function (68). We found that ERα and ERβ were activated during the innate immune response in the spleen and BPA has been found to increase IFNγ in mouse bone marrow cells and isolated $CD11b^+$ cells via ERα (65), suggesting that BPA could be acting through ERs located directly on immune cells to shift the cytokine profile in the heart.

We found that BPA exposure significantly decreased ERα and increased ERβ expression in the heart of females during myocarditis (**Figure 5B**). A recent study in a rat model assessed the effect of BPA on the uterus and found that BPA treatment downregulated ERα and upregulated ERβ mRNA in a manner similar to our findings (16). Another study in mice found BPA increased ERβ levels which promoted cardiac arrhythmias and worse cardiac handling, while ERα was protective (13, 14). The opposing action of the two main ERs in these studies was investigated and it was found that ERα and ERβ transcriptionally cross-regulate each other (69, 70). But it is also thought that ERRγ, which BPA strongly binds to, can regulate ERα by heterodimerizing leading to transcriptional repression (71).

Additionally, BPA can act as a ERβ antagonist rather than an agonist and prevent estrogen-driven non-genomic signaling pathways that could possibly be protective (70).

IL-17A, but not IFNγ, has been shown to promote remodeling and fibrosis and progression from acute myocarditis to chronic myocarditis and DCM in the CVB3 and experimental autoimmune models of myocarditis in male mice and in male patients with myocarditis (54, 63, 72). Activation of TLR4, caspase-1, IL-1β, and mast cells are also known to increase myocarditis and promote remodeling, fibrosis, and DCM in CVB3 myocarditis in male mice (44, 54) suggesting that BPA exposure could increase the risk of progression from myocarditis to DCM and heart failure in women. Importantly, ERα has been found to modulate TLR expression including TLR 2, 7, 8, and 9 (11, 27, 73, 74) while ERα has been found to decrease TLR4 (35).

Numerous studies have found that BPA and other endocrine disruptors activate mast cells leading to degranulation and release of histamine, leukotrienes and other mediators (68). Typically women have a reduced risk of progressing to DCM after myocarditis and are more likely to recover without the need for a heart transplant (4, 6). Other studies have found that BPA increases TLR expression in neonates (75) and promotes cardiac fibrosis (76, 77). Our findings in this study suggest that BPA exposure hastens the onset of remodeling and fibrosis, which usually takes several weeks to develop. In this study mice had not yet developed DCM even though they had pericardial and myocardial fibrosis.

We examined the effect of plastic cages/ water bottles to control for BPA that may leach from the plastic caging. We were surprised to find that exposure to plastic caging alone was able to increase the number and activation of mast cells and lead to pericardial degranulation and fibrosis. cKit is the receptor for stem cell factor which is a marker used to detect mast cells and stem cells (78). Previously, we found that mast cell degranulation was associated with increased CVB3 myocarditis, pericarditis, DCM, and heart failure in male BALB/c mice (54, 79). Mast cells leave the bone marrow as undifferentiated cells and migrate to the heart where they differentiate to form two types of resident mast cell populations: one type contains tryptase and chymotrypsin (also called Serpin A3n) granules and is termed a *TC mast cell* and the other type of mast cell contains only tryptase granules and is termed a *T mast cell* (80). It is not known whether one type of mast cell resides along the pericardium and the other in the myocardium, but this is a possibility. To determine whether BPA exposure altered degranulation of mast cells located in the myocardium we examined the degranulation state of myocardial mast cells using histology. Mast cells that occur in the circulation are known as basophils. Basophils can be recruited to sites of inflammation (81). Infiltrating mast cells or basophils would be expected to be found near vessels. Mast cells found near vessels have been termed "vessel-associated" mast cells for this analysis and are assumed to be mast cells recruited to the heart from the circulation rather than resident mast cells. Mast cells in the myocardium that were not near vessels or evidence of red blood cells were defined as "myocardial" mast cells. It is likely that there is a certain amount of overlap between "myocardial" and "vessel-associated" mast cells due to the inability to detect vessels depending on the cut of the histology section. To determine whether BPA exposure from drinking water or plastic cages altered degranulation of mast cells located near vessels we examined the degranulation state of "vessel-associated" mast cells using histology. Previously, we found that pericardial mast cell degranulation was associated with increased pericarditis, immune complex deposition along the pericardium, DCM and, heart failure in mice (54, 79). BPA exposure at concentrations relevant to human exposure, like in this study, have been found to enhance histamine and leukotriene release from bone marrow-derived mast cells but not did not require ER signaling for this effect.

Overall, our findings suggest that BPA exposure at higher doses increases the risk for women to develop myocarditis following CVB3 infection. Exposure to BPA that has leached from plastic containers activates mast cells in the heart in the context of viral infection, particularly along the pericardium. Future studies will be needed to determine whether the increase in myocarditis caused by BPA is due primarily to ER activation/alteration on immune or cardiac cells.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

Mice were used in strict accordance with the recommendations in the Guide for the Care and Use the Laboratory Animals of the National Institutes of Health. Mice were maintained under pathogen-free conditions in the animal facility at the Johns Hopkins School of Medicine and at Mayo Clinic Florida, and approval obtained from the Animal Care and Use Committee at Johns Hopkins University and Mayo Clinic Florida for all procedures. Mice were sacrificed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

AUTHOR CONTRIBUTIONS

KB and DF designed the study, generated the figures, and wrote the paper. KB, JM, AY, JF, AJS, HG, FM, MG, GC, AB, AM-L, AH, AM, DD, AC, and ARS performed the experiments. KB, JS, and DF analyzed the data. KB, AM-L, AH, AM, DD, JS, and DF edited the paper.

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REVIEW
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Influences of Sex and Estrogen in Arterial and Valvular Calcification

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Vascular and cardiac valvular calcification was once considered to be a degenerative and end stage product in aging cardiovascular tissues. Over the past two decades, however, a critical mass of data has shown that cardiovascular calcification can be an active and highly regulated process. While the incidence of calcification in the coronary arteries and cardiac valves is higher in men than in age-matched women, a high index of calcification associates with increased morbidity, and mortality in both sexes. Despite the ubiquitous portending of poor outcomes in both sexes, our understanding of mechanisms of calcification under the dramatically different biological contexts of sex and hormonal milieu remains rudimentary. Understanding how the critical context of these variables inform our understanding of mechanisms of calcification—as well as innovative strategies to target it therapeutically–is essential to advancing the fields of both cardiovascular disease and fundamental mechanisms of aging. This review will explore potential sex and sex-steroid differences in the basic biological pathways associated with vascular and cardiac valvular tissue calcification, and potential strategies of pharmacological therapy to reduce or slow these processes.

Keywords: age-related cardiovascular parameters, aging, aortic valve stenosis, cardiovascular calcification, epidemiology, estrogen, hemodynamics, testosterone

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BACKGROUND

Ectopic calcification in cardiovascular tissue was once considered to be a passive consequence of cardiovascular disease processes with increasing age. The association-based clinical observations driving this model painted a remarkably appealing picture, with vascular calcification being evident in roughly 25% of patients at age of 50 years, and soaring to over 60% in patients over the age of 75 years (1). While the incidence of aortic valve calcification was slightly lower, the overall trend for dramatic, age-associated increases was equally robust. From such population-based studies, risk factors for vascular and valvular calcification quickly emerged, include aging, metabolic syndrome, smoking, and male sex (2–5).

The site-specific mechanisms of ectopic calcification within the tissues of the cardiovascular system are incompletely understood. At present, calcification in either the coronary arteries or cardiac valves is often considered an organized, regulated, and active pathological process, with evidence of many molecular pathways paralleling those observed in bone/orthotopic ossification. Despite this apparent conservation of core osteoblastic signaling pathways, mature bone matrix is rarely found in calcifying cardiovascular tissues (6–8). Critically, however, upstream mechanisms regulating the induction and amplification of these signaling pathways are likely to be

fundamentally different between cardiovascular and orthotopic tissues, since exposure to oxidative stress amplifies osteogenic signaling in calcifying cardiovascular cells but markedly suppresses calcification in bone-derived osteoblasts (9). Furthermore, the contribution of dystrophic tissue calcification—where amorphous accumulation of calcium occurs in the absence of bone matrix, functional osteogenic signaling, or presence of osteoblast-like cells—remains remarkably elusive in the pathogenesis of aortic valve stenosis.

In recent years, tissue fibrosis emerged as a potential and major contributor to aortic valve dysfunction in experimental animals. In particular, genetically altered mice with a propensity for both hypertension and hyperlipidemia developed hemodynamically-significant aortic valve stenosis associated with structural and molecular changes consistent with activation of fibrogenic signaling, and critically, in the absence of substantive changes in valvular calcification. Consequently, investigation into the role of fibrosis as a clinically-meaningful determinant of the degree of valvular stenosis is an exciting and emerging field.

THE CLINICAL JUSTIFICATION FOR EXPLORING THE ROLE OF SEX IN CAVD

While a number of retrospective studies led many to conclude that "women are protected against aortic valve stenosis by estrogens" (10–12), recent work suggests that the pathobiology underlying the disease process may be fundamentally different. For instance, in aortic valve disease—where calcification was once thought to be the primary and near exclusive driver of valve dysfunction–men had more calcification than women at any given level of valvular stenosis (even after normalizing for body size or aortic root size) (13–15), suggesting that valvular fibrosis may play a greater role in determining cusp movement in women compared to men. Similarly, the site of cardiovascular calcification seems to play an important role in predicting mortality in a sex-dependent manner, with thoracic aortic calcification being a strong predictor of mortality secondary to coronary events predominantly in women (16), whereas thoracic aortic and abdominal aortic calcification are strong predictors of all-cause mortality in men (17).

Collectively, the observation that men have higher prevalence of calcification in atherosclerotic lesions and cardiac valves compared to women at any given decade of life has been an interesting clinical observation, and the biological underpinnings and collective clinical implications of these observations are likely to be of great value in developing sex-specific pharmacological treatments to prevent clinically significant valvular and vascular pathology and dysfunction (15, 18, 19). Herein, we aim to highlight potential cellular mechanisms modulated by sex and sex steroid hormones contributing to key sex differences in cardiovascular calcification, with an overall aim of driving dialogue around critical unanswered questions in the field.

MOLECULAR SIGNALING INVOLVED IN CARDIOVASCULAR CALCIFICATION: AN INITIAL SEX AND SEX HORMONE AGNOSTIC PERSPECTIVE

While calcification in the cardiovascular system is often considered an active, regulated process with activation of many fundamental osteogenic signaling cascades being conserved between ectopic cardiovascular calcification and orthotopic bone ossification, upstream mechanisms contributing to the induction, sustained activation, and amplification of these pathways can differ markedly. Interestingly, even during formation of micro-calcific deposits there is expression of proteins which are usually absent (e.g., osteopontin) and/or overexpression of proteins which are usually very low in local tissue (e.g., matrix Gla protein), suggesting that maladaptive processes may be initiated even in the earliest stages of the disease (4, 5, 14, 20, 21). Several of the major molecular signaling pathways involved in regulation of ectopic calcification are ubiquitously active in both males and females (**Figure 1**).

Transforming Growth Factor-β (TGF-β) Signaling

One of the most extensively studied pathways in calcific vascular and valvular disease is TGF-β pathway. While the downstream effects are remarkably context dependent (and *in vitro* are dependent on substrate stiffness), TGF-β most often induces cell migration, proliferation, and extracellular matrix protein elaboration. Critically, on stiff substrates and matrices, TGFβ robustly induces apoptosis and dystrophic calcific nodule formation in aortic valve interstitial cells from a variety of species, suggesting that the matrix accumulation and sclerosis occurring in early stages of valvular heart disease may shift the phenotypic consequences of increased TGF-β across the spectrum of the disease and contribute to both fibrosis and dystrophic calcification to different extents during the evolution of disease (22, 23).

BMP Signaling

Bone morphogenetic proteins (BMPs), members of the TGF-β superfamily, are significantly increased in ectopic calcification lesions within the cardiovascular system including valvular and vascular tissues (22, 24, 25). Initiated by seminal observations from Demer et al., nearly two decades of work have generated compelling data that BMP signaling plays an integral role in the initiation and progression of cardiovascular calcification. Most paradigms implicating BMP signaling suggest that these morphogens serve as a paracrine signal from nearby resident cells to drive osteogenesis via a BMP-Msx2-Wnt cascade (26–28). Importantly, mechanical stimuli—including non-laminar blood flow patterns exacerbated by multiple disease states–induce both oxidative stress and BMP elaboration from vascular and valvular endothelial cells (29, 30). Furthermore, BMP2- and BMP4-driven osteogenic signaling can be further augmented in conditions

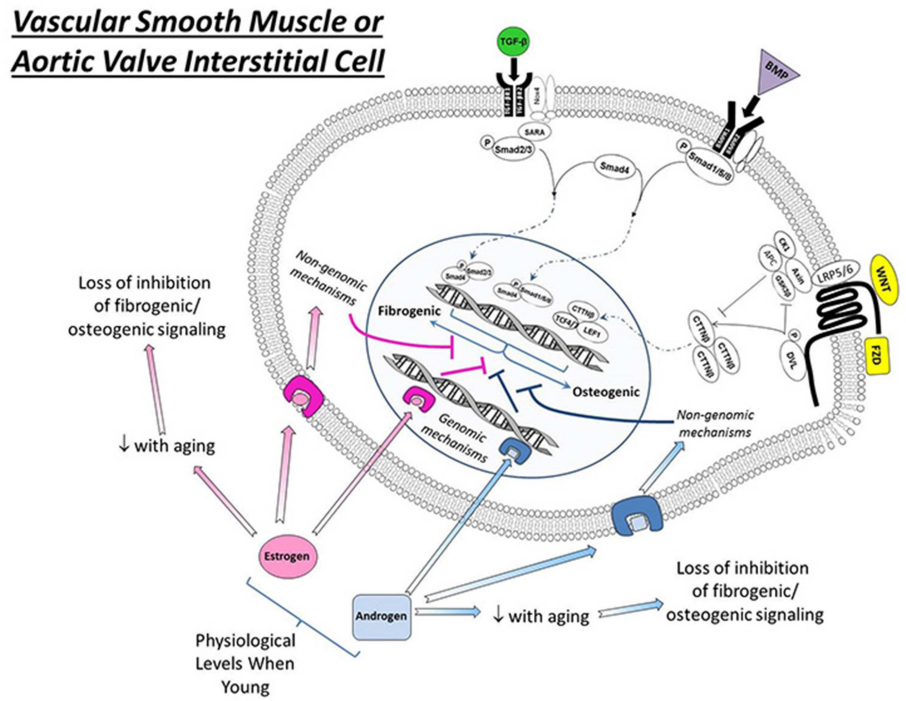

FIGURE 1 | Effects of estrogens and androgen signaling on multiple cellular processes implicated in the regulation of cardiovascular calcification. Note that estrogen can bind to estrogen receptors (ER, resulting in nuclear translation), G-protein coupled estrogen receptors (GPER, eliciting cytosolic signaling), and estrogen binding proteins (EBPs, eliciting cytosolic signaling) to exert a variety of effects—both positive and negative—on molecules influencing ectopic calcification. In general, androgens bind to androgen receptors (AR) and have a smaller number of signal transducing elements compared to estrogens. Abbreviations and impact on calcification: RANKL, receptor activator of nuclear factor κB ligand (promotes calcification); PPAR γ, peroxisome proliferator-activated receptor-γ (prevents calcification); NFκB, nuclear factor κ B (promotes inflammation/calcification); NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase (increases oxidative stress/calcification); SOD, superoxide dismutase (reduces oxidative stress/calcification); $O_2\bullet^-$, superoxide anion (increases calcification); p53, tumor protein 53 (promotes inflammation/calcification).

where endogenous inhibitors (such as matrix Gla protein) are reduced or absent in a variety of disease states (31).

It is noteworthy that not all bone morphogenetic proteins drive ectopic tissue calcification. For example, BMP-7, which is found in human vascular calcification, slows the progression of arterial calcification in both human and mice with diabetes and hyperlipidemia (32, 33). It is also important to note that while the context dependence of TGF-β signaling has been well-defined by numerous investigators, the role of cell-substrate interactions in the phenotypic consequence of BMP signaling has received much less attention in the literature.

Wnt/β-Catenin Signaling

While increased TGF-β superfamily signaling is a near ubiquitous finding in calcifying cardiovascular tissues, numerous investigators have reported upregulation of other signaling pathways central in bone ossification in diseased vascular and valvular tissues (34). One such pathway is Wnt/β-catenin signaling, where multiple reports have documented increases in Wnt ligand elaboration, low-density lipoprotein receptor-related protein (LRP) receptor components, hyperactivation of canonical β-catenin signaling components, and upregulation of β-catenin transcriptional targets (35, 36).

The Role of Extracellular Vesicles in Cardiovascular Calcification

Recently, numerous studies have implicated extracellular vesicles in the initiation and progression of cardiovascular calcification (37–39). While their precise role remains largely unclear, several studies reported accumulation of nanoparticles that appear to precede (or occur concomitantly) with induction of osteogenic signals in cardiovascular tissue, and aggregation of such vesicles can contribute to formation of larger calcific masses at multiple cardiovascular sites (40–42). Unlike bone, however, where matrix vesicles are derived largely from chondrocytes and osteoblasts, extracellular vesicles accumulating in the cardiovascular system appear to be derived from vascular smooth muscle cells and/or immune cells/macrophages (43, 44). While the composition of each vesicle subset has yet to be comprehensively characterized, it is likely that the cell origin, mechanism/driver of release, vesicle contents, and target tissue in which deposition occurs are all likely determinants of phenotypic/biological outcomes (45, 46).

The Site-Specific Role of Inflammation in Ectopic and Orthotopic Calcification

Tumor necrosis factor-α (TNF-α) is a major cytokine involved in driving both local and systemic inflammation in a variety

of cardiovascular pathologies. Thus, it is not surprising that upregulation of TNFα has been shown to augment multiple pathophysiological intracellular signaling cascades involved with vascular and valvular calcification, including interactions with BMP signaling, Msx2-dependent gene transcription, and both canonical and non-canonical Wnt/β-catenin signaling) (35, 47–49). For example, the presence of a TNF-α-Msx2–Wnt/β-catenin cascade acts as a major driver of calcium deposition in aortic valve interstitial cells *in vitro* (48, 49) and in atherosclerotic lesions in hyperlipidemic mice *in vivo* (50). While additional mechanistic studies have suggested that antibody-mediated neutralization of TNFα may be effective at slowing initiation or progression of plaque calcification through attenuation of Wnt/β-catenin signaling (3, 50), systemic, long-term suppression of TNFα also puts patients at risk of being immunocompromised, and development of fatal infections.

IMPACT OF SEX HORMONES ON PROCESSES OF CALCIFICATION

Although the overall lifetime incidence of atherosclerosis, aortic stenosis, and cumulative death from cardiovascular diseases are remarkably comparable between men and women (51, 52), the primary contributor to the perception of reduced CVD burden in women is the delay in prevalence of atherosclerosis and/or aortic valve stenosis in women compared to men at each decade of life (53, 54). Our understanding of the impact of sex hormones on cardiovascular calcification is compounded by several factors, including marked differences in sex hormone levels over the lifespan of men and women, the effect, type, and timing of hormone replacement on cardiovascular biology in women, and the general paucity of appropriately powered clinical trial data evaluating differential efficacy and effectiveness of drug interventions on men and women. This understanding is complicated further by the fact that many pre-clinical studies have not actively considered the role of sex in the biological pathways being interrogated (via the exclusion of female animals) or have not faithfully recaptured changes in hormones similar to that observed over the lifespan of humans. Given these caveats, we will address the potential roles of both estrogens and androgens on cardiovascular calcification by first providing limited insights from clinical observations, followed by mechanistic insights gleaned largely from pre-clinical animal models.

ROLE OF ESTROGENS IN THE REGULATION OF CARDIOVASCULAR CALCIFICATION

Clinical Observations

Several seminal studies reported that post-menopausal estrogen treatments may reduce the risk of cardiovascular calcification when administered within the first 5 years of menopause (55, 56). While several recent studies suggest that initiation of estrogen repletion outside of this time period may not confer optimal protection against a myriad of CV complications, the timing, type, and dosing regimens of estrogen that confer vasculo-/valvulo-protection remains a very active field of investigation (57, 58).

Pre-clnical Observations

Given the increased prevalence of subclinical and clinical CVD occurring within first decade following menopause (59, 60), a large amount of effort has been put into understanding the interplay between exposure to either endogenous or exogenous estrogens and several of the abovementioned pathophysiological signaling cascades.

in vitro, estrogen signals through its binding to cytosolic estrogen receptors (such as estrogen receptor α or β), estrogen binding proteins(EBPs), or membrane G-protein-coupled estrogen receptors(Gpr30) (see **Figure 2**) (61–63). Through a myriad of genomic and non-genomic effects, estrogens can suppress a variety of molecular processes known to drive cardiovascular calcification, including repression of receptor-activator of nuclear factor κB ligand (RANKL) (47, 64) and NFκB signaling, suppression of NADPH oxidase activity in resident cells and inflammatory infiltrates (65, 66) and suppression of p53 (67). Importantly, estrogens do not exert their effects solely by negative regulatory mechanisms, and treatment of cells or animals with exogenous estrogens can drive expression of antioxidant enzymes (in cytosolic, mitochondrial, and lysosomal compartments), and increase nitric oxide synthase activity and expression, both of which have been implicated as key protective mechanisms in cardiovascular calcification (**Figure 2**) (68–70).

in vivo, endogenous estrogen levels are critical for protection against cardiovascular calcification at multiple sites, as surgical ovariectomy in young mice results in accelerated development of advanced calcified lesions in both aortic and aortic valve tissue (14). It is likely that a major mechanism whereby estrogen exerts its protective effects is via the suppression of TGF-β-dependent extracellular matrix production and accumulation and downregulation of non-collagenous proteins (71) in cardiovascular lesions, both of which likely serve to prevent increases in micro-environmental stiffness that increase the propensity for apoptosis in response to sustained elevations in TGF-β (72). The interactions between sex hormones and TGF-β signaling are remarkably complex and context dependent, however, and entire reviews have focused on this topic and unanswered questions in the field (73). Furthermore, given previous work suggesting that TGF-β can reciprocally inhibit estrogen receptor signaling via a canonical smad4 interaction (74, 75), the extent to which systemic estrogens can be increased to win over this interplay remains unclear. Given clinical observations of disproportionately augmented TGF-β signaling and fibrosis in women (which is not associated with increases in deposition of dystrophic calcific deposits) (15), it is evident that the net impact of sex hormones on the molecular and phenotypic sequelae of TGF-β signaling will be paramount to the advancement of pharmacotherapies targeting valvular stenosis.

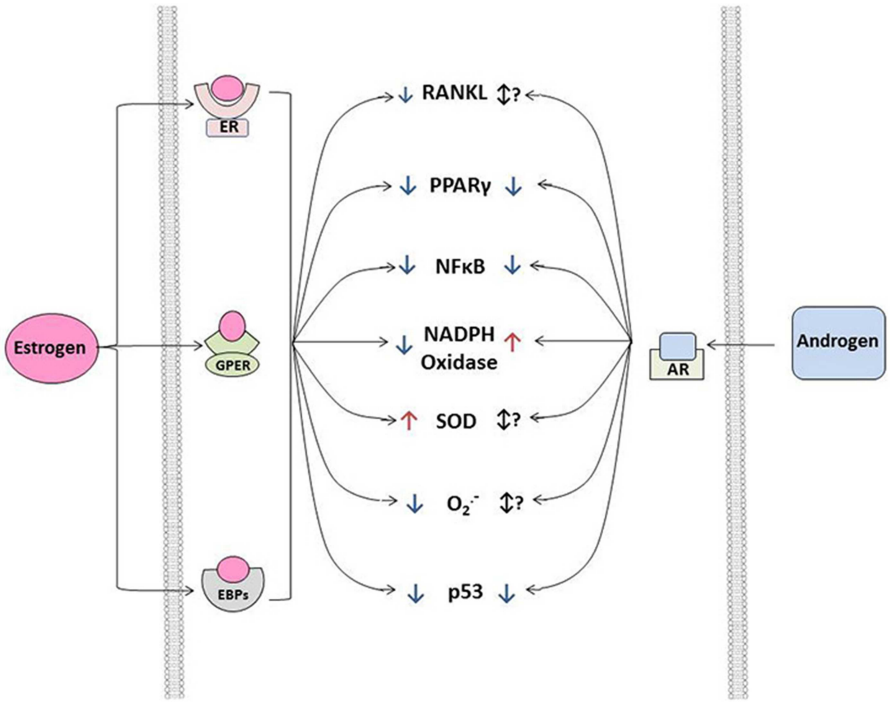

FIGURE 2 | Interactions between estrogen signaling, androgen signaling, and osteogenic signaling in vascular smooth muscle or aortic valve interstitial cells exposed to physiological levels of sex hormones. Note that—in general—both estrogens and androgens suppress osteogenic signaling via both genomic and non-genomic mechanisms in both cell types at physiological levels in relatively early to mid-life stages. Importantly, the therapeutic harnessing of these mechanisms requires substantial research into the context dependence of sex hormone signaling (e.g., timing relative to menopause, level to which hormones should be restored for optimal therapeutic benefit, etc.). TGF-β, transforming growth factor β; BMP, bone morphogenetic protein; Wnt, wingless-related integration site; TGFβR1/2, transforming growth factor beta receptor 1 or 2; BMPR1/2, bone morphogenetic protein receptor 1 or 2; Nox4, NADPH oxidase 4; SARA, smad anchor for receptor activation; Smad, Suppressor of Mothers Against Decapentaplegic; LRP5/6, Low-density lipoprotein receptor-related protein 5 or 6; CK1, Casein kinase 1; DVL, Disheveled protein; Axin, Axin 1 protein; APC, adenomatous polyposis coli protein; GSK3, Glycogen synthase kinase 3; CTTNβ, beta-catenin protein; TCF 4/7, Transcription factor 4 or 7; LEF1, Lymphoid Enhancer Binding Factor 1; FZD, Frizzled receptor.

These estrogenic effects are independent of the Y chromosome, as atherosclerosis, vascular calcification, and bone growth were accelerated in a man with estrogen receptor dysfunction (76). Since testosterone is converted to estrogen by aromatase in both females and males, additional insights related to the role of estrogenic signaling in men can be gleaned from studies in which aromatase inhibition was administered. Here, inhibition of aromatase reduced vascular dilatation in men (77, 78), suggesting the net impact of endogenous "testosterone-derived" estrogens is protective in men. Furthermore, we are not aware of additional clinical evidence that short term use of aromatase inhibitors bring benefit for reducing cardiovascular disease incidence in the elderly male patients with low levels of androgen (i.e., attempts to restore testosterone levels through prevention of its degradation) (78, 79). Complementing these data supporting a net protective effect of aromatase-derived estrogens, administration of aromatase inhibitors for 5 years in women (to reduce the recurrence of estrogen-receptor positive breast cancer), also appears to increase incidence of cardiovascular disease (80).

ROLE OF ANDROGENS IN THE REGULATION OF CARDIOVASCULAR CALCIFICATION

Clinical Observations

Numerous studies have shown that, in general, testosterone levels decline relatively linearly after the third decade of life, and can be reduced by more than 50% beyond the sixth decade of life. While the decline in free testosterone is coarsely and inversely related to cardiovascular event rates, causal relationships between changes in testosterone levels and cardiovascular disease remains complicated and highly context dependent (81, 82).

Currently, the vast majority of scientific literature would suggest that supraphysiological levels of testosterone—such as those observed in athletes aiming to improve performance—results in significantly higher levels of coronary artery atherosclerosis compared to non-users of the same age (83–85). Reciprocally, hypogonadal men (testosterone levels <300 ng/dL) have an increased risk of numerous cardiovascular events and complications (86, 87). Of the handful of controlled clinical trials completed to date, most suggest that restoring testosterone levels

to mid-normal range does not increase cardiovascular event rates during most follow-up periods (82, 88). Such interventions may, however, increase the volume of non-calcified coronary lesions (89), suggesting that normal levels of testosterone may be pro-atherogenic but not pro-osteogenic/-calcific.

Pre-clinical Observations

Androgens

Numerous studies have probed the interactions between androgen signaling and a variety of pathophysiological signaling cascades in cardiovascular tissues, which have in part been a significant contributor to the controversy surrounding their net impact on cardiovascular diseases. In the context of regulating valvular and vascular disease, several studies have demonstrated a clear role for androgens in the promotion of calcific nodule formation through increasing levels of reactive oxygen species (66, 90–92), repressing PPARγ signaling (93–95), and increasing osteogenic signaling (88, 96, 97) (**Figure 2**). In line with the aforementioned clinical observations in hypogonadal men, however, physiological levels of androgens may reduce vascular calcification by sustaining eNOS activity (23, 98, 99), reducing TGFβ signaling (100, 101), through the suppression of p53-dependent cellular senescence (67, 102), prevention of cellular apoptosis (91, 103, 104), reducing RANKL signaling (105), suppression of local inflammatory signaling (106–110), and attenuation of pro-thrombotic factor activity (111, 112) (**Figure 2**).

PROTECTIVE ANDROGENS vs. PROTECTIVE ANDROGEN-DERIVED ESTROGENS

Similar to aging humans, lower serum testosterone is associated with increased risk of cardiovascular calcification in experimental animals and is attenuated by long-term androgen repletion (98, 113). The biological interpretation of this effect is complicated, however, given the fact that both endogenous and exogenous testosterone can be converted to estrogen by aromatase enzymes (114, 115). Seminal studies showing that androgen receptor-dependent signaling has a deleterious impact on CV calcification (via genetic inactivation of the androgen receptor) combined with reports of augmented vascular calcification in men with spontaneous loss-of-function mutations in estrogen receptors also suggests that testosterone-derived aromatases are an underappreciated factor when considering the net impact of androgen signaling on advanced vascular disease (79, 80, 96).

HORMONE-INDEPENDENT EFFECTS OF ORGANISMAL SEX: THE ROLE OF THE CHROMOSOMAL COMPLEMENT

Sex hormones aside, the sex chromosomes–the most fundamental and intrinsic determinant of organismal sex–is also likely to be a significant determinant of propensity for cardiovascular calcification. Both X and Y chromosomes have strong linkage associations with cardiovascular disease risk factors such as hypertension, cardiovascular inflammation, immune biology and macrophage function, and organismal metabolism (116–120). Perhaps most critically, cells derived from XX or XY organisms which are treated with identical *in vitro* conditions show differences in proliferation, fibrosis, and apoptosis in response to various agonists (121, 122). These changes are not restricted solely to vascular tissues, as osteogenic signaling and responses to various agonists also differ in aortic valve interstitial cells from XX and XY animals (123). Thus, while the Y chromosome may be referred to as a "non-recombining desert" in some biological circles (124), its sustained phenotypic impact is of undeniable importance in cardiovascular tissues.

