Angiotensin Converting Enzyme 2 Contributes to Sex Differences in the Development of Obesity Hypertension in C57BL/6 Mice

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Objective—Obesity promotes hypertension, but it is unclear if sex differences exist in obesity-related hypertension. Angiotensin converting enzyme 2 (ACE2) converts angiotensin II (AngII) to angiotensin-(1–7) (Ang-[1–7]), controlling peptide balance. We hypothesized that tissue-specific regulation of ACE2 by high-fat (HF) feeding and sex hormones contributes to sex differences in hypertension.

Methods and Results—HF-fed females gained more body weight and fat mass than males. HF-fed males exhibiting reduced kidney ACE2 activity had increased plasma angiotensin II levels and decreased plasma Ang-(1–7) levels. In contrast, HF-fed females exhibiting elevated adipose ACE2 activity had increased plasma Ang-(1–7) levels. HF-fed males had elevated systolic and diastolic blood pressure that were abolished by losartan. In contrast, HF-fed females did not exhibit increased systolic blood pressure until females were administered the Ang-(1–7) receptor antagonist, D-Ala-Ang-(1–7). Deficiency of ACE2 increased systolic blood pressure in HF-fed males and females, which was abolished by losartan. Ovariectomy of HF-fed female mice reduced adipose ACE2 activity and plasma Ang-(1–7) levels, and promoted obesity-hypertension. Finally, estrogen, but not other sex hormones, increased adipocyte ACE2 mRNA abundance.

Conclusion—These results demonstrate that tissue-specific regulation of ACE2 by diet and sex hormones contributes to sex differences in obesity-hypertension. (Arterioscler Thromb Vasc Biol. 2012;32:1392-1399.)

Key Words: angiotensin-(1–7) ■ blood pressure ■ losartan ■ ovariectomy

Obesity is a primary risk factor for the development of hypertension in the United States. Based on cross-sectional data, men demonstrate a steeper increase in blood pressure with age compared with women, which is reversed after menopause when the prevalence of obesity increases in females.1–3 It is unclear if obesity exerts similar mechanisms to promote hypertension in both men and women. The renin-angiotensin system (RAS) is activated in human and experimental obesity-associated hypertension.4–7 Angiotensin converting enzyme 2 (ACE2) balances the activity of the RAS through catabolism of the vasoconstrictor peptide, angiotensin II (AngII), to form the vasodilator peptide, angiotensin-(1–7) (Ang-[1–7]). Expression of ACE2 by various cell types regulates actions of these peptides locally, as well as contributes to changes in the systemic balance of AngII/Ang-(1–7). Reductions in ACE2 expression and function, as has been demonstrated in adipose tissue of male obese mice,5 shifts the balance toward increased levels of AngII, favoring the development of hypertension.1

A variety of tissues express ACE2, including kidney, heart, lung, and adipose tissue.5,8 Adipose tissue possesses a local RAS capable of synthesizing AngII and forming Ang-(1–7) from ACE2.9 Previous studies in our laboratory demonstrated that ACE2 is regulated in adipose tissue of male mice fed a high-fat (HF) diet.5 Initial stimulation of ACE2 in adipose tissue of HF-fed males was lost when male mice exhibited robust obesity associated with increased plasma levels of AngII and obesity-induced hypertension. These results suggest that HF feeding in male mice shifts the ACE2-regulated AngII/Ang-(1–7) balance to favor high AngII in the development of obesity-associated hypertension. Because both adipose tissue1 and kidney5,6,10 express ACE2, obesity-induced regulation of ACE2 may differ between tissues and according to sex. Moreover, although several studies support sex differences in the RAS, which contribute to differences in blood pressure control,6–19 it is unclear whether sex differences extend to the development of obesity-related hypertension.

Recent results demonstrated that female spontaneously hypertensive rats exhibited a lower level of hypertension in...
response to AngII infusion than males, which was reversed by administration of an Ang-(1–7) receptor antagonist. These results suggest greater conversion of AngII to Ang-(1–7), presumably resulting from ACE2, in kidneys of female compared with male rats. Previous studies demonstrated that estradiol positively regulated renal ACE2 in a rat model of hypertensive renal disease. Taken together, these results suggest that differences in blood pressure responses to AngII between males and females may result from estrogen-mediated increases in ACE2 and increased production of the vasodilator, Ang-(1–7). It is unclear whether estrogen exerts tissue-specific regulation of ACE2 expression, and the role of estrogen regulation of ACE2 in the development of obesity-hypertension.

