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# ACE2 and Microbiota: Emerging Targets for Cardiopulmonary Disease Therapy

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## Abstract

The health of the cardiovascular and pulmonary systems is inextricably linked to the renin-angiotensin system (RAS). Physiologically speaking, a balance between the vasodeleterious (ACE/Ang II/$AT_1R$) and vasoprotective (ACE2/Ang-(1–7)/MasR) components of the RAS is critical for cardiopulmonary homeostasis. Upregulation of the ACE/Ang II/$AT_1R$ axis shifts the system toward vasoconstriction, proliferation, hypertrophy, inflammation, and fibrosis, all factors that contribute to the development and progression of cardiopulmonary diseases. Conversely, stimulation of the vasoprotective ACE2/Ang-(1–7)/MasR axis produces a counter-regulatory response that promotes cardiovascular health. Current research is investigating novel strategies to augment actions of the vasoprotective RAS components, particularly ACE2, in order to treat various pathologies. While multiple approaches to increase the activity of ACE2 have displayed beneficial effects against experimental disease models, the mechanisms behind its protective actions remain incompletely understood. Recent work demonstrating a non-catalytic role for ACE2 in amino acid transport in the gut has led us to speculate that the therapeutic effects of ACE2 can be mediated, in part, by its actions on the gastrointestinal tract and/or gut microbiome. This is consistent with emerging data which suggests that dysbiosis of the gut and lung microbiomes is associated with cardiopulmonary disease. This review highlights new developments in the protective actions of ACE2 against cardiopulmonary disorders, discusses innovative approaches to targeting ACE2 for therapy, and explores an evolving role for gut and lung microbiota in cardiopulmonary health.

## INTRODUCTION

The circulating renin-angiotensin system (RAS) is a hormonal system that plays a prominent role in regulating blood pressure (BP) and maintaining fluid balance in the body[1], whereas local expression of the RAS in organs and tissues promotes growth, differentiation, and

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inflammation[2,3]. Angiotensin II (Ang II), a potent vasoconstrictor and the major effector peptide of this system, is formed as a result of enzymatic cleavage of Angiotensin I (Ang I) by Angiotensin converting enzyme (ACE) (Figure 1). Ang II exerts its actions via binding to two distinct G-protein coupled receptors: angiotensin type 1 receptor ($AT_1R$) or angiotensin type 2 receptor ($AT_2R$). Apart from regulating BP, overactivity of the RAS has been implicated in the development and progression of numerous cardiovascular (CV) pathophysiologies[4]. In fact, ACE inhibitors (ACEi) and angiotensin receptor blockers (ARB) are the first line of treatment for patients with hypertension[5] and other cardiovascular diseases (CVD)[6] . These agents are effective for BP lowering[7], as well as for attenuation of ventricular remodeling, reduced incidence of myocardial infarction (MI), and prevention of the development of heart failure (HF)[8].

The recent discovery of several new RAS components has altered the way RAS is perceived in the field. Of particular significance was the identification of Angiotensin converting enzyme 2 (ACE2)[9,10], a monocarboxypeptidase, which cleaves Ang II into Angiotensin-(1–7) [Ang-(1–7)]. Ang-(1–7) is a vasodilatory heptapeptide that acts through the Mas receptor (MasR) to mediate anti-proliferative, anti-fibrotic, and anti-inflammatory effects[11–13]. These observations have led to the proposal that the angiotensin system is composed of two opposing arms, with ACE, Ang II, and $AT_1R$ representing the deleterious axis [ACE-Ang II-$AT_1R$], whereas ACE2, Ang-(1–7), and MasR represent the beneficial axis [ACE2-Ang-(1–7)-MasR] (Figure 1). Under physiological conditions, there exists a balance between these two axes, which aids in maintenance of cardiopulmonary homeostasis. Upregulation of the vasodeleterious axis leads to vasoconstriction, hypertrophy, inflammation, and cardiac remodeling[14]. In contrast, stimulation of the vasoprotective RAS axis either by genetic overexpression[13,15,16], peptide infusion[17], or administration of synthetic molecules[18] yields valuable cardiopulmonary effects. Despite these beneficial actions of the vasoprotective axis in restoring cardiopulmonary function, the mechanism(s) by which ACE2 mediates its protection are not completely understood. In this review, we present evidence of the direct and indirect actions of ACE2 on the cardiopulmonary system. ACE2-mediated effects on cardiac and pulmonary tissues include vasodilation, anti-hypertrophy, anti-proliferation, and anti-fibrosis (Figure 2). Furthermore, ACE2 treatment has been shown to improve endothelial function[19], facilitate angiogenesis[20,21], and enhance progenitor cell function[22], all of which promote cardiopulmonary health. Additionally, ACE2 exerts favorable actions on the bone marrow (BM), brain, and gastrointestinal system, which provide indirect benefit to the cardiopulmonary system. With regards to its actions on the BM, ACE2 improves the migratory potential of stem/progenitor cells to aid regeneration of the injured cardiopulmonary tissue. ACE2 also increases nitric oxide (NO) production in BM-derived progenitor cells[23] and enhances vascular repair[22,23]. Similarly, ACE2 acts on the brain to improve baroreflex sensitivity[24], reduce sympathetic outflow[25], and decrease BP[26]. It is generally believed that the benefits of ACE2 on the heart, lungs, brain, and progenitor cells are primarily mediated by a reduction in Ang II and subsequent elevation in Ang-(1–7) levels. However, other factors may be involved as well. Recent work has highlighted non-catalytic roles for ACE2 in amino acid transport and expression of antimicrobial peptides in the gut. Along with this, the role of gut and lung microbiota in promoting cardiorespiratory health is rapidly emerging. Thus, we propose that ACE2 might also exhibit cardiopulmonary

