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Abstract

Emerging evidences indicate that diminished activity of the vasoprotective axis of the renin–angiotensin system, constituting angiotensin-converting enzyme 2 (ACE2) and its enzymatic product, angiotensin-(1-7) [Ang-(1-7)] contribute to the pathogenesis of pulmonary hypertension (PH). However, long-term repetitive delivery of ACE2 or Ang-(1-7) would require enhanced protein stability and ease of administration to improve patient compliance. Chloroplast expression of therapeutic proteins enables their bioencapsulation within plant cells to protect against gastric enzymatic degradation and facilitates long-term storage at room temperature. Besides, fusion to a transmucosal carrier helps effective systemic absorption from the intestine on oral delivery. We hypothesized that bioencapsulating ACE2 or Ang-(1-7) fused to the cholera nontoxin B subunit would enable development of an oral delivery system that is effective in treating PH. PH was induced in male Sprague Dawley rats by monocrotaline administration. Subset of animals was simultaneously treated with bioencapsulaed ACE2 or Ang-(1-7) (prevention protocol). In a separate set of experiments, drug treatment was initiated after 2 weeks of PH induction (reversal protocol). Oral feeding of rats with bioencapsulated ACE2 or Ang-(1-7) prevented the development of monocrotaline-induced PH and improved associated cardiopulmonary pathophysiology. Furthermore, in the reversal protocol, oral ACE2 or Ang-(1-7) treatment significantly arrested disease progression, along with improvement in right heart function, and decrease in pulmonary vessel wall thickness. In addition, a combination therapy with ACE2 and Ang-(1-7) augmented the beneficial effects against monocrotaline-induced lung injury. Our study provides proof-of-concept for a novel low-cost oral ACE2 or Ang-(1-7) delivery system using transplastomic technology for pulmonary disease therapeutics.

Keywords

chloroplast, molecular farming, plant-made pharmaceuticals, pulmonary hypertension, renin–angiotensin system

Disciplines

Dentistry

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Abstract

Emerging evidences indicate that diminished activity of the vasoprotective axis of the renin–angiotensin system, constituting angiotensin-converting enzyme 2 (ACE2) and its enzymatic product, angiotensin-(1-7) [Ang-(1-7)] contribute to the pathogenesis of pulmonary hypertension (PH). However, long-term repetitive delivery of ACE2 or Ang-(1-7) would require enhanced protein stability and ease of administration to improve patient compliance. Chloroplast expression of therapeutic proteins enables their bioencapsulation within plant cells to protect against gastric enzymatic degradation and facilitates long-term storage at room temperature. Besides, fusion to a transmucosal carrier helps effective systemic absorption from the intestine on oral delivery. We hypothesized that bioencapsulating ACE2 or Ang-(1-7) fused to the cholera nontoxin B subunit would enable development of an oral delivery system that is effective in treating PH. PH was induced in male Sprague Dawley rats by monocrotaline administration. Subset of animals was simultaneously treated with bioencapsulaed ACE2 or Ang-(1-7) (prevention protocol). In a separate set of experiments, drug treatment was initiated after 2 weeks of PH induction (reversal protocol). Oral feeding of rats with bioencapsulated ACE2 or Ang-(1-7) prevented the development of monocrotaline-induced PH and improved associated cardiopulmonary pathophysiology. Furthermore, in the reversal protocol, oral ACE2 or Ang-(1-7) treatment significantly arrested disease progression, along with improvement in right heart function, and

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H.D and M.K.R share senior authorship.

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Disclosures

None.

decrease in pulmonary vessel wall thickness. In addition, a combination therapy with ACE2 and Ang-(1-7) augmented the beneficial effects against monocrotaline-induced lung injury. Our study provides proof-of-concept for a novel low-cost oral ACE2 or Ang-(1-7) delivery system using transplastomic technology for pulmonary disease therapeutics.

Keywords

chloroplast; molecular farming; plant-made pharmaceuticals; pulmonary hypertension; renin–angiotensin system

Pulmonary hypertension (PH) is a devastating lung disease characterized by elevated blood pressure in the pulmonary circulation, which eventually leads to right heart failure and death.¹ Although significant advances have been made in recent years to improve the quality of life of patients with PH, none of the current treatments are successful in reversing PH or decreasing mortality. This has led to the realization that novel mechanism-based therapies must be developed to accomplish this goal.²

It is well-recognized that activation of the vasodeleterious axis of the renin–angiotensin system (RAS), comprising of angiotensin-converting enzyme (ACE), angiotensin II, and angiotensin type I receptor (AT1R), is involved in the development of PH.^{3,4} However, the clinical use of ACE inhibitors or AT1R blockers have yielded mixed results, thereby failing to reach a consensus opinion about their use for PH therapy. Nonetheless, the recent discovery of a close homolog of ACE, ACE2 has resulted in the reevaluation of the role of RAS in PH.^{5,6} ACE2 is widely expressed in the lungs,⁷ predominantly on the pulmonary vascular endothelium, and catalyzes the conversion of angiotensin II to Angiotensin-(1-7) [Ang-(1-7)]. Ang-(1-7) is a vasoactive heptapeptide that mediates its effects by stimulating the Mas receptor.⁸ Thus, ACE2–Ang-(1-7)–Mas receptor constitutes the vasoprotective axis of the RAS, which counterbalances the deleterious actions of the ACE–angiotensin II–AT1R axis.

Recent reports indicate that decreased tissue and circulating levels of ACE2 are associated with lung diseases in humans.^{9,10} However, restoration of ACE2 through genetic overexpression, administration of recombinant protein, or use of pharmacological ACE2 activators resulted in cardiopulmonary protective effects against animal models of pulmonary diseases.^{11–15} These findings provided compelling evidence for initiating clinical trials with recombinant ACE2 or Ang-(1-7) in treating pulmonary disorders. Although clinical trials are currently underway, the cost of manufacturing, protein stability, repetitive intravenous dosing, and patient compliance pose major impediments in realizing full therapeutic potential of this therapy. We think that development of a plant-based oral delivery system will be an ideal approach to overcome these aforementioned challenges and achieve clinical success.

Therefore, our objective in this study was to develop a low-cost oral delivery system for administering ACE2 or Ang-(1-7) and test its efficacy in an experimental model of PH. We took advantage of transplastomic technology that enables chloroplasts to generate high levels of therapeutic proteins within plant leaves.^{16–21} This technology presents minimal

risk of human pathogen or endotoxin contamination, eliminates complex protein purification steps, and abolishes cold chain and sterile delivery requirements that are commonly associated with protein therapy.^{22–24} Efficacy of plant-based pharmaceuticals has been validated by the fact that Food and Drug Administration has recently approved the use of taliglucerase alfa (Trade name: Elelyso) in the treatment of Gaucher disease.²⁵ This study provides evidence for the development of an oral delivery system to administer ACE2 and Ang-(1-7) using transplastomic technology and demonstrates its efficacy in an established rat model of monocrotaline-induced PH.

