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MECHANISTIC PATHWAYS OF SEX DIFFERENCES IN CARDIOVASCULAR DISEASE

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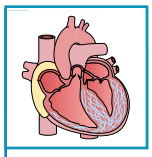

Regitz-Zagrosek V, Kararigas G. Mechanistic Pathways of Sex Differences in Cardiovascular Disease. *Physiol Rev* 97: 1–37, 2017. Published November 2, 2016; doi:10.1152/physrev.00021.2015.—Major differences between men and women exist in epidemiology, manifestation, pathophysiology, treatment, and outcome of cardiovascular diseases (CVD), such as coronary artery disease, pressure overload, hypertension, cardiomyopathy, and heart failure. Corresponding sex differences have been studied in a number of animal models, and mechanistic investigations have been undertaken to analyze the observed sex differences. We summarize the biological mechanisms of sex differences in CVD focusing on three main areas, i.e., genetic mechanisms, epigenetic mechanisms, as well as sex hormones and their receptors. We discuss relevant subtypes of sex hormone receptors, as well as genomic and nongenomic, activational and organizational effects of sex hormones. We describe the interaction of sex hormones with intracellular signaling relevant for cardiovascular cells and the cardiovascular system. Sex, sex hormones, and their receptors may affect a number of cellular processes by their synergistic action on multiple targets. We discuss in detail sex differences in organelle function and in biological processes. We conclude that there is a need for a more detailed understanding of sex differences and their underlying mechanisms, which holds the potential to design new drugs that target sex-specific cardiovascular mechanisms and affect phenotypes. The comparison of both sexes may lead to the identification of protective or maladaptive mechanisms in one sex that could serve as a novel therapeutic target in one sex or in both.

I. INTRODUCTION

In many frequent diseases, significant differences exist between men and women in epidemiology, clinical manifestation, pathophysiology, treatment and outcomes. A part of these differences is due to biological differences between men and women and is commonly designated as sex differences, whereas differences between men and women that depend on the interaction of the individual with the environment and society are referred to as gender differences. It is well known that sex hormones influence behavior and lifestyle. More recent findings demonstrate that environmental influences, such as lifestyle, nutrition, different forms of stress, dust, and heat, lead to epigenetic modifications in the developing fetus, the child, and the adult. These can be transmitted through the germline and affect the development of disease or determine resistance against disease. The psychosocial mechanisms contributing to disease are summarized as gender effects. Sex and gender effects are frequently interrelated and interact in many diseases **(FIGURE 1)**.

This review focuses on the contribution of biology to sex differences in cardiovascular diseases (CVD) and on mechanistic pathways of sex differences in CVD. We first introduce CVD with significant differences between men and women that call for clinical attention. So far, this topic has been neglected by many regulatory, funding, research, and industrial policies, and the knowledge is rather limited in many aspects. We then discuss how experimental animals can serve as models for these diseases and the sex differences therein, acknowledging that these models are hampered by limited comparability between humans and animals regarding age and duration of disease manifestations, comorbidities, hormonal cycle, and pathophysiological mechanisms. Nevertheless, genetic manipulation of experimental animals provides mechanistic insights into disease processes. The main focus is to analyze genetic and epigenetic mechanisms leading to sex differences, the contributions of sex hormones and their receptors to sex differences in biological processes of cardiovascular cells.

II. CLINICAL BACKGROUND

A. Ischemic Heart Disease

Men and women are prone to develop different types of ischemic heart disease (IHD). While men suffer most fre-

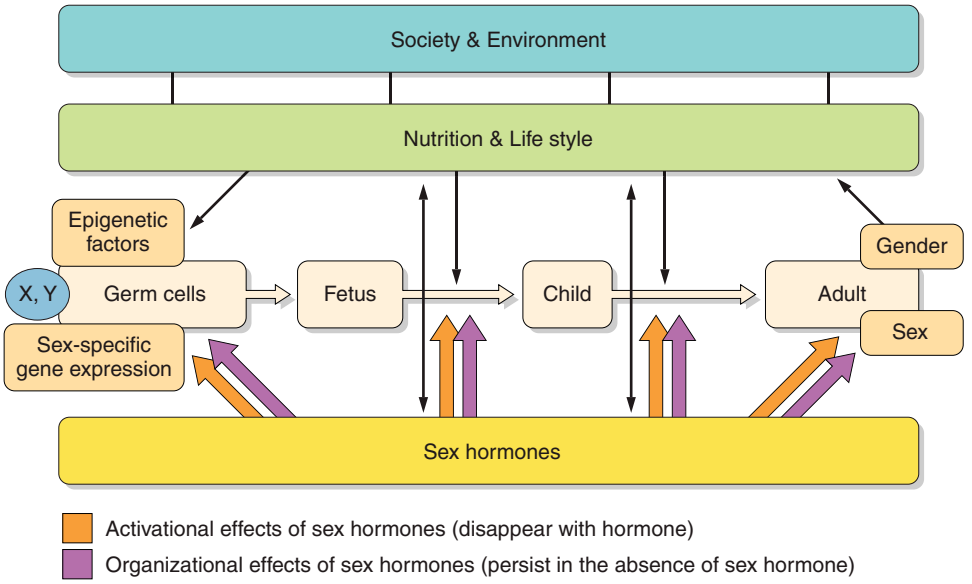

FIGURE 1. Interaction of sex and gender during development and adulthood. X- and Y-linked genes determine the formation of ovaries or testes and subsequent production of sexual hormones. These, in turn, exert direct activational effects that are reversible after removal of sex hormones. In contrast, organizational effects, based on epigenetic modifications of DNA, persist for longer time periods in the absence of sex hormones. Other factors, such as nutrition and environmental factors, contribute to complex interactions.

quently from occlusive coronary artery disease (CAD), women exhibit more frequently a nonobstructive CAD or microvascular dysfunction that is better described with the term *IHD*. The term *IHD* recognizes that the main pathophysiological problem is myocardial ischemia due to a disturbed balance between oxygen supply and demand of the myocardium (48). This may be located in the epicardial coronary arteries, due to atherosclerosis, which is more common in men and known as CAD, or pathological vasoreactivity, such as spasm and endothelial dysfunction, which is more common in women (29, 387). Perfusion problems may also arise from microvascular dysfunction, which appears to be more common in women. Women with recurrent chest pain syndromes without obstructive CAD but with microvascular dysfunction have a twofold increased risk to develop CAD events in the following 5-8 years and have a four times higher risk for rehospitalizations and recurrent angiograms after an acute event than women without these symptoms (171, 338). Pathophysiology of microvascular dysfunction is incompletely understood and needs more systematic investigation.

Other manifestations of IHD with sex-specific prevalence are spontaneous coronary artery dissection and the Takotsubo syndrome. Spontaneous coronary artery dissection occurs preferentially (>90%) in women below 60 years of age. It is frequently associated with immunologic and connective tissue diseases (295). Three-quarters of the manifestations of this syndrome occur in pregnancy (329, 345, 395). The relative high prevalence of this syndrome in pregnancy is the reason that acute coronary events in women that need coronary interventions should be treated with stents and not with thrombolysis (329). Furthermore, the Takotsubo syndrome affects predominantly women (196, 300, 355, 424). It manifests as an acute coronary syndrome and accounts for up to 8% of the acute coronary syndromes in women, but its etiopathology is not clear. The postmenopausal decrease in estrogen levels probably contributes to an increased sensitivity of the heart to circulating catecholamines. Takotsubo is often preceded by massive acute psychological or physical stress. The patients mostly recover with normalized ejection fraction. However, according to the most recent data, mortality is 8% per year, and recurrence is estimated at 5% (380).

Men develop CAD earlier and usually present with more severe atherosclerosis in their coronary arteries than women. As a consequence, myocardial infarction (MI) in general appears 10 years earlier and is associated with a more widespread CAD localization in men than in women. The reason for the relative protection of women against the development of atherosclerosis before menopause is poorly understood. A more beneficial lipid profile may contribute, and some protection seems to be conferred by sex hormones, since women with hormonal disturbances, such as polycystic ovarian syndrome, develop earlier atherosclerosis and MI than healthy women (219, 413). The role of estrogen and its receptor (ER) is substantiated by the fact that even men with a disruptive ERα (*ESR1*) mutation have early CAD (318, 374).

For unknown reasons, acute mortality in the first days after MI is greater in younger women than in age-matched men (397). Cardiac rupture at acute MI has been reported more frequently in women than in men in studies from the United States, Europe, and Japan. These studies have suggested that women have a higher mortality rate than men, even when controlled for age, and die less often from arrhythmia but more often from cardiac rupture independent of whether thrombolytic therapy is used or not (59, 151, 269, 433). In contrast, ischemic sudden death due to arrhythmia occurs more frequently in men than in women.

The risk of heart failure (HF) following MI is higher in women than men (211). Women also have more rehospital-

ization for acute coronary syndrome after MI than men (242). In some but not all studies, a higher total in-hospital mortality rate after MI in women than in men was accounted for on the basis of differences in age and comorbidities (207).

B. Pressure Overload

Female hearts adapt to pressure overload differently from male hearts. In particular, pressure overload-induced hypertrophy in women is associated with smaller internal cavity and relatively larger wall thickness than men (13, 80, 81). Women, independently of left ventricular size, more frequently preserve better ejection fraction and myocardial contractility than men during progression of aortic stenosis (AS) (55, 80, 99, 407). The better systolic function in women with AS may be due to a less pronounced induction in collagen remodeling than in men (406, 407). In our own study of patients undergoing aortic valve replacement, similar percentages of women and men had increased left ventricular (LV) diameters, but women more frequently exhibited LV hypertrophy than men (312). Increased LV diameters persisted 1 wk after surgery in 34% of men but only in 12% of women. LV hypertrophy reversed more frequently in women than in men. In LV tissue samples from AS patients, men had significantly higher collagen I (*COL1A1*) and III (*COL3A1*) and matrix metalloproteinase 2 (*MMP2*) gene expression than women (312). Less fibrosis prior to aortic valve replacement may enable faster regression following surgery (311). Overall, AS leads to sex-specific myocardial remodeling **(FIGURE 2)** with a more concentric form of myocardial hypertrophy, less fibrosis, and a better reversibility after unloading the ventricle by aortic valve replacement in women than in men (311, 312).

C. Hypertension

The number of hypertensive individuals in younger age groups is greater in the male population than in the female population, whereas in the elderly the percentage of hypertensive women doubles the numbers of men (290). Sex differences in hypertension are related to the renin-angiotensin system (RAS) and the bradykinin and nitric oxide (NO) system. Hypertensive LV hypertrophy regresses less well in women under RAS inhibition than in men (128). Hypertensive women sustain higher LV ejection fraction and other measures of systolic function than hypertensive men. Nevertheless, they have an estimated threefold higher risk of developing HF or stroke compared with men (223). Hypertensive women develop more vascular and myocardial stiffness than men at older ages and more often have isolated systolic hypertension, reflecting increased aortic stiffness (254).

D. Exercise-Induced Cardiac Hypertrophy

Cross-sectional studies of endurance athletes suggest that women develop a lower maximal oxygen uptake and less LV hypertrophy than men undergoing a similar training

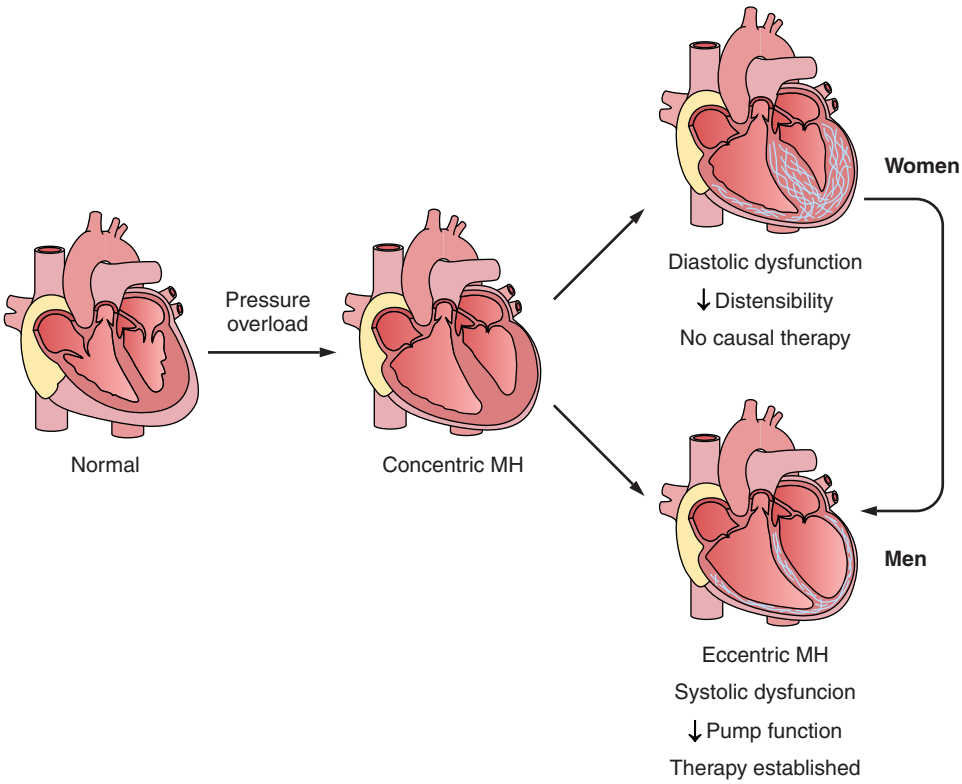

FIGURE 2. Paradigmatic changes in male and female hearts under pressure overload. Both men and women respond primarily with concentric myocardial hypertrophy (MH), but women stay more in concentric MH with maintained systolic function, whereas men develop more easily eccentric MH.

program (429). However, cross-sectional studies are limited by the different forms of competition, exercise, and training performed by male and female athletes. Only recently, a small longitudinal study was published that focused on sex differences in physiological adaptation using identical 1-year endurance training programs in six men and five women (152). The small sample size limits the power of this study; however, the use of cardiac magnetic resonance imaging for analyzing LV mass and the longitudinal character of the study may partially offset this disadvantage. As a first surprising result, the study found sex differences in metabolic adaptation. Women experienced a major reduction in body fat already after 6 mo of training, whereas men experienced a reduction in body fat only at 12 mo of training. Second, ventricular compliance and distensibility improved similarly in men and women. However, men demonstrated greater enhancement in the Frank-Starling mechanism. Third, men exhibited a greater increase in oxygen uptake and a greater increase in LV mass in month 12 of training. Noteworthy, women had the same or even greater increase in LV mass as men after 3 mo, but no further increase occurred up to month 12, leading to much lower total increase of LV mass over the 12-mo period. Myocardial hypertrophy in men could partially be due to testosterone, which is known to increase with regular exercise training (409). In women, estrogen may influence cardiac hypertrophy via pathways, such as phosphatidylinositol 3-kinase/AKT signaling or the β-catenin pathway that can act as a prohypertrophic as well as an antihypertrophic stimulus (discussed later) (143, 188). Premenopausal women are known to exhibit greater cardiac AKT activity than men with subsequent greater antihypertrophic effects (51). Nevertheless, no good mechanistic explanations are provided so far by rodent models, since in these models, female animals develop greater LV hypertrophy than males (discussed later) (108, 198). In summary, the cardiovascular adaptation to endurance training occurs differently between men and women, even with identical training programs. Clearly more data and larger studies are needed in this field.

