

The chronic blockade of angiotensin I-converting enzyme eliminates the sex differences of serum cytokine levels of spontaneously hypertensive rats

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Abstract

Sex hormones modulate the action of both cytokines and the renin-angiotensin system. However, the effects of angiotensin I-converting enzyme (ACE) on the proinflammatory and anti-inflammatory cytokine levels in male and female spontaneously hypertensive rats (SHR) are unclear. We determined the relationship between ACE activity, cytokine levels and sex differences in SHR. Female (F) and male (M) SHR were divided into 4 experimental groups each (n = 7): sham + vehicle (SV), sham + enalapril (10 mg/kg body weight by gavage), castrated + vehicle, and castrated + enalapril. Treatment began 21 days after castration and continued for 30 days. Serum cytokine levels (ELISA) and ACE activity (fluorimetry) were measured. Male rats exhibited a higher serum ACE activity than female rats. Castration reduced serum ACE in males but did not affect it in females. Enalapril reduced serum ACE in all groups. IL-10 (FSV = 16.4 ± 1.1 pg/mL; MSV = 12.8 ± 1.2 pg/mL), TNF- α (FSV = 16.6 ± 1.2 pg/mL; MSV = 12.8 ± 1 pg/mL) and IL-6 (FSV = 10.3 ± 0.2 pg/mL; MSV = 7.2 ± 0.2 pg/mL) levels were higher in females than in males. Ovariectomy reduced all cytokine levels and orchiectomy reduced IL-6 but increased IL-10 concentrations in males. Castration eliminated the differences in all inflammatory cytokine levels (IL-6 and TNF- α) between males and females. Enalapril increased IL-10 in all groups and reduced IL-6 in SV rats. In conclusion, serum ACE inhibition by enalapril eliminated the sexual dimorphisms of cytokine levels in SV animals, which suggests that enalapril exerts systemic anti-inflammatory and anti-hypertensive effects.

Key words: SHR; Enalapril; Sexual dimorphism; Angiotensin I-converting enzyme; Cytokines IL-10, IL-6 and TNF- α

Introduction

Angiotensin II (Ang II) is directly involved in various biological functions including cell growth and proliferation, mediation of oxidative stress (1), and key events associated with the inflammatory process (2,3). Ang II increases vascular permeability, recruits inflammatory cells into tissues, and directly activates infiltrating immunocompetent cells (3). Ang II also acts as a potent proinflammatory mediator, promoting the generation of cytokines and free radicals (2). Furthermore, sex hormones modulate the action of cytokines (4) and the renin-angiotensin system (RAS) (5).

Previous studies have demonstrated that differences in autoimmune disease susceptibility between sexes may

be due to differences in cytokine production after autoantigen-specific stimulation (6). Estrogens have been shown to inhibit mRNA expression levels of platelet-derived growth factor-A, IL-1, and IL-6 mRNA in human vascular smooth muscle cells, a finding that suggests the anti-inflammatory effects of estrogens (7). Similarly, *in vitro* evidence has shown that testosterone may influence the expression of proinflammatory and anti-inflammatory cytokines (8-10). Differences between males and females have been found in components of the RAS, such as differences in the concentrations of prorenin, Ang II, and angiotensinogen in humans (11) and animals (12,13). In the hypertensive rat model (mRen(2).Lewis rat), angio-

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tensin I-converting enzyme (ACE) activity was higher in males than in females (12), and sexual dimorphisms in prorenin levels have been observed in humans (prorenin levels in men were significantly higher than in women) (11).

The pleiotropic anti-inflammatory effects of the ACE inhibitors have been well established (13,14). However, the effects of ACE on proinflammatory and anti-inflammatory cytokine levels in male and female SHR are not clear. Therefore, the present study was designed to investigate: 1) if there are sex differences in inflammatory biomarkers (IL-10, IL-6, TNF- α) in spontaneous hypertension, 2) if sex hormones play a role in these sex differences, and 3) if an ACE inhibitor enalapril interferes with inflammatory biomarkers in male and female SHR.

Material and Methods

Animals

The study was conducted on 12-week-old male and female SHR that initially weighed 150 ± 5 g (females) and 230 ± 5 g (males) and that had free access to food and water. The rats' body weight was monitored during the experiments. The animals were housed in standard plastic cages at a constant temperature of 22°C and on a 12-h light-dark cycle.

