

## REVIEW | *Sex Differences in Cardiovascular and Cerebrovascular Physiology, Disease, and Signaling Mechanisms*

### Sex differences in endothelial function important to vascular health and overall cardiovascular disease risk across the lifespan

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**Stanhewicz AE, Wenner MM, Stachenfeld NS.** Sex differences in endothelial function important to vascular health and overall cardiovascular disease risk across the lifespan. *Am J Physiol Heart Circ Physiol* 315: H1569–H1588, 2018. First published September 14, 2018; doi:10.1152/ajpheart.00396.2018.—Diseases of the cardiovascular system are the leading cause of morbidity and mortality in men and women in developed countries, and cardiovascular disease (CVD) is becoming more prevalent in developing countries. The prevalence of atherosclerotic CVD in men is greater than in women until menopause, when the prevalence of CVD increases in women until it exceeds that of men. Endothelial function is a barometer of vascular health and a predictor of atherosclerosis that may provide insights into sex differences in CVD as well as how and why the CVD risk drastically changes with menopause. Studies of sex differences in endothelial function are conflicting, with some studies showing earlier decrements in endothelial function in men compared with women, whereas others show similar age-related declines between the sexes. Because the increase in CVD risk coincides with menopause, it is generally thought that female hormones, estrogens in particular, are cardioprotective. Moreover, it is often proposed that androgens are detrimental. In truth, the relationships are more complex. This review first addresses female and male sex hormones and their receptors and how these interact with the cardiovascular system, particularly the endothelium, in healthy young women and men. Second, we address sex differences in sex steroid receptor-independent mechanisms controlling endothelial function, focusing on vascular endothelin and the renin-angiotensin systems, in healthy young women and men. Finally, we discuss sex differences in age-associated endothelial dysfunction, focusing on the role of attenuated circulating sex hormones in these effects.

androgens; atherosclerosis; endothelin; endothelium; estradiol; estrogens; menopause; renin-angiotensin system

#### INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality among both men and women in developed countries, although CVD is becoming more prevalent in developing countries as well. However, men develop overt CVD at an earlier age compared with women. This sex disparity persists until women reach menopause, at which time the incidence of new onset CVD in women climbs rapidly until it outpaces that of men (180). This rapid increase in the devel-

opment of CVD coincides with the decline of female sex hormones, characteristic of the menopausal transition, and has long been explained by the loss of cardioprotective effects of estrogens. Furthermore, it is often proposed that androgens are detrimental. However, the roles of sex hormones in cardiovascular health are significantly more complex and are influenced by age, sex differences in the tissues exposed, and route of administration. In this report, we have largely focused on the conducting vessels, as these are the vessels that have been studied most in this field. However, it is important to note that resistance and visceral vessels, while largely unexplored in the context of mechanistic sex differences, may be distinct in their responses and regulation with regard to sex hormones and their receptors.

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A healthy endothelium, described as an appropriate balance of endothelial vasoconstrictor and vasodilator factors, is important for vascular homeostasis. Conversely, reductions in endothelial function are detrimental and predict and precede the development of overt CVD. The hallmark of a healthy endothelium is appropriate nitric oxide (NO) synthesis and release by vascular endothelial cells in response to a vasodilatory stimulus. While NO synthesis and downstream NO-mediated dilation are a barometer of healthy endothelial function, there are additional signaling pathways, such as those mediating constrictor tone, that regulate endothelial function and differ between men and women. These include the intracellular angiotensin and endothelin-1 (ET-1) systems. These mechanisms, which contribute to sex differences in endothelial function in young healthy women and men, may explain the cardioprotective phenotype of premenopausal women and the accelerated progression of CVD in men relative to women before menopause.

In this review, we aim to summarize the literature on sex differences in endothelial function. First, we address female and male sex hormones and their receptors and how these interact with the endothelium in healthy young men and women. Second, we discuss sex differences in sex steroid receptor-independent mechanisms controlling endothelial function, focusing on the intracellular vascular ET system and renin-angiotensin system (RAS), in healthy young women and men. Finally, we present sex differences in age-associated endothelial dysfunction, focusing on the role of attenuated circulating sex hormones in these effects in older adults.

#### FEMALE AND MALE SEX HORMONES AND RECEPTORS

Sex hormones influence endothelial function through their effects on agonists and contribute to key functional differences between women and men related to endothelial function and CVD risk. In women, estrogen receptors (ERs) promote NO release via endothelial NO synthase (eNOS) activation, whereas engagement of the androgen receptor (AR) may result in impaired, agonist-triggered endothelial NO release.

In this section, we provide a brief introduction to sex differences in estradiol, androgen (testosterone), and progesterone signaling in the vasculature. For a more comprehensive examination of this subject, the reader is referred to an excellent review recently provided by Boese et al. (17). It has long been proposed that estrogens play a role in cardioprotection in women (189), whereas androgens may contribute to cardiovascular risk in young women in conditions such as polycystic ovary syndrome (PCOS) (48, 242, 243) or in transgender men receiving testosterone treatment (131, 178, 214). In men, androgens may induce increases in blood pressure via mechanisms including reactive oxygen species (ROS) and decreased NO bioavailability. However, androgens also play a cardioprotective role in men either through direct effects or through conversion to estrogen (157). Thus, the assumption that estrogens are cardioprotective and androgens are detrimental is oversimplified. The effects of these hormones are very much dependent on their environments (124).

#### *Estrogen*

Estrogens contribute significantly to the regulation of vaso-motor tone, although the receptor- and ligand-specific signal-

ing pathways still need further study. The most well-established ERs are ER $\alpha$  and ER $\beta$ , and both of these receptor subtypes are expressed on endothelial cells and vascular smooth muscle cells (VSMCs) (137). While the distinct and specific roles of each subtype continue to be elucidated, both ER $\alpha$  and ER $\beta$  contribute to healthy vascular function (141). Receptor expression is dependent on tissue type (72, 141); each subtype is sensitive to circulating plasma estrogen levels (93), and each subtype is affected differently depending on tissue and concentration (141, 165).

The classical view of ER $\alpha$  and ER $\beta$  is as ligand-activated nuclear receptors that bind to estrogen response elements in the promoter and regulatory regions of their target genes and so take hours or days to induce their effects. However, ERs and the signaling cascades initiated by ERs can also mediate nongenomic responses (30). These rapid, nongenomic vascular estrogen effects were established in studies showing that estradiol infusion induced vasodilation within 15 min (10, 185). Also, binding sites for ERs were identified on human endothelial cell membranes (24). More recent investigations have demonstrated increases of vascular cAMP content within 30 min of 17 $\beta$ -estradiol (E<sub>2</sub>) exposure, occurring concomitantly with vasodilation (10, 154), and thus too rapid to be explained by genomic mechanisms. G protein-coupled receptor 30 (GPR30), a G protein-coupled estrogen receptor (GPER), was identified and shown to bind estradiol and also induce rapid signaling events (179), albeit with lower affinity than ER $\alpha$  or ER $\beta$ . GPER-estradiol binding leads to rapid and transient activation of a number of intracellular signaling pathways in the reproductive system but also plays a role in the vasculature and in estrogen-mediated endothelial function and thus in estradiol-mediated effects on hypertension and atherosclerosis.

The most well-established vascular effect of estradiol is the production of NO, which occurs via both genomic and non-genomic mechanisms. E<sub>2</sub> binding to both ER $\alpha$  and ER $\beta$  subtypes triggers signaling cascades that include activation of the kinases c-Src (61, 119), ERK (104, 197), phosphatidylinositol 3-kinase (PI3K) (184), and Akt (PKB) (204). E<sub>2</sub> also increases expression of eNOS mRNA and protein expression in endothelial cells (85, 128). In humans, E<sub>2</sub> also induces rapid eNOS activation, indicating nongenomic mechanisms, which play an important role in the regulation of vascular function (Fig. 1) (30, 82). Importantly, endothelial cell membranes contain binding sites for E<sub>2</sub> leading to the initial activation of eNOS (190). Estrogen binding to activated eNOS promotes the production of NO (103) in the endothelial cell caveola (143) and causes vascular relaxation and endothelial-dependent vasodilation [for the mechanistic details of these pathways, see Orshal and Khalil (166)]. Generally, E<sub>2</sub> exposure in women increases vascular relaxation and endothelial-dependent vasodilation, increasing blood flow in numerous vascular beds. Estradiol may also increase sensitivity to vasodilatory factors, such as acetylcholine or prostaglandins, thus reducing the concentrations required to evoke similar vasodilatory responses (142, 230). Recent evidence in human endothelial cells has also demonstrated that GPERs contribute to E<sub>2</sub>-induced endothelium-dependent vasodilation and NO formation (64).