E. Genetic Cardiomyopathies and Arrhythmia

Genetic cardiomyopathies due to autosomal gene variations are expected to occur with the same prevalence between men and women. Nevertheless, recent large-scale genome-wide studies associating genetic profiles with disease risk have revealed significant sex differences in genetic variation-disease associations (230, 276, 379). Furthermore, dilated cardiomyopathy and hypertrophic cardiomyopathy (HCM) have a greater prevalence in men than in women (10, 20, 75, 78, 129). Thus compensation for the genetic defect in these syndromes appears to be more efficient in women than in men. Sudden arrhythmic cardiac death is a frequent thread in HCM, and sudden death in young athletes is frequently attributed to undiagnosed HCM. Noteworthy, sudden cardiac death in young and middle-aged athletes affects almost only men (255).

Genetic defects leading to long QT syndromes (LQTS) are located on autosomes. Mutations in 13 genes have been associated with LQTS, but most of the LQTS are due to mutations in three ion channels (342). LQTS-induced tachycardia occurs with equal frequency in boys and girls. However, after puberty, arrhythmias are more frequent in women than men. Women are at higher risk than men for torsades de pointes with LQT type 1 and type 2, but LQT type 3 occurs in equal frequency between men and women (342). It has been hypothesized that testosterone contributes to shortening of the QT interval in men, whereas estrogen has smaller, QT prolonging effects in women (314, 449).

F. Heart Failure

HF is a typical clinical syndrome arising from different pathophysiological conditions, defined by clinical symptoms and signs that has a high prevalence in old age, affecting more than 10% of those above 70 years in western societies and typically more women than men. We now differentiate between HF with reduced ejection fraction, affecting typically men, and HF with preserved ejection fraction, affecting more women (73). In both syndromes, women have better clinical outcomes than men (222, 258). The heart of men and women also adapts differently in HF. In a large population-based German cohort, men with moderate or severe LV dysfunction developed a stronger increase in LV mass than women (236). In HF with preserved ejection fraction, women develop less ventricular dilation than men, but they have smaller and stiffer ventricles (330, 332), which may be due to different fibrous tissue composition or different relaxation kinetics due to sex differences in calcium (Ca^{2+}) handling (97, 330). Men and women with HF with preserved ejection fraction differ mainly in their comorbidities. Aging, over- and undernutrition, diabetes, hypertension, salt loading, as well as inflammatory or autoimmune diseases are significant risk factors, which manifest differently between men and women and contribute to HF with preserved ejection fraction in a sex-dependent manner (133, 210, 262).

III. SEX DIFFERENCES IN EXPERIMENTAL ANIMALS AS MODELS FOR HUMAN CARDIOVASCULAR (PATHO)PHYSIOLOGY

The above described clinically relevant sex differences in many CVD await pathophysiological clarification that may lead to the development of sex-specific therapeutic strategies. Since studies in humans are limited, use of experimen-

tal animals that mimic human disease are necessary to advance our understanding (248). A number of models have been used to mechanistically investigate sex differences in pathophysiology and outcomes. In particular, models for ischemia, pressure overload, hypertension, exercise-induced hypertrophy, and cardiomyopathies have been developed and investigated for sex differences in their effects on myocardial remodeling in rodents (331). For heart failure with reduced ejection fraction, animal models have been developed for the underlying disease conditions, but they have frequently not been studied for sex differences. Due to the multifactorial and largely unknown origins of heart failure with preserved ejection fraction in humans, no good animal models are available for this syndrome. Therefore, sex differences in animal models do not always offer a one-to-one representation of sex differences in humans.

A. Myocardial Ischemia

Animal models for myocardial ischemia that investigated sex differences focused mainly on infarct size and on outcomes in the first days or weeks after acute occlusion of a coronary artery in vivo in mice, rats, rabbits, dogs, and pigs with and without reperfusion. Furthermore, an ex vivo system, the isolated beating heart, i.e., the Langendorff model, has been frequently used. In the Langendorff system, better postischemic recovery of LV function and smaller infarct sizes were found in females than in males (19). Isolated perfused female rat hearts have a better recovery and smaller infarct size than male hearts (46, 175). Female hearts also had improved recovery of contractility ($+dP/dt$) and compliance ($-dP/dt$) after ischemia and reperfusion and less necrosis compared with male hearts (123, 419).

In vivo studies of ischemia revealed smaller infarct size and less apoptotic cell death in female than in male rabbits (41). After coronary occlusion, female rats developed a concentric hypertrophy with no additional cavity dilation and no measurable scar thinning, while males showed eccentric hypertrophy, cavity dilation, and scar thinning (166). Smaller infarct size in females was also confirmed in a dog model (175, 218). Most studies agree that under ischemic stress, female mice, rats, and rabbits have a better survival than males in the first days after MI. This seems partially due to smaller infarct sizes and to lower rates of cardiac rupture in females than in males in the first 5 days after MI (58). Males have delayed myocardial healing, resulting in early cardiac rupture, and the survivors have poorer cardiac function and pronounced maladaptive remodeling, while females show a better outcome and less development of HF. Greater wall stress, partially due to greater volumes, greater inflammation and matrix metalloproteinase activation in males seem to be contributing causes of these sex differences (57, 113). Testosterone enhances early cardiac eccentric remodeling after MI, causing rupture and degrading cardiac function, while estrogen seems to have no significant protective effect in the acute phase after MI (58).

Most of these results were obtained in small studies that could not consider the effects of genetic background, age, body temperature, or other conditions. In a very large analysis of determinants of infarct size in mice that included these variables, female sex was still associated with reduced infarct size after ischemia/reperfusion (139). In our own large study with 400 mice, we also found that female sex improves early survival after MI, and we showed that males and females responded differently to a novel therapy with a transgenic approach (396). The whole study group and the subgroup of males responded with improved survival after acute coronary occlusion to the transgenic overexpression of melusin (*Itgb1bp2*) in the heart, but survival in the female subgroup was not affected by this treatment (396). However, chronic remodeling was positively affected in both sexes. Together, these results corroborate that sex has a major impact on infarct size and survival after MI in rodent models, and this needs to be taken into account when designing a study of MI in mice (139).

B. Pressure and Volume Overload

Pressure overload in mice may be introduced by constriction of the aorta at different levels **(FIGURE 3)**. Male rats with chronic transverse aortic constriction (TAC) develop an unfavorable form of myocardial hypertrophy, i.e., more eccentric hypertrophy and fibrosis, whereas females develop less and more concentric myocardial hypertrophy

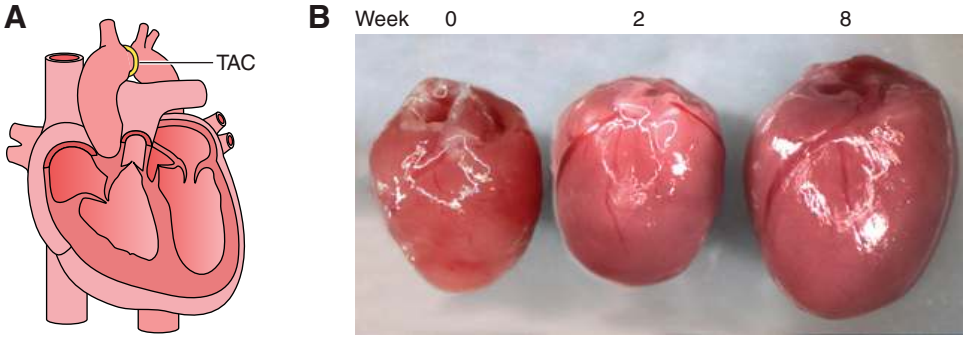

FIGURE 3. Mouse model of myocardial hypertrophy and heart failure. Transverse aortic constriction (TAC) (*A*) leads to concentric myocardial hypertrophy with rounded apex after 2 wk and eccentric myocardial hypertrophy with LV dilation after 8 wk (*B*) in mice.

(98). Contractile reserve and Ca^{2+} handling are better preserved in females than in males subjected to TAC. Furthermore, males develop HF at an earlier stage than females (426). This pattern is similar to human AS as discussed above. In line, our own studies have also revealed significant sex differences in a mouse model of TAC and found a more pronounced increase in myocardial hypertrophy and fibrosis in male animals. In contrast, female animals exhibited less downregulation of genes related to mitochondrial function and respiration (119). The changes in the transcriptional profile induced by pressure overload differed significantly between the sexes (119, 432).

Sex differences in volume overload models have been less frequently studied so far. Female rats with atrioventricular shunt have less HF, maintain cardiac function, and have lower chamber size than males (127). Proapoptotic pathways are increased in males but not in females (91). Males have a greater mortality than females (25 vs. 3%), despite a similar degree of volume overload. Interestingly, ovariectomy abolished the biological advantage of females, and estrogen treatment restored the sex-associated patterns of remodeling in this model (45).

C. Hypertensive Models

Further animal models for other complex human CVD, such as hypertension, have also been analyzed for sex differences. In the spontaneous hypertensive rat (SHR) model, cardiac function declined faster in male than in female animals (313). Female SHR maintained normal cardiac dimensions and function, whereas males developed LV dysfunction and HF. At the age of 12 mo, female SHR have greater ejection fraction and cardiac index and smaller end-diastolic and -systolic volumes than males, despite similar systolic blood pressure values between the sexes (313). Female SHR also developed less LV hypertrophy and fibrosis than males, which was independent of blood pressure differences and was associated with greater generation of NO (339).

In the deoxycorticosterone (DOCA)-salt hypertension model, a model characterized by hyperaldosteronism, a blood pressure-independent sexual dimorphism was confirmed (190). Males developed more LV hypertrophy than females, and this was associated with greater calcineurin activation (140, 190).

D. Exercise

In models of voluntary or forced exercise, female mice develop more cardiac hypertrophy than males (88, 108, 120, 198). Surprisingly, female mice run on a cage wheel longer distances than males (88, 198). However, the greater hypertrophic response in females persists after normalization of cardiac mass to running distance (198). Our own recent studies revealed that the greater increase in physiological myocardial hypertrophy in females is mediated by induction of protein kinase B (PKB, also known as AKT) signaling, mitogen-activated protein kinase (MAPK) pathway, protein synthesis, and mitochondrial adaptation in an ERβ-dependent manner (108). Thus a veritable biological difference between the sexes in the hypertrophic response to exercise must be assumed. Another study also found increased cardiac hypertrophic responses to exercise in female mice and reported that this was associated with increased plasma free fatty acid levels and augmented adipose tissue lipolysis (120). In parallel, myocardial glucose uptake was reduced in female mice after exercise, analyzed by positron emission tomography, while cardiac glucose uptake was unaltered after exercise in males. Expression of genes involved in fatty acid uptake was increased in female compared with male mice. Thus sex differences in exercise-induced cardiac hypertrophy are associated with changes in cardiac substrate availability and utilization with a shift to greater use of fatty acids in females (120). Sex differences were also found in other species and exercise forms. In rats, females subjected to chronic swimming exhibited a marked increase in absolute heart mass associated with increased contractile performance compared with male mice (271, 347, 348). However, in studies of effects of exercise, the accompanying stress response, i.e., catecholamine liberation, must be considered, since mechanical load and catecholamine liberation may exert different effects on cardiac adaptation (349).

E. Genetic Models Leading to Sex Differences in Cardiac Function

Sex differences have also been found in animal models of genetic diseases leading to myocardial hypertrophy and HF. In most of these models, male mice appear more sensitive to genetic interventions than females, since female animals display a lower mortality, less severe hypertrophy, and better preserved function than males **(TABLE 1)** (102). Some of these models mimic human HCM. Mutations in the cardiac myosin heavy chain gene can cause familial HCM. In a transgenic model (missense R403Q allele), males develop more LV systolic dysfunction than females (294). Genetic deletion of *Fkbp1b*, encoding a sarcoplasmic reticulum (SR) protein that regulates cardiomyocyte Ca^{2+} handling, results in increased cardiac mass in male but not in female mice (436). Ablation of phospholamban (*Pln*), another gene involved in cardiac SR Ca^{2+} handling regulation, exacerbates ischemic injury to a lesser extent in female than male mice (82). Female mice with fourfold overexpression of phospholamban do not exhibit LV hypertrophy and mortality at 15 mo, while males do (87). Genetic deletion of peroxisome proliferator-activated receptor alpha (*Ppara*), a gene involved in cellular energy metabolism, led to cardiac lipid accumulation and death in all male mice, but only in 25% of females (95). This lipid accumulation could be prevented by estrogen administration (95). In another study, overex-

Table 1. *Sex-specific genetic models of CVD*

Modification	Gene	Sex-Specific Phenotype	Reference Nos.
Genetic deletion	*Fkbp1b*	Cardiac mass increased in males but not females	436
	Pln	More ischemic injury in males than females	82
	Ppara	Cardiac lipid accumulation and death in all males, but only in 25% of females	95
Transgenic expression	*Myh6*	More LV systolic dysfunction in males than females	294
	Pln	LV hypertrophy and mortality at 15 mo in males but not females	87
	Hdac5	Death in males but not females	85
	Tnf	HF and increased mortality predominantly in males	167

pression of a histone deacetylase (HDAC) in cardiomyocytes caused death in male but not in female mice (85). The transgene effect may have been mediated by alterations in mitochondrial function (85).

Modulation of inflammatory pathways also lead to sex-specific effects. Overexpression of tumor necrosis factor-α (*Tnf*), a proinflammatory cytokine, caused HF and increased mortality predominantly in males (167, 177). This may be due to sex differences in the activation of proinflammatory pathways including TNF-α signaling in the heart. Sex hormones may lead to sex-specific activation or suppression of these pathways (438) and will be discussed later.