Materials and Methods

The online-only Data Supplement includes detailed descriptions of all the methods and additional supporting data.

Results

Creation and Characterization of CTB-ACE2 and CTB-Ang-(1-7) Expressed in Plant Chloroplasts

The native human ACE2 cDNA and synthetic Ang-(1-7) DNA sequences were cloned into the chloroplast transformation vector (pLDutr; Figure 1A). For efficient delivery of the proteins into circulation, a carrier protein, cholera nontoxic B subunit (CTB), was fused to the N terminal of both therapeutic proteins (Figure 1A), which facilitates their transmucosal delivery by binding to monosialotetrahexosylganglioside receptors (GM1) present on the intestinal epithelial cells. Hinge (Gly-Pro-Gly-Pro) and furin cleavage site (Arg-Arg-Lys-Arg) were placed between CTB and therapeutic proteins (Figure 1A) to eliminate steric hindrance and aid systemic release of these therapeutic proteins after they are internalized via ligand–receptor complex formation on the surface of epithelial cells. The expression of the fusion genes was driven by light regulated strong chloroplast *psbA* promoter, and the transcripts were stabilized by placing the *psbA* untranslated region at the 3' end of the fusion genes (Figure 1A). To select the chloroplast transformed with the fusion genes, aminoglycoside-3'-adenylyl-transferase gene (*aadA*), driven by the chloroplast ribosomal RNA promoter (*Prrn*), was incorporated into the expression cassette to confer the transformants resistance to spectinomycin (Figure 1A). This expression cassette was flanked by DNA sequences of isoleucyl-tRNA synthetase (*trnI*) and alanyl-tRNA synthetase (*trnA*) genes, identical to the native chloroplast genome at both flanks (Figure 1A). The flanking sequences serve to facilitate transgene integration into the chloroplast genome (Figure 1A) via double homologous recombination. Chloroplast transformation vectors expressing the ACE2 and Ang-(1-7) genes were coated onto gold particles and delivered into chloroplasts using the biolistic particle delivery system.²⁶ The bombarded plant leaves were then grown on spectinomycin-containing plant regeneration media. The shoots regenerated from the media were investigated for the site-specific integration of the transgenes into the chloroplast genome and homoplasmy of the transgenes (absence of untransformed genomes) using Southern blot analysis with the radioisotope-labeled probe spanning *trnI* and *trnA* flanking sequences.²⁶ HindIII-digested chloroplast genomic DNA from 3 independent transplastomic lines for each transplastomic line showed 2 hybridizing fragments at 8.59 and

3.44 kb for CTB-ACE2 because of an internal Hind III site of ACE2 (Figure 1A) and a fragment at 9.71 kb for CTB-Ang-(1-7), which confirm the absence of untransformed chloroplast genomes (Figure 1B and 1C). Thus, stable integration of the transgenes was confirmed, and the homoplasmic lines were used for further studies. The confirmed homoplasmic lines were multiplied using another round of antibiotic selection under aseptic conditions. Then they were cultivated in a controlled greenhouse for increasing biomass. CTB-ACE2 expression varied between 1.69% and 2.14% of the total leaf proteins (Figure 1D), depending on the harvest time because this transgene is regulated by light via the chloroplast *psbA* promoter. Similarly, the expression level of CTB-Ang-(1-7) varied between 6.0% and 8.7% of total leaf proteins (Figure 1E), at different durations of illumination, reaching maximum expression at the end of the day. Hence, for performing in vivo experimental studies, the therapeutic leaf materials were harvested at 6 PM and powdered in liquid nitrogen.

Both the therapeutic proteins were fused to the transmucosal carrier, CTB. The B subunit has a single intrasubunit disulfide bond that stabilizes the CTB monomer.²⁵ The monomers then assemble to form ring-shaped pentameric structure via intersubunit interactions including hydrogen bonds, salt bridges, and hydrophobic interactions. Upon oral administration, only the pentameric form of CTB binds to the gut epithelial GM1 receptor for internalization.²⁷ Hence, we investigated the proper formation of pentameric structure of the CTB-fused proteins and their binding affinity to GM1 receptor using GM1-ELISA. The binding affinity between CTB pentamers and the receptor was measured spectrophotometrically as a function of absorbance at 450 nm. The therapeutic proteins from the fresh leaf materials showed comparable absorbance to CTB (Figure 1F), confirming that chloroplasts form disulfide bridges, fold, and assemble these fusion proteins. We also lyophilized the leaves expressing ACE2 and Ang-(1-7) and evaluated their affinity to the GM1 receptor (Figure 1F). Lyophilization not only maintained proper folding, disulfide bond, and pentamer assembly but also facilitated long-term storage at room temperature (Figure 1F). Furthermore, the Western blot assay performed under nonreducing conditions without dithiothreitol and boiling showed that there was no monomeric form or cleaved fragments of CTB-Ang-(1-7) (Figure 1G). In the Western blot image, the major bands for pentameric assembly of CTB were detected around ≈ 50 kDa (Figure 1G, arrow head), and the expected bands for pentameric assembly of CTB-Ang-(1-7) were detected (Figure 1G, arrow). Therefore, these results confirm that the therapeutic proteins expressed in chloroplasts exist in an intact and pentameric form.

Oral Feeding of Bioencapsulated ACE2 or Ang-(1-7) Prevents Monocrotaline-Induced PH

Oral gavage of the frozen powdered leaves (500 mg in sterile phosphate-buffered saline) from untransformed wild type, CTB-ACE2 or CTB-Ang-(1-7) transplastomic plants was performed twice daily for 4 weeks in monocrotaline-challenged rats. Monocrotaline injection caused robust elevation in right ventricular systolic pressure (RVSP; Figure 2A) that was associated with the development of RV hypertrophy (RVH; Figure 2B). In contrast, monocrotaline animals gavaged with either ACE2 or Ang-(1-7) showed considerable reduction in RVSP and RVH (Figure 2A and 2B). Furthermore, measurement of hemodynamic parameters in monocrotaline animals revealed increases in RV end-diastolic

