Supplement to Research Letter

'Kidney and Lung ACE2 expression after an ACE inhibitor or an Ang II receptor blocker: implications for COVID-19'

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METHODS

Animal Models

All studies were conducted with the review and approval of the Institutional Animal Care and Use Committee of Northwestern University. Organs from two genetic models of ACE kidney ablation (ACE.4 and ACE 8/8) were generously provided by Drs Hong D. Xiao and Kenneth Bernstein. ACE.4 mice have the somatic ACE promoter replaced by the kidney androgen-regulated protein promoter which in these mice is essentially non-functional[1]; in the absence of exogenous androgens the levels of kidney ACE are less than 1% of normal and no ACE is detected in other organs ; this model is overtly hypotensive [1]. The effect of localized kidney ACE deficiency on kidney ACE2 levels was also examined in ACE8/8 mouse which is a model lacking ACE in the kidney or vascular endothelium, but with 100-fold normal cardiac ACE levels[2]. In addition, ACE8/8 mice have significant levels of ACE activity in the lung and in the blood plasma [2]. In this animal model, kidney represents a major change from wild-type mice as this organ normally expresses a substantial amount of ACE activity in both vascular endothelium and proximal tubular epithelium[2]. The ACE8/8 mice have a near normal blood pressure and do not exhibit any gross abnormalities in kidney function[2]. Both ACE models used in this study have a mixed C57/129 background[1, 2].

To examine the effect of pharmacological ACE inhibition on ACE2, two groups of 12-14 weeks old C57BLKS/J mice were assigned to drink either tap water (n=8) or tap water with an ACE inhibitor, captopril, (n=8) at a dose of 120 $mg*kg^{-1}*day^{-1}$ for 14 days. Two other experimental groups of 12-14 weeks old C57BLKS/J mice were randomly assigned to drink either tap water (vehicle, n=6) or tap water with an angiotensin II receptor antagonist, telmisartan (Boehringer Ingelheim), at a dose of 2 $mg*kg^{-1}*day^{-1}$ (n=6) for 14 days. Before captopril and telmisartan

administration, mice were weighted and the daily fluid intake per mouse was recorded to estimate the concentration of the compound needed to be added to the drinking water. Also during the drug administration, water consumption and body weights were controlled to make appropriate adjustments.

RNA isolation and reverse transcriptase real-time PCR

RNA was isolated from kidney cortex with Trizol reagent (GIBCO Invitrogen). Quantitative real-time PCR was performed using the TaqMan Gold RT-PCR kit and ABI Prism 7700 (Applied Biosystems) sequence-detection system. Primers and probes for ACE were designed using Primer Express software (Applied Biosystems). The sequences of forward, reverse primer, and probe for ACE, ACE2 and Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were the same as described before[3]. Reverse transcription was carried out for 30 min at 48°C. The ACE and ACE2 mRNA levels of the samples were normalized to their G3PDH contents. Experiments were carried out in triplicate for each data point.

Protein extraction and measurement of enzymatic activity for ACE2

For total cell lysates, tissues (kidney cortex and lungs) were homogenized in a buffer consisting of (in mmol/l) 50 HEPES, pH 7.4, 150 NaCl, 0.5% Triton X-100, 0.025 $ZnCl_2$, and 1.0 phenylmethylsulphonyl fluoride and then clarified by centrifugation at 6,000g for 15 min. For membrane fraction isolation, a previously employed protocol was used[4] but without EDTA addition. After measuring protein concentration, tissue samples were diluted in a buffer (50 mmol/l 4-morpholineethanesulfonic acid, 300 mmol/l NaCl, 10 μmol/l $ZnCl_2$, and 0.01% Triton-X-100, pH 6.5), containing EDTA-free tablets. The plates were read using a fluorescence plate reader FLX800 (BIOTEK Instruments) at an excitation wavelength of 320 nm and an emission

wavelength of 400 nm. All reactions were performed at ambient temperature in microtiter plates with a 100 µl total volume [5, 6].