The procedures were carried out in compliance with the guidelines for the ethical use of animals in scientific research and were approved by the Ethics Committee of Universidade Federal do Espírito Santo (#023/2009). All surgical procedures were carried out under ketamine (70 mg/kg, *ip*) and xylazine (10 mg/kg, *ip*) anesthesia.

Experimental design

Castration. Female rats were subjected to a skin incision of 1 to 1.5 cm and a muscular incision to open the peritoneal cavity for the connection of the uterine tubules and ovary removal. The peritoneal cavity was then cleaned and sutured. The estrous cycle of the sham rats was monitored continuously with vaginal smears. This group was subjected to the experimental protocol on the day of proestrus. Orchiectomy was performed through an anterior median incision in the scrotum and each testicle was exposed through the surgical incision. The ductus deferens was isolated, ligated, and severed, allowing the testicle to be removed. The incision was then closed and sutured with 3-0 chromic catgut.

The rats were divided into 4 experimental groups ($n = 7$) of female and male rats as follows: sham-operated vehicle (SV), sham-operated treated with enalapril (SE), castrated + vehicle (CV), and castrated and treated with enalapril (CE). The treatments were started 21 days after castration and continued for 30 days. Enalapril maleate (Sigma, USA) was dissolved in water, and 10 mg enalapril/kg body weight was administered daily by gavage. A dose of $10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ has been shown to

reduce blood pressure in male SHR of this age (15). The rats' systolic blood pressure (SBP) was measured at the onset of the experiment and after 7 weeks of the experimental period (21 days after castration and after 28 days of treatment).

Blood pressure determination

The SBP of conscious rats was measured using a tail-cuff manometer manufactured by IITC Life Science Inc. (USA). The animals were placed inside a warming chamber (about 34°C) for 30 min before the measurements. The aim of the procedure was to calm the animals and dilate the blood vessels in the tail. The arterial blood pressure of each animal was measured at least three times. Any changes in pressure were reported as baseline value variation.

Determining estrous cycle phase

Daily vaginal smears were taken from each female rat as previously described (16) to confirm that their estrous cycles were proceeding normally. The vaginal epithelial cells were examined by microscopy for at least 7 consecutive days before the experiment. The swabs were performed between 8:00 and 10:00 am to maintain consistency. The females exhibiting normal estrous cycles were killed at proestrus between 9:00 am and 1:00 pm.

Measurement of cytokine levels, ACE activity and hormone levels. After measuring the SBP, the rats were decapitated without anesthesia, and 8 mL blood was collected into empty tubes to obtain serum (4 mL) for the determination of cytokines and plasma (4 mL) for the determination of ACE activity. The blood samples were centrifuged at 4°C and 1000 g (Eppendorf, Germany) for 15 min. Serum and plasma were stored at -80°C. IL-6, IL-10, and TNF- α levels were measured by ELISA according to manufacturer specifications (Biosource International, USA). The experiments were performed in duplicate.

The proteolytic activity assay that was used to determine ACE activity in plasma utilized Abz-FRK(Dnp)P-OH (Aminotech Pesquisa e Desenvolvimento, Brazil) as a substrate, which is ideal for studies of enzyme kinetics and the analysis of the somatic activity of the C- and N-terminal domains of ACE. The assay methodology was adapted to a fluorescence plate reader with a 96-well format and utilized excitation at 320 nm and emission at 420 nm. ACE activity is reported in arbitrary fluorescence units (AFU) (17).

Testosterone and estradiol concentrations were determined by ELISA. Testosterone (10 μ L serum sample; AccuBind™, Monobind Inc., USA; sensitivity of 0.038 ng/mL) and estradiol (25 μ L serum sample; AccuBind™, Monobind Inc.; sensitivity of 0.0065 ng/mL) were measured in duplicate. The uterus was removed and weighed.

Statistical analysis

Data are reported as means \pm SE. Statistical analysis

was performed by repeated measures two-way ANOVA followed by the Fisher least significant differences *post hoc* comparison for all statistical analyses. A P value <0.05 was considered to be statistically significant. The statistical analyses were performed with the GB Stat software (USA).

Results

Blood pressure and body weight

Table 1 shows the SBP of all of the groups studied. The initial SBP of the male (M) groups (MSV, MCV, MSE, and MCE) was higher than that of the female (F) groups (FSV, FCV, FSE, and FCE). After the experimental period, the SBP of male rats (MSV and MCV) continued to be higher than the SBP of female rats (FSV, FCV). Castration did not alter the blood pressure of either sex. As expected, treatment with enalapril reduced the SBP in both the non-castrated (MSE and FSE) and the castrated (MCE and FCE) groups, resulting in similar blood pressure levels in all of these groups. However, the observed percent decline was greater in males (SE = -30%, CE = -33%, $P < 0.05$) than in females (SE = -19%, CE = -25%).

