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Effects of Two Soluble ACE2-Fc Variants on Blood Pressure and Albuminuria in Hypertensive Mice: Research Letter

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Abstract

Background: Angiotensin-converting enzyme 2 (ACE2) hydrolyzes angiotensin (Ang) II to Ang-(1-7), promoting vasodilatation, and inhibiting oxidative stress and inflammation. Plasma membrane ACE2 is the receptor for all known SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) viral variants. In COVID-19 infection, soluble ACE2 variants may act as decoys to bind and neutralize the coronavirus, reducing its tissue infectivity. Furthermore, soluble ACE2 variants have been proposed as potential therapeutics for kidney disease and hypertensive disorders.

Objective: Soluble ACE2 variants conjugated to human Fc domains and selected for high-potency viral SARS-CoV-2 neutralization were prepared and evaluated for ACE2 activity in vitro. Lead candidates were then tested for systemic ACE2 activity, stability, and effects on blood pressure and albuminuria in mice with Ang II-induced hypertension.

Methods: ACE2 activity of 10 soluble ACE2 variants was first assessed in cell-free conditions using a fluorogenic substrate, or by Ang II hydrolysis to Ang-(1-7). Hypertension was induced in male or female mice by implantation of osmotic minipumps containing Ang II. Two lead ACE2 variants were injected intravenously (i.v.) into hypertensive mice, followed by measurements of blood pressure (tail-cuff plethysmography), albuminuria, and tissue ACE2 activity and protein (immunoblots).

Results: Soluble ACE2-Fc variants demonstrated significant ACE2 enzymatic activity, with kinetics comparable with human recombinant ACE2. In hypertensive mice, single dose i.v. injection of ACE2-Fc variant K (10 mg/kg) significantly decreased systolic blood pressure at 24 hours, with partial lowering sustained to 48 hours, and tendency to reduce albuminuria at 72 hours. By contrast, ACE2-Fc variant I had no effect on blood pressure or albuminuria in hypertensive mice; ACE2-Fc variant K was detected by immunoblotting in plasma, kidney, heart, lung, liver, and spleen lysates 72 hours after injection, associated with significantly increased ACE2 activity in all tissues except kidney and spleen. Angiotensin-converting enzyme 2-Fc variant I had no effect on plasma ACE2 activity.

Conclusions: Soluble ACE2-Fc variant K reduces blood pressure and tends to lower albuminuria in hypertensive mice. Furthermore, soluble ACE2-Fc variant K has prolonged tissue retention, associated with increased tissue ACE2 activity. The results support further studies directed at the therapeutic potential of soluble ACE2-Fc variant K for cardiovascular and kidney protection.

Abrégé

Contexte: L'enzyme de conversion de l'angiotensine 2 (ACE2) hydrolyse l'angiotensine (Ang) II en angiotensine (Ang)-(1-7), ce qui favorise la vasodilatation et inhibe le stress oxydatif et l'inflammation. L'ACE2 de la membrane plasmique est le récepteur de tous les variants connus du SARS-COV-2. Dans les cas d'infection à la COVID-19, les variants solubles de l'ACE2 peuvent agir comme leurres pour lier et neutraliser le coronavirus, et réduire ainsi son infectiosité dans les tissus. Des variants solubles de l'ACE2 ont également été proposés comme agents thérapeutiques potentiels pour l'insuffisance rénale et les troubles liés à l'hypertension.

Objectif: Des variants solubles de l'ACE2 conjugués au domaine Fc humain ont été sélectionnés pour leur fort potentiel neutralisant du virus SARS-COV-2, puis préparés et évalués pour la mesure de l'activité de l'ACE2 in vitro. Les meilleurs candidats ont ensuite été testés chez des souris souffrant d'hypertension induite par l'Ang II afin de mesurer l'activité d'ACE2, ainsi que leur stabilité et leurs effets sur la pression artérielle et l'albuminurie.

Méthodologie: L'activité de 10 variants solubles de l'ACE2 a d'abord été évaluée en conditions acellulaires à l'aide d'un substrat fluorogène, ou par hydrolyse de l'Ang II en Ang-(1-7). L'hypertension a été induite chez des souris mâles ou

femelles par l'implantation de minipompes osmotiques contenant de l'Ang II. Deux des meilleurs variants de l'ACE2 ont été injectés par voie intraveineuse (i.v.) à des souris hypertendues, puis des mesures de la pression artérielle (pléthysmographie par manchon caudal), de l'albuminurie, de l'activité de l'ACE2 dans les tissus et des protéines (immunobuvardage) ont été effectuées.