pressure (153%), $+dP/dt$ (88%), and $-dP/dt$ (107%). Conversely, treatment with ACE2 or Ang-(1-7) restored all these parameters to near-control levels (Figure 2C–2E). Echocardiography of monocrotaline rats revealed an increase in the ratio of RV to left ventricle end-diastolic area, implying dilation of the right heart (Figure 2F; Figure S1A and S1B in the online-only Data Supplement), which was accompanied with a decrease in ejection fraction (EF), measured as a ratio of RV to left ventricle EF (Figure 2G; Figure S1C and S1D). In addition, the pulsed Doppler blood flow measurement revealed decreased flow rate in the RV outflow tract (Figure 2H). Furthermore, video of the echocardiography revealed mal-adaptive structural remodeling in monocrotaline rat hearts as compared with controls (Movies SI and SII). However, oral delivery of ACE2 or Ang-(1-7) exhibited improved cardioprotective effects. Both ACE2 and Ang-(1-7) were effective in decreasing RV dilation (Figure 2F), increasing EF (Figure 2G), and preventing monocrotaline-induced decrease in RV outflow tract blood flow (Figure 2H). These beneficial effects were associated with reduced cardiac remodeling as evidenced by echocardiography videos (Movies SIII and SIV). Concurrently, RV fibrosis and pulmonary vessel wall thickness were also decreased (Figure 3A and 3B). Oral ACE2 feeding was associated with ~37% increase in circulating ACE2 activity compared with monocrotaline alone rats (Figure 3C) and a 2-fold increase in circulating levels of Ang-(1-7) (Figure S2). Interestingly, ACE2 or Ang-(1-7) did not alter the basal systemic blood pressure (control, 120 ± 5 ; monocrotaline, 123 ± 7 ; monocrotaline +ACE2, 118 ± 2 ; monocrotaline+Ang-(1-7), 116 ± 4 ; $n=5$ / experimental group).

Oral ACE2/Ang-(1-7) Treatment Arrests the Progression of Established PH

We next tested whether oral feeding of ACE2 or Ang-(1-7) after the initiation of PH could arrest the disease progression. We observed that 2 weeks of monocrotaline challenge induces significant elevation in RVSP (>45 mm Hg) compared with controls (Figure S3A). Hence, for this study, oral therapy was initiated after 2 weeks of monocrotaline challenge, and the treatment continued for additional 15 days. This regime of treatment with ACE2 or Ang-(1-7) inhibited further elevation in monocrotaline-induced RVSP and RVH (Figure 4A and 4B) and was associated with increased circulating levels of Ang-(1-7) (Figure S2). Improvements in hemodynamic parameters with regard to lowering RV end-diastolic pressure, decreasing $+dP/dt$, and reducing $-dP/dt$ were also observed (Figure S3B–S3D). In addition, ACE2/Ang-(1-7) therapy decreased RV dilation (Figure 4C; Figure S1A and S1B) and increased RVEF (Figure 4D; Figure S1C and S1D), which was supported by echocardiography video (Movies SV and SVI). Subsequently, blood flow in the RV outflow tract was also improved (Figure 4E). Finally, RV fibrosis and pulmonary vessel wall thickening were significantly attenuated in the ACE2/Ang-(1-7)-treated animals (Figure 4F and 4G).

Combination Therapy With Oral ACE2 and Ang-(1-7) Feeding Rescues Established PH

Next, we evaluated the effects of a combination therapy with ACE2 and Ang-(1-7), wherein 500 mg or 250 mg each of ACE2 and Ang-(1-7) plant material was combined. Reversal protocol was followed for this study, wherein the combination therapy was initiated after 2 weeks of monocrotaline challenge, and the treatment continued for the next 15 days. As expected, we observed better protective effects with the 500 mg combination. This combination showed 18% more reduction in RVSP and 25% additional decrease in RV/LV

+S) ratio compared with the 250 mg combination (Figure 5A and 5B). Similarly, enhanced beneficial effects of the 500 mg combination were observed for other hemodynamic parameters such as RV end-diastolic pressure, $+dP/dt$, and $-dP/dt$ (Figure 5C–5E). Both doses of the combination therapy were effective in decreasing RV dilatation (Figure 5F; Figure S1A and S1B) and increasing EF (Figure 5G; Figure S1C and S1D), which was accompanied by greater RV outflow tract blood flow (Figure 5H). All these observations were supported by echocardiography video (Movies VII and VIII). Consistent with this were the improvement in RV fibrosis and pulmonary vessel wall thickness after combination therapy (Figure 6A and 6B).

Beneficial Effects of ACE2/Ang-(1-7) Oral Therapy Involve Inhibition of Proinflammatory Cytokines and Autophagy

We hypothesized that oral delivery of ACE2 or Ang-(1-7) would correct RAS imbalance and inhibit proinflammatory cytokines. Data in Figure 7 support this hypothesis. Monocrotaline rats revealed increased pulmonary mRNA levels of ACE and AT1R (Figure 7A and 7D), which resulted in 8-fold and 4-fold increases in the ACE/ACE2 and AT1R/AT2R ratios, respectively (Figure 7C and 7F). Conversely, mRNA levels of ACE2 and AT2R were increased, whereas that of AT1R was decreased in the ACE2 or Ang-(1-7)-fed monocrotaline rats, resulting in decreased ACE/ACE2 and AT1R/AT2R ratios. Furthermore, monocrotaline-challenged animals showed increased mRNA levels of tumor necrosis factor- α (4-fold), transforming growth factor- β (4-fold), and toll-like receptor-4 (5-fold), all of which were markedly reduced by ACE2 or Ang-(1-7) treatment (Figure 7G–7I). Recent reports indicate that the autophagic protein degradation pathway is activated in monocrotaline-challenged animals.²⁸ Accordingly, we observed that the lung LC3B-II protein, an autophagy marker, was significantly increased in monocrotaline-challenged rats (Figure 7J). However, ACE2 or Ang-(1-7) decreased LC3B-II levels, implying inhibition of autophagy. Similar results with respect to RAS modulation, anti-inflammatory properties, and inhibition of autophagy were observed in the reversal protocol with monotherapy [either ACE2 or Ang-(1-7)] or combination therapy (Figure 8A–8J).

Discussion

The most significant finding of our study is that we have developed a system to generate human ACE2 and Ang-(1-7) within plant chloroplasts using transplastomic technology, which when orally administered to rats attenuates PH. Although previous genetic interventions with ACE2/Ang-(1-7) have demonstrated beneficial effects in animals,^{11,12} there are several challenges that limit the clinical development of such approaches. The incidence of PH is increasing among the elderly global population, necessitating affordable medication for the masses. Although drugs made in plant cells have been approved by the Food and Drug Administration and are currently marketed,²⁵ targeted gene therapy is still in the experimental stage and far away from clinical applications. Even if gene therapy is approved as a valid approach, it would still be accessible to <1% of the global population because of the limited expertise available in hospitals for gene therapy. In contrast, oral delivery of plant capsules containing therapeutic proteins is feasible and much affordable. So, drug delivery is as important as drug discovery, and this study focuses on the

development of a novel low-cost delivery system for administering therapeutic proteins like ACE2/Ang-(1-7), which have been found to be effective against experimental models of lung diseases, but not yet clinically approved. Injectable delivery of ACE2/Ang-(1-7) poses some unique challenges with respect to cost of manufacturing, protein stability, cold storage, shelf life, sterile delivery, and requirement of health professionals/hospitals for their administration. Most of these concerns are easily eliminated by orally delivering therapeutic proteins bioencapsulated in plant cells. Currently produced injectable protein drugs are not affordable to more than half of the global population, despite decades of optimization of their process development. By developing an oral delivery system for administering ACE2 and Ang-(1-7), as reported here, we have made tremendous advancement to move the field forward and take ACE2/Ang-(1-7) toward translational/clinical research for the treatment of pulmonary diseases.