IV. MECHANISMS OF SEX DIFFERENCES

The large number of sex differences in humans and experimental animals have led to the study of these differences in more detail and to the search for underlying mechanisms. Obviously, (epi)genetic mechanisms, based on the differences in sex chromosomes, are expected to play a major role, as well as sex hormones and their receptors. The share of these mechanisms in contributing to sex differences is not easy to evaluate and frequently surprising. We discuss the most prominent concepts and findings.

A. Genetic Mechanisms

1. Male specific Y-chromosomal gene expression

Sex differences in the transcriptome may arise from the expression of Y-encoded genes and lead to male-specific cardiovascular phenotypes **(FIGURE 4*A*)**. In the 2000s, observations linking gene variants on the Y chromosome to hypertension were reported, which could contribute to the higher incidence of CVD in males compared with females (62). Further results indicated that a locus on the Y chromosome may influence low-density lipoprotein (LDL) levels, independent of testosterone levels (61). Notably, it was reported that a severe form of CAD in men was linked to a Y-chromosomal gene variant possibly through interactions of immunity and inflammation (60). Next, it was suggested that the previously identified association between haplogroup I and CAD was not mediated by an abnormal regulation of sex steroids (33). In summary, these data demonstrate that gene variants on the Y chromosome contribute to cardiovascular phenotypes in men.

2. Incomplete X-chromosomal gene inactivation

Escape of X-chromosomal genes from X-inactivation may contribute to a sex-specific imbalance in gene expression **(FIGURE 4*B*)**. Under normal conditions, a balanced gene expression dosage between males (XY) and females (XX) is achieved by X inactivation. Mammals have evolved a compensatory mechanism to randomly inactivate one of the female X chromosomes, resulting in equalizing gene expression between males and females in the best case. However, despite this chromosome-wide silencing, a number of genes escape X inactivation. This is species-specific; in humans, ~15% of X-linked genes are bi-allelically expressed and in mice, ~3%. Expression from the inactive X allele varies from a few percent of that from the active allele to near equal expression. While most genes have a stable inactivation pattern, a subset of genes exhibit tissue-specific differences in escaping from X inactivation. Escaping genes appear to be protected from the repressive chromatin modifications associated with X inactivation. Differences in the identity and distribution of escape genes between species and tissues suggest a role for these genes in the evolution of sex differences in specific phenotypes.

The contribution of X-chromosomal genes to sex differences in cardiovascular phenotypes may have been underestimated, since genome-wide association studies have frequently not included the X chromosome. In fact, the X chromosome represents one potential source for the "missing heritability" for complex phenotypes, which so far has remained underanalyzed in genome-wide association stud-

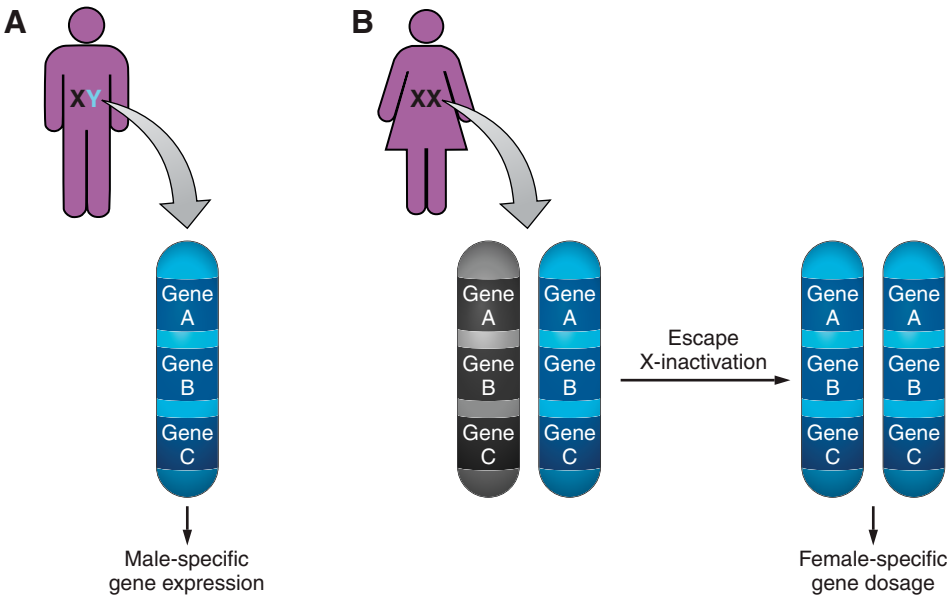

FIGURE 4. Sex chromosome-dependent gene regulation. Y chromosome-specific genes lead to male-specific gene expression (*A*), thereby resulting in male-specific traits. X chromosome inactivation (ca. 15% of genes) leads to female-specific gene dosage effects (*B*), thereby affecting (patho)physiology in a sex-specific manner. Only a part of the chromosome is indicatively shown with selected regions containing 3 genes.

ies. The results of a recent study have provided the first link between phenotypic variation in a population sample and a X chromosome inactivation-escaping locus (394). Interestingly enough, X chromosomal gene variation is associated with slow progression to AIDS in HIV-1-infected women (360). A single nucleotide polymorphism located at Xq21.1 in a conserved sequence element was identified as a significant genetic determinant of disease progression in women but not in men. These data provide a clear motivation for including the X chromosome in large-scale genetic studies of complex diseases and traits.

3. Sex-specific heterochromatizing effects of sex chromosomes

The presence of a large heterochromatic sex chromosome may alter the availability of heterochromatizing factors and thereby alter gene expression from autosomes. This is a theoretical mechanism that has not been conclusively shown in humans yet. Research suggesting a role for X and Y chromosome heterochromatin in regulating epigenetic states of autosomes has highlighted unorthodox mechanisms of gene regulation. They have been postulated to contribute to sex-specific susceptibilities to autoimmune and neurological diseases (361).

4. Sex-specific effects of autosomal genetic variants

Surprisingly, some genetic variants at autosomes act in a sex-specific manner. Newly identified genetic loci link adipose and insulin biology to body fat distribution. In a large study of more than 200,000 individuals, 49 loci were found to be associated with waist-to-hip ratio after adjustment for body mass index (359). Twenty of the 49 waist-to-hip ratio loci show significant sexual dimorphism, 19 of which display a stronger effect in women. The identified loci were enriched for genes expressed in adipose tissue and for putative regulatory elements in adipocytes. Pathway analysis implicated adipogenesis, angiogenesis, transcriptional regulation, and insulin resistance as processes affecting fat distribution, providing insight into potential pathophysiological mechanisms, but this analysis did not give an explanation why they had a stronger effect in women (359). Furthermore, common genetic polymorphisms and haplotypes of the chymase gene (*CMA1*) were associated with LV mass only in male but not in female patients with symptomatic AS (295). Polymorphisms in the bradykinin type 1 receptor (*BDKRB1*), a factor involved in the renin-angiotensin system, have also been associated with sex-specific effects (434).

5. Genome-wide expression profiling and proteomics

Genome-wide expression profiling has been used to analyze sex differences in gene expression. Under healthy conditions, genes located on sex chromosomes are usually the ones that exhibit sex-specific expression. Male-specific expression of Y-linked genes, such as *DDX3Y*, *EIF2S3* (Y-linked), and *KDM5D* (also known as JARID1D), is generally observed in mouse hearts, as well as in the human myocardium. Higher expression levels of X-linked genes are detected in female mice, e.g., *Xist*, *Timp1*, and *Ca5b*, and in women, e.g., *XIST*, *EIF2S3* (X-linked), and *GPM6B*. Nevertheless, genes on autosomal chromosomes encoding cytochromes of the monoxygenase family, such as *Cyp2b10*, carbonic anhydrases, such as *Ca2* and *Ca3*, and natriuretic peptides, such as *Nppb*, have also been reported with sex-specific expression levels (164).

In diseased conditions, such as new-onset HF, females usually have higher expression of genes related to energy metabolism than males (35, 116, 142, 148), indicating that

females may be able to maintain their metabolic function in response to a disease stimulus. Mouse models of MI (68) or dietary manipulation (76) have also revealed sex-specific gene regulation. Furthermore, in patients with AS, we recently reported significant sex differences in genes involved in the regulation of fibrosis and inflammation with a significant repression of these processes in women (184), indicating some protection in the female sex against deleterious effects of persistent fibrosis and inflammation. Importantly, rodent models of pressure overload demonstrate sex differences in a similar pattern, including apoptosis, cytoskeletal integrity, fibrosis, and metabolism (119, 185, 186, 425, 432), thereby making a case for the conservation of specific regulatory mechanisms between humans and rodents.

Sex differences were also found in gene expression in human umbilical vein endothelial cells (233, 367). This regulation is believed to reflect mainly the effect of fetal sex, but it could also be affected by the mother's hormones or those hormones produced by the fetus itself. However, these differences are small and under debate (205, 398), while in vitro experiments are performed after 3–4 wk of culturing; therefore, the hormonal effects are thought to be small. These interesting observations can be used to generate hypotheses on relevant pathways that contribute to sex differences in cardiovascular gene expression.

Proteomic approaches have also been taken to investigate cardiovascular sex differences. In a targeted approach, we analyzed extracellular matrix proteins in LV samples of individuals free of cardiovascular disease, which demonstrated an age-dependent sex-specific regulation (107). Overall, the levels of these proteins in younger individuals were lower in women than men, while in older individuals they were higher in women than men (107).

Other studies focusing on mechanisms involved in metabolism and oxidative stress regulation identified several proteins with sex-specific pattern, including members of the apolipoprotein family, carbonic anhydrase 2, desmin, nitrilase 1, and peroxiredoxin 2 (94), most of which are mitochondrial proteins with antioxidant function. Higher female-specific levels of such proteins may contribute to mechanisms resulting in better adaptation under (patho)physiological conditions and give females an advantage.

Considering the estrogen-dependent induction of antioxidant, longevity-related genes (408) and the significantly high levels of phytoestrogens in human diet (30), another study assessed the effect of phytoestrogens on the cardiac proteome also revealing significant sex differences under healthy conditions. In particular, the authors found that several enzymes of the fatty acid metabolism and their transcriptional regulators varied differentially between males and females (353). The role of phytoestrogens in males or females is still poorly understood, but they can influence cardiac mass in ovariectomized mice (281, 282). Nevertheless, an activation of mechanisms in males leading to deleterious effects has been reported. In particular, increased levels of enzyme species involved in oxidative phosphorylation and generation of reactive oxygen species (ROS) were accompanied by decreased amounts of antioxidants in male mice receiving genistein compared with male mice maintained on a phytoestrogen-free basic chow used as control, which have been previously associated with various pathological conditions (353). Similarly, in a model of endothelin-1 overexpression and endothelial NO synthase knockout (KO), cardiac proteome analysis revealed that the protein abundance of the oxidative stress related enzyme superoxide dismutase presented with sexual dimorphism potentially leading to decreased male-specific antioxidant capacity (405).

Recently, we determined global changes in protein abundance due to sex and ERβ in pressure overload and found major sex and ERβ-dependent differences together with a complex interaction of the two factors (185). The pathways involved include metabolism, p38 MAPK signaling, and cytoskeletal regulation revealing a better adaptation of female than male mice to pressure overload. Importantly, our study revealed previously unrelated proteins to the development and progression of pressure overload-induced LV hypertrophy. These include cofilin 2 (Cfl2) and pyruvate kinase 2 (Pkm2). Given the lack of information between regulation of these proteins and LV hypertrophy, we have postulated that the induction of these proteins in female mice deficient of ERβ might underlie a better structural and metabolic adaptation to pressure overload.

B. Epigenetic Mechanisms

A number of epigenetic DNA and histone modifications have been described that arise at specific time points in development and modulate gene expression. Environmental factors can modify epigenetic marks in a sex-specific manner leading to sex differences in CVD in the individual and following generations. We discuss epigenetic mechanisms that play an essential role in cardiovascular phenotypes, such as myocardial hypertrophy and HF. **FIGURE 5** illustrates a novel hypothetical model of sex-specific DNA and histone modifications, which may also be affected by sex hormones and their receptors.

1. Histone and DNA modifications

Epigenetic changes include methylation of cytosines in the primary DNA sequence, or histone modifications, such as acetylation and methylation. These modifications can last long term, such that epigenetic modulation early in development can alter the phenotype much later in life (448). Some epigenetic modifications can persist in the germ cells and can influence subsequent generations (138, 275). A

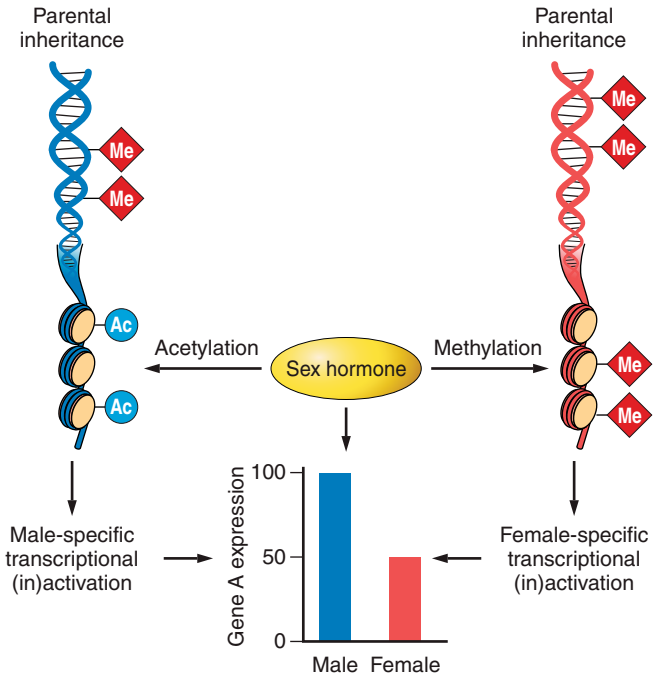

FIGURE 5. Sex- and sex hormone-specific DNA and histone modifications. Sex-specific epigenetic marks from the parent are transmitted to the offspring leading to sex-specific transcriptional (in)activation. Sex hormones may directly regulate DNA and histone-modifying enzymes to modulate the epigenetic profile in a sex-specific manner.

