RAPID REPORT | The Pathophysiology of COVID-19 and SARS-CoV-2 Infection

Sex steroids skew ACE2 expression in human airway: a contributing factor to sex differences in COVID-19?

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1Department of Pharmaceutical Sciences, School of Pharmacy, College of Health Professions, North Dakota State University, Fargo, North Dakota; 2Department of Anesthesiology and Perioperative Medicine, Mayo Clinic, Rochester, Minnesota; and 3Department of Physiology and Biomedical Engineering, Mayo Clinic, Rochester, Minnesota

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Kalidhindi RS, Borkar NA, Ambbore NS, Pabelick CM, Prakash YS, Sathish V. Sex steroids skew ACE2 expression in human airway: a contributing factor to sex differences in COVID-19? Am J Physiol Lung Cell Mol Physiol 319: L843–L847, 2020. First published September 30, 2020; doi:10.1152/ajplung.00391.2020.—The incidence, severity, and mortality of ongoing coronavirus infectious disease 19 (COVID-19) is greater in men compared with women, but the underlying factors contributing to this sex difference are still being explored. In the current study, using primary isolated human airway smooth muscle (ASM) cells from normal males versus females as a model, we explored the effect of estrogen versus testosterone in modulating the expression of angiotensin converting enzyme 2 (ACE2), a cell entry point for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Using confocal imaging, we found that ACE2 is expressed in human ASM. Furthermore, Western analysis of ASM cell lysates showed significantly lower ACE2 expression in females compared with males at baseline. In addition, ASM cells exposed to estrogen and testosterone for 24 h showed that testosterone significantly upregulates ACE2 expression in both males and females, whereas estrogen downregulates ACE2, albeit not significant compared with vehicle. These intrinsic and sex steroids induced differences may help explain sex differences in COVID-19.

airway smooth muscle; estrogen; SARS-CoV-2; sex difference; testosterone

INTRODUCTION

Coronavirus disease 19 (COVID-19) is an infectious disease caused by the recently discovered severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a member of the family of coronaviruses that caused ailments such as the common cold to Middle East Respiratory Syndrome (MERS) and SARS-CoV-induced acute respiratory syndrome (2). The lung is a critical target organ in COVID-19 with the virus amplifying itself using host cells and eventually destroying lung structural cells leading to respiratory distress and its sequelae (2). The main point of cellular entry for SARS-CoV-2 is via SARS-CoV-2 spike protein 1 (S1)-bound angiotensin-converting enzyme-2 (ACE2) (25, 40, 41). Thus, mechanisms that regulate cellular ACE2 expression and functionality have the potential to influence the progression and outcomes of COVID-19.

Emerging clinical and epidemiological evidence suggests sex/gender disparities in the incidence and severity of COVID-19 and its associated mortality (8, 10, 14, 19, 20, 23, 29, 34, 35). While the incidence is influenced by many underlying factors, severity and mortality are clearly higher in males compared with females (1), which may reflect intrinsic sex differences or more intriguingly a potential role of sex steroids in the pathophysiology of COVID-19. Accumulating evidence suggests that sex steroid levels are altered in COVID-19 patients (13, 26) but their functional significance is not clear. Here, sex steroid effects on structural cells are likely important in the context of chronic sequelae of COVID-19 leading to chronic lung disease. The relevance of these cell types lies in the potential for SARS-CoV-2 to target and damage epithelial layers with subsequent influences on underlying mesenchymal cells [airway smooth muscle (ASM), fibroblasts], leading to altered airway reactivity, inflammation, and fibrosis towards long-term sequelae. A recent study in normal human bronchial epithelial (NHBE) cells reported that estrogen downregulates ACE2 mRNA (36). However, to date, no studies have explored ACE2 in ASM cells, or the influence of sex steroids in the context of COVID-19 pathophysiology.

Our hypothesis is that sex steroids such as estrogen and testosterone influence airway ACE2 expression, thereby contributing to the observed sex differences in COVID-19. In this report, using human ASM from males versus females, we show that 1) ACE2 is expressed in human ASM; 2) ACE2 has lower baseline expression in ASM of females compared with males; and 3) estrogen downregulates ACE2 expression, whereas testosterone upregulates ACE2 in human ASM cells.

