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Sex-specific modulation of blood pressure and the renin angiotensin system by angiotensin converting enzyme 2

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Abstract

We showed angiotensin-converting-enzyme 2 (ACE2) is higher in the kidney of male (M) compared to female (F) mice. To further investigate this sex difference, we examined the role of ACE2 in angiotensin (Ang)-[1–8]-induced hypertension and regulation of the renin angiotensin system (RAS) in the kidney of wildtype (WT) and Ace2 knockout (KO) mice. Mean arterial pressure rose faster in WT male (WT-M) than WT female (WT-F) mice after Ang-[1–8] infusion. This sex difference was attenuated in ACE2-KO mice. Ang-[1–8] infusion reduced glomerular angiotensin type-1 receptor (AT_1R) binding in WT-F mice by 30% and deletion of Ace2 abolished this effect. In contrast, Ang-[1–8] infusion increased glomerular AT_1R binding in WT-M mice by 1.2-fold and this effect of Ang-[1–8] persisted in Ace2 KO-M mice (1.3-fold). ACE2 also had an effect on renal protein expression of the neutral endopeptidase neprilysin (NEP), the enzyme that catabolizes Ang-[1–10], the precursor of Ang-[1–8]. Ang-[1–8] infusion down-regulated NEP protein expression by 20% in WT-M whereas there was a slight increase in NEP expression in WT-F mice. Deletion of ACE2 magnified the effects of Ang-[1–8] in both sexes resulting in reduced renal expression of NEP. These findings suggest sex specific ACE2 regulation of the RAS contributes to female protection from Ang-[1–8]-induced hypertension. These findings have ramifications for the current COVID-19 pandemic, especially in hypertension since ACE2 is the SARS-CoV-2 receptor and hypertension is a major risk factor for poor outcomes.

Summary

This study shows that ACE2 regulates the RAS in a sex-specific manner. The key finding is that ACE2 contributes to female protection from Ang-[1–8]-dependent hypertension by regulating plasma Ang-[1–8], lowering glomerular AT_1Rs and preventing Ang-[1–8]-induced down-

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Disclosures

None.

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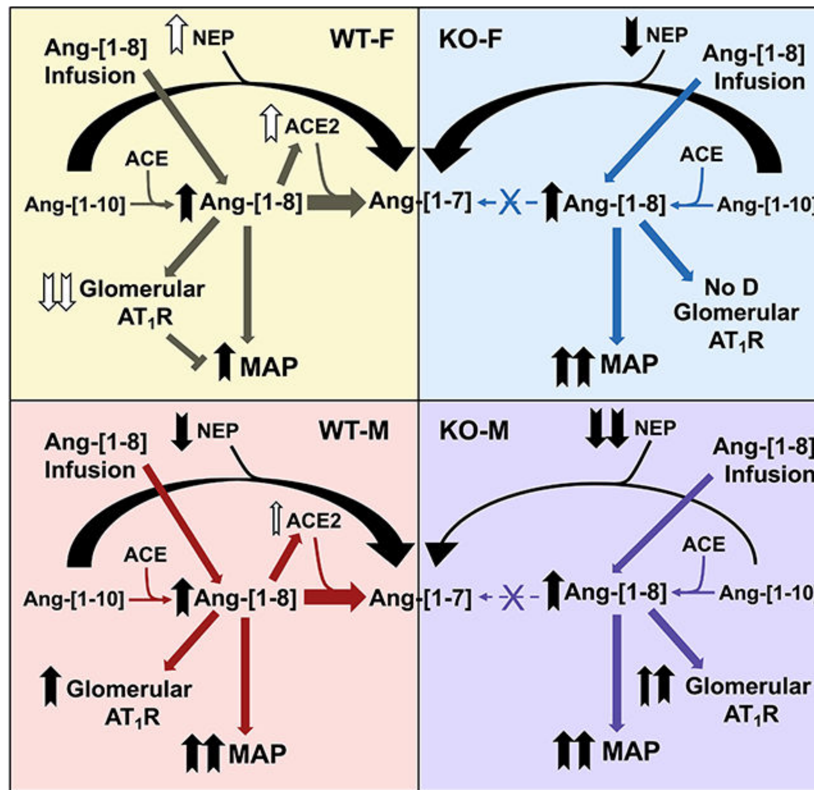
regulation of renal NEP. ACE2 also plays a protective role in the male, though to a lesser extent, by attenuating the Ang-[1–8]-induced down-regulation of renal NEP and up-regulation of glomerular AT_1Rs.

Graphical Abstract



Keywords

angiotensin II; heart rate; gender differences; sexual dimorphism

The renin angiotensin system (RAS) is a complex system that controls blood pressure and water and electrolyte homeostasis. A key component of the RAS is the octapeptide hormone angiotensin (Ang)-[1–8] (also known as Ang II), which primarily regulates blood pressure through the vasoconstrictor actions of the angiotensin type 1 receptor (AT_1R), in the kidney. Ang-[1–8] is synthesized from Ang-[1–10] by angiotensin converting enzyme (ACE) and catabolized by angiotensin converting enzyme 2 (ACE2) to form the vasodilator heptapeptide Ang-[1–7]. Much has been learned about ACE regulation since the discovery of this enzyme in 1956.[1] In contrast, much less is known regarding ACE2, which was only discovered in 2000 through 5' sequencing of a human heart failure ventricle cDNA library.[2] While ACE is widely expressed in many tissues, ACE2 is expressed primarily in the kidney, heart, and testis, with the highest expression occurring in the kidney.[2]

The onset of hypertension occurs earlier in men than women, with similar observations in both genetic and induced animal models of hypertension.[3] Elucidating the mechanisms that

underlie these sex differences could have profound implications for the prevention and treatment of hypertension and its comorbid outcomes. We have shown Ace2 mRNA and ACE2 protein expression and enzyme activity are higher in the kidney of male compared to female MF1 mice under basal conditions.[4] To further investigate the impact of biological sex on ACE2, we investigated the role of ACE2 in Ang-[1–8]-induced hypertension and its effect on RAS components in the kidney using wild type (WT) mice and mice in which the Ace2 gene was knocked out (Ace2 KO).[5]

In 2003, ACE2 was discovered to be the SARS-CoV receptor.[6, 7] Molecular modeling studies indicate the structure of SARS-CoV-2, the virus underlying the current COVID-19 pandemic, has even higher affinity for ACE2 than SARS-CoV.[8] Furthermore, hypertension is a major risk factor for COVID-19 morbidity and mortality.[9] Thus, not only is it worth investigating the impact of sex on ACE2 in hypertension, it is also important to elucidate ACE2 regulation because it is the SARS-CoV-2 receptor and because antihypertensives that target the RAS can modulate ACE2.[10] Moreover, there is accumulating data from many countries including the United States indicating that men have worse outcomes including hospitalization, intensive care admission, progression to pneumonia and fatality from COVID-19 than women.[11]

Methods

The data that support the findings of this study are archived within Georgetown University, Washington, DC in a www.box.com account and are available from the corresponding author upon reasonable request.

Mice

Source—ACE2$^{+/-}$ female mice on the a C57BL6/J background were supplied by Dr. Eric Lazartigues (Louisiana State University, New Orleans, LA) and bred at Georgetown University. ACE2-deficient mice were generated by Dr. Susan Gurley at Duke University (Durham, NC) by replacing the Zn-binding signature motif in the exon of the murine Ace2 gene with a NEO/URA3 cassette.[5] Heterozygous ACE2$^{-/+}$ females on the C57BL6/J background were backcrossed for at least six successive generations on a C57BL6/J genetic background. To confirm specificity, we measured Ace2 mRNA in the kidneys of Ace2 KO mice.[5] Under these conditions, the amount of mRNA obtained was less than 2% in the female compared to the WT mouse.

Diet and Housing—All mice were maintained on a diet composed of 24% protein, 13% fat, and 62% carbohydrates (#5053, LabDiet, St. Louis, MO). All mice were housed 4–5/ cage. All maintenance, breeding and experimental methods were approved by the Georgetown University Animal Care and Use Committee.