In the present study, we hypothesized that male and female mice exhibit sex differences in the development of obesity-induced hypertension because of differential tissue-specific regulation of ACE2 and a shift in the AngII/Ang-(1–7) balance. We focused on regulation of kidney versus adipose ACE2 activity in relation to plasma levels of AngII versus Ang-(1–7) in male and female mice made obese from consumption of a HF diet. Moreover, we defined the relative role of AngII versus Ang-(1–7) as mechanisms to initiate males or protect females, respectively, against obesity-associated hypertension. To define the mechanistic role of ACE2, we examined the effects of ACE2 deficiency in the development of obesity-associated hypertension in males versus females. Finally, we quantified effects of estrogen on ACE2 mRNA expression in adipocytes, and then defined effects of removal of female sex hormones on adipose ACE2 and the development of obesity-associated hypertension.

Methods

Animals and Diets

The institutional Animal Care and Use Committee at the University of Kentucky approved all procedures using animals. Male and female C57BL/6 mice (aged 2 months; The Jackson Laboratories, Bar Harbor, ME) were fed a low-fat (LF; 10% kcal as fat; D12450B; Research Diets Inc, New Brunswick, NJ; n = 10) or HF diet (60% kcal as fat; D12492, Research Diets Inc; n = 10) for 16 weeks. During week 16 of HF feeding, the angiotensin type 1 receptor (AT1R) antagonist, losartan (10 mg/kg per day), was administered in the drinking water of diet. Moreover, we defined the relative role of AngII versus Ang-(1–7) as mechanisms to initiate males or protect females, respectively, against obesity-associated hypertension. To define the mechanistic role of ACE2, we examined the effects of ACE2 deficiency in the development of obesity-associated hypertension in males versus females. Finally, we quantified effects of estrogen on ACE2 mRNA expression in adipocytes, and then defined effects of removal of female sex hormones on adipose ACE2 and the development of obesity-associated hypertension.

Tissue ACE2 Measurements

ACE2 activity was measured in homogenates of kidney and adipose tissue using a commercial substrate (Mca-YVADAPK-[Dnp]-OH, R & D Systems, Minneapolis, MN). Standard curves were generated with the reaction product, Mca-Pro-Leu-OH (Bachem, Torrance, CA), and data are expressed in specific activity (relative fluorescence units per minute per microgram).

Statistical Analysis

Data are expressed as mean±SEM. All data were analyzed using SigmaStat (Version 11, 2008, Systat Software, Inc., Chicago, IL). For 2 factor analysis, a 2-way ANOVA was used to analyze end-point measures followed by Tukey test for post hoc analysis. Significance was accepted at P<0.05.

Results

The Development of Obesity in HF-Fed Male and Female Mice

Male and female mice exhibited significant increases in body weight (Figure 1A, P<0.05) in response to the HF diet. Male HF-fed mice exhibited a 24.6 g increase in body weight, representing an 95% increase. By comparison, LF-fed males gained 7.5 g, representing a 31% increase. HF-fed female mice gained 21.1 g, representing an 117% increase. LF-fed females gained only 3.8 g, representing a 20% increase. After correcting for age-matched increases in body weights in LF-fed mice of each sex at week 16, female mice fed the HF diet gained more weight (97%) than males (64%). Body weight increased significantly in both sexes by week 4 of HF feeding. At study end point, body weights of HF-fed males were significantly increased compared with HF-fed females (Figure 1A).

In LF-fed mice, fat mass was significantly greater in male compared with female mice (Figure 1B). Fat mass increased significantly in both male and female mice fed the HF diet compared with LF-fed controls (Figure 1B). In addition, fat mass was significantly greater in HF-fed males compared with females (P<0.05). Compared with age-matched LF-fed controls, male HF-fed mice exhibited an 86% increase in fat mass (LF, 6.5±2.2; HF, 18.6±0.9 g, P<0.05). By comparison,
female HF-fed mice exhibited a 236% increase in fat mass compared with age-matched LF-fed controls (LF, 2.2 ± 0.2; HF, 9.6 ± 0.6 g, \( P < 0.05 \)). Thus, female mice exhibited a relatively greater increase in body weight and fat mass in response to the HF diet compared with males.

Both male and female HF-fed mice exhibited impaired glucose tolerance (area under the curve for glucose tolerance tests; Figure 1 in the online-only Data Supplement; \( P < 0.05 \)) compared with LF controls. The magnitude of glucose intolerance was similar in HF-fed male (2.30-fold increase) and female (1.95-fold increase) mice compared with LF controls.