ACE2 and Ang-(1-7) were expressed in plant chloroplasts as fusion proteins with CTB. Although Ang-(1-7) is not a gene product, a synthetic gene encoding for Ang-(1-7) was used in this study.²⁹ Chloroplasts are ideal bioreactors for producing human therapeutic proteins. The total copy number of the transgene per cell is expected to be 10 000, resulting in hyperexpression (70% of total leaf protein).¹⁶ A single plant cell has ~100 chloroplasts, and each chloroplast has ~100 copies of chloroplast genome. So, 10 000 copies of transgene in each transformed plant cell is observed. This copy number has been quantified by quantitative polymerase chain reaction in our previous studies.³⁰ However, the most practical way in the literature to evaluate homoplasmy (transformation of all chloroplast genomes in each plant cell) is by showing the absence of native untransformed chloroplast genomes as shown in Figure 1B and 1C. Moreover, this system allows for appropriate disulfide bond formation, proper protein folding, and assembly.^{17,18} Also, bio-encapsulation of proteins within plant cells protects them from gastric degradation. Most importantly, when fused with trans-mucosal carriers such as CTB, therapeutic proteins are effectively released into systemic circulation by microbes colonizing the gut, which lyse plant cells, after CTB-GM1 interaction.^{18–21} GM1 is also present on the cell membrane of the retina and nervous system and hence can be targeted by CTB-fused therapeutic proteins, as reported in our recent study.^{31,32} Chloroplasts are capable of producing high level expression of foreign genes. However, the expression levels of ~93 kDa of ACE2 were below our expectations compared with Ang-(1-7) (Figure 1C and 1D). The prokaryotic nature of chloroplast is not well-suited for the expression of eukaryotic human genes because of the bias in codon preference and usage.^{33,34} For example, native prokaryotic genes from *Bacillus anthracis* and *Bacillus thuringiensis* were expressed 45.3% of the total soluble protein in chloroplasts.^{16,35} Small native human genes such as insulin like growth factor-1 (~7.8 kDa),³⁶ proinsulin (~12 kDa),¹⁶ and interferon- α 2b (~21.5 kDa)³⁷ were expressed in chloroplasts at high levels. However, larger human native genes like blood clotting factors were expressed in chloroplasts at lower levels.²⁰ From the analysis of the ACE2 nucleotide sequence, it was observed that 349 codons of 805 were rare codons (43.5%), which would readily exhaust the corresponding tRNA pools, leaving relatively abundant tRNAs unused. Therefore, for clinical development of ACE2, codon optimization and expression in an edible system (lettuce) should be considered.

We have used CTB as the transmucosal carrier to facilitate the uptake of ACE2 and Ang-(1-7) into circulation. Both CTB fusion proteins are disulfide bonded, form pentamers, and properly folded, as observed for other CTB fusion proteins.^{17,18} CTB is an approved adjuvant³⁸ that has been used in several clinical settings. Administration of CTB-fused antigen (Behcet's disease peptide) in humans with autoimmune eye disorders induced immunologic tolerance by suppressing abnormal T-cell reactivity against the peptide.³⁹ Also, immune suppression to autoantigens (proinsulin and factor IX) linked to CTB has been observed in animal studies after oral administration.^{19,20} Likewise, other studies have shown immune-suppressive effects when CTB was fused to autoimmune or allergic causative agents.⁴⁰ The GM1 receptors present on intestinal epithelial cells make CTB the most appropriate carrier for transporting therapeutic proteins into systemic circulation because this receptor is widely distributed over the intestinal mucosa^{41,42} with a rapid turnover rate.⁴³

The half-life of native Ang-(1-7) is short.^{44,45} However, in this study, the stability of Ang-(1-7) was found to increase in sera. In plant cells, CTB stabilizes Ang-(1-7) by formation of pentamers (Figure 1G) and thus confers protection from plant proteases. However, only monomers are observed in sera after delivery into the sera. Although furin cleavage site (NH₂-R-R-K-R-COOH) should facilitate removal of CTB, efficiency of cleavage depends on the flanking amino acid sequence of the fused protein.⁴⁶ Ang-(1-7) fused to CTB did not provide optimal furin cleavage site because it is not flanked by furin preferred basic amino acids at N-terminal side and serine-valine at C-terminal side. Therefore, it is anticipated that furin cleavage will not be rapid or efficient. This offers greater N-terminal protection to Ang-(1-7) and extends its stability for several hours in the sera compared with injectable Ang-(1-7). Actually, continued treatment with bioencapsulated Ang-(1-7) showed significant increases of the circulating levels of the peptide (Figure S2), which suggests that an oral gavage twice daily of bioencapsulated Ang-(1-7) results in sustained elevated plasma levels of Ang-(1-7) in the treated animals. Ang-(1-7) concentration in frozen leaf materials was found to be 584 µg/g, which translates to 292 µg in 500 mg. This dose compares well with previous studies, wherein 750 to 1000 µg/kg of Ang-(1-7) peptide was administered to rats (≈300 µg per 300 g rat).

Oral feeding of bioencapsulated ACE2 or Ang-(1-7) prevents the development and, most importantly, retards the progression of monocrotaline-induced PH. An upregulation of the deleterious ACE-angiotensin II-AT1R axis and downregulation of the protective ACE2-Ang-(1-7)-Mas axis contributes to PH pathogenesis.⁴⁷ Thus, maintaining equilibrium between these 2 axes is crucial for preserving pulmonary vascular homeostasis. Oral delivery of ACE2 or Ang-(1-7) increased ACE2/ACE and decreased AT1R/AT2R ratios, signifying improvement in pulmonary RAS balance. It was interesting to note that the serum ACE2 activity in monocrotaline animals was decreased compared with controls. This, however, did not affect the circulating levels of Ang-(1-7), which could possibly be because of alternate endopeptidase pathways of Ang-(1-7) generation through neprilysin or thimet oligopeptidase. However, oral ACE2 feeding prevented the decrease in serum ACE2 activity of monocrotaline animals and was associated with a 2- to 3-fold increase in the circulating levels of Ang-(1-7) (Figure S2). In fact, ACE2 fed rats exhibited 37% increase in the enzymatic activity compared with monocrotaline alone animals. Most importantly, this

