

## Review Article

# Sex differences in hypertension: lessons from spontaneously hypertensive rats (SHR)

 Ahmed A. Elmarakby<sup>1</sup> and  Jennifer C. Sullivan<sup>2</sup>

<sup>1</sup>Department of Oral Biology and Diagnostic Sciences, Augusta University, Augusta, GA 30912, U.S.A.; <sup>2</sup>Department of Physiology, Augusta University, Augusta, GA 30912, U.S.A.

**Correspondence:** Ahmed A. Elmarakby (aelmarakby@augusta.edu)



Although numerous clinical and experimental studies have clearly identified a sexual dimorphism in blood pressure control, the mechanism(s) underlying gender differences in blood pressure remain unclear. Over the past two decades, numerous laboratories have utilized the spontaneously hypertensive rats (SHR) as an experimental model of essential hypertension to increase our understanding of the mechanisms regulating blood pressure in males and females. Previous work by our group and others have implicated that differential regulation of adrenergic receptors, the renin–angiotensin system, oxidative stress, nitric oxide bioavailability and immune cells contribute to sex differences in blood pressure control in SHR. The purpose of this review is to summarize previous findings to date regarding the mechanisms of blood pressure control in male versus female SHR.

## Introduction

Hypertension is a slowly developing, progressive disease that currently affects approximately 50% of adults in the United States [1,2]. Hypertension is the primary modifiable risk factor for cardiovascular disease morbidity and mortality globally, and uncontrolled hypertension can lead to stroke, heart attack, heart failure and aneurysm [3]. Hypertension is also the second leading cause of renal failure. Failure to control blood pressure (BP) in hypertension leads to renal failure as a result of either glomerular ischemia due to damage in pre-glomerular arterioles or hypertension-induced glomerular hyperperfusion, which subsequently leads to glomerular injury and loss of renal function [4,5]. The high prevalence of hypertension coupled with poor BP control rates highlights the need to learn more about the mechanisms controlling BP.

## Sex and gender differences in hypertension

There are many predisposing factors for hypertension such as hereditary, age, sex/gender, race, lack of physical activity, weight gain and stress [6]. In particular, clinical data clearly demonstrates gender differences in the incidence and progression of hypertension. Men typically have higher BP and a greater prevalence of hypertension compared with age-matched premenopausal women [7–9] until the sixth decade of life, when the prevalence of hypertension among women begins to exceed that in men. A recent study using sex-stratified analyses of longitudinal BP data attributed the switch in the greater prevalence of hypertension among men to women to a faster rate of progressive BP elevation among women beginning in their 30s [10]. Consistent with clinical findings, we and others have previously shown sex differences in the incidence and progression of hypertension in numerous experimental models of hypertension where young females have a lower BP compared to males [8,11–16].

Human essential hypertension is a complex multifactorial disease. Various models of experimental hypertension have been developed to mimic hypertension in humans. The spontaneously hypertensive rat (SHR) is a commonly employed model of hypertension that exhibits progressive increases in BP as they age similar to what is observed clinically, with established hypertension in young adulthood [17,18]. Similar to the clinical features of essential hypertension, the full development of hypertension in SHR

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**Table 1 Most cited and published literatures supporting a sex difference in BP in SHR**

Authors and reference number	Journal	Year
Ganten et al. [14]	<i>J. Hypertens.</i>	1989
Chen and Meng [12]	<i>Life Sci.</i>	1991
Reckelhoff et al. [44,45,63]	<i>Hypertension</i>	1998, 2000, 2001
Maris et al. [24]	<i>Clin. Exp. Pharmacol. Physiol.</i>	2005
Fortepiani and Reckelhoff [13]	<i>J. Hypertens.</i>	2005
Sullivan et al. [22]	<i>Am. J. Regul. Integr. Comp. Physiol.</i>	2007
Sullivan et al. [20]	<i>Hypertension</i>	2010
Martin et al. [25]	<i>Mol. Cell Biochem.</i>	2012
Moulana et al. [28]	<i>Physiol. Rep.</i>	2014
Elmarakby et al. [27]	<i>Physiol. Rep.</i>	2016
Onishi et al. [26]	<i>Physiol. Genomics</i>	2018
Elmarakby et al. [23]	<i>Prostaglandins Other Lipid Mediat.</i>	2018
Abdelbary et al. [114]	<i>Hypertension</i>	2019

starts with mild elevations in BP followed by a progressive phase of hypertension and finally a sustained hypertensive phase [19]. Importantly, SHR are also used extensively to study sex differences in BP. Sex differences in BP in SHR were first reported in 1989 [14]. Table 1 summarizes most cited and published literatures supporting a sex difference in BP in SHR. We and others have used the SHR model to examine the mechanisms underlying sex differences in BP over the last two decades [20–28]. Increasing our understanding of the mechanisms, controlling BP in both males and females is necessary to lower the incidence of hypertension-associated morbidity and mortality.