Résultats: Les variants solubles ACE2-Fc ont montré une activité enzymatique significative, avec une cinétique comparable à celle de l'ACE2 recombinante humaine. Chez les souris hypertendues, l'injection i.v. d'une dose unique (10 mg/kg) du variant K ACE2-Fc a abaissé significativement la pression artérielle systolique après 24 heures—une réduction partielle s'étant prolongée jusqu'à 48 heures—et a montré une tendance à réduire l'albuminurie après 72 heures. En revanche, le variant I ACE2-Fc n'a eu aucun effet sur la pression artérielle ou l'albuminurie des souris hypertendues. Après 72 heures, le variant K ACE2-Fc a été détecté par immunobuvardage dans le plasma, ainsi que dans des lysats de reins, de cœur, de poumon, de foie et de rate, ce qui a été associé à une augmentation significative de l'activité de l'ACE2 dans tous les tissus sauf dans les reins et la rate. Le variant I ACE2-Fc n'a montré aucun effet sur l'activité de l'ACE2 dans le plasma.

Conclusion: Le variant soluble K ACE2-Fc abaisse la pression artérielle et tend à diminuer l'albuminurie chez les souris hypertendues. Il présente en outre une rétention tissulaire prolongée, laquelle est associée à une plus grande activité de l'ACE2 dans les tissus. Ces résultats appuient d'autres études portant sur le potentiel thérapeutique du variant soluble K ACE2-Fc dans la protection cardiovasculaire et rénale.

Keywords

hypertension, kidney, ACE2, albuminuria, angiotensin

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Introduction

Angiotensin-converting enzyme 2 (ACE2) is a transmembrane monocarboxypeptidase, with high expression in the kidney.1 By removing 1 amino acid at the C-terminal of the vasoconstrictor angiotensin (Ang) II, ACE2 generates Ang-(1-7), which promotes vasodilation and anti-fibrotic/anti-inflammatory responses.1 In mice, gene deletion of ACE2 leads to accelerated glomerulosclerosis,2 supporting a protective effect of ACE2 in progression of chronic kidney disease.

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) uses the extracellular domain of ACE2 as receptor to invade host cells. SARS-CoV-2 promotes internalization of ACE2 and proteolytic cleavage of its extracellular domain, decreasing local ACE2 activity and contributing to tissue injury.1 The development of soluble ACE2 variants to neutralize SARS-CoV-2 and increase tissue ACE2 activity represents a therapeutic strategy in COVID-19. Recently, a human soluble ACE2 variant with an albumin-binding domain was shown to retain enzymatic activity, reduce blood pressure in mice treated with Ang II, and neutralize SARS-CoV-2 in kidney organoids.3 Furthermore, soluble human recombinant ACE2 lowers blood pressure and reduces albuminuria and histologic kidney injury in diabetic mice.4 Thus, soluble ACE2 variants could have therapeutic potential in cardiovascular and kidney disease.

The aim of the present study was to determine the in vitro and in vivo ACE2 enzymatic activities of novel ACE2-Fc variants, designed for high-affinity viral spike (S) protein binding and neutralization of SARS-CoV-2 infection. We selected 2 candidates for in vivo ACE2 activity to test the hypothesis that these variants lower blood pressure and albuminuria in mice with Ang II-induced hypertension.

Methods

ACE2-Fc Variants and Enzymatic Activity

Engineered ACE2-Fc variants were produced recombinantly in the National Research Council of Canada laboratories by transient transfection in Chinese Hamster ovarian cells and purified by protein A affinity chromatography followed by preparative size exclusion chromatography. ACE2 activity was determined by the ability to cleave the fluorogenic ACE2 peptide substrate Mca-APK(Dnp) (Anaspec, Fremont, CA, USA), in the presence or absence of the ACE2 inhibitor MLN-4760 (Calbiochem, San Diego, CA, USA), as we described.5 Angiotensin-(1-7) was measured by enzyme linked immunosorbent assay (ELISA; BMA Biomedicals, Augst, Switzerland).