Western blot analysis

Total cell lysates and membrane preparations from kidney cortices and lungs were isolated as specified above and subjected to Western blot analysis as previously described[6]. For detection of ACE, nitrocellulose membranes were incubated with mouse monoclonal antibody (5C4, kind gift from Dr. Sergei Danilov). ACE2 protein was detected using our affinity purified rabbit anti-ACE2 antibody[5, 6]. Signals on Western blots were quantified by densitometry (Eagle Eye, Stratagene) and corrected for GAPDH (total cell lysates) or β-actin (membrane fraction) loading control.

Statistical analyses

The data were expressed as relative to the control (vehicle or wild-type) animal groups, assigning a value of 100% to the control baseline mean. The data were reported as mean ± standard error (SE). Significance was defined as $p < 0.05$. For comparisons between two independent means, the t-test was used.

Results

In ACE.4 mice, kidney ACE mRNA was about 7±4% of the mRNA levels in WT mice as previously reported [1]. Membrane kidney lysates from ACE.4 mice kidney cortex were examined for the presence of ACE and ACE2 protein (Figure S1A). The signal for ACE protein in kidneys from WT mice was detectable as a single band at the expected molecular weight of about 170 kD. In ACE.4 mice, ACE protein was barely detectable (equivalent of 3±1% of the WT) (Figure S1A). In ACE.4 mice, ACE2 protein abundance in kidney cortex total cell lysates and in isolated

membranes was reduced to 70 % and to 42% of the WT, $p<0.05$, respectively (Figure S1B and S1C).

Concordant with ACE2 protein expression, ACE2 activity was also reduced significantly in kidneys from ACE.4 mice as compared to WT controls (58.3±12% of the WT) (Figure S1D). By contrast , kidney cortex ACE2 mRNA levels in ACE.4 mice were not significantly different from those of their respective WT littermates (1.24±0.71 vs. 0.69±0.18 gene expression units, respectively) (Figure S1E).

ACE2 in kidneys from ACE8/8 mice, a model of kidney ACE deficiency and cardiac ACE over-expression

Consistent with results from Xiao et al.[2], in kidney cortex from ACE8/8 mice there was no detectable ACE protein expression (Figure S2A), whereas it was clearly present in WT kidneys (Figure S2A). ACE2 protein expression in isolated kidney membranes from ACE8/8 was significantly reduced (38±9% of WT mice) (Figure S2B). In ACE8/8 mice, ACE2 activity likewise was lower than in the wild-type but this difference was small and not statistically significant (90±17% of the WT) (Figure S2C). ACE2 mRNA levels in kidney cortex from ACE8/8 mice were not different from those of their respective WT littermates (1.02±0.04 vs. 1.00±0.07, respectively).

References

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Figure S1. ACE2 protein, enzymatic activity and mRNA in kidneys from ACE.4 mice and wild type controls.