MATERIALS AND METHODS

Chemical and reagents. Hanks’ balanced salt solution (HBSS), Dulbecco’s phosphate-buffered saline (DPBS), Dulbecco’s modified Eagle’s medium with F-12 (DMEM/F12), trypsin EDTA and antibiotic-antimycotic were purchased from Thermo Fisher Scientific (Waltham, MA). Fetal bovine serum (FBS) was procured from Millipore-Sigma (Burlington, MA). 17β-Estradiol (E2) and testosterone (Tes) were procured from Tocris (Bristol, UK). Anti-ACE2 antibody (cat no. sc-390851) was procured from Santa Cruz Biotech (cat no. sc-390851, Dallas, TX) and Novus Biologicals (cat no. NB2-67692, Colorado). Anti-β-actin monoclonal antibody (cat no. G043) was obtained from Abcam (Cambridge, MA). Anti-β-actin monoclonal antibody (cat no. G043) was obtained from Abcam (Cambridge, MA).

Tissue and cells. Acquisition of human lung samples and the isolation and culturing of human ASM cells have been previously described.
Briefly, third- to sixth-generation human bronchi were obtained from lung specimens incidental to patient thoracic surgeries at Mayo Clinic (focal, noninfectious indications; typically lobectomies, rarely pneumonectomies). Formalin-fixed, paraffin-embedded sections of lung tissue were used for immunofluorescence studies. ASM cells were enzymatically dissociated and maintained under standard conditions of 37°C (5% CO₂, 95% air) and serum deprived for 24 h before experimentation. The initial review of patient histories with complete de-identification of samples for storage and subsequent usage was approved by Mayo Clinic’s Institutional Review Board and written informed consent was obtained. We used ASM samples from both males and females and limited the culturing to <5 passages to conserve smooth muscle phenotype.

**Immunofluorescence.** Standard techniques were applied to 5 μm thick lung sections. Briefly, sections were baked, processed for antigen retrieval with citrate buffer and rehydrated, permeabilized using 0.1% Triton X-100 in PBS, blocked with 10% goat serum, and exposed to antibodies against ACE2 and α-smooth muscle actin (α-SMA). Secondary antibodies were AlexaFluor-488 for ACE2 and AlexaFluor-555 for α-SMA, respectively, with DAPI counterstain for nuclei. Super-resolution Z-stack images were captured using a Zeiss confocal microscope.

**Cell treatments.** Serum-deprived human ASM cells were exposed to vehicle, E₂ [1 nM (5, 7)], and Tes (10 nM (22,)) in DMEM/F12 (FBS free) for 24 h followed by protein collection for Western analyses (4, 5, 22).

**Western analysis.** Previously described standard techniques were used (4, 5, 22). Total protein content was measured using DC Protein Assay kit (Bio-Rad) and a minimum of 25 μg equivalent protein from each group was loaded in 4–15% gradient gels (Criterion Gel System; Bio-Rad), followed by transfer to 0.22 μm PVDF membranes (Bio-Rad Trans-Blot Turbo), blocking with 5% BSA, and overnight exposure to ACE2 (Santacruz Biotech) and β-actin primary antibodies. Bands were detected on Li-Cor Odyssey CLx system using LiCOR near-red conjugated anti-mouse-800 secondary antibodies. Densitometric analysis was performed using Image Studio software. The data are represented as ACE2/β-actin expression.

**Statistical analysis.** For each group, a minimum of 4–12 independent patient samples with a minimum of two repeats for each experiment were done. Statistical analysis was performed using two-tailed unpaired t test or one-way ANOVA with Dunnett’s post hoc test as applicable using GraphPad Prism software (GraphPad Software, San Diego, CA). Data are expressed as minimum to maximum with center line depicting mean and statistical significance tested at $P < 0.05$ level.

**RESULTS**

**ACE2 expression in human lung tissue.** In human lung sections, ACE2 was found to be expressed across different lung cell types as shown in Z-stacked three-dimensional immunofluorescence images (Fig. 1, A, B, C, and D). We found ACE2 to be substantially colocalized with α-SMA (Fig. 1E), indicating that human ASM expresses ACE2.

**ACE2 expression with respect to sex.** Western analysis of primary human ASM cell lysates from normal males and females indicated a significantly ($P < 0.01$) lower baseline expression of ACE2 in females compared with males (Fig. 2).

![Fig. 1](https://example.com/fig1.png)

Fig. 1. Angiotensin-converting enzyme 2 (ACE2) is expressed in human airway tissue as indicated by immunofluorescence study. Panels showing various angles of three-dimensional (3D) Z-stack images (A, B, and C) and 2.5D image showing intensity of each fluorophore in independent pixels (D). Colocalization of ACE2 (green, AF-488) in airway smooth muscle (ASM) using α-smooth muscle actin (αSMA, red, AF-555) as an ASM-specific marker, where colocalization can be seen as yellow pixels in the scatterplot (E). DAPI was used to stain nucleus (blue). Yellow arrow indicates ACE2 expression in ASM. Representative images of $n = 5$ independent patient samples.
 Effect of sex steroids on ASM ACE2 expression. In lysates of human ASM cells exposed to vehicle, E2 or Tes, we found that E2 exposure resulted in slightly downregulated ACE2 expression in both males and females, although this was not statistically significant. Interestingly, Tes exposed human ASM cells from males and females showed significantly up-regulated ($P < 0.05$ for males and females) ACE2 expression compared with respective vehicles (Fig. 3 A and B).