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In vivo Studies

Male and female C57BL/6 mice (10-14 weeks) were implanted with osmotic minipumps subcutaneously (s.c.) containing Ang II or saline (controls) (Alzet, Cupertino, CA, USA). Ang II was administered for 3 weeks at 1000 ng/kg/min.6 Mice received a single intravenous (i.v.) injection of ACE2-Fc variants (I or K) (10 mg/kg), recombinant human (rh)ACE2 (2.5 mg/kg), or phosphate-buffered saline (PBS), 2.5 weeks after osmotic pump insertion. Seventy-two hours after injection, urine samples were obtained and mice were euthanized, with collection of plasma, kidneys, heart, lung, liver, and spleen. Urine albumin (μg/ml) was measured by ELISA (Bethyl Laboratories Inc., Montgomery, TX, USA), and corrected for creatinine concentration. Systolic blood pressure (SBP) was measured by tail-cuff plethysmography (BP-2000; Visitech Systems, Apex, NC, USA).

Immunoblotting

Immunoblots were performed with goat anti-ACE2 (1:1000, R&D systems, Minnesota, MN, USA), or horseradish peroxidase (HRP)-donkey anti-human immunoglobulin G (IgG) Fcγ (1:10 000, Jackson ImmunoResearch, West Grove, PA, USA).

Data Analyses

Data are presented as mean ± SEM, with statistical analyses using Prism 9.3.0 for Windows (GraphPad Software, San Diego, CA, USA). Statistical comparisons were made by 1-way analysis of variance (ANOVA), followed by either Tukey's or Dunnett's post-test. Significance was considered as $P < .05$.

Results

ACE2 Enzymatic Activity of ACE2-Fc Variants

The novel ACE2-Fc variants in this study carry structural modifications that allow high-affinity binding to SARS-CoV-2 S proteins. Briefly, ACE2-Fc variants consist of an N-terminal ACE2 catalytic domain (amino acids 18-614) linked to a C-terminal human IgG1 Fc fragment (CH2 and CH3 domains) via a GGGGS linker and a human IgG1 hinge region. Angiotensin-converting enzyme 2-Fc variants differ from each other by point mutations in the ACE2 domain and/or in the hinge region.

All 10 ACE2-Fc variants (labeled B-K) showed cell-free ACE2 activity comparable with rhACE2 ($P < .001$ vs negative control [rhACE], n = 5-6). Generation of Ang-(1-7) from Ang II for all variants was significantly higher than the negative control rhACE ($P < .001$, n = 5-6). Variants H, I, J, and K exhibited catalytic efficiencies (Kcat/km, $m^{-1}s^{-1}$) similar to rhACE2, whereas the remaining variants had lower performances, ranging from 34.1% to 83.5% of the catalytic efficiency for rhACE2 (n = 2).

In vivo Studies

The 2 lead candidates for i.v. injection (I and K) were selected based on performance in vitro and structural characteristics. In mice with Ang II administration, rhACE2 transiently lowered SBP (up to 6 hours) (Fig. 1A). ACE2-Fc variant K significantly reduced SBP up to 24 hours, and this effect was sustained for 48 hours (Fig 1A, $P < .05$ vs Ang II alone, n = 3-6). Surprisingly, ACE2-Fc variant I had no significant effect on SBP. A non-significant increase in urine albumin:Cr ratio was observed in Ang II-treated mice (288.2 ± 88.4 μg/mg) (n = 10). While neither rhACE2 (495.0 ± 400.3 μg/mg, n = 3) nor ACE2-Fc variant I (311.6 ± 169.5 μg/mg, n = 4) decreased urine albumin:Cr, mice treated with ACE2-Fc variant K had the lowest value (37.4 ± 12.9 μg/mg, n = 6), a non-significant difference from saline control (25.5 ± 4.6 μg/mg, n = 9) (Fig 1B).

Tissue ACE2 Activity and Protein

At 72 hours after administration of ACE2-Fc variant K, plasma ACE2 activity was >5-fold higher than in rhACE2-treated mice (Fig. 1C, $P < .0001$, n = 3-6). Angiotensin-converting enzyme 2-Fc variant I did not increase plasma ACE2 activity. Immunoblots confirmed the presence of variant K in plasma at 72 hours, with only weak detection of variant I (Fig. 1D). ACE2 activity was significantly increased in heart, lung, and liver lysates from mice treated with ACE2-Fc variant K, at 72 hours ($P < .05$, n = 3-6). Immunoblots showed retention of ACE2-Fc variant K in heart, lung, and liver (n = 3-6 mice per group). There was no significant difference in ACE2 enzymatic activity in kidney or spleen lysates amongst all groups of mice. Notably, immunoblots for ACE2 in kidney showed substantial levels of endogenous ACE2.