It is important to note that not only have studies identified clear sex differences in the magnitude of the activation of pathways known to contribute to hypertension but also sex-specific activation of distinct pathways. These findings underscore the fact that a ‘one-size-fits-all’ approach to hypertension is unlikely to be effective. Despite these findings and the fact that half of the hypertensive population is female, the gender of the patient is not included in the most recent Guidelines for the Management of Hypertension. In the current review, we will summarize previous findings to date regarding the mechanisms of BP control in male versus female SHR. We will emphasize the progress made in understanding why male SHR have higher BP versus females

The terms “sex” and “gender” will be used throughout this review as defined by the Institute of Medicine Committee on Understanding the Biology of Sex and Gender Differences [29]. Gender includes an individual’s self-representation as male or female based on socially constructed characteristics. Therefore, clinical studies will refer to gender differences. Sex is classified based on reproductive organs determined by the chromosomal complement. Thus, experimental animal studies will refer to sex differences.

### Can sex hormones explain sex differences in BP in SHR?

Female SHR maintain a lower BP than males prior to 10–12 months of age [30]. Female SHR stop cycling at 10–12 months of age and estradiol levels drop similar to what is observed in postmenopausal women [30]. Once female SHR stop cycling, BP increases more rapidly in females than in males [31,32]. As a result, by 16–18 months of age the sex difference in BP in SHR is abolished. In contrast, BP remains fairly stable after 8 months of age in male SHR [32,33]. These experimental data fit with clinical results from the National Health and Nutrition Examination Survey (NHANES III) showing a greater increase in the prevalence of hypertension among women after the onset of menopause compared to the rate of increase of hypertension among age-matched men [34]. While these data have widely been interpreted to indicate that the increase in BP in postmenopausal women occurs over time after the ovary becomes senescent [35], recent work showing a steeper increase in BP in women versus men beginning in their 30s calls this thought into question [10]. More work is needed over the full lifespan of women to better understand the role of hormones in the control of BP.

Although the mechanisms responsible for the increased BP in women after menopause are not known, many investigators believe that female sex hormones are the main driving force for sex differences in hypertension. This conclusion is based on the early findings that menopause is associated with an increase in the prevalence of hypertension in women and estrogen replacement therapy in postmenopausal women markedly reduces the risk of cardiovascular diseases [36]. However, there is little clinical evidence supporting a direct role for female sex hormones in BP control. Data from the impact of estrogenic preparations on BP are inconsistent, and include reports of BP decreasing [37–40], increasing [41] or remaining unchanged [42]. In addition, even those studies indicating a decrease in BP with female

sex hormones, the BP decrease is minimal ( $\sim 2$  mmHg). Consistent with these data, as discussed below ovariectomy of female SHR has no impact on BP, suggesting that sex hormones do not directly control BP in female SHR.

In contrast, male sex hormones do modulate BP in SHR. Testosterone promotes the development of hypertension in male SHR. Ganten et al. were the first to report that both castration and testosterone receptor antagonism attenuate the development of hypertension in young male SHR [14]. Follow-up studies confirmed that the sex difference in hypertension in SHR is dependent on male but not female sex hormones [12]. Orchidectomy of male SHR at 4 weeks of age significantly attenuated elevations in BP, whereas ovariectomy at 4 weeks of age had no effect on the development of hypertension in female SHR [12]. Furthermore, testosterone administration to gonadectomized rats increased BP in both sexes, indicating that testosterone promotes hypertension regardless of sex of the animal [12]. Additional studies further confirmed that the pro-hypertensive effects of testosterone and sex differences in BP are only apparent in mature SHR [43]. BP in male and female SHR is similar between the sexes until  $\sim 12$  weeks of age when serum testosterone levels peak and BP becomes significantly higher in males than in female SHR [44].

Numerous studies have examined the mechanisms underlying testosterone-induced increases in BP in male SHR. In particular, the kidney has been implicated in sex differences in BP in SHR [33,44,45]. There is a hypertensive shift in the pressure-natriuresis relationship in male SHR versus females and testosterone-induced reductions in pressure-natriuresis and a rightward hypertensive shift in the pressure-natriuresis relationship contribute to the sex difference in hypertension in SHR [43]. Interestingly, testosterone attenuates the acute pressure-natriuresis relationship in both males and females, indicating a generalized reduction in the ability of the kidney to produce a natriuretic response in the presence of testosterone [44]. This finding was supported by the fact that testosterone administration to ovariectomized females also blunted pressure-natriuresis to levels comparable to those measured in the gonad-intact males [44]. Potential mechanisms driving the hypertensive shift in the pressure-natriuresis relationship in SHR include increased proximal tubular reabsorption leading to a tubuloglomerular feedback-mediated afferent vasodilation, which in combination with the increase in BP, results in glomerular hypertension and renal injury. Indeed, testosterone not only drives an elevation in BP in male SHR versus female but also hormone-dependent increase in albuminuria which is an early sign of renal injury [22].

It is important to note that hormones are not the only physiological factor differentiating the sexes. There are also differences in sex chromosomes and there are experimental studies in mice that have examined the role of chromosomes in BP control. Although role of chromosomes in modulating BP in SHR has not been directly studied, the Y chromosome has been suggested to contribute to the elevated BP in male SHR [17]. When SHR were mated with normotensive WKY and BP in the offspring were measured, the authors found that BP was higher in male offspring when WKY females were mated with SHR males as compared with male offspring derived from SHR females mated with WKY males [17]. Importantly, there were no differences in BP in the female offspring from the different mating pairs, supporting a role for the Y chromosome and not parental imprinting on BP [17]. These findings are consistent with clinical reports supporting a Y chromosome effect on BP [46,47], with the Sry locus proposed to be critical to BP modulation [48]. However, additional studies are needed to more fully define how the Y chromosome controls BP both clinically and experimentally.

