Impact of Salt Intake and Renin-Angiotensin-Aldosterone System Blockade on Lung Severe Acute Respiratory Syndrome Coronavirus 2 Host Factors

Xiaoli Zhang\textsuperscript{a, b, c} Liping Liu\textsuperscript{a} Mohamed M.S. Gaballa\textsuperscript{c, d} Ahmed A. Hasan\textsuperscript{b, c} Yingquan Xiong\textsuperscript{c, e} Li Xie\textsuperscript{a} Thomas Klein\textsuperscript{f} Denis Delic\textsuperscript{c, f} Burkhard Kleuser\textsuperscript{b} Bernhard K. Krämer\textsuperscript{c} Jian Li\textsuperscript{a} Berthold Hocher\textsuperscript{a, c, g, h}

\textsuperscript{a}Key Laboratory of Study and Discovery of Small Targeted Molecules of Hunan Province, School of Medicine, Hunan Normal University, Changsha, China; \textsuperscript{b}Institute of Pharmacy, Freie Universität Berlin, Berlin, Germany; \textsuperscript{c}Fifth Department of Medicine (Nephrology/Endocrinology/Rheumatology/Pneumology), University Medical Centre Mannheim, University of Heidelberg, Heidelberg, Germany; \textsuperscript{d}Faculty of Veterinary Medicine, Benha University, Toukh, Egypt; \textsuperscript{e}Department of Nephrology, Charité - Universitätsmedizin Berlin, Campus Mitte, Berlin, Germany; \textsuperscript{f}Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany; \textsuperscript{g}Reproductive and Genetic Hospital of CITIC-Xiangya, Changsha, China; \textsuperscript{h}Institute of Medical Diagnostics, IMD, Berlin, Berlin, Germany

What is already known about this subject?

- Angiotensin-converting enzyme 2 (ACE2) and the transmembrane protease serine type 2 (TMPRSS2) play pivotal roles in cell entry for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus causing coronavirus disease 2019 (COVID-19).
- ACE2 is part of the renin-angiotensin-aldosterone system (RAAS), a hormone system that maintains salt balance and thus regulates blood pressure. Dietary salt consumption is involved in the regulation of the RAAS.
- SARS-CoV-2 infection is particularly dangerous for people with pre-existing renal and cardiovascular disease treated according to international guidelines with drugs that interfere with the RAAS.

What does this study add?

- This study offers biological support regarding the safety of ACE inhibitors as well as angiotensin II receptor blockers prescribed to COVID-19 patients with renal and/or cardiovascular comorbidity.
- High salt intake increases the expression of ACE2 in the lung and hence might adversely affect COVID-19 outcome.

What impact this may have on practice or policy

- Our preclinical data should stimulate clinical studies addressing the impact of salt intake on SARS CoV-2 infection.
Keywords
ACE2 · TMPRSS2 · High-salt diet · Renin-angiotensin-aldosterone system blockade · Lung

Abstract

Introduction: The angiotensin-converting enzyme 2 (ACE2) as well as the transmembrane protease serine type 2 (TMPRSS2) have been found to play roles in cell entry for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus causing coronavirus disease 2019 (COVID-19). SARS-CoV-2 infection risk and severity of COVID-19 might be indicated by the expression of ACE2 and TMPRSS2 in the lung.

Methods: A high-salt diet rat model and renin-angiotensin-aldosterone system (RAAS) blockade were used to test whether these factors affect ACE2 and TMPRSS2 expression in the lung. A normal (0.3% NaCl), a medium (2% NaCl), or a high (8% NaCl) salt diet was fed to rats for 12 weeks, along with enalapril or telmisartan, before examining the lung for histopathological alteration. Using immunofluorescence and qRT-PCR, the localization as well as mRNA expression of ACE2 and TMPRSS2 were investigated.

Results: The findings provide evidence that both TMPRSS2 and ACE2 are highly expressed in bronchial epithelial cells as well as ACE2 was also expressed in alveolar type 2 cells. High-salt diet exposure in rats leads to elevated ACE2 expression on protein level. Treatment with RAAS blockers had no effect on lung tissue expression of ACE2 and TMPRSS2.

Conclusions: These findings offer biological support regarding the safety of these drugs that are often prescribed to COVID-19 patients with cardiovascular comorbidity. High salt intake, on the other hand, might adversely affect COVID-19 outcome. Our preclinical data should stimulate clinical studies addressing this point of concern.

© 2022 The Author(s).
Published by S. Karger AG, Basel

Introduction

There is some evidence that the severity of coronavirus disease 2019 (COVID-19) is affected by the initial viral load in the respiratory tract. In fact, there are data that patients with vicious COVID-19 are infected with a higher viral load in their respiratory tract, and the virus persists longer in their bodies, in contrast to patients with less severe disease [1, 2]. In the context of influencing viral load and infection, the viral host receptor (angiotensin-converting enzyme 2 [ACE2]) and molecules involved in cell entry (e.g., TMPRSS2) expression levels are expected to have an influential role to play. A severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection begins when the virus binds to host ACE2 on upper or lower respiratory tract epithelial cells. Subsequently, after binding, the viral spike protein then must be cleaved and primed by the host transmembrane protease serine type 2 (TMPRSS2) to facilitate the virus to enter into cells [3]. Thus, different levels of ACE2 and TMPRSS2 expression in the lining epithelium of the lung might likewise affect the susceptibility to SARS-CoV-2.