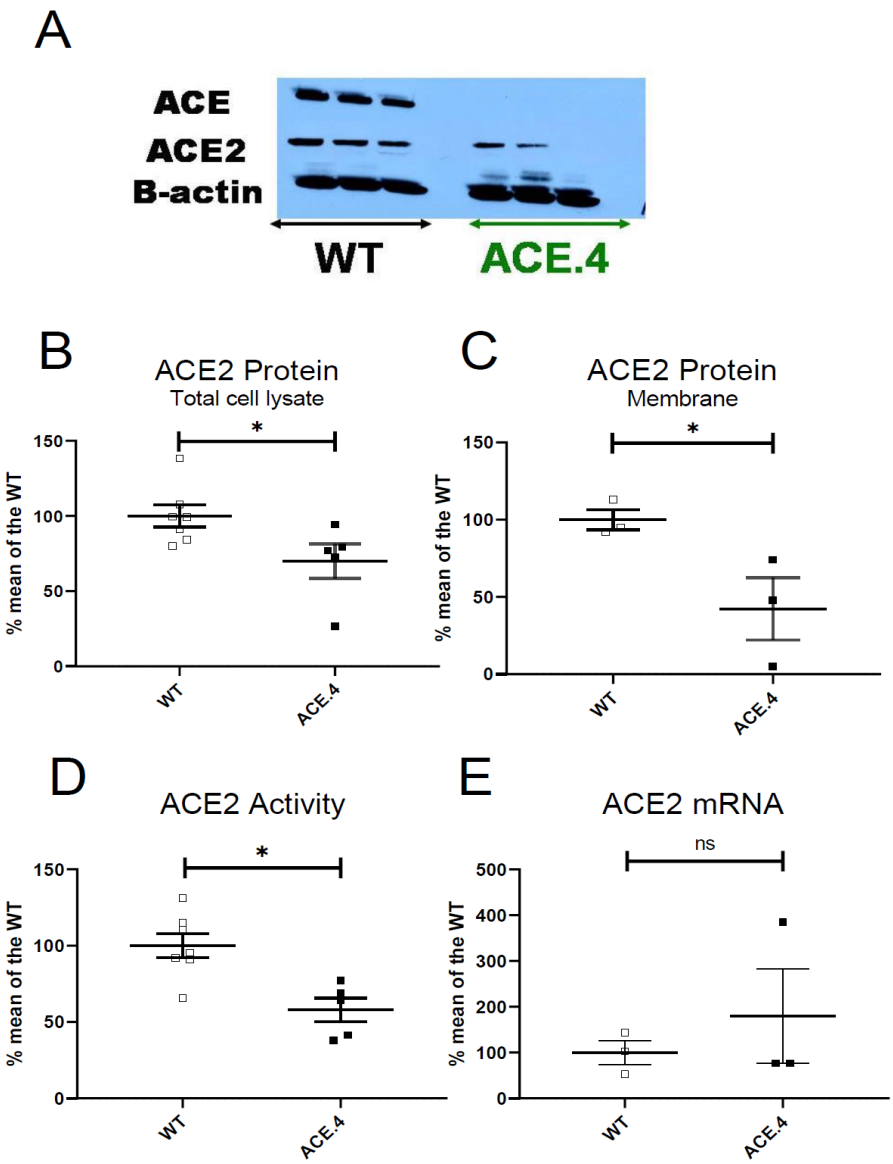


Figure S2. ACE2 protein, activity and m RNA in kidneys from ACE8-8 mice and wild type controls.