CONCEPTUAL GAPS AND CONTROVERSIES IN THE FIELD OF CARDIOVASCULAR CALCIFICATION

While tremendous advances have been made in our understanding of cardiovascular calcification over the past several decades, several major gaps remain in our efforts to translate and apply both biological and clinical discoveries to the care of an individual patient.

As appropriate with the scope of this review, one could readily argue that the field's greatest gap relates to our understanding of the impact of biological sex and the sex steroid hormonal milieu on phenotypic and clinical outcomes in diseases where vascular, valvular, or microvascular calcification are of clinical importance. As the role of this critical context becomes clearer with appropriately controlled and sex-balanced pre-clinical and clinical investigation (125–127), we will undoubtedly gain deeper insights into both the pathobiological underpinnings and potential efficacious therapeutic interventions in men and women suffering from calcific cardiovascular diseases.

Perhaps the greatest controversy in the field of cardiovascular calcification—which is not necessarily exclusive from of our understanding of biological sex—is the contexts in which ectopic calcification is driven by non-osteogenic or osteogenic mechanisms. More specifically, while an overwhelming body of evidence suggests that the osteogenic signaling cascades described in this review are present in calcifying tissues from the vast majority of patients with cardiovascular calcification, clinical observations at the time of surgery or autopsy suggest that bone matrix is only evident in a relatively small fraction of this patient population (e.g., 15–25%) (128). Thus, how the cellular decision is made to initiate maladaptive, osteogenic "response to injury" at the earliest stages of microcalcific nodule formation (129) that propagates to true "ectopic bone" or alternatively expands due to progressive and persistent cellular apoptosis to form an amorphous, calcific deposit (130–133) with merely associative increases in osteogenic signaling remains remarkably elusive.

Finally, the role of the biological context of organismal age (and its fundamental biological determinants including changes in sex steroid hormones) in dictating these decisions is only beginning to be understood. While there has been a longstanding association between cellular senescence and tissue calcification [stemming from seminal work by Shanahan et al.

(134–137)] more recent work suggests that the pharmacological targeting and/or clearance of senescent cells may also be a viable strategy for slowing progression of vascular calcification and dysfunction (136). The postulate that targeting fundamental biological mechanisms of aging may be a viable strategy to delay onset or prevent progression of cardiovascular calcification is supported by intriguing observation that biomarkers of biological age (e.g., telomere length) are stronger predictors of incidence of valvular heart disease than chronological age (138).

PERSPECTIVES ON THE FUTURE OF THE FIELD OF CARDIOVASCULAR CALCIFICATION AND STRATEGIES FOR ADDRESSING THE EFFECTS OF SEX

While the field of cardiovascular calcification has made tremendous strides in advancing our understanding of osteogenic and non-osteogenic mechanisms contributing to ectopic calcium accrual over the past two decades, it is our opinion that the greatest advances—both scientifically and clinically—will be made in the near future by exploring the context of sex and sex hormones in these phenomena. While many may consider this to be too bold of a statement, it stands on a firm foundation of both clinical and biological reports demonstrating clear sex- and sex hormone-driven differences in the progression of calcific cardiovascular diseases and a glaring lack of success in viable pharmacological strategies to mitigate cardiovascular calcification in elderly persons.

While the National Institutes of Health now mandates consideration of sex as a biological variable in all studies that receive funding (e.g., from pre-clinical animal investigation to human trials), there have not been consistent requirements from publishers and journals requiring reporting of data by sex. Consequently, we would make the following additional suggestions to drive discussion of true "best practices" and accelerate development of critical insights into mechanisms underlying cardiovascular calcification in future studies. First, we suggest that *in vitro* studies should include independent cell lines derived from each sex of the species being studied, which will allow for characterization of the impact of chromosomal complement and differing epigenetic demarcations in the absence of the sex-steroid and systemic hormonal millieu. With the emergence of evidence that cell line immortalization can drive X and Y chromosomal reconfiguration (139) and the absence of data demonstrating whether such phenomena eliminates sex-dependent molecular responses to exogenous stressors, we also advocate for the use of primary cell lines until comprehensive characterizations of cell phenotype and response are available. Second, we suggest that all sex-disaggregated data should be available within manuscripts and/or online supplements, and that studies should be designed to detect sex differences with appropriate statistical power in an a priori manner. In clinical conditions in which a disease occurs predominantly in one sex vs. the other, we feel a minimum recommendation of having the experimental sample composition be reflective of the sex distribution within the patient population of interest is reasonable. Finally, execution of appropriate hormonal depletion, repletion, or crossover studies would represent a major advance in the field. By appropriate, we refer not only to absolute hormonal levels but also to timing of repletion/depletion (i.e., initiation of the insult later in life, similar to what occurs in humans).

While we believe few investigators would argue that the abovementioned recommendations directly align with the foundational principles of scientific rigor, we are acutely aware that the logistics of implementing such recommendations must be addressed. While doubling the scope and scale of ongoing research projects is neither feasible nor sustainable, we would argue that generation of pre-clinical and clinical datasets that do not inform either the pathobiological underpinnings or clinical care of half of the world's population reduces the relevance and impact of scientific investigation around the globe. At present, we believe there are few viable arguments against the intentional and appropriate inclusion (and subsequent disaggregated presentation) of data from both sexes in studies of cardiovascular calcification, as the number of samples required for demonstrating sex differences should not increase dramatically when truly qualitatively different trends are uncovered. We acknowledge that discovery of such dichotomous sex responses can often spur new lines of investigation that are beyond the scope of the existing project and subsequently require support through additional funding mechanisms. Critically, we commend NIH and several other funding entities for creation of supplemental research awards devoted to supporting more detailed investigation of mechanisms underlying unexpectedly discovered sex differences, as well as creation of recent RFA's that prioritize identification of novel sex differences in cardiovascular calcification and disease.

Ultimately, we feel that appropriately designed and executed clinical studies—including both men and women potentially with independent outcome measures in each sex (e.g., not only valvular function as a primary outcome but predominantly calcification in men and fibrosis in women as secondary outcomes) is an essential step in ensuring that the utility of sex in predicting therapeutic efficacy and effectiveness across the translational spectrum. While beyond the scope of this review [and covered recently in Sritharen et al. (14)], emerging data strongly suggest that incoming risk profiles, outcomes for surgical valve replacement, outcomes for transcatheter valve replacement, and comorbid condition frequency following disease diagnosis differ robustly amongst men and women, and can serve as a critical catalyst for driving impactful investigation in the biological mechanisms underlying such observations.

SUMMARY

The clinical presentation, biological underpinnings, and molecular interactions with sex hormones and biological sex, and ultimate strategies to therapeutically prevent or slow

progression of cardiovascular calcification differ dramatically between men and women. While in the United States, the National Institutes of Health (NIH) mandate for inclusion of both sexes in research will undoubtedly serve to advance our understanding of these differences, it is our hope that this review will spur additional genuine interest in understanding critical biological and clinical contexts—including, but not limited to organismal sex—and drive transformative advances in the strategies and tools needed to reduce the growing global burden of calcific vascular and valvular diseases.

AUTHOR CONTRIBUTIONS

All authors contributed to the drafting and revision of the manuscript and concepts expressed herein.

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REVIEW
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Non-genomic Effects of Estrogen on Cell Homeostasis and Remodeling With Special Focus on Cardiac Ischemia/Reperfusion Injury

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This review takes into consideration the main mechanisms involved in cellular remodeling following an ischemic injury, with special focus on the possible role played by non-genomic estrogen effects. Sex differences have also been considered. In fact, cardiac ischemic events induce damage to different cellular components of the heart, such as cardiomyocytes, vascular cells, endothelial cells, and cardiac fibroblasts. The ability of the cardiovascular system to counteract an ischemic insult is orchestrated by these cell types and is carried out thanks to a number of complex molecular pathways, including genomic (slow) or non-genomic (fast) effects of estrogen. These pathways are probably responsible for differences observed between the two sexes. Literature suggests that male and female hearts, and, more in general, cardiovascular system cells, show significant differences in many parameters under both physiological and pathological conditions. In particular, many experimental studies dealing with sex differences in the cardiovascular system suggest a higher ability of females to respond to environmental insults in comparison with males. For instance, as cells from females are more effective in counteracting the ischemia/reperfusion injury if compared with males, a role for estrogen in this sex disparity has been hypothesized. However, the possible involvement of estrogen-dependent non-genomic effects on the cardiovascular system is still under debate. Further experimental studies, including sex-specific studies, are needed in order to shed further light on this matter.

Keywords: estrogen, non-nuclear estrogen receptors, cardiomyocytes, vascular cells, ischemia/reperfusion, myocardial infarction, sex, gender

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INTRODUCTION

Cardiovascular diseases (CVD), including acute myocardial infarction (MI), represent leading causes of morbidity and mortality worldwide in both sexes. However, in past years, the risk of CVD was underestimated in women due to the mistaken belief that women could somehow be protected (1, 2). Although it was observed that women develop coronary artery disease about 10 years later than men, they show a higher prevalence of cardiovascular risk factors at the same times of their lifespan (3). Even currently CVD continue to be perceived as predominantly male pathologies, leaving women vulnerable to CVD due to an inadequate prevention. However, even if women in their reproductive age have a lower risk of cardiovascular events, their advantage decreases after

menopause, so that CVD are the leading cause of death in women older than 65 years (4). In fact, in Europe, CVD cause a greater proportion of deaths among women than men (5, 6), also representing a critical economic burden (7).

The mechanisms leading to MI are due to a blocked blood flow resulting in various biochemical and metabolic alterations within the myocardium, i.e., in its main cell components: the cardiomyocytes (CMs). These cells undergo a series of well-characterized alterations, including mitochondrial dysfunction and, if prolonged, the death of cardiomyocytes (CMs). Obviously, ischemic events also induce damage in vascular cells and cardiac fibroblasts (CFs). The ability of cardiac tissue to recover after these events is carried out through a complex process of remodeling, orchestrated by CFs, inflammatory cells and cardiomyocytes (8). A number of complex cellular and molecular pathways, including antioxidant pathways and hormones, have been demonstrated to be able to counteract the damage. Imbalance or failure of these pathways leads to adverse remodeling of the heart and poor prognosis. However, the precise mechanisms of cardiomyocytes molecular injury after MI are still to be elucidated in detail (9). Some of these determinants are of interest of this work and are listed here below.

Following a MI, the left ventricle undergoes a remodeling that involves the removal of the necrotic tissue that is replaced by extracellular matrix proteins. The removal of necrotic tissue is carried out by the immune cells that polarize and release enzymes, such as matrix metalloproteinases (MMPs) and reactive oxygen species (ROS) (10). It was observed that infiltrating leukocytes release cytokines and growth factors such as pro-inflammatory interleukin IL-1β and reparative transforming growth factor β (11) that contribute to microenvironment alteration. This inflammatory state has been shown to be different in males and females either in animal models or in humans. In particular, females have a more moderate response to inflammatory stimuli; for example, in sepsis and atherosclerosis they have lower pro-inflammatory leukocyte-mediated inflammation and a faster resolution of inflammation compared with males (12, 13). Although it is known that XX cells have a more pronounced antioxidant capability (14–17), this matter should be better investigated in post-ischemic MI-associated damage.

It has been observed that early restoration of coronary blood flow after MI plays an important role in minimizing myocardial tissue injury through various types of therapy, such as thrombolytic therapy, coronary artery bypass grafting or primary percutaneous intervention (18). However, reperfusion may further contribute to newer myocardial damage defined as myocardial ischemia/reperfusion (I/R) injury, in which oxidative stress plays a critical role igniting ROS generation eventually leading to necrotic, apoptotic or autophagic cell death (19). Accordingly, current anti-apoptotic agents have generally been reported to safeguard the heart from I/R injury (20–22). However, increasing evidence also indicates that modulation of autophagy, that can be considered as a cytoprotective mechanism that leads to cell death only once all the energy supply derived by intracellular materials are exhausted, is now considered as a novel therapeutic strategy in myocardial I/R injury (23).

Although sex steroid hormones, particularly estrogens, appear to be involved through genomic and non-genomic effects in cell remodeling, molecular mechanisms remain still unknown (24, 25). Females undergo more efficient cardiac remodeling after ischemia/reperfusion injury most likely due to the cytoprotective effects of estrogen via an unknown mechanism. The regulatory effects of estrogen in cardiac sensitivity to I/R injury could have in fact many potential therapeutic implications, e.g., influencing strategies in acute coronary syndrome management. Tamargo and co-workers shed some light on this matter discussing in detail the efficacy and safety of several drugs of common use in cardiovascular diseases taking into account both sexes (6).

ESTROGEN AND ESTROGEN RECEPTORS

Several estrogens, including estrone (E1), 17β-estradiol (E2), and estriol (E3) are present in the adult bloodstream, where E2 is the most represented and exerts many effects in both physiological and pathological conditions including cancer (26). In addition to its production in the ovaries of fertile women, E2 can be produced in other tissues as a product of enzymatic conversion of testosterone by aromatase (27). This enzyme is expressed in different extragonadic tissues, such as fat, bone and brain (28). Furthermore, increasing lines of evidence also demonstrate the local production of aromatase by heart and blood vessels of both sexes (29, 30).

E2 biological activities pass through its interaction with the estrogen receptors ERα and ERβ. Moreover, several polymorphisms that could be of relevance in CVD have been reported for these receptors (31–33). Initially identified into cytosol and nucleus, ERα and ERβ have more recently been described also at the level of the different intracellular compartments like endoplasmic reticulum, Golgi and mitochondria, other than plasma membrane (34, 35). Indeed, the different intracellular localization of these receptors impacts their specific signaling cascades and their ability to control cell growth, differentiation, survival or death (36–38). Besides ERα and β, an additional E2 binding responsive receptor, named G-protein-coupled estrogen receptor (GPER) has been identified (39). GPER is a member of the family of 7-transmembrane G protein-coupled receptors (GPCRs) and, besides plasma membrane, it has been localized in various intracellular organelles where it mediates several E2 effects (40).

Abbreviations: Ang II, angiotensin II; CF, cardiac fibroblasts; CMs, cardiomyocytes; CVD, cardiovascular diseases; CSE, Cystathionine-γ-lyase; DRP1, dynamin-related protein 1; EC, endothelial cells; eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; E1, estrogen; E2, 17β-estradiol; E3, estriol; ER, estrogen receptor; GPER, G protein-coupling estrogen receptor; FIS1, fission protein 1; HF, heart failure; IL, interleukin; I/R, ischemia/reperfusion; KO, knockout; LV, left ventricle; MI, myocardial infarction; MMPs, matrix metalloproteinases; MISS, membrane-initiated steroid signal; MPTP, mitochondria permeability transition pore; MFN, mitofusins; CSC, multipotent cardiac stem cells; NF-κB, nuclear factor kappa B; NO, Nitric oxide; OPA1, optic atrophy protein 1; PGC-1α, peroxisome proliferator-activated receptor-γ co-activator 1-α; ROS, reactive oxygen species; VSMC, vascular smooth muscle cells.

Signaling Pathways of Estrogen Receptors in Brief

Estrogen receptors transmit hormonal signals through three different pathways. The first one, known as "classic" or genomic, regulates the expression of target genes by DNA binding at specific response elements (EREs). Upon E2 binding, ERs dissociate from the complex formed with some heat shock proteins (like HSP70 and HSP90) in the cytosol, change their conformation and migrate as homo- or hetero-dimers into the nucleus (41).

The second signaling is controlled by an indirect ER binding to DNA, mediated by different co-factors (like SP-1, AP-1, and NF-κB) that exert their transcription regulation by physical interaction with DNA (42). Finally, in the non nuclear pathway, E2 induces very rapid cellular effects, acting through receptors localized at the cell membrane, cytoplasm, and mitochondria. Soon after binding E2, the membrane receptors interact with the Gα and Gβγ proteins to stimulate rapid signals (cAMP and cGMP) and trigger the activation of several transduction pathways (43, 44). The activation of kinases phosphorylates ER or other transcription factors resulting in gene expression regulation (45). As far as GPER is concerned, after E2 binding, it mediates a rapid membrane response involving the activation of kinases, ion channels and second messengers (46). In particular, in the endoplasmic reticulum, GPER activation induces calcium release and PI3K-Akt pathway activation, thus inducing cell proliferation (39, 40). Moreover, although still debated, it seems now clear that GPER does not physically associate with the mitochondria but, instead, its ability to regulate intracellular calcium levels indirectly affects mitochondrial function, including the so-called mitochondrial-induced cell death (47). Earliest studies on GPER also suggest how this receptor, although indirectly, regulates gene expression via an importin-dependent mechanism (48, 49). A schematic picture of possible estrogen action by genomic and plasma membrane ER/GPER signaling pathways is reported in **Figure 1**.

Expression of Estrogen Receptors in the Cardiac Tissue

First evidence of ERα and ERβ expression in the cardiac tissue comes from a study performed in both female and male rat cardiac myocytes and fibroblasts (50). Subsequently, both the ERs were described in the human heart tissue (51) Later, ERα was localized in the nucleus and in the sarcolemma and intercalated discs of human cardiomyocytes (52). Additional data obtained from female and male mice hearts showed that ERα was mainly localized to the sarcolemma whereas ERβ to the nucleus and cytosol of the ventricular and atrial cells (53). ERβ was also described in human cardiac mitochondria (54). More recently, isolated mouse cardiomyocytes showed the presence of all the three ERα isoforms (ERα66, ERα46, and ERα36) in the nucleus (55). However, conflicting evidence also exists as concerns ERβ expression and localization in cardiomyocytes. Of course, the use of antibodies of doubtful specificity (56) as well as the wide variability of animal models and samples analyzed (e.g., whole heart or isolated cardiomyocyte lysates) caused the production of mutually contradictory data. For example, the presence of ERβ in human cardiac mitochondria (54) is still debated (57) and some reports have documented the total absence of ERβ in isolated cardiomyocytes (55). More recently, in a study exclusively conducted at mRNA level in rat cardiovascular tissues, high expression levels of ERα were detected, followed by GPER in terms of abundance, whereas ERβ appeared as nearly undetectable (58). Finally, in line with these results, the implication of ERβ in heart functional recovery after treatment with specific agonists in different animal models of heart failure also appears as still unclear (59–61).

As regards vascular smooth muscle cells (VSMCs), ERα was found to localize to the nuclei and to the plasma membrane in combination with caveolin-1, whereas ERβ was predominantly nuclear (62, 63). Both estrogen receptors have been described also in human adult aortic VSMCs (64). In these cells ERα and ERβ appear as localized at the level of caveolae where a direct binding to striatin is essential for their membrane localization (65). Also GPER was detected in numerous cardiac compartments of the human heart (66) and in coronary artery VSMCs (67). During myocardial hypoxia due to infarction, GPER seems to be upregulated in cardiomyocytes (68).

The question whether the beneficial actions offered by estrogen are due to ERα or to ERβ stimulated a large number of *in vivo* studies (69, 70). These studies were conducted in genetically modified mice and the use of selective agonists or antagonists of these receptors. However, which ER could play a major protective role against I/R injury is still under debate. In fact, a role either for ERα (71–74) or for ERβ (61, 75–77) has been hypothesized. This discrepancy could be due to different models of I/R and/or to different doses and timing of treatments taken into consideration.

Estrogen Receptors: Genetically Modified Mice

As mentioned above, experimental studies involving animal models contributed to delineate the mechanisms involved in sex-related differences in cardiac tolerance to ischemia. In particular, most information derives from the study of genetically modified animals (see **Table 1**). Unfortunately, several studies have been performed almost exclusively on male animals, without taking into account the differences in hormonal fluctuations between sexes (127). In particular, studies based on different ERα gene targeting in murine models have defined the role specifically played by this receptor with particular reference to the different functional domains that compose the protein. As a matter of fact both estrogen receptors are composed by six functionally distinct protein regions like a DNA binding domain (DBD), a ligand-binding domain (LBD), a central region containing a nuclear localization sequence (NLS) and two regions acting as transcriptional activators (AF1 and AF-2), respectively located at the carboxy- and amino-terminal ends (128). The protein region responsible for the activity of E2 in the vascular system and in the metabolic function was identified in the AF2 domain (96), while the AF1 domain seems to be mainly involved in the reproductive function (98). In the same way, it was demonstrated that the localization at the plasma membrane of the receptor

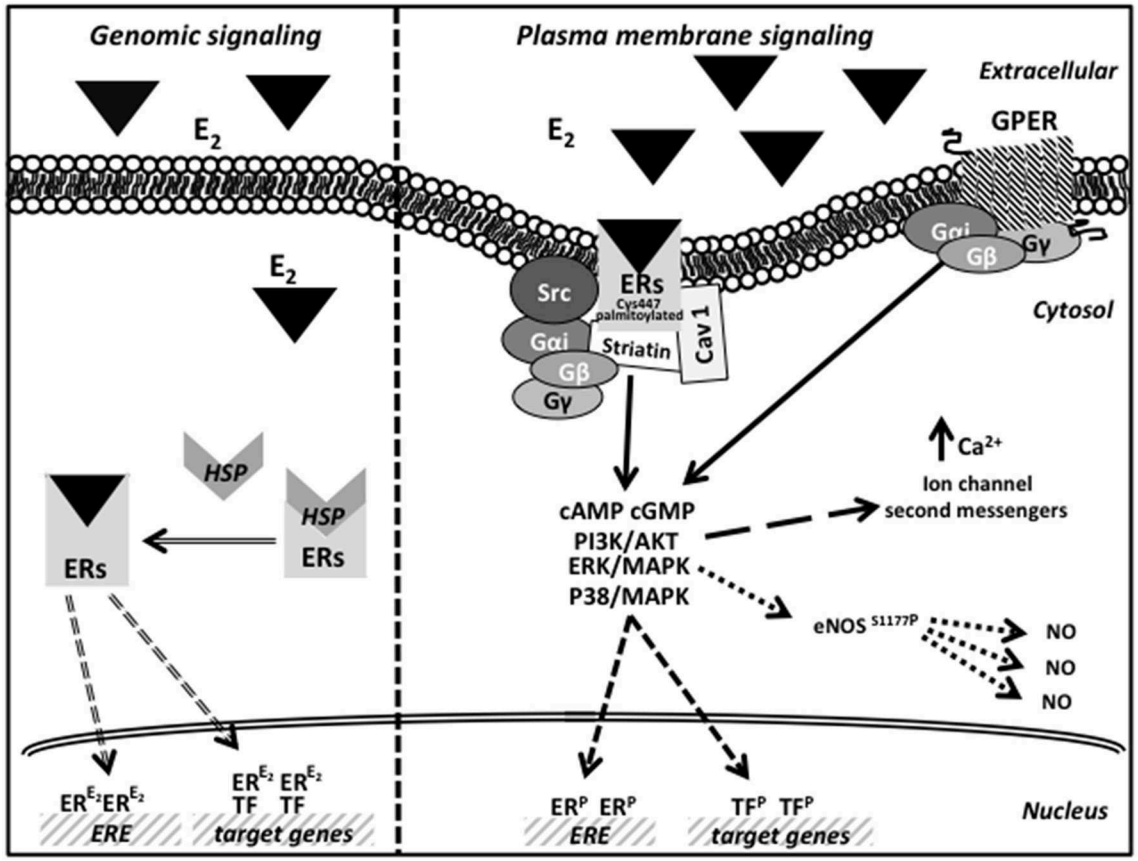

FIGURE 1 | Schematic picture of estrogen action by genomic and plasma membrane ER/GPER signaling pathways.

was closely dependent on its palmitoylation, which in turn favors its association with caveolin-1 in the lipid rafts (99, 100). Indeed, any mutation blocking one of these events effectively abrogates the migration of the receptor to the cell membrane and the stimulation of the membrane specific signaling pathway (129). The importance of striatin in mediating ERs correct localization at plasma membrane was also demonstrated since disruption of ER-striatin interactions abrogated E2-mediated protection against vascular injury (101). More recently, the central role of estrogen-mediated plasma membrane signaling in EC proliferation and migration was further demonstrated by the generation of a mutant version of ERα (KRR ERα), specifically defective in this rapid signaling pathway (103).

Furthermore, some mouse models have been created in order to dissect the different pathways triggered by the nuclear and the non-nuclear ER signaling. The MOER mouse model (98), expressing only the membrane domain (LDB-AF2 domain), showed a phenotype that was very similar to that of ERα$^{-/-}$. However, these mice were still able to regulate some metabolic pathways in response to estrogen treatment (130). On the other hand, in murine models expressing only ERα nuclear mutant (e.g., NOER) the beneficial vascular effects of estrogen were lost (99). More recently, further studies on a different ERα knockout (KO) mouse model have allowed to better define the role played by the nuclear (ERαC451A) and non-nuclear (ERαAF2°) estrogen signaling in arterial protection (97).

ERβ KO mouse models have also been proposed in order to better define the metabolic and vascular activity of ERβ receptors (81, 106). Although showing a less severe phenotype compared with ERα KO, these mice were characterized by abnormalities of heart morphology (109), increased severity of heart failure (HF) after MI as well as less functional recovery after I/R, especially in female mice (75, 110). However, other studies failed to reveal a specific protective role of ERβ in atherosclerosis (112) or in vascular injury (107). Since these two murine models displayed alternative splicing transcripts, additional KO models were also generated (113, 114). Indeed, in these mice the expression of a portion of ERβ in the prostate was observed, suggesting the presence of some still active minor transcripts (114). However, despite being sterile, these KO mice showed a correct development of the main organs and a normal homeostasis of the different body systems. In particular, Antal and coworkers reported the absence of heart abnormalities in 16-month-old male mice (113).

As regards GPER KO, four different mouse models were generated. However, no evident phenotype changes in terms

TABLE 1 | Roles played by estrogen receptors in cardiac function in response to hormonal stimuli: studies in genetically modified animals.

Mouse model	Genetic feature	Vascular phenotype and estrogen response
ERα-Neo-KO	Insertion of neomycin resistance cassette into ESR1 exon 1 resulting in an ERα mutant form lacking the functional AF-1 (78).	Protection of carotid arterial from injury (79). Preserved endothelial NO production (80). Heart functional recovery after I/R in ERαKO female hearts similar to that in WT (75). More severe cardiac damage following I/R injury in male mice (73). Cardiac growth unresponsive to E2 treatment (74).
ERα $^{-/-}$	Insertion of neomycin resistance cassette into ESR1 exon 2 resulting in complete deletion of ERα (81).	Loss of re-endothelialization process (82). Inhibition of NO production in aorta (83). No protection in vascular injury (84). Reduced coronary capillary density associated to decreased VEGF expression and signaling (85).
ER1KO	Targeted mutation of ERα (71)	Decreased heart functional recovery in female ER1KO in comparison to female WT (71).
NERKI $^{+/-}$ or ERα$^{-/A}$ (KI)	Mutated allele in DBD (E207A/G208A, or AA) introduced onto the ERα$^{-/-}$ background (86, 87).	Not determined
ENERKI	Mutation in LBD domain of ERα (G525L) (88).	Not determined
KIKO	Generated by crossing NERKI$^{+/-}$ with ERα $^{+/-}$ mouse model (89).	Not determined
ERα($^{EAAE/EAAE}$) transgenic (KI)	Mutation of four amino acid in the DNA recognition helix (Y201E, K210A, K214A, R215E) (90, 91).	Not determined
H2NES ERα mutant	Insertion of some point mutations in the NLS combined with a nuclear export signal (NES) in the D-domain (92, 93).	Not determined
ERαAF-1^0	Deletion of AF1 domain (amino acids 2-148) (94).	Preserved endothelial NO production and re-endothelialization process and prevention of atheroma (94). Inhibition of neointimal hyperplasia protection in VSMC ERα AF-1 (95).
RαAF-2^0	Deletion of AF2 domain (aa 543–549) (96).	Preserved endothelial repair but failed atheroprotective action (96). Unresponsive to estrogens for beneficial arteriolar effects (97).
MOER	Expression of the ERα E domain (LBD-AF2) containing multiple palmitoylation sites in an ERα$^{-/-}$ background (98).	Not determined
NOER or C451A-ERα	Mutation of palmitoylation site of ERα.	Absence of eNOS phosphorylation, vasorelaxation, acceleration of endothelial healing (99, 100). Fully responsive to prevent atheroma and Ang II–induced hypertension (97).
DPM	Overexpression of the Disrupting Peptide Mouse (DPM) (aa 176–253) to inhibit ERα interaction with striatin (101).	Inability to stimulate EC migration and to inhibit VSMC growth *in vitro*. Loss of protection against vascular injury *in vivo* (101).
(KRR$^{ki/ki}$)	Mutated ERα (KRR) introduced onto the ERα$^{-/-}$ background under the control of the endogenous ERα promoter (102).	Not determined (102). EC (KRR ERα) inability to proliferate and migrate (103).
csERα-OE	Conditioned cardiomyocyte-specific overexpressing ERα (csERα-OE).	Increased LV mass, LV volume and cardiomyocytes length in both sexes. Attenuated fibrosis and increased angiogenesis and lymphangiogenesis in female ERα-OE after MI (104).
csERα$^{-/-}$	Cardiomyocyte-specific ERαKO (csERα$^{-/-}$).	Sex-differences in multiple structural parameters of the heart, with minimal functional differences. Identification of different gene networks potentially involved in cardiac biology (105).
ERβKO	Insertion of neomycin resistance cassette into exon 3 of ESR2 (81, 106). Expression of several transcript variants lacking exon 3.	Conserved inhibition of VSMC proliferation and increase in vascular medial area (106, 107). Vasoconstriction and VSMC abnormalities (106, 108). Defects in heart morphology and increased hypertension with aging (106, 109). More severe heart failure with increased mortality after MI in female KO mice (106, 110). Less heart functional recovery after I/R in ERβKO female hearts compared to WT (75, 106). Loss of inhibition of Ang II-induced hypertrophy (106, 111). Conserved accelerated re-endothelialization in female mice (81, 82). Absence of atherosclerosis protection (112).
ERβKO	Deletion of exon 3 by Cre/LoxP-mediated excision (113, 114). Residual deleted ERβ protein in the prostate tissue (114).	No abnormalities of heart morphology, morphometry, and ultrastructure in 16-month-old males (113). No vascular phenotype determined (114). No cardioprotective effects of E2 on LV hypertrophy (115).
csERβ-OE	Conditioned cardiomyocyte-specific overexpressing ERβ (csERβ-OE).	No differences in heart structure and function compared with WT mice. Improved survival and cardiac function in both sexes compared to the WT counterparts after MI. Attenuated cardiac fibrosis in males csERβ-OE mice (116).

