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Gender-Specific Protection from Microvessel Rarefaction in Female Hypertensive Rats

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Abstract

Epidemiologic studies reveal that women have a significantly lower age-adjusted morbidity and mortality from cardiovascular disease than men, suggesting that gender is a cardiovascular disease risk factor. The mechanism

of the “gender protection” is unknown. In this study, we investigated the microvascular remodeling in reduced renal mass plus a high salt (4.0% NaCl) diet model of hypertension (RRM + HS). We hypothesized that women would be protected from the increase in blood pressure and from the microvascular rarefaction associated with RRM + HS hypertension. Studies were designed to determine whether female rats were less susceptible to changes in microvessel density during RRM + HS. Microvessel density was measured in male and female low salt (0.4% LS) sham-operated controls (Sham + LS) and after 3 days or 4 weeks of RRM + HS hypertension. The microcirculation of hind limb (medial and lateral gastrocnemius, plantaris, soleus) muscles was visualized using rhodamine-labeled *Griffonia simplicifolia I* lectin. Tissue sections were examined by videomicroscopy and microvessel density was determined by quantitative stereology. As shown previously, mean arterial pressure increased to 160 ± 8 mm Hg and microvessel density decreased (>30% decrease in all beds) in male RRM + HS. In contrast, mean arterial pressure of female RRM + HS rats was modestly increased from 101 ± 2 to 118 ± 4 mm Hg. Despite previous results showing a reduction in microvessel density of both normotensive and hypertensive male rats on a high salt diet, microvessel density of female RRM + HS rats was not reduced at either time. These results suggest that gender protection in the RRM rat extends beyond an attenuation of the increase in pressure to an immunity from microvascular rarefaction.

Keywords

High blood pressure, sodium intake, gender, Sprague-Dawley rat, diet

Epidemiologic studies, most notably the Framingham studies[1] have described a greater age-adjusted mortality and morbidity from cardiovascular disease in men when compared to women. On the basis of these and other epidemiologic studies, gender (ie, being male) is identified as a risk factor for developing cardiovascular disease.[2] A similar age-adjusted sexual dimorphism exists with regard to the prevalence of hypertension.[3]The prevalence of hypertension is greater in men of all ages when compared to women. This gender difference exists until women reach or exceed 50 years of age, and coincides with the “postmenopausal” years when plasma estrogen and progesterone levels are decreased. Furthermore, postmenopausal women who receive hormone replacement therapy (ie, estrogen) have a 30% to 50% decrease in cardiovascular mortality and a 50% reduction in cerebrovascular mortality rate when compared to untreated women.[4]

A sexual dimorphism also exists in a variety of animal models of hypertension, including both genetic[5,6] and experimental models.[7,–9] Although these differences have been the focus of several investigations, they remain unexplained. To date these gender differences cannot be fully explained by changes in other known cardiovascular disease risk factors, for example, plasma lipid levels,[10] by changes in plasma vasopressin concentrations,[11] or changes in peripheral vascular receptor sensitivity.[12]

One of the most striking morphologic changes associated with many forms of hypertension is a remodeling of the microcirculation. This remodeling includes both a reduction in vessel density (microvessel rarefaction) and histologic changes. Decreases in microvessel density (arterioles and capillaries), as well as degenerative changes that include vascular smooth muscle cell atrophy and attenuation of the endothelium, have been well described by a number of investigators.[13,,,,,,,,–23] For example, in the reduced renal mass plus a high sodium intake (RRM + HS) model of hypertension, the elevated arterial pressure is accompanied by a 10% to 30% anatomic rarefaction of the microcirculation in skeletal muscle.[16,17] The association of an increase in mean arterial pressure (MAP) and a loss of microvessel density is not unique to the RRM model or to animal models alone. Rather, microvessel rarefaction occurs in many different animal models of hypertension, including the SHR,[14,18,20,24] 2 kidney-1-clip,[19] and DOCA-salt[22]hypertension. Rarefaction has also been demonstrated in patients with essential hypertension.[25] However, all studies of remodeling of the microcirculation in hypertension to date have been

confined to men. Because it is now well established that premenopausal women exhibit an apparent resistance to the development of hypertension, a finding supported by animal models, we hypothesized that the gender protection in terms of elevated pressure in female RRM + HS rats would extend to protection of the microcirculation from rarefaction.