## Mechanisms mediating sex differences in BP in SHR

### Differential regulation of the adrenergic receptor

The role of adrenergic receptor activation in the regulation of vascular tone is well studied, especially at the level of small resistance vascular beds [49]. Hypertension is often associated with altered adrenergic responses in the vasculature as the prevalence and interaction of  $\alpha$ -adrenergic (mainly constrictor) and  $\beta$ -adrenergic receptors (mainly dilator) can change overall vascular resistance and raise BP [50]. Although presynaptic  $\alpha_2$  adrenergic receptor activation contributes to BP control by reducing sympathetic outflow and norepinephrine release [51], post synaptic  $\alpha_2$  adrenergic receptors in the kidney induce vasoconstriction and increase proximal tubular sodium reabsorption leading to increases in BP [52].

Previous findings suggest that the presynaptic  $\alpha_2$  adrenergic receptors are dysfunctional in male SHR versus females [53]. Thus, functional presynaptic  $\alpha_2$  adrenergic receptors in female SHR are likely to have a protective effect to decrease sympathetic outflow and catecholamine release and may contribute to the lower BP in young female SHR [53]. In contrast, male SHR have more than two times higher renal  $\alpha_{2B}$ -adrenergic receptor mRNA expression than female SHR [54], which could contribute to the elevated BP in male SHR vs. females. Sex differences in  $\alpha_{2B}$ -adrenergic receptor expression may be hormonal-dependent as castration of male SHR reduces renal  $\alpha_{2B}$ -adrenergic mRNA levels, whereas ovariectomy slightly increases renal  $\alpha_{2B}$ -adrenergic receptors mRNA levels in female SHR [54]. Furthermore, testosterone treatment increases  $\alpha_{2B}$ -adrenergic receptor mRNA levels of gonadectomized male and female

SHR to a similar level of intact males suggesting that testosterone regulation of renal  $\alpha_{2B}$ -adrenergic receptor gene expression may contribute to the higher BP in males [54].

Vascular reactivity to  $\beta$ -adrenergic stimulation in SHR is also known to be impacted by sex. For example, sex specific differences were reported in the expression and function of  $\beta_1$ - and  $\beta_3$ -adrenergic receptors in aorta of SHR. Females express more dilatory  $\beta_1$ - and  $\beta_3$ -adrenergic receptors than males with a predominant endothelial localization which could counteract the constrictor effects of  $\alpha$ -adrenergic receptor stimulation [55,56]. This sex difference is functionally relevant in young adult SHR and is attenuated by aging, at least in part, via blunting endothelial nitric oxide (NO) production [55,56]. There is also evidence to suggest a sex difference in the response to chronic  $\beta$ -adrenergic stimulation in SHR [57]. Chronic stimulation of  $\beta$ -adrenergic receptor with isoproterenol increased left ventricular-end diastolic (LVED) diameter in sham-operated, but not in castrated male SHR [57]. In females, isoproterenol slightly increased LVED diameter; however, ovariectomy did not significantly change LVED diameter suggesting that testosterone could be the driving factor for  $\beta$ -adrenergic-induced left ventricular remodeling [57]. Taken together, differential regulation of  $\alpha$ - and  $\beta$ -adrenergic receptors in SHR may contribute to sex differences in BP control in SHR.

## Renin–angiotensin system

The renin–angiotensin system (RAS) is a multi-enzymatic hormonal system that controls electrolyte balance, body fluid volume and BP [20,58]. The ‘classical’ RAS pathway involves the conversion of angiotensin I (Ang I) to angiotensin II (Ang II) by angiotensin converting enzyme (ACE). Ang II interaction with angiotensin II type 1 ( $AT_1$ ) receptors results in vasoconstriction, aldosterone and vasopressin release, salt and water retention, sympathetic activation, increased oxidative stress, sodium reabsorption, cell proliferation and vascular hypertrophy [20,58]. The non-classical RAS consists of ACE2, angiotensin 1-7 (Ang 1-7), the angiotensin II type 2 ( $AT_2$ ) receptor, and the MAS receptor that oppose  $AT_1$ -mediated effects causing vasodilation, increases in blood flow and enhanced pressure-natriuresis [20,58].

There are numerous reports of sex differences in RAS components in SHR. Males have greater expression of classical RAS components. For example, plasma renin activity (PRA) and hepatic angiotensinogen mRNA levels are higher in intact male SHR than in females [59]. Male SHR exhibit higher cardiac ACE activity and hypertrophy compared with females [60], as well as higher renal, aortic and mesenteric artery  $AT_1$  mRNA expression [20,22,61]. In contrast, accumulating evidence suggests that the non-classical RAS is enhanced in female SHR. Females have greater renal and vascular  $AT_2$  receptor expression versus males [62], such that the  $AT_1/AT_2$  ratio is less in females compared with males [61]. We have also previously shown that although female SHR have greater plasma Ang II levels and similar levels of renal cortical Ang II versus males [22], females have higher levels of Ang 1-7 in the kidney [20]. Sex differences in the expression of RAS components have been linked to sex hormones. In particular, studies suggest an androgen dependent elevation in classical RAS in male SHR [60]. Orchidectomy lowers plasma renin, renal and hepatic angiotensinogen mRNA levels, and cardiac ACE and ACE2 expression [60,61]. The role for testosterone to mediate these effects have been confirmed using testosterone replacement in both males and ovariectomized female SHR [59]. In contrast, female sex hormones have been shown to attenuate aortic and mesenteric  $AT_1$  mRNA expression and promote  $AT_2$  expression [61].