(Continued)

TABLE 1 | Continued

Mouse model	Genetic feature	Vascular phenotype and estrogen response
GPER KO1-4	Deletion of GPER30 open reading frame to generate KO1 (117) KO2 (118) and KO3 (119). Insertion of full-length lacZ transcript insertion, retaining the C-term portion of the protein in KO4 (120).	Absence of beneficial effects on vascular tone and blood pressure (117, 121). Increased atherosclerosis progression (117, 122). Abrogated vasodilator response (117, 123). Increased blood pressure and vascular resistance with aging (118). Loss of cardioprotection against I/R injury in male mice (118, 124). Impaired LV cardiac function in male KO mice (119, 125). No evident blood pressure problems in younger mice (120).
csGPER-KO	Cardiomyocyte-specific GPER KO.	Alterations of cardiac structure and functional impairment. LV dimension more affected in male KO mice compared to female ones. Differential gene expression profiles affecting multiple transcriptional sex-related networks (126).

of viability or reproductive function were observed. Three of them (117–119) did not express GPER, whereas the fourth mouse model synthesized a lacZ reporter fused with the C-terminal portion of GPER, leaving open the question whether this truncated protein could play a functional role (120). Several vascular problems, in terms of increased blood pressure and atherosclerosis, were shown in the first two models of GPER KO (117, 118, 121–124).

In order to avoid systemic influence on ERs protective effects on the heart, different mouse models were generated characterized by genetically modified cardiomyocytes. Therefore, CMs overexpressing ERα (104) or with defective expression of ERα (105) were established. They demonstrated an important role of ERα in cardiac mass development in both sexes. In particular, ERα gain of function showed a more efficient cardiac repair in female mice in comparison with male mice after ischemic injury (104). As concerns ERβ, mice overexpressing this receptor in the cardiomyocytes (116) showed an improved survival after a MI in both sexes, compared with the wild type counterparts. In addition, a more recent mouse model carrying cardiomyocyte-specific GPER-KO showed structural and functional cardiac alterations in both sexes with LV defect more pronounced in the male mice characterized by an inadequate heart remodeling (126). As extensively discussed in a very recent review (131), ER cardioprotective potential should be investigated in more detail in order to more precisely define the role played by each receptor in the heart integrity and function.

Estrogen Regulatory Role on the Heart

Cardiovascular repair and regeneration is reached by a series of mechanisms that include, on one hand, the reduction of inflammation and the formation of new vessels, on the other the survival and protection of cardiomyocytes (CMs), the activation of a cardiomyogenic process and a sort of cellular anti-aging program, i.e., an antioxidant activity. In this regard, E2 exerts many pleiotropic effects, some of which have a beneficial role on vascular endothelial cells as well as on smooth muscle and cardiac cells.

The role played by estrogens in cardioprotection against I/R injury pass through nitric oxide (NO) production (132). NO seems to play several potential beneficial roles in the cardiovascular system. Estrogen increases NO bioavailability in the vascular system through both the signaling pathways (genomic and non-genomic). Through the non-genomic signaling, E2 binding to ERα lead to endothelial nitric oxide synthase (eNOS) phosphorylation and activation. Upon estrogen binding, caveolae membrane-associated ERα activates Src family tyrosine kinases, PI3K/AKT kinase, and ERK1,2 to stimulate eNOS in NO production (133, 134). In line with these *in vitro* studies, an increase of eNOS activity together with a decreased number of leukocytes normally accumulating on the vascular wall after I/R injury has been observed in mice treated with estrogen. Accordingly, treatment with inhibitors of PI3K or eNOS abolished estrogen vascular protective effect (135). It has also been reported that, in human EC, calcium ions, of great importance in the regulation of nitric oxide synthase activity, increase rapidly at physiological estrogen concentrations (136). This modulation of Ca^{2+} homeostasis is ERα-dependent as demonstrated by using ERα KO cells (137). More recently, an estrogen-dendrimer conjugate (EDC) was reported to selectively activate extra-nuclear ER, in both EC and CMs. However, it seems able to attenuate infarct size in mice lacking ERα expression in CMs but not in mice lacking ERα expression in EC (138). This suggests that a different mechanism may be responsible for cardioprotection in CMs and EC.

As far as VSMC was concerned, it was observed that their proliferation was strictly controlled by kinase-mediated signal transduction. This kinase activity was in turn regulated by a balance between phosphorylation and dephosphorylation events. Indeed, the estrogen-mediated phosphatase activation determines the inhibition of several kinases leading to cell proliferation and migration block. In particular, VSMC proliferation was inhibited by phosphatase 2A, whose activation was mediated by interaction with ERα (139). More recently, in a mouse model with the selective blockade of the membrane-initiated ER signaling ($KRR^{ki/ki}$) the central action of PP2A in metabolic homeostasis has been reported (102).

Non-genomic signaling pathways seem to have a key role in mediating the regulatory action of estrogens in all the cellular components of the cardiovascular system. As a matter of fact, the blockade of the non-genomic signaling impaired the transcriptional response of genes involved in the vascular

function, indicating that the rapid estrogen signaling may contribute to physiological vascular gene regulatory activity (101, 103). Nonetheless, a strong cross talk between the genomic and non-genomic estrogen pathways has been hypothesized.

As concerns GPER, its vasodilatory effect was analyzed by using GPER agonists *in vitro* (140) or in KO mouse models, as discussed above (117, 118, 121). Furthermore, accumulating literature indicates that GPER vasorelaxation *in vivo* could be mediated by both endothelium-dependent and endothelium-independent mechanisms. In the former case, as in the arteries' relaxation, estrogen binds to GPER and leads to the production of nitric oxide in coronary EC by eNOS activation (140). In the endothelium-independent way, the E2-GPER effect on smooth muscle cells relaxation is mediated by the stimulatory activity of calcium- and voltage-activated potassium channels (67). The observed antiproliferative effect of GPER on EC (141) may provide an optimal balance for the opposite effects exerted by ERs on these cells. For example, in rat aortic EC, E2 elicits opposite effects depending on whether the signal depends on ERα or GPER (142). In fact, as for VSMC, GPER seems to act in concert with ERs in inhibiting proliferation and stimulating the differentiation rate of these cells (121, 143, 144). A GPER-mediated paradoxical effect of estrogen in vascular function (relaxation vs. contraction) was also described in porcine coronary arteries, involving the signaling pathway that passes through the transactivation of EGFR (145).

Several studies have shown that estrogen prevents cardiac hypertrophy, in particular through ERβ signaling (146). Firstly, it has been shown how ERs stimulate the production of the myocyte-enriched calcineurin-interacting protein (MCIP1), an inhibitor of calcineurin activity via PI3K. In this way, ERβ signaling blocks the angiotensin II (Ang II)- or endothelin-1 (ET-1)- mediated stimulation of key hypertrophy and ventricular remodeling genes in CMs (146). Thereafter, E2 inability to prevent Ang II-induced hypertrophy and fibrosis in ERβ KO mice was also demonstrated, underscoring the relevance of ERβ in counteracting cardiac hypertrophy (111, 147). Accordingly, the same authors demonstrated that E2 exerted regulatory effects on the synthesis, localization and function of histone deacetylase (HDA) class I (pro-hypertrophic) and class II (anti-hypertrophic), important modulators of cardiac hypertrophy. In this context, ERβ activation suppressed Ang II-induced HDAC2 (class I) production and de-repressed the opposite effects of Ang II on HDAC4 and HDAC5 (class II) (148). The key role of ERβ on hypertrophy was confirmed *in vivo* in hearts derived from ERβ KO mice (111, 147, 148). It is well-known that Ang II stimulates cardiac hypertrophy, in part by inhibiting KLF15 expression. In turn, E2 binding to ERβ appears able to reverse Ang II action, allowing KLF15 transcriptional regulation activity on cardiac hypertrophic gene expression (149). Furthermore, ERβ plays an anti-fibrotic role influencing cardiac fibroblast homeostasis down-modulating TGFβ expression and signaling, otherwise stimulated by Ang-II (150). As regards cardiac fibroblasts, it has very recently been hypothesized that E2, either via ERα or ERβ signaling, could exert opposite effects on the synthesis and secretion of key components of the extracellular matrix, i.e., collagen I and III, by these cells (151). Regarding the debated question dealing with the possibility that GPER could or not activate an autonomous signal, it has been observed that estradiol treatment of infarcted rats improved ventricular remodeling triggering both GPER and ERα activity. Indeed, both receptors activate their membrane-specific signaling that converged into the common PI3K/AKT/eNOS pathway (152). As regards CMs, GPER was suggested to activate signaling of PI3-kinase contributing to cardioprotection in females (153). Interestingly, the PI3K pathway seems to be strictly related to autophagic processes involved in cardioprotection (154), and it has been very recently reported that GPER could counteract CM hypertrophy by up-regulating the PI3K-AKT-mTOR signaling pathway, therefore modulating autophagy (155). A further mechanism of estrogen-induced cardioprotection involving GPER was investigated using its agonist (called G1) in a mouse model of I/R injury. Both G1 and E2 exerted a cardioprotective activity by inhibiting mitochondria permeability transition pore opening that normally leads to apoptotic cell death of CMs after I/R injury (47, 156). A further study demonstrated that post-ischemic GPER activation, preserving mitochondrial structural integrity, decreased ROS production and mitophagy, resulting in reduced myocardial infarct size in both sexes (157). As discussed before, specific GPER KO cardiomyocytes exhibited left ventricular dysfunction and adverse remodeling more pronounced in male KO mice than in female. Furthermore, DNA microarray analysis revealed gene expression differences between sexes, with particular reference to the mitochondrial and inflammatory pathways (126). Finally, the pivotal role of GPER and the involvement of Notch1 pathway in mediating physiopathology of female rat hearts were hypothesized (158).

The effects of E2 on myocyte regeneration have also been investigated. Several studies focused on cardiomyogenesis have established that the genesis of new cardiomyocytes from the preexisting cardiomyocyte pool occurs at a low rate (159, 160). The presence of multipotent cardiac stem cells (CSCs), normally residing within the cardiac niche, has extensively been studied (161, 162) as well as CSC induction to proliferate, migrate, and undergo lineage commitment in response to infarction injury (163). Accordingly, it has been demonstrated that CSCs isolated from adult rodent hearts express stem cell surface markers (c-Kit/Sca-1) and display several stem cell functions (161, 164, 165). Indeed, c-Kit+ precursor cells, which accumulate in the infarcted area, showed increased ERα expression, suggesting a direct effect of E2 on cardiac progenitor cells *in situ* (166).

Estrogen-replacement therapy and acute myocardial infarction were evaluated in a rat experimental model. It has been observed that estrogen-replacement therapy increases the homing of bone marrow stem cells into myocardium and stimulate angiogenesis enhancing ERα and ERβ expression (167). The possibility of ERα-mediated paracrine cardioprotective function has been proposed as one of the major mechanism used by post-infarct cardiac c-kit+ cells (i.e., inducing CM survival). Accordingly, infusion of E2 treated-CSCs into the isolated mouse hearts after acute I/R gave rises to a powerful protective effect probably due to a major production of CSC-derived protective factors (168).

MITOCHONDRIA AS SUBCELLULAR TARGETS OF ESTROGEN

Mitochondria drive different cellular processes by providing chemical energy and they are particularly important in heart muscle cells where mitochondrial dysfunction is associated with important pathological changes leading to impaired cardiac function (169). In fact, dysfunctional mitochondria would ultimately lead to myocardial cell apoptosis and death during I/R injuries.

On the other hand, autophagy, characterized by the formation of autophagosomal vesicles containing degenerating cytoplasmic contents, is considered primarily as a cytoprotective process. Particularly, mitophagy, a selective form of autophagy, represents a protective mechanism that contributes to eliminate damaged mitochondria thus reducing mitochondria-mediated apoptosis and necrosis in the myocardium (170). Accordingly, it has been suggested that autophagy counteracts mitochondrial dysfunction by autophagosome formation, possible embedding of damaged mitochondria in autophagolysosomes and their digestion. This allows the cells to remove injured mitochondria that often represent a source of ROS. During I/R, mitochondria suffer a deficiency to supply the CMs with chemical energy also contributing to oxidative stress and to the cytosolic ionic alterations, especially of Ca^{2+} (171). Interestingly, it has been hypothesized that different types of cardiomyocyte calcium channels could exhibit a marked sexual dimorphism and that their function could be regulated by ERα, ERβ, and GPER, i.e., by non-nuclear estrogen receptor signals (131).

Sex plays a pivotal role in the cardiac tolerance to I/R injury, and it has been reported that male myocardium is more sensitive than the female one. Recent studies have suggested that mitochondria are a major target of cardioprotective signaling (31, 172). Furthermore, numerous studies have suggested that in females mitochondria could be modified and less sensitive to I/R injury. In addition, it was reported that mitochondria from females undergo several posttranslational modifications of enzymes involved in the redox metabolism generating less ROS during the reoxygenation phase following ischemia (173–176).

In particular, Colom and co-authors (174) demonstrated a significant sex difference in the function of cardiac mitochondria. Female rats showed minor cardiac mitochondria content and produced less H_2O_2 than male rats. On the other hand, male myocytes, thanks to the higher density of β-adrenergic receptors, are more responsive to β-adrenergic stimulation than females. This induces an increase in the influx of Ca^{2+} in cardiac cells. Male myocytes are thus particularly prone to calcium overload (177). According with this, it was observed an improved survival of CMs overexpressing ERβ isolated from mice of both sexes, together with a significant reduction of the maladaptive remodeling and the recovery of cardiac function after MI in comparison with wild type CMs. These effects seem to be associated to a better maintenance of Ca^{2+} homeostasis and to less cardiac fibrosis following MI (116).

Mitochondria isolated from hearts of adult male and female rats differ in the sensitivity of the permeability transition pore (MPTP) to the calcium load. In particular, mitochondria isolated from female animals appear more resistant to swelling induced by high Ca^{2+} concentration. It can be hypothesized that the higher ischemic tolerance of female myocardium may be related to the lower sensitivity of MPTP to the calcium induced swelling. Accordingly, it has been observed that a specific ERβ agonist reduced mitochondria-mediated apoptosis and contribute to the preservation of mitochondrial integrity after I/R injury (178).

Bcl2 protein, located at mitochondrial membranes, provides protection against pro-apoptotic stimuli (179), and its expression level is associated with improved recovery of cardiac function after I/R, and reduced infarction area due to a reduced apoptotic cell death (180). Moreover, Bcl2 prevents permeabilization of the outer mitochondrial membrane (181) after I/R thus preventing the release of cytochrome c from mitochondria and subsequent apoptosis. To note, the expression of Bcl2 was found controlled by ERβ (182). It was also reported that the cardioprotection observed in female sex may be related to a greater protein expression of the sarcolemmal and mitochondrial K(ATP) channels. According with this, the blockade of K(ATP) channels significantly increased the damage in the female heart after I/R (183, 184).

Mitochondrial dynamics (i.e., fission/fusion processes) is critical for a correct mitochondrial function, and alterations of mitochondrial dynamics have been associated with neuropathies, non-alcoholic fatty liver disease progression, type 2 diabetes, and CVD (185–187). Very recently, an uncontrolled balance of mitochondrial dynamics was shown to contribute to cardiac dysfunction during I/R injury (188). Several proteins are involved in mitochondrial dynamics: for instance, mitofusins (MFN) and optic atrophy protein 1 (OPA1) participate to mitochondrial fusion process, while mitochondrial fission is manly orchestrated by dynamin-related protein 1 (DRP1) and fission protein 1 (FIS1) (185). Alterations in this mitochondrial dynamics produce altered mitochondria in their shape and size: a prevalence of fusogenic mechanisms favors the formation of a large mitochondrial network; on the contrary, if fission mechanisms prevail a mitochondrial fragmentation occurs (189). In different *in vivo* and *in vitro* models of ischemia or I/R, it was observed that the inhibition of DRP1 selectively blocks DRP1-dependent mitophagy, which is triggered to eliminate mitochondria damaged during the early phase of ischemia in the brain (190). After the inhibition of DRP1, CMs were found to show a significant decrease of oxygen consumption with a negligible alteration of ATP production after I/R (191). Accordingly, in I/R-induced alterations of CMs, mdivi-1, a chemical inhibitor of the mitochondrial fission protein DRP1 that induces mitochondria elongation (192), preserved the mitochondrial structure and significantly reduced the myocardial infarction area (193, 194). Current studies thus indicate that several chemical compounds prevent the alterations of mitochondrial dynamics. However, further toxicological and pharmacokinetic studies are needed before their clinical use.

A very interesting role of sex hormones was reported in mitochondrial biogenesis occurring in the right ventricle after the heart failure associated with pulmonary hypertension. In particular, Liu and co-workers, by studying ovariectomized female rats, found that estrogen therapy counteracted the

loss mitochondrial mass and maintain the cardiac oxidative metabolism. They therefore hypothesized that estrogen could prevent maladaptive remodeling of the right ventricle that often lead to the severe dysfunction frequently associated with pulmonary hypertension (195). Furthermore, it has also been suggested that E2 induces mitochondriogenesis in H9c2 cultured cardiomyocytes through the increase of PGC-1α expression. This effect seems to be mediated by GPER, since specific agonists of this receptor mimic the activity of estrogen (196). However, it should be underlined that other authors (197), in a study in a murine model of hemorrhagic trauma, reported that the effect exerted by estrogen on mitochondrial biogenesis and function at the cardiac level is mediated by both ERα and ERβ.

Although a direct or indirect influence of estrogens on mitochondrial dynamics has not yet been observed in cardiac models, it was found that I/R injury increased ROS production, mitochondrial fission, and increased levels of DRP1 in cardiomyocytes (198). Moreover, in a DRP1 KO mouse model, a cardiac-specific impairment of left ventricular functions has also been observed. These mice died within 13 weeks through the suppression of autophagic flux, thus underlining the pivotal role of autophagy or mitophagy in CM homeostasis (e.g., maintaining the ionic equilibrium) (199). A schematic picture suggesting the possible sequence of events at mitochondrial level after I/R injury in response to activation of the putative non-nuclear ER pathway is reported in **Figure 2**.

ESTROGEN REGULATED miRNAs AND THEIR EFFECT ON MYOCARDIUM AND CARDIAC VASCULAR SYSTEM

Estrogens/estrogen receptor interaction regulates cardiovascular function through either gene expression or epigenetic mechanisms. This last mechanism of action is also dependent on miRNA action (200). miRNAs are highly conserved short non-coding RNAs (19-25 nucleotides) that control many developmental and cellular processes in eukaryotic organisms by post-transcriptional regulation of mRNAs by binding to their 3' untranslated regions, thus triggering their translational inhibition with or without RNA degradation. miRNA expression is strongly regulated at different levels, e.g., during development and for tissue specific functions (201).

Of relevance is, in fact, the role of miRNAs in regulating vascular cell aging, which in women after menopause appears similar to that detected in men (202). This obviously supports the knowledge of a regulatory role of estrogen in fertile woman. Notably, this different regulation might also rely on sex-linked miRNAs. Actually, about 120 miRNAs have been identified on human X chromosome, whereas only 4 on the Y chromosome. This appears as an intriguing result *per se*. In addition, although random X-inactivation should equilibrate female and male expression levels, a number of unbalancing mechanisms have emerged so far. In fact, genes escaping X chromosome inactivation could play a critical a role. Moreover, the number of these genes increases with age and it has been suggested that this could lead to an increased susceptibility of women to inflammatory and autoimmune disease (203). In this field, the interesting study of Florijn and co-workers remarked the harmful effects of the X-linked miRNAs in cardiovascular disease suggesting that the sex-biased miRNA network could play a key role in heart failure with preserved ejection fraction observed in women (202). This hypothesis is only partially in accord with the suggested protective effects of estrogen regulated miRNAs reported elsewhere. Furthermore, estrogens modulate miRNA profiles also during their maturation pathway (204). Numerous lines of evidence underline the importance of estrogen therapy in postmenopausal women to restore the correct level of miRNA expression among many other aging-related physio-pathological aspects. Estrogen protective action on cardiac vascular system has prevalently been associated with the ERα signaling that is responsible of vasodilation, inhibition of inflammation and regulation of the oxidative stress also blocking apoptosis. All these processes play a role in preserving the correct function of endothelial cells, modulating vasoconstriction and inhibiting proliferation of VSMC. Specific miRNA signatures have been associated with cardiac and vascular aging under estrogen control (202). Some estrogen regulated miRNAs and their effect on cardiac and vascular cells are reported in **Table 2**.

MI is consequent to a protracted ischemic injury of vasculature and hypoxic conditions that are characterized by continuous deficit of cardiomyocyte oxygenation and inflammation in the infarcted area. This picture is amplified by increased oxidative stress, i.e., ROS production, and cardiac muscle cell death (222). In the attempt to reduce tissue damage, the infarcted heart undergoes a cardiac self-remodeling that frequently results in increased fibrosis, dilated cardiomyopathy and heart failure (HF) (223). In this context, miRNA roles have broadly been investigated using both cardiac cell cultures and mouse models of cardiac infarction. It is now clear that miRNAs are implicated in cardiac proper functions as well as in pathogenesis of cardiac cell injury, leading to HF. For instance, some miRNAs have directly been associated with estrogen cardioprotective action against oxidative stress. The cystathionine-γ-lyase (CSE), the enzyme involved in cardioprotective H2S generation (224), is indirectly regulated by miR-22 levels as miR-22 specifically down-regulates the Sp1 transcription factor, involved in CSE transcription. Indeed, 17β-estradiol treatment determines down-modulation of this miRNA by ERα action, thus reconstituting Sp1 levels both in cultured cardiomyocytes and in ovariectomized rat hearts (225).

An independent risk factor for HF, and consequently for cardiovascular morbidity and mortality, is cardiac hypertrophy, either concentric or eccentric. The former consists of an increase in ventricular wall thickness without chamber enlargement. The latter promotes chamber dilation with no increase or even decrease of left ventricular wall thickness (226). This remodeling is characterized by age-specific relative changes in LV mass, volumes, and chamber performance during diastolic and systolic function. Differences of this remodeling between pre- and post-menopausal women suggest a key role for estrogen. Indeed, E2 deficiency in the heart of ovariectomized mice increases the age-related ventricular concentric remodeling that,

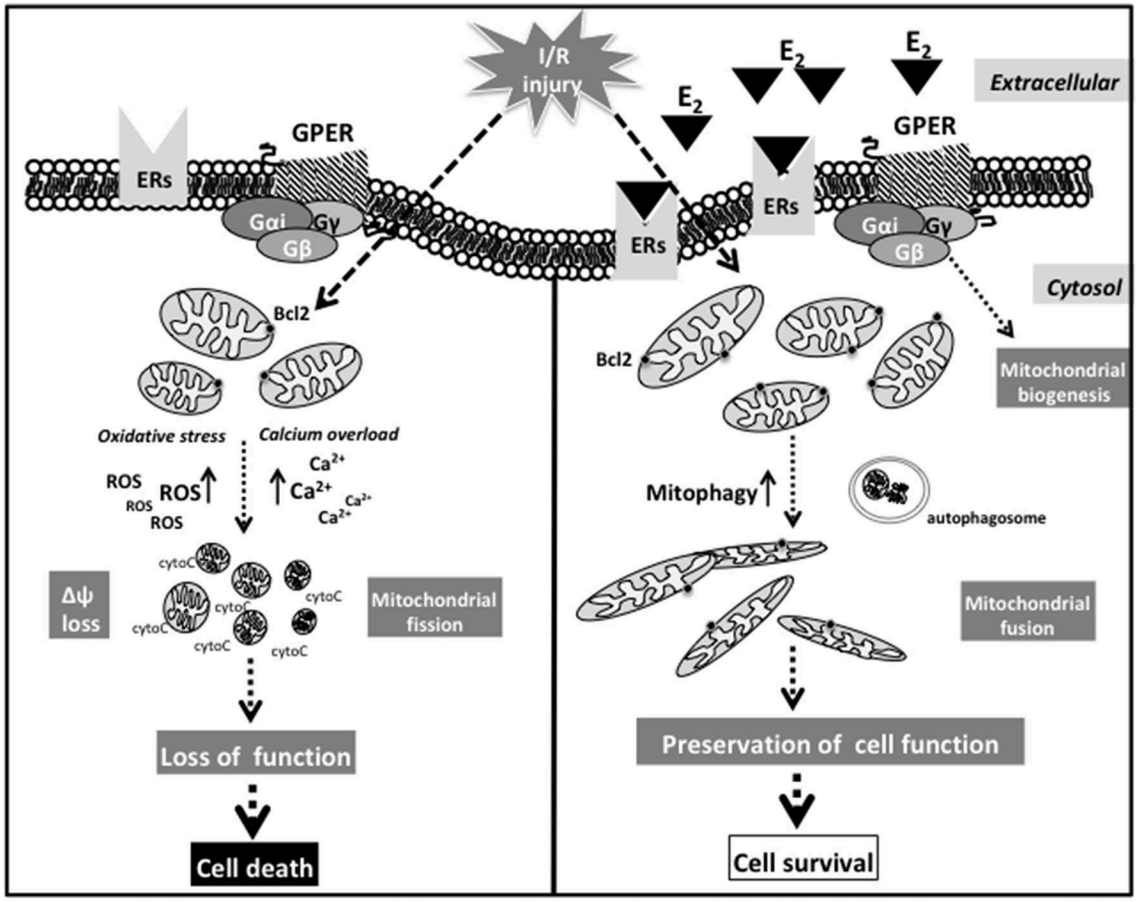

FIGURE 2 | Schematic picture suggesting the possible sequence of events at mitochondrial level after I/R injury in response to activation of the putative non-nuclear ER pathway.

at sub-cellular level, is underlined by the functional impairment of mitochondria.

The molecular mechanisms associated with this ventricular dysfunction have also been correlated to miR-23a levels. In absence of estrogens, miR-23a high level in cardiomyocytes directly targets peroxisome proliferator-activated receptor-γ co-activator 1-α (PGC-1α) down-regulating its expression. This protein is a modulator of mitochondrial function and its heart-specific deletion has recently been associated to cardiac dilation with LV thinning (227). However, as indicated by Sun and colleagues, the E2 deficiency might mediate a possible role of PGC-1α also in concentric remodeling through the miR-23a dependent reduction (228).

A rat model of myocardial ischemia showed that mortality was increased when accompanied with estrogen deprivation (due to ovariectomy). This elevated mortality was associated with miR-151-5p down-regulation. This miRNA binds to the 3′UTR of FXYD1, the gene codifying for phospholemman protein (PLM, an important regulator of ion transport and a substrate for protein kinases A and C), inhibiting its expression. PLM is known to alter cardiac membrane excitability. Thus, in the ovariectomized myocardial ischemic group of animals, the absence of estrogen, reducing miR-151-5p levels favored PML increase, with Ca^{2+} accumulation in cardiomyocytes eventually exacerbating cardiac malfunction (229).

Finally, a recent miRNA specific microarray study on cardiomyocytes treated or not with estrogen showed an increased expression of a further miR: the miR-494. The authors correlated the expression of this miRNA with estrogen dependent cardioprotection and identified in the nuclear factor kappa B (NF-κB) repressing factor (NKRF) the specific target of this miR in cardiomyocytes. In brief, miR-494 overexpression could mimic the estrogen specific cardioprotection reducing the oxidative stress-induced injury (230).

CONCLUSIONS

In this paper we summarized some molecular mechanisms that lead to favorable or unfavorable evolution of remodeling of

TABLE 2 | Some estrogen regulated miRNAs and their effect on myocardium and cardiac vascular system cells.

miRNA	Vascular cells	Functions	Targets	References
miR-126-3p	Endothelial cells	Migration Angiogenesis	Spred1	(205)
miR-221&-222	Endothelial cells	Inflammation	ETS-1	(206)
miR-106b	Endothelial cells VSMC	Apoptosis	PTEN	(207)
miR-143/-145	VSMC	Proliferation Contraction	ACE	(208)
miR-30	Endothelial cells VSMC	Angiogenesis Apoptosis Inflammation	Ang2	(209)
miR-203	VSMC	Proliferation	SRC, ERK	(210)
miR-144	Endothelial cells VSMC	Inflammation Metabolism	COX2 Rac1 ABCA1	(211) (212) (213)
miR-146a	Endothelial cells	Inflammation Senescence	TRAF6, IRAK1 NOX4	(214) (215)
miR-21	VSMC Fibroblast	Inflammation Proliferation	PPARα, Spry1 NF1B,CDC25A	(216) (217) (218)
miR-125	Endothelial cells	Angiogenesis	RTEF-1, VEGF	(219)
miR-34	Endothelial cells	Senescence Inflammation	SIRT-1	(220, 221)

the heart after injury, e.g., in I/R, and how these mechanisms may depend on the effect of sex hormones, of estrogen non-genomic effects in particular. On the basis of the results described above, it appears well-documented that all cell components of the cardiovascular system (such as cardiomyocytes and fibroblasts, as well as endothelial and vascular smooth muscle cells) of males and females, also in virtue of their hormonal differences, differently counteract exogenous or endogenous insults. In this context, the emerging role of non-genomic effects of estrogen on cardiovascular cell homeostasis and remodeling could represent a formidable, novel challenge for this field of investigation. The idea that a prompt, very rapid, i.e., in seconds, response could be played out in order to counteract an injury appears fascinating as well as conceivable: the "classical" genomic activity of hormones appears, in our mind, as too slow in order to face damage and to survive. However, apart from the possible role of this mechanism in the evolution of the species (which should merit a specific discussion), the influence of the estrogen hormone and its pathways in determining cardiovascular cell homeostasis appears as pivotal and should merit more targeted analyses.

A last point deals with sex-specific studies. Although many experimental studies dealing with the analysis of sex differences in the cardiovascular system, either in physiological or in pathological conditions, have been published in the recent years, the molecular mechanisms whereby sex specificities may influence the remodeling and the adaptive response to injury are still to be defined in detail. As a general rule, these studies suggested resilience as a milestone of the female sex, including cellular and tissue responses to environmental insults. Experimental studies, e.g., in freshly isolated cells from males and females are, however, quite complicated. The use of "typical" cultured cells is in fact useless in this field since the great majority of cell lines derive from cancer cells or from established highly proliferating cell lines and we know that these models do not adequately apply to the study of vascular or cardiac cells. Hence, the main bias in the study of the different response of XX and XY cells is the availability of strong and effective cell models. Thus, the influence of hormones, sex hormones in particular, on cardiovascular cell system homeostasis in males and females represents a complex challenge that should properly be investigated in the next years by using cell pathology approaches in parallel with *in vivo* analyses. One further important issue should be referred to hormone variations in the lifespan of men and women that, due to its peculiarities, can be fully investigated neither *in vitro* nor *in vivo*. Consequently, translation of the results obtained in these experimental studies into clinical practice cannot be performed or it should be performed, when appropriate, very carefully. Notwithstanding this, experimental studies appear indispensable: clinical data are often descriptive rising questions to which mechanistic studies could try to answer. To do this, preclinical studies that incorporate both sexes will be crucial

to allow the translation of information from basic research to clinical practice.