increase was sufficient to exert beneficial effects against PH pathophysiology. Previous experimental studies have shown that exogenous administration of recombinant ACE2 increases serum ACE2 activity to exert therapeutic efficacy in several disease models.^{48–50} Increasing serum ACE2 levels is also clinically significant because abnormally low levels of serum ACE2 have been associated with PH.¹⁰ Surprisingly, pulmonary ACE2 mRNA levels were increased with oral ACE2 feeding. We speculate this increase to be a positive feed forward mechanism because similar increases in ACE2 have been reported previously.^{14,15} Also, AT2R levels were increased with ACE2 treatment, which is consistent with previous studies showing a protective role of this receptor in cardiopulmonary disease.⁵¹ Furthermore, the favorable RAS modulation by ACE2 or Ang-(1-7) was associated with reduced lung inflammatory cytokines. Proinflammatory cytokines contribute to thickening of the pulmonary arterioles leading to heightened pulmonary pressure.⁵² In line with these findings, we observed marked increases in vessel wall thickness in monocrotaline-challenged animals. However, ACE2 or Ang-(1-7) treatment significantly inhibited medial wall thickness. The observed effects of ACE2/Ang-(1-7) could be attributed to reduction in proinflammatory cytokines, as well as direct antiproliferative actions on the vascular smooth muscle cells, a contention supported by earlier studies.⁵³ Recent studies have implicated autophagy in PH.²⁶ We observed an increase in LC3B-II, an autophagy marker, in monocrotaline rats, which was significantly decreased with ACE2 or Ang-(1-7) treatment.

Collectively, aforementioned findings suggest that oral delivery of ACE2 or Ang-(1-7) corrects a dysregulated pulmonary RAS, reduces inflammation, decreases vascular remodeling, and inhibits autophagy to exert lung-protective effects. Importantly, ACE2/Ang-(1-7) treatment did not lower basal systemic blood pressure, which is important because induction of systemic hypotension can be detrimental in patients with PH. Similar phenomenon has also been observed in other studies, wherein chronic administration of Ang-(1-7) fails to decrease systemic blood pressure in a variety of models of hypertension.^{54–56} One possibility may be related to pulmonary vasculature being more sensitive to Ang-(1-7) or that abundant receptors for Ang-(1-7) is present on the pulmonary vessels. Furthermore, a combination of ACE2 and Ang-(1-7) treatment produced beneficial effects on the cardiopulmonary system. We observed that the higher dose combination yielded better effects than the lower dose.

Of particular interest are the cardioprotective effects of oral ACE2/Ang-(1-7) therapy. Sustained pressure overload on the right heart induces ventricular remodeling and dysfunction.⁵⁷ Echocardiography of monocrotaline rats revealed prominent structural changes in the heart. The RV assumed a round shape, with a shift in the intraventricular septum causing RV dilation with reduced EF. In addition, the pulmonary artery flow was significantly lowered in the monocrotaline group. All these changes were associated with development of RVH, increased interstitial fibrosis, and cardiac dysfunction. However, ACE2 or Ang-(1-7) treatment restored normal heart structure, inhibited RV dilatation, and improved EF. Also, RVH and interstitial fibrosis were significantly reduced, along with preserved cardiac function. Moreover, the combination therapy with ACE2 and Ang-(1-7) was found to exert superior cardioprotective effects.

Perspectives

Evidences have implicated the therapeutic potential of the ACE2/Ang-(1-7) axis in multiple diseases such as PH, lung-fibrosis, heart failure, renal diseases, and diabetic retinopathy. This study provides proof-of-concept for a low-cost plant-based oral delivery system for ACE2 or Ang-(1-7) bioencapsulated in plant chloroplasts. This transplastomic technology offers the potential to scale-up and generate bulk quantities of therapeutically active ACE2/Ang-(1-7) toward undertaking translational studies with a simple oral delivery system. In this direction, we have lyophilized leaves expressing ACE2 or Ang-(1-7), which have concentrated these therapeutic proteins by ≈ 20.5 - and 14.3-fold, respectively, with no loss of the binding affinity to GM1 receptor (Figure 1F). The concentration effect is because of the packaging of more proteins per milligram of lyophilized leaves as a result of elimination of water by dehydration. So this process does not change the percentage of ACE2/Ang1-7 in the total leaf protein between fresh and lyophilized plant leaves. This freeze-drying method is also advantageous in that it reduces the cost associated with protein purification, eliminates microbial contamination, and facilitates long-term storage of these therapeutic proteins in capsules at room temperature, which are all important considerations for clinical development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Novelty and Significance

What Is New?

- This study provides evidence for the first time the development of a low-cost oral delivery system for the administration of angiotensin-converting enzyme 2 and angiotensin-(1-7) using transplastomic technology that provides impressive protection against pulmonary hypertension.

What Is Relevant?

- Angiotensin-converting enzyme 2 and angiotensin-(1-7) have tremendous therapeutic potential for treating pulmonary hypertension. However, cost of manufacturing, protein stability, repetitive intravenous dosing, and patient compliance pose major impediments for successful clinical therapy. Development of a plant-based oral delivery system will overcome these challenges to realize full therapeutic potential of these proteins.

Summary

Oral delivery of angiotensin-converting enzyme 2 or angiotensin-(1-7) bioencapsulated in plant cells provides protection against pulmonary hypertension and associated cardiopulmonary pathology.