The initial and final body weights (BW) of the male groups (MSV, MCV, MSE, MCE) were different from those of the respective female groups (FSV, FCV, FSE, FCE). All groups had an increase in their final BW after the experimental period. No differences were detected in the initial weight of the female groups; however, after the experimental period, the ovariectomized females (FCV and FCE) exhibited an increase in final BW compared to the sham females (FSV and FSE; Table 1).

ACE activity

Male rats exhibited higher ACE activity than female rats (MSV = 1500 ± 131 AFU; FSV = 1234 ± 80 AFU).

In comparison with the sham groups (MSV and FSV), castration greatly reduced the ACE activity of male rats (MCV = 790 ± 58 AFU) but did not affect the ACE activity of female rats (FCV = 1161 ± 39 AFU). When compared to vehicle treatment, enalapril treatment reduced the ACE activity in the non-castrated (MSE = 59 ± 20 AFU, FSE = 21 ± 4 AFU vs MSV and FSV, respectively) and castrated (MCE = 8 ± 5 AFU, FCE = 32 ± 3 AFU vs MCV and FCV, respectively) rats of both sexes (Figure 1).

Cytokines

IL-10. Figure 2A shows the sex differences in serum IL-10 levels. The male sham vehicle group had a lower IL-10 level than the female sham vehicle group (MSV = 12.8 ± 1.2 pg/mL vs FSV = 16.4 ± 1.1 pg/mL). In com-

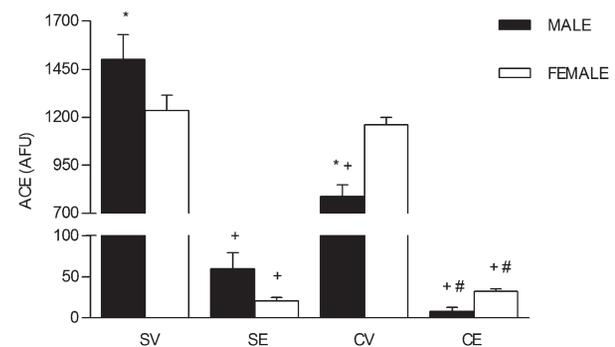


Figure 1. Plasma levels of angiotensin-converting enzyme (ACE) activity (arbitrary fluorescence units, AFU) in SV (sham + vehicle), SE (sham + enalapril), CV (castrated + vehicle), and CE (castrated + enalapril) groups. Data are reported as means \pm SE. * $P < 0.05$ vs females of the same group; + $P < 0.05$ vs sham vehicle animals of the same sex; # $P < 0.05$ vs castrated animals of the same sex (two-way ANOVA and Fisher test).

Table 1. Effect of castration and/or enalapril treatment on body weight (BW), systolic blood pressure (SBP), serum testosterone and estradiol, and uterine weight/body weight (UW/BW) ratio.

Group (n = 7)	Initial BW (g)	Final BW (g)	Initial SBP (mmHg)	Final SBP (mmHg)	Serum testosterone (ng/mL)	Serum estradiol (ng/mL)	UW/BW (mg/g)
MSV	232 \pm 8*	278 \pm 4* ^{&}	219 \pm 5*	222 \pm 5*	179 \pm 14	—	—
FSV	158 \pm 4	187 \pm 3 ^{&}	177 \pm 3	188 \pm 4	25 \pm 3	9.0 \pm 0.15	1.43 \pm 0.21
MCV	239 \pm 6*	280 \pm 6* ^{&}	223 \pm 4*	221 \pm 3*	15 \pm 3 ⁺	—	—
FCV	153 \pm 3	217 \pm 5 ⁺ ^{&}	177 \pm 2	192 \pm 2	18 \pm 2	6.5 \pm 0.12 ⁺	0.36 \pm 0.05 ⁺
MSE	224 \pm 5*	264 \pm 7* ^{&}	215 \pm 4*	153 \pm 5 ⁺	200 \pm 20	—	—
FSE	152 \pm 5	183 \pm 4 ^{&}	181 \pm 3	152 \pm 4 ⁺	28 \pm 4	9.2 \pm 0.10 [#]	1.51 \pm 0.21 [#]
MCE	236 \pm 3*	264 \pm 5* ^{&}	222 \pm 3*	148 \pm 4 ⁺ [#]	9.8 \pm 2 ⁺	—	—
FCE	157 \pm 5	216 \pm 3 ⁺ ^{&}	175 \pm 2	139 \pm 3 ⁺ [#]	19 \pm 3	6.3 \pm 0.31 ⁺	0.32 \pm 0.05 ⁺