Genotyping—Genomic DNA was extracted from mouse tails, as described previously.[12] PCR was used to genotype Ace2 KO mice. The forward primer was 5’- GGG CCA GAG TAT CTG CCC AG-3’. The common reverse primer was 5’-GTG TCC CAT CTC GTG ATG GGC-3’. PCR cycling was as follows: 94°C for 4.3 min then 33 cycles of 94°C for 30 sec, 66°C for 45 sec, 72°C for 30 sec, and 1 elongation cycle at 72°C for 5 min. The Ace2 WT

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and disrupted alleles in the Ace2 KO generated amplicons of 380 bp and 190 bp, respectively.

Mean arterial pressure (MAP) and heart rate (HR)

At 14–18 weeks of age, radio transmitters (#TA11PA-C10, Data Sciences Int. St. Paul, MN) were implanted into WT and Ace2 KO mice, as described previously.[13] Recording of MAP and HR began on days 5–7 after transmitter implantation. Recordings were taken at 30 second intervals every 10 minutes from 6 pm to 6 am and are presented as daily midnight averages for up to two weeks using a Data Acquisition and Analysis System (Data Sciences Int.). Three animals died due to surgical complications from radio transmitter implants: WT-F (1); WT-M (2).

Ang-[1–8] infusion

After recording a stable basal MAP for at least 3 days, Alzet osmotic minipumps (model #1002; 100 μl reservoir vol; 0.25 μl/h; Durect Corporation, Cupertino, CA) filled with Ang-[1–8] (#A9525; Sigma, Saint Louis, MO) dissolved in saline were implanted under isoflurane anesthesia. Since we previously showed no effect on body weight following two weeks of Ang-[1–8] infusion[14], any animals with more than 10% weight loss were removed from the study.

Plasma Ang-[1–7] and Ang-[1–8]

Plasma Ang-[1–7] and Ang-[1–8] were measured in the basal state and two weeks after Ang-[1–8] infusion. In initial studies, we attempted to measure plasma Ang-[1–7] under basal and Ang-[1–8] infusion conditions (350 μl/sample) as we previously described using the RAS Fingerprint™ high pressure liquid chromatography tandem mass spectroscopy (HPLC-MS/MS) assay.[15] However, the levels of this peptide were undetectable (< 2 pg/ml) under our experimental conditions. We were able to detect plasma Ang-[1–8] after Ang-[1–8] infusion using this HPLC-MS/MS method; however, we chose the far less expensive enzyme immunoassay (EIA) assay for this study.

First, a previously reported method[5] was optimized for Ang-[1–8] recovery and stability. In brief, blood (1 ml) was obtained through cardiac puncture under isoflurane anesthesia and collected into ice-cold tubes containing final concentrations of the following: EDTA (25 mM) and the peptidase inhibitors, o-phenanthroline (0.44 mM), p-hydroxymercuribenzoic acid (1 mM) and pepstatin A (0.12 mM). Plasma (0.5 ml) was recovered by centrifugation (3000g x 20 min, 4°C), diluted to 2 ml in phosphate buffer (50 mM sodium phosphate, 1 mM EDTA, 0.25 mM thimerosal, 0.25% BSA) containing peptidase inhibitors at above final concentrations and extracted onto a phenyl solid-phase cartridge (Agilent Technologies, Santa Clara, CA). After washing columns twice with water (1 ml), Ang-[1–8] was eluted with methanol (750 μl). The dried eluant was resuspended in 500 μl Enzyme Immuno Assay (EIA) buffer (Cayman Chemical Co., Ann Arbor, MI) and Ang-[1–8] was measured by EIA in duplicate representing 200 μl of plasma/sample. One sample was lost due to insufficient quantity for assay purposes in the WT-F (1).

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Renal ACE and ACE2 enzyme activity

ACE and ACE2 enzyme activity were measured in the basal state and 3 days after Ang-[1–8] infusion by fluorogenic assay. In brief, renal homogenates were prepared as described[4]. ACE and ACE2 activity were measured using the internally quenched fluorogenic substrate Abz-Phe-Arg-Lys(Dnp)-Pro-OH (GenScript, Piscataway, NJ) in which fluorescence appears when Abz/EDDnp are separated by substrate hydrolysis. Abz is o-aminobenzoic acid and Dnp is 2,4-dinitrophenyl where Abz and Dnp are flanked by natural amino acids. Both ACE and ACE2 enzymes cleave this peptide by splitting off the COOH-terminal Dnp moiety via resonance energy transfer, unleashing the fluorescence inherent in Abz from quenching by Drp. Reactions were carried out in 96 well microtiter plates in a total volume of 100 µl Reaction Buffer (1 M NaCl, 0.5 mM $ZnCl_2$, 75 mM Tris, Ph 7.5) containing 5 µg (ACE) or 10 µg (ACE2) renal protein and 10 µl of a 300 µM stock solution of Abz-Phe-Lys(Dnp)-Pro-OH (Bachem, Torrence, CA). Total peptidase enzyme activity was measured in the presence of vehicle (Reaction buffer). Product formation was determined at 37°C by following the fluorescence as a function of time using a fluorescence plate reader (FLUOstar Omega, BMG LABTECH, Inc., Cary, NC) at an excitation wavelength of 320 nm and an emission wavelength of 410 nm. Initial velocities were determined from the rate of fluorescence increase over the 40–100 min time course corresponding to the linear range. Specific ACE activity was defined as total peptidase activity minus nonspecific ACE activity, which was peptidase activity in the presence of the ACE inhibitor, captopril (10 µM) (Sigma Chemical Co). Specific ACE2 activity was defined as non-ACE activity minus nonspecific ACE2 activity, which was total peptidase activity in the presence of captopril (10 µM) and the ACE2 inhibitor MLN-4760 (10 µM) (Millennium Pharmaceuticals, Cambridge, MA). For ACE2 activity, one sample was lost due to experimental error (KO-M); for ACE activity, two samples were lost due to experimental error in WT-M-Basal group.

Renal NEP protein expression

NEP protein expression was measured in the basal state and 3 days after Ang-[1–8] infusion by Western blot. In brief, protein was extracted from whole mouse kidney by heating (95°C, 10 min) in lysis buffer containing phosphate-buffered saline (pH 7.4), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mmol/l sodium orthovanadate, 1 mmol/l phenylmethylsulphonyl fluoride, 1% Nonidet-P-40, and the protease inhibitors (1 mg/ml each), aprotinin, leupeptin, and pepstatin. The protein concentration was determined using the Bradford method (Bio-Rad Laboratories Inc., Hercules, CA). After electrophoresis on Criterion precasting Tris-HCl gels (Bio-Rad Laboratories) and blotting onto polyvinylidene difluoride membranes, the membranes were incubated with specific goat polyclonal antibodies against mouse NEP (1: 1000; cat. #AF1126: R&D Systems, Minneapolis, MN) and a rabbit anti-goat horseradish peroxidase coupled second antibody (1: 10,000 dilution; cat. #14–13-06: Kirkegaard and Perry Laboratories, Inc, Gaithersburg, MD). Bands were visualized by chemiluminescence (Clarity™ Western ECL Substrate, Bio-Rad Laboratories) and quantified by densitometry using recombinant mouse NEP (#1126-ZNC-010, R&D Systems) as the positive control. Nonspecific binding was blocked with nonfat dry milk (5%). NEP protein was expressed in arbitrary units of immunoreactive protein normalized to β-actin.

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Isolation of glomeruli

Glomeruli were isolated from whole mouse kidneys as we described previously[16] in the basal state and 3 days after Ang-[1–8] infusion. In brief, glomeruli were isolated with the following modifications: mesh sizes for mouse kidneys were 125 μm (mesh size #120; Thermo Fisher Scientific, Waltham, MA) for initial sieving followed by two stacks of sieves with 150 μm (mesh size #100) on the top and 63 μm (sieve size #230) on the bottom. The glomeruli were collected in a glass beaker from the bottom mesh by rinsing the mesh with ice-cold phosphate buffered saline in the presence of protease Inhibitors (Final concentrations: 0.2 μM phenylmethylsulfonyl fluoride; 1 μg/mL leupeptin; 2 μg/mL antipain; 1 unit/L aprotinin). The glomeruli were then centrifuged in 50 mL tubes at 3,000 rpm for 15 min. The supernatant was removed by vacuum aspiration and the pellet was washed with phosphate buffered saline and recentrifuged in a 1.5 mL microcentrifuge tube at 12,000 rpm for 10 min. The final pellet was resuspended in homogenization buffer (50 mM TRIS-HCl, 1 mM EDTA buffer, pH 7.4) supplemented with protease inhibitors (as above). Protein concentrations were determined by the Bradford method using bovine serum albumin as the standard (Bio-Rad Laboratories).