Importantly, sex differences in the expression of classical and non-classical RAS components align with sex differences in BP. BP in SHR is sensitive to RAS inhibition. Treatment with the ACE inhibitor enalapril reduces BP in both male and female SHR and abolishes the sex difference in BP [63]. Similarly, treatment of with the  $AT_1$  receptor blocker losartan abolishes both the hypertension and sex difference in BP in SHR [64], suggesting that the RAS is central in mediating the sex difference in BP in SHR. BP in male SHR is also more sensitive to exogenous Ang II. Male SHR exhibit greater increases in BP following acute [27] and chronic infusion of Ang II [20] compared with females. Male SHR are also more sensitive to Ang II-induced renal injury as acute Ang II infusion resulted in greater decreases in glomerular filtration rate in males [27] and chronic Ang II resulted in greater increases in proteinuria and renal injury [20].

While the above data are largely interpreted as greater classical RAS-mediated increases in BP in male SHR, our data further support a key role for the non-classical RAS to limit increases in BP in female SHR. Pretreatment with the MAS receptor blocker A-779 abolished candesartan-mediated decreases in BP in females, but not males, indicating that greater Ang (1-7) levels in females contribute to angiotensin receptor blocker-mediated decreases in BP in female SHR [65]. A-779 pre-treatment also exacerbated Ang II hypertension only in female SHR, abolishing the sex difference in the BP [20]. Consistent with our results, female SHR are more sensitive to acute  $AT_2$  receptor stimulation-induced renal vasodilation and sodium excretion independent of an effect on BP relative to males [62]. These data support

the hypothesis that enhanced expression of the non-classical RAS in females actively antagonizes the classical RAS to limit increases in BP and contributes to the ability of female SHR to maintain a lower BP versus age-matched males.

In addition to the contribution of RAS in the sex differences in BP control, there is also evidence to support a role for the RAS in modulating vascular function. Endothelial dysfunction is a term that covers diminished production/availability of NO and/or an imbalance in the relative contribution of endothelium-derived relaxing and contracting factors [66]. Endothelial dysfunction is a key factor in the development of cardiovascular disease in men and women [67,68] and endothelium-dependent vascular relaxation to acetylcholine is impaired in patients with hypertension and in various experimental models of hypertension [67,69]. We have previously shown that male SHR have impaired aortic endothelial function and a decrease in basal NO release compared with females [66]. The RAS has been implicated in the development of endothelial dysfunction in SHR and male SHR have greater endothelial dysfunction compared with females [61,64]. A role for the RAS in mediating endothelial dysfunction in SHR is supported by the finding that endothelial function in male and female SHR is improved with the AT<sub>1</sub> receptor blocker losartan [61,64]. However, whether these effects were mediated by blockade of the AT<sub>1</sub> receptor or decreases in BP was not examined. There are also sex differences in vascular sensitivity to exogenous Ang II in SHR. Aorta and mesenteric micro-vessels from female SHR are less responsive to Ang II in comparison to vessels from male SHR [61]. Enhanced vascular function in female SHR has been linked to greater AT<sub>2</sub> receptor expression, increased NO production, and female sex hormones [61]. Indeed, greater NO availability in female SHR have been hypothesized to explain sex differences in endothelial function in SHR; the role of NO will be discussed in greater detail below.