Materials and Methods

All protocols and procedures were approved by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin. Male and female Sprague-Dawley rats (Sasco, Madison, WI) arrived 7 to 10 days before the date of surgery to permit adaptation to their new environment. All animals were fed a low sodium diet (0.4% NaCl, AIN-76A, Dyets Inc., Bethelem, PA) and water ad lib.

Surgical Procedure

A reduction in renal mass (RRM) of approximately 75% was achieved using a two-stage surgical procedure as previously described.[17,26] All surgeries were performed with intramuscular ketamine (100 mg/kg) and acepromazine anesthesia (1 mg/kg) using sterile techniques. Briefly, the right kidney was exposed by a flank incision and the two poles removed. After a 2-week recovery period, the entire left kidney was removed. Sham-operated (Sham) control rats were handled similarly, the kidneys exposed, cleared of perirenal fat, and returned to the abdominal cavity. All rats were kept on a low salt diet for an additional week after the second surgery. Animals were randomly divided into experimental groups. In the first group, male and female Sham + LS (low salt 0.4%) and RRM + HS (high salt 4.0%) rats were fed high or low salt diets (AIN-76A, Dyets Inc.) for 3 days. A total of 29 animals were used. In the second group, male and female Sham + LS and RRM + HS were fed high or low salt diets for 4 weeks. A total of 32 animals were used. In a third group, male (n = 14) and female (n = 5) RRM + HS were fed high salt diets for 4 weeks.

Surgical Implantation of Catheters and Acute Arterial Pressure Measurements

Rats were lightly anesthetized with a combination of intraperitoneal sodium pentobarbital (15 mg/kg) and ketamine (50 mg/kg). Catheters (microbore Tygon and Dural tubing) were surgically implanted into the femoral arteries of rats, as previously described.[27] The catheter was flushed, filled with diluted heparin in saline and connected to a COBE pressure transducer (Arvada, CO). Direct arterial pressures were recorded continuously for 30 to 60 min as previously described.[26] The arterial pressure signal from the transducer was digitized and the data stored for later analysis. Mean arterial pressure was analyzed using data acquisition software (CODAS, Dataq Instruments Inc., Akron, OH; sampling rate of 60 Hz) and stored as minute averages. A single mean ± SE was calculated for MAP for each individual animal. The animal was then euthanized with an overdose of sodium pentobarbital.

Surgical Implantation of Catheters and Chronic Arterial Pressure Measurements

To eliminate any difference in depth or response to anesthesia in the measurement of blood pressure, male and female RRM + HS rats (n = 19) were surgically prepared with chronic indwelling catheters as previously described in detail.[27] Briefly, specially designed catheters (microbore Tygon and Dural tubing) were surgically implanted into the femoral arteries of anesthetized rats (ketamine, 100 mg/kg and intramuscular acepromazine, 1 mg/kg) using sterile techniques. The catheter was tunneled subcutaneously to exit in the subscapular region and protected by a light weight spring. The spring and catheter were connected to a swivel mounted on top of the cage. Rats were given 3 to 5 days of recovery. Morning pressures were measured after the catheter was flushed,

filled with diluted heparin in saline, and connected to a COBE pressure transducer (Arvada). All animals remained in their home cages and were untouched during the procedure. Pressures were recorded for 1 h and a single mean calculated for each animal as described above. Because of the potential influence of the indwelling chronic cannula on distal extremity blood flow, vessel density was not determined in these animals.

Vessel Density Determination

Microvessel density was determined using *Griffonia simplicifolia I* lectin, a probe specific for vessels of the microcirculation with high-affinity binding to vessels of diameters 20 µm or less.[17] The gastrocnemius-plantaris-soleus muscles were removed and rinsed in physiologic salt solution (PSS). The tissue was immediately sectioned using a microtome.[28] The two 75-µm thick longitudinal sections were immersed in 25 µg/mL rhodamine-labeled *Griffonia simplicifolia I* (GS-I) lectin (Sigma Chemical Corp., St. Louis, MO) for 45 min to define the microvascular bed. After immersion, the tissue was rinsed in PSS three times and the sample was mounted on a microscope slide with a water-soluble mountant (Cytoseal 280, Stephens Scientific, Riverdale, NJ). Labeled sections were visualized using a video fluorescent microscope system (Olympus ULWD CD Plan, 20× objective, 1.6 working distance, and 0.4 numerical aperture, Lake Success, NY) with epiillumination. Images were obtained using a single-stage intensified Newvicon camera (model 7010, Olympus, Cohu, California) with 12 representative images from each tissue used for quantifying vessel density. Each image was digitized and processed to determine microvessel density as we have previously described elsewhere.[29]