Data are reported as means \pm SE. M = male; F = female; SV = sham-operated vehicle; SE = sham-operated rats treated with enalapril ($10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$); CV = castrated + vehicle; CE = castrated and treated with enalapril. * $P < 0.05$ vs females of the same group; + $P < 0.05$ vs sham vehicle animals of the same sex; # $P < 0.05$ vs castrated animals of the same sex; & $P < 0.05$ vs initial BW of the same group (two-way ANOVA and Fisher test).

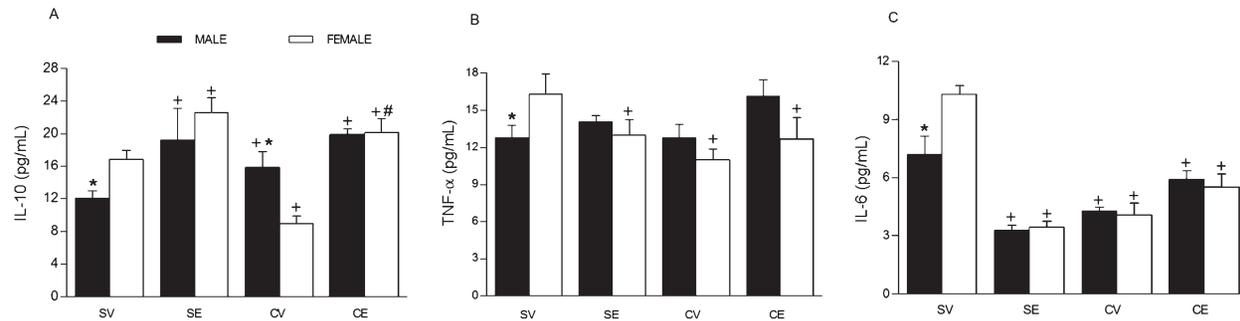


Figure 2. Cytokine levels. A, IL-10, B, TNF- α , and C, IL-6 levels of SV (sham + vehicle), SE (sham + enalapril), CV (castrated + vehicle), and CE (castrated + enalapril) groups, as measured by ELISA. Data are reported as means \pm SE. * $P < 0.05$ vs females of the same group; † $P < 0.05$ vs sham vehicle animals of the same sex; # $P < 0.05$ vs castrated animals of the same sex (two-way ANOVA and Fisher test).

parison with the sham groups, orchiectomy increased (MCV = 15.9 ± 2 pg/mL) and ovariectomy reduced (FCV = 8.9 ± 0.9 pg/mL) IL-10 levels. When compared to the vehicle treatment, enalapril treatment increased the serum IL-10 concentrations in the male (MSE = 19.2 ± 3.1 pg/mL) and female (FSE = 23.5 ± 1.8 pg/mL) sham groups. Enalapril treatment also increased the IL-10 levels of the castrated groups of male (MCE = 19.8 ± 0.7 pg/mL) and female (FCE = 20.1 ± 1.8 pg/mL) rats.

TNF- α . We observed different TNF- α concentrations (Figure 2B) between the male (MSV = 12.8 ± 1 pg/mL) and female sham vehicle groups (FSV = 16.6 ± 1.2 pg/mL). Castration did not alter the TNF- α concentration in males (MCV = 12.7 ± 1 pg/mL), but decreased it in female rats (FCV = 10.9 ± 0.8 pg/mL) relative to the sham vehicle group. Enalapril treatment did not change the TNF- α concentration in males (MSE = 14.1 ± 0.5 pg/mL), but it decreased the TNF- α concentration in the female rats (FSE = 13 ± 1 pg/mL) relative to the sham vehicle group. Treating castrated animals with enalapril did not alter the concentration of TNF- α in males (MCE = 14.2 ± 1.2 pg/mL) or females (FCE = 12.7 ± 1.6 pg/mL) relative to the CV groups, but it eliminated the sexual dimorphism.