Several studies demonstrated an association of dietary salt intake and a variety of comorbid COVID-19 conditions such as hypertension, cardiovascular disease, and kidney disease [4, 5]. There is extensive evidence that diets containing high levels of salt have been shown to activate the renin-angiotensin-aldosterone system (RAAS). ACE2 is part of the RAAS, a hormone system that plays a prominent role in salt balance and thus in blood pressure regulation in humans [6]. Renal ACE2 expression in animal models is strongly influenced by salt intake [7]. As a consequence of excessive sodium intake, renal cortical expression of ACE increased, and ACE2 expression decreased [8]. An elevated sodium intake also decreased expression of cardiac mRNA levels of ACE2, enhanced the local RAAS which promoted hypertension [9].

Given the main effects of the RAAS on the kidney and heart, before the eruption of the COVID-19 pandemic, the regulation of the pulmonary ACE2 with regard to salt intake was not the focus of nutritional studies addressing the effect of salt intake on the RAAS. RAAS-blocking medications such as angiotensin II receptor blockers (ARBs) as well as ACE inhibitors (ACEIs) belong to the guideline-based medication of patients with hypertension and/or chronic kidney disease [10]. SARS-CoV-2 infection is particularly dangerous for people who have pre-existing cardiovascular disease and frequently receive drugs that interfere with the RAAS system. Some studies suggest that ARBs and ACEIs could cause ACE2 compensatory upregulation in the cardiovascular system [11, 12]. Given that ACE2 is the main host receptor for SARS-CoV-2 infection, the effects of RAAS-blocking agents such as ARBs and ACEIs on ACE2 expression gained huge interest [13–16]. It has been intensively discussed whether ARBs and ACEIs are safe and effective for COVID-19 patients [17].

The activity of the RAAS and hence the expression of ACE2 – also in the lungs – depends on two key factors: salt intake and use of RAAS-blocking agents. The interaction of these two key factors on lung ACE2 expression, however, remains to be investigated. Hence, a rat model mimicking these dietary (salt intake) and pharmacologi-
Salt Intake and RAAS Blockade in COVID-19

Kidney Blood Press Res 2022;47:565–575
DOI: 10.1159/000525368

Cal conditions (ARB or ACEI treatment) often seen in patients at high risk for adverse outcome of COVID-19 was established to analyze the influence of these factors on the expression of lung SARS-CoV-2 host factors potentially influencing the risk of COVID-19 outcome.

Materials and Methods

Animals

An adult male Sprague-Dawley (SD) rat, 6 weeks old was obtained from Hunan SJA Laboratory Animal (Changsha, China). Rats were acclimated to the new environment for 1 week before being housed in temperature-controlled cages with 12-h light/dark cycles. Ad libitum food and water were available to all rats. Hunan Normal University’s Experimental Animal Center approved the experimental protocols (Permit number: D2020008).

Study Design

Nine groups of 105 SD male rats were randomly assigned and treated for a period of 12 weeks: (1) rats were fed a 0.3% NaCl control diet (n = 10); (2) rats were fed 2% NaCl medium-salt diet (n = 10); (3) rats were fed 8% NaCl very high-salt diet (n = 15); (4) rats were fed control diet and treated with telmisartan (5 mg/kg) (n = 10); (5) rats were fed 2% NaCl diet and treated with telmisartan (5 mg/kg) (n = 10); (6) rats were fed 8% NaCl diet along with telmisartan (5 mg/kg) (n = 15); (7) rats were fed control diet along with enalapril (10 mg/kg) (n = 10); (8) rats were fed 2% NaCl diet along with enalapril (10 mg/kg) (n = 10); (9) rats were fed 8% NaCl diet along with enalapril (10 mg/kg) (n = 15). Composition of control diet (D10001), 2% NaCl diet (RD20072101), and 8% NaCl diet (D05011703, Shenzhen Ruidi Biotechnology Co., Ltd.) is shown in Table 1. Telmisartan (J20090089, Boehringer Ingelheim Pharma GmbH & Co. KG) and enalapril (H32026567, Yangtze River Pharmaceutical Group Co., Ltd.) were given by gavage. Rats in first three groups were given the same amount of physiological saline by gavage as placebo. By the end of the study, the CODA tail-cuff (Kent Scientific Corp., Torrington, CT, USA) blood pressure system was used to measure the systolic blood pressure (SBP). In the 12th week, the animals were sacrificed by intraperitoneal injection of a solution of sodium pentobarbital 3% (wt/vol). Blood and lung tissue were collected and analyzed for biochemical, morphological, and molecular analysis.

Lung Morphology

Lung samples were fixed, sectioned, and stained with H&E for histopathologic evaluation. Specifically, lung injury was assessed by two independent investigators based on the following factors: hemorrhage, alveolar congestion, leukocyte infiltration or aggregation of neutrophils, and thickness of the alveolar wall. A modified histologic scoring criterion for the above factors was used as previously described [18, 19]: score 0, normal lungs; score 1, mild (<25%); score 2, moderate (25–50%); score 3, severe (50–75%), and score 3, very severe (>75%). A summation of the scores of factors was obtained, and then a mean ± SEM was generated (5 sections/lung, 10 lungs/group). ImageJ software was used to measure the alveolar airspace and circumference. A total of 20 fields per section were selected randomly and examined. The mean alveolar