Statistical analysis

Data are presented as mean ± standard error. The vessel density estimates determined from each of the 12 different images obtained from each tissue were averaged to represent the vessel density for a single animal. Vessel density estimates of each individual animal were then used to compute group data. Group MAP was determined using the average of the 1-h recording. One-way or two-way analysis of variance were used to determine significant effects. Differences in cell means were determined by a Duncan's multiple range test (significance, $P < .05$).

Results

The descriptive data of all experimental groups is shown in Table 1. Sham + LS males had a small but significantly lower initial (presurgical) body weight and were 9 days younger than rats in the RRM + HS group. Final body weights of male rats were significantly greater than females in all groups, and in both experiments. There were no other significant differences between groups. The percent of renal mass remaining calculated after surgical reduction was not significantly different between male and females in either the 3-day or 4-week studies.

Table 1. Age, body weight (initial and final), and percent of renal mass remaining after surgical reduction in preparation of the reduced renal mass (RRM) + high salt diet model of hypertension in rats

			Body Weight (g)		
	Age (days)	**N**	**Initial**	**Final**	**% Renal Mass Remaining**
Group one: Effect of 3 days of diet					
Male					
RRM + HS	85 ± 1[*]	13	239 ± 14[†]	396 ± 8[†]	27 ± 2
SHAM + LS	76 ± 2	6	198 ± 2	366 ± 6[†]	NA
Female					

RRM + HS	85 ± 1	6	194 ± 3	250 ± 5	28 ± 1
SHAM + LS	81 ± 2	7	200 ± 1	278 ± 6	NA
Group two: Effect of 4 weeks of diet					
Male					
RRM + HS	111 ± 4	13	219 ± 9	460 ± 10†	25 ± 5
SHAM + LS	109 ± 4	7	215 ± 7	476 ± 12†	NA
Female					
RRM + HS	107 ± 1	6	207 ± 3	272 ± 5	22 ± 1
SHAM + LS	107 ± 1	6	199 ± 1	297 ± 8	NA

Data are presented as mean ± 1 SE.
*Significantly greater than male sham-operated, low salt control, $P < .05$.
†Significantly greater than matched female group, $P < .05$.

A 75% reduction of renal mass in combination with 4 weeks of a high salt intake resulted in a significant increase in MAP in both male and female RRM + HS rats as summarized in Figure 1. However, in males, the increase in arterial pressure was more rapid, that is, MAP was significantly increased after 3 days of high salt (134 ± 6 mm Hg) when compared to sham-operated, low salt control (103 ± 3 mm Hg). Arterial pressure was further increased after 4 weeks of high salt in males (134 ± 6 to 160 ± 8 mm Hg). In contrast, arterial pressure of female RRM + HS rats was not significantly different than controls at 3 days. Arterial pressure of female RRM-HS rats increased significantly from 111 ± 3 after 3 days to 118 ± 4 mm Hg after 4 weeks of high salt intake. At both 3 days and 4 weeks, arterial pressure of male RRM + HS rats was significantly greater than that of female RRM + HS. There was no significant difference in the MAPs obtained using the acute and chronic techniques (Table 2). There were no differences using these two techniques for either male or female rats.

Mean arterial pressure (MAP) measured in anesthetized male and female reduced renal mass + high salt (RRM + HS) and sham-operated + low salt controls (SHAM + LS) after either 3 days or 4 weeks of diet. MAP was significantly increased in male RRM + HS rats at 3 days, and was further increased at 4 weeks when compared to SHAM + LS. MAP was significantly greater in male RRM + HS when compared to females. #Significantly different from female RRM + HS, $P < .05$. *Significantly different from same gender SHAM + LS control, $P < .05$.