AT_1R and angiotensin type 2 receptor (AT_2R) glomerular binding

AT_1R and AT_2R binding were measured in the basal state and 3 days after Ang-[1–8] infusion by radioligand binding. In brief, ^{125}I-Sar^1,Ile^8-Ang-[1–8] (^{125}I-SI-Ang-[1–8]) receptor binding to glomerular membranes (10 μg protein/tube) was determined at a concentration of 0.4 nM (approximately 400,000 cpm/300 μl). Specific AT_1R binding was defined as total ^{125}I-Sar^1,Ile^8 Ang-[1–8] binding in the presence of 10 μM PD123319 (to block the AT_2R) minus non-specific binding determined in the presence of 10 μM PD123319 and 10 μM losartan (to block both the AT_1R and AT_2R). Specific AT_2R binding was defined as total ^{125}I-Sar^1,Ile^8 Ang-[1–8] binding in the presence of 10 μM losartan (to block the AT_1R) minus non-specific binding determined in the presence of 10 μM losartan and 10 μM PD123319.

Statistics

Data are expressed as means ± SEM. Student's t test was used to determine differences between two groups. A one sample t test was used to determine if there was an effect of Ang-[1–8] over basal conditions. The effect of sex and genotype or sex and Ang-[1–8] treatment were assessed by two-way ANOVA followed by Bonferroni's post hoc test. All statistical tests were conducted using Prism software (version 8, GraphPad Software, San Diego, CA). Significance was defined as $p<0.05$.

Results

Effect of ACE2 on MAP and HR responses to Ang-[1–8] infusion in female and male mice

To determine the role of ACE2 in a widely used model of RAS-dependent hypertension, we measured MAP by telemetry in female (F) and male (M) WT and Ace2 knockout (KO) mice during chronic infusion of Ang-[1–8]. Basal MAP was indistinguishable between WT-F and WT-M mice (Fig. 1A) and between Ace2 KO-F and Ace2 KO-M mice (Fig. 1B).

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Furthermore, there was no effect of knocking out the Ace2 gene on basal MAP in either sex (Fig. 1A–D).

Ang-[1–8] infusion rapidly increased MAP in WT-M mice. After peaking by 3 days, the MAP remained elevated over the following 9 days (Fig. 1A). The time course of the MAP response to Ang-[1–8] infusion was slower in WT-F mice with the peak occurring by day 9. A multiple comparison test showed significant sex differences in MAP on days 2–4 of the infusion. Although two-way ANOVA revealed significant differences in the MAP response between KO-F and KO-M mice, the sex differences on days 2–4 were no longer significant by Bonferroni multiple comparison (Fig. 1B). Ace2 deletion, however, increased the MAP response to Ang-[1–8] infusion in both KO-F (Fig. 1C) and KO-M (Fig. 1D) mice.

Basal HR was indistinguishable between WT-F and WT-M mice (Fig. 2A). Ang-[1–8] infusion evoked an immediate drop in HR in both WT-F and WT-M mice; however, after 3 days, HR returned to basal levels (Fig. 2A). Knocking out the Ace2 gene had little effect on HR responses to Ang-[1–8] in either sex (Fig. 2B–D).

Effect of ACE2 on plasma Ang-[1–7] and Ang-[1–8] in female and male mice

Since ACE2 catabolizes Ang-[1–8] to Ang-[1–7], we measured basal plasma Ang-[1–7] and Ang-[1–8] in WT and Ace2 KO mice under basal conditions and at the end of the Ang-[1–8] infusion period. However, plasma levels of Ang-[1–7] were undetectable under basal or after Ang-[1–8] infusion. Plasma Ang-[1–8] was also below the limits of assay detection in all four groups under basal conditions. In contrast, Ang-[1–8] was measurable in the plasma after Ang-[1–8] infusion [(pg/ml): WT-F (n=8), 37.4 ± 7.8; WT-M, 61.5 ± 12.8 (n=8); *KO-F, 148 ± 33 (n=9); KO-M, 57.5 ± 5.8 (n=8); *p<0.05 vs WT-F, via post hoc analysis]. There was a significant effect of genotype ($p_g < 0.05$) but no effect of sex (p_s=0.11) by two-way ANOVA. However, there was a significant interaction between sex and genotype ($p_{sg} < 0.01$) due to the large increase (4-fold) in plasma Ang-[1–8] observed in KO-F but not KO-M mice compared to their WT counterparts.

Impact of biological sex on renal ACE2 enzyme activity

We previously showed renal Ace2 mRNA, ACE2 protein expression and enzyme activity were significantly higher in the kidneys of male MF-1 mice compared to female MF-1 mice[4]. To determine if this sex difference in renal ACE2 was also observed in the C57BL6/J mouse strain, we measured ACE2 activity in both sexes. Renal ACE2 activity was 1.6-fold higher in the kidney of WT-M compared to WT-F mice (Fig. 3A). As expected, no ACE2 enzyme activity was observed in the Ace2 KO mice (*data not shown*).

We also measured renal ACE2 activity on day 3 after Ang-[1–8] infusion, which was the time point when the difference in MAP was greatest between WT-F and WT-M mice (Fig. 1A). In contrast to the sex difference observed under basal conditions, renal ACE2 activity was not significantly different between WT-F and WT-M mice after Ang-[1–8] infusion (Fig. 3B). Furthermore, while Ang-[1–8] infusion increased renal ACE2 activity in both WT-F (1.8-fold) and WT-M (1.4-fold) mice, there were no significant sex differences in the magnitude increase induced by Ang-[1–8] infusion (Fig. 3C).

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Effect of ACE2 on renal ACE activity in female and male mice

ACE catabolizes Ang-[1–10] to Ang-[1–8]. To determine if ACE2 regulates renal ACE, we measured ACE activity in the kidney of male and female WT and Ace2 KO mice (Fig. 3D). There was a significant interaction between sex and genotype due to WT-F mice having 1.6-fold higher ACE activity in the kidney compared to WT-M mice; however, this sex difference was lost in Ace2 KO mice.

Three days after Ang-[1–8] infusion, renal ACE activity was higher in WT-F (1.2-fold) and Ace2 KO-F (2.1-fold) mice compared to the WT-M and KO-M mice, respectively (Fig. 3E). There was no effect of genotype nor an interaction between sex and genotype. There was, however, an interaction between sex and genotype on the effect size of Ang-[1–8] infusion (Fig. 3F); Ang-[1–8] increased renal ACE activity in both the WT-F (1.4-fold) and KO-F (1.6-fold) mice but not in WT-M or KO-M mice.

Effect of ACE2 on renal NEP protein expression in female and male mice

NEP catabolizes Ang-[1–10] to Ang-[1–7] thereby bypassing ACE and the synthesis of Ang-[1–8]. To determine if ACE2 regulates renal NEP, we measured NEP protein expression in the kidney by Western blot (Fig. 4A). NEP protein expression was higher in the WT-F (1.4-fold) and Ace2 KO-F (1.2-fold) compared to the WT-M and KO-M mice, respectively (Fig. 4B). Ace2 deletion slightly increased NEP protein expression in both sexes. There was no interaction between sex and genotype.

Three days after Ang-[1–8] infusion, NEP protein was higher in the WT-F (1.9-fold) and KO-F (1.6-fold) kidney compared to WT-M and KO-M mice, respectively (Fig. 4C). There was a trend towards a significant interaction between sex and genotype due to the larger sex difference observed in the WT compared to the KO mice.