AUTHOR CONTRIBUTIONS

RP expert in the field of experimental model studies, i.e., animal studies. GMat looked at the aspects referred as to the role of micro RNA. PM followed the aspects dealing with the role of mitochondria in cellular remodeling. GMar contributed for the clinical aspects of cardiovascular disease. WM and AC conceived the work and supervised the manuscript.

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The Effect of Estradiol Administration on Muscle Mass Loss and Cachexia Progression in Female *Apc*$^{Min/+}$ Mice

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Cancer cachexia is a multifactorial muscle wasting condition characterized by severe body weight and muscle mass loss which is secondary to chronic disease. The mechanistic examination of cachexia has predominately focused on the male phenotype and created significant gaps in understanding cachexia progression in the female. Female hypogonadism can accompany cancer cachexia and is characterized by reduced circulating 17ß-estradiol and uterine atrophy. Estrogen has known functions in skeletal muscle homeostasis involving the regulation of muscle protein turnover, cellular stressors, and oxidative metabolism. However, 17ß-estradiol's ability to regulate cachexia progression in the female is not known. The purpose of this study was to determine the effect of gonadal function and estradiol administration on muscle mass loss and cachexia progression in female *Apc*$^{Min/+}$ mice.

Methods: Female C57BL/6 (B6; $N = 82$) and *Apc*$^{Min/+}$ (MIN; $N = 88$) mice were used in two separate experiments. In experiment 1, mice were sacrificed at either 12 ($N = 20$) or 20 ($N = 41$) weeks of age. Body weight and estrous cycle presence was determined weekly. In experiment 2, B6 and MIN mice were randomly allocated to: Control ($N = 17$), received E2 pellet (E2, $N = 18$), ovariectomy surgery (OVX; $N = 19$) or ovariectomy surgery with E2 pellet (OVX + E2; $N = 21$). 17ß-estradiol was administered through an implanted slow-releasing pellet (0.1 mg). In estrogen and ovariectomy experiments, food intake, and functional outcomes were recorded 1 week prior to sacrifice.

Results: We report that E2 administration prevented body weight loss, muscle mass loss, cage inactivity, and grip strength loss associated with cachexia. In skeletal muscle, E2 reduced skeletal muscle AMPK phosphorylation, improved mTORC1 signaling, and prevented mitochondrial dysfunction.

Conclusion: Our results demonstrate a role for 17ß-estradiol for the prevention of skeletal muscle mass loss in female tumor bearing mice. Furthermore, 17ß-estradiol prevented cachexia's disruption in skeletal muscle signaling involving AMPK and mTORC1, in addition to improving mitochondrial function in female tumor bearing mice.

Keywords: muscle wasting, cachexia, 17ß-estradiol, hypogonadism, physical activity

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INTRODUCTION

Cancer cachexia, which occurs in roughly 50% of all cancer patients, is commonly characterized by severe body weight loss. This condition is also associated with several systemic imbalances including anemia, insulin resistance, gonadal dysfunction, and elevated systemic inflammation secondary to chronic disease (1, 2). Patient age is associated with poor prognosis following cancer diagnosis and treatment (3), and the average age of cachexia diagnosis is 68 years (4). Most females are post-menopausal by 55 years of age (5, 6), suggesting that cancer cachexia progression could be influenced by both age and menopausal status. Sex hormones are physiologically relevant throughout the life span, present in almost all tissues, and are linked to disease progression (7, 8). Sex differences have been reported in pre-clinical cancer cachexia models (9, 10) and in cachectic patients (11–14). While females often present a less severe phenotype than males in both pre-clinical models and human cachexia patients, gonadal function has rarely been accounted for in these studies. Furthermore, the mechanistic understanding of cachexia progression has mainly occurred in the male (15, 16). Ovarian function has been reported to be disrupted prior to the initiation of body weight loss and be associated with muscle inflammatory signaling in tumor bearing mice (10). Available evidence clearly demonstrates that further investigation is warranted to mechanistically examine ovarian function's role in cancer cachexia progression.

Estrogen's physiological role has often been investigated in pre-clinical studies through the loss of function or removal of the ovaries (17). Reduced circulating estrogen can decrease bone mineral density, increase abdominal adiposity, increase inflammatory expression, and reduce voluntary activity (18–21). Circulating estrogens can signal through classical or non-genomic activation (22), and regulate skeletal muscle protein turnover and mitochondrial oxidative capacity, which are widely investigated drivers of cancer-induced muscle wasting (20, 23, 24). Moreover, estrogen can regulate skeletal muscle cellular signaling involving Akt, mTORC1, the ubiquitin proteasome system, and 5'-Adenosine monophosphate activated protein kinase (AMPK) (16). Estrogen receptor signaling is also needed for the maintenance of skeletal muscle contractile properties (25). Estrogen combined with progesterone can improve muscle fatigue and voluntary wheel running distance, which are affected by cancer (21, 26). Estrogen's documented effects on skeletal muscle function and cellular processes that regulate protein turnover and metabolism provide justification for further investigation to determine its utility as a therapeutic target for treating muscle wasting in non-hormone sensitive cancers.

The $Apc^{Min/+}$ (MIN) mouse is an established pre-clinical cachexia model. MIN mice are heterozygotes for adenomatous polyposis coli (*Apc*) gene mutation that causes spontaneous development of intestinal and colon polyps. MIN mice develop non-metastatic intestinal polyps at ∼4 weeks of age, which continues until 12–14 weeks of age. Polyp size can continue to increase after 12 weeks of age and is associated with cachexia progression (27). MIN mice initiate body weight loss between 14 and 16 weeks of age (28). We have reported that male MIN mice are hypogonadal (15), have reduced cage activity (29, 30), altered skeletal muscle protein turnover (31), and chronically elevated AMPK (32). Furthermore, male MIN mice demonstrate an induction of skeletal muscle E3 ligase Atrogin-1 during the initiation of bodyweight loss (28, 31). Female MIN mice demonstrate estrous cycle cessation (acyclicity) that corresponds with reduced cage activity, increased fatigue, and muscle mass loss (10). Acyclicity in MIN mice is associated with decreased uterine mass (10). Therefore, the purpose of this study was to determine the effect of gonadal function and estradiol administration on muscle mass loss and cachexia progression in female $Apc^{Min/+}$ mice. We hypothesized that estradiol administration would prevent cachexia progression and skeletal muscle mass loss in female MIN mice.

METHODS

Animals

Female C57BL/6 (B6; $N = 82$) and $Apc^{Min/+}$ (MIN; $N = 88$) mice were bred at the University of South Carolina Animal Resource Facility. MIN mice were initially purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were kept on a 12:12 h light/dark cycle beginning at 7:00 a.m. and were given rodent chow *ad libitum* (Harlan Teklad Rodent Diet, #8604, Harlan, Indianapolis, IN, USA). All experiments were approved by the University of South Carolina Institutional Animal Care and Use Committee.

Experimental Designs

Experiment 1: To determine if the cachectic phenotype in female MIN mice are presented early or late, we sacrificed B6 and MIN mice at 12 ($N = 20$) or 20 ($N = 41$) weeks of age (**Table 1**). Mice were weighed weekly and we determined the presence (cycling) or absence (acyclicity) of an estrous cycle. In the large cohort of female MIN mice aged 20 weeks, we categorized these mice by cachexia severity to determine the effect of cachexia progression on gonadal function. Mice were stratified by change in body weight from peak: weight stable (0%), initiated (0 to −5%), moderate (−5 to −10%), or severe (<-10%) (**Table 2**) (10). Then, to further elucidate the importance of estrous cycle presence, MIN mice were stratified based on the presence or absence of the estrous cycle.

Experiment 2: To determine the effect of 17ß-estradiol administration and ovariectomy surgery on cachexia progression (**Figure 2A**). At 8 weeks of age, B6 and MIN mice were randomly allocated to either; Control ($N = 17$), 17ß-estradiol pellet (E2; $N = 18$), underwent ovariectomy surgery (OVX; $N = 19$) or ovariectomy surgery and received an 17ß-estradiol pellet (OVX + E2; $N = 21$). At 11 weeks of age mice were anesthetized under isoflurane for 5 min for E2 pellet implantation (E2), 30 min to undergo ovariectomy surgery (OVX), E2 pellet implantation and ovariectomy surgery (OVX+E2), or mice were anesthetized under isoflurane for 30 min to receive a SHAM OVX surgery (Intact). A 60-day slow releasing 0.1 mg/pellet of 17ß-estradiol was purchased from Innovative Research of America and used for estrogen administration. Protein expression and mitochondrial respiration was analyzed in B6 and MIN mice that received

TABLE 1 | Animal characteristics in female B6 and MIN mice at 12 and 20 weeks.

Genotype	B6		MIN		*p-value*		
					Age	Genotype	Interaction
Age (weeks)	12	20	12	20	<0.001	0.395	0.803
N	11	32	9	41			
Sacrifice BW(g)	19.8 (0.3)	21.4 (0.3)	18.4 (0.2)	19.2 (0.3)	0.008	<0.001	0.372
Peak BW(g)	19.8 (0.3)	21.7 (0.3)	18.4 (0.2)	20.7 (0.2)	<0.001	0.002	0.503
BWΔ from Peak (%)	0.0 (0.0)	−0.5 (0.2)	0.0 (0.0)	−6.3 (1.0)^	0.010	0.027	0.027
Percent Cycling (%)	100	95.1	100	53.7			
Total Polyp Number	0	0	41 (7)	53 (4)	0.333	<0.001	0.333
Total Large Polyps (> 2 mm)	0	0	19 (4)	34 (4)	0.102	<0.001	0.102
Hindlimb Muscle (mg)	212 (4)	230 (3)	196 (3)@	181 (5)@*	0.837	<0.001	0.027
Soleus (mg)	5.9 (0.5)	6.7 (0.3)	5.9 (0.5)	6.3 (0.2)	0.080	0.421	0.467
Gastrocnemius (mg)	91.2 (2.3)	98.0 (1.4)	82.6 (1.6)	75.9 (3.1)	0.967	<0.001	0.073
Tibialis Anterior (mg)	34.7 (0.6)	37.4 (0.8)	32.9 (0.9)	29.3 (0.9)@*	0.815	<0.001	0.029
EDL (mg)	7.4 (0.6)	7.6 (0.3)	7.3 (0.8)	7.1 (0.3)	0.356	0.897	0.408
Spleen (mg)	93 (8)	78 (2)	159 (20)	415 (18)^	<0.001	<0.001	<0.001
Heart (mg)	103 (4)	104 (3)	90 (1)	107 (2)	0.031	0.212	0.068
Epididymal Fat (mg)	255 (16)	265 (15)	196 (36)	86 (14)^	0.045	<0.001	0.017
Tibia (mm)	16.2 (0.1)	16.6 (0.1)	16.1 (0.1)	16.5 (0.1)	0.001	0.405	0.938

*Data is expressed as mean (standard error of the measurement). Mice were sacrificed around 12 or 20 weeks of age. Hindlimb muscle mass includes the sum of the soleus, gastrocnemius, plantaris, tibialis anterior, EDL, and rectus femoris. g, grams; mm, millimeters; mg, milligrams; %, percent; Δ, change; EDL, Extensor Digitorum Longus. Symbols for Interactions: ^Different than all groups, *Different than B6 12 week, @ Different than B6 20 weeks. p-values for main effects of Age (12 vs. 20 weeks) and Genotype (B6 vs. MIN), and Interactions are listed to the far right of each variable.*

E2 pellet (E2) or were anesthetized under isoflurane but did not receive a pellet (control). One week prior to sacrifice, grip strength, cage activity, and food intake were recorded in estrogen treated and control mice. At 18 weeks of age, mice were sacrificed following a 5 h fast.

Cycle Presence

At 10 weeks of age, female B6 and MIN mice were tracked weekly for the presence or absence of an estrous cycle until mice were euthanized (Experiment 1). Herein we have used a modified methodology to limit pseudopregnancy caused by pipette tip insertion (10). Briefly, the mouse was grasped by the base of the tail, and following urination roughly 25–50 μl of PBS was aspirated into the vaginal canal without inserting the pipette tip to avoid pseudopregnancy as previously described (33). Cycle presence was determined by vaginal smears. We examined the presence of squamous epithelial cells. If we observed the absence of an estrous cycle, we continued the vaginal lavage every other day for 1 week to verify a cycle. If a mouse was in diestrus, presented by mostly the presence of leukocytes, vaginal lavages were completed every other day until we observed squamous epithelial cells. Vaginal lavages were completed at least once a week from 10 to 20 weeks of age. Cyclic mice were given a score of 1 and acyclic mice were given a score of 0; allowing us to quantify an estrous cycle index.

Tissue Collection

Following a 5 h fast, mice were euthanized with a subcutaneous injection of a ketamine-xylazine-acepromazine cocktail (1.4 ml/kg body weight) (34). Hindlimb muscles (soleus, plantaris, extensor digitorum longus, gastrocnemius, tibialis anterior, and rectus femoris) and organs were rapidly excised, cleared of excess connective tissue, rinsed in PBS, weighed, and snap frozen in liquid nitrogen to be analyzed at a later date.

Intestinal Polyp Quantification

Intestinal segments were excised, cleaned with PBS, cut into equal segments, and stored in 10% neutral formalin until tumor count analysis. Intestinal polyps were analyzed after a deionized water rinse and 0.1% methylene blue staining. Total polyp counts were performed using dissecting micro-scope (model SMZ168, Motic, Xiamen, China) by an investigator blinded to the treatment groups as previously described (28, 35).

Western Blotting

Western blot analysis was performed as previously described (36). Briefly, frozen gastrocnemius muscle was homogenized in lysis buffer and protein concentration was determined by the Bradford method. Crude gastrocnemius muscle homogenates were fractionated on 10% SDS-polyacrylamide gels and transferred to PVDF membranes. Membranes were stained with Ponceau red to verify equal loading and transfer. Membranes were then blocked at room temperature for 1–2 h in 5% non-fat milk Tris-buffered saline with 0.1% Tween-20 (TBST). Commercially available phosphorylated and total protein primary antibodies for rpS6, 4E-BP1, and AMPK were raised in rabbit. RpS6, 4E-BP1, and AMPK phosphorylation antibodies were expressed relative to total protein on the same gel and

TABLE 2 | Female MIN mice characteristics stratified by cachexia severity (Experiment 1).

Genotype	MIN				
	WS	**Initiated**	**Moderate**	**Severe**	***p*-value**
N	12	10	9	10	
Sacrifice BW(g)	20.8 (0.4)	19.6 (0.4)	18.8 (0.7)$^{+}$	17.0 (0.3)^	<0.001
Peak BW (g)	21.3 (0.4)	20.4 (0.3)	20.5 (0.4)	20.5 (0.4)	0.341
BWΔ from Peak (%)	−0.1 (0.1)	−2.4 (0.4)	−7.5 (0.7)$^{+}$	−15.3 (1.8)$^{+**}$	<0.001
Percent Cycling (%)	100	80	22	0	
Total Polyp	46 (8)	48 (8)	66 (8)	68 (10)	0.141
Total Large Polyps (>2 mm)	32 (7)	31 (9)	39 (9)	36 (8)	0.826
Hindlimb Muscle (mg)	215 (7)	198 (7)	170 (12)$^{+}$	137 (8)^	<0.001
Soleus (mg)	6.4 (0;5)	6.6 (0.4)	6.9 (0.7)	5.6 (0.4)	0.125
Gastrocnemius (mg)	90.8 (3.2)	85.4 (2.6)	69.9 (6.4)$^{+}$	54.5 (3.7)$^{+**}$	<0.001
Tibialis Anterior (mg)	34.8 (1.9)	31.1 (1.9)	27.3 (1.9)$^{+}$	23.8 (1.2)$^{+**}$	<0.001
EDL (mg)	8.5 (0.5)	7.2 (0.5)	6.4 (0.9)	5.3 (0.5)$^{+}$	0.005
Ovaries (mg)	12 (1)	12 (1)	13 (2)	9 (7)	0.462
Uterus (mg)	43 (4)	36 (4)	33 (5)	38 (3)	0.381
Spleen (mg)	388 (39)	383 (43)	428 (57)	432 (30)	0.357
Heart (mg)	109 (4)	99 (4)	106 (85)	108 (7)	0.501
Epididymal Fat (mg)	156 (29)	118 (25)	51 (51)$^{+}$	5 (5)^	<0.001
Tibia (mm)	16.7 (0.1)	16.4 (0.2)	16.6 (0.1)	16.4 (0.1)	0.108

*Data is expressed as mean (standard error of the measurement). Female MIN mice were sacrificed at ∼20 weeks of age and stratified by body weight change from peak. Weight stable (0%), initiated (0 to −5%), moderate (−5 to −10%), and severe (< −10%). Hindlimb muscle mass includes the sum of the soleus, gastrocnemius, plantaris, tibialis anterior, EDL, and rectus femoris. g, grams; mm, millimeters; mg, milligrams; %, percent; Δ, change; EDL, Extensor Digitorum Longus; and WS, Weight Stable. Symbols for Multiple Comparisons: ^Different than all groups, + Different than WS, **Different than Initiated. P-values are listed to far right for each variable.*

quantified as phosphorylation to total ratio. Phosphorylated rpS6 (S240/244) (cat#2215, 1:1000), total rpS6 (cat#2708, 1:2000), phosphorylated 4E-BP1 (T37/44) (cat#2855, 1:1000), total 4EBP1 (cat#9452, 1:2000), phosphorylated AMPK (T172) (cat#, 1:2000), and total AMPK (cat#2603, 1:1000) primary antibodies were purchased from cell signaling. Commercially available total protein primary antibodies for MuRF-1 and Atrogin-1 were raised in rabbit. MuRF-1 and Atrogin-1 proteins were corrected for GAPDH protein expression on the same gel and quantified as total protein expression to GAPDH ratio. Atrogin-1 (cat# AP2041, 1:1000) and MuRF-1 (MP3401; 1:2000) primary antibodies were purchased from ECM biosciences. GAPDH was purchased from cell signaling (cat# 14C10, 1:4000). All primary antibodies were incubated overnight in 5% TBST milk. Membranes were then incubated in 5% milk-TBST containing anti-rabbit (cat#7074, 1:4000) IgG horseradish-peroxidase conjugated secondary antibody purchased from cell signaling for 1 h at room temperature. Enhanced chemiluminescence (ECL) (GE Healthcare Life Sciences, Piscataway, NJ) was used to visualize the antibody-antigen interactions. Membranes were stripped and re-probed for total or GAPDH protein expression. Immunoblot images were collected using a digital imager (SynGene GBox, Frederick, MD) and quantified by densitometry using imaging software (Image J; NIH).

Mitochondrial Respiration

A randomly selected cohort of five to six mice from control and 17ß-estradiol groups were used for analysis of mitochondrial function. Mitochondrial respiration was measured polarographically in a respiration chamber (Hansatech Instruments, Oxygraph) maintained at 37°C as described previously (37, 38). A 7–10 mg piece of tibialis anterior (TA) muscle was mechanically tweezed with forceps under a dissecting microscope in ice-cold buffer X (mM: 60 K-Mes, 35 KCl, 7.23 K_2EGTA, 2.77 CaK_2EGTA, 20 imidazole, 0.5 DTT, 20 taurine, 5.7 ATP, 15 phosphocreatine and 6.56 $MgCl_2$, pH 7.1). The fiber bundle was then incubated in 50 µM saponin for 30 min and washed three times for 5 min in respiration buffer (mM: 105 K-Mes, 3 KCl, 1 EGTA, 10 K_2HPO_4, 5 $MgCl_2$, 0.005 glutamate, 0.002 malate, 0.05% BSA, and 20 creatine, pH 7.1). Fiber bundles were then placed into the oxygraph machine in 20 mM creatine respiration buffer at 37°C and provided with 5 mM of pyruvate and 2 mM of malate to measure complex I-mediated mitochondrial respiration (39, 40). Two minutes after pyruvate and malate, 0.25 mM of ADP was injected into the chamber to induce state three respiration for 5 min. Oligomycin (10 µg ml^{-1}) was then injected to induce steady state four respiration for 10 min. The respiratory control ratio (RCR) was calculated by dividing state three by state four respirations. All samples were normalized to dry weight.

Plasma Estrogen and Progesterone

Immediately prior to sacrifice, blood was collected via retro-orbital sinus with heparinized capillary tubes, placed on ice, and centrifuged (10,000 × g for 10 min at 4°C). The supernatant was removed and plasma 17ß-estradiol (range, 3–300 pg/ml)

and progesterone (range, 0.15–40 ng/ml) concentrations were determined by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core Facility.

Statistical Analysis

All results are reported as means ± standard error of measurement (SEM). To compare sacrifice characteristics of 12 and 20 week B6 and MIN mice, 2 (Age; 12 vs. 20 weeks) × 2 (Genotype; B6 vs. MIN) ANOVA's were used. To compare body weight over time in 20 week B6 and MIN mice, 2 (Genotype; B6 vs. MIN) × 9 (12–20 weeks) repeated measures ANOVA was used. To compare sacrifice characteristics, food intake, and grip strength in MIN mice that had E2 treatment and/or ovariectomy surgery, 2 (Treatment; Control or E2) × 2 (Condition; Intact or OVX) ANOVA's were used. To compare sacrifice characteristics, food intake, and grip strength in B6 mice that had E2 treatment and/or ovariectomy surgery, 2 (Treatment; Control or E2) × 2 (Condition; Intact or OVX) ANOVA's were used. To compare skeletal muscle signaling, mitochondrial respiration, and cage activity in E2 treated B6 and MIN mice, 2 (Genotype; B6 or MIN) × 2 (Treatment; Control or E2) ANOVA's were used. If there was a significant interaction, unpaired student's *t*-test were used to find differences between variables. One-way ANOVA's were used to compare MIN mice sacrifice variables stratified by cachexia severity. Tukey's *post hoc* analysis was used when appropriate. Unpaired Student's *t*-test were used to compare cycling and acyclic MIN mice. Statistical analysis was performed using GraphPad (Prism 8 for MAC OS X, La Jolla, Ca). Level of significance for all measures was set at $p \leq 0.05$.

Study Approval

All experiments were approved by the University of South Carolina's Institutional Animal Care and Use Committee.

RESULTS

Cachexia in Female Mice (Experiment 1)

Since cachexia progression in the female mouse has not been characterized as well as the male, we first compared B6 and MIN mice at 12 and 20 weeks of age to determine the phenotypic characteristics prior to and during the progression of cachexia (**Table 1**).

Body Weight

We examined body weight change over time in the 20-week-old female mice (**Figure 1A**). MIN mice at 20 weeks of age demonstrated body weight loss which was decreased in the MIN between 16 and 18 weeks of age. Additionally, B6 body weight at 16 weeks of age was greater than the MINs at this age. MIN mice did exhibit a change in body weight at 20 weeks of age when compared to all groups (**Table 1**). Regardless of genotype, mice grew after 12 weeks of age. Body weight at 20 weeks of age was increased when compared to 12-week-old mice (**Table 1**). However, there was a main effect of genotype for MIN peak bodyweight to be less than B6 peak bodyweight.

Skeletal Muscle and Fat Mass

Total hindlimb muscle mass in 20-week-old MIN mice was decreased when compared to 20-week-old B6 mice (**Table 1**). We also examined individual hindlimb muscles to examine the effect of muscle phenotype and wasting. Regardless of age, MIN gastrocnemius muscle mass was decreased in MIN mice compared to B6 mice. MIN tibialis anterior muscle mass at 20 weeks of age was reduced when compared to 20-week-old B6 mice. Interestingly, neither the soleus nor extensor digitorum longus muscles demonstrated any differences between treatment groups. Epididymal fat mass in the 20-week-old MIN mice was decreased compared to all other treatment groups.

Other Cachexia Related Variables

Total intestinal and colon polyp number and the large size polyp number were not different between 12 and 20-week-old MIN mice (**Table 1**). Spleen mass in the 20-week-old MIN mice was increased when compared to all other treatment groups (**Table 1**).

Characterization of Cachexia Progression (Experiment 1)

Cachexia progression is often characterized by the body weight change from the peak body weight measured during the study. However, we have previously reported that female MIN mice exhibit more variable body weight change at any given age when compared to male MIN mice. Therefore, we stratified 20-week-old MIN mice by the degree of body weight change and examined phenotype characteristics. Twenty-week female MIN mice were stratified by their body weight change from peak body weight (**Table 2**). Mice were classified as weight stable, initiating cachexia, moderate cachexia, or severe cachexia (10). As expected, body weight change in this cohort of mice exhibited significant differences in the cachexia severity classification. There was a difference in body weight change between the weight stable and moderate cachexia groups. The body weight change in the severe cachexia group was larger than the change in the weight stable and initiating cachexia groups. There were no differences between groups for peak body weight and tibia length, suggesting that body size was not altered by cachexia. Interestingly, there were no differences in either total polyp number or large polyp number between groups (**Table 2**). Hindlimb muscle mass in the moderate cachexia group was decreased compared to weight stable mice, and mice with severe cachexia had less hindlimb muscle mass when compared to all other groups. Hindlimb muscles were also analyzed individually, and exhibited differences related to mass loss during the progression of cachexia. Both gastrocnemius and tibialis anterior muscle mass were decreased in the moderate cachexia group when compared to weight stable mice. In the severe cachexia group gastrocnemius muscle mass and tibialis anterior muscle mass were reduced compared to both weight stable and initiating cachexia groups. Extensor digitorum longus muscle mass was decreased with severe cachexia when compared to weight stable mice. Interestingly, soleus muscle mass was not altered by the cachexia classification. These results suggest that skeletal muscle mass loss in the cachectic female occurs

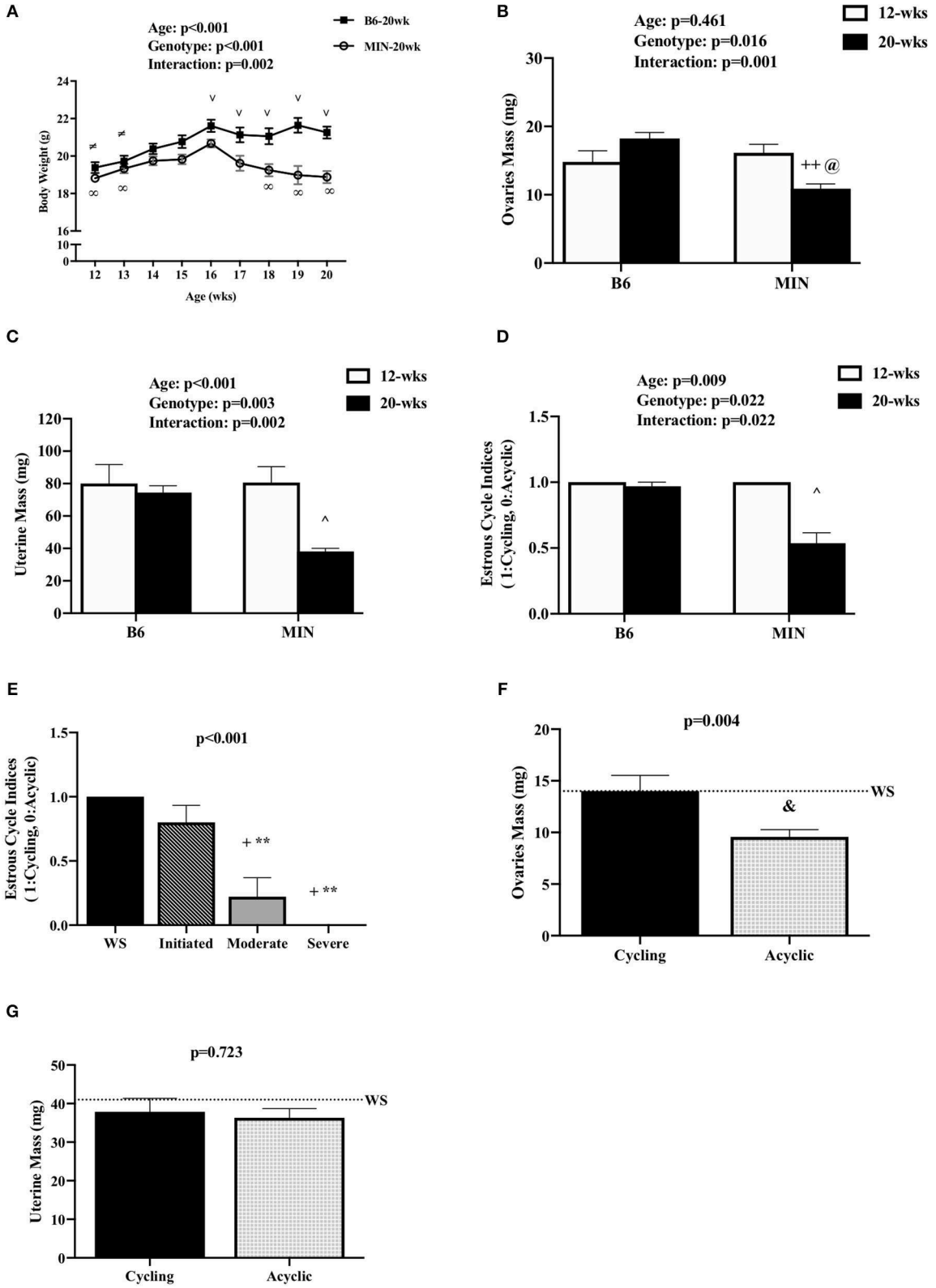

FIGURE 1 | Effect of Cachexia Progression on Hypogonadism. Data is expressed as mean (standard error of the measurement). **(A)** Body weight from 12 to 20 weeks of age in female MIN mice. **(B)** Ovaries mass in 12-week and 20-week B6 and MIN mice. **(C)** Uterine mass in 12-week and 20-week B6 and MIN mice. **(D)**

(Continued)

FIGURE 1 | Estrous cycle index in 12-week and 20-week of age. **(E)** Estrous cycle indices in female MIN mice sacrificed at 20 weeks of age stratified by severity. **(F)** Ovaries mass in cycling and acyclic MIN mice. **(G)** Uterine mass in cycling and acyclic MIN mice. Dashed line is a reference point for female Weight Stable (WS) MIN mice. WS, weight stable; mg, milligrams; %, percent; mm, millimeters. Symbols for statistical significance: ∞Different than MIN at 16 weeks, ≠ Different than B6 at 16 weeks, and ⋁ Different than MIN at given time point, ++Different than 12 week MIN, @Different than 20 week B6, ^Different than all groups, +Different than WS, **Different than Initiated, $^{\&}$Different than Cycling.

TABLE 3 | Ovariectomy and 17ß-estradiol treatment characteristics of female MIN mice (Experiment 2).