Figure 1.

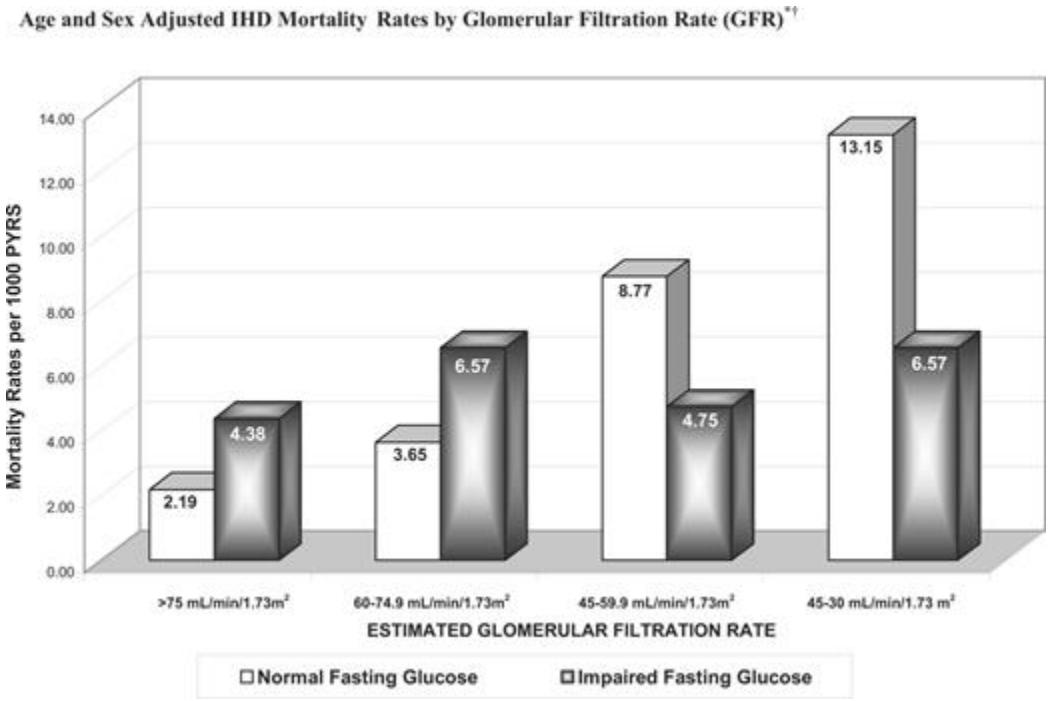


Table 2. Mean arterial pressure in conscious and anesthetized male and female reduce renal mass (RRM) + high salt rats after 4 weeks of high salt diet

Group Three	Conscious	N	Anesthetized	N
Male				
RRM + HS	152 ± 5*	14	160 ± 8*	6
Female (nonestrous)				
RRM + HS	121 ± 2	5	118 ± 4	8

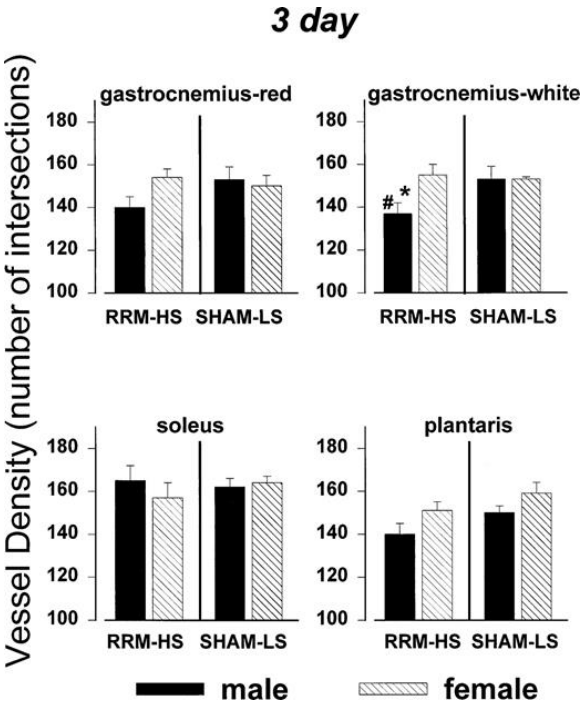
Values are mean ± SEM.
*Indicates significant difference from female under same condition, *P* < .05.