*Estrogens in women.* As described above, premenopausal women have a lower risk of CVD than men of similar age, but they lose this advantage with menopause. Chronic E<sub>2</sub> supplementation results in an upregulation of eNOS expression and

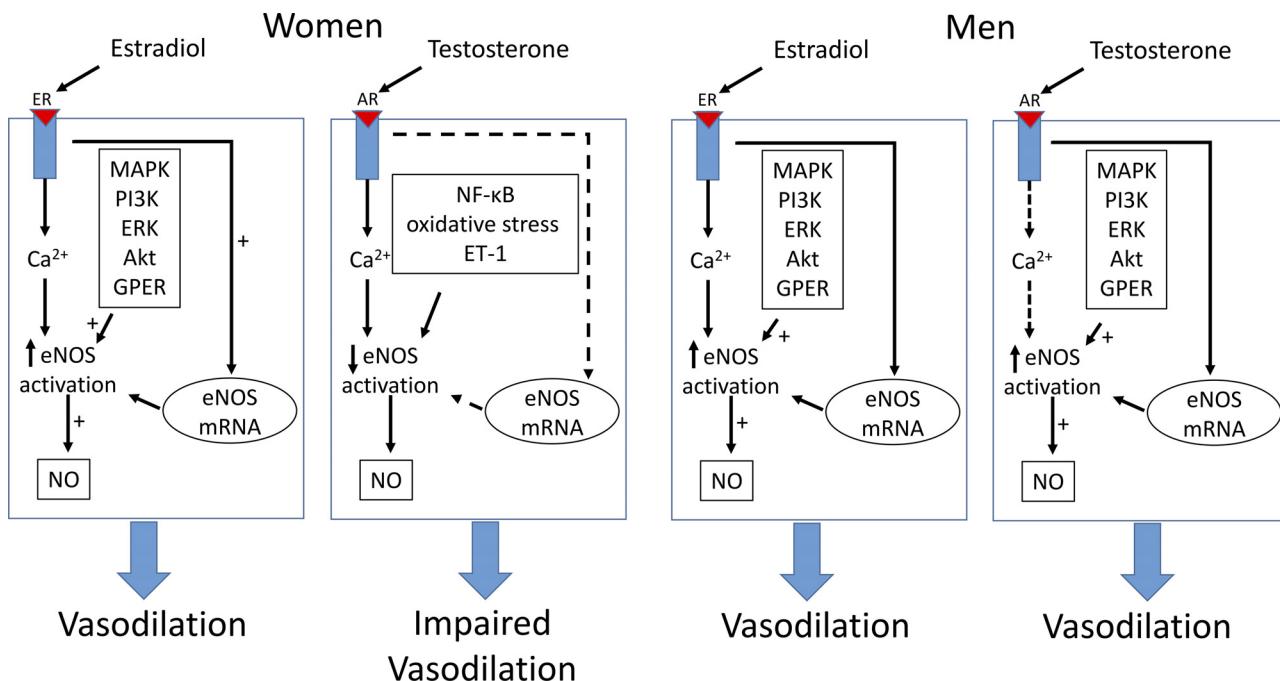


Fig. 1. Sex differences in the signal transduction pathways of endothelial nitric oxide (NO) synthase (eNOS) activation in endothelial cells by estradiol and testosterone and the subsequent vascular response. Estradiol is generally thought to increase NO availability through genomic and nongenomic pathways in both men and women, resulting in vasodilation. Testosterone has similar effects in men. Testosterone works through a separate pathway in women, resulting in reduced NO availability and impaired vasodilation. The dashed line indicates effects that are uncertain. ER, estrogen receptor; AR, androgen receptor; PI3K, phosphatidylinositol 3-kinase; GPER, G protein-coupled estrogen receptor 1.

an increase in available NO in isolated vessels (135). In healthy young women, estradiol is associated with increases in flow-mediated dilation (FMD), a measure of conduit artery endothelial function mediated primarily by NO (1, 136, 144). Similarly, short-term ethinyl estradiol administration increases FMD in women with stage 1 hypertension (1), an effect that is mediated by increases in NO (80, 100).

Ethinyl estradiol also attenuates vasoconstrictor responses to norepinephrine infusion in perimenopausal women (215). This effect is likely mediated by reduced  $\alpha$ -adrenergic concomitant with greater  $\beta$ -adrenergic receptor contribution to vascular tone (60, 255). However, after menopause, there is a reduction in  $\beta$ -adrenergic receptor contribution to vascular tone, which contributes to increased risk of hypertension in postmenopausal women (77). In ovariectomized (OVX) female rat mesenteric arteries, E<sub>2</sub> administration attenuated vasoconstriction during phenylephrine infusion (255) and enhanced vasodilatory responses to isoproterenol infusion (60). Thus, the mechanisms contributing to the greater vascular relaxation by E<sub>2</sub> include enhanced NO bioavailability coupled with greater  $\beta$ -adrenergic and lower  $\alpha$ -adrenergic sensitivity.

**Estrogens in men.** It is not clear whether estrogens provide similar cardioprotection in men as in women. For a discussion of overall estrogen effects in men, we refer the reader to a recent review, *Estrogens in male physiology* (35). Here, we address the impact of estrogen exposure on the male cardiovascular system. Men express ER $\alpha$  and ER $\beta$  (70, 75) and GPER (47) as well as aromatase (70, 75) in their cardiovascular system. GPERs appear to have cardioprotective actions in male rats (47). Consistent with the rapid effects associated with GPERs, E<sub>2</sub>-mediated vasodilation occurs within minutes in men (140). Estradiol increases vasodilation in men via both

prostacyclin and NO mechanisms and so are endothelial dependent and independent (105, 256). An early study (16) demonstrated that acetylcholine induced coronary flow with high levels of equine estrogen infusion, suggesting that estrogens can improve acute endothelial function and avoid ischemia. Testosterone is the major source of E<sub>2</sub> in men through its conversion to E<sub>2</sub> by aromatase, so circulating E<sub>2</sub> depends on concentrations of both testosterone and aromatase. Aromatase is expressed in endothelial cells and VSMCs of male mice (95) and in humans (35, 118). Consistent with effects of E<sub>2</sub> on endothelial function, aromatase inhibition decreases FMD in men (118). Estradiol may also have a cardioprotective effect in men by limiting proliferation and migration in VSMCs (40) via both ER $\alpha$  and ER $\beta$  mechanisms (86). Conversely, ER $\beta$  and E<sub>2</sub> exposure increased atherosclerosis in coronary arteries harvested from men, suggesting a role for E<sub>2</sub> in early coronary atherosclerosis (123).

#### Androgens

Androgens are synthesized in testicular Leydig cells and ovarian theca cells and secreted by the testes and adrenal glands. The primary androgen in men is testosterone, which can be converted to dihydrotestosterone (DHT), a more potent form and one not converted to estrogen by aromatase. Like estrogens, androgens are steroid hormones that regulate gene transcription when ligand activated. Androgens bind to ARs in the cytoplasm, undergo conformational changes, and bind to androgen response elements, which move to the nucleus in the target gene to regulate gene expression. As with estrogens, androgens may also induce rapid activation via kinase signaling cascades and mechanisms independent of transcription (9,

17, 83, 177), including activation of intracellular  $\text{Ca}^{2+}$  (17), MAPK (83), Akt (9), and PKA/PKC (63) (Fig. 1). ARs are expressed in cells throughout the cardiovascular system, including endothelial cells (226) and VSMCs (125). Transcription-independent mechanisms may also be important for androgen effects on endothelium-dependent vasodilation. Although androgen actions on the endothelium are primarily mediated by ARs, some effects of androgens on the cardiovascular system are mediated indirectly through  $E_2$  (157) after testosterone conversion to  $E_2$  by aromatase (226). These effects on the endothelium may be ER and NO mediated. Estradiol exposure varies based on both  $E_2$  and aromatase levels, and concentrations of these fluctuate in men.

**Androgens in men.** Men are at higher risk for developing CVD relative to women of similar age until menopause (89). However, whether this is reflective of negative effects of androgens in men or positive effects of estrogen in women is not entirely clear. While estrogens may play a cardioprotective role in women, normal physiological levels of androgens in men reduce the risk of atherosclerosis (17, 51, 124). Low androgen concentrations are associated with increased risk of coronary heart disease in older men (87, 115), and there is a link between androgen deficiency and atherosclerosis (57, 188). Testosterone administration improves endothelium-mediated vasodilation in some male animal models (34, 36), and knockout of the AR gene increases atherosclerosis in male and female mice (19, 59). Interesting recent data using EAhy926 endothelial cells identified specific androgen-stimulated genes intrinsic to the atheroprotective process (126). In contrast, and adding to the complexity, androgen administration can also blunt endothelium-mediated vasodilation in hypogonadal men by reducing NO availability (12), and testosterone exposure can worsen endothelial function (92) and hypertension in some animal models (155, 208, 251). For example, administration of DHT to male rats induces hypertension, associated with other markers of oxidative stress (155, 208, 251). The variability in these findings is the result of the variations in the concentrations and types of hormones given among the various studies and the numerous cellular [e.g.,  $\text{Ca}^{2+}$  fluxes,  $\text{Ca}^{2+}$ -activated  $K^+$  channels (97), NF- $\kappa B$  activation, etc.] and hormonal (testosterone metabolism to estrogen by aromatase vs. DHT) mechanisms involved (211).

