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REVIEW ARTICLE

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An update on ACE2 amplification and its therapeutic potential

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Abstract

The renin angiotensin system (RAS) plays an important role in the pathogenesis of variety of diseases. Targeting the formation and action of angiotensin II (Ang II), the main RAS peptide, has been the key therapeutic target for last three decades. ACE-related carboxypeptidase (ACE2), a monocarboxypeptidase that had been discovered 20 years ago, is one of the catalytically most potent enzymes known to degrade Ang II to Ang-(1-7), a peptide that is increasingly accepted to have organ-protective properties that oppose and counterbalance those of Ang II. In addition to its role as a RAS enzyme ACE2 is the main receptor for SARS-CoV-2. In this review, we discuss various strategies that have been used to achieve amplification of ACE2 activity including the potential therapeutic potential of soluble recombinant ACE2 protein and novel shorter ACE2 variants.

KEYWORDS

ACE2, ACE2, Angiotensin II, Angiotensin-(1-7), Covid-19, Therapeutic

1 | INTRODUCTION

The renin-angiotensin system (RAS) in its traditional view entails an enzymatic cascade of reactions leading to the generation of angiotensin II (Ang II), the main peptide of the RAS, which has a variety of biological effects.[1-9] Angiotensin II is a potent vasoconstrictor and also promotes renal sodium retention, actions that sustain blood pressure and are part of the stress response triggered to maintain circulating volume when survival is threatened by bleeding and other hypovolaemic situations.[7-9] In addition, Ang II has haemodynamic actions that are key to maintain the renal circulation. This peptide, however, exerts a myriad of actions at the tissue level that can be deleterious particularly when sustained chronically.[10-12] Such adverse actions include pro-inflammatory, pro-proliferative and pro-atherosclerotic effects that are independent of its effect on systemic blood pressure and renal haemodynamic actions.[13-17] Angiotensin II also increases the production of reactive oxygen species (ROS). This action results from activation of nicotinamide adenine dinucleotide phosphate (NADPH). The increase in ROS contributes to the unwanted effects of this peptide.[18,19]

RAS blockade based on inhibiting the formation of Ang II with ACE inhibitors or blocking the activation of the Ang II type 1 (AT1) receptor is a widely used therapy for kidney and cardiovascular disease. Pathways that regulate the degradation of Ang II may also be important for determining levels of Ang II, particularly at the tissue level.[20] Until recently, however, little attention had been paid to enhancing Ang II degradation as a way to counteract RAS overactivity which is usually present in kidneys from experimental models of diabetic kidney disease and likely in patients with many causes of CKD. Several enzymes are involved in the degradation of Ang II (Figure 1).

In this review we will focus on an ACE-related carboxypeptidase (ACE2), a homolog of ACE, described in 2000.[21,22] ACE2 shares 42% homology with the metalloprotease catalytic domains of ACE.[22] ACE2, unlike ACE, contains only one active domain.[21,22] In its full-length form ACE2 has 805 amino acids, whereas the soluble form of

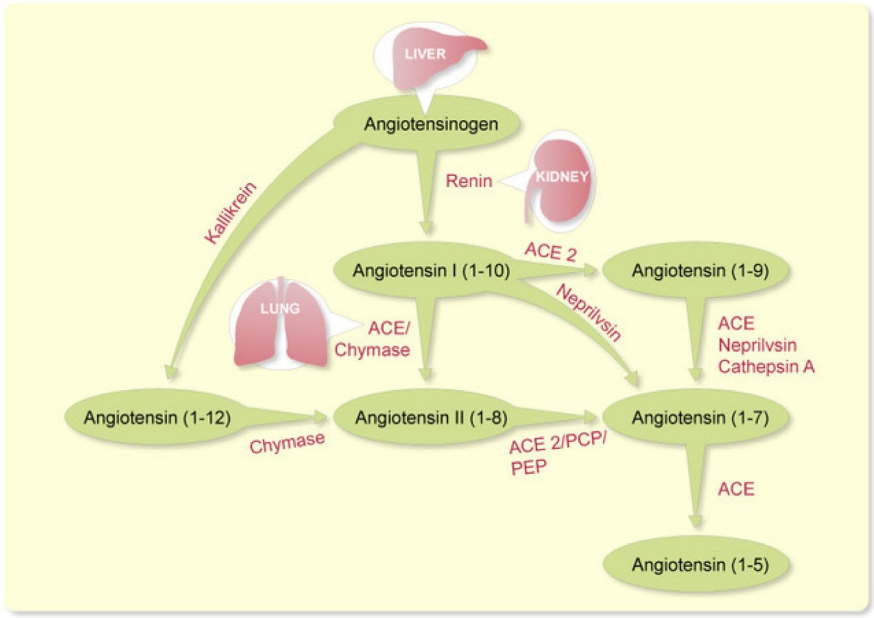

FIGURE 1 Schematic representation of actions of ACE2 and other enzymes involved in the metabolism of Angiotensin peptides; ACE, angiotensin I–converting enzyme, PCP, prolyl-carboxypeptidase, PEP, prolyl-endopeptidase (also known as POP)

ACE2 has only 740 amino acids. ACE2 is not inhibited by any of the existing ACE inhibitors. ACE2 acts by removing single amino acids from the C terminus of its peptide substrates.[23,24] It is one of the more catalytically potent enzymes known to convert the vasoconstrictor Ang II into Ang-(1-7) [21,23,25] Ang 1-7 is increasingly accepted to have vascular-protective and reno-protective properties that oppose and counterbalance those of Ang II, such as vasodilation and oxidative stress.[26-29] Within the renin angiotensin system, the other known target peptide for ACE2 cleavage is Ang I with the subsequent formation of Ang-(1-9).[22-24,30] Recent interest in ACE2 has increased dramatically as a result of the recognition that it is the main receptor for SARS-CoV2, the coronavirus responsible for the current COVID-19 pandemic.

Studies in experimental models of either genetically or pharmacologically induced ACE2 ablation have generally reported deleterious effects in various organs. Therefore, it is not surprising that over the past several years approaches aimed at augmentation of ACE2 activity had gained a significant interest for their therapeutic potential in a variety of pathological conditions. In this review, we will focus on presenting research done in our laboratory and others using various strategies to achieve amplification of ACE2 activity and discuss its therapeutic implications.