protection via alterations in the gut and/or lung microbiomes, which may open new avenues for therapeutic interventions. In this review, we will discuss our current understanding of the role of ACE2 in cardiopulmonary diseases, discuss various strategies to target this enzyme for translational applications, and explore the concept that the beneficial effects of ACE2 on cardiopulmonary disorders may be mediated by changes in microbiota. For this purpose, an extensive search was performed for literature from the last 20 years, utilizing PubMed and other scholarly search bibliographic databases with a variety of terms that included ACE2, renin angiotensin system, pulmonary hypertension, CV disease, and microbiota.

## THE CARDIOVASCULAR SYSTEM

### ACE2 Polymorphisms in Hypertension and Ventricular Hypertrophy

A multitude of research has focused on the role of ACE2 in the CV system. Given that ACE2 maps to a defined quantitative trait locus on the X chromosome in different animal models of hypertension, several studies were carried out to investigate whether common single-nucleotide polymorphisms in the human ACE2 locus correlate with changes in BP and cardiac function. It was observed that the ACE2 variant G8790A was significantly associated with essential hypertension in females and in Han-Chinese males[27]. Furthermore, Patel et al.[28] reported that genetic variation in ACE2 in Caucasians with type 2 diabetes was associated with hypertension and reduced systolic function in men, whereas ACE2 variation in women was associated with hypertension and increased left ventricular (LV) mass. This gender-specific data is especially interesting in light of new evidence that a partial loss of X-linked ACE2 enhances susceptibility to heart disease (HD) in response to CV stressors[28]. Furthermore, in line with the above findings, several other groups have shown that polymorphisms in the ACE2 gene are linked to cardiac hypertrophy[29,30]. However, discrepancies exist between various studies regarding the association of ACE2 polymorphisms with hypertension and cardiac function. These inconsistencies may arise from differences in the gender, ethnicity, or etiology of subject populations under consideration[31]. Nonetheless, all of these observations emphasize the importance of the ACE2 gene in regulating CV function.

### ACE2 in Cardiovascular Disease: Lessons from Animal Models

CVD is the leading cause of death for both men and women in the United States[32]. While the etiology of this disease is complex, it is well-established that the development and progression of CVD is accelerated by the actions of Ang II. One mechanism by which ACE2 is thought to prevent end-organ damage is via degradation of Ang II. In this regard, reduced ACE2 gene expression should favor increased levels of Ang II and promote tissue injury, a contention supported by several animal studies. For instance, increased concentration of cardiac Ang II[33,34] and reduced levels of myocardial Ang-(1–7)[34] were observed in ACE2 knockout (ACE2-KO) mice. These gene-ablated mice displayed enhanced susceptibility to cardiac problems when subjected to chronic Ang II infusion or pressure overload[33–35]. Furthermore, these studies revealed that ACE2-KO mice exhibited maladaptive cardiac remodeling in terms of increased ventricular hypertrophy[33–35], excessive fibrosis[34,35], heightened superoxide production[34,35], and decreased cardiac contractility[33]. In a separate study, Trask et al. [36] demonstrated that chronic ACE2 inhibition

resulted in excessive accumulation of cardiac Ang II and worsening of ventricular remodeling in (mRen2)27 transgenic rats. Subsequently, a report by Sahara et al.[37] revealed that ACE2 deletion promoted the development of atherosclerosis and arterial neointima formation, highlighting the importance of ACE2 in conferring vascular protection. Conversely, overexpression of ACE2 in the myocardium or treatment with recombinant human ACE2 protein (rhACE2) prevented cardiac remodeling following chronic Ang II infusion[35,38], pressure overload[39], or MI[40]. Preliminary studies in our lab have demonstrated that global ACE2 overexpression in mice reduces cardiac injury and remodeling following MI (unpublished data). These findings are consistent with previous reports illustrating that adenoviral-mediated overexpression of ACE2 resulted in decreased ACE and Ang II in the myocardium[40,41]. Likewise, treatment with rhACE2 caused enhanced plasma Ang-(1–7) levels along with reduced plasma and myocardial Ang II[35]. These data suggest that ACE2 expression is essential for CV protection.

## ACE2: A Biomarker for Heart Failure

ACE2 is expressed in various cellular compartments of the heart including the cardiac endothelial cells and vascular smooth muscle cells, as well as in cardiomyocytes[42]. It has been observed that the expression of ACE2 is highly regulated by pathological stimuli and tissue insult. In this regard, animal studies have revealed increased levels of ACE2 mRNA and protein following myocardial injury[42,43]. Consistent with these findings, Zisman et al.[44] demonstrated that ACE2 protein and activity were significantly elevated in failing human hearts, resulting in increased Ang-(1–7) and decreased Ang II levels. ACE2 was also found to be upregulated in idiopathic dilated and ischemic cardiomyopathies[31]. However, due to the complexity of CVD etiology, ACE2 expression in failing human hearts remains controversial[42,45–48]. Nonetheless, these observations suggest that an elevation in cardiac ACE2 levels could serve as a compensatory mechanism to impede progression of HF.