**Androgens in women.** As with men, the impact of androgens on the cardiovascular system in women is also an exciting area of study. Whereas estrogens may play a cardioprotective role, androgens may induce detrimental outcomes on the cardiovascular system in women (51, 124). In general, the engagement of androgen and the AR results in impaired agonist-triggered endothelial NO release in women. This impairment is likely a key causative link between sex differences, infertility, and endothelial dysfunction. Women with high androgen levels, such as women with PCOS, exhibit elevated circulating inflammatory cytokines, mild hypertension, oxidative stress, and NF- $\kappa B$  activation (48), which may reduce NO and lead to endothelial dysfunction (243) and mild hypertension (251). Women with high-androgen PCOS are at risk for CVD (48), and it is likely that androgens drive this risk. In women, serum total testosterone concentration is associated with greater risk of diabetes and related cardiovascular comorbidities (50), all of which are characterized by the presence of endothelial dysfunction (134, 169, 214a). Longitudinal studies of women have

supported an association between PCOS and CVD, which is apparent in premenopausal women in their 40s (27), independent of insulin resistance, obesity, or fertility status (27). Androgen excess is associated with endothelial dysfunction in women, including elevated plasma ET-1 levels. In early menopause, when both androgens and estrogens may be fluctuating (116), androgens may be associated with coronary artery calcification (37, 167) and carotid intima media thickness (13), but these findings are not supported by all studies (28, 107).

To examine administration of high doses of testosterone to women, a recent meta-analysis examined androgen (DHT) treatment in female-to-male transgender patients, some of whom also received estrogen and progesterone as well (131). These treatments induced increases in serum triglyceride (TG) and low-density lipoprotein-cholesterol and decreases in high-density lipoprotein-cholesterol at 3, 6, and 24 mo of testosterone treatment (131). Interestingly, cross-sex hormone therapy with testosterone induced blood pressure increases, insulin resistance, and dyslipidemia (131), but cardiovascular morbidity was not yet apparent in these young transgender men (214). None of these studies have yet followed these transgender men into the aging process as CVD risks accelerate, so the data on testosterone administration on cardiovascular morbidity, including changes in the endothelium, are limited.

Methods to study effects of sex hormones and their receptors and between the sexes on the endothelium have proved challenging. Approaches using female mice lacking either ER $\alpha$  or ER $\beta$  or male mice lacking the AR may help to define signaling pathways involved in endothelial cell function as well as sex differences in the functioning of these receptors in endothelial cells. For example, both ER $\alpha$  knockout and ER $\beta$  knockout mice have been generated and survive into adulthood and have been used to demonstrate the key role that estrogen (via ER $\alpha$ ) plays in VEGF regulation in the coronary circulation (96). Furthermore, female mice show increased insulin resistance and obesity compared with male mice (53) after 5 $\alpha$ -reductase-1 knockout, indicating a more important role for the AR and glucocorticoids relative to androgens in female as opposed to male mice. Such studies are important not only in elucidating mechanisms but also for demonstrating subtle but important sex differences in the functioning of these receptors. These studies have not been performed specific to endothelial cells but would be of great interest in the future.

#### Progesterone

Although estrogens have received the majority of attention regarding cardioprotection in women, there is evidence that progesterone also has important cardiovascular effects (147), which are NO (203, 207) and cyclooxygenase (245) mediated. Progesterone administration is associated with increased eNOS in the rat aorta (203) and human endothelial cells (207). The rapid effects of progesterone may be mediated by both nuclear and membrane progesterone receptors (224). Natural progesterone stimulates NO synthesis via transcriptional and nontranscriptional pathways in human endothelial cells (207, 224) and, when added to  $E_2$ , increases the stimulatory effects of  $E_2$  on eNOS (207) that impact vasodilation and vascular tone. Interestingly, synthetic medroxyprogesterone (MPA) does not stimulate eNOS in endothelial cells and may even begin to degrade  $E_2$  effects on eNOS expression (207). This suggests that the

difference in human endothelial cells is that MPA-bound progesterone receptors do not activate the ERK pathway and so do not subsequently recruit PI3K (207). Thus, the entire signaling pathway for natural versus synthetic progestin is different and may explain the difference in a number of clinical outcomes (207), including mineralocorticoid and inflammatory responses and cancer (192).

Progesterone also impacts VSMCs, inducing rapid  $\text{Ca}^{2+}$  influxes, and progesterone receptors have been identified on both endothelial cells and smooth muscle cells. In studies of healthy young women, progesterone enhanced cutaneous adrenergic responsiveness in skin microvessels, which was mediated by prostanoids (245). These findings were similar to earlier studies showing that some progestins can negate vasodilatory effects of  $E_2$  (254), and micronized progesterone may attenuate  $E_2$  vasodilation during FMD (144). Clearly, much work needs to be done to understand mechanisms by which progesterone may induce vasodilation in some environments but vasoconstriction in others as well as how progesterone and MPA or other progestins interact with  $E_2$  on the vasculature in men and women. The few human studies of progesterone effects on the vasculature have been conducted in women, and there have not been studies examining sex differences in the role of progesterone in endothelial function.

#### SEX DIFFERENCES IN SEX STEROID RECEPTOR-INDEPENDENT MECHANISMS CONTROLLING ENDOTHELIAL FUNCTION

Aside from the marked role(s) of sex steroids and their receptors in the control of endothelial function in men and women, sex differences exist in many additional mechanisms that impact or control endothelial cell biology and function. In particular, sex-specific differences in the RAS and the ET-1 signaling pathway both play large roles in sex differences observed in endothelial function. These sex differences are directly and indirectly mediated by differences in the gonadal hormone milieu, which lead to sex differences in protein expression and/or activity at endothelial cells and VSMCs. In this section, we review sex differences in the role of the RAS and ET-1 signaling in endothelial function in healthy young men and premenopausal women.

##### *ET-1 and Sex Differences in Endothelial Function*

The ET-1 system is an important mediator of sex differences in vascular physiology and pathophysiology. ET-1 is a potent vasoconstrictor, and overactivation or dysfunction of the ET system plays a role in the development and progression of CVD in both experimental and clinical studies. ET-1 is produced from its precursor prepro-ET-1 in a two-step enzymatic pathway governed by the constitutively expressed endothelin-converting enzyme (ECE). ET-1 is continuously synthesized and released and acts primarily through autocrine or paracrine signaling at the level of the tissue in which it is synthesized. In the vasculature, the vasoconstrictor effects of ET-1 are mediated by ET-1 binding to the G protein-coupled ET type A receptor (ET<sub>AR</sub>) and ET type B receptor (ET<sub>BR</sub>) subtypes on the vascular smooth muscle. ET<sub>BR</sub>s are also expressed on the vascular endothelium and, upon ET-1 binding, induce the release of endothelium-derived vasodilators such as NO. As such, the net effect of ET-1-mediated vascular tone derives

from the balance of vascular smooth muscle ET<sub>AR</sub>- and ET<sub>BR</sub>-mediated vasoconstriction and endothelial ET<sub>BR</sub>-mediated vasodilation. ET-1 also induces the production of growth factors, ROS, and inflammatory mediators, which contribute to vascular pathology associated with overactivity of the ET-1 system. In this section, we focus on sex differences in ET-1-mediated endothelial function. Aside from the vascular effects of ET-1, ET-1 synthesis and signaling are important in a number of other physiological processes, including the renal and immune systems, and sexual dimorphism exists in these systems as well. As these systems are outside the scope of this review, we refer the reader to one of many excellent reviews on these topics (69, 110, 228).

In the vasculature, ET-1 is produced by ECE within the endothelial cell and diffuses out of the cell to act on membrane-bound ET<sub>BR</sub>s on the endothelium (autocrine) or ET<sub>AR</sub>s and ET<sub>BR</sub>s on the vascular smooth muscle (paracrine). Circulating ET-1 is cleared by endothelial ET<sub>BR</sub>s and in the lungs by endocytosis and degradation (133). ECE activity and mRNA expression are augmented in the vasculature of OVX rats (221) and attenuated by treatment with  $E_2$  or phytoestrogens (186). Similarly,  $E_2$  inhibits ET-1 mRNA expression and ET-1 release in human endothelial cells (15) and VSMCs (220), whereas testosterone increases ET-1 mRNA expression in human aortic endothelial cells (171). Evidence points to a role for circulating female hormones lowering circulating ET-1 in women compared with age-matched men (178). During the menstrual cycle, plasma ET-1 concentrations fluctuate and are lowest during the luteal phase, when both  $E_2$  and progesterone are elevated. Creatas et al. (38) observed a decrease in plasma ET-1 in primary amenorrheic female teenagers after exogenous  $E_2$  treatment. Pregnancy, another condition in which endogenous estrogens and progesterone are elevated, is associated with decreases in circulating ET-1 (127). Conversely, women who have preeclampsia, a hypertensive disorder of pregnancy, have attenuated plasma progesterone and elevated plasma ET-1 compared with healthy pregnant women, and progesterone supplementation attenuates ET-1 secretion in human umbilical vascular endothelial cells exposed to serum from preeclamptic women (106). Finally, women with PCOS exhibit elevated circulating androgens and display higher plasma ET-1 than healthy age-matched women (49, 50). These data suggest that ET-1 production in the vascular endothelium is attenuated in the presence of estrogens and progesterone. Complementary to these sex hormone-mediated attenuations in ET-1 synthesis, increases in endothelial ET<sub>BR</sub> protein expression with estrogen and progesterone may also increase clearance of circulating ET-1 (133). In general, women have a greater ET<sub>BR</sub> protein expression compared than men, and these differences likely also contribute to sex differences in circulating ET-1 by increasing local ET-1 clearance. Similarly, increases in circulating ET-1 in women with altered estrogen and/or progesterone (i.e., preeclampsia or PCOS) may reflect changes in clearance of ET-1 consequent to changes in ET<sub>BR</sub> expression. Collectively, sex hormone-mediated differences in both ET-1 synthesis by ECE and clearance through ET<sub>BR</sub>s contribute to circulating ET-1 concentrations and are reflected in the differences in plasma ET-1 of men compared with women.