Table 1. Component and energy of control diet, 2% NaCl, and 8% NaCl diet

| Ingredient                  | Control diet | 2% NaCl diet | 8% NaCl diet |
|-----------------------------|--------------|--------------|--------------|
| Protein                     | 20.3 g%      | 19.9 g%      | 18.7 g%      |
| Carbohydrate                | 66.0 kcal%   | 64.7 kcal%   | 60.7 kcal%   |
| Fat                         | 5.0 g%       | 4.9 g%       | 4.6 g%       |
| Total                       | 100 g%       | 100 g%       | 100 g%       |

| Ingredient                  | g% kcal     | g% kcal     | g% kcal     |
|-----------------------------|-------------|-------------|-------------|
| Casein                      | 200 g 800 kcal | 200 g 800 kcal | 200 g 800 kcal |
| DL-methionine               | 3 g 12 kcal  | 3 g 12 kcal  | 3 g 12 kcal  |
| Corn starch                 | 150 g 600 kcal | 150 g 600 kcal | 150 g 600 kcal |
| Sucrose                     | 500 g 2000 kcal | 500 g 2000 kcal | 500 g 2000 kcal |
| Cellulose, BW200            | 50 g 0 kcal  | 50 g 0 kcal  | 50 g 0 kcal  |
| Corn oil                    | 50 g 450 kcal | 50 g 450 kcal | 50 g 450 kcal |
| Mineral Mix S10001          | 32 g 0 kcal  | 32 g 0 kcal  | 32 g 0 kcal  |
| Sodium chloride             | 3 g 0 kcal  | 20 g 0 kcal  | 87 g 0 kcal  |
| Potassium                   | 3 g 0 kcal  | 3 g 0 kcal  | 3 g 0 kcal  |
| Vitamin Mix V10001          | 10 g 40 kcal | 10 g 40 kcal | 10 g 40 kcal |
| Choline bitartrate          | 2 g 0 kcal  | 2 g 0 kcal  | 2 g 0 kcal  |
| Total                       | 1,003 g 3,902 kcal | 1,020 g 3,902 kcal | 1,087 g 3,902 kcal |
### Table 2. Comparison of body weight, lung weight, blood pressure, and histology of lung among different groups

| Parameters                        | ND + PBO | 2% SD + PBO | 8% SD + PBO | ND + TELM | 2% SD + TELM | 8% SD + TELM | ND + ENAL | 2% SD + ENAL | 8% SD + ENAL |
|-----------------------------------|----------|-------------|-------------|-----------|-------------|-------------|-----------|-------------|-------------|
| **Body weight and lung weight**   |          |             |             |           |             |             |           |             |             |
| BW (0 weeks)                      | 336.9±4.15 | 321.0±3.64  | 331.7±4.07  | 335.1±4.24 | 321.6±4.48  | 319.03±3.97 | 324.92±6.08 | 327.44±5.19 | 320.44±3.7  |
| BW (2 weeks)                      | 393±4.42  | 380.18±7.33 | 355.25±4.62**| 367.93±8.41**| 379.21±3.81 | 342.71±4.06 | 344.18±8.27**| 349.08±6.93 | 337.47±4.23 |
| BW (4 weeks)                      | 484.29±5.33 | 465.24±11.19 | 423.17±6.00**| 407.41±6.03**| 452.73±7.27 | 407.92±4.43 | 373.66±1.01**| 446.12±7.26 | 415.81±5.35 |
| BW (6 weeks)                      | 543.7±7.34 | 514.83±10.7**| 450.91±6.48**| 452.41±4.95**| 482.92±10.9 | 442.57±4.51 | 424.09±1.73**| 491.76±7.82 | 445.35±5.3  |
| BW (8 weeks)                      | 593.9±11.54 | 557.93±11.6**| 469.35±6.99**| 483.21±4.08**| 532.62±12.13 | 469.77±5.77 | 456.54±1.43**| 532.42±9.87 | 458.01±8.89 |
| BW (10 weeks)                     | 620.99±11.01 | 591.23±12.73**| 522.93±8.28**| 507.67±3.92**| 565.62±15.49 | 519.37±5.52 | 485.34±1.93**| 568.32±10.06 | 506.53±7.33 |
| BW (12 weeks)                     | 645.3±11.58 | 607.5±13.46**| 548.92±9.98**| 517.17±12.09**| 587.32±16.23 | 550.43±6.92 | 513.34±15.3**| 595.86±11.85 | 539.51±7.66 |
| Lung weight, g                    | 2.57±0.17 | 2.29±0.07   | 2.22±0.07   | 2.12±0.08* | 2.21±0.07   | 2.16±0.06   | 2.07±0.08** | 2.32±0.06   | 2.17±0.04   |
| **Blood pressure**                |          |             |             |           |             |             |           |             |             |
| Heart rate, bpm                   | 387.5±17.89 | 364.5±9.63  | 340.67±6.98**| 405.8±8.9 | 384.8±7.43  | 372.73±8.35**| 383.32±11.06 | 365.1±7.73 | 378.10±15.00**|
| SBP, mm Hg                        | 131.00±2.38 | 137.1±4.05  | 140.86±3.78* | 94.8±2.92** | 103.33±3.00## | 122.33±3.1## | 99.7±3.26** | 118.3±1.93## | 128.8±2.34## |
| **Histology of lung**             |          |             |             |           |             |             |           |             |             |
| Lung injury score                 | 1.3±0.21 | 1.6±0.16    | 1.47±0.13   | 1.4±0.16  | 1.6±0.16    | 1.27±0.23   | 1.22±0.15  | 1.44±0.18  | 1.14±0.18   |
| Alveolar wall thickness, µm       | 7.61±0.17 | 7.34±0.11   | 7.24±0.14   | 6.8±0.1  | 6.87±0.14   | 6.93±0.23   | 7.35±0.15  | 6.98±0.13  | 7.12±0.22   |
| Alveolar airspace area ratio, %   | 43.84±3.37 | 43.46±3.43  | 38.04±2.23  | 42.56±3.45 | 38.23±3.32  | 39.04±2.98  | 46.14±2.45 | 37.28±2.61 | 44.49±2.28   |
| Circumference of alveoli in the unit area, µm/µm²×100% | 8.15±0.29 | 7.72±0.28   | 7.53±0.21   | 7.55±0.28 | 7.25±0.17   | 7.55±0.18   | 7.9±0.24   | 7.55±0.22   | 7.81±0.25    |
| Fibrosis area, %                  | 8.51±0.64 | 8.46±0.55   | 9.28±0.51   | 8.34±0.5  | 7.68±0.66   | 8.37±0.65   | 9.49±0.84  | 9.7±0.66   | 9.12±0.72    |