Dutch study showed that famine-induced epigenetic modifications occurred in a sex-specific manner (389). For 6 of 15 loci studied, significant differences in DNA methylation after famine exposure during pregnancy were observed. This association differed by sex for three loci (*INSIGF*, *GNASAS*, and *LEP*) with stronger methylation in men (389). In the Dutch birth cohorts from Amsterdam, Rotterdam, and Leiden examined at age 59 years, there was a moderate increase in systolic blood pressure and prevalent hypertension in men and women with prenatal famine exposure compared with unexposed controls (368). However, more studies are needed to establish robust associations between famine during pregnancy and clinical conditions (238).

Steroid hormones can induce, among others, modification of histones. Androgen or estrogen receptors act by binding to hormone response elements in the DNA and attract various cofactors that have inherent histone acetyltransferase or methyltransferase activity. This is particularly known for the CREB binding protein (CBP) and E1A binding protein p300 (EP300) (12, 121, 122, 134, 214, 215). The histone-modifying enzymes alter the epigenetic state of gene promoters to which the nuclear receptors bind, thereby changing gene expression.

The induction of DNA or histone demethylation or histone deacetylation has been implicated in CVD-dependent gene regulation. Histone acetyltransferases and deacetylases control cardiac hypertrophy, and a dysregulation of histone methylation profiles is found in HF (179, 392, 446). These mechanisms bear the potential for sex-specific regulation, since DNA modifying enzymes, e.g., histone acetyltransferases CBP and EP300, are recruited to the DNA by estrogen and androgen receptors. The X and Y chromosome encode some DNA demethylases, such as the Jarid family and others, that are activated by estrogen. Some of these genes undergo incomplete X inactivation or are only expressed from the Y chromosome and have therefore the potential to cause different effects between males and females. We therefore hypothesize that sex-specific DNA deacetylation or demethylation controls sex-specific gene transcription in the development of CVD and its course, thereby determining sex- and disease-specific cardiovascular phenotypes.

2. Non-coding RNA

Non-coding RNA species represent recently discovered mechanisms that control gene transcription by binding of RNA fragments to regulatory DNA sequences. Different types of non-coding RNA may contribute to sex differences. MicroRNAs (miRNAs) are small non-coding RNAs of ~22 nucleotides that inhibit gene expression pairing to the 3' untranslated region (3' UTR) of target messenger RNAs (mRNAs). The expression of several 100s different miRNAs has been described in the mouse heart, many of which are regulated during the development of LV hypertrophy (53, 70, 346, 378, 390, 401). However, so far only few data exist about sex-specific regulation of miRNAs and their role in the observed sex differences in heart disease.

Our group has proposed sex-specific and estrogen-dependent regulation of miRNAs as a potential mechanism that may lead to sex differences particularly in fibrosis. Recently, we showed for the first time that TAC led to the sex-specific regulation of cardiac miRNAs (320). In particular, a large number of miRNAs were upregulated in males but not in females. In fact, we obtained evidence for the sex-specific expression of functionally related miRNA-21, -24, -27a, -27b, 106a, and -106b and the regulation of their expression by estrogen and ERβ (320). Functional target sites for these miRNAs are located on three repressors of the MAPK signaling pathway, i.e., Rasa1, Rasa2, and Spry1, which may all lead to cardiac fibrosis. miRNA-21 has already been linked to fibrosis (386). Our data suggest that the sex-specific expression of specific miRNAs is related to sex differences in fibrosis under pressure overload.

Recently, we also aimed at the analysis of cardiac miRNA regulation by sex and ERβ and their target proteins related to mitochondrial metabolism (343). After TAC, we found 34 miRNAs upregulated, 31 of which were induced only in males. Pathway enrichment analysis of potential targets of male-specific upregulated miRNAs identified mitochondrial metabolism, MAPK signaling, and extracellular ma-

trix organization. Six mitochondrial proteins, i.e., Auh, Crat, Decr1, Hadha, Hadhb, and Ndufs4, carrying putative binding sites for the male-specific induced miRNAs were reduced only in males under pressure overload. The deletion of ERβ induced miRNAs in unstressed animals of both sexes. However, in ERβ-deficient mice, the upregulation of miRNAs in the males under pressure overload was abolished. Thus, under pressure overload, the regulation of miRNA expression and of relevant targets differs significantly between the sexes. ERβ plays a major role in this process by modifying pressure overload-induced regulation of miRNA in a sex-specific manner.

C. Sex Hormones and Sex Hormone Receptors

1. Synthesis and metabolism of sex hormones

Sex hormones are synthesized early in embryonic development and act through a number of different mechanisms. Sex hormones belong to a large family of endogenous signaling molecules that can modulate cellular processes via gene regulation and protein modification. In fact, androgens, estrogens, and progesterone affect the cardiovascular system leading to sex differences. The bulk of sex hormones is synthesized in the gonads, but extragonadal synthesis in cardiomyocytes or neurons, among others, also occurs. In particular, testosterone may be metabolized via aromatase to estrogen, thereby allowing estrogen to contribute to pathophysiology in males. In this review, we will mainly focus on estrogen and its receptors and to a lesser extent on androgens, while progesterone will not be addressed.

Androgens are mainly produced in the Leydig cells of the testis but also to a lesser extent by the adrenal gland, in the adipose tissue and bone (34, 268). In fact, it has been suggested that the rise in cardiovascular mortality in women following menopause may be due to an increased ovarian production of testosterone, which is in part stimulated by high levels of circulating gonadotropins (364). Nearly all organs, including the brain and cardiovascular tissues, express the androgen receptor (AR) and are responsive to androgens (4, 86, 257, 260).

Testosterone is the most important natural androgen. Testosterone's highly active metabolite is dihydrotestosterone (DHT), which also exerts its effects through binding to the AR, particularly with a two- to-fivefold higher binding capacity to the receptor (15). DHT is the most potent androgen in the body being ~10-fold more potent in inducing AR-mediated signaling than testosterone (15, 371, 439). In fact, the main physiological role of DHT is expected to be the amplification of testosterone-dependent actions, such as the formation of reproductive organs (244).

DHT is converted from testosterone by isoforms 1–3 of the enzyme 5-α-reductase, which are expressed at similar levels in hearts of male and female mice (15, 431). However, it was recently shown that isoform 3 of 5-α-reductase (Srd5a3) is the predominant cardiac isoform (451). Nevertheless, it was reported that the expression of all 5-α-reductase isoforms is increased in human and mouse hypertrophic hearts, thereby leading to increased abundance of DHT (451). Similarly, increased levels of DHT were found in preparation of microsomes from human hypertrophic hearts (385). However, epidemiological studies and clinical trials have shown contradictory effects, where testosterone appears to improve functional exercise capacity in patients with HF (252, 296, 391). On the other hand, long-term anabolic-androgenic steroid use has been associated with myocardial hypertrophy and LV dysfunction (21, 341). A current hypothesis is that testosterone may be beneficial, while DHT may exert deleterious actions (40).

The major circulating estrogen is 17β-estradiol (E2), which binds equally to both ERα and ERβ (197). Other naturally occurring estrogens include estrone and estriol, the latter being the weakest of all estrogens, while E2 has the strongest potency. The primary sources of production of E2 are the theca and granulosa cells of the ovaries (136). However, E2 can also be produced locally as a result of the conversion of testosterone by the enzyme aromatase (362). Aromatase is present in a number of extragonadal tissues, such as the adipose tissue, bone, brain, heart, and the vasculature in both sexes (27, 370). Notably, there is a highly significant increase in E2 levels associated with inflammation and obesity. This increase results from aromatase conversion of androgen to estrogen in adipose tissue contributing significantly to the circulating pool of E2 (280). Consequently, in men, E2 is produced in significant quantities by local tissue aromatization of androgenic precursors from the testes and adrenal glands (241). In fact, in obese men, there is a marked increase of E2 production (74), and elderly men may have higher concentrations of E2 compared with age-matched women (54).

2. Sex hormone receptors in the cardiovascular system: relevant subtypes and regulation

Sex hormone-activated receptors, i.e., AR, ERα, ERβ, and the G protein-coupled receptor GPR30, affect a number of genomic and nongenomic pathways in a cell- and sex-specific manner. Regulation of these receptors in the cardiovascular system in different localizations and disease conditions is still not well understood.

The AR, ERα, and ERβ are members of the evolutionary related superfamily of nuclear steroid hormone receptors (206, 415). They are intracellular proteins that regulate gene expression in a hormone-dependent fashion upon their activation (47, 112, 206, 415). Like all steroid receptors, the AR, ERα, and ERβ have a conserved zinc finger-based DNA-binding domain (DBD) (C region), which contains regions that mediate dimerization, a COOH-terminal

ligand-binding domain (LBD) (E region), which contains the ligand-dependent activation function (AF-2) domain and mediates ligand binding, and they bind to specific nucleotide sequences (112, 393). Additionally, they contain an NH_2-terminal A/B domain with constitutive activation function (AF-1) and a hinge domain (D region) (393).

Several ER isoforms have been identified. The full-length 66-kDa ERα protein is composed of six domains (A to F), while there is a physiologically expressed 46-kDa isoform lacking the entire A and B domains, thereby being devoid of AF-1 (26). Alternative splicing events can generate a 55-kDa isoform, whose levels are enough to mediate several actions of E2 in the vessel wall of mice (307), as well as a 61-kDa isoform, whose transactivation capability can be as high as 75% of the WT ERα in uterine tissue of mice (201). Several polymorphisms for human ERα and ERβ have also been reported. In particular, ERα polymorphisms correlate with increased risk of MI (351, 356), while ERβ polymorphisms are associated with increased LV mass and LV wall thickness (309). In line, the human AR located on the X chromosome also exhibits polymorphisms, which are associated with higher maximal LV wall thickness (227).

Functional ERα and ERβ have been identified in the vascular endothelium, vascular smooth muscle cells, cardiac fibroblasts, and cardiomyocytes of male and female individuals (135, 189, 192, 234, 265, 402). Of interest, levels of ERα mRNA are similar in hearts of both men and women, while ERβ mRNA levels are higher in male than in female human hearts, including hearts without recognizable pathology that were not suitable for transplant due to technical reasons (286). We also demonstrated the implication of both ERs in human cardiac disease through the upregulation of mRNA levels of both receptors in the myocardium of patients with AS (286) and the elevated mRNA and protein levels of ERα in end-stage failing hearts (247). We also visualized a significant change in the intracellular localization of ERα in failing hearts, away from the intercalated disk, where it is also usually found in healthy hearts (247). We speculate that this may lead to mechanical instability of the intercalated disk in HF. Mechanistically, our studies identified that the nuclear factor κB (NF-κB) pathway is a part of the regulatory mechanism involved in ERα expression in the human heart (249) and atrial natriuretic peptide precursor A (NPPA) interacting with ERα in an E2-dependent manner (251). Studies in the vasculature revealed that ERα levels are lower in atherosclerotic coronary arteries compared with normal arteries of premenopausal women (234).

A pool of ERs is localized at the plasma membrane, which mediates the rapid extranuclear activity of E2 (11, 435). In addition to the membrane and nuclear localization of ERs, ERα and ERβ have also been detected in mitochondria (66, 440). Notably, in response to ischemia/reperfusion, myocardial mitochondria of ovariectomized rats and ERα KO mice showed noticeable ultrastructural damage and a decrease in mitochondrial respiratory chain function (444, 445). Treatment with E2 reversed the mitochondrial dysfunction (324, 376).

The first mouse models of ERα gene disruption included a global model showing effects on fertility and the reproductive system (106, 235), while the use of conditional site-specific recombination system enabled tissue-specific manipulation of ERα (264). These models have been extensively used to understand cardiovascular (patho)physiology, and several examples will be discussed in subsequent sections. Newer models have been generated to investigate potentially different functions specific to different localizations. In particular, to explore the role of the two activation functions (AFs), AF-1 and AF-2, mice lacking ERαAF-1 or ERαAF-2 were generated (1, 7, 31). More recently, a mouse model with a point mutation of the palmitoylation site of ERα (C451A-ERα) that leads to membrane-specific loss of function of ERα was generated, resulting in the abrogation of E2 vascular actions, such as rapid dilation, acceleration of endothelial repair, and endothelial NO synthase phosphorylation (3).

A third receptor, the G protein-coupled receptor (GPR30), has been proposed to be an ER mediating nongenomic effects of E2 (117, 118, 335), whose role, though, as an actual ER binding E2 in a direct manner or an interacting partner of the classical ER has been debated (221, 297). GPR30 has been shown to be expressed in certain endothelial cells (163); however, its role in mediating E2 actions in the endothelium is currently not understood mainly due to conflicting reports using several ER gain- or loss-of-function models (44, 141, 304).

3. Genomic actions of sex hormone receptors

The genomic actions of sex hormones are mainly mediated by their receptors that function as ligand-activated transcription factors. Following the diffusion of the sex hormone into the cell, it binds to the ligand-binding domain of the receptor, leading to the dissociation of the receptor from its cytoplasmic chaperones (365). Following nuclear translocation, the formation of a homodimer or a heterodimer ensues (79). The hormone-receptor complex then binds to hormone response elements situated in the promoter or enhancer region of target genes (279). The binding to hormone response elements is either direct or indirect through tethering with other transcription factor sites, such as activator protein 1 (AP-1) or specificity protein 1 (SP-1) (92). Interestingly, the interaction of the ER with NF-κB has been shown to mediate E2-dependent transcriptional regulation of genes that lack estrogen response elements (326). Recruitment of cell- and sex-specific cofactors is necessary and determines their action. In particular, interactions of the hormone-receptor complex with other transcriptional co-

factors, i.e., co-activators and co-repressors, facilitate modulation of transcription of target genes (144, 251, 357). Our previous studies have shown that E2 regulates gene transcription in cardiac tissue and cells and that this regulation occurs in a sex-specific manner (182, 183, 188, 312). For example, E2 induces a female-specific increase in progesterone receptor levels, which might confer females protection (182). On the other hand, E2 induces a male-specific increase in myosin regulatory light-chain interacting protein (MYLIP), which leads to reduced contractility in males (183).