Genotype	MIN						
	MIN Intact		MIN OVX		*p-value*		
Treatment	Control	*E2*	OVX	OVX ± *E2*	Condition	Treatment	Interaction
N	8	9	10	11			
BW Pre-Treatment (g)	18.4 (0.5)	17.9 (0.4)	18.8 (0.3)	18.6 (0.4)	0.216	0.482	0.757
BW Mid-Treatment (g)	18.6 (0.4)	20.3 (0.4)	22.7 (0.5)^	20.1 (0.4)	<0.001	0.383	<0.001
BW End-Treatment (g)	18.7 (0.5)^	21.4 (0.5)	21.3 (0.2)	21.0 (0.5)	0.021	0.009	0.002
Peak BW (g)	20.4 (0.2)	21.7 (0.4)	22.7 (0.3)$^{\$\$}$	21.3 (0.5)	0.054	0.873	0.003
Total Polyp Number	77 (13)	57 (7)	53 (14)	52 (18)	0.288	0.440	0.491
Total Large Polyps (> 2 mm)	53 (10)	29 (6)	30 (3)	39 (5)	0.500	0.407	0.081
Progesterone (ng/ml)	1.8 (0.2)	2.3 (0.6)	5.2 (0.7)	4.3 (1.2)	0.001	0.796	0.392
Liver (mg)	1150 (72)	1606 (100)	1281 (44)	1552 (97)	0.644	<0.001	0.268
Spleen (mg)	422 (47)	520 (52)	342 (37)	400 (57)	0.052	0.124	0.691
Heart (mg)	100 (7)	116 (10)	115 (3)	108 (4)	0.591	0.454	0.069
Tibia (mm)	16.5 (0.1)	16.2 (0.1)	16.3 (0.1)	15.9 (0.1)	0.061	0.017	0.982

Data is expressed as mean (standard error of the measurement). Female MIN mice were sacrificed at 18 weeks of age following a 5-h fast. MIN mice were randomly assigned to intact control, received 17ß-Estradiol pellet (intact E2), underwent ovariectomy surgery (OVX) or ovariectomy surgery and received a 17ß-Estradiol pellet (OVX + E2). Intact mice underwent a SHAM OVX surgery. g, grams; mm, millimeters; mg, milligrams; %, percent; ng, nanograms; E2, 17ß-Estradiol; OVX, Ovariectomy. Symbols for Interactions: ^Different than all groups, $$ Different than intact control. P-values for main effects of Condition (intact vs. OVX) and Treatment (Control vs. E2), and Interactions are listed to the far right of each variable.

after the initiation of cachexia, and muscle phenotype impacts the degree of mass loss. Epididymal fat mass was decreased by moderate cachexia when compared to weight stable mice, and severely cachectic mice had less epididymal fat mass than all other cachexia classifications. Taken together, our results suggest that there is a clear phenotypic distinction that occurs during the progression of cachexia in the female MIN mouse. While fat loss continued to decline with greater body weight change, muscle mass loss occurred prior to severe weight loss. Lastly, the initiation of cachexia provides a critical window for intervening to slow muscle and fat mass loss in the female.

Hypogonadism and Cachexia Progression (Experiment 1)

We examined hypogonadism during the progression of cachexia. Gonadal mass and estrous cycle index were examined in 12 and 20-week-old mice. The 20-week old MIN mice exhibited hypogonadism. Ovary mass was decreased in 20-week-old MIN mice when compared to the same age B6 mice and 12-week-old MIN mice (**Figure 1B**). Uterine mass in the 20-week-old MIN mice was decreased compared to all other groups (**Figure 1C**). The estrous cycle index was also decreased in the 20-week-old MIN when compared to all other groups (**Figure 1D**). To determine if hypogonadism preceded cachexia development, we examined gonadal function in 20 week female MIN mice stratified by cachexia severity (**Table 2**). Neither ovary nor uterine mass were impacted by cachexia severity, demonstrating that hypogonadism occurs before bodyweight loss. The estrous cycle index was reduced in moderate and severely cachectic mice when compared to weight stable and mice initiating cachexia (**Figure 1E**). These data suggest that the estrous cycle is maintained until moderate body weight loss occurs.

Given that estrous cycle absence is a possible indicator of cachexia progression, we examined the phenotypic differences in the presence or absence of an estrous cycle. Mice were stratified by cyclicity ($N = 22$) or acyclicity ($N = 19$). We found that the change in body weight from peak was greater in acyclic mice when compared to cyclic mice [cyclic: −1.2 (0.38%), acyclic: −11.4 (1.4%); *data not shown*]. Hindlimb muscle mass [cyclic: 209 (5 mg), acyclic: 150 (7 mg); *data not shown*] and epididymal fat mass [cyclic: 145 (23 mg), acyclic: 26 (15 mg); *data not shown*] were also decreased in acyclic mice when compared to cyclic mice. Ovary mass decreased in acyclic compared to cyclic mice (**Figure 1F**). Total polyp number (data not shown) and uterine mass (**Figure 1G**) were not affected by estrous cyclicity, which is in agreement with previous published data that rigorously examined the difference in acyclic and cyclic female MIN mice (10). Collectively, our data suggest that estrous cycle cessation occurs after a mouse has achieved 5% body weight loss, and acyclic mice exhibit a more severe cachectic phenotype.

Ovariectomy and 17ß-estradiol Administration and Cachexia Progression (Experiment 2)

We investigated the importance of gonadal hormones in the regulation of cachexia progression. The effect of ovariectomy and 17ß-estradiol administration on cachexia progression in female MIN mice was examined (**Table 3**). At 8 weeks of age, mice were randomly to either; SHAM OVX surgery (intact control), 17ß-estradiol pellet administration (intact E2), ovariectomy (OVX control), or ovariectomy and 17ß-estradiol pellet administration (OVX+E2). At 11 weeks of age, mice underwent surgery and/or pellet implantation and were sacrificed at 18 weeks of age (**Figure 2A**). Uterine mass was increased in ovary intact MIN mice receiving E2 when compared to intact control and OVX control. Uterine mass was increased in OVX mice that received E2 compared to all other groups (**Figure 2B**). There was a main effect of treatment for E2 mice to increase circulating 17ß-estradiol compared to control mice (**Figure 2C**). There were main effects of treatment for E2 to have reduced MIN mouse body weight change (**Figure 2D**), increased epididymal fat mass (**Figure 2F**), increased relative food intake (**Figure 2G**), increased relative grip strength (**Figure 2H**), and increased liver mass (**Table 3**) compared to control MIN mice. There were main effects of condition for OVX to increase circulating progesterone (**Table 3**) and increase epididymal fat mass (**Figure 2F**) compared to intact MIN mice. While there were no differences in pre-treatment body weights (**Table 3**), OVX mid-treatment (14 weeks) body weight was increased when compared to all groups. Hindlimb muscle mass was decreased in the intact control MIN mice when compared to all other groups (**Figure 2E**). There were no differences between treatment groups in total polyp number, large polyp number, spleen mass, and heart mass. Taken together, 17ß-estradiol was able to prevent body weight loss in the MIN mouse and improve indices of cachexia without altering total polyp burden.

We also examined the effect of ovariectomy and 17ß-estradiol administration in female B6 mice (**Table 4**). There were main effects of treatment for E2 mice to have increased uterine mass, increased circulating 17ß-estradiol, and decreased relative grip strength compared to control mice (**Table 4**). There was also a main effect of condition for OVX in B6 mice to decrease uterine mass, increase circulating progesterone, and decrease relative grip strength compared to intact B6 mice (**Table 4**). Intact E2 mice had a greater body weight at the pre, mid, and end time points. Peak body weight was also greater in the intact E2 B6 mice when compared to intact control B6 mice. Hindlimb muscle mass was not altered by either OVX or E2 treatments in B6 mice. Interestingly, epididymal fat mass was decreased in OVX+E2 B6 mice when compared to either intact or OVX control mice. Epididymal fat mass was also decreased in intact E2 B6 mice when compared to intact control mice. Relative food intake was reduced in the intact control pellet mice when compared to all other treatment groups. Intact E2 mice had reduced relative food intake compared to either B6 OVX and OVX + E2 mice. Taken together, these results validate our 17ß-estradiol administration and OVX treatments and identify differences when examining the effects of ovariectomy or E2 administration in either healthy or tumor bearing mice.

17ß-Estradiol Administration and Skeletal Muscle Signaling (Experiment 2)

We determined the effect of E2 administration on skeletal muscle signaling associated with cancer cachexia. Skeletal muscle AMPK has been widely investigated for its regulatory role in protein turnover and metabolism. AMPK is chronically activated in cachectic male MIN mouse skeletal muscle and further increased by high levels of circulating IL-6. Skeletal muscle mTORC1 signaling through downstream targets 4E-BP1 and rpS6 is also suppressed by cachexia and IL-6 (32). Protein expression was examined in the gastrocnemius muscle. MIN control mice had decreased gastrocnemius muscle mass when compared to all other groups (**Figure 3A**). E2 treatment increased MIN gastrocnemius muscle mass to the level of the B6 mouse. In B6 mice, muscle AMPK (T172) phosphorylation to total ratio was not different than B6 mice administered E2 (**Figure 3B**). As expected, muscle AMPK (T172) phosphorylation to total ratio was increased in MIN control pellet mice compared to both B6 groups; E2 treatment in the MIN mouse suppressed this induction (**Figure 3B**). There was a main effect of genotype for MIN mice to have increased total muscle AMPK protein expression compared to B6 mice (**Figure 3C**). 4E-BP1 (T37/44) phosphorylation to total ratio was decreased in B6 E2 treatment mice compared to B6 control mice. The 4E-BP1 (T37/44) phosphorylation to total ratio was decreased in MIN control mice when compared to B6 control mice. E2 treatment in MIN mice increased 4E-BP1 phosphorylation to total ratio compared to MIN control mice (**Figure 3D**). Interestingly, E2 treatment to B6 mice increased total 4E-BP1 above all other groups (**Figure 3E**). Muscle rpS6 (S240/244) phosphorylation to total ratio was increased by E2 treatment in MIN mice compared to all other groups (**Figure 3F**). There were no differences in total rpS6 protein expression (**Figure 3G**). The protein expression of muscle E3 ligases involved in ubiquitin proteasome degradation were also examined. We report differential sensitivity of MuRF-1 and Atrogin-1 protein expression to either cachexia or E2 stimuli. There was a main effect of treatment for E2 mice to increase muscle MuRF-1 protein expression compared to control mice (**Figure 3H**). We report a doublet of MuRF-1 which the scientific literature seems to be equivocal on whether MuRF-1 is expressed as a singlet or doublet and as to the importance of the doublet, research is warranted to further define the biological significance of the MuRF-1 doublet. Published studies suggest that the MuRF-1 double band can be associated with the gel percentage used, animal phenotype, or injury recovery (41–45). However, the specific rationale for the cause of the double bands has not been firmly established and warrants further investigation. There was a main effect of genotype for MIN mice to have increased muscle Atrogin-1 protein expression compared to B6 mice (**Figure 3I**). While we report the ratio of phosphorylated to total protein to represent activity, we also quantified muscle AMPK, rpS6, and 4E-BP1 phosphorylation separately (**Supplemental Figure 1**). There was a main effect of

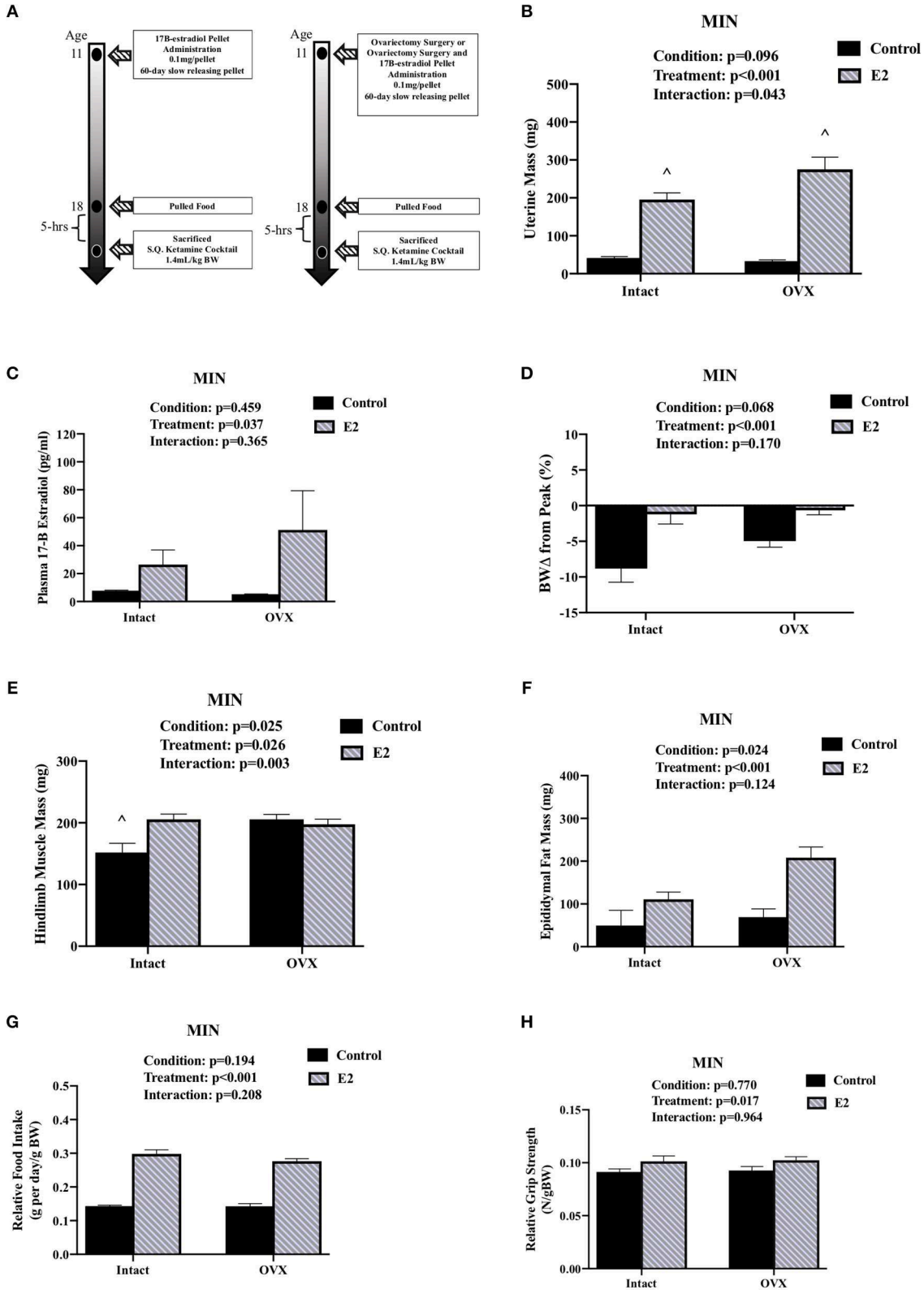

FIGURE 2 | Ovariectomy and 17ß-Estradiol Administration on Cachexia in Female MIN Mice. Data is expressed as mean (standard error of the measurement). All female mice analyzed correspond to **Table 3**. **(A)** Study Design. At 11 weeks of age female MIN mice were randomly allocated to intact control, 17ß-Estradiol pellet

(Continued)

FIGURE 2 | (E2), ovariectomy surgery (OVX), or ovariectomy surgery followed by 17ß-estradiol pellet (OVX + E2). Intact mice underwent a SHAM OVX surgery. **(B)** Uterine mass. **(C)** Plasma concentration of 17ß-Estradiol. **(D)** Body weight change from peak. **(E)** Hindlimb muscle mass. **(F)** Epididymal fat mass. **(G)** Relative food intake. **(H)** Relative grip strength. E2, 17ß-estradiol; mg, milligrams; pg/ml, picograms per milliliter; g, grams. Food intake was collected on a subset of mice ($N = 5–7$ per group). *p*-values for main effects of Condition (intact vs. OVX) and Treatment (Control vs. E2), and Interactions are listed above the figures. Symbol for an Interaction: ^Different than all groups.

TABLE 4 | Ovariectomy and 17ß-estradiol treatment characteristics of female B6 mice (Experiment 2).

Genotype	**B6**						
	B6 Intact		**B6 OVX**				
Treatment	**Control**	***E2***	**OVX**	**OVX ± *E2***		***p-value***	
					Condition	**Treatment**	**Interaction**
N	9	11	9	10			
BW Pre-Treatment (g)	17.5 (0.2)	18.5 (0.3)$^{\$\$}$	18.1 (0.3)	17.7 (0.3)	0.687	0.330	0.010
BW Mid-Treatment (g)	19.1 (0.3)	20.0 (0.3)$^{\$\$}$	20.9 (0.3)	20.2 (0.3)	0.004	0.952	0.026
BW End-Treatment (g)	20.4 (0.2)	21.5 (0.5)$^{\$\$}$	22.2 (0.4)	21.6 (0.3)	0.021	0.584	0.037
Peak BW (g)	20.5 (0.2)	21.5 (0.5)$^{\$\$}$	22.5 (0.4)	21.7 (0.3)	0.009	0.788	0.040
Uterus Mass (mg)	81 (6)	238 (11)	39 (4)	203 (17)	0.001	<0.001	0.770
Hindlimb Muscle Mass (mg)	218 (5)	220 (4)	225 (6)	220 (5)	0.457	0.790	0.461
Epididymal Fat (mg)	324 (24)	166 (33)$^{\$\$}$	349 (21)	72 (24)$^{\$\$*}$	0.233	<0.001	0.045
Liver (mg)	894 (33)	1011 (26)	1173 (42)^	1015 (27)	<0.001	0.550	<0.001
Heart (mg)	86 (1)	99 (13)	96 (3)	104 (4)	0.035	0.005	0.431
Tibia (mm)	16.4 (0.1)	16.5 (0.1)	16.7 (0.1)	16.4 (0.1) *	0.302	0.142	0.010
Relative Food Intake (g/g BW)	0.14 (0.00)^	0.19 (0.01)^	0.20 (0.01)	0.20 (0.01)	<0.001	<0.001	<0.001
Relative Grip Strength (N/g BW)	0.11 (0.00)	0.10 (0.00)	0.09 (0.00)	0.08 (0.00)	<0.001	0.018	0.911
Plasma 17-B Estradiol (pg/ml)	5.3 (0.3)	163 (41)	3.0 (0.8)	99 (43)	0.298	<0.001	0.334
Plasma Progesterone (ng/ml)	3.6 (1.0)	4.5 (0.8)	6.6 (1.3)	5.5 (1.1)	0.048	0.933	0.217

*Data is expressed as mean (standard error of the measurement). Female B6 mice were sacrificed at 18 weeks of age following a 5-h fast. B6 mice were randomly assigned to intact control, received 17ß-Estradiol pellet (intact E2), underwent ovariectomy surgery (OVX) or ovariectomy surgery and received a 17ß-Estradiol pellet (OVX + E2). Intact mice underwent a SHAM OVX surgery. g, grams; mm, millimeters; mg, milligrams; %, percent; ng, nanograms; E2, 17ß-Estradiol; OVX, Ovariectomy. Food intake was collected on a subset of mice (N = 5–7 per group). Symbols for Interactions: ^Different than all groups, $$ Different than intact control, *Different than OVX. P-values for main effects of Condition (intact vs. OVX) and Treatment (Control vs. E2), and Interactions are listed to the far right for each variable.*

genotype for MIN mice to have increased muscle AMPK (T172) phosphorylation compared to B6 mice. However, muscle 4E-BP1(T37/44) phosphorylation in MIN control mice was reduced when compared to B6 control mice. MIN E2 treated mice had higher rpS6 (S240/244) phosphorylation when compared to MIN control or B6 E2 treated mice. Taken together, these data suggest that increased plasma 17ß-estradiol can prevent muscle mass loss and improve some aspects of disrupted AMPK and mTORC1 skeletal muscle signaling in female tumor bearing mice.

17ß-Estradiol Administration and Muscle Mitochondrial Respiration (Experiment 2)

We examined the effect of 17ß-estradiol administration on skeletal muscle mitochondrial respiration from isolated tibialis anterior fiber bundles. Estrogen has established beneficial effects on mitochondrial function. Furthermore, mitochondria dysfunction has been widely investigated as a driver of cancer-induced muscle wasting. Tibialis anterior muscle mass was sensitive to both cachexia and E2 treatment (**Figure 4A**). There was a main effect of genotype for MIN mice to have decreased tibialis anterior muscle mass compared to B6 mice. There was a main effect of treatment for E2 mice to have increased tibialis anterior muscle mass compared to control mice. Mouse cage activity level has been shown to be suppressed during the progression of cancer cachexia, and extended periods of inactivity can impact skeletal muscle oxidative metabolism. Mouse cage activity was impacted by the cancer environment and E2 treatment. There was a main effect of genotype for MIN mice to have decreased cage activity compared to B6 mice and a main effect of E2 treatment of have increased cage activity compared to control mice (**Figure 4B**). There was a main effect of genotype for MIN mice to have decreased STATE 3 respiration (**Figure 4C**) and decreased respiratory control ratio (**Figure 4E**) compared to B6 mice. However, there was a main effect of treatment for E2 mice to have decreased STATE 4 respiration (**Figure 4D**) and increase the muscle respiratory control ratio (**Figure 4E**) compared to control mice. Collectively, these data suggest that 17ß-estradiol increased cage activity and prevented mitochondrial respiration dysfunction in female MIN mice.

DISCUSSION

There is evidence to link hypogonadism and cancer cachexia progression in the MIN mouse (10, 15). There is also sexual

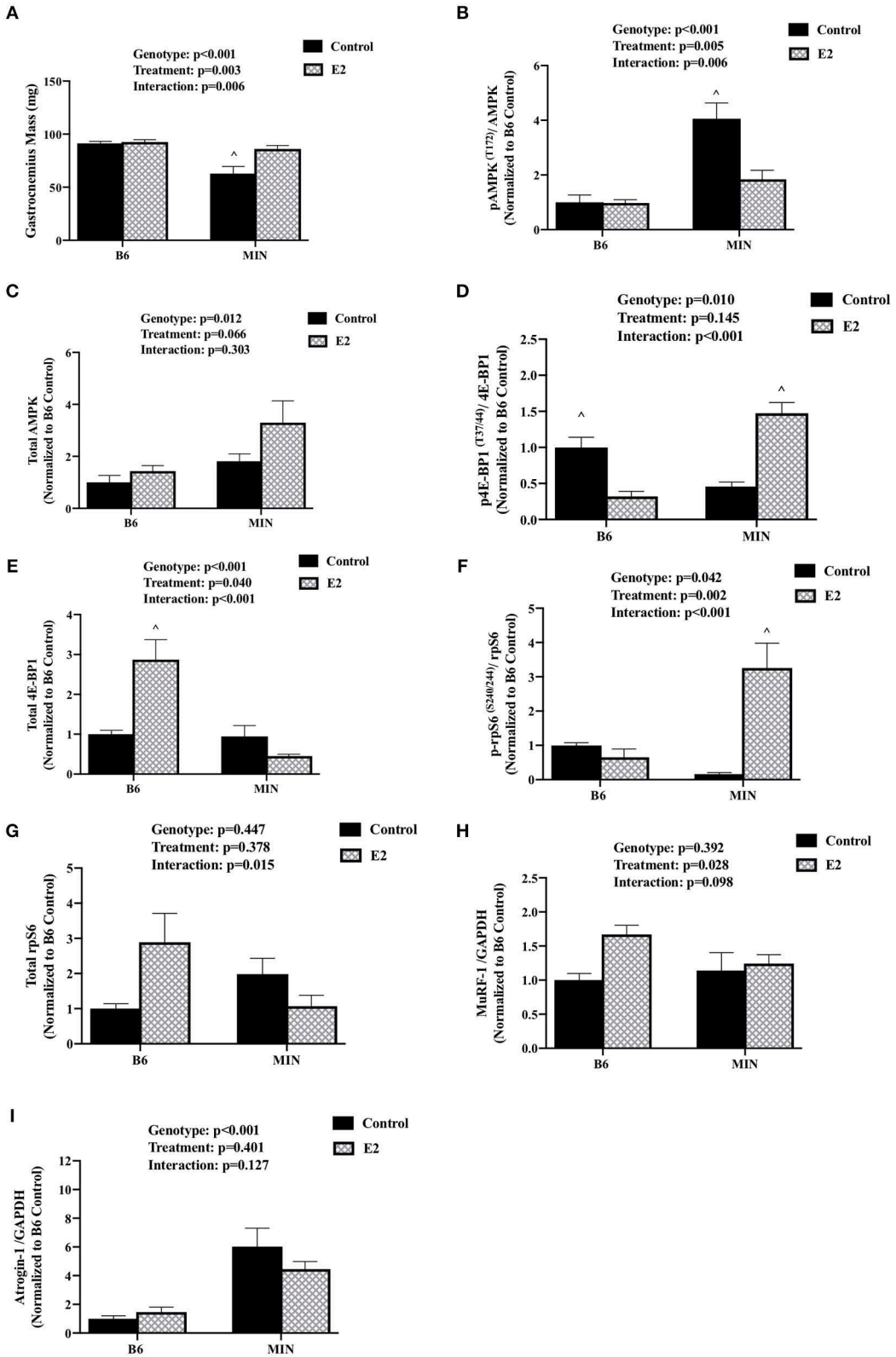

FIGURE 3 | Effect of 17ß-estradiol administration on skeletal muscle signaling. Data is expressed as mean (standard error of the measurement). At 11 weeks of age female MIN mice were randomly allocated to control or 17ß-Estradiol pellet (E2) in B6 and MIN mice. **(A)** Gastrocnemius muscle mass. **(B)** Phosphorylation at site

(Continued)

FIGURE 3 | T172 to total ratio of AMPK. **(C)** Total AMPK protein expression. **(D)** Phosphorylation at site T37/44 to total ratio of 4E-BP1. **(E)** 4E-BP1 total protein expression. **(F)** Phosphorylation at site S240/244 to total ratio of rpS6. **(G)** rpS6 total protein expression. **(H)** MuRF-1 to GADPH ratio. **(I)** Atrogin-1 to GAPDH ratio. Data was normalized to B6 control for all protein analysis. E2, 17ß-estradiol; mg, milligrams; AMPK, 5-AMP-activated protein kinase; 4E-BP1, 4E-Binding Protein 1; rpS6, ribosomal protein S6; MuRF-1, Muscle Ring Finger Protein-1; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase. *p*-values for main effects of Genotype (B6 vs. MIN) and Treatment (Control vs. E2), and Interactions are listed above the figures. Symbols for Interaction: ^Different than all groups.

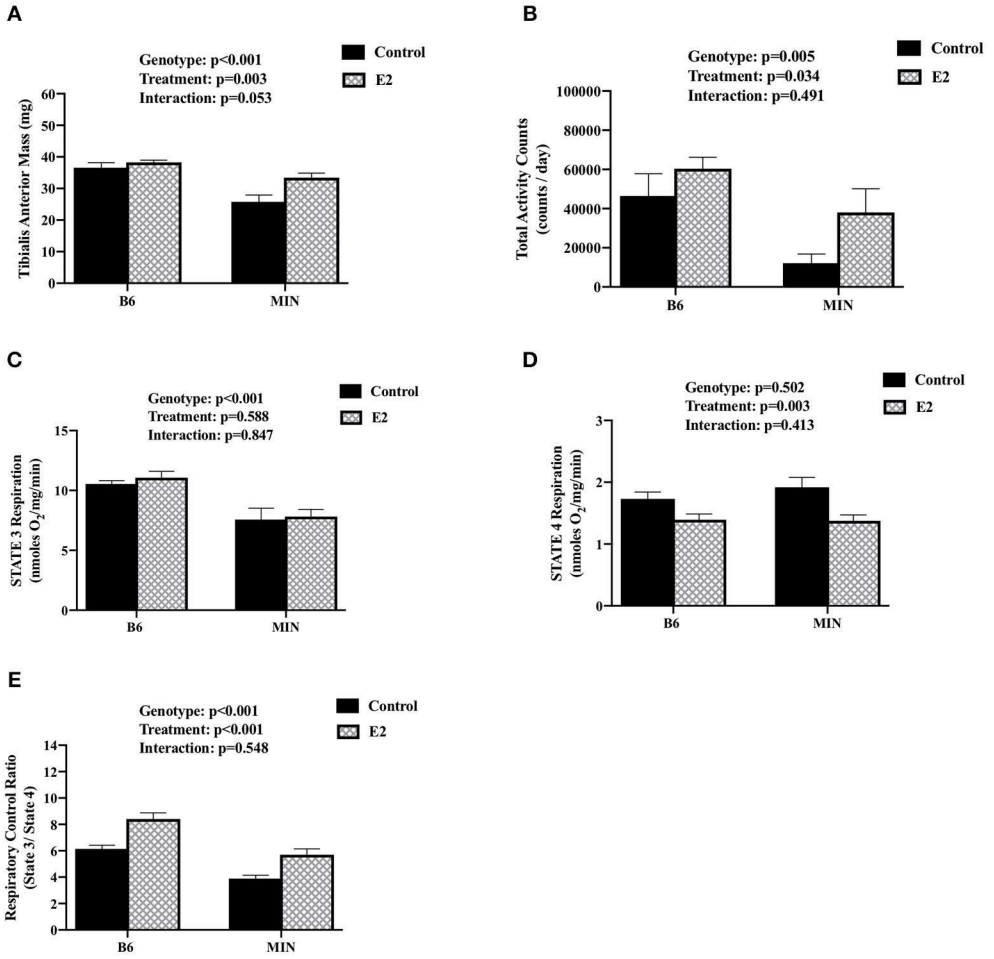

FIGURE 4 | Effect of 17ß-estradiol administration on skeletal muscle mitochondrial respiration. Data is expressed as mean (standard error of the measurement). At 11 weeks of age female MIN mice were randomly allocated to control or 17ß-Estradiol pellet (E2) in B6 and MIN mice. **(A)** Tibialis anterior muscle mass. **(B)** Total ambulatory cage activity counts for 24-h. **(C)** STATE 3 mitochondrial respiration. **(D)** STATE 4 mitochondrial respiration. **(E)** Mitochondrial respiratory control ratio (STATE 3/STATE 4). Physical activity was collected on a subset of mice ($N = 5$–6 per group). Mitochondrial respiration was collected on a subset of mice ($N = 5$–6 per group). E2, 17ß-estradiol; mg, milligrams; counts/day, cage activity counts per day; nmoles/0_2/mg/min, nanomoles of 0_2 per milligram of muscle per minutes. P-values for main effects of Genotype (B6 vs. MIN) and Treatment (Control vs. E2); and Interactions are listed above the figures.

dimorphism in the progression of cancer cachexia that has only just begun to be investigated (46). We extended these findings by elucidating the therapeutic potential of estrogen on the progression of cachexia in tumor bearing mice. In female MIN mice, 17ß-estradiol administration prevented muscle mass loss and blocked whole body indices of cachexia progression involving grip strength loss and decreased cage activity. Interestingly, 17ß-estradiol administration increased relative food intake in MIN mice, which was likely involved in the prevention of muscle mass loss. However, food intake has not been reported to be reduced in male and female MIN mice undergoing cachexia. The beneficial effects of 17ß-estradiol administration in ovariectomized MIN mice suggest that ovarian function is not necessary for the beneficial effects on cachexia progression, and has significant therapeutic implications since most woman that develop cachexia are hypogonadal (47).