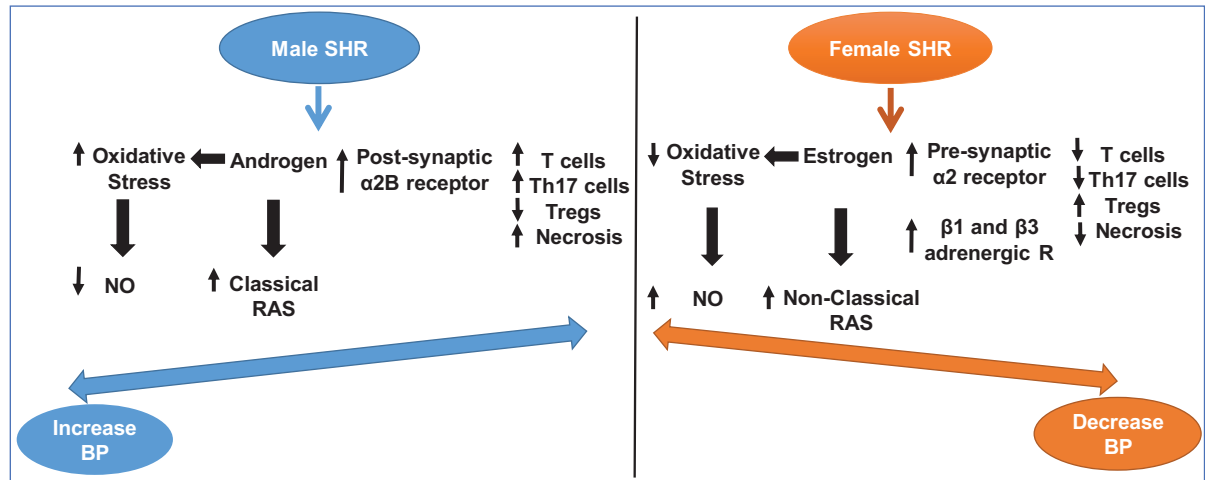
The studies discussed above were all performed in young SHR. As noted above, the sex difference in BP in SHR is lost with aging. Despite the loss of the sex difference in BP, BP in aging males remains more dependent on the classical RAS. Sixteen-month-old male SHR exhibit greater decreases in BP with losartan treatment than age-matched females, despite no sex differences in PRA, renal AT<sub>1</sub> receptor expression or ACE [58], supporting the hypothesis that there are sex differences in the downstream signaling pathways following Ang II receptor activation. Indeed, losartan resulted in a decrease in oxidative stress only in aged males. Regardless, there is abundant evidence supporting the hypothesis that differential regulation of the RAS contributes to the sex difference in BP in SHR where males have greater classical RAS activation whereas females have greater activation of the non-classical RAS. Better understanding of the pathways activated in a sex-specific manner downstream of the AT<sub>1</sub> and AT<sub>2</sub> receptors (for example, oxidative stress, NO and inflammation) will likely provide additional and novel insight into BP control in both sexes.

## Oxidative stress

Oxidative stress is an imbalance between reactive oxygen species (ROS) production and antioxidant defense [11,70]. ROS are produced not only by mitochondrial energy production but also by enzymes such as NADPH oxidase, uncoupled NO synthase (NOS) and xanthine oxidase [11,70]. Increases in ROS production can be mediated by either increased expression or activation of ROS generating enzymes or impaired activity of antioxidant defense systems. The main antioxidant pathways include superoxide dismutase (SOD), catalase, hemeoxygenase and glutathione peroxidase [13,70,71]. Under normal physiological conditions, SOD reacts with superoxide to produce H<sub>2</sub>O<sub>2</sub>, which is subsequently metabolized by catalase or glutathione peroxidase to produce water [13,70,71]. In pathophysiological conditions such as hypertension, increased superoxide production exceeds the levels than can be scavenged by SOD and hence promotes vascular injury.

There are sex differences in oxidative stress in SHR [11,13,21–23,70,72–74]. Male SHR have greater renal superoxide production, F<sub>2</sub>-isoprostane levels and H<sub>2</sub>O<sub>2</sub> excretion compared with females [21,22,70,75,76]. Consistent with our previous findings [23], renal TBARs are also significantly greater in male SHR compared to female SHR and normotensive WKY (Figure 1). Males also exhibit greater increases in renal measures of oxidative stress in response to chronic Ang II infusion than females [11]. Indeed, greater increases in oxidative stress in male SHR vs. females could be secondary to higher activation of classical RAS in males as Ang II activation of the AT<sub>1</sub> receptor is known to increase NADPH oxidase dependent superoxide production [22,77]. Consistent with this hypothesis, greater oxidative stress in mesenteric arteries from male SHR versus females is abolished by losartan [73]. However, not all studies and not all markers of oxidative stress report a sex difference. There were no differences in plasma F<sub>2</sub>-isoprostane between male and female SHR [75]. Sex differences in oxidative stress have also been linked to sex hormones as male sex hormones promote oxidative stress while female sex hormones offer protection [21].

Sex differences in measures of oxidative stress may be related to sex differences in anti-oxidant pathways. Male SHR have greater SOD and catalase activity in the renal inner medulla than females, although plasma SOD activity is higher in females [21]. Consistent with these findings, in separate studies using the whole kidney, male SHR exhibit greater expression of antioxidant enzymes, Mn-SOD, Cu, Zn-SOD, glutathione peroxidase and catalase than females



**Figure 1. Schematic diagram showing mechanisms underlying sex differences in blood pressure in spontaneously hypertensive rats (SHR)**

[76]. Total antioxidant capacity, SOD and catalase are also significantly greater in erythrocytes from male versus female SHR [78]. The greater expression and activity of antioxidant defense enzymes in male may be a compensatory mechanism to buffer greater oxidative stress production in male SHR versus females.

Oxidative stress has been implicated in BP control and in the sex differences in BP in SHR. A role for oxidative stress in hypertension was demonstrated in early studies showing that infusion of a fusion-protein containing human Cu/Zn-SOD or administration of the xanthine oxidase inhibitor oxypurinol reduced BP in male SHR [76,79]. Consistent with male SHR being more dependent on the classical RAS to increase BP, and the fact that AT<sub>1</sub> receptor activation leads to increases in oxidative stress, BP in male SHR has been reported to be more sensitive to oxidative stress than females [11,13,45,75]. Indeed, treatment with the NADPH oxidase inhibitor apocynin attenuated Ang II-induced increases in BP only in male SHR, thereby abolishing the sex difference in the Ang II-BP response [11]. However, treatment with apocynin alone for 1 week had no effect on BP in male or female SHR and treatment with the SOD mimetic tempol for 2 weeks does not alter baseline BP in male or female SHR [72]. Tempol treatment with for 6 weeks reduced BP only in male SHR [13] and terminal BP measured via femoral catheter indicated a decrease in BP in only male SHR following 7 days treatment with apocynin [76]. These findings support enhanced BP sensitivity to oxidative stress in male SHR and suggest that greater Ang II-mediated increased in oxidative stress are central to the enhanced BP response to Ang II in male SHR.