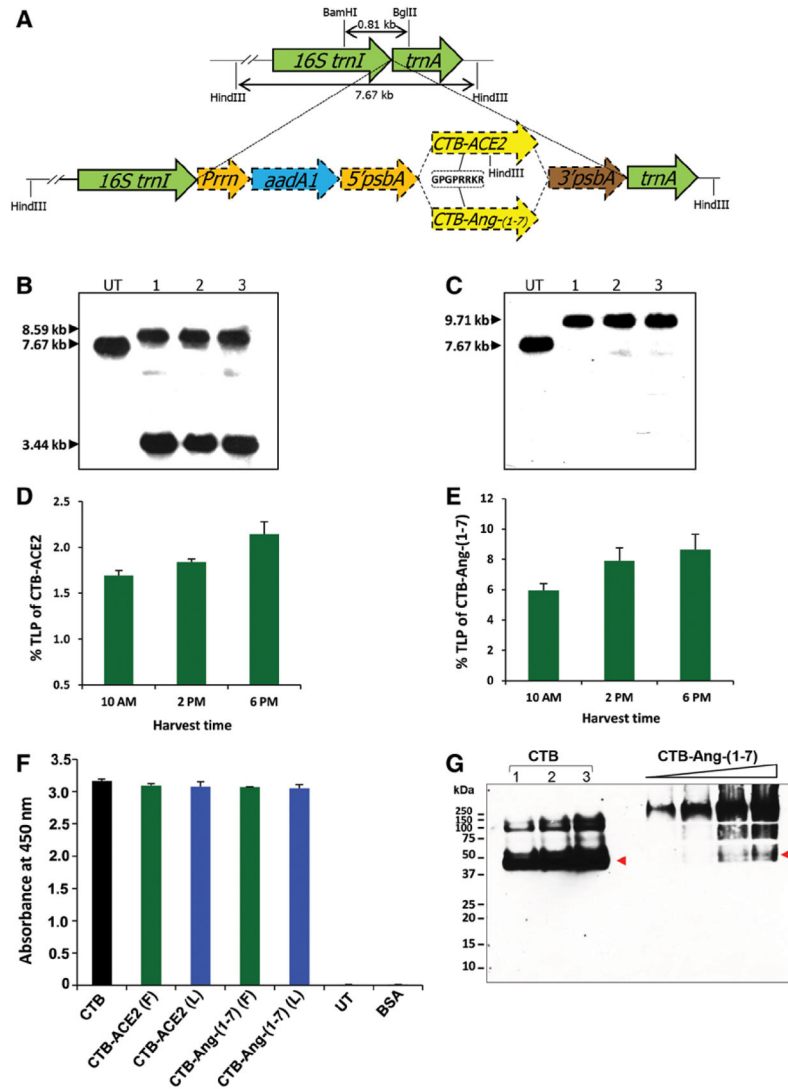


Figure 1. Characterization, concentration, and evaluation of pentameric structure of cholera nontoxin B subunit (CTB)-angiotensin-converting enzyme 2 (ACE2) and CTB-angiotensin-(1-7) [Ang-(1-7)] expressed in plant chloroplasts. **A**, Schematic representation of CTB-ACE2 and CTB-Ang-(1-7) gene cassettes and flanking regions. Southern blot analysis of **(B)** CTB-ACE2 and **(C)** CTB-Ang-(1-7) transplastomic lines. HindIII-digested untransformed (UT) and transformed (lane 1, 2, and 3) genomic DNA was probed with P³²-labeled flanking sequence. Quantification of **(D)** CTB-ACE2 and **(E)** CTB-Ang-(1-7) as a percentage of the total leaf proteins (TLP). **F**, GM1-binding assay of CTB-ACE2 and CTB-Ang-(1-7). **G**, Western blot analysis of CTB-Ang-(1-7) in nonreducing condition without boiling and dithiothreitol. Lanes 1, 2, and 3: 10, 15, and 20 ng of CTB; total homogenate of CTB-Ang-(1-7): 0.2, 0.4, 0.8, and 1.6 μ g. The pentameric structures for the CTB alone and the fusion protein are indicated by arrow head and arrow, respectively. Data shown are mean \pm SD of 3 independent experiments. F indicates fresh; L, lyophilized; and BSA, 1% wt/vol.

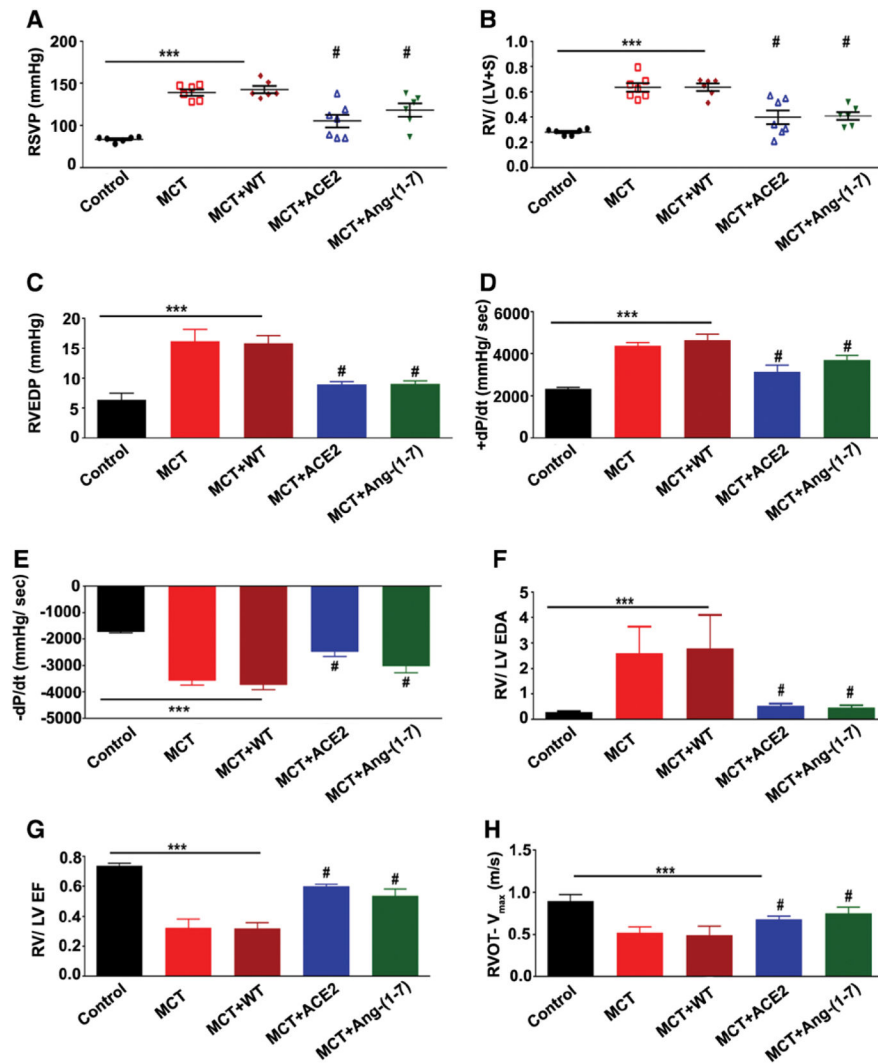


Figure 2.

Oral administration of bioencapsulated angiotensin-converting enzyme 2 (ACE2) or angiotensin-(1-7) [Ang-(1-7)] prevents monocrotaline (MCT)-induced pulmonary hypertension. **A**, Measurement of right ventricular (RV) systolic pressure (RVSP) in normal controls and MCT-challenged rats that were either untreated or orally fed with wild-type (WT) leaf material or gavaged with bioencapsulated ACE2/ Ang-(1-7). **B**, RV hypertrophy, measured as the ratio of RV to left ventricle (LV) plus interventricular septum (S) weights [RV/(LV+S)]. Measurement of **(C)** RV end-diastolic pressure (RVEDP), **(D)** +dP/dt, and **(E)** -dP/dt. Echocardiography data representing **(F)** ejection fraction, **(G)** ratio of the right to left end-diastolic area, signifying right heart dilation, and **(H)** the blood flow rate in the RV outflow tract (RVOT). Data shown are mean \pm SEM. *** P <0.001 vs control rats and # P <0.05 vs untreated or WT leaf-fed MCT rats. n=6 to 8 animals/group.