Ang-[1–8] infusion down-regulated NEP protein by 20% in WT-M mice whereas there was a slight increase in NEP expression in WT-F mice (Fig. 4D). Deletion of Ace2 magnified the effects of Ang-[1–8] in both sexes resulting in lowered NEP expression in both KO-F and KO-M mice.

Effect of ACE2 on glomerular AT_1R and AT_2R binding in female and male mice

AT_1Rs in glomeruli play a key role in blood pressure regulation by binding to Ang-[1–8] and inducing vasoconstriction. Thus, to determine if ACE2 regulates glomerular AT_1Rs by a feedback mechanism, we measured the binding of ^{125}I-SI-Ang-[1–8] to AT_1Rs in glomeruli isolated from WT-F and WT-M and their corresponding Ace2 KO mice. ^{125}I-SI-Ang-[1–8] binding to glomerular AT_1Rs was similar in WT-F and WT-M mice (Fig. 5A). There was a significant interaction between sex and genotype due to the large sex difference observed in the KO but not the WT mice. Deletion of Ace2 reduced AT_1R binding in the KO-F by 55% compared to the KO-M kidney. In contrast, there was no effect of Ace2 deletion on AT_1R binding in the male kidney.

Three days after Ang-[1–8] infusion, glomerular AT_1R binding was 39% lower in the WT-F compared to the WT-M mice (Fig. 5B). There was a small interaction between sex and genotype due to a larger sex difference in the Ace2 KO mice.

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Ang-[1–8] infusion lowered glomerular AT_1R binding by 30% in the WT-F (Fig. 5C). This effect was attenuated in Ace2 KO-F mice. In contrast, Ang-[1–8] infusion increased glomerular AT_1R binding by 1.2-fold in male mice regardless of genotype.

Angiotensin type 2 receptors in the kidney play a protective role in blood pressure regulation by binding to Ang-[1–8] and inducing vasodilation[17]. Thus, to determine if ACE2 regulates renal AT_2Rs by a feedback mechanism, we measured the binding of ^{125}I-SI-Ang-[1–8] to AT_1Rs in WT-F and WT-M renal homogenates and in their corresponding Ace2 KO mice. However, AT_2R binding under these conditions was below the detection limits of the radioligand binding assay.

Discussion

The major finding from this study was that biological sex has a major impact on ACE2 modulation of Ang-[1–8] pressor action. The WT-F mice exhibited a slow pressor response to Ang-[1–8] infusion compared to the WT-M mice. This sex difference in the time course of the pressor response was attenuated in Ace2 KO mice (Fig. 1). These findings indicate that ACE2 contributes to sex differences in Ang-[1–8]-dependent hypertension. Furthermore, comparison of MAP responses to Ang-[1–8] within each sex suggests ACE2 plays a greater protective role in Ang-[1–8]-dependent hypertension in the female than the male mouse. The magnitude difference we observed in the Ang-[1–8] pressor response between the WT-M and KO-M is smaller than previously reported by Gurley et al.[5] We suspect genetic drift has impacted genetic modifiers of blood pressure in the ACE2 KO mouse line over the 14+ years since the ACE2 KO mice were first created in 2006. Genetic drift is unavoidable in mouse strains and we have previously shown pressor responses to Ang-[1–8] in a mouse KO line drifted over years even when the mice were housed and maintained in the same environment.[18] Kawada et al.[19] showed that a slow pressor response to Ang-[1–8] is likely due to a late rise in renal vascular resistance with a fall in glomerular filtration rate after the initial increase in post-glomerular vascular resistance occurs. It will be interesting in future studies to determine if similar mechanisms underlie the slower pressor response to Ang-[1–8] in WT-F mice.

Female WT mice had lower plasma levels of Ang-[1–8] than the WT-M at the end of the Ang-[1–8] infusion period. These findings are similar to our findings in the MF-1 mouse[14] indicating this sex difference is a not specific to one mouse strain. Knocking out Ace2 led to a large magnitude increase in plasma Ang-[1–8] in KO-F compared to KO-M mice. These findings suggest ACE2 plays a greater role in regulating plasma levels of Ang-[1–8] in the female mouse in contrast to the male mouse. Further support for this contention is the trend we observed towards sex differences in the effect of Ang-[1–8] infusion on renal ACE2 activity; the WT-F (1.8-fold) mouse increased renal ACE2 to a larger extent than the WT-M (1.4-fold) after Ang-[1–8] infusion (Fig. 3A). Assessing the impact of ACE2 on renal Ang peptides is clearly warranted in future studies since mechanisms regulating intrarenal Ang-[1–8] are distinct from those that regulate circulating Ang-[1–8].[20]

In addition to catabolizing Ang-[1–8], ACE2 could potentially lower plasma Ang-[1–8] by negative feedback on the Ang-[1–8] synthetic pathway; however, knocking out ACE2 had

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little effect on renal ACE activity in either sex under basal conditions (Fig. 3D) or after three days of Ang-[1–8] infusion (Fig. 3E). Still it will be worth investigating if ACE2 modulates ACE in other key target tissues like the heart and brain. Catabolism of Ang-[1–8] by ACE2 generates the vasodilatory heptapeptide, Ang-[1–7]. Thus, increased levels of this vasodilator could also contribute to female protection from Ang-[1–8]-induced hypertension. Although Ang-[1–7] was below the detection limits of our assay under our experimental conditions, it will be important to further study the role of this vasodilator using more sensitive methods of detection[21] such as sample concentration before HPLC-RIA[22] or a hybrid approach involving pre-absorption to an Ang-[1–8] antibody followed by HPLC-MS/MS.[23]

NEP catabolizes Ang-[1–10] to form Ang-[1–7] thereby bypassing the synthesis of Ang-[1–8].[24] Our findings that renal NEP protein expression was higher in WT-F compared to WT-M mice while Ace2 KO-F and KO-M mice had lower levels of renal NEP protein than their WT counterparts support previous studies demonstrating renal NEP activity was greater in the female compared to the male congenic mRen(2).Lewis rat, a genetic model of hypertension due to increased renin activity.[25] These authors also concluded, as we do, that NEP contributes to greater female protection from Ang-[1–8]-dependent hypertension (Fig. 4). This suggests a positive feedback mechanism exists in which ACE2 up-regulates renal NEP protein expression. The finding that Ang-[1–8] infusion down-regulated NEP in the WT-M but not the WT-F indicates ACE2 modulation of NEP could contribute to female protection from Ang-[1–8]-dependent hypertension through maintaining renal NEP conversion of Ang-[1–10] to Ang-[1–7] and thereby contributing to lower plasma levels of Ang-[1–8]. Further support for this concept is a study showing sex-specific regulation of renal NEP activity in diabetes. While female mRen2.Lewis rats had higher levels of renal NEP activity than male rats, in the presence of diabetes, renal NEP activity decreased to levels observed in the male diabetic and control kidneys.[26]

The RAS plays a central role in most forms of hypertension. Therefore, it is not surprising that the regulation of the RAS is impacted by biological sex[27] given the fact that sex differences in blood pressure are a robust finding across species and experimental models of hypertension including both induced and genetic models.[28] In this regard, male mice were shown to have higher systolic blood pressure on a high fat diet compared to female mice and these differences in systolic blood pressure were attenuated in Ace2 KO mice.[29] It would be interesting to further study the effects of ACE2 KO on the components of the RAS in this model of obesity-associated hypertension. It will also be important to continue to interrogate the role of ACE2 in other models of hypertension, particularly since Ang-[1–8] infusion might overwhelm the normal role of renin and angiotensinogen in regulating blood pressure.

Accumulating studies suggest that the vasoconstrictor arm of the RAS is more active in males than females, suggesting a male-sex bias exists towards the vasoconstrictor arm.[30] We found glomerular AT_1R binding was higher in male compared to female WT mice under basal conditions. This finding in mice supports our previous work in rats showing AT_1R binding is higher in the male kidney compared to the female.[31] Ang-[1–8] infusion down-regulated the glomerular AT_1R receptor in WT-F but not in WT-M mice (Fig. 5). This ability to down-regulate the glomerular AT_1R was not observed in female Ace2 KO mice

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suggesting that ACE2 plays a protective modulatory role in female renal responses to Ang-[1–8] through a negative feedback mechanism that results in AT_1R down-regulation.