4. Nongenomic effects of sex hormone receptor activation

In addition to the classical genomic effects, sex hormones have been demonstrated to exert rapid nongenomic effects. These actions are mediated either by the classical receptors located in or adjacent to the plasma membrane or by other plasma membrane-bound receptors (89, 92). These effects are so rapid that phenotypical changes may occur in a matter of minutes following exposure to the sex hormones, and normally they affect signaling. For example, the nongenomic activities of estrogen include the activation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) and Src, as well as the increased phosphorylation of c-Jun-NH_2-terminal protein kinase (JNK) (246, 289, 366). Particularly in the myocyte, estrogen modulates signaling through phosphoinositide 3-kinase (PI3K), PKB, glycogen synthase kinase 3β (GSK3β), β-catenin, calcineurin, mechanistic target of rapamycin (mTOR), ERK1/2, p38 MAPK, JNK, and others. Together, these data demonstrate that estrogen interferes with a vast number of cytoplasmic signaling pathways through rapid nongenomic mechanisms, thereby altering cellular function. Notably, the nongenomic actions of sex hormones are not dependent on changes in gene expression for their action. However, the rapid induction of gene expression has also been reported (89, 246).

5. Manipulation of sex hormones in (patho)physiological animal models

To understand sex differences in animal models, several studies embarked on the analysis of the contribution of sex hormones to sex differences employing animal models and incorporating ovariectomy (OVX), orchiectomy, along with hormone substitution protocols. Most studies have focused on the effects of estrogen and related compounds, while testosterone has been less frequently studied. It is important, however, to consider the way of administration of the hormone, the hormonal levels reached in the circulation, and the limitation that these hormones are usually given transdermal or intramuscular to young animals, which is different to the situation in humans.

In ischemia, E2 administration has been shown to reduce infarct size and to improve postischemic myocardial function in a number of different animal models, including rabbits, mice, and rats (36, 37, 145, 216, 283, 303). The specificity of this effect was frequently documented by its blockade by the ER antagonist ICI182780 (36, 104). Furthermore, hearts from OVX mice and rats exhibited a greater infarct size, impaired functional recovery, and worse remodeling, which were reversed by E2 administration in physiological doses (195, 231, 283).

E2 supplementation has also been shown to exert antihypertrophic effects in different pressure overload models, and several mechanisms have been identified. In particular, E2-mediated inhibition of LV hypertrophy and prohypertrophic gene expression in the TAC model was reported (399). It was demonstrated that E2 exerts profound antihypertrophic effects, which were mediated through the regulation of atrial natriuretic factor (ANF) and myosin heavy chain beta (MHCβ) (16, 302). In another study, changes observed in OVX rats, such as a significant increase of LV hypertrophy, cardiomyocyte diameter and heart weight-to-body weight ratio, and a decrease in fractional shortening and ejection fraction, were largely reversed by administration of E2 (83). Our own studies have aimed to identify the receptor involved. We administered E2 and the selective ERα agonist 16α-LE2 for 9 wk after induction of pressure overload by TAC. Both slowed the progression of LV hypertrophy leading to reduced systolic dysfunction and fibrosis (427). We therefore concluded that ERα inhibits myocardial fibrosis in female mice under pressure overload.

While estrogen exerts antihypertrophic effects in the diseased heart, its role in the healthy heart is less studied. Recently, we found that long-term oral E2 administration induced physiological hypertrophic growth in the healthy C57BL/6J mouse heart, and this was characterized by an increase in nuclear β-catenin (188). In a model of cardiac β-catenin deletion, our surprising finding was that E2 had the opposite effects in WT littermates with a C57BL/6N background (188). Thus E2 exerts contradictory effects on postnatal cardiac growth in mice with distinct genetic backgrounds through the regulation of β-catenin.

In a mouse model of pressure overload-induced hypertrophy, treatment with finasteride, which inhibits the 5-α-reductase-dependent DHT generation, reduced mortality, inhibited pathological hypertrophy and fibrosis, LV dilation, and dysfunction in both male and female mice (451). Similarly, the removal of androgens through orchiectomy prevented adverse myocardial remodeling and dysfunction in response to several stressors (56, 126, 225, 272). Collectively, these data suggest that increased androgen levels play a major role in cardiac pathophysiology of both sexes.

6. Role of sex hormone receptors in cardiovascular disease models

To verify whether ERα and/or ERβ mediates the beneficial effects of E2, ERα- and ERβ-selective agonists have been used. A number of studies point to a cardioprotective effect of ERα agonists. Treatment with the ERα agonist 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) and E2, but not with the ERβ agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN), resulted in a significant reduction of infarct size in a rabbit model (38). Along the same lines, ERα agonism with PPT or ERA-45 reduced infarct size and accompanying inflammation (169, 287). In another model of MI, administration of PPT and a membrane-impermeable estrogen-albumin construct revealed that cardiac fibrosis was attenuated by inhibiting RhoA/ROCK/cofilin signaling through a membrane-bound ERα-mediated mechanism (217). Comparing ERα agonism with PPT and ERβ agonism with DPN in the same setting showed that both conferred protection following ischemia/reperfusion (412). However, agonism of ERβ also mediates strong cardiovascular effects. The ERβ agonist DPN improved postischemic recovery of isolated perfused hearts (283, 442). In a rat model of trauma-hemorrhage, DPN, as well as E2, protected against the consequences of ischemia (153).

Data on the potential role of GPR30 have also been emerging with postischemic contractile dysfunction and infarct size being significantly reduced in animals treated with the highly specific GPR30 agonist G1 compared with untreated controls (93). Assessment of the underlying mechanisms suggests that G1-mediated activation of GPR30 improves functional recovery and reduces infarct size in isolated rat hearts following ischemia/reperfusion through a PI3K-dependent, sex-independent mechanism (93). Interestingly, GPR30 seems to regulate contractile function through PI3K, among other signaling pathways, also in skeletal muscle (209).

7. Genetic deletion of sex hormone receptors

Hormone receptors have also been modulated genetically in models of myocardial ischemia, pressure overload, and others. Frequently, global KO models for sex hormone receptors have been used. However, such global KO models reflect effects in the whole body associated with counterregulatory effects, thereby being of limited use for cell-specific hypothesis testing. Therefore, cell-specific KO models have been developed more recently.

Several studies tried to verify the role of ERα and ERβ in mediating beneficial effects of E2 during LV hypertrophy using mice with genetic deletion of ERα (ERKO) and/or ERβ (BERKO). Early studies suggested a protective role for ERβ (17). In other studies, genetic deletion of ERα did not affect LV hypertrophy in TAC models, while deletion of ERβ did (363), suggesting a greater role for ERβ in attenuating the hypertrophic response to pressure overload. Due to these diverging findings, we investigated the effect of ERβ deletion under TAC conditions in males and females and found that WT males had more LV hypertrophy, greater cardiomyocyte hypertrophy, and more fibrosis (119). Deletion of ERβ increased LV hypertrophy and fibrosis in a different manner between males and females (119). In particular, we concluded that ERβ promotes fibrosis in males, but it inhibits fibrosis in females. ERβ limits cardiomyocyte hypertrophy and inhibits apoptosis in both sexes, but with a greater antiapoptotic effect in male hearts that actually exhibit more apoptosis than females. Thus, under pressure overload, the loss of ERβ is detrimental for both males and females but for different reasons.

Similarly, we found recently that sex differences in exercise-induced physiological myocardial hypertrophy are also modulated by ERβ (108). Physical exercise induces physiological myocardial hypertrophy. The sex-specific response of the heart to exercise is mediated by sex-specific regulation of PKB and MAPK signaling pathways, protein synthesis, and mitochondrial adaptation in an ERβ-dependent manner (108). In line, sex-specific modulation of adipose fatty acid metabolism during exercise may result in alterations of circulating free fatty acids, which leads to sexual dimorphic changes in cardiac substrate utilization and cardiac hypertrophy (120). Our recent studies have demonstrated that E2 also induces physiological cardiac growth via β-catenin signaling in an ERα-dependent manner (187, 188).

Several studies have also attempted to determine the role of ERα or ERβ in mediating the beneficial actions of E2 in ischemia/reperfusion using ERKO and/or BERKO mice. Under hypercontractile conditions, female BERKO mice exhibit a significantly greater degree of ischemia/reperfusion injury than ERKO or WT female mice (123), suggesting a protective role for ERβ. It was also shown that E2 treatment resulted in smaller infarct size in OVX ERKO mice than in OVX BERKO mice (18), again pointing towards an important role for ERβ. Similarly, deletion of ERβ in OVX mice subjected to chronic MI increased mortality and aggravated biochemical markers of HF (306). In another study, although no significant differences in overall mortality, infarct size, and parameters of LV remodeling were found when comparing infarcted ERKO and BERKO mice with infarcted WT mice, it was found that ERβ deficiency resulted in prolonged ventricular repolarization and decreased ventricular automaticity in female mice with chronic MI (200). These observations support a relevant role for ERβ in mediating an attenuated response to cardiovascular tissue injury in females.

Other studies have reported that the cardioprotective effects of E2 are mediated by ERα. For example, it was shown that female ERKO mice subjected to ischemia/reperfusion

had a similar recovery with WT and ERKO males, while WT females had a worse recovery (417). In a similar study, it was demonstrated that the deletion of ERα is associated with more severe cardiac damage following ischemia/reperfusion injury (444). Endothelial ERα was also shown to play a crucial role in the E2-induced prevention of endothelial dysfunction after ischemia/reperfusion. In fact, it was demonstrated that targeting endothelial protection per se can confer cardiomyocyte protection under ischemia/reperfusion conditions (115). Although these studies utilizing ERKO and/or BERKO mice were not able to provide a clear consensus regarding which ER mediates the protection against cardiac injury, they actually suggest that both ERs may be involved in the cardioprotective effects of E2.

8. Overexpression of sex hormone receptors

Our own recent studies with a unique cardiac-specific overexpressing (OE) model of ERα show that this receptor protects the heart against ischemic injury (250). At baseline, unstressed male and female ERα-OE mice showed increased LV mass, LV volume, cardiomyocyte length, increased expression of markers of LV hypertrophy, as well as reduced markers of mitochondrial function and sarcoplasmatic Ca^{2+} transport compared with WT mice. Nevertheless, while ERα-OE mice exhibited a similar infarct size with WT mice, they had a lower increase in LV volumes and smaller loss in wall thickness, as well as a lower induction of profibrotic genes than WT mice. ERα-OE mice also exhibited enhanced expression of angiogenesis and lymphangiogenesis markers and neovascularization in the peri-infarct area in both sexes. Thus cardiomyocyte-specific ERα induces neovascularization and attenuates profibrotic gene expression probably through paracrine actions.

Recently, we have also overexpressed ERβ in cardiomyocytes **(FIGURE 6)** (352). We found that 2 wk after MI, ERβ-OE males and females showed improved survival. ERβ-OE was associated with attenuated LV dilation, smaller increase in heart weight and less lung congestion, as well as improved systolic and diastolic function in both sexes. Two potential pathways for ERβ-mediated myocardial protection were identified. First, male and female ERβ-OE mice had a lower reduction of the cardiac SR Ca^{2+}-ATPase (SERCA2a) expression after MI, suggesting less reduction in diastolic Ca^{2+} reuptake into SR post MI. Second, male ERβ-OE revealed attenuated cardiac fibrosis in the remote LV tissue and expression of fibrosis markers, such as collagen type I and III, periostin and miRNA-21. Thus ERβ-OE led to improved survival, reduced maladaptive remodeling, improved cardiac function, and less HF development after MI in both sexes. These effects seem to be related, at least in part, to a better maintenance of Ca^{2+} cycling and a lower induction of cardiac fibrosis after MI.

9. The four core genotype model: chromosomal versus hormonal effects

Even though a lot of meaningful results have been obtained by the aforementioned animal models, a lot of questions still remain open. Most studies have suggested that female sex, as well as estrogens, are protective. This has led the field to develop more sophisticated models to address whether differences in chromosomes or hormones mainly account for the observed sex differences. To this extent, the four core genotype (FCG) model has been developed and suggested that chromosomes, as well as hormones, play a major role and interact in a complicated manner.

In the FCG mouse model, gonadal sex and sex chromosome complement are uncoupled (90). In particular, the testis-determining gene, *Sry*, has been moved from the Y chromosome to an autosome. Consequently, FCG mice comprise XX and XY gonadal males (XXM and XYM) and XX and

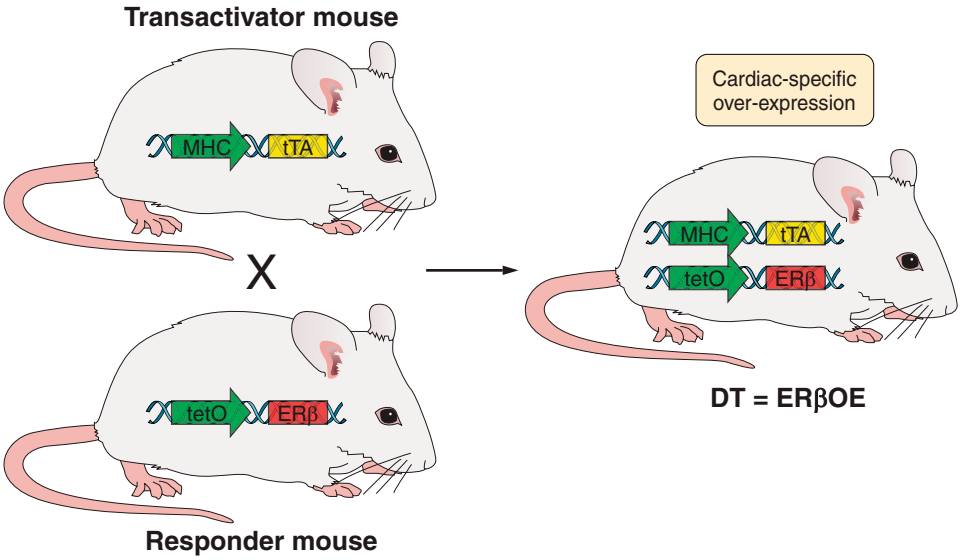

FIGURE 6. Generation of an inducible cardiac-specific overexpression (OE) mouse model of ERβ. Crossing of a transactivator (tTA) mouse line that expresses a transactivator under a cardiomyocyte-specific promoter, i.e., myosin heavy chain (MHC), with a responder mouse that expresses ERβ under the control of the transactivator results in a double-transgenic (DT) ERβOE mouse line.

XY gonadal females (XXF and XYF) (243). Therefore, differences can be studied in XX versus XY mice that have the same sexual phenotype, same type of gonads, and same sex hormone levels. Consequently, it can be used to dissociate the effects of sex hormones from the sex chromosomes.