2 | ACE2/ANG II/ANG (1-7) AXIS

Two studies in 2000 reported the existence of a new enzyme that was termed ACE2.[21,22] This discovery now 20 years ago created an interest in the ACE2/Ang-(1-7) axis. In general, ACE2 and Ang (1-7) are felt to exert beneficial actions that are organ protective.[21,22,27] ACE2 and two other peptidases, prolylcarboxipeptidase (PRCP) and prolylendopeptidase (POP), are the known enzymes responsible for the formation of Ang 1-7 from Ang II (1-8).[31-34] These three enzymes cleave the phenylalanine amino acid from the C-terminal end of Ang II-(1-8) to form Ang 1-7 (Figure 1). The relative importance varies from tissue to tissue. For instance, ACE2 is very critical for this action in the kidney, whereas POP is the dominant enzyme in lungs and systemic circulation.[33] Ang I (1-10) can also contribute to the formation of Ang (1-7). The conversion into Ang 1-7 I from ANG I is produced by neprilysin.[28,35,36] ACE2 contributes to the formation of Ang (1-7) not only by enhancing the formation Ang (1-7) from Ang II (1-8) but also by increasing the formation of Ang-(1-9) from Ang I. Angiotensin (1-9) could be then converted into Ang 1-7 by ACE,[22,32] neprilysin [37] or cathepsin A.[38] Interestingly, Ang (1-9) could also contribute to the protective effects since this peptide has been proposed to activate the AT2R [39] (Figure 2).

While this review is limited to the formation of Ang-(1-7) from Ang II by ACE2, it should be noted that ACE2 hydrolyzes several other peptide substrates. Those include apelins (ie apelin-13 and apelin-36), the opioid peptide dynorphin A, the kallikrein-kinin-system peptide des-^{9}Arg bradykinin and ghrelin, a growth hormone secretagogue.[23] In addition to the catalytic properties of ACE2 this protein may also exert non-enzymatic functions.[40] Of great interest, the membrane-bound ACE2 is known to be the receptor for the severe acute respiratory syndrome-associated coronavirus (SARS-COV)[41] and recently for SARS-COV2, the coronavirus responsible for COVID-19 [42,43](Figure 2). Given the rapid emergence of the COVID-19 pandemic, there is increasing interest in ACE2 and its tissue distribution. This enzyme is only present in small amounts in the circulation but heavily expressed in certain organs such as kidneys and in the

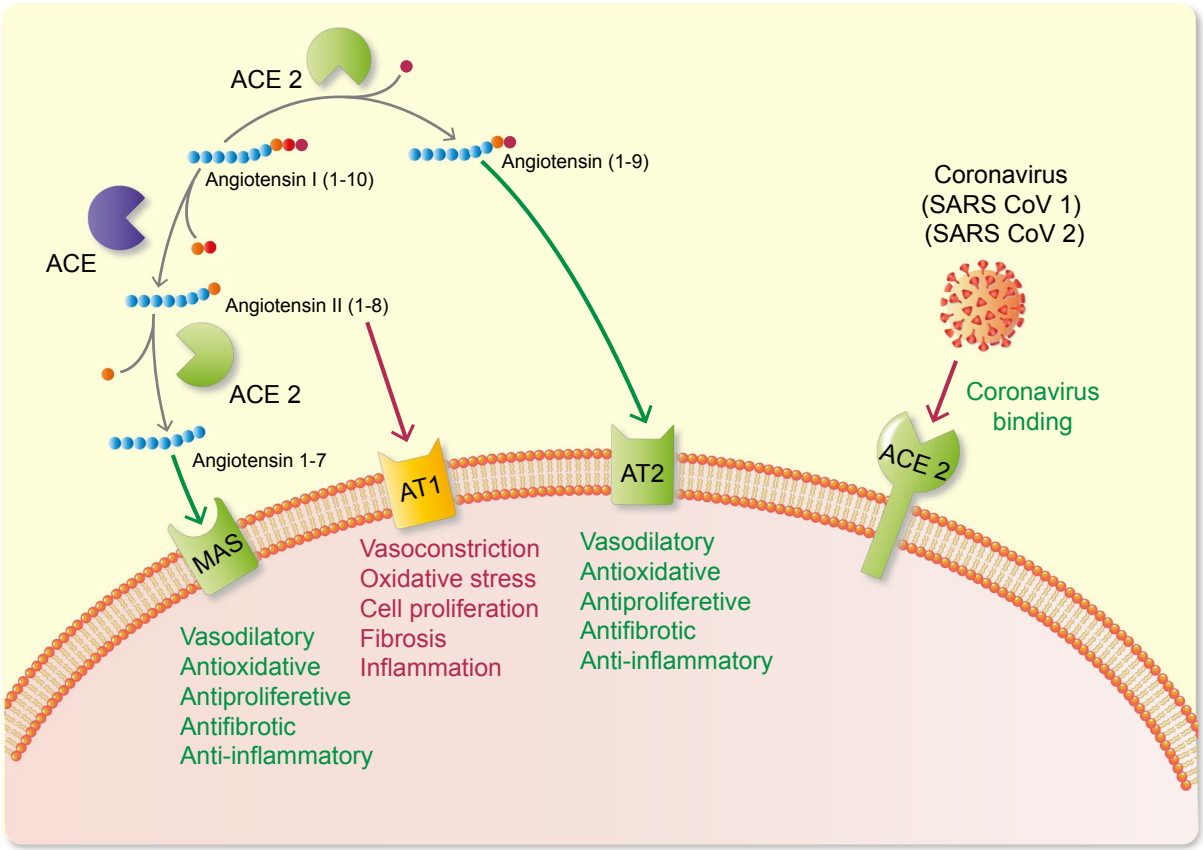

FIGURE 2 Different receptors in the cell wall and the myriad of actions elicited when activated by the various peptides. ACE2 acts as receptor for the SARS-CoV-2 leading to cell viral entry and replication

intestines.[33] In comparison with these organs, the expression of ACE2 in the lungs is low[33] but present in alveolar type II pneumocytes.[44] Unlike the full-length ACE2, which is anchored to the plasma membrane, the soluble form of ACE2 circulates but in very small amounts.[25] Theoretically, the administration of soluble ACE2 protein, in sufficient amounts, by binding to the spike protein of COVID-19, could reduce attachment to the full-length ACE2 in the plasma membrane.[45] This could be used therapeutically as a way to reduce infectivity in COVID-19–treated patients.