Values are given as mean±SEM. ND, normal diet; SD, salt diet; PBO, placebo; TELM, telmisartan; ENAL, enalapril. *p < 0.05 versus ND + PBO. **p < 0.01 versus ND + PBO. ***p < 0.01 versus 2% SD + PBO. ☆p < 0.05 versus 8% SD + PBO. ☆☆p < 0.01 versus 8% SD + PBO.
circumference was calculated according to the peripheral lengths of each empty alveolus in the unit area using the same images to measure the thickness of the alveolar wall. Approximately 60–80 lines per field were drawn perpendicular to the narrowest segment of alveolar septa, and the mean length of lines was calculated [18]. Lung samples were stained with Sirius Red for fibrosis assessment and were immunostained for ACE2 (mouse monoclonal anti-ACE2 antibody, 1:100; sc-390851, Santa Cruz, CA, USA), TMPRSS2 (rabbit polyclonal anti-TMPRSS2 antibody, 1:50; ab92323, Abcam, Cambridge, UK), and surfactant protein C (SFTPC) (rabbit polyclonal anti-SFTPC antibody, 1:50; 10774-1-AP, Proteintech, USA). The fluorescent images were captured by a BZ 9000 microscope (Keyence, Neu-Isenburg, Germany) (20 images for each sample). Then, the intensity of immunofluorescence signal in each image was measured by Image J software as previously described [20].

Real-Time Quantitative PCR
Expression levels of Ace2 or Tmprss2 in lung tissues were assessed by real-time quantitative PCR using a standard protocol. Briefly, total RNA was isolated from the lung using Trizol reagent (TaKaRa, Dalian, Liaoning, China). The cDNA was synthesized using oligo-dT and random primers (TaKaRa). Primer sequences designed using the Primer 5 software were as follows: Ace2 forward 5′-TGCGTATGAATGGACGACA-3′, reverse 5′-CTGCTTCTAGCACATGGA-3′; and β-actin forward 5′-CTGGCTTCTAGCACCACATGGA-3′, reverse 5′-AAAACGCGAGCTGAACACGTC-3′. qRT-PCR was performed using a Bio-Rad CFX96 cycler (Bio-Rad Laboratories, USA). Data analysis was performed using Applied Biosystems Comparative CT method.

Statistics
Unless otherwise noted, all data are shown as means ± SEM. By using the Shapiro-Wilk test, the distribution of data was checked for normality. The minimal significant difference test was employed to all data after one-way analysis of variance. We regarded <0.05 as a significant p value. GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA) and SPSS version 22.0 (SPSS, Chicago, IL, USA) were used for data analysis.

Results
Body Weight, Lung Weight, and Blood Pressure
Body weight and lung weight of rats in each group are indicated in Table 2. The body weight of rats in the 8%
NaCl diet group was significantly decreased when compared with the control group from the 2nd week, and also in the 2% NaCl diet group from the 6th week. Though body weights mostly were lower in the treatment groups, than in the ND + PBO group, body weight within the telmisartan and enalapril increased with higher dietary sodium content. The lung weight is also lower in 8% SD group than that in control group (Table 2). The rats fed with 8% salt diet depicted increased SBP as compared to rats with control diet (see Table 2). As expected, both telmisartan and enalapril administration for 12 weeks significantly decreased SBP when compared to the ND + PBO and 8% SD + PBO group.

**Lung Morphology**

We next examined the morphological changes in the lung. Lung injury score, alveolar wall thickness, alveolar airspace and circumference, and fibrosis area were assessed, but comparisons among the groups did not find any statistically significant treatment-related differences (Table 2).

**Localization of ACE2 and TMPRSS2 in the Lung of Rats**

Based on the anatomical shape and distribution, we considered that in the control lung tissues, both ACE2 as well as TMPRSS2 were very well expressed in the bronchial epithelium. In addition, ACE2 was also expressed in alveolar type 2 (AT2) cells, which is further confirmed by colocalization of ACE2 and surfactant protein C (SFTPC) – a biomarker of AT2 cells – in the lung of rat (Fig. 1).**

**Effect of High-Salt Diet and RAAS Blockade on ACE2 and TMPRSS2 Protein Expression in the Lung**

The ACE2 protein expression in the lung tissues in 8% SD diet group was significantly increased compared with that in lung tissues in control ($p < 0.05$), but neither telmisartan nor enalapril mitigated this effect (Fig. 2a, d). We also noted that neither telmisartan nor enalapril medication altered lung ACE2 protein expression (Fig. 2b). Among the groups, neither a high-salt diet nor a RAAS blockade affected TMPRSS2 expression (Fig. 3).