Sex hormones may exert acute effects, the so-called activational effects (8). On the other hand, sex hormones may also induce longer lasting effects, such as changes in DNA structure and chromatin remodeling, which are the so-called organizational effects (8). Androgens and estrogens together with their receptors induce sex-specific DNA modifications by recruiting DNA modifying enzymes, which lead to the induction of activational or repressive marks on target genes, ultimately regulating their expression. These marks can persist in the absence of sex hormones during cell division, thereby transmitting epigenetic regulation over the life span of a cell or an organism. If animals are gonadectomized, organizational and activational effects of sex hormones can be distinguished (9). Gonadectomy equalizes the levels of gonadal hormones among groups. Under these conditions, when effects of gonadal sex are found, they are most likely caused by organizational effects of gonadal hormones, i.e., effects of gonads in early development, before gonadectomy (9).

The FCG mouse model has been used to investigate protective mechanisms from cardiac ischemia/reperfusion injury and the role of sex chromosomes (224). It was shown that XX male and female mice are more susceptible to this type of injury compared with XY male and female mice attributed to the extra copy of X chromosome and not the absence of the Y chromosome (224). Similarly, the hearts of XY male and female mice were less vulnerable to coxsackie virus B3 than those of XX male and female mice, thereby developing significantly less myocarditis than XX mice (337). However, the levels of myocarditis measured were significantly higher in XY male mice compared with XX female mice, but the opposite occurred in XY male and XX female mice, which were gonadectomized, thereby suggesting a complex interaction of sex chromosome complement with sex hormones.

D. Sex Differences in Biological Processes in Cardiovascular Cells

As a result of the function of sex chromosomes, sex hormones, and their receptors, several cellular processes differ between male and female cells. Here, we discuss ion handling and rhythmicity, mitochondrial function and energy metabolism, cardiac lipid and carbohydrate metabolism, cell death and survival, inflammation, fibrosis, vascular function, and gene expression. An overview is given in **FIGURE 7**.

1. Ion handling and rhythmicity

There are several electrophysiological differences between men and women, including faster resting heart rates and longer rate-corrected QT intervals in women than in men (2, 263, 325). Women are also more susceptible to drug-induced QT prolongation and torsades de pointes (100, 220, 333). On the other hand, ventricular arrhythmias and sudden cardiac death are more common in men than in women, and male sex is a strong predictor of risk for atrial fibrillation (6, 14, 28, 50, 180). These phenotypes can be caused by pathological mechanisms affecting ion channels or factors related to regulatory pathways of ion channels in a sex-specific manner.

In particular, there are marked sex differences in Ca^{2+} handling. These are expected to be due to sex-specific Ca^{2+} homeostasis protein regulation, thereby altering functional outcomes, such as force development and relaxation (72). To this extent, female rodents demonstrate smaller Ca^{2+} transients and have reduced cardiac reserve, smaller changes in shortening, and less SR Ca^{2+} loading compared with males in response to β-adrenergic stimulation (64, 411). Mechanistically, these sex differences are partly mediated by the cAMP/protein kinase A (PKA) pathway (299), which may be due to the function of sex chromosomes or sex hormones.

In particular, SR Ca^{2+} content and peak Ca^{2+} transient amplitudes are significantly increased in OVX rodents compared with sham-operated rodents, while these effects are reversed by E2 treatment (84, 114, 204, 239). It has also been shown that Ca^{2+} homeostasis is further regulated as a function of the estrous cycle (240).

In addition, *S*-nitrosylation of the L-type Ca^{2+} channel is increased in female hearts following ischemia/reperfusion, leading to reduced Ca^{2+} entry and SR loading, thereby reducing tissue injury (375). Conversely, OVX leads to decreased cardiac endothelial NO synthase levels and increased expression of the L-type Ca^{2+} channel in rats, while E2 treatment reversed these effects (71, 288). Furthermore, E2 reduced the Ca^{2+} current and intracellular Ca^{2+} concentration in guinea pig cardiomyocytes (172), while cardiomyocytes from ERKO mice exhibited an increased expression and activity of the L-type Ca^{2+} channel (174).

The sensitivity of myofilament response to Ca^{2+} is regulated by estrogen administration (49, 71, 384). In particular, myofilament Ca^{2+} sensitivity is increased in hearts of OVX rats compared with sham-operated rats, while this effect is reversed by E2 treatment (49, 422, 423). Similarly, $Ca_V1.2$ protein levels and the gain of excitation-contraction coupling are higher in hearts from OVX rats compared with those of sham-operated rats (71, 114).

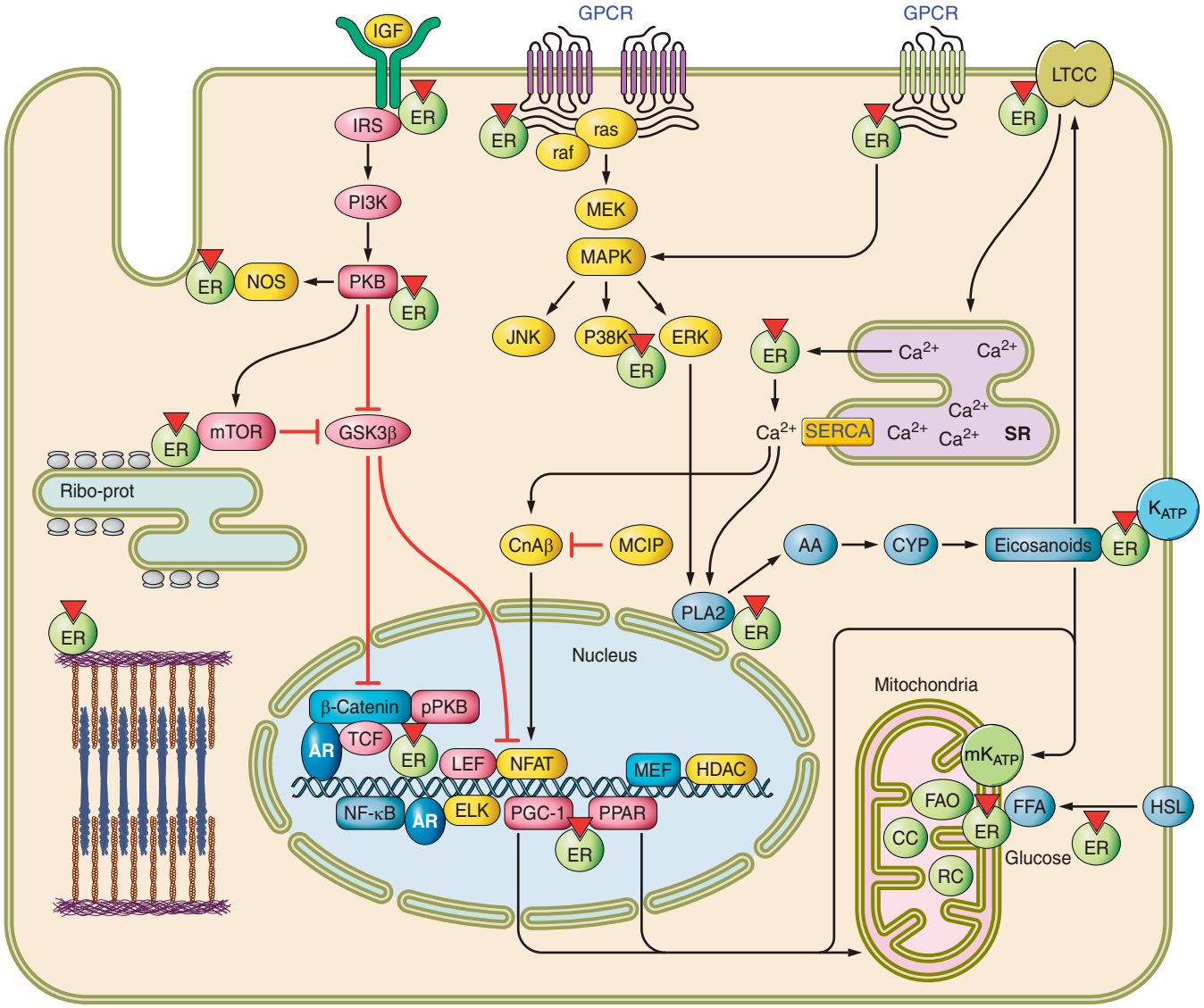

FIGURE 7. Interaction of sex hormones with intracellular signaling relevant for the cardiovascular system. Selected abbreviations: PKB, protein kinase B; CnA-β, calcineurin Aβ; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; GSK3β, glycogen synthase kinase 3β; HSL, hormone-sensitive lipase; IGF, insulin-like growth factor; JNK, c-jun-NH_2-terminal kinase; LTCC, L-type Ca^{2+} channel; MAPK, mitogen-activated protein kinase; MCIP, myocyte-enriched calcineurin interactin protein; MEF2, myocyte enhancer factor 2; mTOR, mammalian target of rapamycin; NFAT, nuclear factor of activated T-cells; NF-κB, nuclear factor κ, B cells; NOS, nitric oxide synthase; PGC-1, peroxisome proliferator-activated receptor gamma coactivator 1; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog.

Testosterone also modulates ion channel homeostasis. For example, in isolated cardiomyocytes, testosterone rapidly stimulates intracellular Ca^{2+} activating phospholipase C and the inositol-3-phosphate pathway (403). Gonadectomy leads to the downregulation of Ca^{2+} channel regulatory proteins in the hearts of male rodents, which is reversed by testosterone treatment (131–132). Along this line, L- and T-type Ca^{2+} currents are induced in neonatal rat cardiomyocytes treated with testosterone in an AR-dependent manner (110, 266). However, chronic testosterone treatment can have opposite effects from acute testosterone treatment on L- and T-type Ca^{2+} channels and Ca^{2+} sparks (110, 266). These contradictory effects are currently poorly understood, and the role of aromatase is not clear. In chronic testosterone treatment, aromatase converting testosterone to estrogen might contribute to the latter repressing L- and T-type Ca^{2+} channels. Testosterone may also shorten action potential duration by activating potassium channels leading to shorter QT interval (22, 232).

Female sex is associated with greater levels of the sarcolemmal and mitochondrial ATP-sensitive potassium (K_{ATP}) channels, whose inhibition during ischemia increases the degree of tissue injury (175, 323). Interestingly, E2 treatment led to increased sarcolemmal K_{ATP} channel levels and

to the protection of cardiac cells against hypoxia/reoxygenation injury (218, 322).

Furthermore, E2 leading to the increased activity of large-conductance Ca^{2+}-activated K^+ channels in mitochondria of rat cardiomyocytes (292) and to the modulation of K^+ currents in male diabetic rat cardiomyocytes by interacting with angiotensin (358) exerts antiarrhythmic effects. In fact, estrogen has been demonstrated to attenuate the occurrence of ischemia/reperfusion-induced arrhythmias through the modulation of NO and Ca^{2+}-activated potassium channels (284, 420). However, the antiarrhythmic actions of acute E2 treatment are more prominent in females than in males (315).

Several genetic models of ion handling proteins also exhibit major sex differences. For example, deletion of the *Fkbp1b* gene, a SR protein regulating the ryanodine Ca^{2+} release channels, led to myocardial hypertrophy in males but not in females (436). Similarly, ablation of phospholamban (*PLN*) exacerbated ischemic injury to a higher extent in males than females (82). On the other hand, overexpression of *PLN* led to ventricular hypertrophy and mortality in male mice aged 15 mo, while there were no such effects in age-matched female mice (87).

2. Mitochondrial function and energy metabolism

Female mitochondria exhibit lower oxidative damage under stress underlain by higher antioxidant gene expression than male mitochondria (39). In cardiomyopathy induced by doxorubicin, which is a member of the anthracycline family and an effective broad-spectrum chemotherapeutic drug targeting topoisomerase-II to trigger cell death (382), male mice exhibit significantly higher mortality than female mice (277). The underlying mechanisms seem to include significant decreases in the levels of total adenosine monophosphate-activated protein kinase and in markers of mitochondrial biogenesis and cardiolipin content only in males (277). In vascular smooth muscle cells isolated from rat aorta, female cells show better survival and seem to be more resistant to oxidative stress than those isolated from males (253).

In addition, female mice displayed improved recovery of cardiac mitochondrial respiratory function and higher ATP levels versus males in response to acute oxygen deprivation, underlain by diminished transcript levels of *Ppara*, muscle-type carnitine palmitoyltransferase 1 (*Cpt1b*) and pyruvate dehydrogenase kinase 4 (*Pdk4*) (111). Cardiac-specific expression of a phosphorylation-deficient cyclic nucleotide regulatory element binding protein (*Creb*) mutant in transgenic mice led to significantly higher mortality and contractile dysfunction in female compared with male mice, which were underlain by a significant decrease in mitochondrial density and deterioration of mitochondrial structure, increased ROS were accompanied by decreases in the expression/activity of the mitochondrial antioxidants manganese superoxide dismutase (MnSOD) and glutathione peroxidase in female mice only (421). Sex differences in the phosphorylation of mitochondrial proteins, such as aldehyde dehydrogenase-2 (ALDH2), also play a major role in mitochondrial sex differences resulting in reduced production of ROS and cardioprotection in females (208).

Mitochondrial function and energy metabolism are also regulated by estrogen (155, 218, 369). Such estrogenic actions are expected to be mediated by the ER, as functional ERα and ERβ have been documented in mitochondria (66, 440). Subsequently, E2 may affect the expression of nuclear and mitochondrial DNA encoded proteins, lead to post-translational modifications of mitochondrial proteins, and control free radical production, thereby affecting mitochondrial function and biogenesis **(FIGURE 8)**.

In particular, E2 treatment reversed the mitochondrial dysfunction associated with menopause and ovariectomy (324, 376). Notably, estrogen regulates the key activator of mitochondrial biogenesis and function peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) (153, 154, 432). We postulate that a female-specific interaction between E2/ER and PGC-1α may at least partially maintain mitochondrial function in female hearts **(FIGURE 8)**. In pathological LV hypertrophy, genome-wide expression profiling revealed less downregulation of metabolic genes dependent on PGC-1α in female hearts than in male hearts (119, 432). PGC-1α is located downstream of HDAC, and it is therefore of interest to study sex-specific HDAC activation. In a transgenic model of HDAC5, where myocyte enhancer factor-2 (MEF2) was repressed, all male mice died early, while female mice survived (85). In particular, male mice exhibited severe defects in mitochondrial number and structure, as well as a repression of PGC-1α and mitochondrial enzymes (85). On the other hand, female mice survived without mitochondrial damage, suggesting that female mice maintain their PGC-1α levels independent of MEF2.