ACE2 interacts with amino acid transporters[46] and integrins.[47] It is therefore possible that some of the pre-clinical benefits of ACE2 amplification described below are beyond the dissipation of Ang II and/or the formation of Ang (1-7). Until more evidence for such other mechanisms become available, however, we are assuming that the conversion of Ang II into Ang (1-7) is a key step on the mechanism of action.[25] In other words, lowering Ang II and increasing Ang (1-7) is a therapeutic goal for most biological actions as depicted in Figure 2. Most of the work so far has been done in experimental animals. In healthy human volunteers recombinant ACE2 reduced the level of Ang II.[48] Even though Ang (1-7) infusion failed to demonstrate vascular effects in humans,[49] Sasaki et al reported that Ang (1-7) infusion increased forearm blood flow in healthy subjects, whereas in patients with hypertension there was a minimal effect. In human adipose and atrial microvessels, Ang (1-7) induces vasodilation via nitric oxide (NO)–dependent and telomerase-dependent processes through MasR, effects that seems to be absent in patients with coronary artery disease.[50]

Finally, ACE2/Ang 1-7/Mas receptor axis undoubtedly seems to have a protective effect on different organ and systems. Interestingly different studies, however, reported that Ang (1-7) has deleterious effects to increase blood pressure and exacerbate cardiac fibrosis in subtotal nephrectomy rats kidney disease model in association with increased cardiac ACE activity.[51,52]

3 | METHODS OF ACE2 AMPLIFICATION

Several approaches can be used experimentally to increase ACE2 activity (Table 1) those range from ACE2 gene delivery via lentivirus, adenovirus or adeno-associated virus, creation of transgenic rodent model, minicircle DNA delivery, administration of recombinant ACE2 proteins and the use of ACE2 activators. All of these approaches have been attempted in rodent models and a human recombinant ACE2 protein has been safely administered to humans. Below, we

TABLE 1 Approaches used experimentally to amplify ACE2 activity for therapeutic purposes

ACE2 gene delivery via lentivirus, adenovirus or adeno-associated virus
Minicircle DNA delivery
Transgenic rodent models
Administration of recombinant ACE2 proteins
ACE2 activators
Novel ACE2 variants of shorter molecular size

summarize these studies and the pre-clinical entities that have been studied.

4 | VIRAL ACE2 GENE DELIVERY SYSTEMS AND TRANSGENIC MICE

Viral delivery systems using adenovirus, adeno-associated virus or lentivirus have been used as proof-of-concept approaches to augment ACE2 expression in vivo at the central nervous system and variety of peripheral tissues.

4.1 | Heart

Huentelman et al[53] first used lentiviral vector encoding mouse ACE2 (lenti-mACE2) to amplify ACE2 activity in the heart. Lenti-mACE2 was injected intracardiac in 5-day-old Sprague-Dawley rats. Angiotensin II administration for 4 weeks to control rats resulted in the expected increase in systolic blood pressure, increased weight to body weight ratio and increased myocardial fibrosis. [54] Transduction with lenti-mACE2 resulted in a decrease in the heart weight to body weight ratio and a reduction in the myocardial fibrosis caused by infusion of angiotensin II. [54] This improvement in cardiac hypertrophy was associated with increased expression of ACE2 in cardiac tissue. Similar to Ang II-induced hypertension, in the spontaneously hypertensive rat (SHR) murine ACE2 gene transfer into the heart using lentiviral transduction attenuated hypertension and the associated pathological changes, such as left ventricular wall thickness and of perivascular fibrosis.[54] In addition, an improvement of cardiac function, as evidenced by an increase in left ventricular end diastolic and end systolic diameters in SHR rats after ACE2 overexpression, was observed.[54]

The use of lentivirus and adeno-associated viral systems also allowed to generate ACE2-overexpressing transgenic animals for studies examining effects of cardiac-specific ACE2 amplification in adult animals. These viral systems, similar to conditional transgenic models, have the advantage of long-term in vivo ACE2 overexpression that could be "turned on" in adult life and therefore avoid the interference from the effect of developmental ACE2 overexpression. In this respect it should be noted that initial study in traditional transgenic mice with cardiac overexpression of human full-length ACE2 was not encouraging.[55] Even though transgenic mice appeared healthy, they died prematurely. Their diminished survival rates correlated with the extent of ACE2 overexpression in two transgenic lines suggesting a transgene dose effect.[55] Hearts from both transgenics, however, were essentially normal, without hypertrophy and similar to wild type. In addition, transgenic and non-transgenic littermates were similar to each other by echocardiography. Moreover, by cardiac catheterization ventricular performance was similar.[55] The mortality of the transgenic mice could ultimately be explained by electrophysiological analyses that revealed conduction disturbances and lethal ventricular arrhythmias in hACE2 transgenic mice. It still remains to be examined whether or not the discrepancy between the cardiac effects by virally-induced ACE2 overexpression in adult animals and those reported in ACE2 transgenic mice are caused by developmental issues.

4.2 | Blood Pressure

Yamazato M et al[56] showed the long-term effect of ACE2 on blood pressure. In this study, SHR rats had a relative deficiency in ACE2 protein expression within the cardiovascular regulatory neurons of the rostral ventrolateral medulla (RVLM) when compared with normotensive Wistar-Kyoto rats. Attempted correction of this deficiency in the SHR rats by ACE2 overexpression by lentivirus injection into RVLM resulted in long-term reduction in blood pressure.[56] Therefore, central ACE2 overexpression could correct its intrinsic decrease in the RVLM and, similarly to the above-discussed cardiac ACE2 overexpression,[54] leading to a substantial blood pressure reduction in the SHR rat.[56]

4.3 | Lungs

In lungs, the involvement of RAS in the pathogenesis of certain conditions such as pulmonary hypertension (PH) has been inferred from the high abundance of ACE in the pulmonary vasculature.[57,58] High ACE levels likely contribute to excessive generation of Ang II[59] Pulmonary hypertension (PH). Is a disease characterized by a sustained increased in pulmonary artery pressure and resistance over time… [59] Lentiviral overexpression of ACE2 within the lungs was attempted in a mouse model of PH induced by monocrotaline (MCT). In this model, lentiviral vector durably and efficiently transduced ACE2 into a wide variety of cells of lung tissue which was associated with marked attenuation of PH and a reversal of PH-induced lung injury suggesting support

for ACE2 as a possible target for upregulating strategies for the treatment of this disease [59]

