SARS-CoV-2 Receptor ACE-2 (Angiotensin-Converting Enzyme 2) Is Upregulated in Colonic Organoids From Hypertensive Rats

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Hypertension is the most common comorbidity associated with unfavorable outcomes in patients with coronavirus disease 2019 (COVID-19). This especially impacts the elderly population with its underlying high rate of hypertension.[1] Emerging evidence also implicates the gastrointestinal tract in COVID-19, with ≈30% to 50% of patients presenting with gastrointestinal symptoms. Nasal, pulmonary, and gastrointestinal epithelia express high levels of ACE-2 (angiotensin-converting enzyme 2), the receptor for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) cell entry.[2] While the primary mode of viral transmission is inhalation of respiratory droplets, the gastrointestinal epithelium is the body site with greatest ACE-2 expression.[2] Furthermore, a critical role for gut in COVID-19 pathophysiology is emerging that is potentially relevant for hypertension-COVID-19 comorbidity[2]: (1) ≈30% to 50% COVID-19 of patients manifest gastrointestinal symptoms, often before respiratory symptoms[1]; (2) infectious SARS-CoV-2 has been detected in stool, with viral RNA shedding in feces for weeks[3]; (3) all patients with COVID-19 show altered fecal microbiome and dysbiosis, even those without gastrointestinal symptoms,[4] and some of those bacterial species adversely influence *ACE2*[4]; (4) gut mucosa exhibits all components of renin-angiotensin system[2]; and (5) the intestinal epithelium supports SARS-CoV-2 replication.[2] These observations led us to hypothesize that increased *ACE2* expression in gut epithelium would predispose hypertension patients to COVID-19 infection. We tested this hypothesis in organoid cultures from spontaneously hypertensive rats (SHR) using Wister Kyoto rats (WKY) as controls.

Results and Discussion

We first established culture conditions and compared basic properties of colon organoids between WKY and SHR. Colon was studied based on our previous data demonstrating colonic pathology and altered epithelial gene profile in the SHR.[5] We observed that SHR had 14% shorter colons than WKY (SHR: 0.062 cm/gm versus WKY: 0.072 cm/gm; $P<0.001$, Figure [A]). This is consistent with observations of decreased colonic length in other chronic inflammatory diseases, such as colitis and could have important implications in absorption of key nutrients and the altered epithelial-microbiota cross-talk we proposed earlier.

Crypts containing colonic stem cells were cultured on the same batch of Matrigel matrix throughout. Organoids reached maximal size in 7 days and there were no differences in organoid size or viability between WKY and SHR (Figure [B]). However, SHR crypts formed 30% fewer organoids than WKY rats (SHR versus WKY, 3 days $P<0.001$, 5 and 7 days $P<0.0001$, Figure [C]). This is consistent with decreased colonic length in SHR and suggests that 3-dimensional organoids in culture maintain in vivo properties. *Ki67* expression, a nuclear protein marker of cell proliferation, was comparable in both strains (Figure [D]). However, *Krt20*, a marker for enterocytes and goblet cells, was increased 2.4-fold in the SHR (Figure [D]). This is in line with RNA-seq data from colonic epithelium (Figure [E]) and suggests that decreased growth in SHR may result from increased differentiation or dysbiosis-enhanced epithelial autophagy, a view that needs further exploration.

Next, we compared *Ace2* and transmembrane protease serine 2 (*Tmprss2*) expression. *Tmprss2* is a membrane-anchored protease that is critical in the activation of SARS-CoV and SARS-CoV-2 spike protein,[2] a necessary step in ACE-2-mediated entry of these coronaviruses into cells. *Ace2* mRNA was ≈2-fold enriched in SHR organoids compared to WKY (Figure [E]), with the increase confirmed by both immunostaining and Western blotting (Figure [F]). High magnification images in Figure [F] indicated *Ace2* localized to the cell surface. Increased *Ace2* mRNA in the SHR epithelium reinforced the preservation of epithelial properties in organoids. *Tmprss2* mRNA levels showed a trend towards an increase in SHR organoids (SHR versus WKY, $P=0.09$, Figure [E]), although this increase was significant in SHR epithelium (Figure [E]). In contrast, B^0AT1 mRNA levels were comparable in WKY and SHR. B^0AT1 is a neutral amino acid transporter of Solute Carrier Family 6 and after forming the complex with ACE-2 acts as the gut's primary transporter of amino acids from the lumen. These observations suggest that increased expression of *Ace2* coupled with *Tmprss2* expression could provide a basis for higher infectivity of SARs-CoV-2 in hypertension.[1,2]