Estrogen treatment of OVX rodents exposed to ischemia/reperfusion injury also restored mitochondrial respiratory function and attenuated mitochondrial and ultrastructural damage in an ERα-dependent manner (444, 445). These protective effects are also associated with E2-dependent induction of proteins involved in the activity of oxidative phosphorylation (OXPHOS) or tricarboxylic citric acid (TCA) cycle (162, 334). In fact, the E2/ER signaling axis is expected to play a major role in the regulation of OXPHOS by enhancing the expression of several nuclear- and mitochondrial-encoded OXPHOS proteins (65, 155) followed by direct binding of the ER to mitochondrial DNA and mitochondrial estrogen response elements in an E2-dependent manner (67, 334). Consequently, these ER-mediated effects on OXPHOS protein regulation protect against mi-

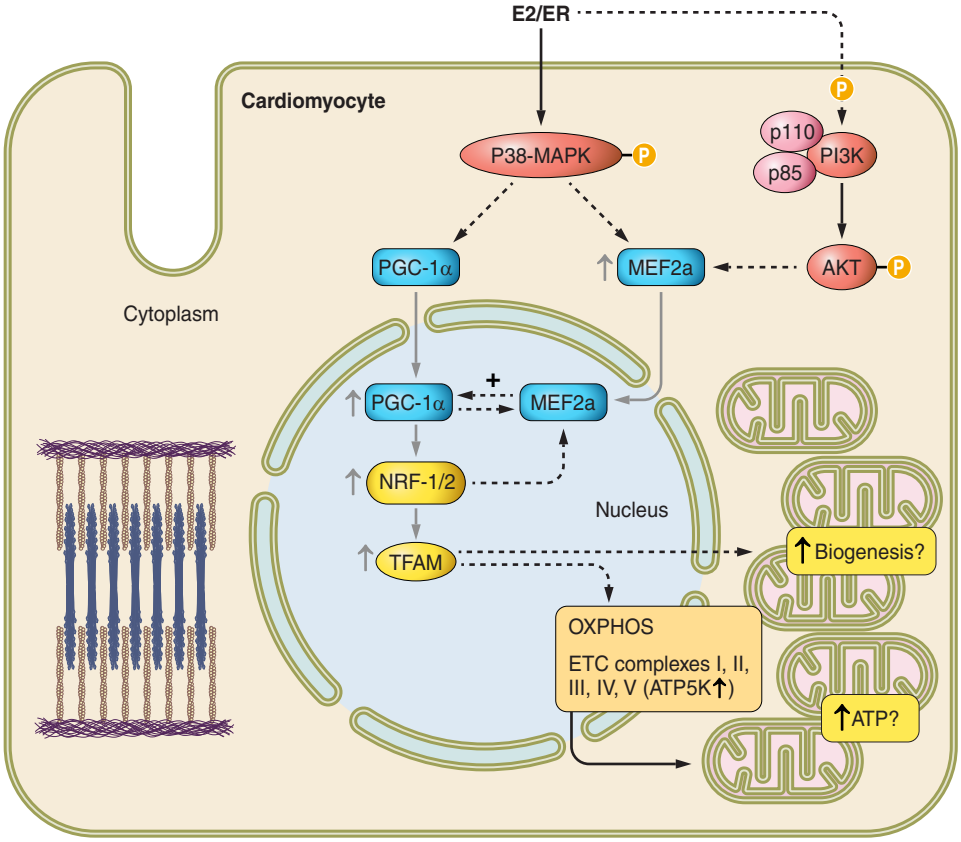

FIGURE 8. Hypothetical scheme for E2/ER-induced activation of PI3K/PKB and p38-MAPK pathways in female hearts leading to increased mitochondrial biogenesis and function.

tochondrial and cellular injury induced by oxidative stress (267, 369).

The protective actions of the E2/ER axis against oxidative stress may also be mediated through mechanisms exerting direct effects on mitochondrial enzymes. In particular, E2 induced the expression and activity of superoxide dismutase (SOD) in vascular smooth muscle cells, thereby diminishing the production of ROS (373). Along this line, E2 stimulated the activity of the mitochondrial antioxidant MnSOD and repressed superoxide generation in neonatal rat cardiomyocytes subjected to oxidative stress (229). In OVX Dahl salt-sensitive rats, the levels of the antioxidative enzymes glutathione peroxidase 1 and 4 that scavenge hydrogen peroxide were reduced, while E2 treatment reversed this effect (447). The induction of these mitochondrial antioxidants by the E2/ER axis detoxifies ROS and confers protection against ROS-induced cytotoxicity (65). E2-dependent posttranslational modifications in the heart, such as *S*-nitrosylation of several proteins, including mitochondrial F1-ATPase (226), have also been reported, thereby modulating mitochondrial bioenergetics. Collectively, these data exhibit the mechanisms that contribute to a sex-specific mitochondrial protein composition with higher levels of enzymes of the respiratory chain in females. Recent resources and tools, such as a functionally validated metabolic network of the human cardiomyocyte (191), will enable further studies of the mechanisms accounting for sex- and sex hormone-dependent regulation of cellular metabolic processes crucial for the maintenance of cardiovascular function.

3. Cardiac lipid and carbohydrate metabolism

Sex differences in cardiovascular pathophysiological processes could also be the consequence of sex hormone-specific alterations in glucose and fatty acid supply and/or changes in the uptake and catabolism of energy substrates in cardiovascular cells. In the cardiomyocyte, fatty acid uptake is mediated by the membrane transporter CD36 and fatty acid transport proteins FATP (237), which seem to be under the regulation of estrogen (199, 381). Free fatty acid levels are also kept low by estrogen (170), and it is expected that E2 directs fatty acids to beta oxidation, ultimately producing energy for high demand cardiac functions (245, 377).

Male transgenic mice lacking *Ppara* and overexpressing lipoprotein lipase (*Lpl*) in cardiac muscle displayed increased mortality, while their female counterparts were not affected (285). Importantly, E2 treatment rescued the lethal phenotype of mice lacking *Ppara* (95). Deletion of the *Ppara* gene resulted in massive cardiac lipid accumulation and death in all males with E2 being able to rescue this phenotype, while merely one-quarter of the females were affected (95). Notably, a reduction in lipid accumulation by estrogen in human macrophages has been reported (259). Estrogen has also been associated with lowered LDL cholesterol and

raised high-density lipoprotein (HDL) cholesterol levels (261, 414), further implicating estrogen signaling in the regulation of lipid metabolism.

Sex-specific regulation of adipose triglyceride lipase (ATGL) and hormone-sensitive lipase contributes further to sex differences in energy metabolism (270, 278). Testosterone has also been shown to affect cardiomyocyte metabolism regulating the activity of hormone-sensitive lipase, thereby altering cardiac balance between lipid and carbohydrate metabolism (212). Notably, female sex is associated with increased rates of myocardial fatty acid oxidation in humans (310) and exercising rodents (120). Female rats also have higher cardiac docosahexaenoic acid (DHA, 22:6*n*-3) levels than males, but the expression of enzymes involved in the biosynthesis of docosahexaenoic acid from short-chain *n*-3 polyunsaturated fatty acids does not differ between the sexes (194), calling for a better understanding of the underlying mechanisms leading to cardiovascular sex differences.

Eicosanoid pathways are also differentially activated between males and females, leading to female protection via epoxyeicosatrienoic acids (EET) and male maladaptation via hydroxyeicosatetraenoic acid (HETE) (149, 278, 428). These pathways also seem to be involved in the actions of testosterone that lead to increased blood pressure in rats, while castration or blockade of the AR attenuate the development of hypertension (125, 158, 327, 328). In male mice with elevated plasma androgens, the CYP4A12 enzyme was induced and caused ω-hydroxylation of arachidonic acid and formation of 20-HETE leading to hypertension (150). Castration prevented the hypertension and enzyme induction, while testosterone treatment restored the hypertension and CYP4A12 enzyme activity (150).

4. Cell death and survival

Cell death also occurs in a sex-specific manner, where cardiomyocyte loss increases in men with aging but not in women (293), and cardiomyocyte death is higher in the male failing heart than in the female failing heart (137). Notably, young women have higher levels of nuclear-localized phosphorylated-PKB compared with aged men or postmenopausal women, which might contribute to the mechanisms conferring protection against cell death in young women (51). The female heart seems to be better protected also against ischemia-induced apoptosis. In particular, the peri-infarcted area displays a 10 times higher apoptosis rate in men than in women (32). In a rabbit ischemia/reperfusion model, apoptotic cell death was significantly higher in males than females (41). Mice subjected to ischemia/reperfusion demonstrated similar responses, where female hearts showed less necrosis compared with male hearts (123, 419). The mechanisms conferring the advantage to females seem to include male-specific reduction of the antiapoptotic protein Bcl2 and female-specific reduction of the proapoptotic protein Bax (63). Furthermore, genetic deletion of mammalian target of rapamycin complex 1 (mTORC1) downstream signaling molecule ribosomal protein S6 kinase 1 (*Rps6kb1*) inhibits cell senescence and favors longevity restricted to female mice (354). Consistent with the genetic deletion of *Rps6kb1*, pharmacological intervention with rapamycin has most prominent effects in female mice (147).

Sex hormones also exert antiapoptotic effects mediated through a number of different mechanisms. E2 treatment of OVX mice with MI reduced cardiomyocyte apoptosis (400). Such antiapoptotic effects of E2 in cardiomyocytes are partly mediated by activation of PI3K/PKB signaling (303), leading, for example, to the improvement of cardiac function following trauma-hemorrhage (441). E2 treatment also induces the nuclear localization of phosphorylated-PKB in cultured cardiomyocytes (51). The antiapoptotic effects of estrogen may also be mediated through the repression of TNF-α in the heart (438). Furthermore, E2 prevented cardiomyocyte apoptosis via the modulation of p38α and -β MAPKs in cultured rat cardiomyocytes (193), along with inhibiting p53 and its translocation to the mitochondria (228). Interestingly, estrogen preserved the integrity of ischemic tissue by augmenting the mobilization and incorporation of bone marrow-derived endothelial progenitor cells into sites of neovascularization (165). Studies with OVX rats and isolated perfused hearts from OVX rats showed that acute administration of E2 and ERα agonist significantly reduced oxidant stress and necrosis following ischemia/reperfusion (169, 287). Together, regulation of these factors by estrogens is a set of different mechanisms mediating antiapoptotic effects in a sex-specific manner.

Testosterone-induced increased PKB phosphorylation has also been reported and may be involved in the observed antiapoptotic effects of testosterone against doxorubicin-induced cardiotoxicity (159). However, depending on the model, the effects of testosterone may be opposing. In fact, in an ischemia/reperfusion model, acute testosterone infusion led to the downregulation of PKB and lower levels of MnSOD (157). Similarly, testosterone led to aggravated cardiac damage in both males and females exposed to ischemia/reperfusion injury, and the mechanism includes the downregulation of the antiapoptotic protein Bcl-xL (213) and the upregulation of the proinflammatory mediators TNF-α, IL-1β, and IL-6 (418).

Sex differences in autophagy have also been reported, where males display a lower level of autophagy than females (77). Stress seems to induce autophagy activity at a higher level in female cells compared with male cells (372). In rat neuronal cells, nutrient deprivation-induced autophagy death was higher in males than in females, with neurons from females surviving longer (101). Evidence for the crosstalk between the E2/ER axis and the autophagy pathway has been provided through the association of the

autophagy mediator Beclin-1 with ERα downregulating E2 signaling, thereby contributing to the development of E2 resistance (173). However, the E2- and sex-specific regulation of autophagy in cardiac cells is currently not understood. In fact, a recent study suggested that the male heart has major constitutive autophagy (52).

E2 further activates signaling pathways that regulate protein metabolism (274), thereby regulating protein synthesis and protein degradation (178). In fact, estrogen regulates cardiomyocyte contractile function modifying the ubiquitin-proteasome system (UPS) (183). Another important effect of estrogen is the degradation of calcineurin, which leads to the attenuation of LV and cardiomyocyte hypertrophy by an ER-dependent pathway (96).

5. Inflammation

Men and women differ in the activation of adaptive and innate immune system. Women appear to be more efficient in fighting a primary pathogenic insult, but on the other hand they are more prone to the development of autoimmunity. A link between the activity of the adaptive immune system and cardiovascular function is illustrated in autoimmune diseases with severe cardiovascular manifestations, such as systemic sclerosis. Cytokine receptors and parts of the inflammasome are regulated in a sex-specific manner. These complex interactions may modify inflammatory reactions in the heart and the vasculature.

In animal models, females seem to be protected against tissue injury through decreased inflammatory cytokine production, such as TNF-α, IL-1, and IL-6 expression (113, 416, 438). As with other transgenic models described previously, overexpression of *Tnf* leads to increased mortality and HF to a greater extent in males than in females (167, 177). The receptor activator of NF-κB ligand (RANKL), its receptor RANK, and the decoy receptor osteoprotegerin also appear to be involved in the inflammatory process (146, 176, 350). RANK expression has been reported in the heart, which is actually one of the highest producing tissues of osteoprotegerin (TNFRSF11B), which, in turn, is expressed in a sex-specific manner (164). Together, these sex differences in inflammatory mediators play a major role in sex-specific inflammatory responses.

Estrogen also exerts a key role in immune responses, regulating proinflammatory cytokine expression through monocyte and macrophage regulation and affecting the expression of target genes (202, 388). In fact, cardioprotective effects of E2 on neutrophil infiltration, oxidant stress, and necrosis in an ER-dependent manner following ischemia/reperfusion have been reported (169). Notably, ERβ seems to be necessary for the strict regulation of the inflammatory response to cardiac insults (186). Monocyte chemoattractant protein (MCP)-1-dependent ROS production in monocytes is closely associated with LV dysfunction, and E2 is directly involved in the regulation of the MCP-1 gene (437). In OVX rats subjected to ischemia/reperfusion, a marked increase in the proinflammatory cytokine TNF-α occurred, while the treatment with E2 reduced TNF-α levels in the myocardium and further decreased its release after ischemia/reperfusion, which was associated with improved functional recovery and a decrease in markers of tissue injury and apoptosis (438).

Based on these findings, we postulate that estrogen and the ER may partly exert their effects via the modulation of key inflammatory mediators and cells, including mast cells, macrophages, and T cells **(FIGURE 9)**. Along this line, in a KO model of ERβ under chronic pressure overload with evident sex differences (119), the presence of ERβ was necessary for the inhibition of inflammatory factors and ROS-mediated NF-κB regulation, and for the maintenance of the cytochrome *P*-450 pathway (186). Interestingly, NF-κB inhibits ERα transcription, but NF-κB may in turn be inhibited by the E2/ER axis (249). Other anti-inflammatory actions of estrogen may include the rescue of PPARγ-expression (388), which in turn functions as a transcriptional repressor of proinflammatory signaling pathways. In contrast, the actions of testosterone are rather conflicting, and it apparently activates the transcription factor NF-κB contributing to inflammatory mechanisms (316).