4.4 | Nervous System

It is also important to mention the protective role of ACE2 on the nervous system. Feng et al[60] studied the effects of ACE2 using a transgenic mouse model with high expression of hACE2 protein in the brain. In this study they concluded that overexpression of ACE2 would attenuate the development of neurologic hypertension as a consequence of attenuation of parasympathetic tone and spontaneous baroreflex sensitivity. Of note, neurological deficits improvement and cerebral infarct size reduction after a neurological ischemic event have been attributed to anti-oxidative and anti-inflammatory effects of ACE2/Ang (1-7)/Mas axis. [61] Therefore, ACE2 could also be a target to prevent and treat ischemic stroke in the future.

4.5 | Kidneys

Various studies have shown a role of ACE2 in kidney disease. Nadarajah et al generated a model of glomerular ACE2 overexpression using a podocyte-specific ACE2 transgenic mice and showed partial protection against the early development of albuminuria.[62] Preservation of podocyte proteins and podocyte number was seen in STZ-induced diabetes transgenic mice with overexpression of the human ACE2 protein.[62] That kidney overexpressed ACE2 can ameliorate glomerular injury in diabetic animals was also suggested by a study using adenoviral kidney ACE2 (Ad-ACE2) overexpression in STZ rats.[63] Compared with control, the Ad-ACE2-treated group showed a reduction in systolic blood pressure and improvement in urinary albumin excretion, creatinine clearance and glomeruli sclerosis index. Ad-ACE2 also had decreased TGF-β1, vascular endothelial growth factor and collagen IV protein expression. [63]No additional benefit of ACE inhibition was noticed in the combined use of Ad-ACE2 and ACEI.[63] Overall these studies suggest that kidney ACE2 amplification may represent a therapeutic target in the treatment of glomerular injury in diabetic kidney disease.

5 | MINICIRCLE DNA DELIVERY

Minicircle DNA vectors consist of a circular expression cassette devoid of the bacterial plasmid DNA backbone that provides sustained transgene expression in quiescent cells/tissues.[64] We studied the effects of murine recombinant ACE2 in streptozotocin-induced diabetes in mice as well as the effect of increasing circulating ACE2 using minicircle DNA delivery. [65] This approach resulted in sustained increase in serum ACE2 activity and enhanced ability to degrade infused angiotensin II (1-8)..[64] In mice with streptozotocin-induced diabetes pre-treated with ACE2, minicircles, plasma ACE2 protein increased as shown by western blot and ACE2 serum activity increased more than 100-fold.[65] Urinary ACE2 activity and kidney ACE2, however, did not Increase despite the profound augmentation of ACE2 in plasma. Moreover, the glomerular lesions and hyperfiltration seen in this diabetic model of experimental kidney disease were not affected at all.[65] From these findings we concluded that targeting kidney ACE2 rather than circulating plasma ACE2 might be necessary to effectively treat early diabetic kidney disease as seen in the STZ model.

6 | RECOMBINANT ACE2

ACE2 in its full-length form is a 110-120kDa-protein comprising 805 amino acids (aa). It is a type I transmembrane protein that contains a major extracellular domain (aa 1-740), and the much smaller: transmembrane region (aa 741-768) and intracellular tail (769-805).[66,67] The extracellular domain of ACE2 (1-740 aa) is enzymatically active as it contains a complete and functional catalytic domain.

7 | HUMAN RECOMBINANT ACE2

Thus far, the form of soluble recombinant ACE2 consisting of the extracellular 740 N-terminal amino acids (aa) has predominantly been used in both pre-clinical and clinical research. Wysocki et al[25] showed that during Ang II infusion, rACE2 administration effectively degrades Ang II and, in the process, normalizes blood pressure in a mouse model of acute Ang II-dependent hypertension. (Figure 3). Imai et al [68] used hrACE2 to examine its effect on experimental acute lung injury. Using aspiration-induced acute lung injury murine model they injected human recombinant ACE2 (hrACE2) into acid-treated Ace2 knockout mice and observed a decrease in the degree of acute lung injury, and pulmonary oedema formation. hrACE2 has also been proposed as a potential candidate to treat diastolic and systolic heart failure.[69] Zhong et al[70] showed that hrACE2 ameliorated pressure overload induced as well as Ang II-induced myocardial remodelling. –hrACE2 has also shown to reduce the level of Angiotensin II in healthy human volunteers. Despite the ACE blockade the levels of Angiotensin II could remain elevated via chymase system.[48] All of the above suggest that therapies using hrACE2 for heart failure seem promising.

Human recombinant ACE2 has been evaluated in human subjects in limited clinical studies. The pharmacokinetics, pharmacodynamics, safety and tolerability of hrACE2 were