Traditionally, ACE2 has been thought of as a tissue-bound enzyme; however, recent reports have detected and measured soluble ACE2 (sACE2) in circulating human plasma. In fact, sACE2 activity was found to be increased in patients with HD[49–53], which positively correlated with disease severity[49–51,53], end systolic diameter[53], end diastolic diameter[53], and infarct size[52], whereas it correlated negatively with ejection fraction[52,53]. Interestingly, sACE2 activity was also elevated in hypertensive patients with impending HF[53]. Thus, sACE2 could be a potential biomarker of cardiac dysfunction in patients with hypertension and HF. However, recent work by Shao et al.[54] appears to run counter to the idea that increased ACE2 activity is suggestive of worsening disease. This study found that a 50% increase in baseline sACE2 levels following intensive medical therapy in acutely decompensated HF patients was predictive of improved clinical outcomes. We speculate that these discrepancies may be due to differences in etiology, patient populations, disease stages, or medical therapies. While these disparities warrant further investigation, the current evidence is highly indicative of a cardioprotective role for ACE2 in humans and suggests that differential regulation of ACE2 may have important functional consequences in CVD.

## Brain ACE2 Effects on Cardiovascular Disease

The role of the brain in development and perpetuation of CVD, namely hypertension and HF, is well established. ACE2 is widely expressed in the brain, particularly in areas involved in regulation of CV function. Whereas sACE2 activity in the plasma appears to be increased in pathological states, ACE2 expression was found to be considerably reduced in the brains of animals with hypertension[24,26,55], neurogenic hypertension[56], and chronic HF[25,57]. This is consistent with studies showing that ACE2 gene deletion from brain regions led to impaired baroreflex and autonomic functions[58]. On the other hand, genetic overexpression of ACE2 in the brain decreased BP[26], reduced sympathetic outflow[25], increased baroreflex sensitivity[24] and attenuated neurogenic hypertension[59,60]. In addition, brain ACE2 overexpression increased expression of endothelial and neuronal nitric oxide (NO) synthase[56], augmented NO release[56], reduced pro-inflammatory cytokines[60], and decreased reactive oxygen species [61], all factors that contribute to lowering of BP. These data support a role for ACE2 in regulation of CV functions via actions on the brain.

## ACE2 in Circulating Cells

It is becoming evident that interplay between organs and BM-derived circulating cells is critical for disease prevention. A number of cardiopulmonary disorders have documented imbalances in the circulating levels of reparative and inflammatory cells (ICs), which contribute to inflammation and impaired vascular repair. We have begun to investigate the central mechanisms involved in the regulation of BM-derived cells. Retrograde neuronal tracing via injection of pseudorabies virus into the BM established a functional connection between the brain and BM[62]. Further studies from our group have demonstrated that the interaction between the autonomic nervous system and BM is dysfunctional in hypertensive rats, a finding associated with increased ICs and decreased endothelial progenitor cells (EPCs)[63]. Progenitor cells, specifically EPCs, are vital for vascular endothelial repair[64], and reduced numbers and function of EPCs have been observed in patients with cardiopulmonary disease[65–68]. The vasoprotective RAS has demonstrated a role in improving the number[69] and function[22,23] of progenitor cells. In fact, animals treated with the ACE2 activator, diminazene aceturate (DIZE), displayed increased circulating EPC levels, improved engraftment of cardiac progenitor cells into the heart, and decreased ICs in the infarcted heart[70]. DIZE also normalized the decrease in EPC proliferation and migration associated with monocrotaline (MCT)-induced pulmonary arterial hypertension (PAH)[71]. Consistent with this, the EPCs of patients with PAH demonstrated a reduction in migratory potential *in vitro*, which was improved with DIZE treatment[71]. Similarly, genetic modification of BM-[72] or umbilical cord blood-[73] derived mesenchymal stem cells (MSCs) with ACE2 yielded cardiopulmonary protective effects. Work from our own group has demonstrated that NO production was significantly increased in BM-derived MSCs overexpressing ACE2 and Ang-(1–7) (Figure 2). Thus, ACE2 is vital for the function and balance of circulating progenitor cells and ICs.

# THE PULMONARY SYSTEM

## Role of ACE2 in Lung Injury

The benefits of the RAS vasoprotective axis are not only limited to the CV system; studies over the past few years have highlighted the salutary effects of ACE2 on lung pathophysiology. With regards to pulmonary disease, ACE2 came into prominence when it was identified as a functional receptor for the SARS coronavirus (SARS-CoV), the etiological agent of severe acquired respiratory syndrome (SARS)[74]. It has been observed that SARS infection reduces pulmonary ACE2 expression and also leads to lung failure and death. This connection between ACE2 expression and lung pathophysiology is further supported by studies conducted on ACE2-KO mice, which demonstrated increased susceptibility to acute lung injury[75]. Recently, ACE2 was also implicated in protection against influenza-induced lung injury. For instance, ACE2 deficiency worsened disease pathogenesis in H7N9 virus-induced lung injury[76]. In addition, ACE2-KO mice displayed increased severity of H5N1-induced lung injury[77]. Consistent with this evidence, H5N1[77] and H1N1[78] infection decreased lung ACE2 expression in wild-type mice. Furthermore, patients with H5N1 exhibited increased levels of serum Ang II, a finding recapitulated in murine models of H5N1 infection[77]. Interestingly, treatment with rhACE2 restored lung function in ACE2 null mice following H5N1-induced lung pathogenesis[77]. Taken together, these data suggest that pulmonary ACE2 is protective and that treatments targeting this enzyme might be effective in attenuating acute lung injury.