Discussion

The present studies reveal that novel ACE2-Fc variants exhibit cell-free ACE2 enzymatic activity, despite the structural modifications to improve affinity with SARS-CoV-2. Lead ACE2-Fc variant K had sustained SBP lowering effects when administered to mice with Ang II-induced hypertension, with a trend for reduction in urinary albumin excretion. Lead variant K (but not variant I) was retained in several tissues 72 hours after i.v. administration, associated with sustained ACE2 activity in plasma, lung, heart, and liver tissue.

One of the therapeutic challenges with delivery of soluble rhACE2 is its relatively short circulatory half-life.3 The stability, pharmacokinetics, and resistance to aggregation of recombinant proteins can be improved by addition of an Fc fusion protein.7 Here, the 2 leads selected for in vivo

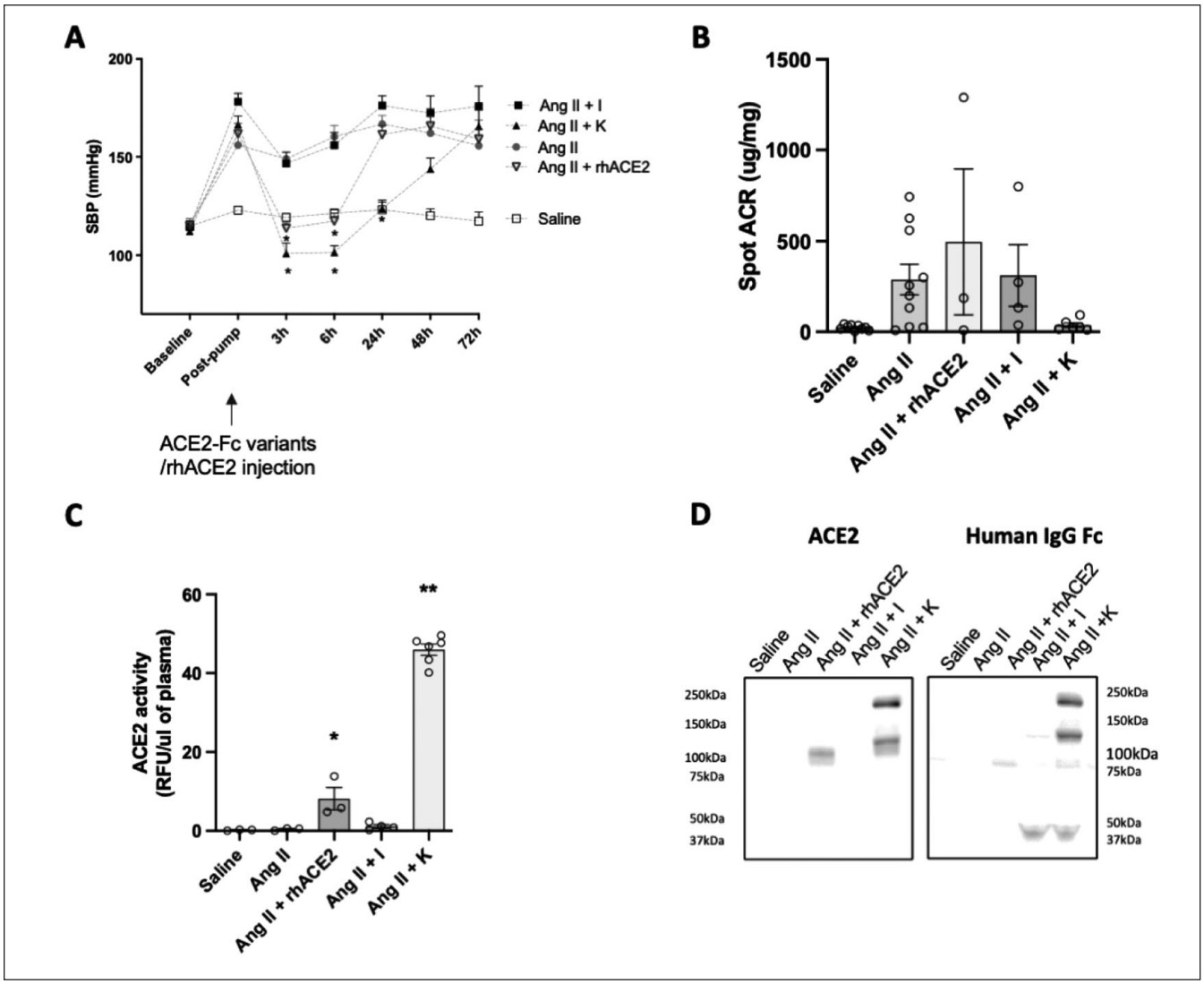

Figure 1. Effect of novel ACE2-Fc variants injected i.v. on SBP, urine ACR, and plasma ACE2 activity and protein levels.
Note. (A) SBP measured by tail-cuff method 3, 6, 24, 48, and 72 hours after administration of ACE2-Fc variants, (B) urine ACR, (C) ACE2 activity in plasma collected at endpoint (72 hours after administration of variants), and (D) Immunoblotting for ACE2 and IgG-Fc in plasma samples, revealing the monomeric and homodimeric forms of ACE2-Fc variant K with both antibodies. Data are means ± SEM, $^{*}P < .05$ vs all other groups and $^{**}P < .0001$ vs all other groups. N = 3 to 10 per group. ACE2 = angiotensin-converting enzyme 2; SBP = systolic blood pressure; ACR = albumin: creatinine ratio; rh = recombinant human. RFU = relative fluorescent units.

administration included the non-covalent homodimeric variants I and K, featuring cysteine-devoid hinges. Of the 10 ACE2-Fc variants studied, variant I, featuring 3 mutations in the spike-binding ACE2 domain, has the highest binding affinity to SARS-CoV-2 S proteins. However, variant I did not reduce SBP in hypertensive mice, nor did it increase plasma ACE2 activity. The rapid loss of enzymatic activity in vivo may be related to formation of aggregates, or interaction with other proteins, reducing enzymatic activity. Variant K features a naturally occurring polymorphism mutation, T92I, in the human ACE2 domain that eliminates glycosylation of amino acid N90. Removal of the ACE2 glycan at N90 has been shown to improve S protein binding.8 In mice with Ang II-induced hypertension, variant K reduced SBP for a prolonged duration, associated with a sustained increase in plasma and tissue ACE2 activity. The mechanisms for differences in performance in vivo between ACE2-Fc variants I and K are unclear and will require further study.