AT_1Rs are known to be regulated by Ang-[1–8] in a tissue-specific manner. Increased plasma Ang-[1–8], as a result of potassium deficiency, led to an increase in AT_1R numbers in uterine and vascular smooth muscle.[32] In contrast, no change in AT_1R binding was observed in the combined diencephalon and midbrain in rats infused intracerebroventricularly with Ang-[1–8].[33] The renal AT_1R is also known to be differentially regulated by changes in plasma Ang-[1–8] levels[34]. Thus, dose-response studies of Ang-[1–8] modulation of AT_1Rs as a function of ACE2 in both sexes and also as a function of target tissue is warranted.

We previously showed ACE2 contributes to the protective effects of estradiol on renal injury in a model of renal wrap hypertension in the female rat.[35] However, we did not study the impact of biological sex on indices of renal injury in these Ace2 KO mice because Ang-[1–8] infusion in the C57BL6/J mouse is not associated with significant renal pathology. In fact, this strain is known to be resilient to renal injury, in general.[36] In contrast, the 129/SvJ strain is more susceptible to glomerulosclerosis induced by remnant kidney and podocyte injury models (e.g., adriamycin). Therefore, investigating the impact of biological sex on ACE2 modulation of renal pathology in the more vulnerable 129/SvJ mouse strain will be of interest.

ACE2 is also present in the brain and plays a central role in cardiovascular function.[37–39] For example, specific pharmacological inhibition of ACE2 in the nucleus tractus solitarius attenuated baroreflex sensitivity for reflex bradycardia.[38] Thus, another question to explore is how biological sex impacts ACE2 contributions to baroreflex control of heart rate in response to increases in arterial pressure.

In summary, our study suggests ACE2 plays a greater role in protecting the female from Ang-[1–8]-induced hypertension than in the male mouse. ACE2 protects the WT-F mouse from Ang-[1–8]-induced hypertension by increasing catabolism of Ang-[1–8], preventing Ang-[1–8]-induced down-regulation of NEP and reducing glomerular AT_1Rs in response to Ang-[1–8] infusion (Fig. 6). These protective effects are lost in the Ace2 KO-F. ACE2 also exerts protective effects in the male mouse, though to a lesser extent than in the female, by attenuating the Ang-[1–8]-induced downregulation of renal NEP and Ang-[1–8]-induced up-regulation of glomerular AT_1Rs. These effects are lost in the Ace2 KO-M.

In conclusion, these studies support the contention that sex differences in the balance of the vasoconstrictor and vasodilatory arms of the RAS is a likely contributor to why the onset of hypertension occurs earlier in men than women. These observations also emphasize the importance of taking into account the sex of the animal in studies of ACE2 modulation of blood pressure. Lastly, these findings in mice may have relevance for observed sex differences in the current COVID-19 pandemic. Studies throughout the world including the United States show male sex is a risk factor for poor outcomes in COVID-19 disease including hospitalizations, admission to intensive care units, progression to pneumonia and fatality.[11] Sex-specific regulation of ACE2 may contribute to these worse outcomes in men

for several reasons. First, if men have higher basal levels of ACE2, then they may have higher viral load compared to women. Second, if ACE2 in women gives them greater ability to down-regulate renal AT_1Rs (and perhaps in other target tissues as well) in the face of Ang-[1–8]-dependent hypertension, this mechanism may act much like AT_1R blockers[40, 41] and protect women from AT_1R-mediated inflammation and other AT_1R-associated COVID-19 co-morbidities.

Perspectives

Our finding that ACE2 plays a greater protective role in Ang-[1–8]-dependent hypertension in the female compared to the male mouse provides another example of biological sex differences that could impact drug action. Although enhancement of ACE2 should have benefit for both sexes, it may be a more effective antihypertensive strategy in females than males given that ACE2 plays a greater role in regulating blood pressure and RAS components in the female mouse. Our findings that ACE2 plays a minor role in protecting the male from Ang-[1–8] -dependent hypertension compared to the female emphasizes the fallacy in assuming mechanisms identified in basic science experiments conducted in males are readily applicable to females. This study also illustrates the need to investigate pathophysiological mechanisms in the female to avoid missing out on effective drug targets for women[42]. Given the growing awareness for sex differences in drug disposition[143], one can imagine a future where drug approval and/or drug recommendations are also based on sex differences in pharmacokinetics and pharmacodynamics. In this regard, women are at increased risk for drug-induced cardiac arrhythmias compared to men due to longer QT intervals[44]. Given that hydroxychloroquine use is associated with prolonged QT intervals in electrocardiograms[45] and is being studied as a potential therapeutic treatment for COVID-19, it is not only important to disaggregate COVID-19 incidence and disease progression by sex[11], it is also critical to disaggregate the response to treatment by sex.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Novelty and Significance

What is New?

- This study is the first to show ACE2 plays a greater protective role in Ang-[1–8]-induced hypertension in the female compared to the male mouse.
- ACE2 plays a greater role in regulating plasma Ang-[1–8] levels in the female compared to male mice after Ang-[1–8] infusion.
- ACE2 prevents Ang-[1–8]-induced down-regulation of renal NEP in the female but has little effect in the male mouse.
- ACE2-mediates Ang-[1–8]-induced down-regulation of glomerular AT_1Rs in the female but has little effect in the male mouse.

What is relevant?

- Sex-specific ACE2 modulation of the RAS may contribute to the delayed onset of hypertension in women compared to men.
- This study illustrates how mechanistic insights from studies in females can not necessarily be extrapolated to males and vice versa.
- This study emphasizes the importance of considering the biological variable of sex in studies investigating the role of ACE2 in COVID-19 pathogenesis and response to treatment, especially in individuals with hypertension and other diseases that involve dysregulation of the RAS.

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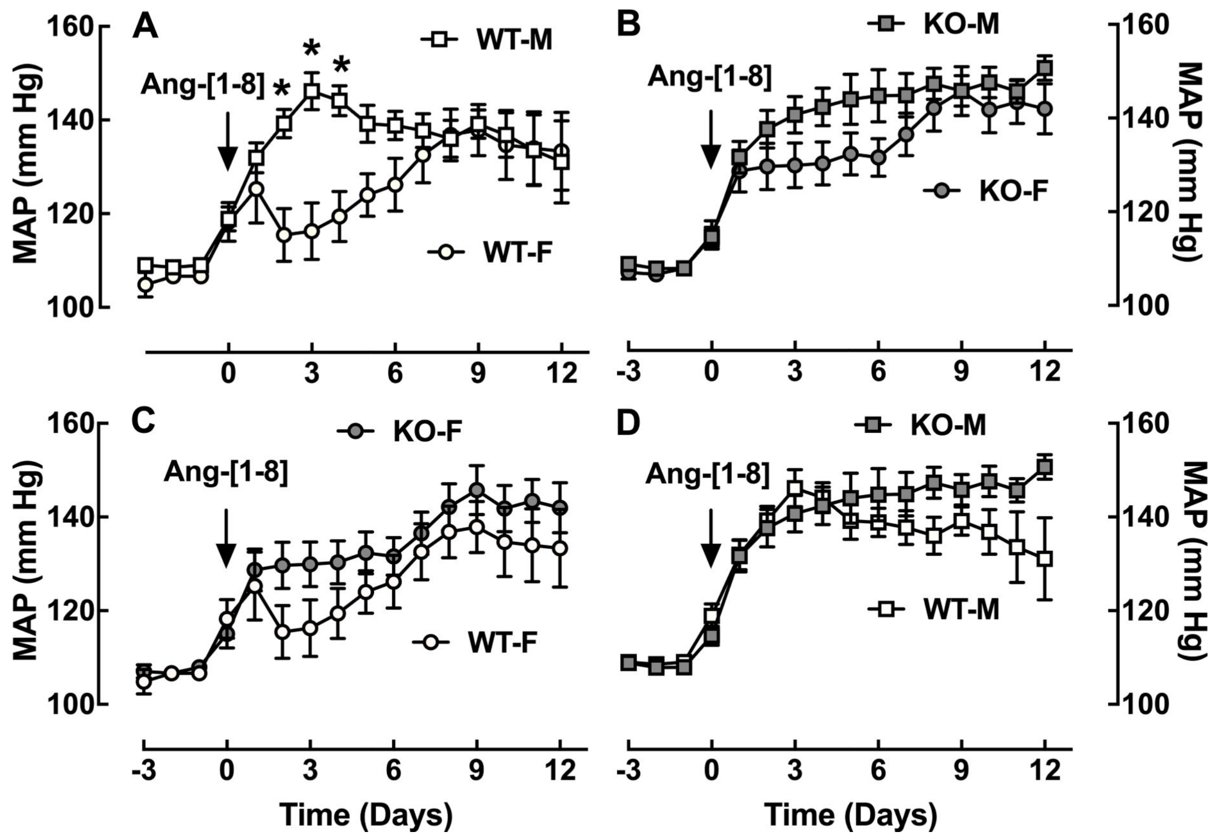

Figure 1.

