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Sex- and age-specific regulation of ACE2: Insights into severe COVID-19 susceptibility

Anissa Viveiros [a,b], Mahmoud Gheblawi [a], Preetinder K. Aujla [a], Deanna K. Sosnowski [c], John M. Seubert [c,d], Zamaneh Kassiri [a], Gavin Y. Oudit [b,e,*]

[a] Department of Physiology, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Alberta, Canada
[b] Mazankowski Alberta Heart Institute, University of Alberta, Edmonton, Alberta, Canada
[c] Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada
[d] Department of Pharmacology, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Alberta, Canada
[e] Division of Cardiology, Department of Medicine, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Alberta, Canada

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ABSTRACT

Aged males disproportionately succumb to increased COVID-19 severity, hospitalization, and mortality compared to females. Angiotensin-converting enzyme 2 (ACE2) and transmembrane protease, serine 2 (TMPRSS2) facilitate SARS-CoV-2 viral entry and may have sexually dimorphic regulation. As viral load dictates disease severity, we investigated the expression, protein levels, and activity of ACE2 and TMPRSS2. Our data reveal that aged males have elevated ACE2 in both mice and humans across organs. We report the first comparative study comprehensively investigating the impact of sex and age in murine and human levels of ACE2 and TMPRSS2, to begin to elucidate the sex bias in COVID-19 severity.

Nonstandard abbreviations

ACE2	Angiotensin-converting enzyme
ADAM17	A disintegrin and metalloprotease 17
CVD	cardiovascular disease
E2	17β-estradiol
miRNA	Micro RNA
RAS	Renin-angiotensin system
SI	Small intestine
TMPRSS2	Transmembrane protease, serine 2

1. Introduction

The COVID-19 pandemic predominately finds aged males experiencing worsened disease severity and adverse outcomes [1]. Current experimental data are limited in explaining this sex bias. The SARS-CoV-2 virus predominantly hijacks two broadly-expressed endogenous proteins to facilitate infection, namely angiotensin-converting enzyme 2 (ACE2) and transmembrane protease, serine 2 (TMPRSS2) [2,3]. A meta-analysis of thirty-six clinical studies found that a higher viral load at symptom onset is associated with disease severity, ICU hospitalization, and mortality [4]. Therefore, it is likely that increased viral load confers susceptibility to severe COVID-19; thus, the sex discrepancy may result from differences in levels of viral entry factors.

ACE2 demonstrates sexually dimorphic expression dependent on sex chromosomes and sex hormones [1]. Interestingly, *Ace2* is an X-linked gene that escapes X-chromosome inactivation, which may confer a "double-dosage" of ACE2 mRNA [1,2]. However, the female hormone 17β-estradiol (E2) reduces ACE2 expression in vitro, but does not affect TMPRSS2 [5]. In the kidney, basal ACE2 activity is reduced in females compared to males in an E2-dependent manner [6,7]. However, E2 upregulates ACE2 in a model of intrauterine growth restriction, thereby complicating the relationship between E2 and ACE2 in disease [8]. Further, ACE2 is regulated downstream by micro RNAs (miRNAs) and proteolytic cleavage [1]. Finally, the TMPRSS2 gene is positively regulated by androgens in the prostate, thus may demonstrate male-biased expression elsewhere [9]. In all, the regulation of ACE2 and TMPRSS2 are complex and organ-specific, and the role of sex and age have yet to be elucidated.

Our recent review highlights the global pattern of sex- and age-bias

* Corresponding author at: Division of Cardiology, Department of Medicine, Mazankowski Alberta Heart Institute, University of Alberta, Edmonton, Alberta T6G 2S2, Canada.
E-mail address: gavin.oudit@ualberta.ca (G.Y. Oudit).

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in COVID-19 severity to favor older males [1]. Here, we aimed to delineate the impact of sex and aging on the expression, protein levels, and activity of viral entry factors ACE2 and TMPRSS2 to begin to explain this discrepancy.

2. Materials and methods

2.1. Animal studies

Animals were maintained at the University of Alberta, and animal protocols were performed in compliance with the guidelines of the Animal Care and Use Committee and the Canadian Council of Animal Care. Kidney, lung, heart, and small intestine (SI) were harvested from male and female wild-type (WT) mice (background C57BL6/J) at three months (young), twelve months (adult), and eighteen months of age (aged) under ketamine-xylazine anesthesia.