Experimental kidney injury induced by Ang II is associated with proteinuria.9 Non-significant increases in albuminuria were observed in all Ang II-treated mice, except for those treated with ACE2-Fc variant K. Deletion of ACE2 aggravates kidney injury in mice with diabetic nephropathy.10 Interestingly, kidney ACE2 activity did not significantly change after injection of soluble ACE2-Fc variant K, although the variant was present on kidney lysate

immunoblots. We hypothesize that high levels of endogenous ACE2 in kidney may mask enzymatic activity induced by exogenous ACE2 variants. Overall, our results suggest that ACE2-Fc variant K may prevent progressive kidney injury associated with activation of the renin-angiotensin system.

Limitations of this study include the lack of in vivo pharmacokinetic analyses or dose comparisons, and small animal numbers, which could impact statistical power. Future studies should address these limitations, and in particular increase numbers to determine the effect of animal sex on responses to ACE2-Fc variants. Our results support the further development of a novel ACE2-Fc variant (K) as a biotherapeutic for hypertension and kidney disease.

Conclusions

Ten novel ACE2-Fc variants showed in vitro ACE2 activity, with performance comparable with rhACE2. While variant I rapidly lost activity in vivo in a mouse model of Ang II-induced hypertension, ACE2-Fc variant K demonstrated enhanced performance, reducing SBP and persisting within tissue lysates 72 hours after i.v. injection. Our results suggest that this ACE2-Fc variant may have important therapeutic potential for cardiovascular and kidney disease.

Ethics Approval and Consent to Participate

Not applicable, since human subjects did not participate in this research.

Consent for Publication

Not applicable.

Availability of Data and Materials

Information on data and materials is available by contacting the corresponding author.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Considerations

All animal procedures were approved by the University of Ottawa Animal Care Committee (protocol 3514), following regulations of the Canadian Council on Animal Care (CCAC).

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