Impact of biological sex on the modulation of MAP by ACE2. A) MAP as a function of time before and after Ang-[1–8] infusion (1000 ng/kg/min) in wildtype (WT) and Ace2 null mice (KO) female (F) and male (M) mice. A) WT-F vs WT-M. B) KO-F vs KO-M. C) WT-F vs KO-F. D) WT-M vs KO-M. Data were analyzed using 2-way ANOVA with the factors sex (s) and its probability of effect (ps; <0.001), genotype (g) and its probability of effect (pg; <0.001) and the probability of an interaction between sex and genotype (psg; <0.001). *p<0.05 vs female, same genotype via post hoc analysis; WT-F (n=11); WT-M (n=10); KO-F (n=12); KO-M (n=12). MAP, mean arterial pressure.

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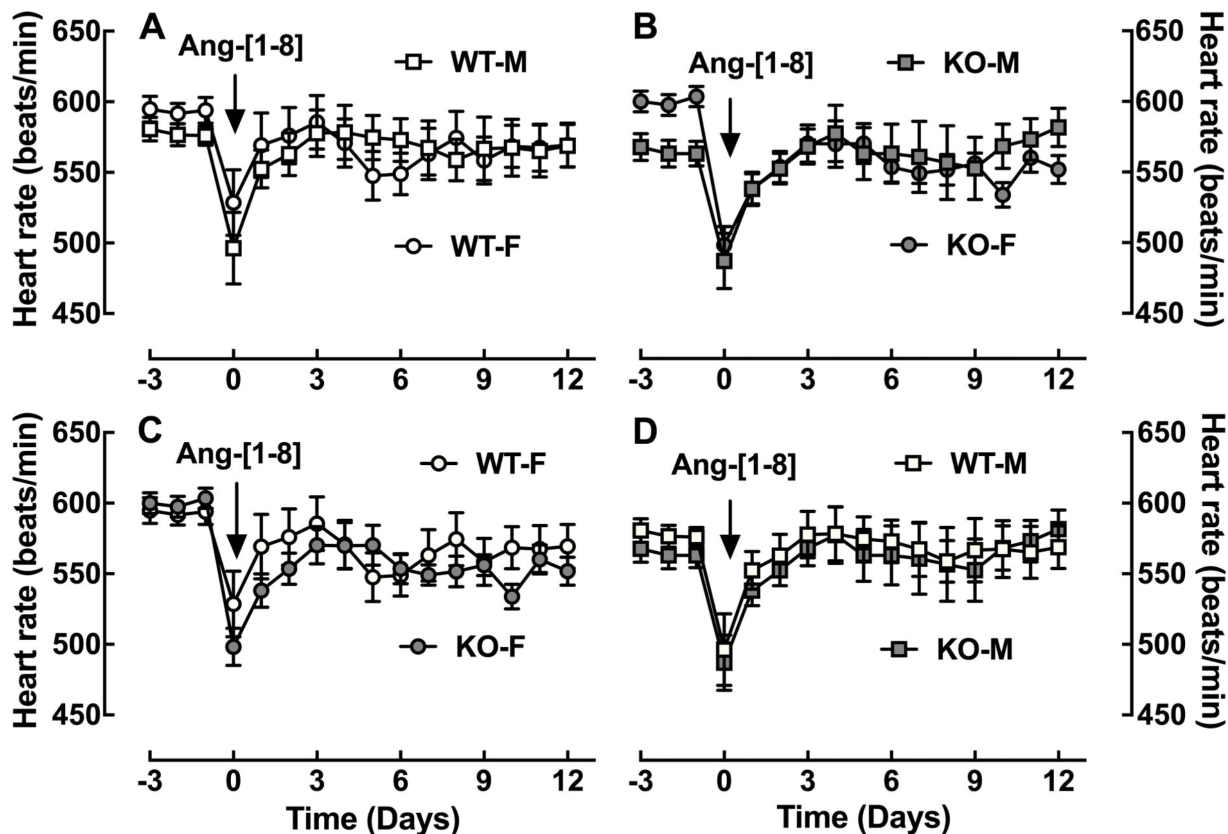

Figure 2.

Impact of biological sex on the modulation of HR by ACE2. A) HR as a function of time before and after Ang-[1–8] infusion (1000 ng/kg/min) in wildtype (WT) and Ace2 null mice (KO) female (F) and male (M) mice. A) WT-F vs WT-M. B) KO-F vs KO-M. C) WT-F vs KO-F. D) WT-M vs KO-M. Data were analyzed using 2-way ANOVA with the factors sex (s) and its probability of effect (ps; <0.05), genotype (g) and its probability of effect (pg; <0.05) and the probability of an interaction between sex and genotype (psg; <0.05). WT-F (n=11); WT-M (n=10); KO-F (n=12); KO-M (n=12).

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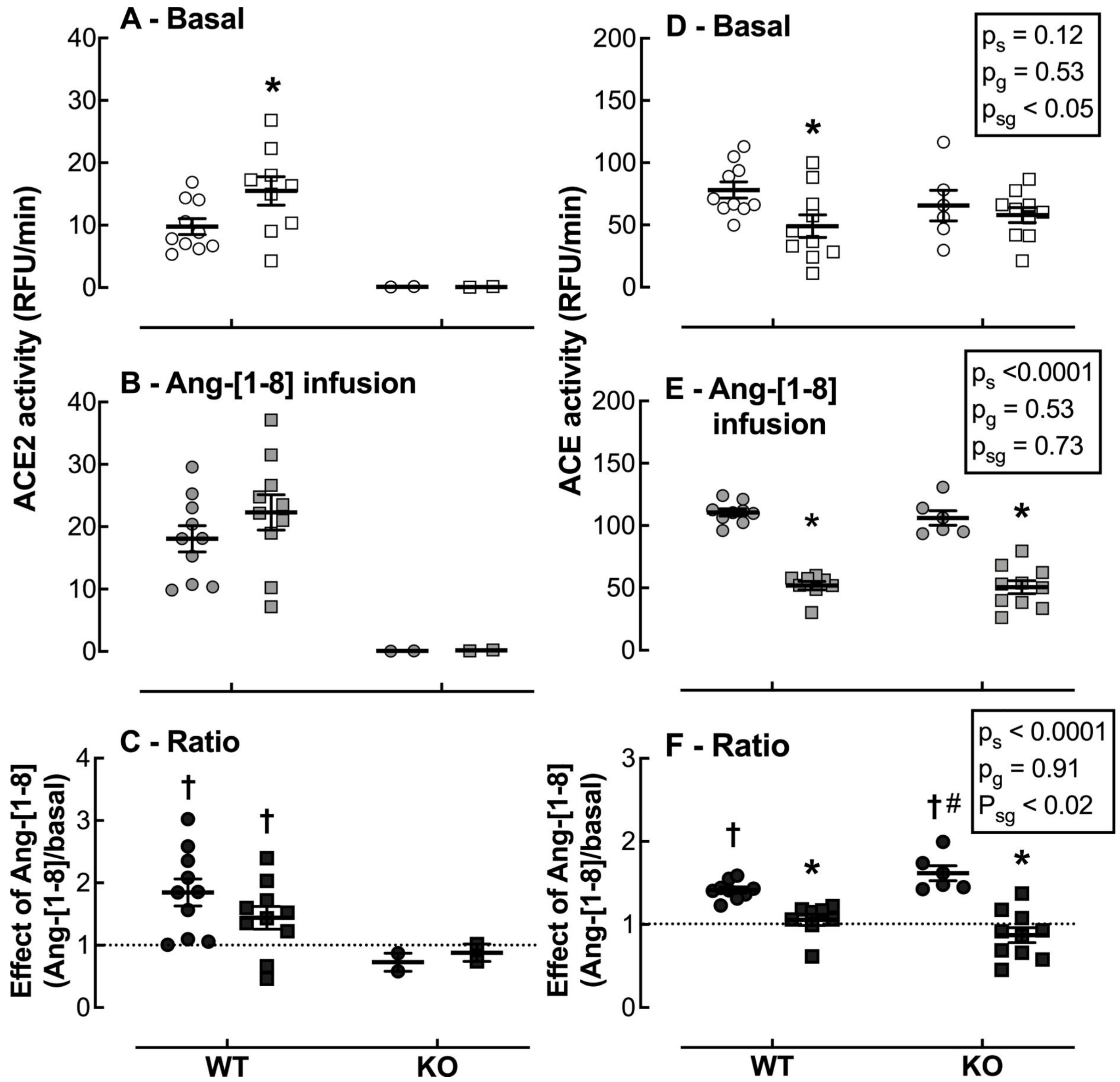