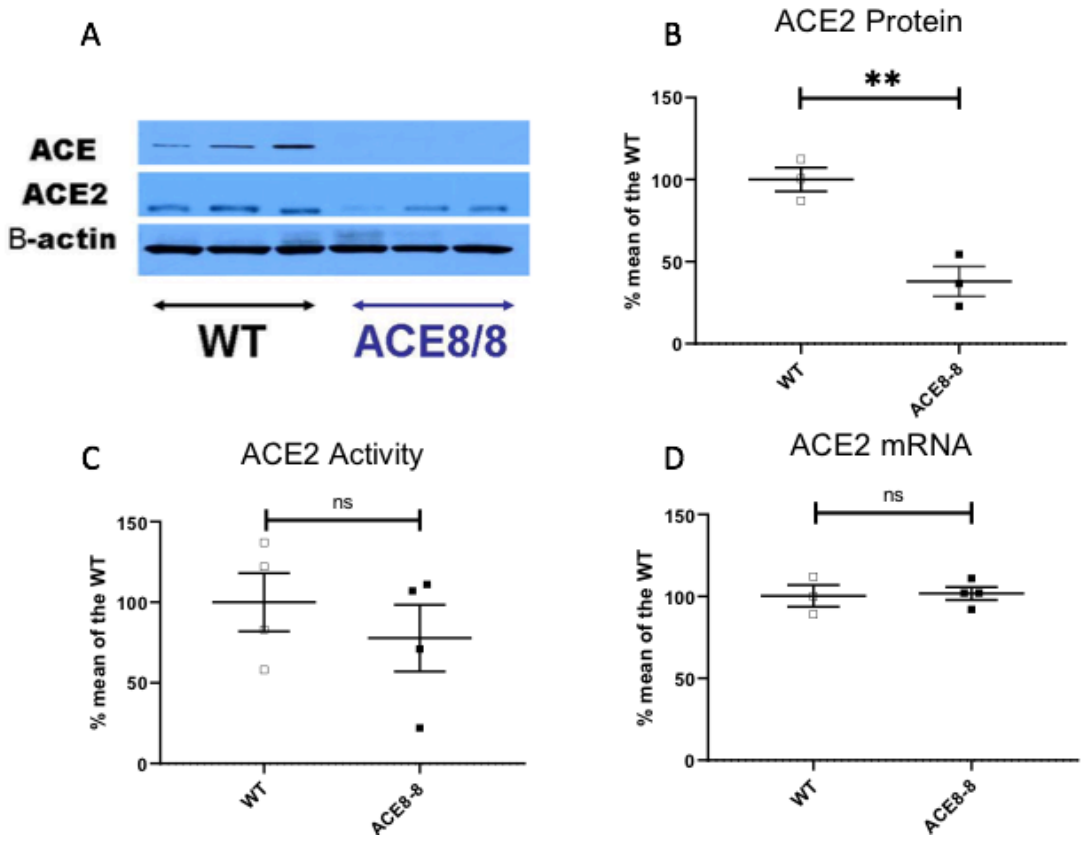
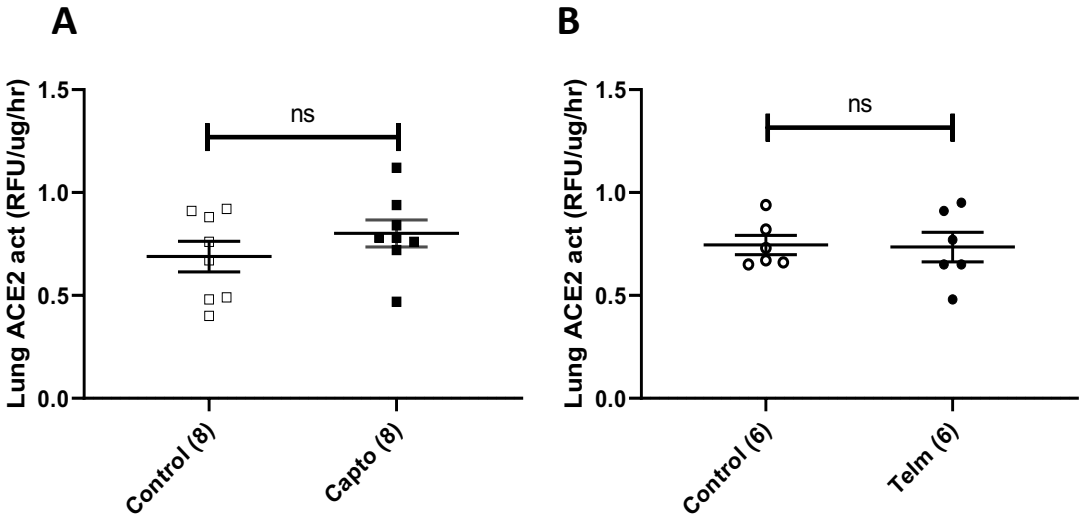


Figure S3. ACE2 activity in total cell lysates from lungs of captopril and telmisartan treated mice.



Supplemental Figure legends

Figure S1. ACE2 protein, enzymatic activity and mRNA in kidneys from ACE.4 mice and wild type controls. Panel A shows a representative Western blot for ACE, ACE2 and β-actin for three mice from each respective group in kidney membrane preparations. ACE2 protein in kidney total cell lysates (panel B), ACE2 protein in isolated membranes (panel C), ACE2 enzymatic activity (panel D) and ACE2 mRNA (panel E) * $p<0.05$.).

Figure S2. ACE2 protein, activity and mRNA in kidneys from ACE8-8 mice and wild type controls. Panel A shows a representative Western blot for ACE, ACE2 and β-actin for three mice from each respective group. Panel B shows ACE2 protein in isolated membranes . Panel C shows ACE2 enzymatic activity level and panel D ACE2 mRNA in cell lysates ** $p<0.01$.

Figure S3 ACE2 activity in total cell lysates from lungs of captopril (A) and telmisartan (B) treated mice. No significant differences were detected.