2.2. Human explanted hearts

Explanted human hearts were obtained following cardioplegic arrest according to the Human Organ Procurement and Exchange (HOPE) program protocol and approved by the Health Research Ethics Board of the University of Alberta. Transmural left ventricle specimens were procured from young (median age 23/23; M/F) and aged (median age 55/56; M/F) human donors with no history of cardiovascular disease (Supplementary Table S1).

2.3. Gene expression

RNA isolation from mouse and human tissue was performed by Trizol-chloroform extraction. Isolated RNA (1 μg) was reverse transcribed with random primers (Invitrogen), and cDNA synthesized using SuperScript® II Reverse Transcriptase (Invitrogen). Real-time quantitative PCR was performed with TaqMan premixed assays (ThermoFisher Scientific) for gene expression in mice for TMPRSS2 (Mm00443687_m1) and ACE2 (Mm01159009_m1), and in humans for ACE2 (Hs00222343_m1).

2.4. Immunoblot

Cross-sectional tissue sections from each organ were homogenized in CelLytic™ Lysis reagent (ThermoFisher) and normalized by bicinchoninic acid protein assay (BCA). Proteins were resolved by SDS-PAGE on a 12% gel and transferred to polyvinylidene fluoride membranes in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol (pH 8.3)). Membranes were blocked in 5% non-fat milk and probed for ACE2 (Abcam) (1:1000) and TMPRSS2 (Abcam) (1:1000) in 5% bovine serum albumin (BSA). HRP-conjugated secondary antibodies (1:4000, CST) were used for detection. Lanes were normalized to MemCode™ total protein staining (ThermoFisher). Immunoreactivity was detected with Clarity™ Western ECL substrate (BioRad), and band densitometry measured with Image Studio (LI-COR Biosciences).

2.5. Immunofluorescence

Immunofluorescence was performed with Optimal Cutting Temperature (OCT)-embedded frozen transmural left ventricle tissue sections from both mice and humans. Sections were fixed in 4% paraformaldehyde, blocked with 4% BSA, and probed for ACE2 (R&D Systems) and NG2 (Abcam). Proteins were visualized with anti-goat Alexa-488 and anti-rabbit Alexa-594 secondary antibodies, respectively. Wheat Germ Agglutinin (WGA) conjugated to Alexa-405 (Invitrogen) was included to delineate the cell membrane.

2.6. ACE2 enzyme activity assay

ACE2 activity assays were performed as previously described with modification for tissue analysis [10]. Briefly, tissue lysates were normalized (2.3), and equal protein (150 μg heart/lung, 50 μg SI/kidney) was diluted in 1× Assay buffer (75 mM Tris, 5 mM $ZnCl_2$, 1 M NaCl, pH 6.5) with protease inhibitors, including 10 μM captopril and 5 μM amastatin. Specificity was assessed by incubating samples in the presence and absence of ACE2 inhibitor DX-600 (Cayman Chemical) for thirty minutes. Fluorogenic Peptide Substrate VI (ES007, R&D Systems) was subsequently added (final concentration of 50 μM in Assay buffer), and kinetic measurements obtained immediately following (350/405 nm). Fluorogenic standards (R&D Systems) were run in parallel to generate a conversion factor (pmol/RFU) to calculate the specific activity of ACE2.

2.7. Statistical analysis

Statistics were performed using SPSS software and graphs plotted with GraphPad Prism. Data are represented as the mean ± SEM. Data that did not follow normal distribution were analyzed as independent samples with the Kruskal-Wallis test with pairwise comparison adjusted by Bonferroni correction. Two-way ANOVA with Tukey post hoc test was used to compare multiple groups. Unpaired student's *t*-test was used to compare only two groups. $*p < 0.05$; $**p < 0.01$, $***p < 0.001$. Alternate symbols may be used to indicate significance among different comparisons, but the number of symbols indicates the level of significance.

3. Results

Gene expression for *Ace2* exhibits a tissue-specific pattern, with the highest expression in the small intestine (SI) and lowest expression in the heart and lungs (Fig. 1A), demonstrating a positive linear relationship with ACE2 protein levels and enzyme activity (Fig. 1B). In contrast, *Tmprss2* mRNA is highest in the kidneys, whereas the highest TMPRSS2 protein level is in the SI, with undetectable mRNA and minimal protein in the heart (Fig. 1C).