The influence of gender on the microvasculature is evidenced by significant changes in microvessel density in male but not in female rats. Vessel density was significantly reduced in the gastrocnemius-white muscle in male RRM + HS rats after 3 days of high salt when compared to male Sham + LS (Figure 2). Vessel density of the gastrocnemius-white muscle was significantly lower in male RRM rats when compared to females. There were no significant differences in the vessel density of any of the skeletal muscles of female RRM + HS. There were no significant differences in the vessel density of any of the skeletal muscles in male and female sham-operated animals.

As shown in Figure 3, vessel density was significantly decreased in *all* hind limb muscles measured in male RRM + HS rats after 4 weeks of high salt when compared to male Sham + LS controls. In contrast, no significant changes in vessel density were observed in female RRM + HS rats. In addition, vessel density was significantly lower in all hind limb muscles of male RRM + HS rats when compared to female RRM + HS after 4 weeks.

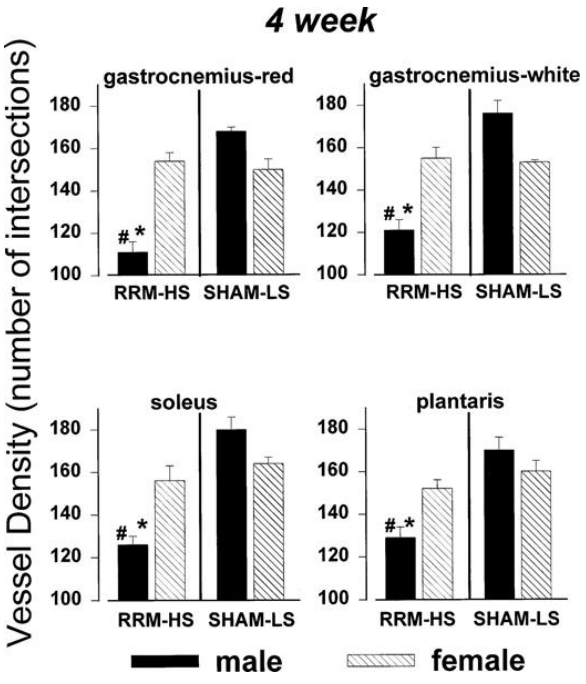
Vessel density in hind limb muscles of male and female reduced renal mass + high salt (RRM + HS) and sham-operated + low salt controls (SHAM + LS) after 3 days of diets. Vessel density was significantly reduced in gastrocnemius-white muscle of male RRM + HS rats when compared to female RRM + HS rats. Vessel density in male RRM + HS rats was significantly lower than in male SHAM + LS controls. #Significantly different from female RRM + HS, *P* < .05. *Significantly different from same gender SHAM + LS control, *P* < .05.

Figure 2.



Microvessel density in male and female reduced renal mass + high salt (RRM + HS) and sham-operated + low salt controls (SHAM + LS after 4 weeks of diets. Vessel density was significantly reduced in gastrocnemius-white, gastrocnemius-red, soleus, and plantaris muscles of male RRM + HS rats compared to female RRM + HS rats. Vessel density was also significantly lower in male RRM + HS rats than in male SHAM + LS controls. #Significantly different from female RRM + HS, *P* < .05. *Significantly different from same gender SHAM + LS control, *P* < .05.

Figure 3.



Discussion

This study is the first to demonstrate that female RRM hypertensive rats are protected from microvascular rarefaction, and have a markedly attenuated increase in MAP. This moderate change in pressure is in contrast to the rapid and significant elevation in arterial pressure and the microvascular rarefaction seen at an equivalent time in hypertensive male RRM + HS rats. Our results also confirm the presence of rarefaction in the postural hind limb muscles of male rats and further demonstrate the progressive loss of microvessels with time in this experimental model.