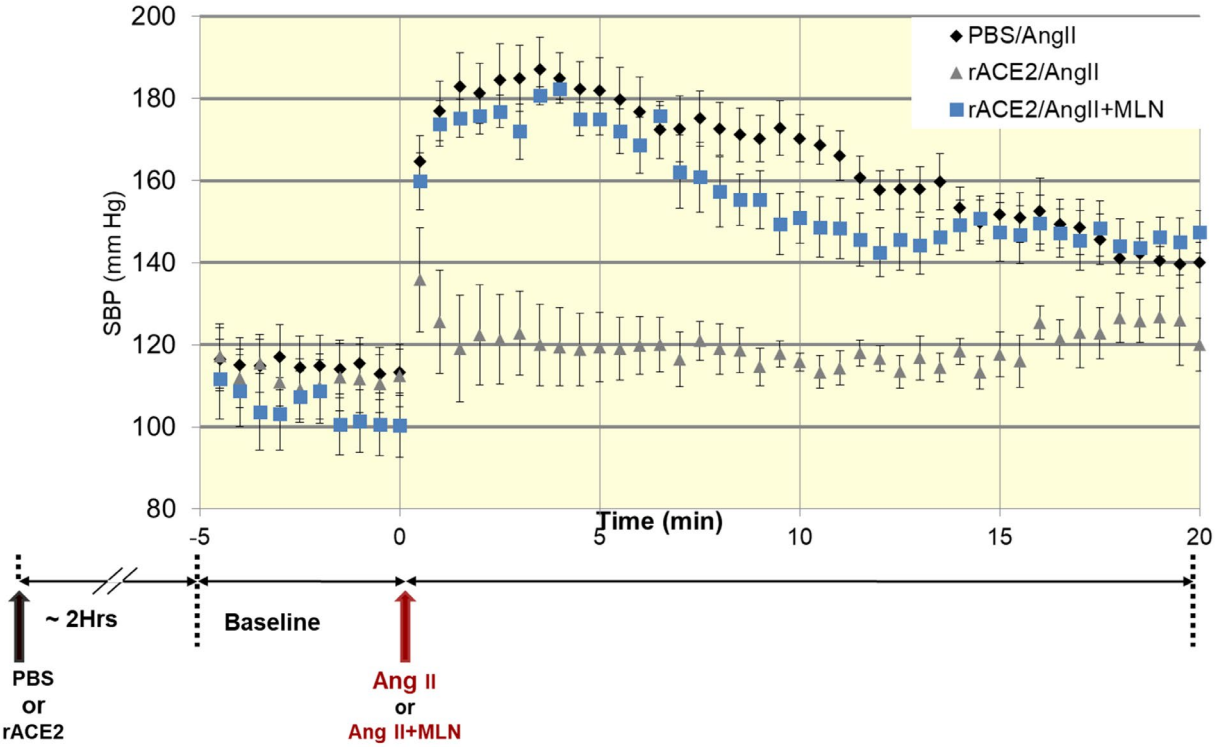

FIGURE 3 Systolic blood pressure changes after angiotensin II infusion. A bolus of Ang II to mice pre-treated with PBS (n = 14) was associated with a rapid increase in SBP. In mice treated with rACE2 prior to Ang II injection (n = 11), the SBP increase was blunted and normalized within the first 5 minutes after Ang II injection. Adapted from Wysocki et al Hypertension 2010[25]

determined in healthy volunteers.[71] This study was randomized, double-blind and placebo controlled. In addition to showing good tolerability of hrACE2 (1-740 AA) by healthy human subjects, this study revealed a suppression of systemic Ang II levels both by single and repeated dosing of hrACE2.[71]

Since ACE2 has been implicated in animal models of acute lung injury,[68,72] it was postulated that administration of hrACE2 could attenuate acute lung injury in human subjects with ARDS.[73] In patients with acute respiratory distress syndrome (ARDS) hrACE2 (GSK2586881) was well tolerated.[73] Human rACE2 caused a decrease of circulating Ang II levels,[73] whereas angiotensin (1-5) and angiotensin (1-7) levels were increased and continued elevated for 48 h.[73] In this exploratory study registered under ClinicalTrials.gov, NCT01597635, surfactant protein D (SP-D) which is considered a beneficial biomarker contributing to normal surfactant structure and inhibition of inflammatory response was increased in hrACE2-treated subjects compared with placebo. However, hrACE2 infusions did not result in improvement in other physiological or clinical measures of ARDS in this small study and the trial was terminated early.[73]

Encouraging but still very preliminary findings were drawn from a recent phase IIa, open-label pilot study which suggested a potential therapeutic role for hrACE2 in pulmonary arterial hypertension.[74] This study found a reduced plasma ACE2 activity in subjects with PH. This was inferred from higher plasma Ang II to Ang (1-7) ratio; however, the ratio is not specific as it can potentially be affected by changes in other enzymes that affect the conversion of Ang II into Ang (1-7). At baseline in subjects with PH, increased expression in six of nine measured cytokines as compared with controls (interleukin (IL)-10, IL-1β, tumour necrosis factor (TNF)-α, IL-13, IL-8 and IL-4) was found. Reduced plasma superoxide dismutase 2 (SOD2), which is considered an anti-oxidant enzyme, and increased oxidant stress parameters were also observed.[74] After hrACE2, cytokines such as IL-10, IL-1β, IL-2 and TNF-α were decreased 2 hours after administration. Human rACE2 administration was reported to also beneficially influence SOD2 levels, and reduce plasma oxidant stress. These findings were based on a limited number of subjects. Further assessment of hrACE2 as a potential therapeutic in PH certainly will require larger studies. Table 2 provides a summary of studies so far that have used human soluble recombinant ACE2.

8 | RODENT RECOMBINANT ACE2

While the human rACE2 is the ultimate form of the protein that can be used in clinical studies, it has some limitations for pre-clinical research in rodents. Human rACE2 can certainly be used in acute studies in rodents. There is a limited value, however, for chronic studies as a result of the development

TABLE 2 Studies in human subjects receiving recombinant soluble ACE2

Title	NCT number	Number of participants	Condition or disease	Intervention/Treatment	Collaborators/ Sponsors	Phase	Status
A two part study to investigate the safety, tolerability, pharmacokinetics and pharmacodynamics of GSK2586881 in patients with acute lung injury	NCT01597635	44 participants	Lung injury, Acute	Drug: Dose 4 GSK2586881 Drug: Placebo (saline)	GlaxoSmithKline	Phase 2	Completed
An open-label, dose-escalation study to evaluate the safety, tolerability, pharmacokinetics and pharmacodynamics of single Doses of GSK2586881 in participants with pulmonary arterial hypertension	NCT03177603	23 participants	Hypertension, pulmonary	Drug: GSK2586881	GlaxoSmiithKline	Phase 2	Completed
The effects of GSK2586881 on the responses to acute hypoxia and exercise	NCT03000686	17 participants	Healthy volunteers	Biological:GSK2586881 Other: Placebo	GlaxoSmithLline	Phase 1	Terminated
A Randomized, open label, controlled clinical study to evaluate the recombinant human angiotensin-converting enzyme 2 (rhACE2) in adult patients with COVID-19	NCT04287686	0 participants	COVID-19	Drug: Recombinant human angiotensin-converting enzyme 2 (rhACE2)	The first affiliated hospital of Guangzhou Medical University	Not applicable	Withdrawn (without CDE approval)

of antibodies that decrease the enzymatic activity of ACE2 after 2 weeks of administration to mouse models.[25,75] Mouse rACE2 should not elicit formation of such neutralizing antibodies when given to mice, Indeed, we documented sustained elevations in serum ACE2 activity after murine rACE2 administration,[75] thereby providing a suitable strategy for ACE2 amplification in chronic studies in mice. One has to keep in mind, however, that mouse and human rACE2 are not identical in terms of their enzymatic effects on RAS peptides. While both mouse and human recombinant ACE2 promote the degradation of Ang II and lead formation of Ang (1-7),[75,76] only human recombinant ACE2 degrades Ang I to form Ang-(1-9).[75,76]