Ovarian estrous cycle cessation is associated with cachexia progression in female MIN mice and has been reported to precede the development of cachexia (10). We extend these findings, reporting that estrous cycle cessation is more prevalent

after the mouse has achieved 5% body weight loss. Interestingly, muscle mass, and epididymal fat loss continue to decline as does body weight, which further emphasizes the importance of gonadal dysfunction in the progression of cancer cachexia. While not affected by cachexia severity, uterine and ovaries mass decreased between 12 and 20 weeks of age in tumor bearing mice, and indices of ovarian cycling were also reduced by 20 weeks of age. Thus, the cancer environment and cachexia differentially affect ovarian function. We have previously shown that the ovaries being present in female tumor bearing mice can protect mice from high levels of circulating IL-6 (10). Interestingly, we report an increase in progesterone in ovariectomized mice. The ovariectomized mice progesterone concentrations were within the normal range as previously reported for ovariectomized female mice (48). Additionally, throughout the estrous cycle progesterone can range from 2 to 8 ng/ml (49) thus the increase in progesterone in ovariectomized mice is most likely negligible. It is possible that estradiol concentrations are different between B6 and MIN mice, but is greatly influenced by the estrous cycle phase which can range from 5 to 60 pg/ml (50), and is most likely a result of the estrous cycle.

Skeletal muscle mass is maintained by the balanced regulation of protein synthesis and degradation (51), and cancer-induced disruption to this regulation is a well-established driver of cachexia (31, 52, 53). We examined the effect of 17ß-estradiol on cachectic skeletal muscle protein turnover. Estrogen can regulate mTORC1 signaling, the ubiquitin proteasome system, autophagy, and AMPK signaling (16). All of these signaling pathways have been reported to be disrupted in cachectic skeletal muscle. We found that 17ß-estradiol inhibited the cachexia induction of AMPK, which coincided with the induction of mTORC1 signaling and suggests a role for 17ß-estradiol being an anabolic stimulus in the catabolic environment. Our results suggest differential regulation of mTORC1 by 17ß-estradiol and the cachexic environment when compared with healthy wild type mice. There was no induction of mTORC1 signaling in wildtype mice receiving 17ß-estradiol, which coincides with reports that postmenopausal woman have no change in protein synthesis following estradiol administration (54, 55). In cultured myotubes estradiol improved protein synthesis further confounding the implications of estradiol in skeletal muscle signaling in a non-diseased state (56). Progesterone has been implicated as an anabolic stimulant (55), whereas estradiol may have greater effects on cellular apoptosis and contractile properties (24). Furthermore, we report that Atrogin-1, not MuRF-1 was induced in MIN mouse skeletal muscle. These results coincide with previous published findings from our lab reporting the induction of muscle Atrogin-1 expression in MIN mice without changes in MuRF-1 expression (28). Additionally, we report MuRF-1 expression was induced by 17ß-estradiol administration. The induction of MuRF-1 following 17ß-estradiol administration is an agreement with previous human studies reporting an induction of MuRF-1, but not Atrogin-1, in post-menopausal women following hormone replacement therapy (57). Further research is warranted to determine if 17ß-estradiol can prevent skeletal muscle mass wasting through direct regulation of AMPK and mTORC1 signaling. Collectively, suggesting a role for sex hormones to minimize the effects of hypogonadism through muscle anabolism.

Skeletal muscle mitochondrial dysfunction has been widely investigated as a critical driver of muscle wasting with cancer and aging (58, 59). Furthermore, mitochondrial dysfunction is associated with AMPK activation in disease and has the potential to disrupt mTORC1 activity in male MIN mice (60). We report that mitochondrial State 3 respiration was decreased in tumor bearing mice whereas state four mitochondrial respiration was decreased in mice that received 17ß-estradiol. These findings suggest that tumor bearing mice have suppressed substrate mitochondrial oxidation and improved proton leak (61). These findings are further supported by increased respiratory control ratio (RCR: state 3/state 4) indicative of mitochondrial function, such that 17ß-estradiol was able to prevent RCR suppression in MIN mice. These results are similar to reported improvements of 17ß-estradiol administration on muscle mitochondrial function in disease free mice (23). Estrogen is an established modulator of muscle mitochondrial biogenesis and mitophagy (62), induces mitochondrial gene expression (23), and improves ATP turnover (63). Reduced physical activity is a common altered behavior in pre-clinical models of cancer cachexia and cachectic patients (30, 64, 65), and decreased activity level can impact mitochondrial function. Improved mitochondrial respiration reported herein coincides with increased activity in the E2 treated MIN mice. 17ß-estradiol administered to MIN mice prevented physical activity loss which accompanies cachexia progression, and it has been reported that in OVX mice, estradiol improved voluntary wheel activity (26), thus having significant clinical implications. Furthermore, cancer cachexia can disrupt normal feeding and physical activity behavior patterns (66, 67) and has been suggested to be a potential driving factor in cancer biology (68). Further research is warranted to determine how estradiol was able to protect skeletal muscle mitochondrial function in the cachectic environment, and if this effect was related to improved feeding and activity behaviors.

In summary, to best of our knowledge this is the first study to examine 17ß-estradiol's effect on cachexia progression in female tumor bearing mice. Our findings support a role for 17ß-estradiol administration in the prevention of cachexia in the female MIN mouse. These findings have important implications for therapeutic treatment options for cachectic patients with non-hormone sensitive cancer. We have shown that 17ß-estradiol administration can prevent skeletal muscle mass loss in female MIN mice with or without the ovaries being present. Furthermore, 17ß-estradiol normalized cachexia's disruption to skeletal muscle AMPK signaling, mTORC1 signaling, and mitochondrial function. Indices of decreased function in cachectic tumor bearing mice involving reduced grip strength and decreased cage activity level were prevented by 17ß-estradiol administration. We also report that the estrous cycle in tumor bearing mice is associated with cachexia progression. Our results suggest a role for hypogonadism in cachexia's progression, and further research is warranted to determine if this is a viable therapeutic target for the management of cachexia with some types of cancers. Further

investigation is needed to determine the mechanisms by which 17ß-estradiol can target muscle fiber intracellular signaling and the associated microenvironment in muscle to alter wasting associated with the progression of cachexia. This additional knowledge should provide valuable insight on the regulation of cachexia progression and the development of efficacious treatments for this wasting disease.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

All experiments were approved by the University of South Carolina's Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

BC and DF contributed to collecting data. BC, DF, and JC contributed to data interpretation and contributed to writing and preparing the manuscript. KH and JC contributed to the conceptual development the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00720/full#supplementary-material

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Regulatory Actions of Estrogen Receptor Signaling in the Cardiovascular System

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Premenopausal females have a lower incidence of death from cardiovascular disease (CVD) than male counterparts, supporting the notion that estrogen is protective against the development and progression of CVD. Although large-scale randomized trials of postmenopausal hormone replacement therapy failed to show cardiovascular benefits, recent ELITE study demonstrated anti-atherosclerotic benefits of exogenous estrogen depending on the initiation timing of the therapy. These results have urged us to better understand the mechanisms for actions of estrogens on CVD. Here, we review experimental and human studies, highlighting the emerging role of estrogen's non-nuclear actions linking to NO-cGMP signaling pathways.

Keywords: estrogen, cardiovascular diseases, receptor signaling, non-nuclear pathway, animal study

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INTRODUCTION

In developed countries, cardiovascular diseases (CVD) are the leading cause of death in males and females. As females are less likely to develop CVD before menopause, the endogenous female hormone estrogen appears to provide protection against CVD (1). Consistently, numerous experimental studies of CVD models affirmed the beneficial effects of estrogen treatment, including the inhibition of the development of atherosclerosis and endothelial dysfunction, and the reduction in myocardial ischemic injuries (2–4). While earlier large-scale randomized clinical studies of postmenopausal hormone replacement therapy showed rather adverse events without significant cardiovascular benefits (4, 5), a recent clinical study revealed that the timing for therapy initiation might be a critical determination factor (6).

Estrogen binds to multiple receptors, including classical nuclear estrogen receptors (ERs), ERα, and ERβ, and a new class of membrane G protein-coupled receptor, GPR30, also referred to as GPER (7). The ERs signal not only via "classical" regulation of gene transcription in the nucleus, but also via regulation of "non-nuclear" signaling pathways on ligand binding to ERs (1, 8). Accumulating evidence has shown a critical role for the non-nuclear ER signaling in maintaining the homeostasis of the cardiovascular system (9).

THE EFFECTS OF ESTROGEN HORMONE THERAPY ON THE CARDIOVASCULAR SYSTEM

Multiple cohort studies consistently revealed the lower risk of CVD with hormone therapy (HT), indicating that estrogen loss enhances the risk of CVD in postmenopausal females (2–4). On the other hand, randomized studies including the Women's Health Initiative (WHI) showed no cardiovascular benefits, but rather found an increased risk of stroke and deep vein thrombosis

(4, 5). These discordant findings could reflect variations in the time between menopause and HT initiation: earlier cohort studies involved younger women who received HT early after menopause, and patients in randomized studies receive HT 10 years after menopause, where vascular endothelium might have lost its responsiveness to estrogen.

Indeed, several recent studies provided supporting evidence for this "timing hypothesis." Estrogen alone reduced total mortality in early (<10 years) postmenopausal females (10). After 10 years of randomized treatment, women who received HT early after menopause showed a significantly reduced risk of mortality, heart failure, and myocardial infarction, without increases in the risk of cancer, venous thromboembolism, or stroke (11). The Early vs. Late Intervention Trial with Estradiol (ELITE) study enrolled postmenopausal females with and without histories of prior hysterectomy surgery (6). Approximately half of the participants were considered early menopause (<6 years; mean age, 55 years), and the other half were later menopause (at least 10 years; mean age, 65 years). Participants were randomized to receive either oral 17 β-estradiol or a placebo, and the carotid intima-medial thickness (CIMT) by ultrasound, an atherosclerosis marker predicting cardiovascular events, was assessed as the primary clinical outcome. Over 6 years, the early menopause participants who were randomized to 17 β-estradiol showed a slower progression of CIMT than those randomized to placebo (6). These results strongly indicate that HT exerts cardioprotective effects when initiated at an ideal time point after menopause.

These recent clinical findings encourage researchers to further investigate molecular and physiological function of ER-mediated signaling in the cardiovascular system (12, 13), which provides meaningful steps forward to the development of a next generation of HT for women that capture the beneficial cardiovascular effects of the hormone while minimizing its potential for harm.

Estrogen Receptors and Cardiovascular Cells

ERα and ERβ exhibit high homology and, as with all steroid hormone receptors, function as transcription factors that alter gene expression when activated (3). Functional ERs are expressed in vascular endothelial cells (EC), vascular smooth muscle cells (VSMC), and cardiomyocytes in humans and animals (3) (**Figure 1**). Some cardiovascular effects might be mediated by a transmembrane G-protein-coupled receptor GPR30 which is extensively reviewed elsewhere (7, 14), and thus this mini review will focus on ERα and ERβ.

Nuclear ER Signaling

In the nucleus, ligand-bound ERs interact with estrogen response elements (ERE) and function as transcription factors, regulating gene expression (1). Nuclear ER-estrogen complexes also modulate the function of other classes of transcription factors through protein-protein interactions; thus, controlling gene expression without direct binding to DNA (1). Cell type-specific recruitment of co-activators and displacement of co-repressors to DNA binding sites determine cellular response to estrogen (3, 15).

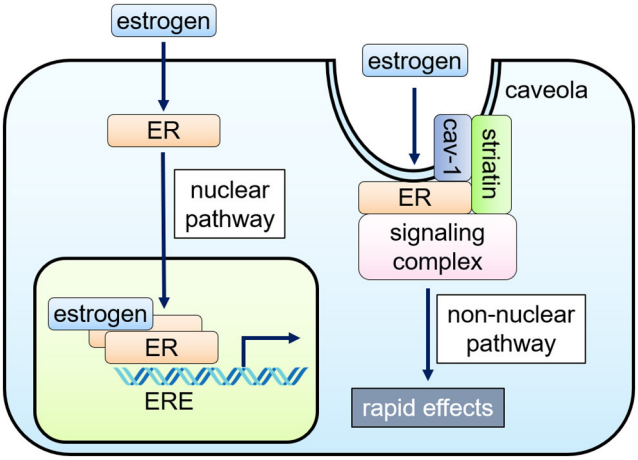

FIGURE 1 | Nuclear and non-nuclear ER signaling pathways. Classically, the hormone-bound estrogen receptor (ER) dimerizes and binds to specific DNA sequences called estrogen response elements (ERE) and activates gene expression (nuclear pathway). Alternatively, ERs localized to caveolae, cell membrane microdomains, can signal without nuclear translocation through inducing a subpopulation of cell membrane–associated ERs to form a signaling complex that results in rapid activation of specific kinases and eNOS in endothelial cells (non-nuclear pathway).

Non-nuclear ER Signaling

Cellular responses to estrogen occur within minutes, which are mediated via enzymatic pathways through the activation of membrane-associated ER, referred to as "rapid" or "non-nuclear" ER signaling (9, 16). Non-nuclear ER signaling has been documented in a variety of cell types *in vitro*, including oocytes, osteoblasts, osteoclasts, breast cancer cells, adipocytes, VSMC, EC, and cardiac myocytes (17–19). The rapid actions originate at the ERs located at caveolae, small invaginations of the cell membrane, activating kinases, or phosphatases to impact cell physiology (16, 20–22).

The non-nuclear ER signaling in the cardiovascular system has been most studied in EC where rapid eNOS activation by estrogen within 15–30 min was initially identified (23, 24). ERα, co-localized with caveolin-1, binds to a scaffold protein striatin, also associated with caveolin-1 (20, 25, 26). Endogenous ERβ was also detected in the EC membrane, particularly at the caveolae (27), but its associated proteins remain undetermined.

ERs-localized to caveolae activate PI3K, Akt kinase, and ERK1/2, enhancing phosphorylation of Ser-1177 of eNOS (22, 28–30). The activation of PI3K involves a complex process. ERα directly binds to the p85α regulatory subunit of PI3K in a ligand-dependent fashion (30), while PI3K activation requires c-Src kinase, whose SH2 domain interacts with phosphorylated Tyr-537 of ERα (31, 32). Gαi is also involved in this ERα complex at the caveolae, and the physical association of ERα with Gαi is required for eNOS activation (33, 34). On the other hand, striatin

serves as a scaffold protein of the ERα complex at the caveolae. Since interruption of ERα-striatin binding, either with a peptide representing ERα amino acids 176-253 or the ERα triple point mutation (lysine 231, arginine 233, and arginine 234 to alanine: KRR), leads to defective non-nuclear signaling without affecting nuclear signaling, striatin also plays a key role to the non-nuclear signaling of ERα (25, 35, 36).

Estrogen-Independent ER Signaling

Interestingly, unliganded ERs or growth factor signaling-mediated ERs play a role in cellular physiology in the absence of estrogen. The presence of unliganded ERα alone decreases EC migration and EC proliferation, and increases SMC proliferation (37). Unliganded ERα also regulates gene expression of ECs. These effects either on physiology or gene expression are reversed by ERα activation in the presence of E2 (37). ERs can be activated in the absence of estrogen by growth factor receptor signaling such as epidermal growth factor and insulin-like growth factor receptors (38), and such estrogen-independent ER activation has been reportedly induced by different intercellular pathways in vascular as well as non-vascular cells (3, 16). Thus, cardioprotective effects of estrogen might be in part attributable to preventing the adverse cardiovascular effects of unliganded ERα by its binding to the receptor and other growth factor signal-mediated ERα activation.

ACTIONS OF ESTROGEN IN CARDIOVASCULAR CELLS

Functional ERs are expressed in multiple cell types which compose the heart. Estrogen regulates diverse cellular functions via nuclear and non-nuclear signaling pathways or ligand-independent signaling pathways; estrogen exerts inhibition of cellular hypertrophy and apoptosis in cardiomyocytes, proliferation of cardiac fibroblasts, proliferation and eNOS signaling activation in vascular endothelium, and anti-proliferative effect on vascular smooth muscle cells. In this section, we review cell-type specific *in vitro* studies, including cardiomyocytes, cardiac fibroblasts, vascular endothelial, and smooth muscle cells.

Cardiomyocytes

Cardiomyocytes express functional ERs, and estrogen regulates expression of cardiac genes, such as connexin 43, β-myosin heavy chain and ion channels (39, 40). Estrogen also regulates calcineurin abundance, cGMP-PKG activation, Akt activation, and miRNAs in cardiomyocytes to inhibit cellular hypertrophy and confer protection against apoptosis, where both nuclear and non-nuclear pathways might be involved (**Figure 2**). Sasaki et al. demonstrated rapid activation of cGMP-PKG in response to estrogen in cardiac myocytes, providing the link between estrogen and cGMP-PKG signaling, both of which are known to play protective roles against diverse cardiac pathologies, including cardiac hypertrophy, failure, and ischemic injuries (41). While cGMP-PKG inhibits activation of calcineurin, a central molecule for pathological hypertrophy, estrogen also enhances the degradation of calcineurin. Estrogen activates Akt and inhibits apoptosis via rapid signaling, while it down-regulates miR22 to activate Sp1 for anti-oxidant induction (42–44). Furthermore, Lin et al. reported that estrogen and ERβ-specific agonist diarylpropionitrile increased S-nitrosylation of heat shock proteins and decreased infarct area after ischemia-reperfusion in isolated mice hearts, while these effects were abolished in ERβ knockout mice or with a NOS inhibitor (45).

Cardiac Fibroblasts

Cardiac fibroblasts are the primary source of myocardial extracellular matrix proteins, matrix metalloproteinases (MMPs), growth factors, and cytokines, all of which contribute to cardiac remodeling on physiological and pathological levels (46). Lee and Eghbali-Webb reported that both ERα and β are expressed in fibroblasts isolated from female rats, where ERβ is predominant with both cytosolic and nuclear localization (47). They also reported that 17 β-estradiol enhances cardiac fibroblast proliferation through mitogen-activated protein kinase-dependent mechanisms (47).

Vascular Endothelial Cells

NO derived from eNOS activation exerts potent cardiovascular effects, including vascular relaxation, growth and migration of ECs and VSMCs, and the antagonism of platelet activation, thrombus formation, and leukocyte-EC adhesion (27, 29, 48–50). Estradiol conjugated to bovine serum albumin (E2-BSA), which does not pass through the cell membrane, was originally used to evaluate the role of cell membrane ER (51), and shown to promote phosphorylation of eNOS in cultured EC. Consistently, an estrogen-dendrimer conjugate (EDC) that activates membrane-associated ER, but is excluded from the nucleus (52), was shown to stimulate cultured EC proliferation and migration via ERα, heterotrimeric G protein Gi, and the activation of eNOS (53) (**Figure 2**).

Recent transcriptome analysis by Lu et al. using EC expressing KRR mutant ER revealed that estrogen regulates 60 genes in ECs expressing wild-type ERα, many of which were associated with cell migration and proliferation identified by pathway analysis. Meanwhile, only 10 genes were regulated by E2 in ECs expressing KRR ERα and thus lacking non-nuclear signaling pathway. These data provide the evidence that non-nuclear signaling also plays a pivotal role for the transcriptional responses of EC to estrogen (35).

Vascular Smooth Muscle Cells

Estrogen exerts anti-proliferative effects on VSMC (54–56). Cell proliferation is considerably regulated by kinase-mediated signal transduction. Treatment with estrogen inhibits phosphorylation of growth-related kinases, such as ERK1/2, JNK, p38, and Akt, which are phosphorylated and activated by growth factor stimulation (54, 55). Estrogen controls the expression and activity of several phosphatases in VSMC (57, 58), including MKP-1, SHP-1, PTEN, and PP2A, balancing the kinases (56, 59, 60). In cultured VSMCs, ERα and PP2A form a complex, and estrogen treatment increases PP2A activity resulting in the inhibition of growth-promoting signal activation (61). The role of non-nuclear ER signaling in VSMC proliferation was evaluated with using

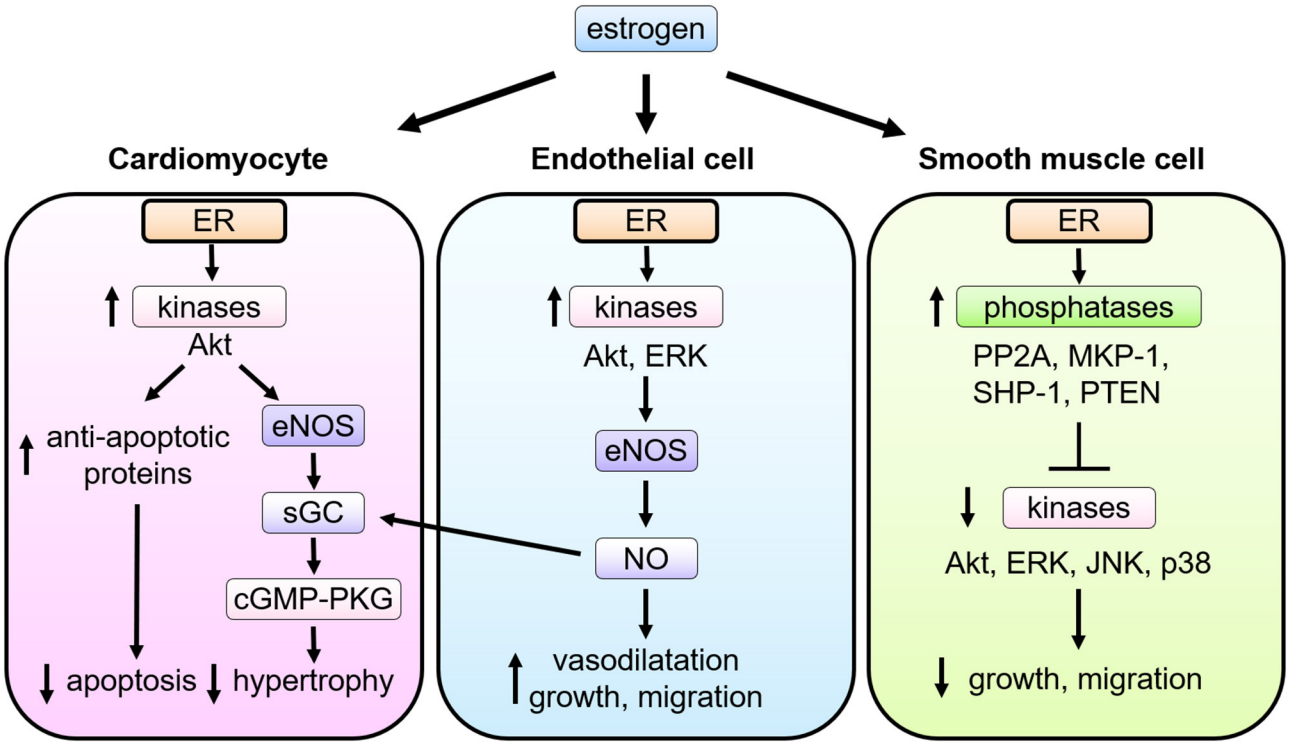

FIGURE 2 | Multiple actions of estrogen-estrogen receptor signaling in cardiovascular cells. In cardiomyocytes, ER signaling leads to the phospho-activation of Akt and eNOS, the latter stimulating soluble guanylate cyclase (sGC) to activate cyclic guanosine monophosphate (cGMP)—PKG (cGMP-dependent protein kinase) signaling pathways and thereby attenuate pathological cardiac hypertrophy. In endothelial cells (EC), upon estrogen binding, ER signaling complex activate the tyrosine kinase Src, the serine/threonine kinase PI3K, and the kinase Akt. Akt then directly phosphorylates eNOS on serine 1177, leading to its enzymatic activation and production of NO, which promotes EC proliferation and migration as well as vasodilatation. NO produced in EC also functions as a stimulator of sGC in cardiomyocytes. In smooth muscle cells (VSMC), upon stimulation of estrogen, ER interacts with and activates several phosphatases including protein phosphatase 2A (PP2A), mitogen-activated protein kinase phosphatase 1 (MKP-1), Src homology region 2 domain-containing phosphatase-1 (SHP-1), and phosphatase and tensin homolog deleted from chromosome 10 (PTEN), and modulates activation of kinases induced by growth stimulation, leading to inhibition of VSMC proliferation and migration.

a transgenic mouse model (Disrupting Peptide Mouse; DPM), in which non-nuclear ER-mediated signaling was abolished by overexpression of the peptide representing amino acids 176–253 of ERα, preventing ER from forming a signaling complex with striatin (62). Estrogen inhibition on VSMC proliferation was lost in VSMC derived from the DPM (61), supporting the theory that the non-nuclear ER signaling pathway is required for the estrogen-mediated anti-proliferative effects in VSMC (**Figure 2**).

ESTROGEN ACTIONS IN ANIMAL MODELS OF CARDIOVASCULAR DISEASES

To date, many studies using experimental models of CVD have been reported to elucidate the role of estrogen in the cardiovascular system. Most studies utilized pharmacological interventions or global gene deletion, whereas studies employing tissue-specific mutant models would provide more insights into our understanding the role of estrogen in CVD.

Ischemic Heart Diseases

ERα and ERβ are both reportedly involved in the cardioprotective effects of estrogen. Global ERβ KO mice revealed increased mortality and exacerbated heart failure after myocardial infarction (63). Consistently, cardiomyocyte-specific ERβ overexpression improved cardiac function and survival after myocardial infarction induced by left anterior descending coronary artery ligation. On the other hand, cardiac fibrosis post-myocardial infarction was attenuated with increased angiogenesis in female mice overexpressing ERα (64, 65).

In an ischemia-reperfusion model of ovariectomized global ERα KO mice, coronary endothelial dysfunction was not reversed by estrogen, while it was normalized in wild types (66). Other studies further revealed ERα KO demonstrated exacerbated ischemia reperfusion phenotype, including markedly impaired cardiac contractility, increased cardiomyocyte death, and mitochondrial damage (67, 68). On the other hand, ERβ KO female hearts exhibited poor functional recovery compared to wild-type or ERα KO mice in an *ex vivo* model of global ischemia-reperfusion (69). Mechanistically, estrogen mitigates reperfusion injuries after ischemia primarily by activating PI3K-Akt, increasing expression of the anti-apoptotic protein BCL-2 and decreasing the expression of the proapoptotic caspase proteins (43). Importantly, neither ischemic recovery nor PI3K-Akt activation was observed in hearts isolated from female

ERβ KO mice (43, 70, 71). Together, ERβ appears to play substantial cardioprotective roles against ischemia reperfusion injury, while the role of ERα seems to differ depending on methodological conditions.

Estrogen might contribute to regenerative process in the heart. Cardiac stem cells treated with estrogen produce protective factors and improve cardiac function and cardiomyocyte survival when injected into hearts after ischemia-reperfusion injury (72). Both ERα and β contribute to estrogen-mediated endothelial progenitor cell activation and mobilization in tissues, helping preserve cardiac function after myocardial infarction (73). ERα selective agonist propylpyrazoletriol reduced apoptosis and increased survival of cardiac cells expressing c-Kit, a potential marker for adult cardiac stem cells, in infarcted hearts; whereas the ERβ selective agonist diarylpropionitrile had no effect (74).

Cardiac Hypertrophy and Failure

The heart develops pathological hypertrophy in response to various pathological stressors, such as genetic, mechanical, or excessive neurohormonal stress. If the stress is sustained, hypertrophy transits to failure. Sex difference has been known to be a modifier of human cardiomyopathy where estrogen also plays a role (75). Genetically-modified mouse models of hypertrophic cardiomyopathy presented sex differences of cardiac phenotype. Mice with a missense mutation (R403Q) in the α-myosin heavy chain exhibited severe biventricular hypertrophy. At 8 months of age, only male mice displayed overt heart failure phenotype (76, 77). Also, transgenic mice with a missense mutation (R92Q) in the cardiac troponin T exhibited more severe fibrosis with marked hypertrophic marker gene induction in males, compared to females (78). Importantly, ovariectomized mutants showed worse phenotype of further impaired contractile function and myocardial energy metabolism, and estrogen supplementation restored these parameters (79), suggesting estrogen's protective effects against cardiac hypertrophy and heart failure in this model.

Studies using global deletion of ERα or ERβ exposed to chronic angiotensin II or pressure-overload have suggested the role for ERβ in the estrogen's protection against hypertrophy and failure, where calcineurin and cGMP-PKG signaling regulation might be significantly involved (80). In particular, the link of estrogen to the latter signaling pathway might be important and deserves further investigation. Myocardial cGMP-PKG signaling pathway is de-activated in human heart failure with preserved ejection fraction (HFpEF), while cGMP-PKG activation is regarded as a new therapeutic strategy to treating heart failure. Considering HFpEF is associated with female sex and increased age independent of obesity and diabetes, it is reasonable to speculate estrogen loss and subsequent cGMP deactivation might contribute to the pathophysiology of HFpEF. Importantly, Sasaki et al. reported that estrogen signal is required in order to activate cGMP-PKG using a PDE5 inhibitor in female cardiac myocytes to ameliorate heart failure in female mice (41, 81, 82). There are several other reported molecular mechanisms including a mammalian target of rapamycin signaling, regulation of phosphorylation of p38 mitogen-activated protein kinase pathway, and regulation of cardiomyocyte histone deacetylases (83–85).

Vascular Injury and Atherosclerosis

Estrogen inhibits excessive responses to vascular injury in a mouse carotid artery injury model. Here, *in vivo* estrogen treatment inhibits the proliferation of VSMC and promotes re-endothelialization (53, 86–92). In ERβ KO mice, estrogen is still protective against vascular injury (86, 93), whereas, in ERα KO mice, estrogen treatment shows no protective effect on the vascular injury response (86, 90). This supports the concept that ERα is responsible for estrogen's protective effect on vasculature.

The role of the non-nuclear ER signaling pathway in estrogen-induced vascular protection was evaluated in a "gain of function" and "loss of function" study. EDC promoted carotid artery re-endothelialization in an ERα-dependent manner and diminished the development of neointimal hyperplasia following vascular injury in a manner equivalent to estrogen treatment (53). Of note, endometrial carcinoma cell growth *in vitro* and breast cancer xenograft growth *in vivo* were stimulated by estrogen, but not EDC (53). This suggests that selective activation of the non-nuclear signaling pathway did not promote cancer growth. Moreover, estrogen significantly decreases injury-induced neointimal hyperplasia while inhibiting VSMC proliferation in wild-type mice. Strikingly, estrogen had no significant effect in DPM mice (62). Taken together, non-nuclear signaling plays a major role in estrogen-induced protection against vascular injury, and compounds that activate non-nuclear signaling might provide a vascular benefit as non-nuclear selective ER modulators without increasing the risk of uterine or breast cancer.