Aside from sex differences in endothelial synthesis of ET-1, men have a greater vasoconstrictor response to exogenous ET-1 administration compared than women. Competitive bind-

ing studies conducted in human saphenous veins have demonstrated a higher ratio of ET-1 binding to both ET<sub>AR</sub>s and ET<sub>BR</sub>s in men, resulting in twofold greater constrictor responses in the veins obtained from men compared with those from women (58). Similarly, vascular sensitivity to ET-1 is reduced in cultured cerebral arteries obtained from women compared with those from men (2). Indeed, animal models have demonstrated that physiological concentrations of estrogens attenuate ET-1-mediated vasoconstriction in vivo, whereas testosterone treatment exaggerates these responses (223).

These sex differences in vascular responsiveness to ET-1 are mediated, at least in part, by differences in the expression and location of ET-1 receptor subtypes. In general, women have a greater proportion of endothelial ET<sub>BR</sub>s relative to vascular smooth muscle ET-1 receptors, favoring more dilation with which to attenuate the overall constrictor tone. Administration of the ET<sub>BR</sub> antagonist BQ-788 into the cutaneous microvasculature of healthy young human subjects demonstrates that ET<sub>BR</sub>s mediate tonic constriction in men but contributes to tonic vasodilation in the microvasculature of women (102). Stauffer et al. (213) showed that increases in forearm blood flow in response to intra-arterial infusion of the ET<sub>AR</sub> antagonist BQ-123 are greater in men, demonstrating that men also have greater tonic ET<sub>AR</sub>-mediated vasoconstriction. In contrast, saphenous veins and cerebral arteries obtained from women demonstrate greater ET<sub>BR</sub> expression than tissues obtained from men (2, 58). These human studies are supported by animal models in which OVX increases the vascular vasoconstrictor response to ET-1 and to ET<sub>BR</sub> antagonism, and this effect is reversed when estrogen is administered (42). Few, if any, studies have examined the role of testosterone in ET-1 receptor subtype location and expression. Interestingly, women with PCOS, who demonstrate hyperandrogenism and consequently elevated circulating testosterone, exhibit attenuated ET<sub>BR</sub>-mediated dilation compared with normally ovulating control women (242). Furthermore, this effect is abolished during androgen suppression (243). Collectively, this body of literature suggests that healthy premenopausal women benefit from a greater endothelial ET<sub>BR</sub>-mediated dilation, mitigating ET-1-induced tonic vasoconstrictor tone in the vasculature (Fig. 2). This sex-dependent protective effect is likely governed by circulating ovarian hormones, since OVX animals and women with hyperandrogenism seem to lack this protection, contributing to relative vascular dysfunction compared with their healthy counterparts (Fig. 3).

Several clinical trials have demonstrated the efficacy of selective ET<sub>AR</sub> and combined ET<sub>AR</sub>/ET<sub>BR</sub> antagonism in reducing blood pressure in humans with hypertension (112, 156, 240, 253). However, despite this clinical potential, none of these antagonists have been approved for the management of primary hypertension because of their side effects, mainly fluid retention. Clinical trials exploring sex differences in the response to ET receptor antagonism in humans are lacking; however, sex hormones may contribute to the differential responses to ET antagonists in male and female animals. Bosentan, a mixed ET<sub>AR</sub>/ET<sub>BR</sub> inhibitor, is more effective at lowering blood pressure in male compared with female DOCA-salt rats (227). This sex-dependent effect is abolished after OVX of female animals and returns with estrogen replacement, along with ET<sub>BR</sub> expression (41). Conversely,

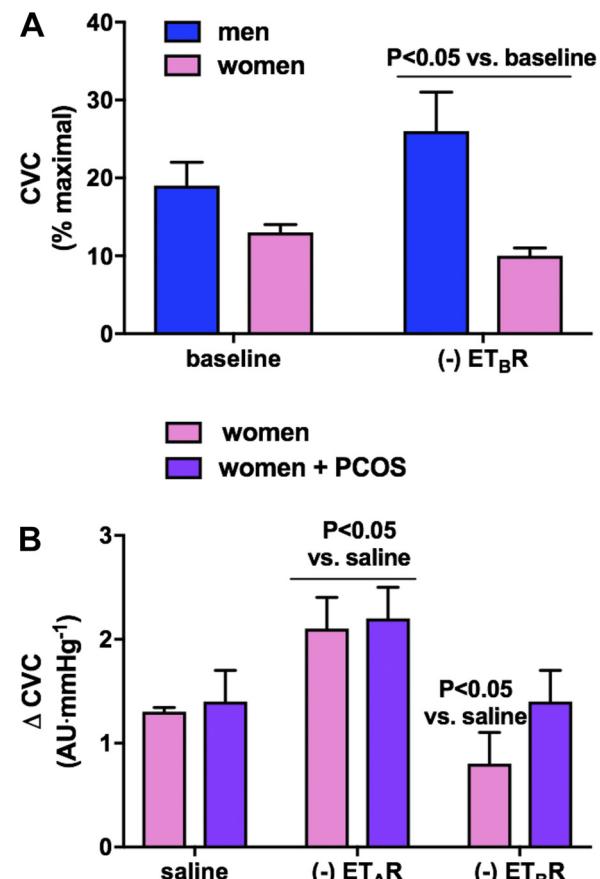
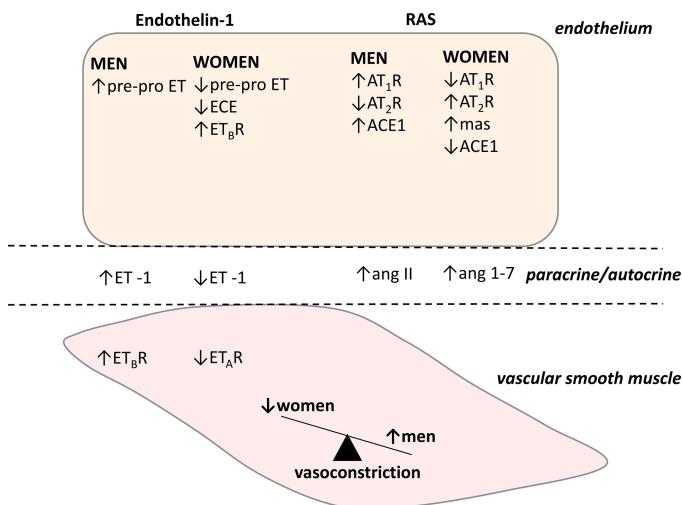


Fig. 2. A: sex differences in cutaneous microvascular responses to endothelin type B receptor (ET<sub>BR</sub>) subtype antagonism. Healthy young men demonstrate an increase in cutaneous vascular conductance (CVC) with ET<sub>BR</sub> blockade, whereas women show a decrease, suggesting that the ET<sub>BR</sub> mediates tonic constriction in men but contributes to vascular relaxation in women [adapted from Kellogg et al. (102)]. B: however, although there are no differences in tonic constriction mediated by the endothelin type A receptor (ET<sub>AR</sub>) between healthy women and women with polycystic ovarian syndrome (PCOS), women with PCOS do not demonstrate a decrease in CVC with ET<sub>BR</sub> antagonism. These data suggest that the ET<sub>BR</sub> does not contribute to vascular relaxation in women with PCOS, which is characterized by elevated circulating androgens [adapted from Wenner et al. (243)]. AU, arbitrary units.

administration of a selective ET<sub>AR</sub> antagonist more effectively lowers blood pressure in female compared with male rats (101). These findings corroborate the proposed protective role of ET<sub>BR</sub>-mediated dilation in female subjects and support the idea that differential activation of ET-1 signaling contributes to sexual dimorphism in ET-1 mechanisms contributing to hypertension. Clinical trials in humans focusing on sex differences in efficacy and outcomes of treatment with different ET antagonists will provide further evidence of these sex differences in human vascular physiology and provide clinical insight into sex-specific management of CVD.