Ace mRNA was also increased in both SHR organoids and epithelium. ACE, unlike ACE-2, is not a receptor for coronavirus entry into cells. However, we can only speculate about the relevance of increases in both ACE and *ACE2* in

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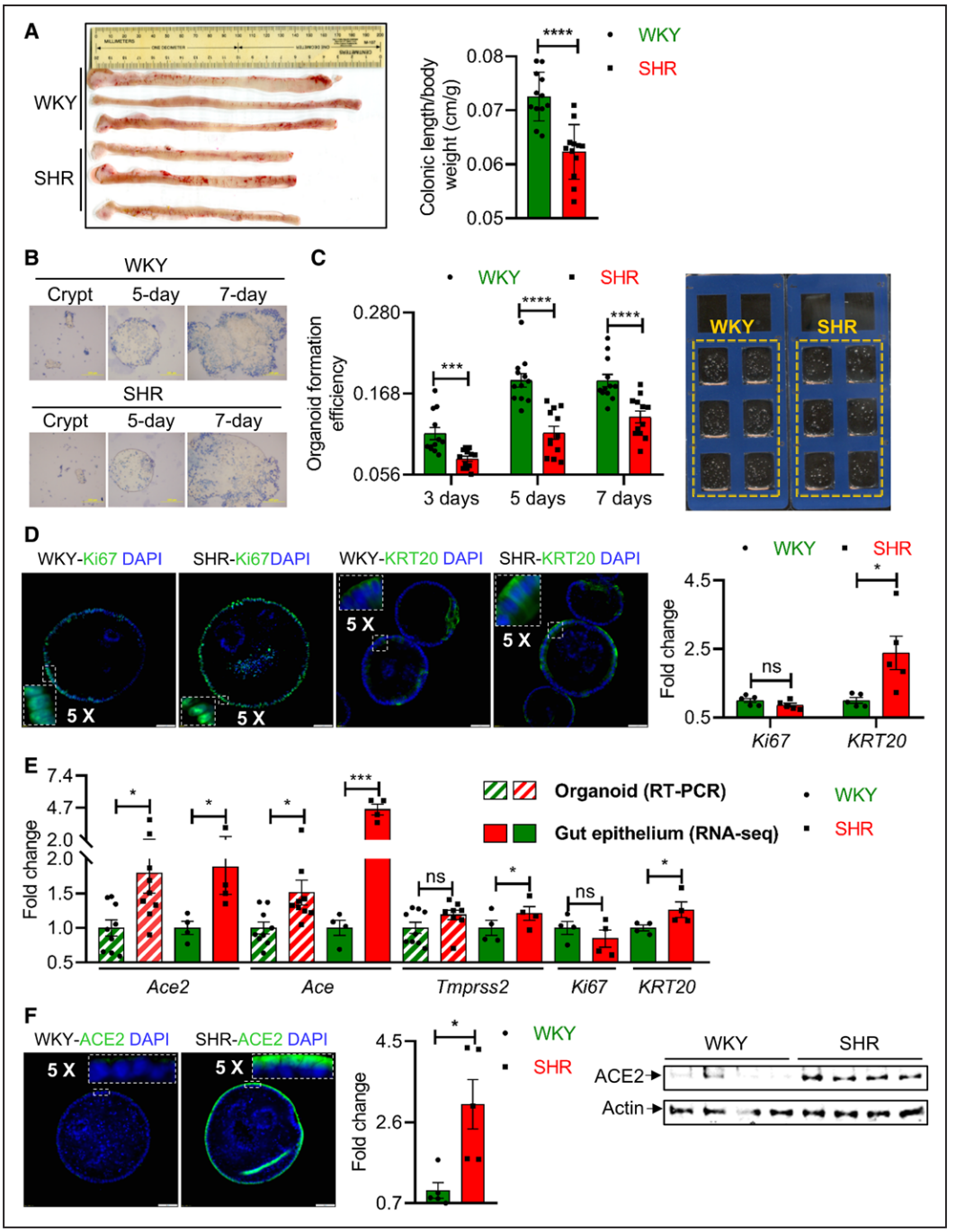