In the present study, MAP of male RRM rats was significantly increased after 3 days of high salt diet and was further increased after 4 weeks. These results are similar to and confirm those previously reported by our group.[16,17,26] However, in female RRM-HS rats, both the magnitude and the time of onset of the pressure increase was significantly less than in male rats. After 3 days on a high salt diet, MAP of female rats increased to 111 ± 3 mm Hg and this increase was not significantly higher than controls. When fed a high salt diet for 4 weeks, MAP in the female rats increased further. Despite a statistically significant increase in MAP in the female RRM + HS rats after 4 weeks on a high salt diet, the increase was only modest (118 ± 5 mm Hg). In contrast, MAP of male RRM + HS rats increased rapidly to 132 ± 6 mm Hg after only 3 days of high salt and was further increased to 160 ± 7 mm Hg at 4 weeks. The large differences in both the rate of increase of arterial pressure and in the magnitude of the pressure changes occurred despite the fact that both groups were fed the same high salt diet and that the percent reduction in renal mass was identical for male and female rats. This effect cannot be explained by differing responses to anesthesia. In our study, acute pressure measurements were not significantly different from measurements made in the conscious state (Table 2). This suggests that gender differences in MAP were not attributable to differences in measurement methods. Gender differences in MAP, such as those we observed, have been reported in the spontaneously hypertensive rats[5,6] and in the DOCA-salt model of hypertension.[7,–9,30]

In addition to a significant effect of gender on arterial pressure, gender appeared to modulate the changes in microvessel density. Microcirculatory beds from skeletal muscle of male rats demonstrated rarefaction, as evidenced by a significant loss of microvessels from the gastrocnemius-white muscle after only 3 days of high salt diet. The magnitude of rarefaction seen is identical to that previously reported.[29] In contrast, rarefaction did not occur in female rats (ie, no changes in vessel density were detectable). High salt diet for 4 weeks resulted in a greater differentiation between male and female rats. In male RRM + HS rats, vessel density was significantly decreased in all of the hind limb muscles: red and white gastrocnemius, plantaris, and soleus muscles. This was not the case in female rats, where no significant changes in microvessel density were seen after 4 weeks on a high salt diet.

In the present studies, differences in vessel density were paralleled by similar gender-related protection from elevations of MAP. In a previous study, we used a mathematical model to predict the increase in microvascular resistance caused by rarefaction.[16] Using that model, approximately one-third to one-half of the increase in pressure seen in male RRM + HS hypertensive rats may have been attributable to an uncompensated increase in microvascular resistance due to rarefaction. Therefore, the absence of rarefaction and the associated increase in microvascular resistance in the female rats may account, at least in part, for the attenuated arterial pressure increase seen in the female rats in the present study.

The mechanisms of the gender differences in arterial pressure and rarefaction in the RRM + HS model are unclear. One explanation could be that female rats did not rarify because arterial pressure changes were nonexistent or small. That is, that changes in arterial pressure drive the microvessel rarefaction rather than vice

versa. We have previously reported a 20% to 25% reduction in microvessel density of the cremaster muscle of normal male rats in response to 3 days of a high salt intake, demonstrating that, in male rats, an increase in salt intake alone induced microvessel rarefaction that was independent of an increase or change in arterial pressure.[31] Boegehold et al[13] have also demonstrated a pressure-independent rarefaction in male rats. Collectively, these results suggest that the absence of rarefaction in female RRM + HS rats is more than just a manifestation of an attenuated arterial pressure response. Furthermore, the data strongly suggest that rarefaction is not secondary to an increase in blood pressure in either male or female rats. In fact, we have recently reported that there are many instances in which microvessel density is either decreased or increased with no change in blood pressure[29,32] Thus, microvessel density is poorly correlated with arterial pressure in an intact animal model in which compensatory mechanisms are available to buffer such changes.