IL-6. The serum concentrations of IL-6 are shown in Figure 2C. We observed sexual dimorphism in the IL-6 concentrations of the sham vehicle groups (MSV = 7.2 ± 0.2 pg/mL vs FSV = 10.3 ± 0.2 pg/mL). Castration decreased the IL-6 concentrations in male (MCV = 4.2 ± 0.2 pg/mL) and female (FCV = 4.0 ± 0.6 pg/mL) rats relative to the sham vehicle groups. Enalapril treatment reduced IL-6 concentrations in the male (MSE = 3.2 ± 0.9 pg/mL) and female (FSE = 3.5 ± 0.2 pg/mL) sham groups and eliminated the difference between sexes. However, the association of enalapril treatment with castration failed to change the IL-6 concentrations in either the male (MCE = 5.9 ± 0.4 pg/mL) or female (FCE = 5.5 ± 0.6 pg/mL) rats relative to the values obtained for the respective CV groups.

Hormone levels and uterine weight

Castration resulted in significant reductions in the levels of estradiol in female rats and in the levels of testosterone in male rats. Enalapril treatment did not alter the serum levels of estradiol or testosterone. As expected, ovariectomy reduced the uterine weight/body weight ratio (Table 1).

Discussion

It has been demonstrated that sex hormones and the immune system are connected (4,8-10) and that differences in hormone levels between sexes can alter cytokine production profiles (18). One of the main findings of the present study was the observation of sex dimorphisms in serum IL-10, TNF- α , and IL-6 levels in SHR. Furthermore, data from clinical and experimental studies have emphasized the important roles of ACE inhibitors in affecting non-hemodynamic, immune-mediated functions such as cytokine production (19,20). In the present study, we investigated the effect of the ACE inhibitor enalapril on systemic cytokine production in SHR and showed that enalapril increased the IL-10 levels and decreased the IL-6 levels of both male and female hypertensive animals.

The data presented in this study demonstrated that male SHR had a higher SBP than female SHR. Castration did not alter this parameter and the differences in SBP levels were maintained across the sexes. Female SHR that were subjected to ovariectomy did not show changes in their blood pressure (21,22). Previous studies have shown that castration lowered the blood pressure of male SHR rats (23-25); however, the initial blood pressure detected in our animals was higher than in previous studies, and the rats were castrated at a younger age (4 or 5 weeks) and observed for a longer period of time in our study (23,25). However, it has not been clearly established that the removal of sex hormones influences the development of hypertension. Furthermore, SBP was evaluated by direct measurements in anesthetized ani-

mals (23-25).

Our results identified sexual dimorphisms in the levels of the cytokines studied. Female SHR had higher levels of both the pro- and anti-inflammatory cytokines than male SHR. Establishing an estrogen-deficient state in female rats by ovariectomy reduced the levels of all the cytokines studied, whereas reducing the testosterone level in male rats by orchiectomy resulted in a different cytokine profile. Indeed, the effect of the female sex hormones appears to be evident regarding cytokine levels; ovariectomy did not change the ACE activity or the SBP but reduced all the cytokines studied when compared to the sham vehicle group. Orchiectomy decreased the ACE activity without changing the SBP in male rats. On the other hand, orchiectomy enhanced IL-10 and reduced IL-6 levels, indicating a relationship between ACE activity and the levels of these cytokines that was independent of the SBP. Thus, in female rats the differences in the cytokine levels between the sham and castrated animals are influenced by the sex hormones independent of ACE activity and SBP. However, in male rats the differences in cytokine levels are influenced by ACE activity, which was reduced after castration. These data suggest that sex hormones influence the serum cytokine levels in SHR, but generalizations cannot be made.

Estrogen appears to have a concentration-dependent effect on immune response polarization as low levels of exogenous estrogen were shown to support a proinflammatory Th1 (IL-1, IFN) response, while high doses of estrogen support a Th2 response by upregulating the production of IL-4, IL-10, and IL-6 (26,27). One of the strengths of the present study was that the cytokine measurements were carried out in sham females during proestrus, the stage with the highest endogenous estrogen concentrations (27). This might be responsible for the higher IL-10, IL-6, and TNF- α concentrations observed in the female sham vehicle group because ovariectomy decreased the cytokine concentrations.

Among the sham groups, male rats displayed lower IL-10, IL-6, and TNF- α concentrations than female rats. A direct relationship between testosterone and ACE activity has been identified (28), and we demonstrated that castration of male SHR reduced ACE activity. Therefore, the increase in IL-10 levels and the decrease in IL-6 levels observed after castration could have been due to ACE inhibition. Castration or blockade of the testosterone receptor with flutamide improved myocardial function after acute ischemia-reperfusion by decreasing the levels of proinflammatory cytokines (29). There are conflicting reports in the literature regarding the relationship between TNF- α and testosterone, with results showing both increases (30) and decreases (8,29,30) in TNF- α levels in the presence of testosterone.