### Effects of Obesity on Plasma AngII and Ang-(1–7) Concentrations and Tissue ACE2 Activity in Male and Female Mice

In LF-fed mice, plasma concentrations of AngII were similar between male and female mice (Figure 2A). With HF feeding, male, but not female mice, exhibited a significant increase in plasma concentrations of AngII (\( P < 0.05 \)). Plasma concentrations of Ang-(1–7) were significantly lower in age-matched LF-fed female compared with male mice (Figure 2B; \( P < 0.05 \)). In male mice fed a HF diet, plasma concentrations of Ang-(1–7) decreased significantly compared with LF-fed controls (Figure 2B; \( P < 0.05 \)). In contrast, HF-fed female mice exhibited a significant increase in plasma Ang-(1–7) concentrations compared with LF-fed controls (\( P < 0.05 \)).

In LF-fed mice, ACE2 protein (Figure II in the online-only Data Supplement) and enzymatic activity (Figure 2C; \( P < 0.05 \)) were significantly greater in kidneys of male compared with female mice. Similarly, ACE2 activity was significantly greater in adipose tissue from LF-fed male compared with female mice (Figure 2C). In HF-fed male mice, kidney ACE2 activity was significantly decreased compared with LF-fed controls (Figure 2C, \( P < 0.05 \)), and kidney ACE2 protein was modestly, but not significantly reduced (Figure IIIA in the online-only Data Supplement, \( P = 0.08 \)). Interestingly, mas receptor protein increased significantly in kidneys from HF-fed male (Figure IV in the online-only Data Supplement), but not female mice (data not shown). There was no effect of HF feeding on kidney ACE2 protein (Figure IIIIB in the online-only Data Supplement) or activity (Figure 2C; \( P > 0.05 \)) in females. Notably, whereas ACE2 activity in adipose tissue was not altered with HF feeding in
male mice, it increased significantly in adipose tissue from HF-fed females compared with LF-fed controls (Figure 2C; \( P < 0.05 \)).


During the light cycle, SBP and diastolic blood pressure (DBP) were similar in LF-fed male and female mice (Figure 3A and 3C). However, during the night cycle, SBP, but not DBP, was significantly lower in LF-fed female compared with male mice (Figure 3B and 3D; \( P < 0.05 \)). As previously reported,\(^5\) HF-feeding male mice exhibited a significant increase in SBP during both the day and night compared with LF-fed controls (Figure 3A and 3B; \( P < 0.05 \)). During the night, DBP was also significantly increased in HF-fed male mice compared with LF-fed controls (Figure 3D; \( P < 0.05 \)). Male HF-fed mice exhibited significantly increased mean arterial pressure compared with LF-fed controls, with no differences in heart rate, pulse pressure, or activity (Table I in the online-only Data Supplement). In contrast, despite the robust development of obesity (Figure 1), female mice fed the HF diet did not exhibit a significant change in SBP or DBP during the day or at night (Figure 3A–3D). Although there was no effect of HF feeding on mean arterial pressure, pulse pressures, or activity, heart rate was significantly increased in HF-fed females compared with LF-fed controls (Table I in the online-only Data Supplement). Administration of the AT1R antagonist losartan had no significant effect on SBP in LF-fed male mice, but it significantly reduced SBP in HF-fed male mice to levels observed in LF-fed males (Figure 4A; \( P < 0.05 \)). Conversely, infusion of the mas receptor antagonist D-Ala-Ang-(1–7) to female HF-fed mice resulted in a significant increase in SBP compared with vehicle (Figure 4B; \( P < 0.05 \)).

**ACE2 Deficiency Increases Blood Pressure in HF-Fed Male and Female Mice**

Based on the above described results, we hypothesized that ACE2 deficiency would increase blood pressure in male HF-fed mice, primarily by increasing AngII/AT1R-mediated effects. In contrast, we hypothesized that female ACE2-deficient mice would develop obesity-hypertension because of an increase in the AngII/Ang-(1–7) balance (Figure 2B). ACE2-deficient male and female mice exhibited significant increases in body weight and fat mass with HF feeding (data not shown). After 16 weeks of HF feeding, body weights of HF-fed ACE2-deficient male and female mice were modestly, but significantly lower than HF-fed wild-type controls (Table II in the online-only Data Supplement, \( P < 0.05 \)).