Figure 3.

Impact of biological sex on ACE2 and ACE activity. (A-C) ACE2 and (D-F) ACE enzyme activity in female (F, circle) and male (M, square) wildtype (WT) mice and Ace2 knockout (KO) mice under (A,D) basal conditions (white) or (B,E) 3 days after Ang-[1–8] infusion (grey). (C,F) Ang-[1–8]-induced change in (C) ACE2 and (F) ACE activity (Ang-[1–8]/Basal) (black). The dashed line in panels C & F represents no change from basal. The data are presented as a scatter plot showing individual data points (n) with the mean and SEM indicated by horizontal and vertical bars, respectively. Data were analyzed using 2-way ANOVA with the factors sex (s) and its probability of effect (p_s) and treatment (t) and its probability of effect (p_t) or genotype (g) and its probability of effect (p_g) and the probability of an interaction between sex and treatment (p_{st}) or between sex and genotype (p_{sg}). *p<0.05 vs female, same genotype via post hoc analysis. †p<0.05 vs 1.0 (no effect of Ang-[1–8]) by

one sample t test. ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2.

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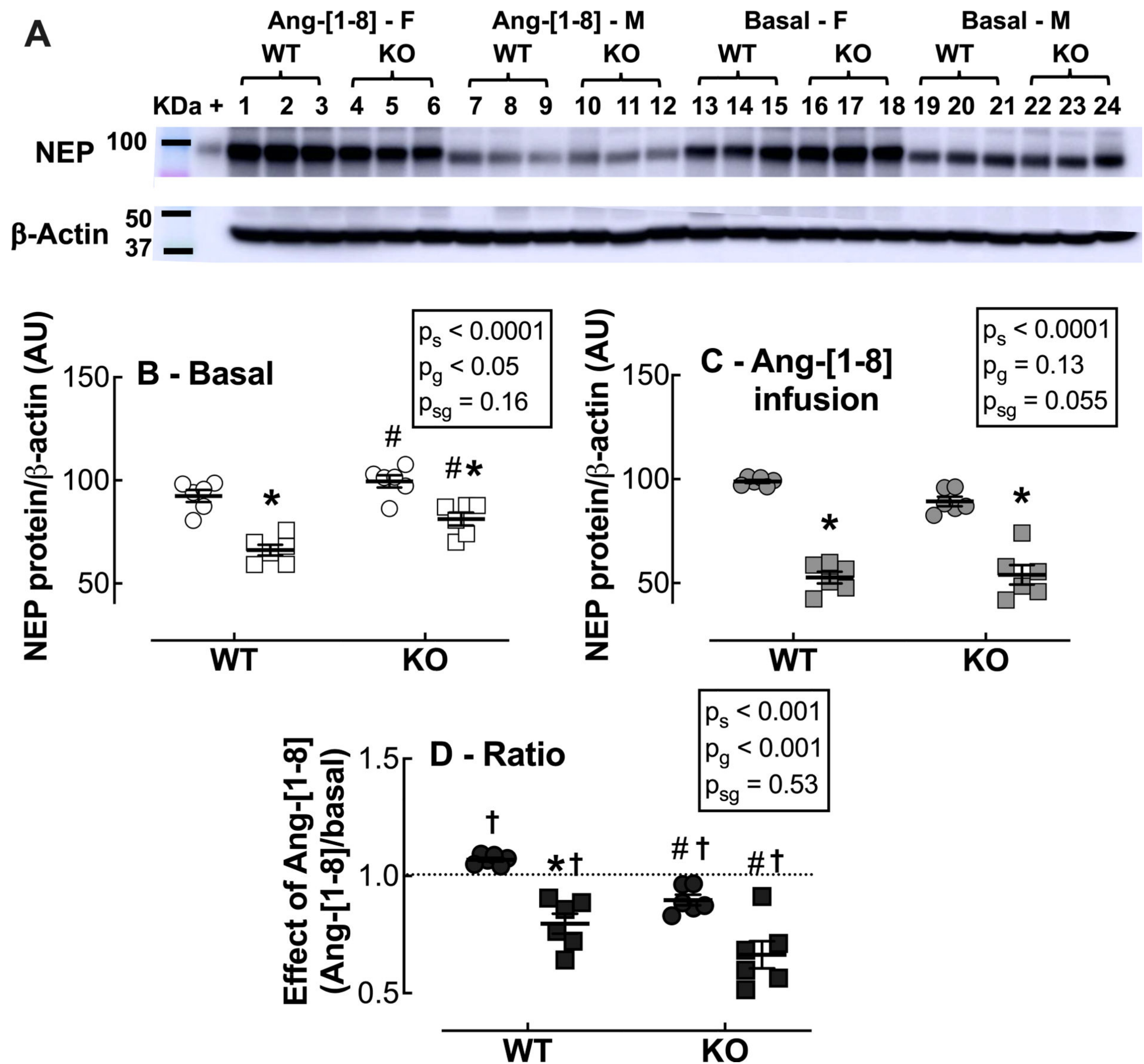

Figure 4.

Impact of biological sex on ACE2 modulation of renal NEP protein expression. A) Representative Western blot of NEP protein expression in female (F) and male (M) wildtype (WT) and Ace2 knockout (KO) mice under basal conditions and after Ang-[1–8] infusion. B) NEP protein in female (F, circle) and male (M, square) WT and KO mice under C) basal conditions (white) or after B) Ang-[1–8] infusion (grey). D) Ang-[1–8]-induced change in NEP protein expression (Ang-[1–8]/Basal) (black). The dashed line in panel C represents no change from basal. The data are presented as a scatter plot showing individual data points (n) with the mean and SEM indicated by horizontal and vertical bars, respectively. Data were analyzed using 2-way ANOVA with the factors sex (s) and its probability of effect (p_s), genotype (g) and its probability of effect (p_g) and the probability of an interaction between sex and genotype (p_{sg}). *p<0.05 vs female, same genotype and $^{\#}$p<0.05 vs WT, same sex via post hoc analysis. †p<0.05 vs 1.0 (no effect of Ang-[1–8]) by one sample t test. NEP, neprilysin.