To analyze the impact of sex and aging across organs, we first examined the molecular signature of ACE2 and TMPRSS2 in mice. In the heart, *Ace2* mRNA was lowered in the aged animals (Fig. 1D) with a lack of a difference in ACE2 protein levels and activities between males and females within age groups; however, ACE2 activity was elevated in aged animals (Fig. 1E–F). In the lungs, *Ace2* mRNA expression was higher in adult females, whereas protein levels and activity were reduced in the aged females compared to males (Fig. 1D–F). In the kidneys, ACE2 levels and activity were higher in males than females across all age groups, thus corroborating studies demonstrating increased renal ACE2 activity in male rats [7]. No differences in ACE2 were observed in the SI. In both organs, *Ace2* mRNA expression did not show a sex-dependent variation (Fig. 1D–F). In contrast, TMPRSS2 mRNA and protein levels did not vary between sexes and with aging in the lungs and SI; however, adult females had increased expression in the kidneys compared to males (Fig. 1D–E).

SARS-CoV-2 infection facilitates the loss of membrane ACE2 following initial infection [2,11]. The high burden of cardiovascular disease (CVD) in COVID-19 patients and the critical role of ACE2 in myocardial and vascular protection highlights this pathophysiological connection; thus, we next focused on examining ACE2 in the heart [2,12]. To determine the age-related pattern of myocardial ACE2 across age groups, we first measured murine protein levels by immunoblot. ACE2 was elevated in aged males and females compared to the younger age groups (Fig. 2A), in accordance with ACE2 activity (Fig. 1F), yet in contrast to mRNA expression (Fig. 1D). Immunofluorescence demonstrated predominant pericyte and vascular distribution of ACE2 (colocalization with NG2) (Fig. 2B) [12]. We next analyzed young and aged

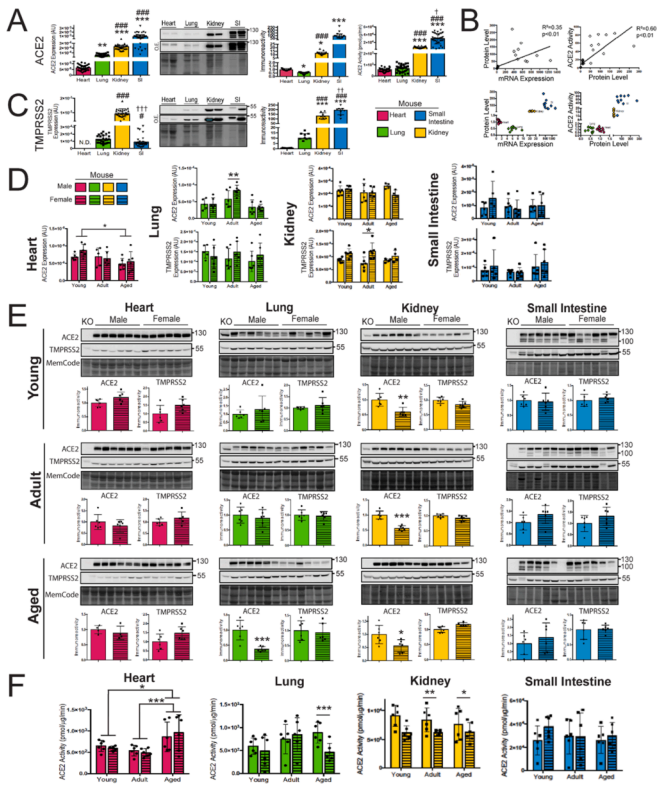

Fig. 1. Assessment of ACE2 and TMPRSS2 across sex and aging.
A. ACE2 mRNA expression ($n = 36$, biological replicates), protein levels ($n = 11$, biological replicates), and activity (n = 36, biological replicates) are positively correlated across all organs in male and female animals (B.). C. TMPRSS2 mRNA (n = 36, biological replicates) and protein levels (n = 11, biological replicates). Data from A-C was generated from C57BL6/J mice using a pooled analysis of males and females. Levels are compiled from all sexes and age groups for expression and activity; however, protein levels are compiled from representative and three replicate gels sampled from a subset of all age groups (Supplementary Fig. S1). All organs were run on the same gel for comparison. Immunoblots were over-exposed (O.E.) to visualize low levels in the lung and heart. Expression and activity data were run one time in the same plate for quantification. Data are represented as median with interquartile range (IQR) and were analyzed as independent samples with the Kruskal-Wallis test with pairwise comparison adjusted by Bonferroni correction; * indicates differences from the heart; # indicates differences from the lung; and † indicates differences from the kidney. $*p < 0.05$; $**p < 0.01$, $***p < 0.001$ where the number of symbols indicates the level of significance. Analysis of mRNA expression (D.), protein levels (E.), and ACE2 activity (F.) of ACE2 and TMPRSS2 across sex and age in the heart (magenta), lung (green), kidney (yellow), and small intestine (SI) (blue) ($n = 6$, biological replicates/group). ACE2 global knockout mice were analyzed as a negative control (KO) for western blots. Raw images are provided in Supplementary Fig. S2-S3. Data are represented as the mean ± SEM and analyzed by two-way ANOVA with Tukey post hoc test (mRNA and activity), or unpaired student's *t*-test (protein levels). For all experiments, young animals ($n = 12$) were obtained from four litters and each litter was collected over two days. Adult and aged animals (n = 12 /group) were obtained from five and three litters respectively, and each litter was collected over two days. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