Plasma concentrations of Ang-(1–7) in HF-fed Ace2\(^{+/+}\) male mice (Ace2\(^{+/+}\): 0.15\(\pm\)0.03 ng/mL) were not significantly different from levels in HF-fed wild-type male mice (Ace2\(^{+/+}\): 0.13\(\pm\)0.02 ng/mL). However, plasma Ang-(1–7) concentrations in HF-fed Ace2\(^{+/+}\) males were lower than those observed in wild-type male mice fed the LF diet (0.59\(\pm\)0.14 ng/mL; Figure 2B; \( P < 0.05 \)). In contrast to findings from wild-type HF-fed females (Figure 2B), HF feeding did not result in an increase in plasma concentrations of Ang-(1–7) in ACE2-deficient females (Ace2\(^{−/−}\): 0.31\(\pm\)0.04; Ace2\(^{−/−}\): 0.25\(\pm\)0.05 ng/mL).
As demonstrated (Figure 3), night cycle SBP was significantly greater in HF-fed male compared with female Ace2<sup>1+/1</sup> mice (Figure 5A; \(P<0.05\)). Similar findings were observed for day cycle SBP (data not shown). ACE2 deficiency significantly increased SBP and mean arterial pressure in HF-fed male and female mice compared with wild-type controls of each sex (Figure 5A; Table II in the online-only Data Supplement; \(P<0.05\)). However, male HF-fed ACE2-deficient mice had significantly greater SBP compared with ACE2-deficient females (Figure 5A; \(P<0.05\)). To determine if male and female ACE2-deficient mice fed the HF diet exhibited increased SBP from elevated systemic AngII effects at AT1R, we administered losartan to Ace2<sup>1+/1</sup> and Ace<sup>−/−</sup> mice. In HF-fed male mice, administration of losartan significantly lowered SBP (24 hours) in both genotypes (Figure 5B; \(P<0.05\)). However, losartan-mediated reductions in SBP were more pronounced in ACE2-deficient mice compared with wild-type male mice (\(P<0.05\)). In HF-fed female mice, losartan had no effect on SBP in Ace2<sup>1+/1</sup> females (data not shown), but significantly lowered blood pressure in Ace2<sup>−/−</sup> females (prelosartan: 137±2; postlosartan: 111±4 mm Hg; \(P<0.05\)).

As demonstrated (Figure 3), night cycle SBP was significantly greater in HF-fed male compared with female Ace2<sup>1+/1</sup> mice (Figure 5A; \(P<0.05\)). Similar findings were observed for day cycle SBP (data not shown). ACE2 deficiency significantly increased SBP and mean arterial pressure in HF-fed male and female mice compared with wild-type controls of each sex (Figure 5A; Table II in the online-only Data Supplement; \(P<0.05\)). However, male HF-fed ACE2-deficient mice had significantly greater SBP compared with ACE2-deficient females (Figure 5A; \(P<0.05\)). To determine if male and female ACE2-deficient mice fed the HF diet exhibited increased SBP from elevated systemic AngII effects at AT1R, we administered losartan to Ace2<sup>1+/1</sup> and Ace<sup>−/−</sup> mice. In HF-fed male mice, administration of losartan significantly lowered SBP (24 hours) in both genotypes (Figure 5B; \(P<0.05\)). However, losartan-mediated reductions in SBP were more pronounced in ACE2-deficient mice compared with wild-type male mice (\(P<0.05\)). In HF-fed female mice, losartan had no effect on SBP in Ace2<sup>1+/1</sup> females (data not shown), but significantly lowered blood pressure in Ace2<sup>−/−</sup> females (prelosartan: 137±2; postlosartan: 111±4 mm Hg; \(P<0.05\)).

**Ovx Reduces ACE2 Activity in Adipose Tissue and Promotes Obesity-Associated Hypertension in Female Mice**

Estrogen has been reported to positively regulate ACE2 activity in female rat kidney. Therefore, we quantified effects of Ovx on tissue ACE2 activity and blood pressure in HF-fed females. Ovx increased body weights of HF-fed females compared with sham-operated controls (Sham, 41±3; Ovx, 50±2 g; \(P<0.05\)). In kidneys from HF-fed females, Ovx had no effect on ACE2 activity (Figure 6A). In contrast, ACE2 activity in adipose tissue from HF-fed females was significantly reduced by Ovx (Figure 6A; \(P<0.05\)). Plasma concentrations of Ang-(1–7) in HF-fed females were significantly decreased by Ovx (Figure 6B; \(P<0.05\)) to levels that were comparable with those in LF-fed female mice (Figure 2B). Notably, SBP (night cycle) was significantly increased by Ovx (HF, sham: 120±1; HF, Ovx: 141±3 mm Hg, Figure 6C; \(P<0.05\)) to levels similar to those observed in HF-fed males (Figure 3). Similar findings were observed in day cycle SBP (data not shown) and mean arterial pressure (Table III in the online-only Data Supplement). To determine if sex hormones regulate adipocyte ACE2 expression, we quantified concentration-dependent effects of estrogen,
In contrast, females exhibit increased plasma Ang-(1–7) of hypertension, and plasma Ang-(1–7) levels are suppressed. Balance toward AngII/AT1R stimulation and the development of obesity-hypertension. In males, HF feeding shifts the diet-induced obesity, and contributes to diverging susceptibility toward obesity-hypertension. In the current study, the AngII/Ang-(1–7) balance is regulated differently in male and female mice with obesity-hypertension. The AngII/Ang-(1–7) balance contributing to sex differences in the development of obesity-associated hypertension.