#### The RAS and Sex Differences in Endothelial Function

The RAS is a key system in controlling body fluid balance and blood pressure, and overactivation of the RAS leads to increases in blood pressure and the progression of CVD and renal disease in men and women. Sex hormones affect the components of the RAS by a number of mechanisms, and the net effect of physiological estrogen concentrations in humans



**Fig. 3.** Sex differences in the cellular endothelin (ET)-1 system and renin-angiotensin system (RAS). Collectively, men synthesize more ET-1 and angiotensin II (ANG II) and have greater vasoconstrictor responses to both ET-1 and ANG II, mediated by sex differences in receptor subtype expression, whereas women have higher circulating ANG-(1-7) concentrations. These differences contribute to an overall greater tonic vasoconstrictor tone in healthy young men compared with healthy young women. ECE, endothelin-converting enzyme; ET<sub>A</sub>R, ET type A receptor; ET<sub>B</sub>R, ET type B receptor; AT<sub>1</sub>R, ANG II type 1 receptor; AT<sub>2</sub>R, ANG II type 2 receptor; ACE1, angiotensin-converting enzyme; mas, Mas receptor.

seems to be an overall inhibition of angiotensin II (ANG II) synthesis and signaling resulting in a blood pressure reduction. Conversely, circulating testosterone may amplify the RAS, resulting in greater renin activity and circulating ANG II in male rats (32, 54); however, these findings have not yet been explored in humans. In this section, we focus on sex differences in RAS effects on endothelial function. For detailed explanations of the role of sex hormones in upstream regulation of the RAS, including renin activity and ANG II synthesis, we refer the reader to one of many excellent reviews on these topics (62, 216).

In response to systemic graded infusions of ANG II, young healthy men may have exaggerated increases in diastolic blood pressure and mean arterial pressure compared with young healthy women (225). However, these findings contradict earlier studies showing that there are no differences in pressor or forearm vasoconstriction responses to ANG II administration between men and women (20), although Gandhi et al. (67) did find that the duration of the pressor response was significantly longer in men than in women. Despite these equivocal results, tissue responsiveness to exogenous ANG II is attenuated in women and female animals. Cultured cerebral arteries from women have an attenuated vasoconstrictor response to exogenous ANG II compared with those obtained from men, despite a lack of difference in ANG II type 1 receptor (AT<sub>1</sub>R) protein expression (2). Human studies of renal vasoconstrictor responses to ANG II are lacking; however, animal studies have consistently demonstrated augmented renal constrictor responses to exogenous ANG II in male compared with female animals (163, 200). Furthermore, these effects have been found to be modulated by circulating sex hormones, such that androgens contribute to exaggerated constrictor responses in male animals (164, 212), and estrogens attenuate these responses in female animals (163, 187, 252). Similarly, in humans, the

degree of hypertension and renal injury closely parallels increases in RAS activation in men, whereas the same relation is not as strong in women (111).

Recently, ANG II has been demonstrated to enhance ET-1-mediated constriction through increased tissue expression of ET-1 (88), upregulation of ET<sub>A</sub>R expression (122), and decreasing ET<sub>B</sub>R function (109), and it is likely that sex differences in the ET-1 system contribute to sex differences in the vascular responses to ANG II. To this end, Kittikulsuth et al. (108) recently demonstrated that ET<sub>B</sub>R blockade during ANG II infusion and high-salt feeding exacerbated the rise in blood pressure in female rats, such that they demonstrated the same increase in blood pressure as that observed in high-salt/ANG II male rats. However, ET<sub>B</sub>R blockade had no effect on male rats, suggesting that the ET<sub>B</sub>R plays a beneficial role in protecting female compared with male rats against ANG II-induced hypertension (108). Intapad et al. (94) recently found that ET<sub>A</sub>R inhibition reduced ANG II-mediated increases in blood pressure in male, but not female, rats, suggesting that the ET<sub>A</sub>R contributes to ANG II-mediated increases in blood pressure in male but not female rats. While this mechanistic interplay of the RAS and ET-1 system has not yet been explored in humans, sex differences in the ET-1 system likely contribute to sex differences in the vascular responses to ANG II and vice versa.

Most of the classical effects of the RAS are mediated at the tissue level, primarily by the binding of ANG II to AT<sub>1</sub>Rs, leading to vasoconstriction, and to some extent by the binding of ANG II to the opposing ANG II type 2 receptor (AT<sub>2</sub>R). The AT<sub>2</sub>R is expressed at low levels in the adult vasculature but elicits downstream effects such as NO generation and vasodilation and has anti-inflammatory and antithrombotic actions, which oppose those of the AT<sub>1</sub>R. Female Sprague-Dawley rats have greater aortic expression of AT<sub>2</sub>Rs than male Sprague-Dawley rats, and testosterone withdrawal by castration significantly elevates AT<sub>2</sub>R expression in male rats, suggesting a role for androgens in downregulating AT<sub>2</sub>Rs and contributing to sex differences in vascular AT<sub>2</sub>R expression (146). Similarly, female Sprague-Dawley rats have an estrogen-dependent increase in AT<sub>2</sub>Rs (193, 195). However, because AT<sub>2</sub>R expression is minimal in the adult human vasculature, and because sex differences in the AT<sub>2</sub>R have not yet been examined in humans, it remains unclear whether sex differences in the AT<sub>2</sub>R contribute to differences in the endothelial response to ANG II in men and women. Similar to circulating renin and angiotensin-converting enzyme (ACE), vascular AT<sub>1</sub>R expression and activity are downregulated in the presence of estrogen and upregulated in the presence of testosterone (33, 45, 159, 160, 168, 187). Downstream of the AT<sub>1</sub>R, the intracellular RAS operates independently of the circulating RAS and contributes to alterations in vascular tone and arterial structure. All of the components of the circulating RAS are expressed in the vascular wall, and all are modulated by estrogens, mainly at the endothelial level. Tissue ACE1 expression and activity are elevated in ovariectomized female animals and decreased with estrogen replacement (45, 66, 222). In contrast, oophorectomized male mice have decreased ACE1 protein expression compared with intact male mice, suggesting a role for androgens in the sex-specific increase in tissue ACE1 expression (65). In humans, atrial tissue slices obtained from men demonstrate downregulated ACE1 expression when treated with

estradiol (23). These sex hormone-specific effects have been demonstrated to be mediated via tissue ERs and ARs in both human and animal tissues. ERs themselves initiate many downstream vasoprotective signaling mechanisms (see above). Endothelial ER-mediated increases in NO and decreases in ROS likely protect the vasculature from ANG II-mediated vasoconstriction, inflammation, and ROS production, representing a female sex-specific protective mechanism in ANG II signaling biology.

Aside from the classical proconstrictor RAS pathway, the more recently described “nonclassical” RAS pathway has gained attention for its ability to antagonize classical RAS signaling, leading to vasodilation and/or mitigation of ANG II-mediated vasoconstriction and inflammation. In the nonclassical RAS pathway, ACE2 catalyzes the production of ANG-(1–7) directly from ANG II and indirectly from ANG I [via ANG-(1–9)]. Similarly, the peptidase neprilysin directly converts ANG I to ANG-(1–7) and plays a major role in ANG-(1–7) formation in the circulation and vascular endothelium (4). In turn, ANG-(1–7) can bind the AT<sub>2</sub>R as well as its own receptor, Mas, to oppose the intracellular actions of ANG II-bound AT<sub>1</sub>Rs. Similarly to the AT<sub>2</sub>R, sex differences in the ANG-(1–7)/Mas receptor axis may also play a role in divergent responses to RAS stimulation between the sexes. In animal models of hypertension, circulating ANG-(1–7) is higher in female than male animals, and antagonism of the Mas receptor abolishes female sex-specific protection from vascular injury (71, 217, 238). Similarly, Brosnihan et al. (22) demonstrated the ability of E<sub>2</sub> to promote ANG-(1–7) production in transgenic hypertensive rats. Human studies of sex differences in ANG-(1–7) are limited, but Sullivan et al. (218) recently reported that healthy premenopausal women have higher circulating plasma ANG-(1–7) concentrations than age-matched healthy men. Women taking oral contraceptives have also been reported to have greater circulating ANG-(1–7) compared with ovulatory women during both the follicular and luteal phases of their ovulatory cycle; however, these increases were accompanied by similarly elevated ANG II and may be compensatory and/or result from a generalized increase in RAS activity (44). Interestingly, women taking oral contraceptives have, on average, ~5-mmHg higher mean arterial pressures compared with ovulatory women not taking hormonal birth control, suggesting that these circulating changes in ANG-(1–7) may not translate to reduced vasoconstrictor tone. Among pregnant women, where circulating E<sub>2</sub> and other hormone concentrations increase, urinary ANG-(1–7) increases, supporting a role for female gonadal hormones in mediating an increase in ANG-(1–7) synthesis (231). Downstream of circulating ANG-(1–7), gonadal hormones likely influence Mas receptor expression and/or sensitivity. Female spontaneously hypertensive rats demonstrate greater renal cortical Mas protein expression than male spontaneously hypertensive rats (194, 217), and normotensive female rats demonstrate a reduced renal blood flow response to Mas receptor blockade, which is absent in their male counterparts (191). Along with its vasodilator role in the endothelium, the ACE2/ANG-(1–7)/Mas axis has also been suggested to reduce inflammation and inhibit vascular and cellular growth mechanisms in cerebral, renal, cardiac, and pulmonary vascular tissues (206). While the full scope of this anti-inflammatory mechanism is outside the scope of this review, it is possible that the anti-inflammatory role of ACE2/

ANG-(1–7)/Mas contributes to sex differences in the incidence of CVD by inhibiting vascular inflammatory processes in premenopausal women. Collectively, although human studies of ANG-(1–7) synthesis and Mas receptor expression are limited, emerging animal data suggest that the ACE2/ANG-(1–7)/Mas receptor axis likely plays a role in sex differences in endothelial function and contributes to the relatively protected vascular phenotype of premenopausal women (Fig. 3).