## ACE2 in Pulmonary Arterial Hypertension

PAH is a fatal lung disease characterized by increased BP in the pulmonary circulation. A growing body of work has focused on the importance of ACE2 in PAH and associated right HF. Decreased lung ACE2 expression[79,80] and enzymatic activity[81], along with increased circulating Ang II levels [82], have been observed in PAH. Additionally, reduction in circulating ACE2 and Ang-(1–7) levels has been observed in patients with PAH from congenital HD[83,84]. Moreover, auto-antibodies to ACE2 predisposed patients with connective tissue diseases to development of PAH[85]. These clinical findings suggest that enhancement of ACE2 could provide pulmonary protection. In this regard, animal studies have shown that overexpression of ACE2[86] or administration of rhACE2[87] attenuated PAH and were associated with improved structural and functional changes in the right heart in several models[16,39,86]. Thus, elevating levels of circulating ACE2 could be a viable option to improve cardiopulmonary function in the clinical setting of PAH.

## ACE2 in Pulmonary Fibrosis

Pulmonary fibrosis (PF) is a devastating disease marked by scarring of the lung tissue, and recent research emphasizes the contributions of ACE2 in protection against PF. Studies by Li et al.[88] were the first to show that lung ACE2 expression and activity were severely downregulated in humans and in animal models of lung fibrosis. Along the same lines, ACE2-KO mice were more prone to bleomycin-induced lung injury[89]. Conversely, pulmonary overexpression of ACE2 by gene delivery or administration of rhACE2 attenuated PF[16,89]. Collectively, these observations suggest a potent anti-fibrotic role for ACE2 in animal models of PF.

## THE GASTROINTESTINAL SYSTEM

The above data clearly indicate that ACE2 is beneficial to the cardiopulmonary system. These favorable effects could be manifested in a paracrine-like manner via alteration of the local RAS. However, the indirect effects of ACE2 on modulation of circulating molecules, including Ang-(1–7), cannot be ruled out. Nevertheless, it appears that the therapeutic potential of ACE2 may be more pronounced than that of Ang-(1–7). Thus, we propose that ACE2 has a multifunctional role in providing cardiopulmonary protection. This view is supported by recent evidence: i) Oral feeding of ACE2 attenuates PAH with only a modest increase in plasma Ang-(1–7)[81], and ii) ACE2 plays a non-catalytic role in gut biology and modulation of gut microbiota composition[90]. This has led us to speculate if the beneficial effects of ACE2 on the cardiopulmonary system are partially mediated by changes in the gut microbiome.

### Role of ACE2 in the Gut

Research in the last few years has highlighted an important non-RAS-related role for ACE2 in amino acid transport and gut microbiota composition. Work by Harmer et al. [91] demonstrating high levels of ACE2 expression within the human gastrointestinal tract suggested a functional role for ACE2 in this system. However, the discovery of Collectrin, a regulator of neutral amino acid transporters in the kidney, provided the first evidence of a non-catalytic role for ACE2[92]. Similar to ACE2, Collectrin is a type 1 transmembrane protein that shares 47.8% identity with the extracellular, transmembrane, and cytosolic domains of ACE2[92]. The physiological role of Collectrin in amino acid reabsorption/transport in the kidneys via its association with the Slc6 family of neutral amino acid transporters[93] raised the question of a similar function for ACE2. One of these neutral amino acid transporters, $B^0AT1$, colocalizes and interacts with ACE2 on small intestine brush border membranes[94], where Collectrin is absent. In fact, ACE2 binds and stabilizes $B^0AT1$ in the small intestine, and the transport activity of $B^0AT1$ is dramatically stimulated by co-expression with ACE2[95]. Important work by Hashimoto et al.[90] linked the amino acid transport function of ACE2 to the microbial ecology in the gut. Their studies were the first to demonstrate that ACE2- KO animals had reduced levels of neutral amino acids in the serum and, specifically, displayed impairment in uptake of tryptophan (Trp). ACE2 mutants also exhibited decreased expression of antimicrobial peptides and showed altered gut microbial composition, which was restored by tryptophan administration[90]. Interestingly, a recent study by Murr et al[96] found that coronary artery disease (CAD) patients with low serum tryptophan concentrations displayed reduced life expectancy. These findings support the idea that ACE2 effects on gut microbiota could positively influence the CV system.

On the other hand, ACE2 cardioprotective actions could be mediated directly via antimicrobial peptides, independent of changes in gut microbiota. Antimicrobial peptides themselves have demonstrated effects on the cardiopulmonary system through functions other than antimicrobial activity. The myocardial expression of LL-37 and β-2 defensin increased following hypoxia, and LL-37 treatment enhanced the migration of human circulating peripheral blood stem and progenitor cells (PBSPCs)[97], raising the possibility that some antimicrobial peptides may aid in the homing of PBSPCs to injured myocardium.

Another antimicrobial peptide, PR-39, has also demonstrated cardioprotection via prevention of leukocyte adhesion and emigration[98], as well as suppression of superoxide release[99]. Interestingly, the role of antimicrobial peptides may not be so straightforward. The alpha defensins, for one, have been linked to atherosclerosis[100–102], CAD[103], and ST-segment elevation MI[103]. In addition, the antimicrobial peptide LL-37 is highly expressed in atherosclerotic plaques and has been implicated in modulation of the vessel inflammatory response[104]. Taken together, these data implicate antimicrobial peptides in both cardiopulmonary health and pathophysiology, and their actions may be one mechanism by which ACE2 mediates its protective effects.