Ligand-bound ERα mediates target gene transcription through the activation function 2 (AF2) domain, located on the C-terminal, and ligand binding domain of ERα. A recent report that used knock-in mice that lacked a functional AF2 domain showed that AF2 is needed to inhibit atherosclerosis (94). In contrast, the effect of estrogen on re-endothelialization after vascular injury was preserved in these mice (94), suggesting an essential role of the non-nuclear ER signaling on endothelial healing after vascular injury.

CONCLUSION

Large-scale, randomized clinical studies from the early 2000s failed to demonstrate cardiovascular benefits associated with post-menopausal estrogen treatment, and rather showed an increased risk of breast cancer and thrombosis. However, recent clinical trials and meta-analyses have suggested that estrogen treatment has beneficial effects for preventing CVD and does not affect mortality if HT is initiated early after menopause (6, 11). In addition to the timing of HT initiation, various factors including treatment duration, dose, formulation, regimen (estrogen or estrogen plus progesterone), and route of administration may affect cardiovascular outcomes. Greater clarity of the molecular mechanisms by which estrogen regulates specific cardiovascular processes is needed to optimize next-generation HT.

Various ERs are expressed in most cardiovascular cell types, including cardiomyocytes, fibroblasts, vascular EC, and VSMC. Each ER is involved in protective effects of estrogen in multiple animal disease models, including ischemic heart disease, cardiac hypertrophy, heart failure, vascular injury, and atherosclerosis. Emerging evidence has indicated the potential importance of the non-nuclear ER signaling on diverse aspects of the cardiovascular systems, supporting the potential opportunity to design pathway-specific selective ER modulators capable of regulating non-nuclear and nuclear effects, assisting with the development of personalized therapies for preventing and treating CVD. Further research will provide more insight into therapeutic approaches that translate basic science findings into clinical practice innovations.

AUTHOR CONTRIBUTIONS

KU and ET wrote the manuscript. YA, PL, and NF critically revised the manuscript and contributed to designing the figures.

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REVIEW
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Female Heart Health: Is GPER the Missing Link?

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The G Protein-Coupled Estrogen Receptor (GPER) is a novel membrane-bound receptor that mediates non-genomic actions of the primary female sex hormone 17β-estradiol. Studies over the past two decades have elucidated the beneficial actions of this receptor in a number of cardiometabolic diseases. This review will focus specifically on the cardiac actions of GPER, since this receptor is expressed in cardiomyocytes as well as other cells within the heart and most likely contributes to estrogen-induced cardioprotection. Studies outlining the impact of GPER on diastolic function, mitochondrial function, left ventricular stiffness, calcium dynamics, cardiac inflammation, and aortic distensibility are discussed. In addition, recent data using genetic mouse models with global or cardiomyocyte-specific GPER gene deletion are highlighted. Since estrogen loss due to menopause in combination with chronological aging contributes to unique aspects of cardiac dysfunction in women, this receptor may provide novel therapeutic effects. While clinical studies are still required to fully understand the potential for pharmacological targeting of this receptor in postmenopausal women, this review will summarize the evidence gathered thus far on its likely beneficial effects.

Keywords: diastolic dysfunction, estrogen, heart failure with preserved ejection fraction, calcium homeostasis, chymase, inflammation, oxidative stress, LV remodeling

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INTRODUCTION

Among measures of cardiac function, diastolic performance is one of the most comprehensive—integrating myocardial relaxation, mitochondrial bioenergetics, cardiomyocyte/myocardial structure, and left ventricular (LV) ejection with respect to proximal aortic distensibility—and is a potential barometer of cardiac health (1). LV diastolic function is impaired by all of the common pathological processes that affect LV function or produce LV hypertrophy or fibrosis, including hypertension, diabetes mellitus, obesity, sleep apnea, ischemia, aortic stenosis, and can occur before development of symptoms or changes in electrocardiogram and wall motion (2). The heart is designed to be a supple, elastic muscle that fills with blood easily at low pressure. Diastolic dysfunction with elevated filling pressures is a central feature of heart failure with preserved ejection fraction (HFpEF) (3) and disproportionally affects women with a sex ratio of about 2:1 (4–6). HFpEF is the most common form of heart failure (3, 7) and is outpacing other forms of heart failure as a result of the expanding elderly population (7–9).

Despite a marked female sex-specific predilection in HFpEF, relatively little is known regarding the mechanisms by which sex hormones, particularly the estrogens, and estrogen receptors (ERs) impact diastolic function. Over the last decade, we and others have explored the roles of the newest estrogen receptor, G protein-coupled estrogen receptor (GPER; previously known as GPR30), in the maintenance of cardiac function and structure after estrogen loss. In this review, the effects of pharmacologic activation of GPER by its specific agonist G1 on mitigating the adverse consequences of estrogen loss on relaxation, mitochondrial function, LV stiffness, and aortic distensibility will be presented. The influence of global and cardiomyocyte-specific GPER gene deletion on function and structure at the cardiomyocyte, whole heart, and conduit vessel levels will also be discussed.

WHAT IS DIASTOLIC DYSFUNCTION?

Diastolic dysfunction denotes a condition whereby the LV cannot fill adequately despite normal filling pressure. Slowing, delayed, and incomplete myocardial relaxation results from alterations in intracellular calcium handling, impairments in energy metabolism, and increases in LV stiffness due to hypertrophic and/or interstitial remodeling. Elevations in LV filling pressure initially compensate, but eventually pulmonary congestion develops as a result of increased left atrial (LA) pressure (10). While a wide range of diastolic function parameters can be obtained by Doppler-echocardiography (11), a simple composite of blood flow and tissue Doppler measures, as reviewed by ourselves and others (12–14) can sensitively detect and predict diastolic dysfunction in humans (15–17), non-human primates (18, 19), and preclinical rodent research models (20–25). The spectrum of diastolic dysfunction is portrayed schematically in **Figure 1**. Essentially, as described in detail by Nagueh and colleagues in the American Society of Echocardiography and European Association of Cardiovascular Imaging Guideline (11), the healthy adult LV fills primarily during the early filling phase of diastole, defined by transmitral Doppler E wave velocity, followed by a small contribution from atrial systole, defined by the late or transmitral A wave. Normally, E is equal to or greater than A. In addition, the longitudinal and radial myocardial fibers adjacent to the mitral annulus elongate and "twist" during early filling, creating a "suction-like" effect that helps propel blood into the LV. This motion of the mitral annulus during diastole is measured using tissue Doppler, and is termed e′. With increasing age (>50 years), in the initial stages of hypertension, and even in asymptomatic ischemia, early filling is slowed, delayed, or impaired and atrial contraction increases to partly compensate and augment ventricular volume. In this scenario, E wave velocity is less than the A wave velocity (e.g., E < A). In addition to the changes in filling dynamics, myocardial relaxation, assessed as e′, is reduced. With progressive worsening of diastolic dysfunction, LA size and pressure increase. Because the LA functions as a reservoir to help maintain an appropriate atrioventricular pressure gradient during diastole, this increase in LA pressure that occurs with progressive deterioration of diastolic function helps "load" blood into the non-compliant LV. In so doing, the transmitral flow velocity profile may appear normal (e.g., E > A); however, given that the mitral annular motion, or e′, remains reduced, the mitral inflow velocity profile represents a "pseudonormal" pattern, indicative of increased severity of diastolic dysfunction. Normally, the LV produces suction in order to fill while in the presence of advanced diastolic dysfunction, the left atrium produces loading in order to compensate and achieve adequate filling.

WHAT EVIDENCE SUPPORTS A ROLE FOR ESTROGEN IN THE MAINTENANCE OF DIASTOLIC FUNCTION?

The increased prevalence of HFpEF in older women compared with men of the same age appears related to the loss ovarian hormones, and primarily estrogens, that occur during menopause (7, 26). Epidemiologic evidence further suggests that premature or early natural menopause (27–29) and a shorter total reproductive duration positively associate with incident heart failure (30). Hall et al. (30) showed that the incidence of HFpEF was higher in postmenopausal women who were nulliparous, further suggesting a role of endogenous estrogens in the pathogenesis of the disease process. Importantly, diastolic dysfunction, the harbinger of HFpEF, was recently described as part of the "postmenopausal syndrome" (31). When compared with premenopausal women, postmenopausal women exhibit a higher prevalence of LV filling abnormalities. Moreover, when older women are compared with their age-matched male counterparts, the likelihood of manifesting more prominent diastolic dysfunction is increased (32–34). Findings from small clinical (35–39) and animal studies, as reviewed by us (40) and others (31, 41), document estrogen therapy efficacy in improving diastolic function and/or limiting increases in LV mass and interstitial remodeling after surgically induced or natural menopause. These data affirm estrogen's role in the preservation of diastolic function in the female heart.

Abbreviations: ACE, angiotensin-converting enzyme; ANF, atrial natriuretic factor; Ang I, angiotensin I; Ang II, angiotensin II; Ang-(1-12), angiotensin-(1-12); β-AR, beta-adrenergic receptor; BNP, brain natriuretic peptide; Ca^{2+}, calcium; CaMKII, calcium/calmodulin-dependent protein kinase II; CICR, calcium-induced calcium release; E_2, estradiol; EKG, electrocardiogram; eNOS, endothelial nitric oxide synthase; ER, Estrogen receptor; ERα, Estrogen receptor subtype α; ERβ, Estrogen receptor subtype β; ET-1, endothelin-1; FBS, fetal bovine serum; GPER, G-protein-coupled estrogen receptor; GPR30, G-protein-coupled receptor; HFpEF, heart failure with preserved ejection fraction; HTN, hypertension; $I_{Ca,L}$, L-type calcium channels $Ca_V1.2$; i.p., intraperitoneal; IL, interleukin; ISO, isoproterenol; KO, knockout; LA, left atrium; LAP, left atrial pressure; LV, left ventricular; MCT, monocrotaline; MnSOD, manganese superoxide dismutase; mtDNA, mitochondrial DNA; NCX, sodium/calcium exchanger; NOX4, NADPH oxidases; OVX, ovariectomy; PAH, pulmonary arterial hypertension; PKA, protein kinase A; PLB, phospholamban; PMCA, plasma membrane calcium ATPase; pPLB, phosphorylated phospholamban; RAS, renin angiotensin system; ROS, reactive oxygen species; RV, right ventricular; RVT, right ventricular free wall thickness; RyR, ryanodine receptor; s.c., subcutaneous; SD, Sprague Dawley; SLN, sarcolipin; SR, sarcoplasmic reticulum; SERCA2a, sarco/endoplasmic reticulum Ca^{2+}ATPase 2a.

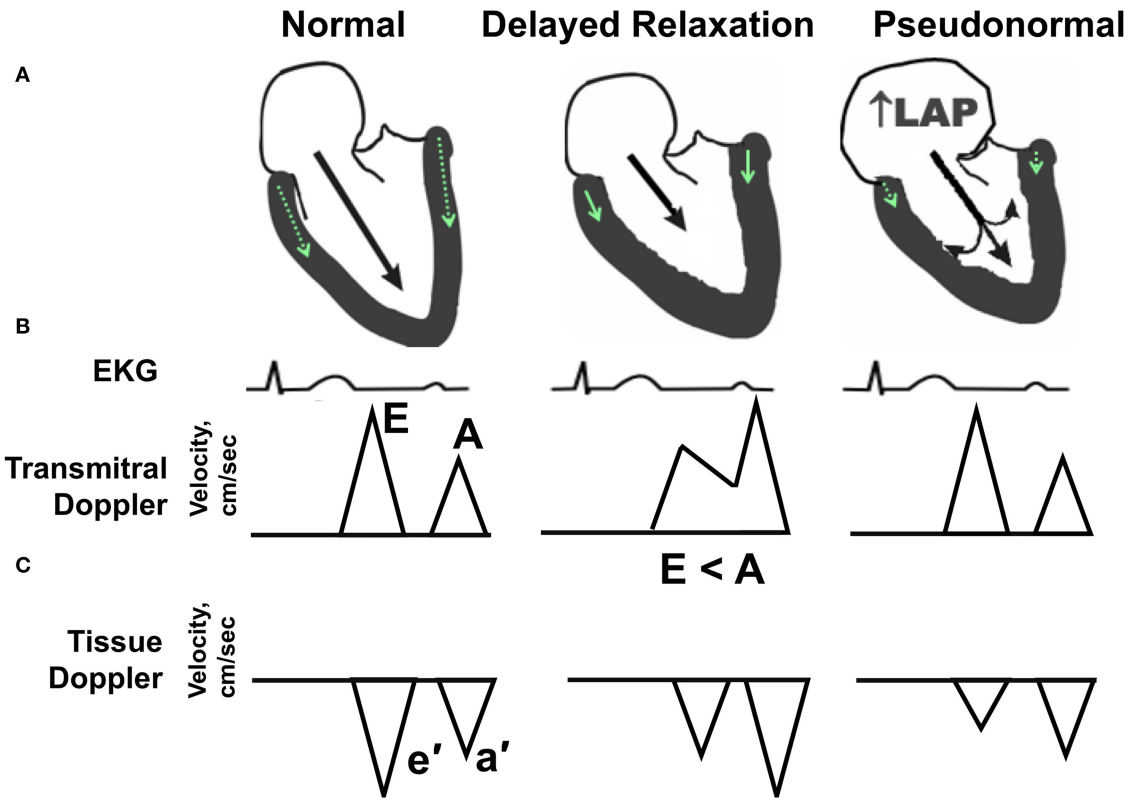

FIGURE 1 | Echocardiographic hallmarks in the spectrum of diastolic dysfunction. **(A)** Schematic long-axis, sagittal view of the left atrium and left ventricle showing transmitral Doppler filling (black arrow) and septal and lateral mitral annular motion (small green arrow within LV wall) during early diastole. **(B)** Graphic representation of early and late transmitral Doppler-derived wave patterns in relation to the electrocardiogram (EKG). **(C)** Graphic representation of early and late tissue Doppler-derived mitral annular wave patterns. (Left) Normal diastolic function. The majority (80%) of left ventricular (LV) filling occurs during the early phase of diastole, as depicted by a relatively long black arrow extending from the mitral leaflets into the LV apex. The longer the arrow, the higher the relative velocity of early filling, or E wave, compared with late filling (A wave). Normally, E velocity is equal to or greater than A velocity. (Middle) Impaired relaxation or stage I diastolic dysfunction. With aging, mild hypertension or pressure overload, and/or ischemia, early filling (E wave) is impaired or reduced as depicted by a shorter extension of the black arrow into the LV apex. Also, late filling (A wave) is increased, due to a more vigorous atrial contraction to partly compensate and augment ventricular volume. The ratio of early-to-late-filling velocity is <1, or E < A, in this stage of diastolic dysfunction. Also, septal and lateral mitral annular velocities (e′) are reduced when compared with "normal" (green arrow). (Right) Pseudonormal pattern or Stage II diastolic dysfunction. With progressive worsening of diastolic dysfunction, LA size, and pressure increase. Because the LA functions as a reservoir to help maintain an appropriate atrioventricular pressure gradient during diastole, this increase in LA pressure (LAP) helps load blood into the non-compliant LV. With progressive worsening of diastolic dysfunction, LA size, and pressure increase. While the transmitral flow velocity profile appears normal, the mitral annular motion, or e′, remains reduced. In this situation, the mitral inflow profile is termed "pseudonormal".

ESTROGEN RECEPTORS IN THE HEART

Estrogen mediates its actions on the heart through three identified ERs. Estrogen receptor subtypes α (ERα) and β (ERβ) are classical nuclear hormone receptors, which bind estrogen and translocate to the nucleus to regulate target gene expression. However, molecular signaling is also induced by estrogen outside of the nucleus. While some intracellular signaling may be initiated by truncated forms of the steroid ERs (42), a membrane-bound ER distinct from ERα and ERβ was identified as the orphan receptor GPR30 before being renamed GPER (43, 44). GPER binds estradiol (E_2) at a similar nanomolar affinity as ERα and ERβ and exerts comparable actions on calcium mobilization and phosphoinositide 3-kinase activation (45). ERα and GPER are expressed at similar levels in cardiac tissue from male and female rodents (46) as well as from humans (47). In contrast, reports of ERβ expression in the heart are conflicting, with ERβ mRNA detected in human cardiac tissue (48) but remaining below detectable levels in rodent cardiac tissue (46, 49). Based on observed improvement in cardiac function in response to E_2 treatment in postmenopausal women (50) and in ovariectomized (OVX) rats (21), researchers have attempted to identify the primary receptor mediating estrogen's cardioprotective effects. Despite the inability to detect ERβ mRNA in some rodent models, administration of a novel ERβ agonist (βLGND2) attenuates angiotensin II-induced cardiac fibrosis (51) and genetic deletion of ERβ removes female sex-based cardioprotection in a model of pressure overload (52). These studies indicate that ERβ may be upregulated in the heart during disease or impact cardiac function through infiltrating cells rather than in cardiomyocytes. While studies using selective ERα and ERβ agonists indicate that both receptors induce cardioprotection (53), direct comparisons

of genetic ERα and ERβ knockout (KO) mice indicate a dominant role for ERβ (54). However, assessing the double ER KO mouse in addition to each receptor KO individually showed no differences in infarct size, suggesting physiological redundancy or compensation (55).

GPER is expressed on the plasma and intracellular membranes of cardiac cells, including cardiomyocytes, cardiac fibroblasts, mast cells, and endothelial cells (56–59). To clarify the roles of GPER in the heart, pharmacologic approaches using the selective agonist G1 and antagonists G15 or G36 are commonly used. In **Table 1**, we summarize the ability of relevant hormones, natural estrogens, and drug molecules to bind to and activate signaling through GPER and ERα/β. G1 is the most commonly used tool for studying GPER. This non-steroidal, high-affinity ($K_d = 11$ nM) and highly selective GPER agonist was developed from a library of 10,000 molecules and does not activate the classical estrogen receptors at concentrations up to 10 μM (63). G15 and G36 are antagonists of GPER with low affinity binding to the classical estrogen receptors (64). While the exact signaling actions and transduction pathways of cardiac GPER are not completely understood, they are likely dependent on the cell type, site of action and the relative levels in comparison with the other estrogen receptors (46). The selective GPER agonist G1 modulates fast transduction pathways in the heart that are involved in (1) controlling intracellular calcium via actions on cardiac channels and pumps, (2) regulating phosphoinositide 3-kinase (PI3Ks) and extracellular signal-related kinases (ERKs), and (3) modulating cyclic adenosine monophosphate (cAMP) (see sections Effects of Estrogen and GPER Activation on $I_{Ca,L}$ and Estrogen, GPER, and SERCA2a and Its Regulatory Proteins below). The rapid signaling events following GPER activation also lead to inhibition of the expression of cell cycle genes, such as cyclin B1 and CDK1, which are involved in cardiac fibroblast and mast cell proliferation and contribute to interstitial remodeling (see sections GPER Inhibits Interstitial Remodeling and GPER and Cardiac Chymase/Ang II below). Moreover, GPER activation by G1 reduces remodeling promoted by hypertrophic regulators, including angiotensin II and endothelin-1, via inhibition of 1/2 ERK signaling and upregulation of PI3K/Akt/mTOR pathways (see section GPER and Anti-hypertrophic Remodeling below).

Since only a limited number of studies have explored the actions of GPER in diastolic function, future studies are needed to deepen our understanding of its effects in the various cardiac cell populations. Elucidation of these cell-type specific signaling mechanisms will help to clarify the therapeutic potential of cardiac GPER activation in preventing and/or halting the progression of cardiac diseases that involve diastolic dysfunction. Herein, where data exists, we include the signaling pathways of an activated GPER that are linked to the physiologic underpinnings of diastolic function preservation in the context of estrogen deprivation (**Figure 2**). Initial work showed that administration of the GPER agonist G1 prevents diastolic dysfunction and LV remodeling in OVX (25) and salt-loaded (65) mRen2.Lewis rats. To tease out more precise information about the functional role of GPER in the heart, we generated a novel conditional mouse model where GPER was specifically deleted in cardiomyocytes (23). Therefore, the remainder of the review will examine the evidence that links GPER to the preservation of myocardial relaxation and LV structure in the female heart after estrogen loss during hypertension, heart failure, and normal aging.

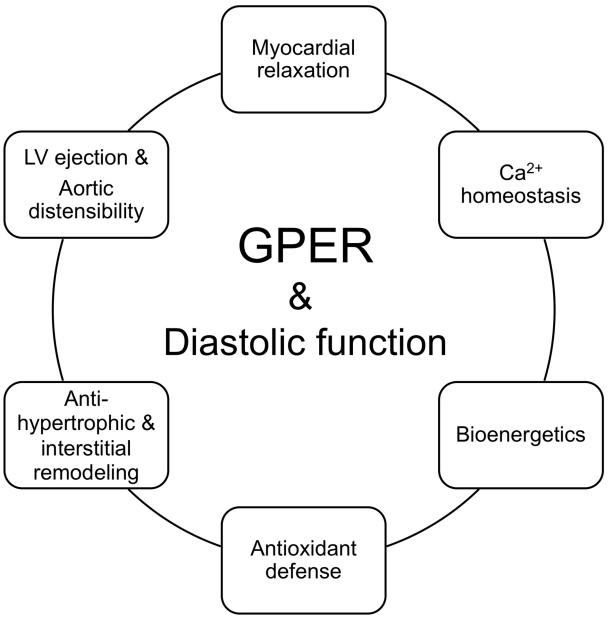

FIGURE 2 | GPER in the functional circle of diastology of the female heart. Diastolic function is an appropriate barometer of overall heart health as it reflects cellular and subcellular events responsible for maintaining myocardial relaxation, cardiomyocyte calcium homeostasis, mitochondrial bioenergetics, antioxidant defense, left ventricular (LV) and myocyte structure, and proximal aortic distensibility. Loss of ovarian estrogens due to aging/menopause or surgery lead to impairments in myocardial/cardiomyocyte relaxation, increased mitochondrial reactive oxygen species and impaired oxidant defenses and bioenergetics, hypertrophic and/or interstitial remodeling, and aortic stiffening. Preclinical studies show that activation of the non-canonical estrogen receptor, GPER, by its agonist G1 or estradiol (E_2) favorably regulates and likely integrates components of relaxation, mitochondrial function, LV structure, LV ejection, and aortic compliance, to preserve diastolic function in the female heart.

GPER AND MYOCARDIAL/CARDIOMYOCYTE RELAXATION

Overview of Cardiac Ca^{2+} Machinery Involved in Contraction and Relaxation

Maintaining cardiomyocyte calcium concentration $[Ca^{2+}]_i$ within a tightly controlled range is critical for normal systolic and diastolic function. In adult cardiomyocytes, the L-type Ca^{2+} channel is the main pathway for Ca^{2+} influx. The Na/Ca^{2+} exchanger is quantitatively the most important pathway for Ca^{2+} efflux out of the cardiomyocyte. The sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps and the sarcoplasmic reticulum (SR) Ca^{2+} release channels (ryanodine receptors) are pivotal in determining $[Ca^{2+}]_i$ and subsequent contraction and relaxation.

TABLE 1 | Ligands of GPER and ERs (60).

	DPN	PPT	17β-Estradiol	Genistein BPA Nonylphenol DDT	Tamoxifen Raloxifene	ICI182,780	G-1	G15 G36	Quercetin (61, 62)
ERα/β	++	++	++	++	+	–	n.d	n.d.	+
GPER	n.d.	+?	++	++	++	++	++	––	++

DPN, diaryl propionitrile; PPT, propylpyrazoletriol; BPA, bisphenol A; DDT, dichlorodiphenyltrichloroethane. Plus (+) symbol denotes weak agonist; Double plus (++) symbol denotes strong agonist; Minus (–) symbol denotes weak antagonist; Double minus (– –) symbol denotes strong antagonist; n.d., no reported data. Question mark (?) denotes interpret with care until confirmed by additional approaches.

At the initiation of myocardial contraction, depolarization of the cardiomyocyte leads to activation of the inward Ca^{2+} current conducted via L-type Ca^{2+} channels $Ca_v1.2$ ($I_{Ca,L}$). This is the main trigger for Ca^{2+} release from the sarcoplasmic reticulum, termed Ca^{2+}-induced Ca^{2+} release (CICR). CICR involves ryanodine receptors (RyR), of which RyR2 is the predominant myocardial isoform. $I_{Ca,L}$ and CICR together increase the concentration of intracellular Ca^{2+} that binds to troponin C on myofilaments to initiate myocardial contraction.

Myocardial relaxation begins when ATP hydrolyzes and actin-myosin cross-bridges unlink. Removal of cytoplasmic Ca^{2+} and subsequent dissociation of Ca^{2+} from troponin is required for myocardial relaxation. This involves multiple components, including Ca^{2+} reuptake into the sarcoplasmic reticulum via SERCA2a (responsible for 70% cytoplasmic Ca^{2+} removal in humans) and activation of the sarcolemmal Na^+/Ca^{2+} exchanger (NCX, 28%) and, to a lesser extent, plasma membrane Ca^{2+}-ATPase (PMCA, 2%) (66). $I_{Ca,L}$ also contributes to the filling status of the SR and myocardial relaxation. Other important processes allowing myocardial relaxation and diastolic ventricular filling include deactivation of the thin myofilaments, modulated by troponin and tropomyosin, and cross-bridge cycling, as recently reviewed (67).

We will briefly discuss the effects of estrogen on components of myocardial Ca^{2+} signaling, with a focus on mechanisms that regulate myocardial relaxation and the known role of GPER. A summary of the proteins/messengers examined in this context is provided in **Table 2**.

Effects of Estrogen and GPER Activation on $I_{Ca,L}$

While being a key contributor to systolic Ca^{2+} increase, $I_{Ca,L}$ is also a main source for refilling SR Ca^{2+} in smooth muscle (78), and directly regulates diastolic Ca^{2+} level in ventricular myocytes (79). Thus, it significantly affects myocardial relaxation. Overall, there is abundant evidence that estrogen inhibits $I_{Ca,L}$ via $Ca_v1.2$ in cardiomyocytes.

The involvement of GPER in controlling myocardial contraction by mediating the inhibitory effects of E_2 on $I_{Ca,L}$ can now be deduced from observations made even before GPER was recognized as an ER. The negative inotropic effect of E_2 was reported in the early 1990s. Indeed, E_2 causes a decrease in cell shortening associated with reduced action potential duration; in patch-clamp and fluorescent measurements, E_2 decreases the peak inward Ca^{2+} current and delays recovery of $I_{Ca,L}$ from inactivation (70). The phytoestrogen resveratrol was later shown to inhibit the amplitude of electrically stimulated Ca^{2+} transients and cell shortening in ventricular cardiomyocytes (73). In line with earlier observations (70, 73), E_2 (0.1–1 nM) reduces heart rate and pressure and cAMP production in the isolated perfused heart treated with isoproterenol; these effects are not inhibited by tamoxifen, an ERα/ERβ antagonist, and at the time were attributed to activation of an unknown membrane receptor (75). We now know that 4-OHT, a metabolite of tamoxifen, is a GPER agonist (45, 80). Later studies confirmed the inhibitory effect of E_2 on $I_{Ca,L}$ and further showed that genetic deletion of ERα or ERβ does not affect this inhibition (71). In the same studies, raloxifene, an antagonist of ERα/ERβ and agonist of GPER with a functional effective dose of 100 nM (44, 80), decreased $I_{Ca,L}$ in cardiomyocytes from wild-type, ERα KO, and ERβ KO animals. Howlett's group also observed that electrically stimulated Ca^{2+} transients are larger in ventricular myocytes from OVX mice compared with sham female mice (74). We now know that a likely explanation for these observations was that they were mediated by GPER. Initial evidence supporting a role of GPER came from Tran's group, and indicated that GPER activation using G1 (0.001–1 μM) suppresses the isoproterenol-stimulated increases in LV contraction, Ca^{2+} signals, and $I_{Ca,L}$ in intact hearts and in ventricular cardiomyocytes freshly isolated from male mice; these effects are associated with inhibition of protein kinase A (PKA)-dependent phosphorylation of $Ca_v1.2$ (69). Taken together, the reported effects of E_2 and GPER activation on cardiomyocyte $I_{Ca,L}$ and Ca^{2+} transients have been consistently inhibitory and suggest that E_2 and GPER prevent excessive cardiac contraction in response to acute stimuli.

In stressed cardiomyocytes, the picture appears to be different. The cardiac $[Ca^{2+}]_i$ regulatory systems are influenced by the activity of the sympathetic nervous system (SNS) via beta-adrenergic receptor (β-AR)-mediated, cAMP-dependent mechanisms. Estrogen alters gene expression of β-ARs and calcium-handling proteins (81). Preliminary data from Cheng's lab demonstrated that chronic *in vivo* G1 treatment restores normal myocyte basal and β-adrenergic receptor (β-AR)-mediated contraction, relaxation, and Ca^{2+} signals, leading to regression of LV dysfunction in a male mouse model of isoproterenol-induced HFpEF (68). These observations are consistent with reduced receptor sensitivity that is typically seen

TABLE 2 | Effect of E_2 and GPER on proteins/messengers involved in Ca^{2+}-dependent cardiac function.