While it is commonly hypothesized that the sex differences in vascular RAS mechanisms play a large role in the relative protection of women against CVD before menopause, the pathophysiological significance of the vascular tissue RAS in clinical CVD is unclear. However, patients with hypertension have been shown to have increased vascular responses to exogenous ANG II (205, 229), and patients treated with ANG II receptor blockers (ARB) or ACE inhibitors demonstrate significant regression of vascular remodeling compared with patients treated with atenolol (where blood pressure was lowered independently of RAS activity) (130, 198, 199). Interestingly, women respond more favorably to ARB treatment, whereas ACE inhibitors are more effective in men. In the China STATUS II study, women showed a greater antihypertensive response to treatment with the ARB valsartan combined with amlodipine compared with men on the same treatment (236). Similarly, survival rates for women after congestive heart failure are better for women treated with an ARB compared with those treated with an ACE inhibitor, and yet the opposite was true for men (90). Although few clinical trials have reported efficacy by sex, these few findings are supported by animal studies in which the antihypertensive effects of ACE inhibitors are greater in young male animals compared with young female animals, whereas ARB treatment has a greater depressor effect on blood pressure in young female compared with male animals. While more human data from clinical trials powered to examine sex differences is warranted, these findings suggest that the depressor arm of the RAS is increased in women, and therapeutic interventions leveraging these sex-specific protective mechanisms are more effective in women than they are in men.

#### *Sex Differences in Sympathetic Nerve Activity*

While a full discussion of sex differences in sympathetic nerve activity directed to the vasculature is outside the scope of this review, it is important to note that these differences likely play a role in the different relative CVD risk observed between men and women. While there is no relationship between muscle sympathetic nerve activity (MSNA) and blood pressure in healthy young men or women, the mechanisms mediating this are different between the sexes. In young men, there is an inverse relationship between MSNA and cardiac output, such that under conditions of high MSNA, cardiac output is lower to maintain mean arterial pressure (78). However, in young women, sympathetic vasoconstriction is attenuated by β-adrenergic-mediated dilation, abolishing the relationship between MSNA and total peripheral resistance to maintain blood pressure (78). In women, changes in circulating concentrations of sex hormones (across the menstrual cycle, with hormonal contraceptive use, or with pregnancy) have also been shown to influence the relationship between MSNA and mean arterial pressure, highlighting a role for sex hormones in mediating sex

differences in these responses. Importantly, changes in vessel stiffness mediated by sex differences in mechanisms contributing to endothelium-dependent dilation and vascular smooth muscle function alter the relationship between sympathetic nerve activity and vascular tone. For further explanation of this topic, we refer the reader to one of many excellent reviews on the topic of sex differences in neurovascular control of blood pressure (7, 21, 79).

#### SEX DIFFERENCES IN AGE-ASSOCIATED ENDOTHELIAL DYSFUNCTION

The prevalence of CVD increases with age in both men and women. As described above, the prevalence of CVD is generally lower in young women than in age-matched men. However, after menopause, women have a higher prevalence of CVD compared with age-matched men. Declines in endothelial function with aging have been well described. Interestingly, the decline in endothelial function begins around the fourth decade of life in men but is shifted ~10 yr in women, such that declines in endothelial function are most noted during the fifth decade of life, which is at the time of menopause (31). Furthermore, the rate of decline in endothelial function is much steeper in women than in men later in life (31). Indeed, within women, there is a progressive decline in endothelial function across the stages of menopause (151). Drastic changes in sex hormones (particularly E<sub>2</sub>) have been traditionally thought to mediate these changes in both endothelial dysfunction and CVD with aging; in other words, the loss of the vasoprotective/vasodilatory effects of E<sub>2</sub> concomitant with unopposed vasoconstriction due to the loss of E<sub>2</sub> and vascular remodeling over time are responsible for the greater decline in endothelial function and the associated rise in CVD in women after menopause.

Given the numerous changes in vascular function with aging, it is challenging to understand what is driven purely by age versus the changing hormonal milieu. Two elegant studies from the laboratory of Dr. Muller-Delp have examined the separate and combined impact(s) of aging and hormonal status in female rats (99, 117). Vascular function was measured in young and old intact female rats, young and old OVX, and young and old OVX rats administered E<sub>2</sub>. In young female rats, vasodilation was blunted in OVX rats compared with intact rats. Vasodilation was similar between intact rats and OVX rats given E<sub>2</sub>. As expected, vasodilation was lower in old female intact rats compared with young female intact rats. However, OVX did not cause a further decline in vasodilation, as there were no differences in vessel relaxation between old intact and old OVX female rats. Estrogen administration to old OVX rats improved vasodilation, such that vessel relaxation was similar to that of young intact rats. Importantly, these studies administered E<sub>2</sub> immediately after OVX in old female rats and were able to completely restore vasodilation to a similar response to that of young female rats. Although not statistically compared, young OVX rats appeared to have similar vessel relaxation to that of old intact female rats (~30%), suggesting a strong influence of ovarian hormones on vasodilatory function. Taken together, these data show the important influence of both age and hormonal status on endothelial function.

#### *Sex Hormone Changes With Aging*

As women approach menopause, E<sub>2</sub> and progesterone generally decline and follicle-stimulating hormone increases (76). However, E<sub>2</sub> can fluctuate widely before menopause (26, 196). Due to the initial loss of ovarian function during the early perimenopausal period, women may initially experience surges in E<sub>2</sub> followed by rapid declines (26, 196). These fluctuations are commonly associated with erratic changes in menstrual cycle length (i.e., shorter, more frequent cycles) and vasomotor symptoms or hot flashes. As women progress through perimenopause, ovarian function continues to decline and E<sub>2</sub> levels decrease. During this time (late perimenopause), menstrual cycle frequency declines such that women experience periods of amenorrhea (>60 days). This is followed by a complete loss of ovarian function and permanent cessation of menses (>1 yr), at which time women are considered postmenopausal. As such, levels of E<sub>2</sub> and progesterone remain low after menopause, and women remain in a hypoestrogenic state permanently. With regard to androgens, studies have shown that testosterone may either increase (138) or remain stable (25) during menopause. Further evidence suggests a rapid decline in testosterone after menopause but that concentrations begin to increase with advancing age (after 70 yr) (116). Importantly, given the large decline in E<sub>2</sub> with menopause, there is an increase in the androgen-to-estrogen ratio in postmenopausal women, which may also have implications for endothelial function and cardiovascular health (174).

As men age, there are declines in circulating androgen concentrations. Although this is generally not as dramatic as the decline in E<sub>2</sub> observed in women after menopause, there are instances where the loss of testosterone can be quite significant. As such, the impact of “low testosterone” on cardiovascular function has gained recent attention over the past decade. There appears to be a link between androgen deficiency and developing atherosclerosis (57, 188). Low circulating testosterone in older men is associated with atherosclerosis compared with age-matched controls with testosterone concentrations similar to those in younger men (8, 188). Given that there are many changes that occur to the cardiovascular system with aging, this association does not necessarily prove “cause and effect.” However, data in animal models using castration (3) or AR knockout models (249) suggest a direct association between testosterone and atherosclerosis. To our knowledge, there are no data in humans that demonstrate a cause-and-effect relationship between testosterone and atherosclerosis but it is an important area of investigation.

#### *Mechanisms Contributing to Endothelial Dysfunction With Aging*

Due to the interactions of sex hormones on multiple pathways regulating vascular function, the mechanisms contributing to endothelial dysfunction with aging are complex, multi-factorial, and not completely understood. As reviewed above, there is a strong and direct influence of E<sub>2</sub> on NO production and release from the endothelium via ER $\alpha$  (see Fig. 1). Thus, declines in E<sub>2</sub> after menopause likely result in endothelial dysfunction due to reduced eNOS activation and a subsequent reduction in NO production or bioavailability. Moreau et al. (68) demonstrated that expression of ER $\alpha$  and eNOS in endothelial cells harvested from peripheral veins of women are

lower in postmenopausal women than in young women (68). Thus, not only does  $E_2$  decline with aging but  $ER\alpha$  receptor expression also declines. Furthermore,  $ER\alpha$  expression and eNOS expression are correlated with flow-mediated vasodilation. These data provide direct evidence in humans that the age-associated decline in endothelial function in postmenopausal women is due in part to a reduction in  $ER\alpha$  expression, loss of eNOS, and subsequent reductions in NO production. NO bioavailability can also be impacted by oxidative stress, and it is well known that estrogens have antioxidant capabilities. Therefore, there may be greater generation of ROS, which can scavenge  $O_2^-$  and reduce NO bioavailability in postmenopausal women due to the declines in  $E_2$  with menopause. Systemic infusion of the nonspecific antioxidant ascorbic acid increased flow-mediated vasodilation in estrogen-deficient postmenopausal women (152). However, in postmenopausal women using estradiol (either oral or transdermal), there was no further increase in flow-mediated vasodilation with the infusion of ascorbic acid (152). These data demonstrate that oxidative stress is a key mechanism contributing to endothelial dysfunction in estrogen-deficient postmenopausal women and that estrogen treatment improves endothelial function in part via reductions in ROS.