---

![Figure 2](image-url)  
**Fig. 2.** ACE2 protein expression in the lung in different groups.  
(a) Photomicrographs of immunofluorescence-stained lungs. Red color indicates ACE2.  
(b) Effect of high-salt diet on the protein expression of ACE2 in the lung.  
(c) Effect of RAAS blockade on the protein expression of ACE2 in the lung.  
(d, e) Comparison of ACE2 protein expression in the lung between rats fed with high-salt diet and treated with RAAS blockade and rats only fed with high-salt diet (magnification: ×20; scale bars: 100 μm). Data are expressed as mean ± SEM. *$p < 0.05$ versus ND + PBO. ND, normal diet; SD, salt diet; PBO, placebo; TELM, telmisartan; ENAL, enalapril.
Effect of High-Salt Diet and RAAS Blockade on Ace2 and Tmprss2 mRNA Expression in the Lung

We investigated whether either Ace2 or Tmprss2 mRNA level are affected by high salt or RAAS blockade. There was no alteration in Ace2 and Tmprss2 mRNA levels in the lung of rats fed with high-salt diet (Fig. 4a, b). Neither telmisartan nor enalapril treatment affected lung Ace2 and Tmprss2 mRNA expression (Fig. 4c, d). Also, no significant difference on Ace2 and Tmprss2 mRNA expression in the lung of rats fed with high-salt diet and treated with RAAS blockade compared with that in rats only fed with high-salt diet was found (Fig. 4e–h).

Discussion

The first critical step in the SARS-CoV-2 infection process is binding of the spike protein of the virus to the cell membrane-bound ACE2 protein in the upper and lower respiratory tract epithelial cells [21]. The ACE2 is a component of the RAAS, a key system regulating renal salt handling in mammals, and salt content of the food vice versa regulates the activity of the RAAS [22]. However, it was unknown so far whether or not salt intake as well as treatment with medications interfering with the RAAS might affect the expression of ACE2 in the respiratory tract. Aside from ACE2, cell entry-associated molecules (e.g., Tmprss2) might also take part in SARS-CoV-2 infection. It seems that their expression in the lung plays a pivotal role in the development of SARS-CoV-2-induced lung damage in animal models [10, 16] as demonstrated, for example, in ACE2 knockout mice who are protected from infection with coronaviruses [23].

Viral persistence along with overall disease severity may be influenced by genetics and behavioral host factors. The present study aimed to address one fundamental question: does salt intake and RAAS blockade alter pulmonary expression of the host factors for SARS-CoV-2 infection ACE2 or Tmprss2? In this experiment, we analyzed the expression of ACE2 and Tmprss2 both on mRNA and protein level in the lung tissue of rats exposed to high-salt diet and treated with RAAS blockade.
The findings of this study offer histological confirmation that ACE2 and TMPRSS2 are highly expressed in bronchial epithelium, and ACE2 is also expressed in AT2 cells. High-salt diet exposure in rats increased ACE2 expression on protein level but not on mRNA level. Treatment with RAAS blockade has no effect on the expression of both ACE2 and TMPRSS2 in lung tissues of rats. These latter findings present biological evidence for the continuous use of RAAS blockers in individuals with hypertension or cardiovascular disease during the COVID-19 pandemic as before. Additionally, our research for the first time provides evidence that high salt intake, as it is frequently used in many countries, even more so during the COVID-19 pandemic, might prove to be a novel risk factor for adverse outcome of SARS-CoV-2 infections. Our study should hopefully stimulate clinical work addressing salt intake in relation to clinical outcome of SARS-CoV-2 infection.

In this study, we found the body weight decreased significantly in rats fed with high-salt diet, which was consistent with earlier studies [24]. Mechanically, some studies clarified that the low body weight in rats with high-salt diet was due to an increase in BAT UCP1 expression and the consequent higher energy expenditure [25]. Several animal studies indicate excessive salt intake chronically may result in pressure overload, and in addition even may trigger blood pressure-independent target organ injury, including cardiac and renal dysfunction [7, 26]. In humans, it has long been known that high salt intake is associated with the aggravation of hypertension [27]. Consistent with these previous reports, our study further confirmed that high-salt diet intake can lead to a rise in blood pressure in a rat model. Mechanically, the structural, functional, and pathophysiological alterations triggered by chronic salt overload could be mediated through the dysregulation of the RAAS activity [28, 29].

A high intake of NaCl in the diet has been linked with cardiac and renal dysfunction, but it is not known whether high levels of NaCl have an effect on the lungs. As a result of osmotic gradients driven by Na, K, and Cl ions in the alveolar epithelium, fluid passes passively from the air spaces to the interstitial spaces. In addition to prevent-

---

Fig. 4. mRNA expression of Ace2 and Tmprss2 in the lung in different groups. a, b Effect of high-salt diet on the mRNA expression of Ace2 (a) and Tmprss2 (b) in the lung. c, d Effect of RAAS blockade on the mRNA expression of Ace2 (c) and Tmprss2 (d) in the lung. e–h Comparison of Ace2 (e, g) and Tmprss2 (f, h) mRNA expression in the lung between rats fed with high-salt diet and treated with RAAS blockade and rats only fed with high-salt diet. Data are expressed as mean ± SEM. ND, normal diet; SD, salt diet. PBO, placebo; TELM, telmisartan; ENAL, enalaprill.
ing alveolar edema after alveolar damage, this process maintains efficient gas exchange [30]. One study reported a low-salt diet might attenuate lung fibrosis triggered by bleomycin [31]. In the current study, we did not observe obvious morphological changes in the lung tissues from rats fed with high-salt diet.