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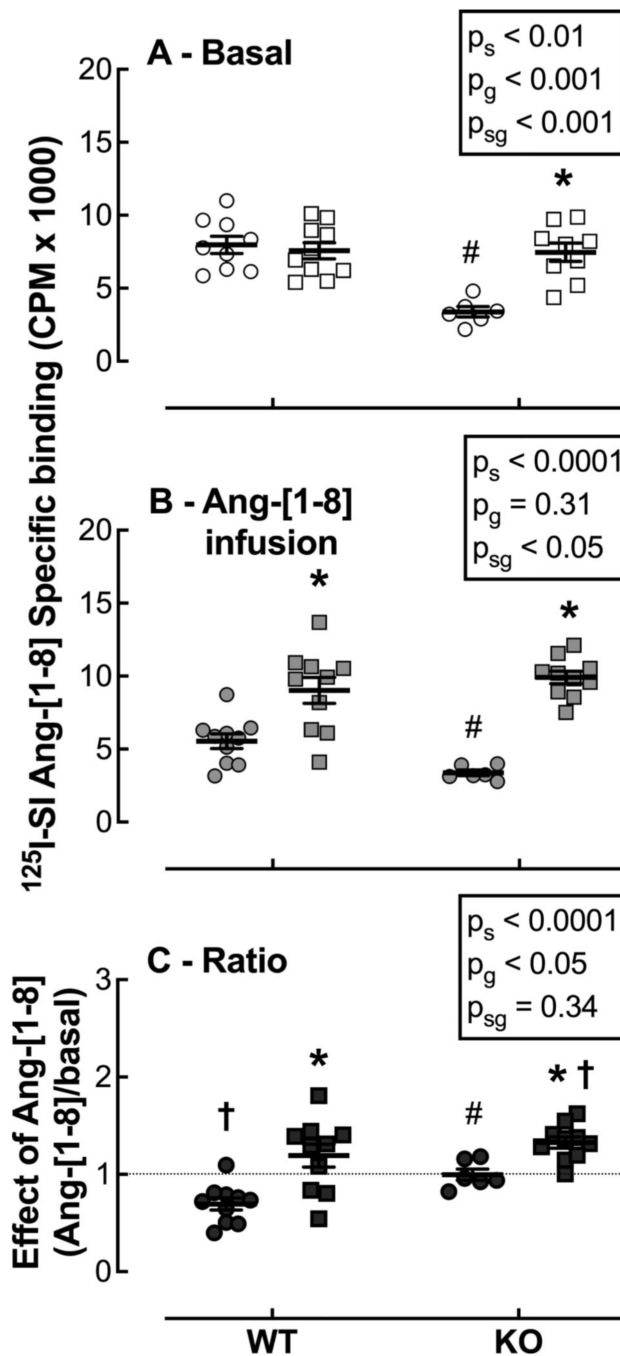

Figure 5.

Impact of biological sex on ACE2 modulation of AT_1R binding. A) AT_1R binding in female (F, circle) and male (M, square) wildtype (WT) and Ace2 knockout (KO) mice under A) basal conditions (white) or after B) Ang-[1–8] infusion (grey). C) Ang-[1–8]-induced change in AT_1R binding (Ang-[1–8]/Basal) (black). The dashed line in panel C represents no change from basal. The data are presented as a scatter plot showing individual data points (n) with the mean and SEM indicated by horizontal and vertical bars, respectively. Data were analyzed using 2-way ANOVA with the factors sex (s) and its probability of effect (p_s),

genotype (g) and its probability of effect (p_g) and the probability of an interaction between sex and genotype (p_{sg}). *p<0.05 vs female, same genotype and #p<0.05 vs WT, same sex via post hoc analysis. †p<0.05 vs 1.0 (no effect of Ang-[1–8]) by one sample t test. AT_1R, angiotensin type 1 receptor.

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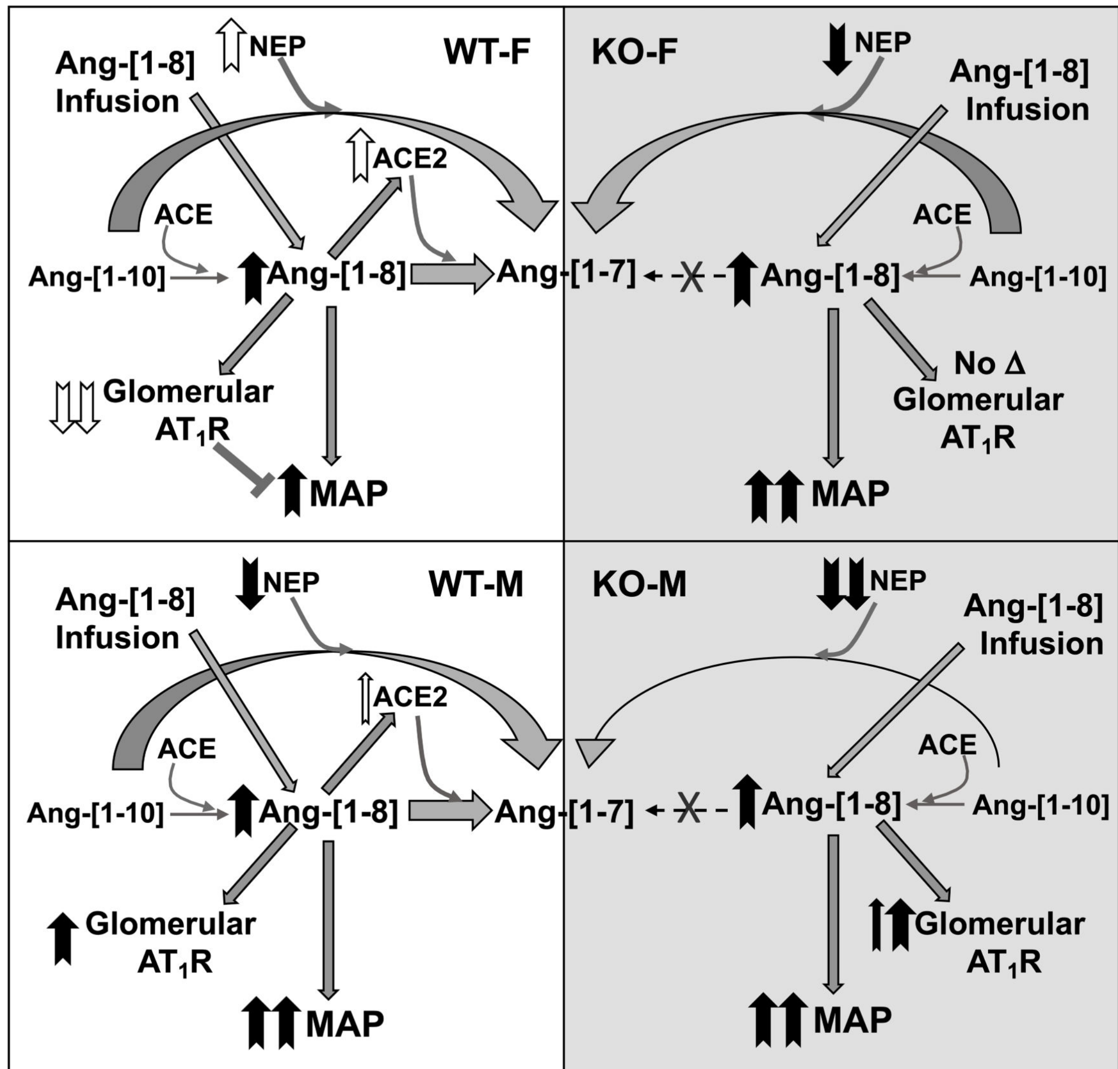

Figure 6.

Role of ACE2 in Ang-[1–8]-induced hypertension in female and male mice. ACE2 protects the wildtype female (WT-F) mouse from Ang-[1–8]-induced hypertension by increasing catabolism of Ang-[1–8], preventing Ang-[1–8]-induced down-regulation of NEP and reducing glomerular AT_1Rs in response to Ang-[1–8] infusion. These protective effects are lost in the Ace2 knockout (KO)-F mouse. ACE2 also exerts protective effects in the male mouse, though to a lesser extent than in the female, by attenuating the Ang-[1–8]-induced downregulation of renal NEP and Ang-[1–8]-induced up-regulation of glomerular AT_1Rs. These effects are lost in the Ace2 KO-M mice. ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2; AT_1R, angiotensin type 1 receptor, NEP, neprilysin.