To our knowledge, this is the first study to examine sex differences in the development of obesity-associated hypertension between male and female mice. In the present study, mice fed HF diets exhibited several characteristics of the metabolic syndrome, including obesity, glucose intolerance, and hypertension, some of which exhibited sex differences. For example, in contrast to male mice, female mice were resistant to the development of obesity-hypertension despite more pronounced fat mass and body weight. Although it is generally considered that females exhibit lower levels of cardiovascular diseases compared with males, the age-adjusted prevalence of uncontrolled blood pressure is higher in females compared with males. In addition, although blood pressure increases with age in both women and men, the age-related increase is more rapid in women after menopause than in men. Results from this study suggest that changes in the AngII/Ang-(1–7) balance, favoring an increase in systemic levels of Ang-(1–7) associated with HF-induced activation of ACE2 in adipose tissue, contribute resistance to the development of obesity-hypertension in female mice. Given that females exhibited greater elevations in fat mass with obesity than males, elevated ACE2 activity in the expansive mass of adipose tissue of HF-fed females would predictably influence the local and systemic RAS. As females gained more weight and fat mass compared to males fed the HF-diet, the lack of hypertension development in females cannot be attributed to diminished development of obesity. Moreover, because glucose intolerance was similar between male and female HF-fed mice, the differences in insulin sensitivity most likely did not contribute to sex differences in the development of hypertension.

In agreement with previous studies, plasma concentrations of AngII were increased in male mice exhibiting obesity-associated hypertension. To our knowledge, this is the first report that obesity results in sex-dependent differences in plasma concentrations of Ang-(1–7). Marked reductions in plasma Ang-(1–7) concentrations in male HF-fed mice favored an increase in the AngII/Ang-(1–7) balance, which was supported by data demonstrating pronounced reductions in ACE2 mRNA abundance in adipose tissue with estrogen, but not other sex hormones, resulting in a concentration-dependent increase in ACE2 mRNA abundance in 3T3-L1 adipocytes. Incubation of differentiated 3T3-L1 adipocytes with progesterone, or testosterone on ACE2 mRNA abundance in 3T3-L1 adipocytes. Incubation of differentiated 3T3-L1 adipocytes with estrogen, but not other sex hormones, resulted in a concentration-dependent increase in ACE2 mRNA abundance (Figure V in the online-only Data Supplement; P<0.05).

**Discussion**

The current study provides evidence that the AngII/Ang-(1–7) balance is regulated differently in male and female mice with diet-induced obesity, and contributes to diverging susceptibilities to obesity-hypertension. In males, HF feeding shifts the balance toward AngII/AT1R stimulation and the development of hypertension, and plasma Ang-(1–7) levels are suppressed. In contrast, females exhibit increased plasma Ang-(1–7) concentrations with obesity and are resistant to the development of obesity-hypertension. AT1R antagonism eliminated obesity-hypertension in HF-fed males, whereas mas receptor antagonism promoted obesity-hypertension in females. ACE2 was demonstrated as a mechanism for differences in obesity-hypertension between males and females, because ACE2 deficiency promoted obesity-hypertension in both sexes. Administration of losartan decreased effects of ACE2 deficiency to promote obesity-hypertension in males and females. Female sex hormones were demonstrated to contribute to tissue-specific regulation of ACE2 and the development of obesity-hypertension, since Ovx of females reduced adipose ACE2 activity and promoted obesity-hypertension. Notably, regulation of adipose ACE2 activity by HF feeding and Ovx in females paralleled blood pressure changes in females, whereas kidney ACE2 activity paralleled blood pressure changes in males. These results support the hypothesis that tissue specific regulation of ACE2 by obesity and female sex hormones results in a shift in the AngII/Ang-(1–7) balance contributing to sex differences in the development of obesity-associated hypertension.
in obesity-hypertension in males administered losartan. As ACE2 converts AngII to Ang-(1–7), we hypothesized that tissue-specific regulation of ACE2 in HF-fed male mice contributed to a shift in the AngII/Ang-(1–7) balance. Indeed, HF-fed male mice, but not females, exhibited reductions in kidney ACE2 protein and activity that likely contributed to elevated plasma levels of AngII and reduced levels of Ang-(1–7). In addition, as plasma levels of Ang-(1–7) in HF-fed male mice were similar to those of ACE2-deficient male mice, the increased blood pressures in ACE2-deficient male mice fed the HF diet most likely resulted from augmented effects of AngII. This conclusion was supported in the present study by more pronounced losartan-mediated reductions in blood pressure in ACE2-deficient male mice compared with controls. Administration of losartan has been demonstrated previously to lower blood pressure to a greater extent in obese compared with lean male rats.7,28 These findings suggest that diet-induced obesity in male mice is associated with a shift in the AngII/Ang-(1–7) balance toward high AngII, potentially resulting from dysregulated kidney ACE2.