Since the epidemic of COVID-19, an expanding number of research studies have shown that too much sodium intake is clearly associated with a variety of COVID-19 comorbid conditions such as hypertension, cardiovascular disease, and renal disease [5]. We assessed the expression levels of ACE2 as well as TMPRSS2 recognizing two important molecules related to COVID-19. Our finding that ACE2 protein expression is increased in the lung of rats exposed to high-salt diet, without change in mRNA level, which is consistent with earlier findings observed in the kidney of mice [32] and a observational study on lung tissues of type 2 diabetes patients [33]. A recent review article demonstrated that the post-transcriptional regulation of ACE2 is essential to determine its protein abundance [34]. Apart from transcript abundance, many factors have an impact on the expression of a protein, including translation rates, protein synthesis delay, regulation of protein half-life, and protein transport [16, 35]. As for ACE2, potential microRNAs [36] and protein shedding should be also taken into account [37].

However, no differences were found in the expression of TMPRSS2 both in mRNA and protein levels in the present study. In addition, we observed TMPRSS2 was predominantly expressed in bronchial epithelium in rat lungs. In contrast, the distribution of ACE2 was more diffuse and equally present in both the bronchioles and AT2 cells. The differences observed in present study indicate that ACE2 and TMPRSS2 may have an independent relationship, although these two proteins are reported to interact previously [38] and often considered together during the COVID-19 pandemic [3]. There is no denying that TMPRSS2 is primarily related to prostate cancer [39].

The controversial hypotheses and evidence regarding the relationship between RAAS blockade and ACE2 expression and COVID-19-linked outcomes lead to a surge of studies on evaluating the safety and effectiveness of administration of ACEIs/ARBs during COVID-19 [40, 41]. Some studies showed that RAAS blockade upregulated the ACE2 expression in patients with comorbidities, which potentially predisposed patients on these medications to severe infection of SARS-CoV-2 [11, 42]. However, some studies support that ACEIs/ARBs have no effect on ACE2 concentration, activity, or expression [16, 43, 44]. Our experimental study found that RAAS blockade does not affect the lung tissue expression of rats’ ACE2 and TMPRSS2.

Some limitations exist in our study. To begin with, there may be differences in ACE2 or TMPRSS2 expression patterns between rats and humans. We identified that ACE2 was prominently expressed in epithelial cells of bronchiole and AT2 cells of the lung in rats. However, in humans, ACE2 was mainly localized to ciliated epithelial cells and was almost completely absent in all cell types except for rare AT2s [45]. In addition, the evidence from single-cell analyses in human pulmonary tissue indicated that ACE2 was principally in bronchial transitory secretory cells [46], club cells, bronchiolar basal cells, ciliated cells in addition to type II pneumocytes [16, 47]. Second, dosing of ACEI (enalapril) and ARB (telmisartan) was based on previous publications. However, given the profound reduction in SBP with both antihypertensives in our study, the dose we used was an effective dose. Finally, we just analyzed male rats. In addition, we analyzed ACE2 and TMPRSS2 expression just based on computer-aided image analysis of immunostaining but not using Western blotting.

**Conclusion**

In brief, our study offers useful information about the SARS-CoV-2 cell entry receptor (ACE2) as well as the essential linked protease (TMPRSS2) expression in the lung of rats exposed to high-salt diet and treated with RAAS blockade. The finding that high salt intake increases pulmonary ACE2 expression raises clinical questions about the link of salt intake and clinical outcome of SARS-CoV-2 infections in humans and might stimulate clinical work addressing this important nutritional question.

**Acknowledgments**

We acknowledge and appreciate our colleagues for their valuable efforts made on this work.

**Statement of Ethics**

All animal experiment protocols were reviewed and approved by Hunan Normal University’s Experimental Animal Center (Permit number: D2020008).
Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This project was supported by the National Natural Science Foundation of China (Grant No. 81833841), Hunan Province Natural Science Foundation (Grant No. 2018JJ3366), Huxiang Young Talents project (Grant No. 2021RC3094), Hunan Province Science and Technology Plan (Grant No. 2014SK3003), and a China Scholarship Council (CSC) grant to the first author Xiaoli Zhang.