We showed that male SHR had a higher serum ACE activity than female SHR and that castration only affected the ACE activity of male rats. Similarly, Pendergrass et al.

(12) showed that male hypertensive mRen(2).Lewis male rats had a higher plasma ACE activity than females, and Lim et al. (28) reported similar findings in mice. Another study consistent with our results demonstrated that normotensive female rats subjected to ovariectomy did not show altered ACE activity (31). Other studies showed that there were no changes in the pituitary (32) or circulating (33) ACE activity levels during the normal estrous cycle and that ACE activity was only reduced after exogenous estrogen administration (32,33). This suggested that ACE activity in plasma was only affected by the administration of exogenous estrogen (31,33).

A sex difference in the bradykinin (BK) system has been reported (34), and the level of BK may have contributed to our results. Although the inhibition of ACE determines the increase in BK levels (35), we cannot assume an important role for the bradykinin system in our results. Consistent with Reckelhoff et al. (21), we demonstrated that there were no differences in blood pressure levels between intact and ovariectomized females. Therefore, because the decrease in blood pressure after enalapril treatment was the same in the ovariectomized and sham rats, despite the reduction in the BK system in ovariectomized rats (36), it is debatable whether the BK system played an important role in the hypotensive effect of enalapril. Additionally, a study by Majima et al. (37) found that the levels of BK needed to reduce the blood pressure of SHR males were 20 to 100 times higher than the level produced by the ACE inhibitor captopril. In another study, inhibition of BK with the BK antagonist was shown to have no effect on the basal blood pressure of SHR or WKY (38).

It is well established that Ang II may contribute significantly to the development of diverse forms of vascular disease by activating the components of the inflammatory response (39) and that ACE inhibitors have pleiotropic anti-inflammatory effects (13), most likely by reducing activation of the nuclear factor-kappaB system and reversing the elevated expression of proinflammatory cytokines (5). We therefore used enalapril to assess the influence of ACE on cytokine levels in both male and female rats. First, we demonstrated that enalapril treatment decreased both the blood pressure and ACE activity of intact and castrated animals. Second, the pharmacological blockade of ACE led to changes in the cytokine levels of the sham animals. The inhibition of ACE decreased IL-6 and TNF- α levels and increased IL-10 levels in female rats and enhanced IL-10 levels, reduced IL-6 levels, and did not change TNF- α levels in male rats. However, castration increased IL-10 levels without affecting the proinflammatory cytokine levels, despite reductions in the ACE activity and blood pressure compared to the vehicle controls. Enalapril treatment markedly affected the serum IL-10 levels in both castrated and non-castrated hypertensive rats. It is known that endogenous IL-10 limits Ang II-mediated increases in IL-6, oxidative

stress, and vascular dysfunction (13). Therefore, enalapril-mediated increases in IL-10 and reductions in IL-6 may protect against Ang II-induced vascular dysfunction and mediate vascular protection during hypertension and other vascular diseases where Ang II plays a major role (39).

The results presented in this report are consistent with studies that demonstrated similar cytokine-modulating effects of ACE inhibitors in various experimental models (13,40). One could argue, however, that the effects of enalapril treatment on the cytokine expression profiles observed were due to a direct effect of blocking Ang II activity or as a consequence of reduced hypertension. Treatment with enalapril exerted important anti-inflammatory activity in normotensive patients (13) and animals (14). Based on our findings, the reduction in ACE activity appears to influence the cytokine levels independently of the reduction in blood pressure. For example, in the

castrated male rats, ACE activity is reduced without a reduction in SBP, yet we observed an increase in IL-10 levels and a decrease in IL-6 levels, which shows that testosterone influences the activity of ACE (27). Enalapril treatment did not alter the proinflammatory cytokines in either male or female castrated rats despite a decrease in blood pressure. Therefore, enalapril treatment only changes the proinflammatory cytokine profile in the presence of the sex hormones, regardless of the reduction in SBP.

This study identified sexual dimorphisms in serum cytokine levels in SHR; sex hormones differentially modulate levels of specific cytokines; male, but not female, sex hormones modulate ACE activity; ACE inhibition produces systemic anti-inflammatory and anti-hypertensive effects in SHR regardless of sex. These data support the evidence that both sex hormones and the RAS affect cytokine expression profiles.

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