Whereas deficiency of ACE2 augmented obesity-hypertension in male mice, it induced obesity-hypertension in female mice. Mechanisms of ACE2 deficiency to induce the development of obesity-hypertension in HF-fed females included reductions in plasma Ang-(1–7) levels. Recent findings demonstrated increased conversion of AngII to Ang-(1–7) and lower blood pressure responses to AngII in female compared with male spontaneously hypertensive rats.9 Our results extend these findings by demonstrating that ACE2 deficiency in female mice eliminates HF diet–induced increases in plasma Ang-(1–7) and induces obesity-hypertension. In addition, because administration of losartan normalized blood pressures of ACE2-deficient HF-fed females, these results suggest that ACE2 deficiency augments obesity-hypertension in male and female mice by increasing the AngII/Ang-(1–7) balance. Recent results demonstrated that a polymorphism in the ACE2 gene is associated with a lower risk for fatal cardiovascular events in females30, potentially related to 2 alleles of the X-linked ACE2 gene in females compared with males. It is unclear whether gene dosage differences in ACE2 expression between male and female mice in this study contributed to resistance to obesity-hypertension in females. However, as plasma Ang-(1–7) levels, as well as kidney and adipose ACE2 levels, were lower in LF-fed females than males, these results do not support a role for gene dosage effects of ACE2 in females. Rather, male and female mice appear to respond differently to consumption of a HF diet in the tissue-specific regulation of ACE2 and AngII/Ang-(1–7) balance. Tissue-specific regulation of angiotensin peptide balance by deficiency of ACE2 has been reported recently to impact pregnancy and gestational weight gain in pregnant female mice.30

Our results indicate that female sex hormones also contribute to protection of females against obesity-hypertension, and that this protection may involve estrogen-mediated regulation of ACE2 in adipose tissue. Previous studies demonstrated that estrogen protects female rats against hypertension by amplifying the vasodilator contributions of Ang-(1–7) and reducing formation of AngII.31,32 Additional studies by this group33 demonstrated that administration of estradiol to ovariectomized apolipoprotein E–deficient female mice decreased kidney ACE2 mRNA abundance. In contrast, in a renal wrap model of hypertension, Ovx decreased kidney ACE2 activity, which was restored by estradiol.20 However, this same group recently demonstrated that Ovx of female mice increased renal ACE2 activity,34 prompting the authors to speculate that estrogen regulation of ACE2 in kidney is different between normal and diseased conditions. Our findings do not support a role for female sex hormones as regulators of kidney ACE2 activity in the diseased condition of obesity. Rather, adipose ACE2 activity and plasma Ang-(1–7) levels were reduced by Ovx of HF-fed females and blood pressure increased, supporting adipose ACE2 as a target of female sex hormones in protection against obesity-hypertension. Further, estrogen, but not other sex hormones, increased ACE2 mRNA abundance in an adipocyte cell culture system, suggesting that in vivo effects of Ovx to decrease adipose ACE2 activity resulted from a lack of estrogen. Previous investigators demonstrated that the ACE2 promoter has 2 putative estrogen response elements.35 Interestingly, obesity is associated with altered effects of estrogen in adipose tissue,35,36 suggesting that estrogen may exert tissue-specific effects on adipose tissue of obese female mice. Moreover, a loss of estrogen in postmenopausal women has been suggested as a contributor to an increased prevalence of obesity in this population.37,38 Indeed, in the present study, Ovx promoted the development of obesity, which may have contributed to increased blood pressure in ovariectomized HF-fed females.

In conclusion, results from this study demonstrate sex differences in the development of obesity-associated hypertension related to ACE2-mediated regulation of the AngII/Ang-(1–7) balance. Male mice exhibited obesity-hypertension associated with enhanced AngII/AT1R effects, whereas protection of female mice against obesity-hypertension was abolished by Ang-(1–7) mas receptor antagonism. Although ACE2 deficiency promoted obesity-hypertension in males and females, tissue-specific regulation of ACE2 by diet and sex hormones may have contributed to sex differences in obesity-hypertension. Regardless of sex differences in obesity-hypertension, these results suggest that stimulation of ACE2 may be beneficial in the treatment of obesity-associated hypertension in both males and females.