References

1 Liu Y, Yang Y, Zhang C, Huang F, Wang F, Yuan J, et al. Clinical and biochemical indexes from 2019-nCoV infected patients linked to viral loads and lung injury. Sci China Life Sci. 2020 Mar;63(3):364–74.
2 Zheng S, Fan J, Yu F, Feng B, Lou B, Zou Q, et al. Viral load dynamics and disease severity in patients infected with SARS-CoV-2 in Zhejiang province, China, January–March 2020: retrospective cohort study. BMJ. 2020 Apr 21; 369:m1443.
3 Hoffmann M, Kleine-Weber H, Schroeder S, Kruger N, Herrler T, Erichsen S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Cell. 2020 Apr 16;181(2):271–e8.
4 de Wardener HE, MacGregor GA. Harmful effects of dietary salt in addition to hypertension. J Hum Hypertens. 2002 Apr;16(4):213–23.
5 Brown RB. Sodium toxicity in the nutritional epidemiology and nutritional immunology of COVID-19. Medicina. 2021 Jul 22;57(8):739.
6 Lan J, Ge J, Yu J, Shan S, Zhou H, Fan S, et al. Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. Nature. 2020 May;581(7807):215–20.
7 Berger RC, Vassallo PF, Crajoinas O, Oliveira ML, Martins FL, Nogueira BV, et al. Renal effects and underlying molecular mechanisms of long-term salt content diets in spontaneously hypertensive rats. PLoS One. 2015;10(10):e0141288.
8 Samuel P, Ali Q, Sabuhi R, Wu Y, Hussain T. High Na intake increases renal angiotensin II levels and reduces expression of the ACE2-AT1(0.4)R MasR axis in obese Zucker rats. Am J Physiol Renal Physiol. 2012 Aug 1;363(3):F412–9.
9 Takeda Y, Zhu A, Yoneda T, Usukura M, Takata H, Yamagishi M. Effects of aldosterone and angiotensin II receptor blockade on cardiac angiotensinogen and angiotensin-converting enzyme 2 expression in Dahl salt-sensitive hypertensive rats. Am J Hypertens. 2007 Oct;20(10):1192–4.
10 Chu C, Zeng S, Han AA, Hocher CF, Krämer BK, Hocher B. Comparison of infection risks and clinical outcomes in patients with and without SARS-CoV-2 lung infection under renin-angiotensin-aldosterone system blockade: systematic review and meta-analysis. Br J Clin Pharmacol. 2021 Jun;87(6):2475–92.
11 Fang L, Karakiulakis G, Roth M. Are patients with hypertension and diabetes mellitus at increased risk for COVID-19 infection? Lancet Respir Med. 2020 Apr;8(4):e21.
12 Guo J, Huang Z, Lin L, Lv J. Coronavirus disease 2019 (COVID-19) and cardiovascular disease: a viewpoint on the potential influence of angiotensin-converting enzyme inhibitors/angiotensin receptor blockers on onset and severity of severe acute respiratory syndrome coronavirus 2 infection. J Am Heart Assoc. 2020 Apr 7;9(7):e016219.
13 Li J, Wang X, Chen J, Zhang H, Deng A. Association of renin-angiotensin system inhibitors with severity or risk of death in patients with hypertension hospitalized for coronavirus disease 2019 (COVID-19) infection in Wuhan, China. JAMA Cardiol. 2020 Jul 1;5(7):825–30.
14 Srimat K, Insal PA. Risks of ACE inhibitor andARB usage in COVID-19: evaluating the evidence. Clin Pharmacol Ther. 2020 Aug;108(2):236–41.
15 Vaduganathan M, Vardeny O, Michel T, McMurray JIV, Pfeffer MA, Solomon SD. Renin-angiotensin-aldosterone system inhibitors in patients with covid-19. N Engl J Med. 2020 Apr 23;382(17):1653–9.
16 Batchu SN, Kaur H, Yerra VG, Advani SL, Kabir MG, Liu Y, et al. Lung and kidney ACE2 and TMPRSS2 in renin-angiotensin system blocker–treated comorbid diabetic mice mimicking host factors that have been linked to severe COVID-19. Diabetes. 2021 Mar;70(3):759–71.
17 Estler M, Estler D. Can angiotensin receptor–blocking drugs perhaps be harmful in the COVID-19 pandemic? J Hypertens. 2020 May;38(5):781–2.
18 Ashour K, Shan L, Lee JH, Schlicher W, Wada K, Wada E, et al. Bombesin inhibits alveolarization and promotes pulmonary fibrosis in newborn mice. Am J Respir Crit Care Med. 2006 Jun 15;173(12):1377–85.
19 Taki T, Masumoto H, Funamoto M, Minakata K, Yamazaki K, Ikeda T, et al. Fetal mesenchymal stem cells ameliorate acute lung injury in a rat cardiopulmonary bypass model. J Thorac Cardiovasc Surg. 2017 Mar;153(3):726–34.
20 Hasan AA, von Websky K, Reichtzeder C, Tsuprykov O, Gaballa MMS, Guo J, et al. Mechanisms of GLP-1 receptor-independent renoprotective effects of the dipeptidyl peptidase type 4 inhibitor linagliptin in GLP-1 receptor knockout mice with 5/6 nephrectomy. Kidney Int. 2019 Jun;95(6):1373–88.
21 Xiao L, Sakagami H, Miwa N. ACE2: the key molecule for understanding the pathophysiology of severe and critical conditions of COVID-19: demon or angel? Viruses. 2020 Apr 28;12(5):491.

Author Contributions

Berthold Hocher designed the study. Xiaoli Zhang, Liping Liu, and Li Xie performed the animal experiments and statistical analysis. Xiaoli Zhang, Mohamed M. S. Gaballa, and Yingquan Xiong performed morphological analysis. Xiaoli Zhang, Ahmed A. Hasan, Jian Li, and Berthold Hocher checked quality of the data. Xiaoli Zhang and Mohamed M. S. Gaballa drafted the manuscript. Thomas Klein, Denis Delic, Bernhard K Krämer, Burkhard Kleuser, Jian Li, and Berthold Hocher contributed to the revisions of the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.
Salt Intake and RAAS Blockade in COVID-19

22 Michaud V, Deodhar M, Arwood M, Al Rihani SB, Dow P, Turgeon J. ACE2 as a therapeutic target for COVID-19; its role in infectious processes and regulation by modulators of the RAAS system. J Clin Med. 2020 Jul 3; 9(7):2096.

23 Kuba K, Imai Y, Rao S, Gao H, Guo F, Guan B, et al. A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. Nat Med. 2005 Aug; 11(8):875–9.

24 Prada P, Okamoto MM, Furukawa LN, Machado UF, Heimann JC, Dolnikoff MS. High- or low-salt diet from weaning to adulthood: effect on insulin sensitivity in Wistar rats. Hypertension. 2000 Jan;35(1 Pt 2):424–9.

25 Coelho MS, Passadore MD, Gasparetti AL, Bibancos T, Prada PO, Furukawa LL, et al. High-salt or low-salt diet and ADAM17 cleavage: ethanol metabolism in rats. Nutr Metab Cardiovasc Dis. 2006 Mar; 16(2): 148–55.