DISCUSSION

As the global death toll due to the novel coronavirus mounts, there is increasing evidence that the incidence and severity of COVID-19 and associated mortality are all greater in men compared with women. Whether these clinical observations reflect intrinsic sex differences in organ-level or whole-body responsiveness to the virus (potentially complicated by comorbidities) or whether sex steroids play a modulatory role is not clear. Given emerging evidence for differences in sex steroid levels in COVID-19 patients (8, 10, 14, 19, 20, 23, 29, 34, 35), we explored whether sex steroids (estrogen and testosterone) modulate specific aspects of SARS-CoV-2 functionality, focusing on ACE2 expression in the lung, the critical target organ of the virus. We focused on the airway, appreciating the fact that following initial infection, progressive involvement of structural cells of the airway can contribute to long-term sequelae of viral infection. While the epithelium is the primary entry point for SARS-CoV-2, following initial infection and cellular damage, the underlying ASM is likely the subsequent target and thus needs to be explored. While some studies have reported the effect of estrogen on ACE2 expression in other cell types (36), there have not been any studies in the airway, or on the role of estrogen and testosterone in regulating ASM ACE2 expression.

Multiple groups, including our own, have shown a role for sex steroids, especially estrogen (3–5, 12, 15–17, 21, 22, 31, 33, 37–39) and testosterone (11, 18, 22, 24, 27) in regulating the pathophysiology of lung diseases such as asthma or COPD as well as the known sex differences in these conditions (9). In this

Fig. 2. Sex/gender differences in angiotensin-converting enzyme 2 (ACE2) expression in primary human airway smooth muscle (ASM) cells from males and females. Data represented as minimum to maximum of $n = 12$ for females and $n = 11$ males and analyzed using two-tailed unpaired $t$ test. **$P < 0.01$ versus males.

Fig. 3. Effect of sex-steroids, estrogen ($E_2$, 1 nM for 24 h) and testosterone ($Tes$, 10 nM for 24 h) on angiotensin-converting enzyme 2 (ACE2) expression in nonasthmatic male (A) and female (B) human airway smooth muscle (ASM) cells. Data represented as line plots for $n = 4$ males and $n = 5$ females and analyzed using one-way ANOVA with Dunnett’s post hoc test. *$P < 0.05$ versus vehicle.
Regard, sex steroids can differentially influence the expression and functionality of multiple signaling pathways in lung cells including ASM, contributing to altered airway reactivity and remodeling. In this novel study, we report that ACE2 is expressed in human ASM. Furthermore, our Western data from cell lysates of female patients show downregulated baseline ACE2 expression compared with males, which may reflect elevated circulating estradiol concentrations in females. Our data in ASM are also consistent with the recent report of lower baseline ACE2 mRNA in airway epithelial cells and the blunting effect of estrogen at supraphysiological concentration (36). Perhaps more interestingly, Tes exposure significantly upregulated ACE2 expression in human ASM cells. Here, it is important to note that E2 and Tes concentrations used in our study are physiological (4, 5, 7, 22, 38, 39). Furthermore, we have previously reported that both male and female human ASM expresses estrogen receptor isoforms (alpha and beta) as well as the androgen receptor (6, 22). Thus, while there is an understandable focus on the “protective” role of estrogens, it is possible that circulating Tes in males in fact contributes to elevated ACE2 expression, thereby increasing susceptibility for SARS-CoV-2. What is not known is the mechanisms by which Tes could upregulate ACE2, and whether such effects also occur in other cell types, particularly epithelium. Conversely, the mechanisms underlying an inhibitory role for estradiol may be important to understand towards approaches to blunt ACE2 expression and reduce viral infectivity.

In conclusion, our novel findings suggest a differential role for male versus female sex steroids in a key aspect of COVID-19 pathophysiology: ACE2 expression in ASM cells. Although not a focus of this report, it may be worthwhile to explore whether factors such as age, and local sex steroid metabolism further influence differential ACE2 expression based on sex. Our data set the stage to understand whether sex steroids also differentially influence downstream aspects of SARS-CoV-2 functionality in terms of viral entry and intracellular expansion.

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