### Gut Microbiota and Cardiovascular Disease

The gut microbiome is emerging as a critical player in health and disease. Reports have drawn associations between intestinal dysbiosis and metabolic diseases, including obesity and type 2 diabetes[105], both of which are considered major risk factors for developing CVD[106]. A recent study by our group has also demonstrated a link between gut dysbiosis and hypertension[107]. These findings are consistent with clinical studies which suggest that consumption of probiotics improves CV health. Namely, probiotics have been reported to reduce BP[108–115], decrease oxidative stress[116–123], positively alter cholesterol concentrations[108,109,124–126], and release ACE-inhibiting peptides[127]. More recently, alterations in the gut microbiota have been directly implicated in the pathogenesis of CVD. For instance, infants with congenital heart defects showed reduced total bacterial count as compared to healthy infants[128], and atherosclerotic plaques were found to house microbiobes[129]. Furthermore, administration of probiotics has been successful in decreasing BP and delaying the development of HF in rats following MI[130]. Interestingly, BP reduction may be mediated by the effects of probiotics on autonomic neurotransmission. In support of this hypothesis, intraduodenal injection of *Lactobacillus johnsonii* resulted in reduced renal sympathetic nerve activity and increased gastric vagal nerve activity[131]. Consistent with these findings, others have reported that communication between the nervous system and microbiota may occur via afferent sensory neurons in the gut[132]. In fact, there is evidence that stress-induced intestinal permeability allows enhanced uptake of bacterial antigens[133], allowing for modulation of the central nervous system through immune and enteric nervous system pathways. It is believed that probiotics may attenuate this stress-induced hyperpermeability[134]. Collectively, these data raise the question of whether the beneficial effects of enhanced ACE2 expression and activity on HF and hypertension are partly a result of altered gut microbiota and subsequent modified autonomic inputs to the CNS.

## LUNG MICROBIOME AND PULMONARY DISEASE

The respiratory tract houses its own microbiome, and Dickson et al.[135] have proposed a model in which inflammation in response to a trigger alters lung conditions and affects the ability of various microbes to immigrate or be eliminated, thus favoring the growth of some microbes over others. This is supported by studies that have demonstrated lung dysbiosis during exacerbations of respiratory diseases[135]. Specifically, progression of idiopathic PF is associated with increased presence of *Staphylococcus* and *Streptococcus* bacteria[136]. Interestingly, pulmonary dysfunction is often related to bowel disease[137]; however, the

connection between the lung and the gut is not completely understood. Recent work investigating microbiomes in infants with cystic fibrosis discovered an overlap in bacterial genera present in the gut and respiratory tract, with some populations that change similarly over time[138]. Additionally, numerous studies have demonstrated that enterically-administered probiotics prevent upper respiratory tract infections[139]. Though these studies have not addressed the mechanisms behind the observed pulmonary benefits, we speculate that probiotics could modulate the gut microbiome to favorably alter the nervous system and circulating cells to exert respiratory protection. In addition, benefits may result from a direct connection between gut and lung microbiomes. It will be interesting to investigate whether the benefits of ACE2 on pulmonary diseases may be mediated via modulation of gut and/or lung microbiota populations.

## CLINICAL PERSPECTIVES OF ACE2-TARGETED THERAPIES

### Recombinant Human ACE2 Protein

Treatment with rhACE2 has demonstrated protection against a variety of experimental disease models that are associated with elevated levels of Ang II or a dysregulated RAS[140]. Encouraging results from animal studies have resulted in the initiation of clinical trials using rhACE2. Single or multiple injections of rhACE2 have been well-tolerated by healthy subjects, with no serious adverse effects or dose-limiting toxicity[141]. Currently, GlaxoSmithKline is testing the therapeutic potential of GSK2586881 (formerly APN01), a soluble form of rhACE2, against acute lung injury. In another trial, rhACE2 is under evaluation for the treatment of PAH (ClinicalTrials.gov; NCT01884051). Although rhACE2 is in clinical trials, there appear to be several limitations that undermine the success of protein therapeutics. One of the major hurdles is the high cost associated with recombinant protein manufacturing. Furthermore, protein stability, repetitive intravenous administration, and patient compliance pose challenges in realizing the true benefits of rhACE2 therapy.

### ACE2 Activators

An effective approach to overcome the limitations of protein therapy would be to identify synthetic compounds that can enhance the activity of endogenous ACE2 protein. In 2008, our group was the first to discover several synthetic activators of ACE2 while employing a structure-based drug design[18]. These small molecule ACE2 activators included resorcinolnaphthalein[18], 1-[[2-(dimethyl amino) ethyl] amino]-4-(hydroxymethyl) - 7-[[(4-methylphenyl) sulfonyl] oxy]-9H–xanthone9 (XNT)[18], and DIZE[142]. Among these three compounds, it was observed that DIZE is the most potent, followed by XNT, and finally resorcinolnaphthalein. DIZE, an antiprotozoan chemotherapeutic agent, was shown to exert beneficial effects against heart and lung diseases. For example, DIZE provided cardioprotective effects in a rodent model of MI[70] and was effective in preventing the development of PAH in the MCT, bleomycin, and hypoxia models[71]. Similarly, chronic treatment of spontaneously hypertensive rats (SHR) with XNT prevented an increase in systemic BP, along with improving cardiac contractility[18]. Furthermore, in the MCT-induced rat model of PAH, administration of XNT resulted in lowering of right ventricular systolic pressure (RVSP)[143], a surrogate marker of pulmonary pressure. This data is supported by studies using another ACE2 activator, resorcinolnaphthalein, which provided