26 Shimoura CG, Lincevicus GS, Nishi EE, Girardi AC, Simon KA, Bergamaschi CT, et al. Increased dietary salt changes baroreceptor sensitivity and intrarenal renin-angiotensin system in goldblatt hypertension. Am J Hypertens. 2017 Jan;30(1):28–36.

27 He Y, Yang W, Liu S, Gan L, Zhang F, Mu C, et al. Interactions between angiotensin-converting enzyme-2 polymorphisms and high salt intake increase the risk of hypertension in the Chinese Wa population. Int J Clin Exp Pathol. 2017;10(11):11159–68.

28 Te Riet L, van Esch HJ, Roks A, van den Mei-racker AH, Danser AH. Hypertension: renin-angiotensin-aldosterone system alterations. Circ Res. 2015 Mar 13;116(6):960–75.

29 Cao G, Della Penna SL, Kouyoundzian NM, Choi MR, Gorzalczyzny S, Fernández BE, et al. Immunohistochemical expression of intrarenal renin angiotensin system components in response to tempol in rats fed a high salt diet. World J Nephrol. 2017 Jan 6;66(1):29–40.

30 Hummeler E, Barker P, Beermann F, Gatzky J, Verdugo C, Boucher R, et al. Role of the epithelial sodium channel in lung liquid clearance. Chest. 1997 Jun;111(6 Suppl 1):113S–116S.

31 Chen W, Pilling D, Gomer RH. Dietary NaCl effects bleomycin-induced lung fibrosis in mice. Exp Lung Res. 2017 Nov–Dec;43(9–10): 395–406.

32 Wysocki J, Ye M, Soler MJ, Gurley SB, Xiao HD, Bernstein KE, et al. ACE and ACE2 activity in diabetic mice. Diabetes. 2006 Jul;55(7): 2132–9.

33 Wijnant SRA, Jacobs M, Van Eeckhoutte HP, Lapauw B, Joos GF, Bracke KR, et al. Expression of ACE2, the SARS-CoV-2 receptor, in lung tissue of patients with type 2 diabetes. Diabetes. 2020 Dec;69(12):2691–9.

34 Sparks MA, South AM, Badley AD, Baker-Smith CM, Battle D, Bozkurt B, et al. Severe acute respiratory syndrome coronavirus 2, COVID-19, and the renin-angiotensin system: pressing needs and best research practices. Hypertension. 2020 Nov;76(5):1350–67.

35 Liu Y, Beyer A, Aebersold R. On the dependence of cellular protein levels on mRNA abundance. Cell. 2016 Apr 21;165(3):535–50.

36 Alavi S, Bouskila D, Nigam S, Jhaveri K, et al. Increased expression of ACE2, the SARS-CoV-2 receptor, in lung tissue of patients with type 2 diabetes. Diabetes. 2020 Dec;69(12):2691–9.

37 Liu Y, Beyer A, Aebersold R. On the dependence of cellular protein levels on mRNA abundance. Cell. 2016 Apr 21;165(3):535–50.

38 Heurich A, Hofmann-Winkler H, Gierer S, Schulz C, et al. RAAS and ADAM17 cleave ACE2 differentially and only proteolysis by TMPRSS2 augments entry driven by the severe acute respiratory syndrome coronavirus spike protein. J Virol. 2014 Jan;88(2):1293–307.

39 Lin B, Ferguson C, White JT, Wang S, Vessella R, True LD, et al. Prostate-localized and androgen-regulated expression of the membrane-bound serine protease TMPRSS2. Cancer Res. 1999 Sep 15;59(17):4180–4.

40 Hanfl TC, Harhay MO, Brown TS, Cohen JB, Moharab AM. Is there an association between COVID-19 mortality and the renin-angiotensin system? A call for epidemiologic investigations. Clin Infect Dis. 2020 Jul 28;71(15):870–4.

41 Sparks MA, South A, Welling P, Luther JM, Cohen J, Byrd JB, et al. Sound science before quick judgement regarding RAS blockade in COVID-19. Clin J Am Soc Nephrol. 2020 May 7;15(5):714–6.

42 Elshafei A, Khidir EG, El-Husseiny AA, Gomaa MH. RAAS, ACE2 and COVID-19: a mechanistic review. Saudi J Biol Sci. 2021 Nov;28(11):6465–70.

43 Wysocki J, Loes E, Ye M, Soler MJ, Battle D. Kidney and lung ACE2 eExpression after an ACE inhibitor or an Ang II receptor blocker: implications for COVID-19. J Am Soc Nephrol. 2020 Sep;31(9):1941–3.

44 Cohen JB, South AM, Shaltout HA, Sinclair MR, Sparks MA. Renin-angiotensin system blockade in the COVID-19 pandemic. Clin Kidney J. 2021 Mar;14(Suppl 1):448–59.

45 Soni S, Jiang Y, Tesfaiyi T, Hornick JL, Catalapo S. Comparative analysis of ACE2 protein expression in rodent, non-human primate, and human respiratory tract at baseline and after injury: a conundrum for COVID-19 pathogenesis. PLoS One. 2021;16(2): e0247510.

46 Lukassen S, Chua RL, Treffner T, Kahn NC, Schneider MA, Muley T, et al. SARS-CoV-2 receptor ACE2 and TMPRSS2 are primarily expressed in bronchial transient secretory cells. EMBO J. 2020 May;18;39(10):e105114.

47 Sunnak W, Huang N, Bécavin C, Berg M, Queen R, Litvinukova M, et al. SARS-CoV-2 entry factors are highly expressed in nasal epithelial cells together with innate immune genes. Nat Med. 2020 May;26(5):681–7.