explanted human heart samples. ACE2 protein levels were elevated in aged males but not in aged females, as confirmed by ACE2 immunofluorescence (Fig. 2C–D). Interestingly, no differences in *Ace2* mRNA expression were discernable; however, ACE2 activity was consistent with the immunoblot results, in which aged males were elevated (Fig. 1F). Discordant mRNA and protein levels also characterized murine hearts (data not shown).

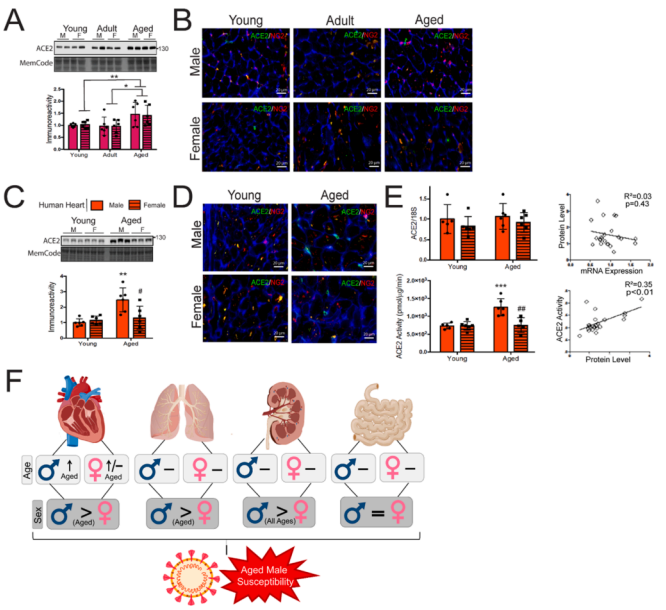

Fig. 2. Sex- and age-differences in ACE2 levels in mouse and human hearts.
A. Representative immunoblot of mouse hearts for ACE2 protein levels ($n = 6$ biological replicates/group). Three gels were compiled for quantification (Supplementary Fig. S4). Data are represented as the mean ± SEM and were analyzed by two-way ANOVA with Tukey post hoc test; $*p < 0.05$; $**p < 0.01$, $***p < 0.001$. For all experiments, young animals ($n = 12$) were obtained from four litters and each litter was collected over two days. Adult and aged animals ($n = 12$ /group) were obtained from five and three litters respectively, and each litter was collected over two days. B. Immunofluorescence of ACE2 (green) and pericyte marker NG2 (red). Wheat Germ Agglutinin (WGA) (blue) was used to delineate the cell membrane. Qualitative images were captured from $n = 2$ biological replicates and $n = 16$ (8 images/animal) technical replicates for each age group. C. Representative immunoblot for ACE2 in human hearts ($n = 6$, biological replicates/group). Two gels were compiled for quantification (Supplementary Fig. S4). Quantification represents the combined result from two western blots. Data are represented as the mean ± SEM and were analyzed by two-way ANOVA with Tukey post hoc test. * indicates differences from young males, and # indicates differences between aged males and aged females; $*p < 0.05$; $**p < 0.01$, $***p < 0.001$ where the number of symbols indicates the level of significance. D. Immunofluorescence of ACE2 (green) and NG2 (red). WGA (blue) was used to delineate the cell membrane. Qualitative images were captured from $n = 2$ biological replicates and $n = 16$ (8 images/donor) technical replicates for each age group. E. ACE2 protein levels and activity are positively correlated, but not mRNA expression. * indicates differences from young males, and # indicates differences between aged males and aged females. $*p < 0.05$; $**p < 0.01$, $***p < 0.001$ where the number of symbols indicates the level of significance. F. Schematic figure showing the multi-organ impact of sex and aging on ACE2 levels as a potential contributor to the increased male susceptibility to severe COVID-19. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