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Disclosures

None.
References


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SUPPLEMENTAL MATERIAL

ACE2 CONTRIBUTES TO SEX DIFFERENCES IN THE DEVELOPMENT OF OBESITY HYPERTENSION IN C57BL/6 MICE
Detailed Methods

**Tissue ACE2 activity measurements.** Kidney and adipose tissues were homogenized on ice in buffer containing 0.1M Tris-HCl, 0.3M NaCl, 10 μM ZnCl₂ and Z-pro-prolinol with 0.5% Triton-X and Complete protease inhibitors without EDTA (Roche, Indianapolis, IN). Samples were centrifuged at 10,000 rpm for 15 minutes at 4°C and a BCA assay (Thermo Fischer, Rockford, IL) was performed on the supernatant to determine protein concentration. Kidney (final concentration, 1.75 μg/well) or adipose protein (final concentration, 7 μg/well) were incubated in ACE2 buffer (75 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 μM ZnCl₂, 100 μM Z-pro-prolinol, 10 μM captopril) for 10 minutes prior to the addition of 50 μM of Mca-YVADAPK-(Dnp)-OH (R & D Systems, Minneapolis, MN). A fluorescent spectrophotometer (Spectramax M2, Molecular Devices, Downingtown, PA) was utilized with an excitation of 320 nm and an emission of 405 nm. Kinetic reactions were performed at 37°C for 2.5 hours and a Vmax was calculated for each sample. Standard curves were generated with the reaction product, Mca-Pro-Leu-OH (Bachem, Torrence CA), and data are expressed in specific activity (RFUs/min/μg).

**Western blot analysis.** Kidney tissue was homogenized on an ice bath using a glass-on-glass homogenizer (Glas-Col, Terre Haute, IN). Whole kidney was homogenized in 0.5 mL of M-PER reagent (cat#78501, ThermoScientific, Rockford, IL). A protease inhibitor cocktail was added to M-PER at a 1:100 dilution (P2714, Sigma, St. Louis, MO). Samples were spun at 10,000 g's for 10 minutes and the supernatant was removed and protein analysis was performed (BCA kit, ThermoScientific) using BSA as a standard (Bio-Rad, Hercules, CA). Kidney protein (25 μg) was treated with Beta-mercaptoethanol (Fischer, Chicago, IL), sample buffer, and heated to 100°C for 5 minutes and then placed on ice to cool. Samples were spun down and then loaded on 7.5% SDS-PAGE gels (for ACE2) or 12% SDS-PAGE gels (for MasR). Both proteins came out at the predicted size (ACE2, 120 kDa; MasR, 40 kDa). Proteins were then blotted to PVDF membrane (RPN303F, GE Healthcare, Piscataway, NJ). Blots were rinsed in PBS/0.1% Tween and then incubated for 1 hour with 5% nonfat dry milk in PBS. Blots were then rinsed and incubated with an anti-goat ACE2 antibody (cat#sc-21834, lot#I2508, 2 μg/mL final concentration, Santa Cruz) or anti-rabbit MasR antibody (cat#NBP1-60091, lot#Rb556-100808-W, 1 μg/mL final concentration, Novus Biologicals) overnight at 4°C. Blots were then rinsed three times and incubated with secondary antibodies for 1 hour at room temperature. Secondary antibodies were donkey anti-goat IgG-HRP (cat#sc-2056, lot#D0309, mouse/human adsorbed, Santa Cruz) or anti-rabbit-HRP (cat#NA934VS, lot#4618762, GE Healthcare). Blots were then rinsed 3 times and then incubated with SuperSignal West PicoChemiluminescent substrate (cat#34080, ThermoScientific) for 5 minutes at room temperature. For beta-actin analysis, blots were stripped with Restore PLUS stripping buffer (cat#46430, ThermoScientific) for 30 minutes at room temperature and then re-blocked in milk. Primary antibody for beta-actin was anti-mouse (Sigma, clone AC-15, lot#030M4788) and incubated for 1 hour at room temperature. A anti-mouse IgG was used as a secondary (cat#NA931VS, lot#4629354, GE Healthcare) and exposed to substrate using the above method.
Effects of estrogen on ACE2 mRNA abundance in 3T3-L1 adipocytes. 3T3-L1 cells were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum. Cells were grown to confluence, and then differentiation was induced with a cocktail of insulin (0.1 µM, Sigma, St. Louis, MO), dexamethasone (1 µM; Sigma, St. Louis, MO) and isobutyl methyl xanthine (0.5 mM; Sigma, St. Louis). On day 8, cells (duplicates/treatment with an n = 4-5 replicates) were incubated with vehicle (ethanol for estrogen, DMSO for testosterone, and chloroform for progesterone)(0.1% vehicle in culture media) or estrogen (cat#E2257, lot#021M8707V), testosterone propionate (cat#T-1875, lot#115K1065) and progesterone (cat#P6149, lot#051M8720V) (1 - 100 nM) for 24 hours and then harvested for RNA extraction and RT-PCR. All hormones were purchased from Sigma Biochemicals (St. Louis, MO). Data are normalized to 18S RNA and analyzed using the 2^−ΔΔCt method.
### Supplemental Table I. Characteristics of LF and HF-fed male and female mice.