Variations in the sensitivity to a high salt intake remains as one possible explanation for the differential responses in arterial pressure and rarefaction. Recently, Calhoun et al[6]reported that nighttime blood pressures of male but not female Wistar-Kyoto (WKY) rats increase in response to high salt intake. Furthermore, gender differences have been reported in all of the models of hypertension that are salt-dependent, DOCA-salt,[7,–9,30]partial nephrectomy salt,[33] and salt-sensitive spontaneously hypertensive rat.[5,6] These results suggest that the gender-mediated differences in hypertension and microvessel remodeling may involve the handling of a sodium load. One possible explanation for the present results could be that male rats consume more salt than did female rats. Although we did not measure intakes in this present study, Fregly and colleagues[34,35]have failed to demonstrate a gender difference in either daily salt intake or salt appetite in DOCA-salt rats. Although male rats consumed more salt on a per gram basis, these differences disappeared when intake was expressed or adjusted for the differences in body weight between genders. A second potential mechanism for a salt effect would be whether there was a difference in the remaining renal mass after surgical reduction. Thus, salt per gram of kidney weight may be important. In this study, we documented the percentage of renal mass remaining in all groups (Table 1). There were no significant differences between male and female rats. This suggests that neither renal mass nor daily salt intake can explain the gender differences found in the present study. However, it does not eliminate the possibility of a gonadal steroid modulation of a undetermined salt-dependent mechanism.

The renin-angiotensin system is an important regulator of fluid and electrolyte homeostasis that responds to changes in sodium intake. Angiotensin has also been shown to induce angiogenesis in vitro and in vivo.[36,37] A number of studies from our group and others have demonstrated that vessel density is highly correlated with plasma angiotensin II levels.[28,31,32,37,38] This is in contrast to a poor relationship between arterial pressure and vessel density. We have demonstrated that the renin-angiotensin system is suppressed and that microvessel density is reduced in normal male rats placed on a high salt diet (4.0%).[28] We have also shown that chronic infusions of angiotensin II at subpressor doses effectively blocked the rarefaction associated with a high salt intake[25] and, in fact, stimulated angiogenesis in a dose-dependent manner. In addition, Wang and Prewitt[39] have shown that administration of oral captopril (an angiotensin converting enzyme inhibitor) suppresses angiotensin levels and results in microvessel rarefaction. Similarly, we recently reported that captopril eliminated the normal angiogenic response to exercise.[40] Collectively, these results suggest that the renin-angiotensin system, or more specifically, angiotensin II affects both angiogenesis and rarefaction. Therefore, it is possible that the renin-angiotensin system of female rats responds differently to salt than that of male rats. These possibilities remain to be investigated.

Finally, it is possible that the gonadal steroids (estrogen, progesterone, or testosterone) have either a direct effect or act indirectly to modulate arterial pressure and microvessel growth. This is supported by studies of Crofton et al in which ovariectomy increased systolic blood pressure in rats.[7,30] Furthermore, our preliminary data in ovariectomized DOCA-salt rats with or without estrogen replacement therapy indicated that, without estrogen, female rats exhibit “male-like” increases in arterial pressure.[38] Recently, Crofton and Share[30] reported

an attenuation of hypertension in intact male DOCA-salt rats treated with 17-β-estradiol and an exacerbation of hypertension in gonadectomized male rats. In these studies, ovariectomy resulted in hypertension in female rats that was essentially blocked by treatment with estradiol. The mechanisms for these gonadal steroid effects remain undetermined. However, there are many interesting possibilities.

Receptors for estrogen have been found on vascular smooth muscle cells[41] and gender differences in an endothelium-dependent contractile response of isolated aortas have been reported.[12] Although the physiologic function of these receptors is as yet undetermined, their presence supports the concept of a direct effect of estrogen on the vasculature.[42] This hypothesis is supported by the inhibition of angiogenesis in chick egg chorioallantoic membrane when antiestrogens are administered.[43] Possibly, the gonadal steroids act by way of a modulation of renin angiotensin system or within the central nervous system to modulate arterial pressure. Androgen receptors have been demonstrated in the cardiovascular blood pressure centers located in the brainstem[25]and in the areas responsible for vasopressin release.[44] Additional studies are required to understand the interactions between the gonadal steroids and the mediators of angiogenesis and the long-term controllers of arterial pressure.

This is the first study to examine the effect of gender on microvascular remodeling. The results suggest that female rats are protected from salt-induced rarefaction of microvessels and that this lack of rarefaction may have contributed to the attenuated increase in arterial pressure seen in female rats in this model of hypertension. Further studies are required to determine the exact mechanism of the protection and to determine whether estrogen and progesterone are independent modulators of microvessel growth, or whether they act through the well-known renin-angiotensin system.

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