Ca^{2+} signaling protein involved	Assay	Model/ Intervention/ Treatment	Tissue/cells examined	Effects of intervention
L-type Ca^{2+} channels ($Ca_v1.2$)	Agonist-induced Ca^{2+} signal	G1 (68) G1 (69) G36 (69)	ISO-induced HFpEF myocytes (68) Male LV myocytes (69) Male LV myocytes (69)	Restored ISO-induced Ca^{2+} transient amplitude (68) ↓ ISO-induced Ca^{2+} signals (69) ↑ ISO-induced Ca^{2+} signals (69)
	Electrically stimulated Ca^{2+} transient	E_2 (70–72) RVT (73) E_2, raloxifene (71) E_2/OVX (72) OVX (74)	Ventricular myocytes (70, 71, 73) $ER\alpha^{-/-}$, $ER\beta^{-/-}$ myocytes (71) LV apical myocytes (72) Ventricular myocytes (74)	↓ amplitude (70, 71, 73), delay recovery from inactivation (70) ↓ cell shortening (71) ↓ amplitude (71) ↑ amplitude (72) ↓ amplitude (74)
	ISO-induced cAMP Basal cAMP	E_2 (75) OVX (72) OVX/E_2 or G1 (72)	Perfused heart (75) LV apex (72) LV apex (72)	↓ cAMP (75) ↓ cAMP (72) Restored to sham level (72)
	$Ca_v1.2$ mRNA immunoblotting	OVX (72) E_2/OVX (72)	LV apical myocytes (72) LV apical myocytes (72)	↓ mRNA (72) Restored to sham level (72)
RyR2	Immunoblotting	G1/ OVX-HTN (25)	LV tissue (25)	No change (25)
	Caffeine-induced SR Ca^{2+} release	OVX (74)	Ventricular myocytes (74)	↑ SR Ca^{2+} release (74)
	$^{45}Ca^{2+}$ flux	OVX (76) E_2/OVX (76) PKA (-)/ OVX (76)	LV myocytes (76) LV myocytes (76) LV myocytes (76)	↑$^{45}Ca^{2+}$ flux (76) Restored to sham level (76) Restored to sham level (76)
SR Ca^{2+}-ATPase (SERCA)	Agonist-induced SR Ca^{2+} accumulation	G1 (24)	Saponin-skinned myocytes (24)	↑ Ca^{2+} accumulation (24)
	SR Ca^{2+} uptake	OVX (77) E_2/OVX (77) Progesterone/OVX (77)	LV tissue (77) LV tissue (77) LV tissue (77)	↓ uptake (77) Restored uptake (77) Restored uptake (77)
	Immunoblotting	OVX (76) E_2/OVX (76) G1/OVX old-aged (24)	LV myocytes (76) LV myocytes (76) LV tissue (24)	No change (76) No change (76) ↑ expression (24)
Phospholamban (PLB)	PLB mRNA PLB immunoblot	G1/OVX-HTN (25) OVX/MCT-PAH (20) OVX/G1/MCT-PAH (20)	LV tissue (25) RV tissue (20) RV tissue (20)	No change (25) ↓pPLB/PLB expression (20) Restored normal expression (20)
	Ser16 PLB phosphorylation	OVX (77)	LV tissue (77)	No change
	Thr17 PLB phosphorylation	OVX (77) E_2/OVX (77) Progesterone/OVX (77)	LV tissue (77) LV tissue (77) LV tissue (77)	↓ Thr17 phosphorylation (77) Restored Thr17 phosphorylation (77) Restored Thr17 phosphorylation (77)
Na^+/Ca^{2+} exchanger (NCX)	Na^+-dependent Ca^{2+} uptake	OVX (76) E_2/OVX (76) PKA(–)/OVX (76)	LV myocytes (76) LV myocytes (76) LV myocytes (76)	↑ Na^+-dependent Ca^{2+} uptake (76) Restored to sham level (76) Restored to sham level (76)

E2, estrogen; ER, estrogen receptor; HTN, hypertension; HFpEF, heart failure with preserved ejection fraction; ISO, isoproterenol; LV, left ventricular; MCT-PAH, monocrotaline-induced pulmonary arterial hypertension; OVX, ovariectomized; pPLB, phosphorylated phospholamban; PKA, protein kinase A; RV, right ventricular; RVT, right ventricular free wall thickness; RyR, ryanodine receptor; SR, sarcoplasmic reticulum.

in heart failure. The ability of G1 to restore these parameters suggest that chronic GPER activation re-sensitizes cardiac β-AR regulation in this HFpEF model (68). These data also mirror those reported in a study of the apical myocardium, in which expression of the $Ca_v1.2\alpha$ subunit and $I_{Ca,L}$ were lower in apical myocytes from male or OVX mice compared with sham female mice, and E_2 treatment of myocytes from OVX animals corrected these differences (72). The reduction in the amplitude of electrically stimulated Ca^{2+} transients in myocytes isolated from OVX female mice were restored by G1, to an extent similar to that achieved with E_2 treatment. Moreover, blockade of GPER with G15 reversed the benefit of E_2 while other ER antagonists had no effect. This data suggests that the protective effects of E_2 on $I_{Ca,L}$ are mediated in part through GPER in this model. Adrenergic stress-induced declines in contraction amplitude and calcium transients in OVX myocytes were also eliminated via E_2/GPER as were decreases in cAMP concentration. Overall, existing data suggest that E_2 and GPER activation reduce electrically stimulated or agonist-induced Ca^{2+} signals and contraction in the normal

myocardium yet prevent the inhibition of these functions in stressed myocardium.

Estrogen, GPER, and SERCA2a and Its Regulatory Proteins

SERCA2a is the main mechanism by which SR Ca^{2+} is refilled during diastole; it also is responsible for removing 70% of cytoplasmic Ca^{2+} in human cardiomyocytes (66). Several factors control SERCA2a activity. Phospholamban (PLB) is a trans-SR membrane protein that directly interacts with SERCA2a and reduces its activity by lowering its Ca^{2+} affinity (82). PLB phosphorylation at Ser16 and Thr17 in its cytoplasmic domain disinhibits SERCA2a. Phosphorylation of PLB at Ser16 is mediated by protein kinase A, while Thr17 is phosphorylated by Ca^{2+}/CaM-dependent protein kinase II (CaMKII) (83, 84). By stimulating PLB phosphorylation, β-AR activation promotes SERCA activity, which increases the rate of Ca^{2+} sequestration in diastole and facilitates myocardial relaxation (83, 85). Sarcolipin (SLN) is another trans-SR membrane protein that regulates SERCA activity; genetic knockout of SLN enhances SR Ca^{2+} uptake and cardiac contractility (86). Similar to PLB, SLN reduces the Ca^{2+} affinity of SERCA2a, though the underlying mechanisms are still a source of debate.

GPER improves LV lusitropy in models of hypertension and aging (24, 25, 65). GPER may increase intracellular Ca^{2+} homeostasis and improve diastolic function by increasing either the expression and/or activity of SR Ca^{2+} regulatory proteins. Past reports showed that estrogen increases the expression of SERCA2a, while its expression is decreased in OVX animal models. We found no evidence for changes in SERCA2, PLB, calmodulin, or RYR2 gene or protein expression in cardiac tissues with chronic G1 treatment (50 μg/kg/day) of OVX mRen2.Lewis rats with systemic hypertension, despite improvements in myocardial relaxation (25). However, chronic activation of GPER with G1 (400 μg/kg/day) attenuates the adverse effects of monocrotaline (MCT)-induced pulmonary arterial hypertension (PAH) on SERCA2a and the ratio of phosphorylated PLB to total PLB in an OVX model (20). G1-mediated improvements in Ca^{2+} regulatory proteins are accompanied by a reversal in PAH-induced LV diastolic dysfunction, pulmonary artery flow, and right ventricular (RV) dysfunction when compared with vehicle-treated counterparts (20). In a normotensive aging model (26-month-old OVX-Brown Norway Fischer 344 rats), 8 weeks of G1 treatment reverses the adverse effects of age and E_2 loss on myocardial relaxation, in part via increases in SERCA2 protein expression (24). In addition to changes in SERCA and PLB expression/phosphorylation profiles, the improvement in cardiac function with G1 treatment in these models could also be due in part to improvements in endothelial nitric oxide synthase (eNOS) activity and vascular tone. The effects of G1 on the vascular endothelium likely involve stimulation of Ca^{2+}/calmodulin signaling network activities (87), including GPER activation *per se* (87, 88), upregulation of the Ca^{2+}-dependent interaction between eNOS and calmodulin (87), improvement in the eNOS phosphorylation profile (87, 89), and optimization of vascular Ca^{2+} signaling via combined effects on influx (90) and efflux pathways (91).

To determine whether specific changes in SR Ca^{2+} uptake by GPER activation are associated with improvements in myocardial relaxation, we performed *ex vivo* studies in saponin-skinned muscle fascicles from 8-month-old female Wistar rats (24). SR Ca^{2+} content was evaluated by caffeine-induced tension under various loading conditions. Compared with vehicle, treatment with G1 increases SR Ca^{2+} accumulation in a concentration- and loading time-dependent manner suggesting that chronic GPER activation may increase cardiac Ca^{2+} mobilization not only by increasing the number of SERCA2 pumps, but by also augmenting SERCA activity (24). These data are consistent with a report on an OVX rat model; in that study, 10 weeks after OVX, SR Ca^{2+} uptake is reduced, with decreased SERCA activity and expression level, and Thr17 phosphorylation of PLB is reduced but Ser16 phosphorylation was unchanged. Interestingly, supplementation with either E_2 or progesterone prevents the OVX-related reductions in cardiac SERCA expression and activity and Thr17 PLB phosphorylation (77). However, Yang et al. (92) reported that cardiomyocytes from OVX guinea pigs have 22% larger SR Ca^{2+} stores and higher frequency of Ca^{2+} sparks and waves than sham animals; addition of E_2 prevents these changes. Similarly, Howlett's group showed that the caffeine-induced SR Ca^{2+} release signal, an indirect indicator of SR Ca^{2+} content, is larger in cardiomyocytes from OVX than from sham C57BL/6 mice (74). While these results appear to be contradictory, it is important to note that, in addition to differences in model species, treatment conditions, and dosing, the loss of progesterone with OVX may also play a significant role in the observed effects in each study, as suggested by data from Bupha-Intr and Wattanapermpool (77). Overall, studies using the specific GPER agonist G1 support a role for GPER in influencing SR Ca^{2+} uptake in the heart to improve diastolic function. Further studies using GPER KO models, especially cardiac-specific $GPER^{-/-}$ cardiomyocytes, will provide further insights.

Estrogen and the Na^+/Ca^{2+} Exchanger

The NCX is responsible for removal of ∼28% of cytoplasmic Ca^{2+} in human cardiomyocytes and as such is an important determinant of diastolic function. However, the effects of estrogen on cardiac NCX are unclear. Estrogen has been reported to increase, have no effect, or decrease NCX expression. In one study, NCX expression was increased by E_2 and was decreased in untreated OVX rats (93). In other studies, no change was observed in the expression of NCX expression by estrogen treatment or OVX (25, 94). Kravtsov et al. (76) determined NCX activity in the heart from OVX rats. In their study, NCX activity, measured as Na^+-dependent $^{45}Ca^{2+}$ uptake, was increased by OVX, and E_2 replenishment abolished this increase (76). Mechanistically, the effect of E_2 loss and restoration was associated with changes in PKA-mediated activation of NCX and not on changes in the expression level of NCX. However, no study to date has specifically examined if GPER activation mediates the effects of E_2 on NCX activity in cardiac tissue.

GPER, BIOENERGETICS, AND MITOCHONDRIAL ROS

Diastolic function is an energy-requiring process in that ATP is necessary for the sequestration of cytoplasmic Ca^{2+} back into the SR during diastole. As the heart possesses the highest content of mitochondria of any tissue (95), even slight alterations in mitochondrial cellular energy production contribute to impairment of myocardial relaxation. Indeed, an increasing body of literature suggests that abnormalities in cardiomyocyte mitochondrial function and structure are important factors in the pathogenesis of HFpEF (96, 97). Specifically, elevated and pathologic reactive oxygen species (ROS) production has been implicated in mitochondrial damage, resulting in a mismatch between ATP production and energy demand, while also activating signaling pathways that further contribute to LV remodeling, all of which lead to diastolic dysfunction.

The role of mitochondrial dysfunction in ROS accumulation and/or alterations in mitochondrial bioenergetics in the estrogen-deficient heart is still emerging, as are the mitochondria-related effects of E_2 that are mediated through GPER (98). In a recent general population study (Flemish Study on Environment, Genes, and Health Outcomes), individuals with normal and abnormal diastolic function were found to have different levels of circulating metabolites indicative of energy substrate utilization and protection again oxidative stress (99). In another cohort from that study (100), mitochondrial DNA (mtDNA), a circulating marker of mitochondrial dysfunction, was positively associated with female sex, while mtDNA levels were reduced in women receiving estrogen/progesterone treatment. With regard to cardiac pathology, one preclinical study using OVX CTnT-Q92 transgenic mice as a model of human hypertrophic cardiomyopathy showed that E_2 replacement reduces oxidative damage and improved decrements in mitochondrial bioenergetics and diastolic function (101). Potential mechanisms underlying the estrogen-mediated protection from mitochondrial-derived oxidative injury include decreasing ROS accumulation by increasing respiratory chain efficiency; reducing apoptotic leakage of cytochrome C; upregulating mitochondrial antioxidant enzymes such as manganese superoxide dismutase (MnSOD), catalase, and glutathione peroxidase; and decreasing NADPH oxidases (NOX4) (102–105). Pharmacologic interventions in isolated cardiomyocytes and in ischemia-reperfusion-challenged hearts indicate that GPER activation by G1 reduces oxidative stress by limiting cytochrome C release and inhibiting mitochondrial pore opening, respectively (106–108). Our data from the novel cardiomyocyte-specific GPER KO female mouse developed in the Groban laboratory extend these findings with more direct evidence of the potential importance of cardiac GPER in the maintenance of mitochondrial processes that counteract ROS accumulation in the female heart (23). In brief, treatment of female GPER KO mice with the mitochondrial antioxidant MitoQ attenuates the adverse effects of cardiomyocyte GPER deletion on myocardial relaxation, filling pressures, interstitial remodeling, and oxidative damage (109). MitoQ also limits the genomic responses to increased oxidative stress and decreases oxidant defense related to cardiomyocyte-specific GPER deficiency (109). Taken together, GPER appears to have a regulatory role in aspects of mitochondrial function that balance ROS formation and antioxidant defense, which in turn has the potential to impact intracellular calcium homeostasis (110), thereby contributing to the maintenance of diastolic function after estrogen loss.

As diastolic dysfunction accounts for half of what drives HFpEF symptoms and adverse clinical events, it is also important to consider the impact of systemic inflammation, coronary microcirculatory disturbances, skeletal muscle weakness, pulmonary disease, and renal dysfunction (111). Indeed, the most current paradigm of HFpEF goes well beyond diastolic dysfunction. For instance, systemic inflammation, oxidative stress, and/or endothelial dysfunction contribute to capillary rarefraction and mitochondrial dysfunction in skeletal muscle and myocardium of HFpEF patients (112–114), impairing oxygen delivery and utilization, and adversely affecting exercise tolerance (115, 116). While it is not entirely clear what role estrogen deficiency and/or GPER deactivation might have on these extracardiac parameters linked to HFpEF (117), future preclinical studies focusing on this paradigm may reveal therapeutic strategies that can be personalized to prevent the development of this disorder in postmenopausal women.

GPER AND LV STRUCTURE

In addition to the cellular mechanics of myocardial relaxation and subcellular mitochondrial energy-producing processes required, the structure of the myocardium at both cardiac muscle cell and LV chamber levels determines LV ventricular distensibility and stiffness. At the cellular level, isolated cardiomyocytes from preclinical models of diastolic heart failure exhibit increases in diameter without changes in length, which correspond to increases in LV wall thickness with normal or near-normal end diastolic volumes (118), a pattern indicative of concentric LV remodeling. *In vitro* cardiomyocyte functional data from patients with HFpEF further indicate increased stiffness and decreased distensibility, with resting tensions two times that of normal cardiomyocytes (119, 120). Translating this to the tissue level, a relatively stiff, non-distensible ventricle requires higher pressures to achieve filling of a given volume. Conventional and tissue Doppler echocardiographic techniques estimate filling pressure (see section What is Diastolic Dysfunction? above and **Figure 1**). The effects of GPER activation on components of LV remodeling, and potential mechanisms in the context of the renin angiotensin system and local inflammatory/immune processes, are presented in **Table 3**.

GPER and Anti-hypertrophic Remodeling

Preclinical studies reveal that GPER activation by E_2 or G1 prevents hypertrophic remodeling, independent of its effects on blood pressure. We have shown that high salt or estrogen deprivation in hypertensive mRen2.Lewis rats increases LV mass, wall thickness, and myocyte size and is attenuated by chronic G1 treatment (25, 65). Moreover,

TABLE 3 | Anti-remodeling effects of GPER activation in cardiac tissue and cells.

Species or cells	Models/Strains	G1 treatment	Effects of intervention
Rat	mRen2.Lewis rats/OVX (25)	s.c., 50 μg/kg/day, for 2 weeks	Limited OVX-induced ↑ LV filling pressure, LV mass, wall thickness, interstitial collagen deposition, and cardiac ANF and BNP mRNA levels
	mRen2.Lewis rats/high salt diet (65)	s.c., 40 nmol/kg/hr, for 2 weeks	Improved myocardial relaxation and reduced cardiac myocyte hypertrophy and wall thickness
	F344BN old-aged rats/OVX (24)	s.c. 100 μg/kg/day, for 8 weeks	Reversed adverse effects of age and estrogen loss on myocardial relaxation and interstitial collagen deposition
	Wistar rats/OVX + monocrotaline-induced pulmonary hypertension (20)	s.c., 400 μg/kg/day, for 14 days after the onset of disease	Limited adverse effects of pulmonary hypertension on RV interstitial fibrosis, RV free wall thickening, and LV diastolic function
	Wistar rats/myocardial infarction (121)	50 μg/kg per day, gastric gavage, for 4 weeks	Attenuated LV hypertrophy, assessed by cardiomyocyte size, to an extent similar to E_2
	Wistar rats/OVX and diabetes mellitus (122)	i.p. injection, 50 μg/kg, every 4 days for 4 weeks	Improved cardiac weight, atherogenic and cardiovascular risk indices; meanwhile GPER antagonism with G15 exacerbated cardiac weight and atherogenic indices
	SD rats/OVX + ISO-induced heart failure (123)	s.c.,120 μg/kg·d, for 14 days	Decreased cardiac BNP, reduced cardiac fibrosis, and enhanced contraction
Mouse	C57BL/6 mice/OVX & myocardial infarction (124)	i.p. injection, 35 μg/kg/d, for 4 weeks	Reduced myocardial fibrosis and infarct area
	$Ramp3^{+/+}$ and $Ramp3^{-/-}$ (125)	s.c., 0.1 mg/kg/day, for 40 days	Reduced perivascular fibrosis and cardiomyocyte area in RenTgMK/$Ramp3^{+/+}$ male mice
Neonatal rat cardiomyocytes	ET-1 (100 nmol/l) for 48 h (126)	10 nmol/l for 48 h	Abolished hypertrophic actions of ET-1, which was reversed by G15; siRNA silencing of GPER inhibited antihypertrophic effect of E_2
	100 nM of Ang II for 24 h (127)	1,000 nM for 24 h	Attenuated Ang II-induced cardiomyocyte hypertrophy and downregulated mRNA levels of ANF and BNP
H9c2 cardiomyocytes	Ang II (10^{-7}M) for 24 h (25)	10^{-7} M for 24 h	Inhibited Ang II-induced hypertrophy, evidenced by reductions in cell size, protein content per cell, and ANF mRNA levels; G15 inhibited protective effects of G1 or E_2
Adult rat cardiac fibroblasts	Growth medium with 10% FBS (57)	0.01–10 μM for 24 h	Inhibited proliferation of rat cardiac fibroblasts

ANF, atrial natriuretic factor; Ang II, angiotensin II; BNP, brain natriuretic peptide; E_2, estradiol; ET-1, endothelin-1; FBS, fetal bovine serum; i.p., intraperitoneal; ISO, isoproterenol; LV, left ventricular; OVX, ovariectomized; RV, right ventricular; s.c., subcutaneous; μg, micrograms; SD, Sprague Dawley.

ventricular hypertrophy assessed by cardiomyocyte size after infarction by coronary ligation in OVX Wistar rats is attenuated to a similar extent by G1 and E_2 (121). Lee et al. (121) further showed that GPER and ERα activation converge to elicit post-ischemic antihypertrophic remodeling via a PI3K/Akt/eNOS-dependent pathway. In cultured primary neonatal cardiomyocytes (127) and H9c2 cells (25), GPER activation by G1 attenuates angiotensin II (Ang II)- and endothelin-1 (ET-1)-induced hypertrophy, respectively, as demonstrated by reductions in atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) mRNA expression levels, cell size, and protein content. The protective effects of GPER involved inhibition of ERK1/2 signaling and an upregulation of the PI3K/Akt/mTOR pathway (127). The latter is known to effect autophagy, which is important in the preservation of cell homeostasis. In another study using neonatal cardiomyocytes, ET-1–induced hypertrophy was prevented by E_2/GPER via inhibition of ERK1/2 signaling (126).

GPER Inhibits Interstitial Remodeling

Alterations in the extracellular matrix, specifically increases in collagen, with corresponding increments in the width and continuity of fibrillar components (128), further contribute to diastolic dysfunction through increases in chamber stiffness. Interstitial and perivascular collagen deposition are enhanced in physiopathologic situations that commonly manifest diastolic dysfunction such as aging, hypertension, pressure overload hypertrophy, and estrogen loss. E_2 or GPER activation with G1 prevents increases in OVX-related effects on profibrotic gene expression, fibroblast proliferation, and collagen deposition in rodent and non-human primate models of normative cardiac aging, hypertension, and pulmonary hypertension (19–21, 24). However, it is worth mentioning that increased cardiac collagen after estrogen loss may not be universal, nor is its effect on increasing passive chamber stiffness (129). Whether length of time of estrogen deprivation, animal species and strain, and the type physiologic stress account for these discrepancies is not entirely clear. Nonetheless, *in vitro* studies demonstrate the

capacity of E_2 to regulate the proliferation of cardiac fibroblasts and their collagen production (130), effects that are deemed to be partly mediated by GPER. We recently confirmed GPER expression in cardiac fibroblasts of male Sprague Dawley rats and further demonstrated the efficacy of G1 on inhibiting cardiac fibroblast proliferation in a dose-dependent manner (57). These findings were confirmed *in vivo* in OVX-mRen2.Lewis females, in which 2 weeks of G1 treatment limits estrogen deficiency-induced increases in LV cardiac fibroblast number, proliferation, and gene expression levels of the cell cycle proteins, CDK1, and Cyclin B1 (57).

GPER and Cardiac Chymase/Ang II

Activation of the renin angiotensin system (RAS) is one mechanism for LV hypertrophic and interstitial remodeling that contributes to LV stiffness and diastolic dysfunction. Indeed, Ang II is involved in tissue remodeling and the induction of fibrosis (131). While RAS blockade is a widely used approach to treat heart failure, including HFpEF (132–134), the clinical benefits gained from RAS blockers in halting or reversing disease progression has fallen short of expectations (135–138). These drugs may have limited ability to suppress Ang II synthesis at the intracellular spaces where Ang II is formed and exerts its trophic and profibrotic actions (139, 140). Findings from the Ferrario lab (140–144) and others (145–148) suggest that chymase, not angiotensin-converting enzyme (ACE), is the major Ang II-forming enzyme in both human and rat hearts, and produces Ang II from the substrate angiotensin I (Ang I) or angiotensin-(1–12) [Ang-(1–12)].

With respect to estrogen status (intact vs. OVX) and LV diastolic dysfunction, we demonstrated a positive relationship between cardiac chymase-forming Ang II and echo-derived filling pressures in normotensive Wistar Kyoto female rats (149) and hypertensive mRen.Lewis rats (150). OVX-related increases in chymase and Ang II expression were further associated with increases in cardiac fibrosis. Because mast cells are a major source of chymase (151, 152) and generate Ang II from Ang I or Ang-(1–12) (153), we also determined the impact of mast cell inhibition by the mast stabilizer cromolyn sulfate on OVX-induced diastolic dysfunction (154). In brief, 8 weeks of cromolyn sulfate administered subcutaneously to OVX-BNF344 rats attenuates the adverse effects of estrogen loss on diastolic function, interstitial collagen deposition, and collagen type 1A mRNA levels. Even though cardiac chymase activity in OVX rats is not overtly reduced by cromolyn ($P < 0.06$), cardiac Ang II content is reduced when compared with OVX vehicle, suggesting a role for mast cell derived-factors and chymase/Ang II in the progression of cardiac aging and diastolic dysfunction after estrogen loss (154). Indeed, E_2 treatments favorably regulate cardiac mast cell number and prevent the adverse effects of OVX on cardiac remodeling and LV function in an Ang II-dependent rodent model of hypertension and LV diastolic dysfunction (150) and in models of surgically induced pressure overload (155) and volume overload (156, 157).

Although the mechanisms by which estrogen regulates cardiac mast cell number are not entirely clear, it appears to be mediated in part through GPER. Findings from us suggest that GPER is expressed in RBL-2H3 mast cells (58) and that GPER activation by its agonist G1 inhibits serum-induced proliferation of these cells through interaction with the cell cycle protein CDK1. GPER blockade by G15, but not by ERα or ERβ antagonists, completely prevents E_2-induced inhibition of mast cell proliferation (58). This effect was confirmed *in vivo* in OVX-mRen2.Lewis rats; 2 weeks of G1 treatment decreases cardiac mast cell number and chymase expression/Ang II levels, and limits gene and protein expression of cell cycle proteins (58). Taken together, these data suggest that the inhibitory effects of GPER on extracellular matrix remodeling may in part involve cardiac mast cell chymase/Ang II modulation.

GPER and Cardiac Inflammation

Another indirect way by which GPER activation could prevent OVX-induced remodeling and diastolic dysfunction is through modulation of local inflammatory defense mechanisms (111, 158).

Using cardiomyocyte-specific GPER KO mice (23), we found an intriguing relationship between loss of cardiac GPER and the NLRP3 inflammasome, which includes NLRP3, caspase-1, interleukin-1β (IL-1β), and IL-18. The NRLP3 inflammasome is formed and activated by various stimuli, including oxidative stress, and participates in the pathogenesis of hypertension, diabetes, atherosclerosis, myocardial infarction, heart failure, and other cardiovascular diseases (159). Characterization of innate immunity gene transcripts in hearts from 6-month-old cardiomyocyte-specific GPER KO mice and their GPER-intact wild-type littermates revealed that expression of NLRP3 and IL-18 are increased nearly three-fold (22). The importance of NLRP3 upregulation in GPER KO-induced heart failure was further confirmed in an *in vivo* study showing that, compared with vehicle-treated KO mice, 8 weeks of treatment with a NLRP3 inhibitor, MCC950 (10 mg/kg, i.p., 3 times per week), significantly limits hypertrophic remodeling and improves LV systolic and diastolic function (22). Consistent with a potential role of GPER in inflammasome deactivation, gene expression levels of key inflammatory genes, and cytokines related to inflammasome biology, including IL-18, IL-33, NLRP3, and caspase-1, were reduced in hearts of OVX-mRen2.Lewis rats treated with G1 compared with vehicle (unpublished data).

Whether G1/GPER-mediated anti-inflammatory responses are related to its effects on mast cells, as discussed previously, is not known. Mast cells are potent innate immune cells that accumulate in chronically inflamed tissues. The IL-1 family of cytokines, and particularly IL-33, activate mast cells and prime them to respond to inflammatory signals (160). If estrogen loss leads to a low-grade, chronic inflammatory state (161) in the female heart, the role of mast cells may evolve and continue to "feed the fire" via ongoing mediator release, such as chymase, thereby contributing to LV stiffness through hypertrophic cardiomyocyte and interstitial remodeling.

GPER, LV EJECTION, AND PROXIMAL AORTIC DISTENSIBILITY

LV ejection with respect to proximal aortic distensibility is another factor that contributes to diastolic function in the female heart. During systole, the long axis of the left ventricle normally shortens by pulling the aortic annulus toward the relatively fixed LV apex (162, 163). Displacement of the aortic annulus and sinotubular junction without concomitant movement of the aortic arch during systole promotes longitudinal stretch of the proximal aorta (162, 164, 165). While the aortic stretch that occurs during systole imposes a systolic load on the heart, it actually enhances early diastolic filling by serving as a reservoir for elastic energy (165). With loss of aortic distensibility due to advancing age, and presumably estrogen deficiency (163), or HFpEF (166) the displacement of the aortic annulus is reduced, as is the longitudinal long axis force or shortening of the left ventricle, leading to less stored elastic energy and impaired LV filling (167). Interestingly, postmenopausal women are more susceptible to the adverse effects of greater proximal aortic stiffness and pulsatile load on diastolic function and ventricular-arterial interaction than men of the same age (168–170). Moreover, the relationship between aortic impedance and diastolic dysfunction and ventricular-arterial coupling in women might be independent of LV remodeling (168), suggesting an additional contribution of aortic impedance to diastolic dysfunction in women.

Although the exact role of estrogen/GPER in aortic–ventricular interactions with respect to diastolic function is not known, recent preclinical studies suggest that GPER activation limits aortic stiffening and remodeling. GPER is expressed in both endothelial and smooth muscle cells of the aorta (59, 171) and GPER activation induces vasodilation similar to that seen with E_2 (89, 172). In contrast to resistance arteries, GPER-induced vasorelaxation in the aorta is less robust (173) and the contribution of endothelial vs. smooth muscle signaling is more variable (59, 171). Interestingly, aortic GPER expression is downregulated in diabetes (174) but is functionally enhanced during pregnancy (171). In contrast to the extensive work assessing aortic reactivity, less is known about the impact of GPER on passive structural properties of conduit arteries. In the mRen2 rat model of hypertension, pharmacological activation of GPER in salt-loaded females significantly decreases aortic wall thickness without impacting blood pressure (175). GPER is also protective during carotid injury, where adenovirus-induced restoration of GPER protein expression is associated with a reduction in wall thickness in both male and female rats (176). While Ang II-induced hypertension is not impacted by GPER deletion, pulse pressure, and aortic wall thickness are significantly greater in female cardiomyocyte-specific GPER KO vs. wild-type mice (177). Therefore, while the role of GPER in proximal aortic distensibility has not yet been directly measured, published studies suggest that it most likely is another important factor impacting diastolic function.

TRANSLATIONAL PERSPECTIVE

The sex-differential in the prevalence and incidence of human HFpEF is stark. Among women age >/=65 years, nearly 90% of new cases of heart failure are HFpEF. In the small number of men who develop HFpEF, the underlying characteristics differ markedly from that seen in women with predominantly ischemic heart disease, mildly dilated LV, and borderline/mild levels of systolic dysfunction. Thus, classic HFpEF is nearly exclusively a disorder of older, post-menopausal women. Despite this overwhelming magnitude of this profound biologic signal, its fundamental basis has not been systematically examined. Doing so could produce major insights into the initiation and progression of human HFpEF. Thus, the emerging data reviewed above has the potential to promote key advances in the understanding of human HFpEF and novel approaches to interrupting the pathways that lead to one of its precursors, diastolic dysfunction. This is greatly needed given the disappointing results of the recent PARAGON trial (178), and the other 7 large randomized trials of HFpEF that failed to achieve their pre-determined primary endpoint (179).

CONCLUSION

Improvements in preventive medicine and health habits by positively lengthening the human life-span have brought to the forefront the impact of the menopause decline in women's cardioprotection. Estrogen-mediated cardiac health in women, using diastolic function as its monitor, is influenced by non-genomic mechanisms through GPER in the heart, in part by counteracting age and/or estrogen loss-dependent abnormalities in myocardial relaxation, cardiomyocyte Ca^{2+} homeostasis, mitochondrial function, and anti-hypertrophic/interstitial processes (**Figure 2**).

AUTHOR CONTRIBUTIONS

LG conceived and designed the manuscript. LG, Q-KT, and SL drafted the manuscript. Q-KT, HW, XS, LG, and SL created the tables and figures. DK provided the translational perspective. DK, CF, and CC were involved in critically reviewing and revising the manuscript for important intellectual content. All authors gave final approval of the manuscript to be published and agreed to be accountable for all aspects of the work.

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