In this study, our data reveal an organ-, sex- and age-dependent difference in ACE2 regulation, with increased ACE2 in the lungs and hearts of aged males, and with a sex- rather than an age-dependent pattern in the kidneys favoring males. These changes can contribute to the increased severity and adverse outcomes reported in male COVID-19 patients (Fig. 2F). As SARS-CoV-2 viral load is predictive of intensive care unit hospitalization and mortality [4,13], we propose that aged males may be at an elevated risk partly due to the higher initial viral burden driven by the increased tissue ACE2, thus subject to pulmonary, myocardial, and renal injury. However, additional studies are necessary to delineate the relationship between viral load and the levels of ACE2 (and TMPRSS2). Further, as we confirmed the low levels of TMPRSS2 in

the heart, other proteases, such as the cathepsins, may also facilitate spike protein priming and are worthy of investigation [3]. Our results also highlight that SARS-CoV-2 could target multiple organs beyond the respiratory system, including the heart, kidneys, and gut, mainly because ACE2 protein levels in these organs far exceed the levels in the lungs. In fact, gastrointestinal symptoms are common in COVID-19 patients and occasionally precede pulmonary manifestations [11].

While a positive relationship exists between protein levels and activity of ACE2, *Ace2* mRNA and protein levels were uncoupled within organs. These findings, coupled with the observation that females have two functional copies of the X-linked *Ace2* gene, suggest a complex mechanism of transcriptional, translational, and proteolytic control of ACE2 that counteracts the predicted female-biased expression [1]. For example, given the impact of E2 on ACE2 levels and activity, the levels of gonadal steroids may also influence the observed sex differences [6]. Besides sex hormone-dependent regulation, ACE2 protein levels are subject to posttranscriptional and posttranslational modulation, such as by proteolytic cleavage and miRNAs.

Proteolytic cleavage of ACE2 is linked to the pathogenesis of CVD [1,2]. Physiologically, ACE2 counteracts the canonical renin-angiotensin system (RAS), which promotes vasoconstriction and inflammation with angiotensin II as the effector molecule. Therefore, ACE2 is protective in CVD, where chronic elevation of the canonical RAS pathway is characterized [2]. A downstream consequence of RAS activity is the activation of a disintegrin and metalloprotease 17 (ADAM17), which cleaves and releases ACE2 from the plasma membrane. Therefore, aberrant canonical RAS and subsequent increase of ADAM17 activity generate soluble plasma ACE2, which is associated with detrimental outcomes in CVD [1,2]. Similarly, ADAM17-dependent ACE2 shedding may contribute to increased COVID-19 severity following initial viral endocytosis [2]. In accordance, increased plasma ACE2 is associated with worsened clinical outcomes in hospitalized COVID-19 patients [2,14].

Finally, another mechanism of posttranscriptional control occurs by miRNAs, which suppress gene expression. Organ-specific miRNAs are predicted in silico to target ACE2 mRNA based on transcript sequences [15], as well as validated experimentally in cardiomyocytes [16]. Therefore, the mechanisms of proteolytic cleavage and miRNAs further complicate the discourse on ACE2 regulation. Additional studies are required to delineate the contribution of miRNAs and proteolytic cleavage on ACE2 expression and activity. In all, the regulation of ACE2 is complex, and the homeostatic mechanisms controlling ACE2 should be assessed for modulation in health versus disease. Given the discordance between mRNA expression and protein levels, we emphasize the complicated regulation of viral entry proteins. Further, we demonstrate the need to assess all levels from transcriptional to posttranslational to understand the biological consequences of all modes of regulation.

5. Conclusion

Our study highlights a novel sex- and age-specific bias in ACE2 protein levels and activity in the kidney, heart, and lung at basal conditions. To our knowledge, we present the first comparative analysis to comprehensively investigate the impact of sex and age on ACE2 and TMPRSS2 in both humans and mice. Given the global burden of the COVID-19 pandemic, our findings expand the knowledge of the tissue distribution of viral entry factors and begin to inform the sex-discrepancy in COVID-19 severity.

Declartion of Competing Interest

The authors have no conflict of interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yjmcc.2021.11.003.

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