Experimentally, mouse intact (1-740) rACE2 had been employed to assess its effects on hypertension induced in mice by Ang II. In this respect, single injection of soluble rACE2 caused a marked increase in circulating ACE2 activity and, after supra physiological dose of Ang II (1ug/g body weight), within few minutes circulating levels of this peptide normalized and blood pressure fell more rapidly than in animals not infused with ACE2.[75] Intact (1-740) of Human Recombinant ACE2 has also been used for similar experiments showing similar results. [25] Of note, the infusion of rodent recombinant ACE2 under baseline conditions does not lower blood pressure.[75]

That the effects of rACE2 are restricted to the circulation was suggested by the observations that its administration did not result in noticeable increase in ACE2 activity within the heart,[25,65] the kidney[25,65] or in the urine [25,65,77] of healthy mice. In diabetic mice with only modest albuminuria rACE2 also did not appear in the urine.[65,77] In contrast, in the Alport mouse model of severely compromised glomerular filtration barrier, we found a massive increase in urinary ACE2 after soluble rACE2 administration.[65] The large molecular size of soluble rACE2 (100-110 kDa) [65,77] renders it not filterable trough an intact or only slightly damaged glomerular barrier. In addition, soluble rACE2 1-740 tends to form homodimers making its actual size even bigger and thus reduce further the likelihood of glomerular basement membrane passage.[76,77]

rACE2 administration to markedly increase circulating levels of soluble ACE2 can effectively obliterate Ang II-induced hypertension [25,75,78] or hypertension caused by renin overexpression mouse models. [79] Therefore, it is conceivable that the use of soluble rACE2 could likely be applied to treatment of conditions associated with systemic RAS activation that secondarily might lead to kidney injury. In contrast, in states where local kidney RAS is expected to be overactive, such as diabetic kidney disease,[80] soluble rACE2 of 1-740 AA might not be effective. Consistent with this notion, we have found that in STZ-treated mice, a diabetic mouse model with local kidney but not systemic RAS overactivity,[65] long-term augmentation of circulating ACE2 activity was not sufficient to beneficially alter albuminuria, GFR or kidney histology.[65]

Glomerular filtration of administered rACE2 is a prerequisite for a subsequent tubular uptake of the protein from the urinary space.[65,80] Kidney targeting of biologics is complex and can be affected by many factors including their molecular size.[81] We reasoned that in forms of kidney disease with an overactive RAS within the kidney,[65,82] ACE2 amplification would require forms of recombinant ACE2 that are short enough to be able to pass the glomerular filtration barrier and be consequently reabsorbable by the kidney to be able to exert any direct kidney-specific therapeutic effect. Based on these considerations, we have generated ACE2 variants of shorter molecular size that still retain enzymatic activity (see below).

9 | NOVEL VARIANTS OF ACE2 OF SHORTER MOLECULAR SIZE

We have generated and tested two novel recombinant mouse ACE2 proteins of a molecular size (~69-71 kD) which are much shorter than the original soluble rACE2 of 100-110 kD. The two short ACE2 variants 1-605 and 1-619 AA have been found to be active systemically and of a molecular size short enough to render them filterable through the kidney glomerular filtration barrier.[65,80] Moreover, we have found that these short variants could by the kidney tubules and thus capable to amplify kidney ACE2 activity and foster the formation of Ang (1-7) from Ang II. This feature should make them attractive to combat RAS activation in a vast array of kidney diseases where the RAS is overactive, such as the acute kidney injury.

The pharmacokinetics of two active short ACE2 proteins 1-619 and 1-605 was examined in mice after iv and ip administration based on measurements of serum ACE2 activity against a fluorogenic substrate Mca-APK-Dnp.[65,80] After intravenous administration, ACE2 1-619 and 1-605 showed a substantially extended circulatory elimination half-life (~4 hours) as compared to the elimination half-life of the original soluble rACE2 (~ 1.4 hours). After ip administration, the elimination half-life of the original soluble rACE2 was similar or slightly longer than that of ACE2 1-619 and 1-605 variants. The area under the curve and the peak plasma ACE2 activity for both short ACE2 proteins after iv injection and after ip administration, however, were considerably higher than that of the original soluble rACE2 suggesting overall improved pharmacokinetic profiles as compared to the original soluble rACE2. Even though the pharmacokinetic data show clear superiority of short rACE2 variants as compared to the original soluble rACE2, it should be cautioned that this effect may be dependent on the artificial substrate used to measure ACE2 activity.[65,80]

Catalytic efficiency towards the fluorogenic substrate Mca-APK-(Dnp) was also found to be much higher for both short rACE2 variants than that of the original soluble rACE2 which might, in part, reflect higher affinity towards this artificial substrate.[65,80] Against the natural substrate, Ang II, the catalytic efficiency was similar for the short ACE2 variants and the original soluble ACE2. Likewise, the two short ACE2 variants 1-605 and 1-619 in vivo exhibited a similar systemic effect on the acute Ang II-induced hypertension as the original soluble ACE2.[65,80] Both small ACE2 variants blunted the peak increase in blood pressure after Ang II infusion which normalized within 5 minutes or less to a similar extent as the original soluble ACE2.[65,80]Therefore, both small ACE2 variants seem to degrade excess of systemic circulating Ang II to a similar degree as original soluble rACE2, thereby enhancing blood pressure recovery when Ang II is infused.