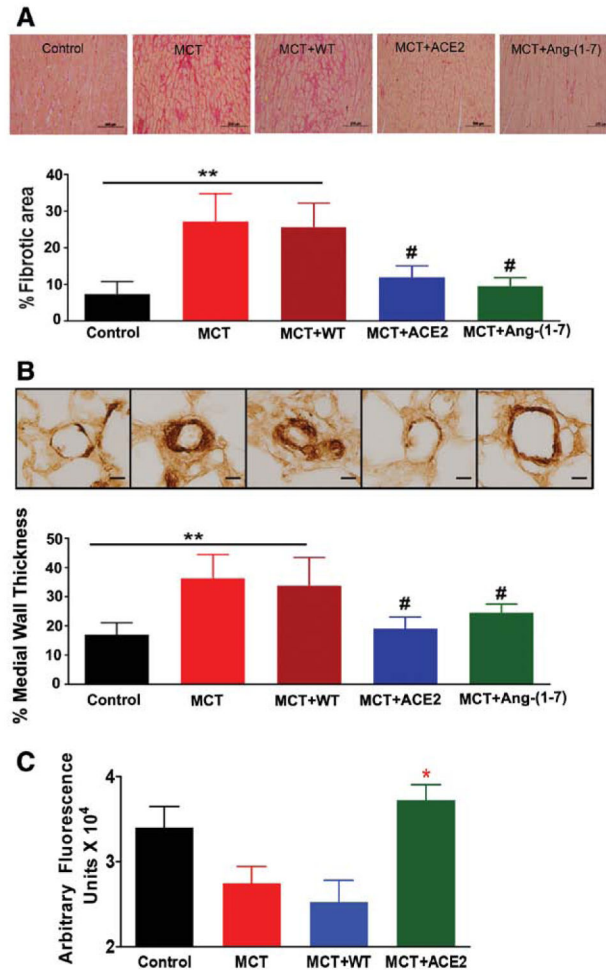
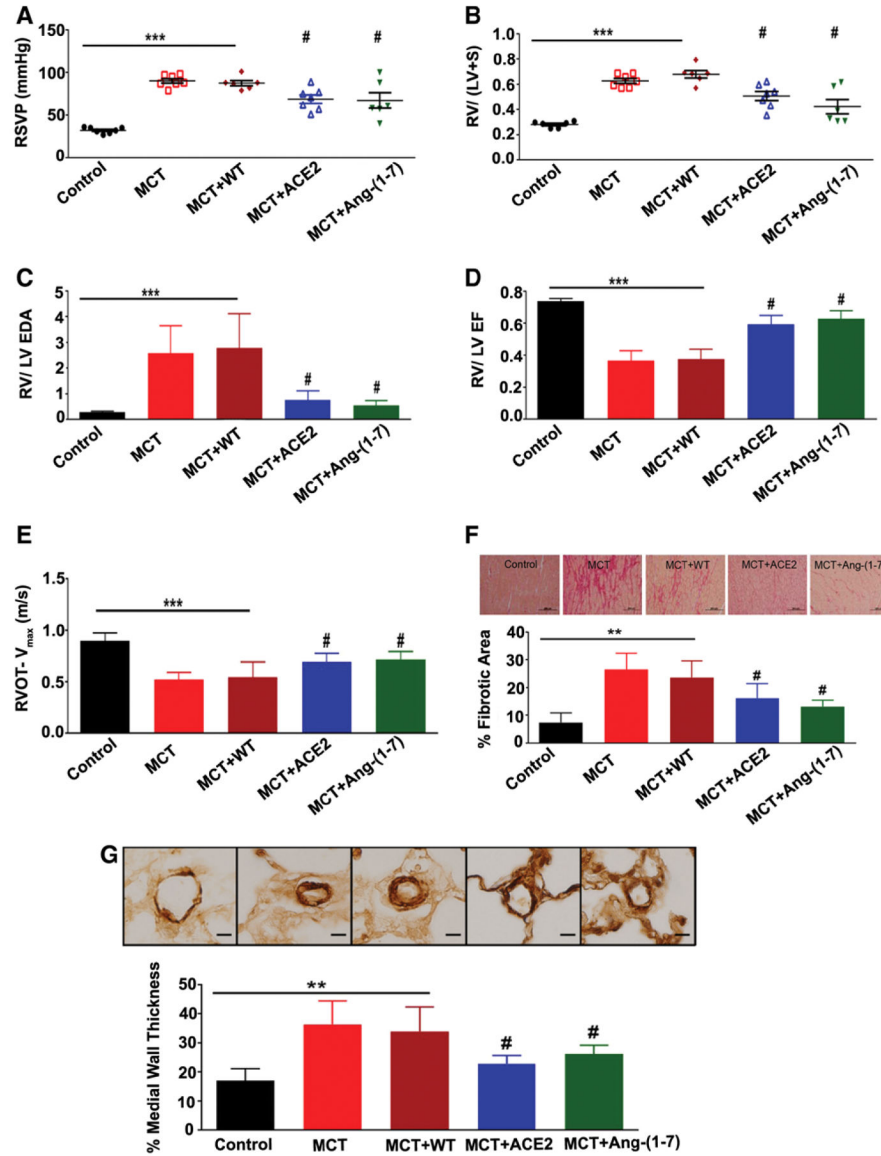


Figure 3. Oral feeding of bioencapsulated angiotensin-converting enzyme 2 (ACE2) or angiotensin-(1-7) [Ang-(1-7)] exerts antifibrotic and antiremodeling effects in the prevention protocol. **A**, Interstitial collagen deposition in the right ventricle. **B**, Staining for α -smooth muscle actin to quantify medial wall thickness of the pulmonary arteries measuring $<50 \mu\text{m}$. Scale bar, $10 \mu\text{m}$. **C**, ACE2 activity was measured in rat sera ($10 \mu\text{L}$) collected from different experimental groups. Data represents mean \pm SEM with $*P < 0.05$ vs other groups, $**P < 0.01$ compared with controls, whereas # representing $P < 0.05$ vs untreated and wild-type (WT) plant material-fed monocrotaline (MCT) rats as assessed by 1-way ANOVA followed by Newman-Keuls test. AFU indicates arbitrary fluorescence units.