The ET system is also impacted by aging. Most studies have shown that plasma ET concentrations are greater in postmenopausal women (129, 241) and older men (52) compared with their younger counterparts. Furthermore, ET-1 protein expression in harvested endothelial cells is greater in older men than in younger men and associated with the decline in endothelial function (52). As mentioned above, there are known sex differences in plasma ET-1 as well as expression of  $ET_{AR}$ s and  $ET_{BR}$ s. For example, brachial artery infusions of an  $ET_{AR}$  antagonist resulted in a greater increase in forearm blood flow in older men compared with women, indicating that  $ET_{AR}$ -mediated vasoconstriction is greater in older men than in women (213). However, when a combination of  $ET_{AR}$  and  $ET_{BR}$  antagonists was infused, blood flow increased more in

women (213), suggesting that older women have greater  $ET_{BR}$ -mediated vasoconstriction. This is in direct contradiction to the primary role of  $ET_{BR}$ s in mediating vasodilation in young women, demonstrating the complex interplay between aging and sex hormones in mediating sex differences across the lifespan. These sex differences in heightened ET-1-mediated vasoconstriction in older adults may also contribute to the age-related declines in endothelial function and explain, in part, the sex differences in endothelial function in older adults. Indeed,  $ET_{AR}$ -mediated vasoconstriction is greater in older men than in younger men (232). To determine the role of this heightened constriction on the age-related declines in endothelial function in men, Westby et al. (246) infused acetylcholine in the presence and absence of the  $ET_{AR}$  antagonist BQ-123 in young and older men. As expected, vasodilatory responses to acetylcholine were blunted in older men. However, coinfusion of BQ-123 improved endothelium-dependent dilation in older men (Fig. 4). These data demonstrate that the reduction in endothelial function in older men is due, in part, to greater  $ET_{AR}$ -mediated constriction (246). ET-1 also contributes to the decline in endothelial function in postmenopausal women (241); however, this decline is mediated by a different receptor mechanism, primarily a shift in  $ET_{BR}$  responses, as the  $ET_{BR}$  antagonist BQ-788 restored vasodilation in postmenopausal women (241). Interestingly, there were no differences in vasodilator capacity during blockade of  $ET_{AR}$ s between young and postmenopausal women. Thus, the decline in endothelial function in postmenopausal women is due, in part, to a loss of  $ET_{BR}$ -mediated dilation (241), whereas  $ET_{AR}$ -mediated constriction contributes to the declines in endothelial function in older men (246) (Fig. 4). Whether these changes in  $ET_{BR}$  function are related to declines in  $ER\alpha$  sensitivity are not known. Taken together, sex differences in the ET-1 system persist throughout aging and contribute to endothelial dysfunction through different receptor mechanisms.

Activation of the RAS occurs with aging and likely contributes to the greater prevalence of hypertension in postmeno-

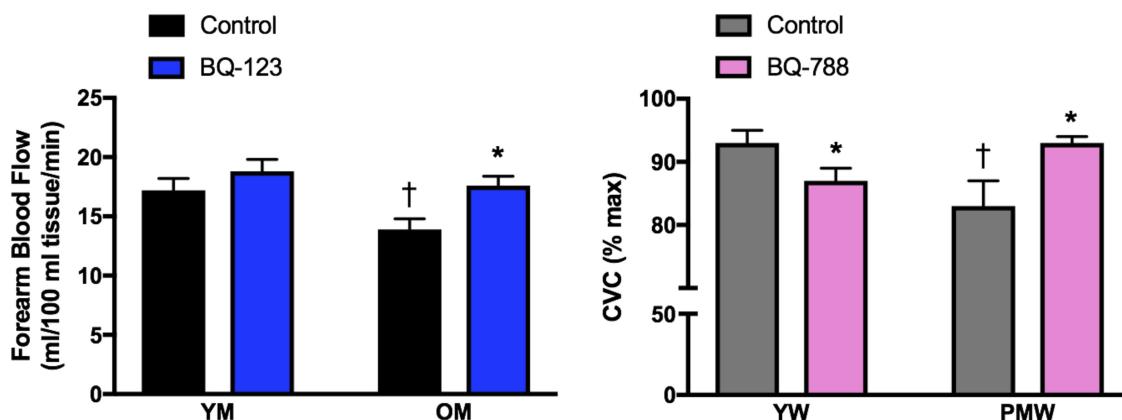


Fig. 4. Age and sex differences in endothelin (ET)-1 receptor responses on endothelial function. Endothelial function in young men (YM) and older men (OM) (*left*) was determined by measuring forearm blood flow responses to acetylcholine during control/saline conditions (black bars) or coinfused with the ET type A receptor ( $ET_{AR}$ ) antagonist BQ-123 (blue bars). Data were adapted from Westby et al. (246) and plotted as blood flow response at the highest dose ( $16 \mu\text{g} \cdot 100 \text{ ml tissue}^{-1} \cdot \text{min}^{-1}$ ) of acetylcholine. The age-associated decline in endothelial function in men was abolished with infusion of  $ET_{AR}$  antagonist BQ-123 (246). In contrast, the age-related decline in endothelial function in women is mediated in part by alterations in the ET type B receptor ( $ET_{BR}$ ; *right*). Cutaneous microvascular conductance (CVC; expressed as %maximal dilation) was measured in young women (YW) and postmenopausal women (PMW) during local heating with microdialysis perfusions of lactated Ringer solution (control; gray bars) or the  $ET_{BR}$  antagonist BQ-788 (pink bars). Data were adapted from Wenner et al. (241).  $ET_{BR}$  mediates dilation in YW. However, the age-associated decline in microvascular endothelial function in PMW was abolished with perfusion of the  $ET_{BR}$  antagonist BQ-788 (241). \* $P < 0.05$  vs. control; † $P < 0.05$  vs. young.

pausal women (120, 121). The balance between ANG II and ANG-(1–7) has gained recent attention as an important pathway for cardiovascular health, and Mas receptor-mediated increases in NO have been proposed to be a new target for improving endothelial function (84, 173). Recent data indicate sex differences in this pathway, showing that the lower pressor response to ANG II in female rats occurs via AT<sub>2</sub>Rs and is mediated by estrogens (193, 195). However, less is known about aging and menopause on the impact on ANG-(1–7) and Mas receptors. In a surgical model of menopause, constricting responses to ANG II were greater and dilator responses to ANG-(1–7) were blunted in OVX rats (56). Furthermore, ovariectomy resulted in a reduction in Mas receptor expression and ACE2 gene expression in heart tissue of mice (18). Thus, it appears that the abrupt reduction in estrogen exposure with OVX, or a surgical model of menopause, alters the vasodilatory ANG-(1–7)/Mas receptor pathway. However, to our knowledge, there is a dearth of information in humans; whether these findings in animal models translate to humans and specifically to control of endothelial function warrants additional study.

Finally, these age-related declines in endothelial function may also be related to a reduction in ER (or AR) function or sensitivity. Moreau et al. (68) demonstrated that ER $\alpha$  expression is lower in postmenopausal compared with young women. To our knowledge, there have been no parallel studies in men to examine AR expression in relation to changes in endothelial function with age. Given the importance of ERs and ARs on cardiovascular function, we refer the reader to a recent elegant review focusing on these signaling mechanisms (17).

#### *Hormone Therapy Effects on Endothelial Function*

Given the potential importance of estradiol on maintaining vascular endothelial health, numerous studies have examined the impact of hormone therapy on endothelial function in postmenopausal women. Menopausal hormone therapy (MHT), but particularly E<sub>2</sub> administration, improves endothelial function in postmenopausal women (91, 152, 234). However, it seems as though the magnitude of improvement depends on the timing of when MHT was initiated. In women who were <5 yr postmenopausal, both acute (sublingual dose) and chronic (3 mo, oral) estrogen administration improved FMD more compared with those women who were >5 yr postmenopausal (235). Changes in ER expression or eNOS expression, as highlighted above, may explain, in part, the reason for this discrepancy. Until recently, the timing of MHT initiation had not been a focus of understanding the vascular consequences of menopause and the subsequent importance of MHT. As such, the data reviewed here do not separate postmenopausal women on the basis of time since menopause but demonstrate the influence of estradiol on the mechanisms regulating endothelial function.

Several lines of evidence in cell and animal models demonstrate that ovarian hormones, particularly E<sub>2</sub>, modulate ET-1. As highlighted above, E<sub>2</sub> lowers ET-1 expression (98), attenuates ET-1-mediated vasoconstriction (223), and increases ET<sub>B</sub>R mRNA expression in coronary vessels (172). In humans, intracoronary infusions of E<sub>2</sub> decrease plasma levels of ET-1 in the coronary vasculature of postmenopausal women (239). Furthermore, administration of hormone therapy to postmeno-

pausal women reduces plasma ET-1 concentrations (14, 247). Although recent data indicate that endogenous fluctuations in ovarian hormones modulate ET-1 receptor function in young women (202), additional data are needed to understand the impact of MHT on ET-1 receptor function in postmenopausal women.