Figure. **A**, Representative images of colon (**left**), and the ratio of colon length/body weight of Wister Kyoto rat (WKY) and spontaneously hypertensive rat (SHR) rats (n=12 per group; **right**) from male 14-wk-old SHR and WKY rats (Charles River), mean systolic blood pressures 211±4 and 137±3 mm Hg, respectively, measured by Tail-Cuff Plethysmography. **B**, Phase microscopic images documenting growth of organoids from isolated colonic crypts of WKY and SHR rats stained with Trypan blue, dead cells are blue. Scale bar: 200 µmol/L. Primary colon organoids were grown from isolated crypts (gentle dissociation reagent [Stem Cell Technologies] in Matrigel [BD Biosciences] and organoid growth medium [Stem Cell Technologies] supplemented with recombinant mouse Noggin [PeproTech] and EGF [BioLegend], recombinant human IGF-1, FGF-basic [FGF-2; BioLegend] and R-spondin1 [R&D], Y-27632 [STEMCELL Technologies], and A83-01 [Tocris] described elsewhere [*Cell Stem Cell*, 2018, 23(6): 787–793. e6]). (n=12 rat colon cultures/group.) **C**, Organoid formation efficiency determined 3, 5, and 7 d after culture of isolated colonic crypts from WKY and SHR rats (n=12 per group; **left**) and 4% PFA-fixed organoids after 5 d in culture (**right**). **D**, Representative confocal immunofluorescence images of Ki67 and KRT20 in organoids from WKY and SHR (**left**, scale bar: 50 µmol/L); quantified and normalized fluorescent intensity (**right**, n=5 per group). Fixed, permeablilized organoids stained with Ki67 and KRT20 specific antibodies (Abcam) and DAPI. **E**, Comparison of mRNA for *Ace2*, *Ace*, *Tmprss2*, *Ki67*, and *KRT20* in colonic organoids by real-time polymerase chain reaction (RT-PCR; **left**, shaded bars, n=9 per group) and in epithelium of proximal colon by RNA-seq (**right**, solid bars, n=4 per group) from WKY and SHR. For organoids, total RNA was purified using RNAeasy plus mini kit (Qiagen) and reverse-transcribed using iScript Reverse Transcription Supermix (Bio-Rad). Finally, RT-PCR (ABI Prism 7600) was performed with Taqman universal PCR master mix and specific probes. RNA-seq method. [5]**F**, Analysis of *Ace2* in organoids by confocal immunofluorescent imaging (**left**, Scale bar: 50 µmol/L) and by Western blot (**right**), quantified and normalized to actin fluorescence intensity (**middle**, n=5 per group). ACE-2 (Angiotensin-converting enzyme) and actin antibodies were from Abcam and Western blots were homogenates of organoids (2% SDS-Tris buffer, pH=7.5), run on 12% TGX precast gels, transferred to PVDF membranes (Biorad) and imaged on Odyssey imaging system with infrared light for Li-Cor Biosciences secondary antibodies. Fold change relative to WKY group. Values are means±SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, and ns P>0.05 vs WKY from SHR group, unpaired t test.

hypertension and COVID-19. It may be that hypertension is driven by ACE-mediated increases in the vasodeleterious renin-angiotensin system axis. This is counterbalanced by an amplification of the protective ACE-2 axis of the renin-angiotensin system during hypertension to maintain homeostasis. But this also increases the receptor for SARS-CoV-2 and, therefore, vulnerability to infection.

In conclusion, we have established organoid cultures from WKY and SHR which demonstrate much of the COVID-19-relevant physiology of the respective in vivo epithelial phenotypes. SHRs show increased *Ace2*, *Ace* and expression of *Tmprss2*. Thus, organoids provide an opportunity to investigate cellular and molecular interactions of components of SARS-CoV-2, the renin-angiotensin system and hypertension. Finally, caution is warranted to prevent over-interpretation of these data before this concept can be validated with our planned studies in organoids from patients with hypertension.

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Disclosures

None.

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