In addition to the systemic effect, both small ACE2 variants have also an added effect on the local urinary and kidney ACE2 activity augmentation which is in contrast to the original soluble ACE2.[65,80] This was recently demonstrated in experiments where short ACE2 variants and the original soluble ACE2 were administered to ACE2-deficient mice. In contrast to original soluble rACE2, both small recombinant variants resulted in a gain in urinary ACE2 activity. L-lysine, a tubular reabsorption blocker,[83,84] further increased urinary ACE2 activity suggesting that the two short ACE2 variants are both filtered and reabsorbed by the tubules. Moreover, it was demonstrated that the short ACE2 protein variant 1-619 is taken up by the kidney in enzymatically active state. This was shown by the presence of ACE2 activity in kidneys isolated from ACE2-deficient mice that had been previously injected with ACE2 1-619 but not from the kidneys injected with equivalent dose of original soluble rACE2. In addition, ex vivo kidney cortex lysates from ACE2 1-619-injected mice were able to form significantly more Ang (1-7) from Ang II than kidney lysates from PBS- or original soluble rACE2-injected mice. In the aggregate these data demonstrate that short ACE2 variants are active, and sufficiently small to be filtered by the kidney and moreover capable to increase kidney ACE2 activity to an extent that Ang (1-7) formation from Ang II is increased.

10 | ACE2 ACTIVATORS

The crystal structure of ACE2 was solved by Hernández Prada et al [85] and three putative small molecule binding pockets were identified. These authors carried out in silico screening of a small molecule library followed by in vitro studies and found that of the three sites, two of them were inhibitor sites and the third one was a presumed activator site. The same investigators[85] identified then two small-molecular compounds as ACE2 activators: XNT and resorcinolnaphthalein. XNT was preferred over resorcinolnaphthalein since XNT solubility properties appeared to be more favourable.[85] Three years later an agent commonly used to treat some parasitizes in animals [86] called Diminazene (DIZE) was proposed as an ACE2 activator.[87]

Both XNT and DIZE have been used experimentally to serve as potential treatment for various conditions such as certain types of hypertension,[85-88] pulmonary hypertension,[89-91] Cardiac and renal fibrosis[85] and glaucoma.[92] The therapeutic benefits of these two components should be attributable to conversion of Ang II into Ang (1-7) as a consequence of ACE2 activation.[86,87,89-92] One of the caveats with many of these studies is, however, that the effect on ACE2 activity was usually not reported *in vivo*.[85,87,89-91,93-100] In these studies, moreover, it was not shown that the use of these presumed ACE2 activators had taken place by demonstrating the enhanced conversion of Ang II into Ang (1-7).

Our group reported low levels plasma ACE2 activity in both vehicle and XNT-infused mice.[65,80] Furthermore, after Ang II infusion, the plasma levels Ang (1-7), the peptide generated by the cleavage of Ang II and plasma levels Ang II, the substrate of ACE2, were not affected by the administration of XNT. Therefore, it was suggested that the effect of XNT on Ang II-induced hypertension was not caused by activation of ACE2. To substantiate this hypothesis, we performed experiments in an ACE2 KO mouse model. Despite the lack of ACE2, XNT was capable to elicit an enhanced recovery from hypertension induced by Ang II infusion similar to the recovery seen in WT mice (45). In addition, XNT and DIZE failed to increase ACE2 enzymatic activity of either mouse or human rACE2 in vitro and ex vivo using mouse and rat kidney tissue. Consistent with these findings, Ang II degradation ex vivo and in vitro was not affected by XNT and DIZE.[78] It should be also noted, however, that XNT and DIZE have been reported to cause a significant increase in ACE2 mRNA expression.[99,101,102] This suggests that these two compounds could upregulate ACE2 gene expression, leading to an ACE2 activity augmentation chronically. Although the acute (minutes) effect of XNT on blood pressure that Haber et al[78] reported in ACE2 replete mice after Ang II infusion seems unlikely to be explained by this upregulation of ACE2 mRNA expression. Recently, an egg white-derived tripeptide IRW (Ile-Arg-Trp) was reported to increase ACE2 activity of hrACE2 in a cell-free system and also to upregulate ace2 mRNA expression in cultured cells.[103] There have been no follow-up studies, to our knowledge, with the IRW compound which would independently assess its potency as an ACE2 activator. As of today there is no convincing evidence, in our opinion, that the presumed ACE2 activators studied exert their otherwise undisputable biological effects by activating ace2 as their main mechanism of action.

11 | POTENTIAL THERAPEUTIC ACTION OF ACE2 AMPLIFICATION

From the forgoing, it is logical to postulate that ACE2 amplification within the kidney is a very attractive therapeutic approach to promote the metabolism of Ang II and reduce its detrimental actions. Some of the potential diseases that could benefit from ACE2 amplification have been mentioned already while discussing the methods for increasing ACE2 activity. As noted earlier, partial kidney protection against STZ-induced diabetic kidney disease could be shown in a transgenic model where ACE2 overexpression was limited to glomerular podocytes.[62] In one study human recombinant ACE2 was shown to improve diabetic kidney disease in Akita mice.[104] When these mice were injected with hrACE2 for 4 weeks, hrACE2 normalized blood pressure and reduced albuminuria. Unlike other models of early DKD, the Akita mouse is hypertensive and is very possible that the improvement in albuminuria noted in this study was related to lowering of blood pressure by ACE2 by decreasing Ang II which is elevated in this model.

In keeping with previous studies in mice by Ye et al,[105] Mizuiri et al [106] found that in patients with diabetic kidney disease expression of ACE and ACE2 was altered. In this study, ACE2 expression was decreased while levels of ACE expression were increased in glomeruli, resulting in a significant increase in the ACE/ACE2 ratio. Reich et al [107] measured ACE2 and ACE mRNA expression in kidney biopsies from patients with type 2 diabetes and associated kidney disease and controls. Glomerular and proximal tubular ACE2 mRNA expression was reduced by more than half while ACE mRNA was augmented in both compartments in diabetic patients compared to controls. This finding suggests that ACE2 gene expression may play an important role in the development and progression of kidney injury in human subjects with diabetes.

Wysocki et al [65] examined the kidney effects of mrACE2 in mice with STZ-induced diabetic nephropathy as well as the amplification of circulating ACE2 using minicircle DNA delivery prior to induction experimental diabetes. Recombinant murine ACE2 given for 4 weeks failed to stop the progression kidney pathology and albuminuria in this model. A markedly increase in ACE2 activity as well as an enhanced ability to metabolize acute load of Ang II resulted from ACE2 minicircle delivery. However, this augmented ACE2 activity achieved by this technique did not affect urine ACE2 activity. Moreover, there were no improvements in albuminuria, glomerular expansion, glomerular cellularity or glomerular size when compared to vehicle-treated diabetic controls (Figure 4). Therefore, this study emphasized the importance of targeting the kidney rather the circulating RAS in order to treat diabetic nephropathy.