Interestingly, BP in female SHR appears to be resistant to oxidative stress. BP is not altered in female SHR even following treatment with molsidomine to increases oxidative stress; molsidomine increases BP in male SHR [13,71,76]. Even in aged female SHR, treatment with tempol fails to lower BP despite pronounced increases in F<sub>2</sub>-isoprostanes [30]. However, vitamins E and C did lower BP and oxidative stress in aged female SHR [30]. The authors speculated that the mechanisms producing oxidative stress differed between the sexes driving the sex difference in BP sensitivity to antioxidants. Because vitamins E and C are generic antioxidants that interact with superoxide, peroxides, and hydroxyl radicals, more studies are needed to test this hypothesis.

## Nitric oxide bioavailability

The NO/NOS pathway is critical in BP regulation [69,80,81], and deficiencies in NO are correlated with the incidence and progression of hypertension [82–84]. The balance between NO production and the scavenging of NO determines NO bioavailability. NO is produced by the enzyme NOS, and there are three NOS isoforms: NOS1 (neuronal NOS, nNOS), NOS2 (inducible NOS, iNOS) and NOS 3 (endothelial NOS, eNOS) [85]. Excess superoxide preferentially binds NO resulting in decrease in NO bioavailability and the production of peroxynitrite [21,70,72]. Decreases in NO bioavailability contribute to endothelial dysfunction and the progression of cardiovascular disease [70].

There are numerous clinical and experimental studies indicating that NO production is greater in females versus males [86,87]. We have reported that there are sex differences in NOS in SHR. Within the kidney, NOS activity and expression are comparable between male and female SHR in the renal cortex and outer medulla [16]. However, female

SHR have greater NOS1 activity and expression and greater NOS3 activity in the renal inner medulla versus males [16]. Interestingly, cGMP levels, an indirect indicator of NO bioavailability, are greater in both the renal cortex and inner medulla of female SHR versus males [16]. Additional studies confirmed female SHR exhibit an increase in NOS1 expression and NOS activity in the renal inner medulla as they sexually mature from 5 to 13 weeks of age [88]. NOS does not increase with age in male SHR [88]. Tetrahydrobiopterin (BH<sub>4</sub>) is a critical cofactor required for NO generation, and decreases in BH<sub>4</sub> as a result of increases in oxidative stress have been implicated in the pathogenesis of hypertension [72]. Indeed, we found higher oxidative stress in male SHR results in a relative deficiency of BH<sub>4</sub> compared with females, resulting in diminished renal NOS activity in male SHR [72]. Therefore, female SHR have both greater NO production and less scavenging of NO resulting in greater NO bioavailability.

Female sex hormones have been shown *in vitro* to increase NOS3 in pulmonary arteries, cultured human endothelial cells and thoracic aorta of rats [89–91]. Thus, female sex hormones may mediate the sex difference in NO in SHR. Consistent with this, we confirmed that the enhanced NOS activity in the renal inner medulla and cortex of female SHR is mediated by estradiol [92,93]. This is further supported by the finding that increased NOS expression and activity in female SHR from 5 to 13 weeks of age is prevented by ovariectomy [88].

Importantly, there are physiological implications for the sex difference in NO bioavailability in SHR; females are more dependent on NOS to maintain BP and vascular function. SHR are highly sensitive to the BP raising effects of NOS inhibition [94]. Although both male and female SHR display a dose-dependent increase in BP to the non-specific NOS inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME), females exhibit a greater increase in BP than males [83]. In additional studies, we found that chronic treatment of male and female SHR with L-NAME significantly increased BP in both sexes; however, prior exposure to L-NAME only increased BP sensitivity to chronic NOS inhibition in females. Interestingly, studies by Dr Reckelhoff's group examined whether the lack of a BP response to increases in oxidative stress in female SHR was due to increases in NO by treating rats with a combination of molsidomine to increase oxidative stress and L-NAME [94]. They confirmed that BP in female SHR is highly sensitive to NOS inhibition, although the combination of molsidomine and L-NAME did not exacerbate L-NAME-induced increases in BP. These data underscore the importance of NOS in the maintenance of BP and the lack of BP sensitivity to oxidative stress in females SHR.

As noted above, a decrease in NO bioavailability leads to endothelial dysfunction and sex differences in endothelial function in SHR have been reported [95]. Consistent with female SHR having greater NO bioavailability, aortic endothelium-dependent relaxation to acetylcholine is greater in female SHR compared with males [95]. Studies by our group have further examined vascular function in aorta and small mesenteric arteries from male and female SHR and WKY [66]. We found that aorta from male SHR exhibit blunted NOS-mediated vasoconstrictor buffering capacity and attenuated endothelium-dependent relaxation versus all other rat groups. Interestingly, there is evidence suggesting that the vascular protection afforded female SHR is lost after cessation of estrous cycling in females, further supporting the hypothesis that female sex hormones mediate enhanced NOS [31].