protection against MCT-induced PAH by improving endothelial function, modulating cytokine release, and reducing right ventricular hypertrophy[79,144]. However, certain issues hamper the successful translation of these ACE2 activators into the clinic. XNT has an undesirable pharmacokinetic profile with poor water solubility and requires acidic pH for solubilization, thus making it an undesirable drug entity[18]. On the other hand, DIZE is associated with severe toxic effects. Chronic DIZE administration can result in renal, hepatic and brain injury, which could be life-threatening[145]. Besides, DIZE is mutagenic, but not teratogenic[145]. Nonetheless, XNT and DIZE could serve as lead molecules that could be structurally modified for clinical use.

A recent report from the Batlle group challenged the idea that the benefits of XNT and DIZE are mediated via activation of ACE2[146]. Using *in vivo* mouse experiments, they have shown that the BP-lowering effects of XNT were not blocked by the ACE2 inhibitor, MLN-4760, and that XNT decreased BP even in ACE2-KO animals. Furthermore, XNT-treated animals demonstrated no difference in kidney or serum ACE2 activity, plasma Ang II levels, or plasma Ang-(1–7) levels as compared with controls. Additionally, neither XNT nor DIZE was capable of increasing ACE2 activity *in vitro* or *ex vivo*[146,147]. This is in stark contrast to data published by our group and others which have demonstrated the ability of these activators to enhance ACE2 enzymatic activity *in vitro* and *in vivo*. A number of publications have recorded *in vitro* increases in the enzymatic activity of rhACE2 following incubation with XNT[18,142,148], DIZE[71,142], or resorcinolnaphthalein[18]. Similar results have been obtained from *in vivo* studies as well, as XNT was shown to increase ACE2 activity in thrombi[149] and cardiac tissue[150] of hypertensive rats. Additionally, administration of DIZE increased ACE2 activity in the retina[151], kidney[152], lungs[71], heart[70] and plasma[70]. Both XNT and DIZE were found to significantly increase plasma Ang-(1–7) levels[148,152], which is a further indication of the direct effects of these small molecules on ACE2. A recent study demonstrated that restoration of impaired endothelial-dependent relaxation (EDR) in *db/db* mice by DIZE treatment was ACE2-mediated. This group showed that Ang-(1–7), but not DIZE, rescued EDR in aortas from ACE2-KO animals[153]. Moreover, the protective effects of XNT on endothelial function[148] and MCT-induced PAH[143] were attenuated by co-administration of the MasR antagonist A-779. Likewise, the benefits of resorcinolnaphthalein on PAH[79] and of DIZE on cerebral ischemia[154] were abolished by blockage of the MasR. Co-administration of the ACE2 inhibitor, C-16, also blocked the positive effects of DIZE on PAH[71]. Furthermore, DIZE failed to elicit beneficial effects on cholesterol metabolism and abdominal aortic aneurysms in ACE2-KO mice[152]. The recent discovery of a novel ACE2 activator, NCP-2454, provides additional support for the idea that ACE2 activators do indeed enhance activation of ACE2 both *in vitro* and *in vivo*[155]. This mountain of data provides evidence that these compounds are, indeed, ACE2 activators. However, this does not rule out potential off-target effects of XNT and DIZE in addition to ACE2 activation. The observed discrepancies may be attributed to differences in dosages, duration of drug administration, and animal strains used in the different experimental protocols.

### Genetic Modification of Stem and Progenitor Cells

Pharmacotherapies including beta blockers, ACEi, ARBs, and anti-mineralocorticoids are used to attenuate maladaptive cardiac remodeling associated with various HD[156]. In addition, interventional or surgical methods are employed to improve heart function. Despite these therapies, the prognosis for patients diagnosed with HF remains poor[157]. As a result, researchers are exploring cell-based therapies as a means of regenerating the damaged myocardium. In this context, administration of EPCs, BM- or adipose-derived MSCs, and umbilical cord blood cells are being evaluated for their potential to offer cardioprotection. However, many hurdles still exist before the benefits of cell therapy in humans may be fully realized. For example, the hostile environment of the injured tissue, characterized by inflammation and high oxidative stress, could impair engraftment of the injected progenitor/stem cells. We believe that overexpression of ACE2 in these cells could improve their survival and enhance their potential to effectively repair tissue injury. In fact, we have shown that ACE2 priming of EPCs not only enhances their function[22,23] but also increases their therapeutic efficacy to render protection against stroke[23]. Recently, umbilical cord- or BM-derived MSCs were more effective in rescuing lung injury compared to MSCs alone[72,158]. Autologous stem cell transfer is attractive for clinical therapy because it eliminates the possibility of an adverse immune response by the host and also overcomes the toxicity of immunosuppressive medications. We believe that improving the function of progenitor/stem cells through genetic modification may hold promise for treating cardiopulmonary disorders.