In other study using Col4a3 -/- mouse model of Alport's syndrome, murine recombinant (mr) ACE2 also decreased markers of kidney injury. ACE2 plasma activity was increased using mrACE2 minipumps leading to an improvement in albuminuria, reduced ERK1/ERK2 signalling and amelioration of inflammation, fibrosis and oxidative stress.[108] In this study renal function did not improve as

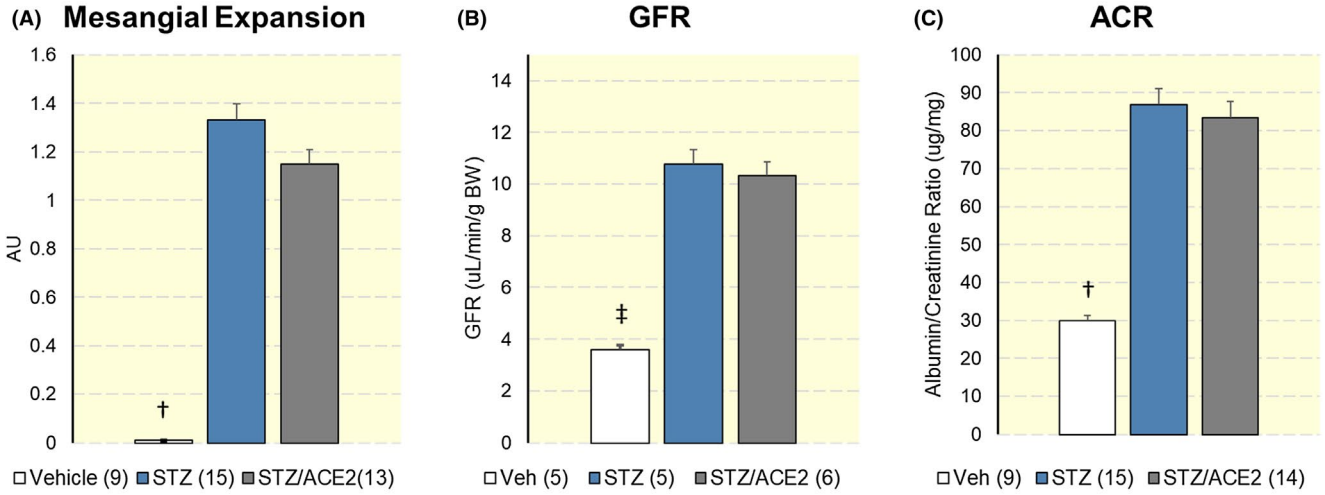

FIGURE 4 A, Mesangial expansion score, B, Glomerular filtration rate (GFR, middle) and C, albumin/creatinine ratio (ACR, right) in mice 20 weeks after diabetes induction with streptozotocin (STZ) and in vehicle-treated (Veh) mice that served as a non-diabetic control group. There were no differences in any of the parameters examined. Between ACE2-treated and untreated animals. †$P < .01$ ‡$P < .001$. Adapted from Wysocki et al, Kidney International, 2017[65]

measured by blood urea nitrogen and GFR was not measured. In this model, the profound defect in glomerular permeability allows the infused rACE2, despite its large molecular size, to get filtered.[65] We think that part of the benefit observed may have reflected local kidney uptake of the infused ACE2. Clearly, in a model of early DKD systemic administration of ACE2 did not prevent the development of hyperfiltration or mild diabetic kidney injury as observed in STZ-treated animals.[65] As pointed out by us[65] and others [109] in order to achieve an effective therapeutic approach, increasing circulating ACE2 levels may not be enough and other strategies that modulate the ACE2/Ang (1-7)/MasR axis using molecules capable to reach the glomerulus should be considered. We are encouraged by the development of novel ACE2 variants of shorter molecular size that can be filtered by the kidney and able to increase the formation of Ang (1-7) from Ang II.[80] The therapeutic potential of these short ACE2 variants is currently being explored in pre-clinical studies for acute and chronic kidney injury. Alterations in the Renin Angiotensin system (RAS) are involved not only in the progression of chronic kidney disease (CKD) [110-114] but also in AKI and are associated with adverse outcomes both in experimental and clinical studies.[115-122] We think that AKI could be an initial target to explore in humans the potential preventative effect of our short ACE2 variants.

Finally, this review comes at a time that the world is grappling with the COVID-19 pandemic caused by SARS-CoV2 virus. From the SARS outbreak in 2003 we have known that SARS-CoV1 spike glycoprotein recognized ACE2 as a receptor on the cell surface for host entry.[41] Consequently, there have already been several reports looking at the association of SARS-CoV2 and ACE2.[43,123] It is known that SARS-COV2 does bind to ACE2 to gain host cell entry causing its cell internalization and likely reducing membrane ACE2 expression.[124] In murine models, the dysregulation of ACE2 is associated with cardiac, pulmonary and kidney alterations. There is preliminary data from COVID-19 subjects in whom elevated levels of plasma angiotensin II correlated with degree of lung injury.[125] Further prior pre-clinical studies in respiratory syncytial virus and avian H5N1 influenza suggested that restoration of ACE2 by recombinant ACE2 administration appeared to reverse worsening lung injury.[73,126,127] Therefore, there is now significant interest in looking at recombinant ACE2 protein to rebalance the RAS network and potentially help mitigate the pulmonary, cardiac and kidney damage done by COVID-19. We have proposed that soluble ACE2 may act as a competitive interceptor of SARS-CoV and SARS-CoV2 by preventing binding of the viral particle to the surface-bound, full-length ACE2[45]. In this context, administration of soluble recombinant human ACE2 proteins might be beneficial as novel biologics to treat the infection caused by coronaviruses that utilize ACE2 as a receptor.

CONFLICT OF INTEREST

D. Batlle and J. Wysocki: co-inventors Patent: 'Active Low Molecular Weight Variants of Angiotensin Converting Enzyme 2'; and have also submitted a patent on the potential use of novel ACE2 proteins for coronavirus infection. D. Batlle: Founder of 'Angiotensin Therapeutics'.

DISCLOSURE

The other authors have nothing to disclose relevant to this manuscript.

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