Overall, NO bioavailability (reflecting both levels of oxidative stress and NO production) is likely a contributing factor to observed sex differences in BP control and overall cardiovascular health. Greater understanding of why females have greater NO levels could be important in improving treatment options for hypertension. In addition, better understanding is needed regarding how the NO/NOS pathway interacts with other pathways that control BP (RAS and immune cell activation). BP control is a complex process that involves the integration of multiple organ systems and pathways. To improve our ability to adequately control BP in both sexes, additional studies are required to interrogate and unravel these complex relationships in a sex-specific manner.

## Role of immune cells and inflammatory cytokines

Hypertension is now well accepted to be a state of inflammation, and there is accumulating evidence in both clinical and experimental studies that hypertension is associated with an increase in renal T-cell infiltration and accumulation [96–101]. There is ample support for a role for the immune system in the development of hypertension in SHR. Interestingly, male SHR have higher levels of renal immune cell infiltration and activation of nuclear factor-kappaB (NFκB) compared with WKY even at 3 weeks of age, well before the development of hypertension [102,103]. As male SHR age, immune cells continue to accumulate as BP begins to increase [102,103]. There is direct support for an inflammatory component to BP control in SHR since manipulating the immune system alters BP. The passive transfer of lymph node cells from male SHR into a male WKY causes the development of hypertension in the recipient rat, while a thymus transplant from a normotensive male WKY into a male SHR reduces BP in the SHR recipient [104,105]. Similarly, anti-thymocyte serum decreases BP in male SHR to normal levels supporting a crucial role for the adaptive immune system in BP control [106,107]. Additional support for lymphocyte control of BP comes from studies using

the immunosuppressive drug mycophenolate mofetil (MMF). Treatment of male SHR with MMF abolishes the hypertension, reducing BP to comparable levels as measured in control WKY rats, and the decreases in BP are associated with reduced renal lymphocyte and macrophage infiltration and decreases in oxidative stress [103,108].

Interestingly, there is evidence to suggest that the immune system underlies sex differences in hypertension, and there are numerous reports of sex difference in the inflammatory profile in SHR. Sex differences in Ang II hypertension are absent in *Rag1*<sup>-/-</sup> mice, and this seems to be mediated by an attenuation of Ang II hypertension in males lacking B and T cells [109,110]. As additional studies in *Rag1*<sup>-/-</sup> mice confirmed that T cells mediate Ang II hypertensive responses [97], our group became interested in the role of the immune system in mediating sex differences in BP control. In particular, we assessed pro-inflammatory CD3<sup>+</sup>CD4<sup>+</sup> Th17 cells which are linked with increases in BP and anti-inflammatory CD3<sup>+</sup>CD4<sup>+</sup> T regulatory cells (Tregs) which limit increases in BP [111,112]. Consistent with sex differences in BP, we found that male SHR have more renal Th17 cells and female SHR have more Tregs [111,113,114].

To begin to gain mechanistic insight into sex differences in renal T cells in SHR, we first examined the roles of sex hormones and BP [113]. Sex hormones could drive sex difference in the T cell profile as previous findings suggest that both estrogen and testosterone impact T cells *in vitro* and *in vivo* [115,116]. Estrogen stimulates Treg production *in vitro* and *in vivo* in CB57BL/6 mice [117]. Castration reduces circulating Tregs [118] and testosterone supplementation to male experimental autoimmune orchitis rats increases Treg expression in the testis [119]. Consistent with these data, gonadectomy increased Th17 cells and decreased Tregs in both sexes, although the sex differences in the T cells were maintained [113]. These data suggest that although sex hormones are anti-inflammatory, hormones alone cannot account for the sex difference in the T cell profile.

To assess the role of hypertension per se in mediating sex differences in the renal T cell profile, male and female SHR were treated with BP lowering drugs to abolish both the hypertension and the sex differences in BP. Interestingly, both preventing the development of hypertension and reversing established hypertension were associated with a decrease in renal Tregs in females to the levels observed in males [113], supporting the hypothesis that females exhibit a compensatory increase in Tregs in response to increases in BP. Indeed, this conclusion is supported by the finding that there were no sex differences in renal Tregs in 5-week-old SHR prior to the development of hypertension [120].

Although renal Th17 cells were not significantly altered by BP lowering drugs in SHR, there was a trend for Th17 cells to decrease in males with decreases in BP [113]. To examine the mechanism driving this effect, we measured necrosis in male and female SHR. Hypertensive stimuli induce cell death [121–123], and cellular necrosis is pro-inflammatory. We found that male SHR had greater levels of renal necrosis versus females [114]. Blocking necrosis with Necrox-5 decreased renal necrosis abolishing the sex difference and attenuated maturation-induced increases in BP in male SHR; BP in female SHR was not altered by Necrox-5 treatment [114]. While Necrox-5 decreased pro-inflammatory renal T cells in both sexes, sex differences were maintained [114]. Therefore, although greater necrotic cell death in male SHR exacerbates maturation-induced increases in BP with age contributing to sex differences in BP, necrosis is unlikely to explain sex differences in the renal T-cell profile.