Oxidative stress is a key feature of vascular aging in both men and women, contributing to endothelial dysfunction (150, 152, 176). Given the antioxidant capacity of E<sub>2</sub>, it may not be surprising that improvements in endothelial function after E<sub>2</sub> administration are due, in part, to reductions in oxidative stress. Women who underwent OVX had reduced vasodilatory responses to acetylcholine, which was restored after infusion of vitamin C (234). However, 3 mo of estrogen replacement therapy in these women also restored endothelial function; since vitamin C infusion no longer increased vasodilation in women receiving E<sub>2</sub>, the authors concluded that E<sub>2</sub> improves endothelial function by reducing oxidative stress (234). Subsequent studies have also shown that vitamin C infusion increases FMD in estrogen-deficient postmenopausal women (152). Interestingly, aerobic exercise, a common lifestyle modification to improve cardiovascular health, increases FMD in older men but not in postmenopausal women, unless they are receiving E<sub>2</sub> therapy (152). One potential mechanism may be related to oxidative stress, as exercise-trained postmenopausal women receiving E<sub>2</sub> had no further increase in FMD during vitamin C infusion (152). Taken together, estrogen deficiency elicits oxidative stress and results in endothelial dysfunction, whereas estrogen replacement appears to mitigate oxidative stress to restore endothelial function.

Hormone therapy for postmenopausal women (particularly oral) has been shown to increase angiotensinogen (201) and plasma renin activity (81), whereas other studies have shown a reduction in ACE activity (161). However, systemic changes in the RAS do not necessarily reflect what is happening at the tissue/cellular level. As stated above, E<sub>2</sub> treatment increased ANG-(1–7) in human umbilical vein endothelial cells (148), and blockade of the Mas receptor in isolated vessels attenuated the vasodilatory effects of estradiol (209). However, E<sub>2</sub> administration to OVX rats attenuated cardiac ANG-(1–7) and Mas receptor expression (237). It is worth noting, however, that these studies were performed in young animals; as mentioned above, it is less clear how aging impacts ANG-(1–7)/Mas receptors and the impact of hormone therapy on this system. Therefore, additional data are needed in both animals and humans to understand the impact of hormone therapy, for both women and men, on an aging vascular system.

#### *Testosterone Therapy*

Testosterone falls with advancing age in men, and low circulating testosterone in older men is associated with atherosclerosis compared with age-matched controls with circulating testosterone concentrations similar to those of younger men (8, 188). In low-testosterone aging rat models, androgen administration can induce compensatory downregulation of androgen synthesis and protection against CVD, depending on dose and timing of administration (181). In humans, testosterone replacement to levels of younger men restores endothelial function (145), although with little impact on markers for

atherosclerosis (11). Interestingly, plasma ET-1 levels are higher in hypogonadal men, and testosterone treatment tends to decrease these levels (113). In contrast to these studies, blood pressure was reduced in castrated male rats but increased with testosterone administration (182); these blood pressure responses are mediated by ARs (183). In humans, androgen administration blunts endothelium-mediated vasodilation in hypogonadal men (12), increases peripheral vascular disease and hypertension (233), and impairs vascular reactivity (233). Similarly, testosterone administration does not enhance endothelium-mediated cutaneous microvascular vasodilation during local warming of the skin in older healthy men (210). Given the conflicting findings in the literature, additional studies are warranted to further understand the mechanisms underlying changes in testosterone and endothelial function.

Testosterone therapy has also become more common for use in postmenopausal women (although in lower doses compared with men), particularly to improve libido. As in men, the literature is conflicting regarding the impact of testosterone therapy on cardiovascular health for postmenopausal women. Some studies have demonstrated a positive correlation between testosterone and CVD risk factors in postmenopausal women (73, 114, 132, 170). Additional studies have reported detrimental effects of testosterone on lipid profiles (46, 114, 153) and atherosclerosis, particularly in carotid arteries of postmenopausal women (74, 175, 248). However, others have demonstrated that endogenous testosterone levels were positively correlated with FMD in postmenopausal women, such that those women with the lowest testosterone had the lowest FMD (149). It is important, however, to consider the ratio of testosterone to E<sub>2</sub>. Testosterone combined with E<sub>2</sub> therapy attenuated the development of foam cells and injury to the endothelium as well as reduced inflammation in cell and animal models (39). Similar findings were also shown in humans. In postmenopausal women currently using MHT, the addition of testosterone increased FMD (250). Although there are conflicting reports in the literature with regard to testosterone therapy in postmenopausal women and little is known about the underlying mechanisms, it appears that an appropriate androgen-to-estrogen ratio is an important consideration for vascular health.

#### SUMMARY AND FUTURE DIRECTIONS

In summary, a complex interplay between circulating sex steroid hormones, their receptors, and sex steroid-independent mechanisms contributes to the overall difference in endothelial function observed between healthy young men and women. The generalized sum of these differences suggests that young men have greater vasoconstrictor tone and attenuated endothelium-dependent dilation compared with premenopausal women, and it is this subclinical reduction in endothelial function that likely contributes to the earlier rise in the incidence of CVD in men. However, aging and the concomitant reduction in circulating sex hormones complicates the delineation of sex differences in older adults, and women may lose cardioprotection compared with age-matched men after menopause. Not all of the mechanistic sex differences that are present in young adults persist into older age, and new differences emerge in this unique period. Collectively, while a great deal of mechanistic work has described the role of sex hor-

mones and other sex differences in endothelial function across the lifespan, further study of these mechanisms is warranted.

One mechanism that has not been fully explored in humans in the context of sex, sex hormones, and relative changes in sex hormones with aging is prostanoid-dependent vascular responses. In particular, the balance between dilating and constricting prostanooids has been demonstrated to shift in numerous CVD states, and the influence of sex on relative protection from or contribution to these shifts has not been examined. OVX rats demonstrate a greater cyclooxygenase-dependent dilation to the endothelial agonist 2-thiazolylethylamine compared with intact or OVX + E<sub>2</sub>-supplemented rats (29). On the constrictor side, studies in GPER-1 knockout animals have shown no effect of GPER-1 deficiency on NO bioavailability but did show an increased VSMC response to endothelium-derived vasoconstrictor prostanooids, suggesting that E<sub>2</sub> signaling via GPER-1 may also contribute to VSMC contraction to cyclooxygenase-derived prostanoid vasoconstrictors (139). Furthermore, E<sub>2</sub> administration to OVX rats attenuated prostanoid-dependent vasoconstriction (5, 43). In humans, prostacyclin-mediated dilation is reduced with aging (158) and menopause (162), but the balance between vasodilator and vasoconstrictor prostanooids is not altered with menopause (162). Additional studies are needed to fully characterize the impact of prostanooids on age- and sex-related declines in endothelial function.

Interestingly, there is sexual dimorphism in renal production of prostanooids in spontaneously hypertensive rats, such that female animals have greater cyclooxygenase-2 expression and produce more prostaglandin and thromboxane in the renal medulla (219). Moreover, prostaglandin production is sensitive to physiological changes in testosterone but not estrogen in this model (219). However, although selective cyclooxygenase-2 inhibition reduces prostanoid production in female spontaneously hypertensive rats, there is no blood pressure lowering effect of this treatment in either male or female rats, suggesting that cyclooxygenase-2 is not a likely contributor to blood pressure control in either sex (55). Prostanoid production is downstream of many vascular signaling mechanisms that are affected by sex and aging (e.g., ANG II and ET-1), further obscuring distinct effects of sex hormones on prostanoid signaling. While this mechanism has been addressed in animal models, additional research is required in humans to determine the interaction between sex hormones and sex differences and the prostanoid system in endothelial function in humans. Thus, the relative contributions of sex hormone effects on prostanoid-dependent vascular responses, as well as their implications for sex differences in long-term CVD outcomes, is an area that requires future research attention.

In addition to understanding sex differences in the control and maintenance of endothelial function and cardiovascular health, additional study focused on harnessing these differences should aim to target sex-specific mechanisms for the prevention or treatment of CVD. As described above, men and women may respond differently to different cardiovascular medications, and these epidemiological results suggest that targeting or leveraging sex-specific mechanisms may improve patient outcomes across a wide variety of CVDs when the patient's sex is considered during treatment. Similarly, hormone replacement therapy for aging men and women may mitigate the progression of CVD and/or prevent some of the

age-related changes in sex-dependent mechanisms. However, further study, particularly related to timing, formulation, and administration of exogenous hormones to these populations, is necessary. Overall, sex differences in the mechanistic control and maintenance of endothelial function play an important role in supporting cardiovascular homeostasis, and identifying sex-specific recommendations for the treatment or prevention of CVD is essential for the mitigation of CVD morbidity and mortality in men and women across the lifespan.

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## DISCLOSURES

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

## AUTHOR CONTRIBUTIONS

A.E.S., M.M.W., and N.S.S. prepared figures; A.E.S., M.M.W., and N.S.S. drafted manuscript; A.E.S., M.M.W., and N.S.S. edited and revised manuscript; A.E.S., M.M.W., and N.S.S. approved final version of manuscript.

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