### Bioencapsulation of ACE2

The most preferred means of drug administration is via the mouth. However, therapeutic proteins such as ACE2 cannot be given orally due to degradation by the acidic environment of the gastrointestinal tract. To overcome this issue, we have developed an oral delivery platform that utilizes transplastomic technology to bioencapsulate ACE2 within plant leaves[81]. Production of therapeutic proteins using transplastomic technology has multiple benefits over conventional transgenic plants and mammalian systems, including high expression of transgenes, the ability to produce in bulk, low maintenance and cost requirements, use of the operon for expression of multiple genes simultaneously, minimized risk of contamination from human pathogens, and improved biological containment due to the maternal inheritance of plastids[81,159,160]. A major advantage of this technology is that chloroplast expression of ACE2 enables bioencapsulation within plant cells, such that it is protected from gastric enzymatic degradation upon oral delivery. We observed that oral feeding of rats with bioencapsulated ACE2 attenuated MCT-induced PAH and was associated with improved right heart function[81]. This oral delivery platform holds great potential for the clinical development of ACE2 therapy.

## CONCLUSION

The evidence presented here reinforces the idea that enhancement of the vasoprotective RAS components, particularly ACE2, will be critical for the future of cardiopulmonary disease therapy. Although the exact mechanism of ACE2 action is unknown, it is likely a consequence of both its catalytic and non-catalytic activity. Current therapies have focused

on improving ACE2 enzymatic activity; however, given the ability of ACE2 to modulate gut microbiota, we anticipate that future therapies may also target the non-traditional aspects of ACE2 function.

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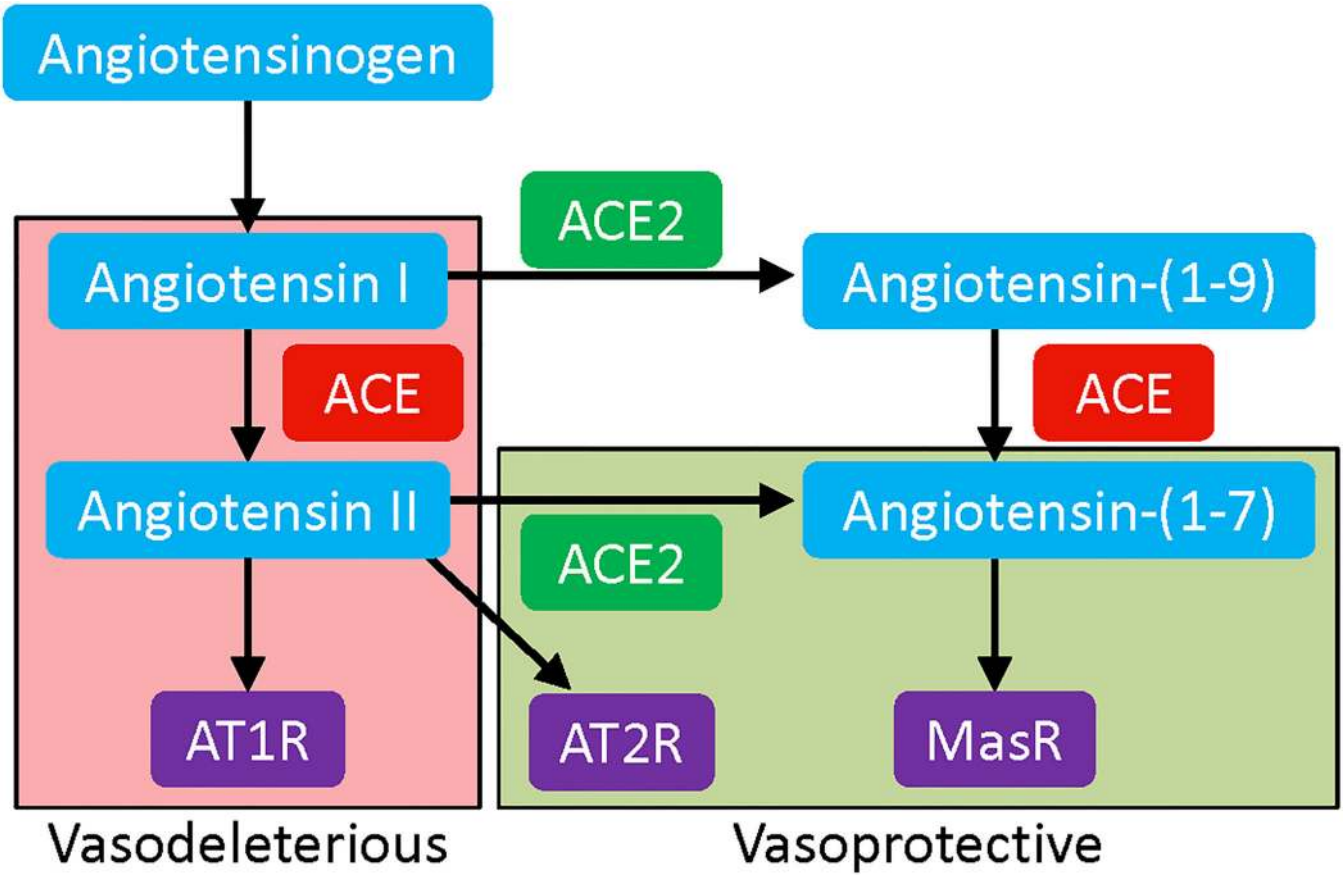