Based on the ability of Tregs to protect against increases in BP, more studies have focused on gaining additional insight into the mechanisms modulating Tregs in female SHR. The local cytokine environment is a critical determinant for T-cell differentiation into the different subtypes [98,116]. Naïve T cells differentiate into Th17 cells in the presence of low levels of transforming growth factor- $\beta$  (TGF- $\beta$ ) and high levels of interleukin (IL)-6 and IL-23. In contrast, high concentrations of TGF- $\beta$  with low IL-6 levels drives Treg formation [98,116]. Importantly, female SHR have greater TGF- $\beta$  expression compared with male SHR [88,96,98,116]. In addition, increases in Tregs from 5 to 13 weeks of age in female SHR are paralleled by increases in TGF- $\beta$ , ovariectomy decreases renal Tregs and TGF- $\beta$ , and the decreases in Tregs with BP lowering drugs are accompanied by a decrease in TGF- $\beta$  in female SHR [115]. However, despite TGF- $\beta$  neutralization decreasing circulating Tregs, renal Tregs were unaffected and BP was not altered suggesting that sex differences in TGF- $\beta$  do not mediate the sex difference in renal Tregs. Because we have also found that male SHR have greater renal levels of IL-6 and IL-23 [98,113], future studies will examine the roles of these cytokines in mediating sex difference in the renal T cell profile.

Additional studies in male and female SHR using the NOS inhibitor L-NAME found that the compensatory increase in renal Tregs in female SHR is blocked in L-NAME hypertension [83], implicating an intact NOS system in the ability of female SHR to increase Tregs. We further tested this hypothesis in female Sprague-Dawley rats treated with L-NAME, Ang II or norepinephrine to increase BP [124]. All three treatments increased BP, although only Ang II and norepinephrine-induced increases in BP were accompanied by an increase in Tregs. Understanding how NOS modulates Treg proliferation and activation will provide novel insight into the innate mechanisms offering young females protection against the development of cardiovascular diseases.



**Table 2** Known mechanisms mediating sex difference in BP in SHR and supporting literatures

Mechanism mediating sex difference in BP	Male SHR	Female SHR	Reference number
1- Sex hormones			
A- Androgen	↑ BP		[12,14,22,43,44]
B- Estrogen		↓ BP	[37–40]
2- Renin–angiotensin system			
A- Classical RAS	↑ BP		[20,22,60,61,63,64]
B- Non classical RAS		↓ BP	[20,61,62]
3- Oxidative stress	↑ BP		[21,22,70,75,76]
4- Nitric oxide		↓ BP	[16,86–89,91]
5- Sympathetic tone			
A- presynaptic $\alpha_2$ receptor		↓ BP	[53]
B- postsynaptic renal $\alpha_2\beta$	↑ BP		[54]
C- $\beta_1$ and $\beta_3$ receptor		↓ BP	[55,56]
6- Immune cells			
A- Th17 cells	↑ BP		[110–113]
B- Tregs		↓ BP	[110–113]
7- Cellular necrosis	↑ BP		[114]

Studies by our group have also confirmed that lymphocytes contribute to hypertension in both male and female SHR [113]. Interestingly, female SHR are more sensitive to the BP lowering effects of MMF versus males. Although not examined, the greater decrease in BP in female SHR may reflect the fact that females maintained more Tregs than males after treatment with MMF. We have previously shown that female rats are more dependent on Tregs to limit DOCA-salt-induced increases in BP compared with males [125]. Indeed, decreasing Tregs with anti-CD25 treatment exacerbated DOCA-salt induced increases in BP only in females [125], and we have unpublished data with similar findings in female SHR supporting a critical role for Tregs in mitigating the development of hypertension in females.

In summary, what we learn from SHR as a model of essential hypertension that sex chromosomes/hormones contribute to the sexual dimorphism in BP either directly or indirectly via differential regulation of sympathetic nervous system, RAS, oxidative stress, NO bioavailability and immune cells. Table 2 summarizes the known molecular mechanisms underlying sex differences in BP in SHR over the last two decades. However, based on the clinical data showing conflicting results of sex hormone antagonists in abolishing sex differences in hypertension, other mechanisms are also involved in sexual dimorphism in hypertension. Thus, more research is needed to determine how sex modulates the important physiological key factors that control BP. While differences in the gonadal steroid profile contribute to the sexual dimorphism in BP control, the sex chromosomes independently of the gonadal hormone milieu also play a crucial role. Not only focusing on gonadal hormone regulation within one sex but also on genes on the Y chromosome that are not found on the X chromosome will likely uncover new insights into BP control in order to develop novel targets for the treatment of hypertension with better efficiency in both sexes.

### Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

### Abbreviations

ACE, angiotensin converting enzyme; Ang, angiotensin; eNOS, endothelial NOS; IL, interleukin; iNOS, inducible NOS; LVED, left ventricular-end diastolic; MMF, mycophenolate mofetil; nNOS, neuronal NOS; NOS, NO synthase; RAS, renin–angiotensin system; ROS, reactive oxygen species; SHR, spontaneously hypertensive rats; SOD, superoxide dismutase; TGF- $\beta$ , transforming growth factor- $\beta$ .

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