6. Fibrosis

Profibrotic pathways exacerbate cardiovascular disease and lead to severe dysfunction. Recent studies have demonstrated that male patients with AS have increased levels of fibrosis mediators compared with female patients (184, 311). ERα, ERβ, and AR control fibrotic pathways, collagen, and matrix-metalloproteinase synthesis in a sex-specific manner **(FIGURE 10)**. In addition, estrogen attenuates the development of cardiac fibrosis. This could be through direct effects of estrogen inhibiting collagen synthesis and directly regulating collagen type I and III levels (103, 251, 312, 450), along with matrix metalloproteinase expression via activation of the ERα and MAPK-ERK1/2 signaling pathway (246). E2-dependent induction of the progesterone receptor might also contribute to the antifibrotic actions of estrogen (182). Furthermore, the E2/ER axis regulates a network of miRNAs, thereby affecting fibrosis (320). Cardiomyocyte-specific ERα expression enhances angiogenesis and the reduction of fibrosis following myocardial infarction (250). Interestingly, the genetic deletion of the AR leads to the exacerbation of angiotensin II-induced cardiac dysfunction and fibrosis (160).

7. Sex differences in vascular function and gene expression

There are major physiological differences between males and females in vascular function, in aging arteries, and in disease.

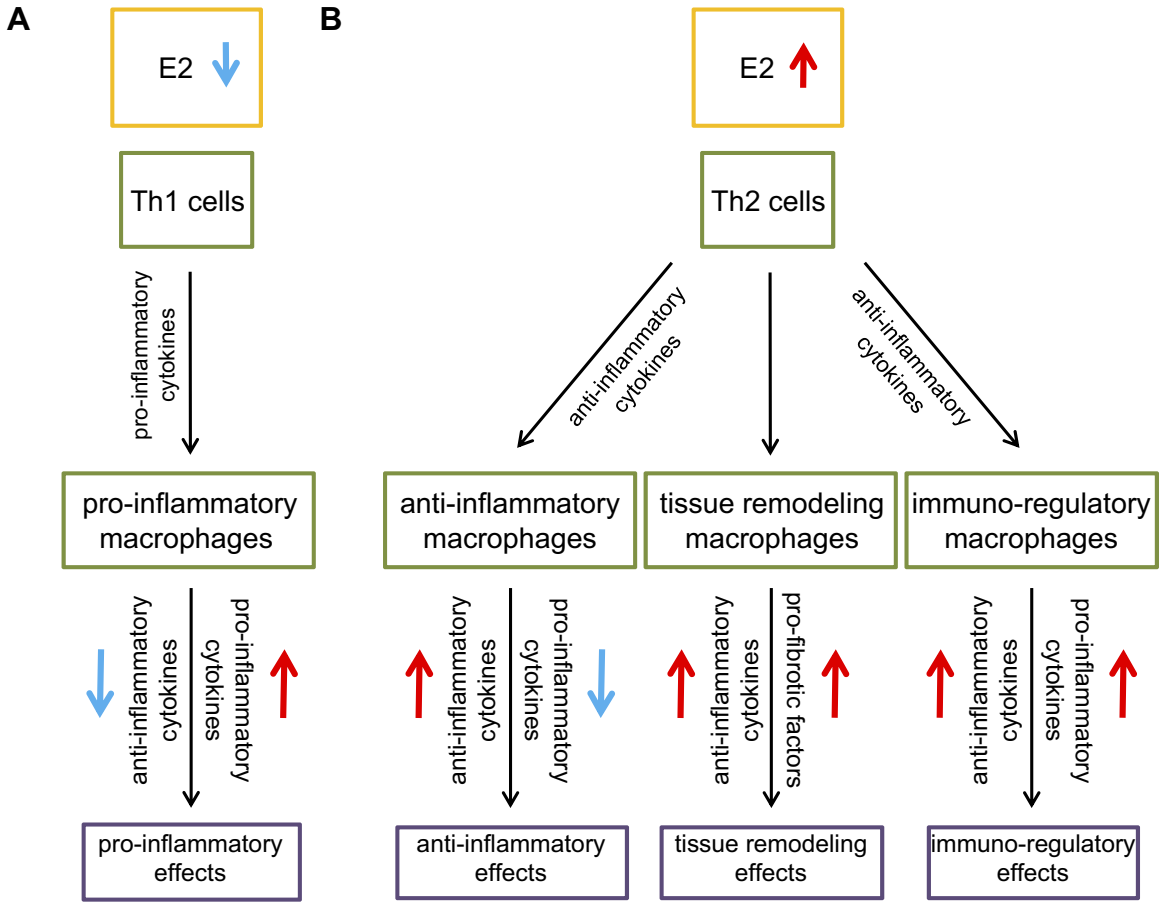

FIGURE 9. Estrogen regulates the inflammatory response. Low E2 levels induce a Th1 response leading to a proinflammatory response (*A*), while high E2 levels induce a Th2 response leading to an anti-inflammatory response (*B*).

Atherosclerosis affects men earlier than women, but microvascular disease and dysfunction are more prominent in women. Notably, microvascular dysfunction may play a major role in the development of diastolic dysfunction. Patients with LV hypertrophy, a precursor of HF, often show an impaired coronary reserve despite angiographically normal coronary arteries (321). This microvascular dysfunction occurs particularly following menopause and has been linked to sex differences in chronic ischemia and diastolic HF (23, 256).

Research on sex differences in the vasculature has been so far largely focused on the effect of sex hormones. However, some studies have also considered the effect of sex beyond the effects of sex hormones when analyzing sex-specific gene regulation (319). The sex-specific effects of estrogen and testosterone on cardiovascular risk, the direct vascular effects of these sex hormones, and how these effects influence the development of atherosclerosis have been reviewed in detail previously (410). Here, we discuss a few interesting findings.

Estrogen attenuates atherosclerotic plaque progression by inducing prostacyclin production through the activation of cyclooxygenase-2 (109); it inhibits smooth muscle cell proliferation and matrix deposition (298); and it promotes angiogenesis (273) and reendothelialization (43, 203). Furthermore, estrogen treatment restores endothelium-dependent NO-mediated vasorelaxation and endothelial NO synthase expression (69, 340, 430). The vasodilatory effects of estrogen may also be partially mediated through its inhibitory actions on the renin-angiotensin system (42, 124) or its inducing effects on epoxyeicosatrienoic acids and vasodilator metabolites of cytochrome *P*-450 (156, 404). Estrogen treatment of OVX mice also rescued vascular PPARγ expression; reduced ROS generation, monocyte recruitment, and atherosclerotic lesion formation; and improved endothelial function (388). A similar E2 treatment of OVX mice abolished progressive growth and decreased severity of angiotensin II-induced abdominal aortic aneurysms through mechanisms acting on smooth muscle cell α-actin and transforming growth factor-β (TGF-β) (383). A study employing ERKO and BERKO mice revealed that ERα- and ERβ-dependent pathways regulate distinct and largely nonoverlapping sets of genes in aortas (291). Notably, while ERα seems to be required for most of the estrogen-mediated increase in gene expression, ERβ mediates the large majority of estrogen-dependent decrease in gene expression (291).

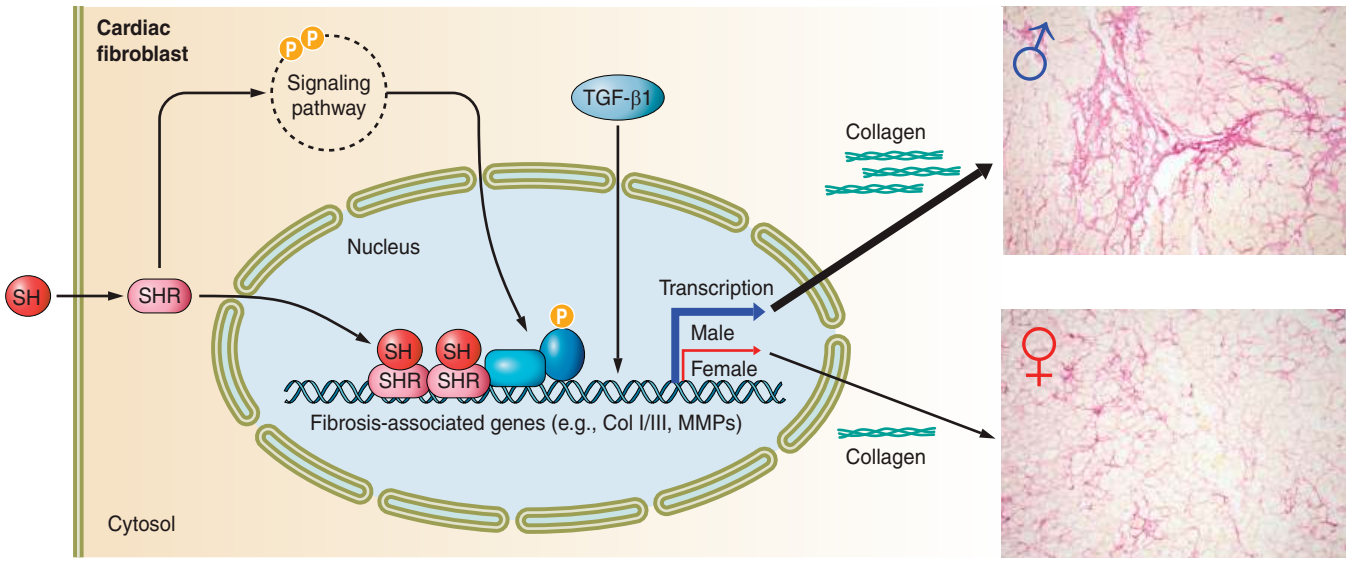

FIGURE 10. Interaction of sex hormones with fibrotic pathways. Sex hormones (SH) bound to their receptors (SHR) interact with other signaling pathways or directly target fibrosis-associated genes in a sex-specific manner. Consequently, there is a stronger transcriptional activation of collagen synthesis in males vs. females, thereby leading to higher collagen deposition and degree of fibrosis in male than in female hearts.

Hormonal regulation of NO production via endothelial NO synthase by estrogen or testosterone has implications in maintaining vascular health in combination with cardiovascular risk factors, such as diabetes and metabolic disorders (105). Reduced bioavailability and/or responsiveness to endogenously produced NO contributes to the development of cardiovascular disease. Interestingly, testosterone also appears to induce rapid vasorelaxation in both large arteries and smaller resistance vessels (308). Conversely, low plasma free testosterone levels are associated with endothelial dysfunction, while testosterone treatment leads to a rapid endothelium-dependent vasodilating effect (5, 443). The dependence on the AR of the testosterone-induced release of NO leading to vasorelaxation is not clear, as conflicting data have been reported (130, 161). However, the antiandrogen compound flutamide, which was used in those studies, has additional effects beyond AR blockade, probably mediated by a rapid-acting AR (24–25).

V. LIMITATIONS AND IMPLICATIONS FOR FUTURE RESEARCH

There is an ongoing debate whether experimental animals are informative models of human sex differences. Furthermore, as recently pointed out, studies of sex differences in permanent cell lines may not reflect sex differences in humans, since sex chromosomes may become modified (336). However, this may be overcome by switching to freshly isolated primary cells or by testing cell lines for sex chromosomes before the study of sex differences.

It is well known that sex differences in animal models mimic those in humans imperfectly. Age, duration of disease exposure, sex hormone and growth hormone profiles, many forms of stressors, and other factors differ between animal models and humans. Furthermore, menopause is a human phenomenon that is difficult to study in animal models, and surgical ovariectomy is an imperfect proxy for physiological menopause. Other models of menopause, such as slow chemical destruction of the ovaries, have been developed to mimic the human situation better, but they still have limitations (181). Nevertheless, these limitations do not apply only to the case of the investigation of sex differences, but rather to the whole research spectrum. Despite these limitations, animal models have been used successfully for drug development for decades. Consequently, we believe that animal models have their value for the understanding of human pathophysiology, including sex differences, and hold the potential for translational use, particularly when results are interpreted with caution and the model-inherent limitations are taken into consideration.

To improve future understanding of the role of sex in pathophysiology, we put forward that investigations in animal models are based on precise hypotheses on sex differences and include all possible confounders, such as housing, diet, environment, stress, and so on (317). This is, however, a requirement that does not apply only to the study of sex differences. Recent studies have shown that, against general belief, variability in female rodents is not bigger than in males, even when females have a proper hormonal cycle. A large amount of variability is due to housing conditions (317). Investigators have to consider this. Effect size may be different between both sexes, but such a finding may be very important for clinical translation and should be followed.

In human studies, various investigations have shown that abnormalities of sex hormone status may influence disease patterns. However, this has not received enough attention so far, and we put forward that hormonal status should be better documented and controlled in clinical studies. This refers to abnormal sex hormone profiles, such as in hypothalamic or stress-induced hypogonadism, polycystic ovary syndrome (PCOS), late menarche, early menopause, andropause, and other forms of sex hormone abnormalities (168, 301, 344). The same is true for pregnancy conditions and complications, particularly eclampsia, pregnancy-induced diabetes, and hypertension (329). Obesity and proinflammatory states, physiological and psychological stressors, environmental toxins or endocrine disruptors, such as bisphenols, affect sex hormone profiles in men and women and should be included in patient documentation (168). Transgender individuals frequently exhibit cardio-metabolic disease, supporting the view that abnormalities in sex hormones lead to CVD. Finally, measuring a proxy of self-estimated gender, which may be different from biological sex, can provide a novel cardiovascular risk factor (305). Considering these variables in clinical and epidemiological studies may be very important steps towards truly personalized medicine or precision medicine.

VI. CONCLUSION

Sex differences have consistently been confirmed throughout a large spectrum of experimental and clinical studies, in different species, and in (patho)physiological conditions. However, a more detailed understanding of sex differences and their underlying mechanisms is needed to design new drugs that target sex-specific cardiovascular mechanisms and affect phenotypes. The comparison of both sexes is essential for the identification of protective or maladaptive mechanisms. A knowledge of such mechanisms in gene transcription, intracellular signaling, organelle function, and interorgan cross-talk will reveal new targets for the activation or inhibition of specific aspects of the cardiovascular system that are linked to sex hormones or other differences between males and females.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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