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
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<tbody>
<tr>
<td></td>
<td>LF</td>
<td>HF</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>107.9 ± 1.9</td>
<td>117.6 ± 1.3*</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>586 ± 5</td>
<td>603 ± 16</td>
</tr>
<tr>
<td>Pulse pressure (mmHg)</td>
<td>30.5 ± 2.6</td>
<td>34.8 ± 3.1</td>
</tr>
<tr>
<td>Activity (counts/min)</td>
<td>5.5 ± 0.3</td>
<td>4.8 ± 0.4</td>
</tr>
</tbody>
</table>

Data are mean ± SEM from n = 10-15/group.

*, P<0.05 compared to LF within sex.
Supplemental Table II. Characteristics of HF-fed Ace2<sup>+/+</sup> and Ace2<sup>−/−</sup> mice.

<table>
<thead>
<tr>
<th></th>
<th>Ace2&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Ace2&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td><strong>Female</strong></td>
<td><strong>Male</strong></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>50.5 ± 1.1</td>
<td>39.2 ± 1.3</td>
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<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>117.6 ± 1.3</td>
<td>105.6 ± 2.4</td>
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<tr>
<td>Heart rate (bpm)</td>
<td>603 ± 16</td>
<td>639 ± 7</td>
</tr>
<tr>
<td>Pulse pressure (mmHg)</td>
<td>34.8 ± 3.1</td>
<td>29.1 ± 1.7</td>
</tr>
<tr>
<td>Activity (counts/min)</td>
<td>4.8 ± 0.4</td>
<td>8.1 ± 1.4</td>
</tr>
</tbody>
</table>

Data are mean ± SEM from n = 10-15/group.

*, P<0.05 compared to Ace2<sup>+/+</sup> within sex.
### Supplemental Table III. Characteristics of HF-fed sham and Ovx female mice.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Ovx</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean arterial pressure (mmHg)</strong></td>
<td>105.6 ± 2.4</td>
<td>116.1 ± 3.1*</td>
</tr>
<tr>
<td><strong>Heart rate (bpm)</strong></td>
<td>639 ± 7</td>
<td>621 ± 9</td>
</tr>
<tr>
<td><strong>Pulse pressure (mmHg)</strong></td>
<td>29.1 ± 1.7</td>
<td>31.9 ± 1.7</td>
</tr>
<tr>
<td><strong>Activity (counts/min)</strong></td>
<td>8.1 ± 1.4</td>
<td>5.2 ± 0.4</td>
</tr>
</tbody>
</table>

Ovx, ovariectomy

Data are mean ± SEM from n = 10-15/group.

*, P<0.05 compared to Sham.
Supplemental Figure I. Glucose tolerance is impaired in male and female HF-fed mice compared to LF controls. Top, Area under the curve (AUC) for glucose tolerance tests in male LF and HF-fed mice. Bottom, AUC for glucose tolerance tests for female LF and HF-fed mice. Data are mean ± SEM from n = 10-15/group. *, P<0.05 compared to LF.
Supplemental Figure II. ACE2 protein levels in kidneys from LF-fed males versus females. Data are mean ± SEM from n = 4 mice/group. *P<0.05 compared to females.
Supplemental Figure III

**Supplemental Figure III.** ACE2 protein levels in kidneys from LF and HF-fed male (A) and female (B) mice. Data are mean ± SEM from n = 3-4 mice/group.
**Supplemental Figure IV.** Mas receptor (MasR) protein levels in kidneys from male mice fed a LF or HF diet. Data are mean ± SEM from n = 4 mice/group. *P<0.05 compared to LF.
Supplemental Figure V. Estrogen (E), but not progesterone (P) or testosterone (T), results in a concentration-dependent increase in ACE2 mRNA abundance in 3T3-L1 adipocytes. 3T3-L1 cells were differentiated to adipocytes, and on day 8 incubated with vehicle or different concentrations of sex hormones for 24 hours. Data are mean ± SEM from n = 3 independent experiments. *P<0.05 compared to vehicle.