**Figure 1.**
Components of the vasodeleterious and vasoprotective axes of the RAS.

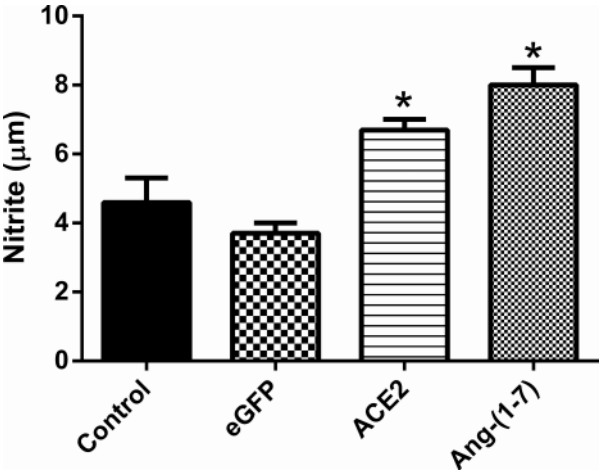


**Figure 2.**

Cultured rat MSCs were infected with 50 multiplicity of infection (MOI) of lenti-eGFP, lenti-ACE2, or lenti-Ang-(1–7). Control was uninfected MSCs. Lenti-eGFP served as viral transduction control. Data are expressed as mean ±SEM. *p<0.05 vs Control & eGFP groups.

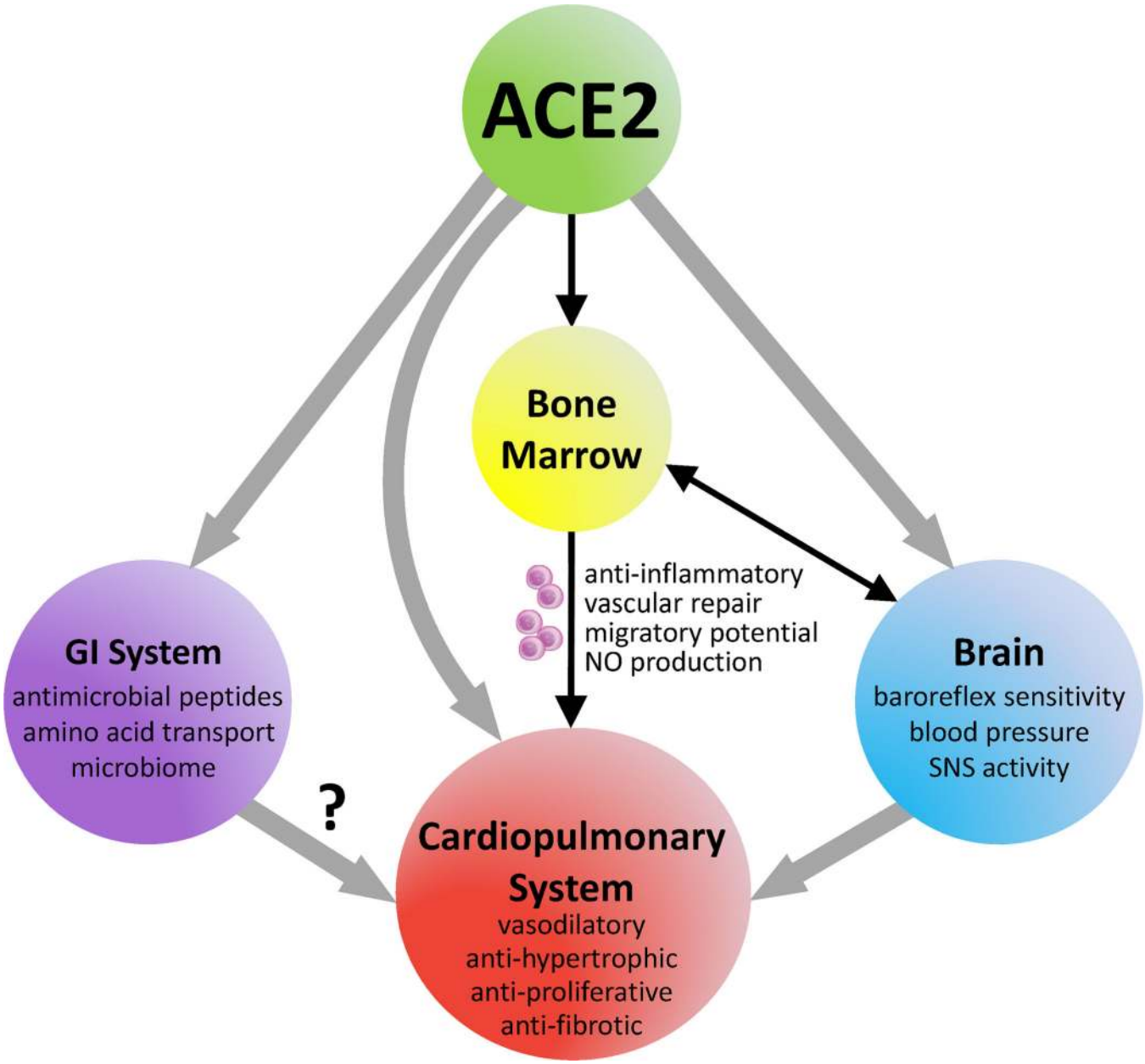


**Figure 3.**
Influences of ACE2 on body systems and the subsequent implications on cardiopulmonary health. SNS = sympathetic nervous system.