**Figure 4.**

Oral treatment with angiotensin-converting enzyme 2 (ACE2) or angiotensin-(1-7) [Ang-(1-7)] arrests disease progression and attenuates cardiopulmonary remodeling. **A**, Individual values of the right ventricle systolic pressure (RVSP) from different experimental groups of the reversal protocol. **B**, Ratio of RV to left ventricle (LV) plus interventricular septum (S) weight [RV/(LV+S)] values from individual animals, denoting right heart hypertrophy. Echocardiography data representing (C) ratio of the right to LV end-diastolic area, (D) ejection fraction (EF), and (E) the blood flow rate in the RV outflow tract (RVOT) of the different experimental groups. **F**, Representative photographs and quantification of interstitial fibrosis. **G**, Measurement of vessel wall thickness after α -smooth muscle actin staining of the pulmonary arteries (<50 μ m). Scale bar, 10 μ m. Data shown are mean \pm SEM. ** P <0.01, *** P <0.001 vs control rats and # P <0.05 vs untreated or wild-type (WT) leaf-fed monocrotaline (MCT) rats. n=6 to 8 animals/group.

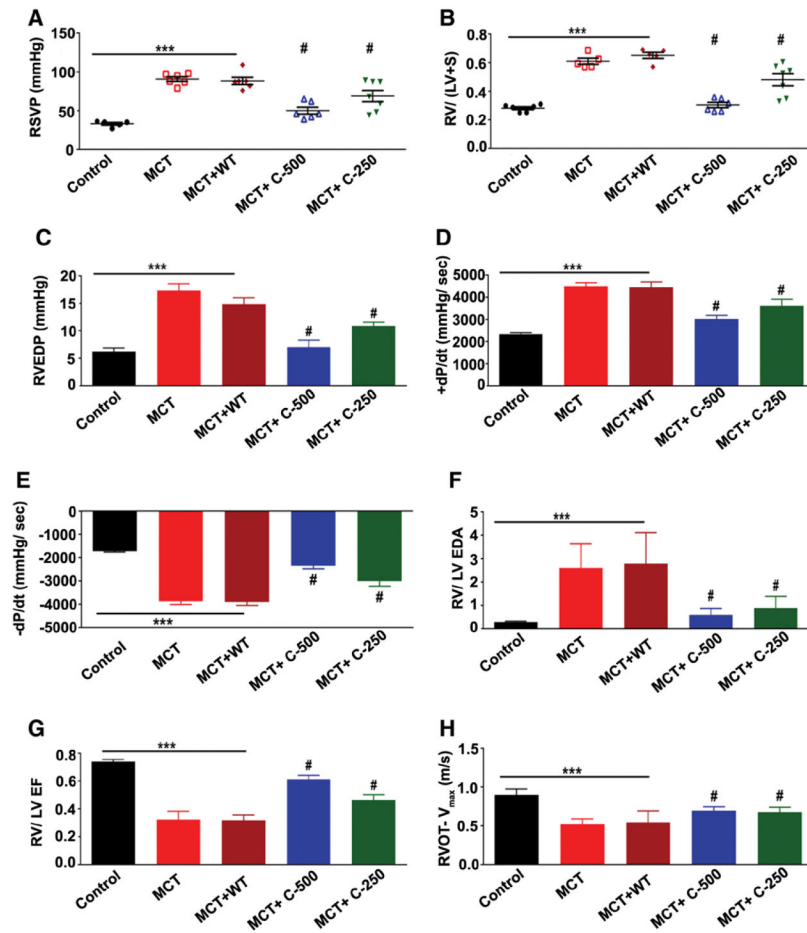


Figure 5. Combination therapy with angiotensin-converting enzyme 2 (ACE2) and angiotensin-(1-7) [Ang-(1-7)] rescues established pulmonary hypertension. **A**, Measurement of right ventricular systolic pressure (RVSP) in monocrotaline (MCT) rats treated with a combination of either 500 mg or 250 mg each of ACE2 and Ang-(1-7). **B**, Data representing right ventricular hypertrophy as a ratio of RV/(LV+S). Measurement of (C) right ventricular end-diastolic pressure (RVEDP), (D) +dP/dt, and (E) -dP/dt from the combination study. Echocardiography data representing (F) ejection fraction (EF), (G) ratio of the right to left end-diastolic area, and (H) the blood flow rate in the right ventricular outflow tract (RVOT). Data shown are mean \pm SEM. *** P <0.001 vs control rats and # P <0.05 vs untreated or wild-type (WT) leaf-fed MCT-rats. n=6 to 8 animals/group.

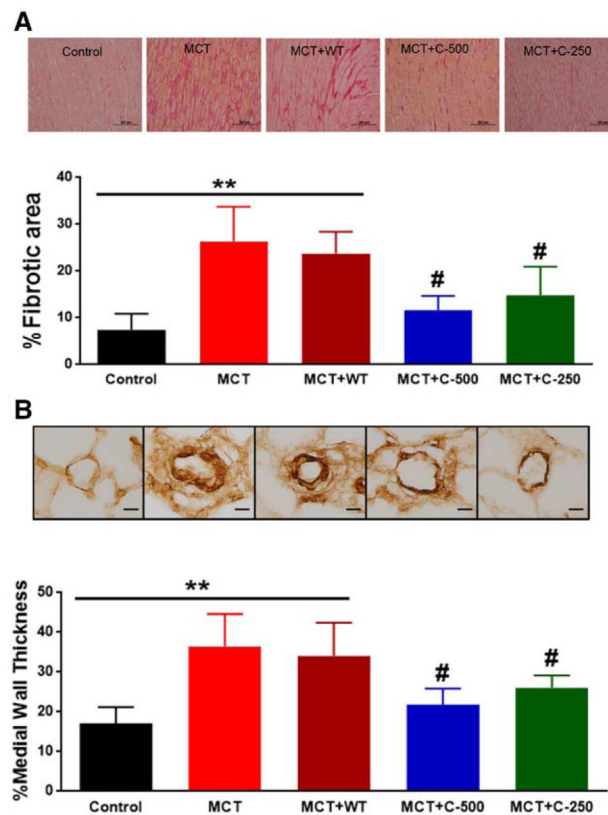


Figure 6. Combination of angiotensin-converting enzyme 2 (ACE2) and angiotensin-(1-7) [Ang-(1-7)] decreases ventricular fibrosis and attenuates pulmonary vascular remodeling. **A**, Representative photographs of collagen staining and quantitative analysis of right ventricular fibrosis after 2 week treatment with combination therapy. **B**, Measurement of vessel wall thickness of the pulmonary arteries (<math><50 \mu\text{m}</math>). Scale bar, 10 μm . Data are expressed as mean \pm SEM; ** $P<0.01$; vs controls and # $P<0.05$ vs untreated and wild-type (WT) plant material-fed monocrotaline (MCT) rats. $n=5$ to 7 animals per experimental group.

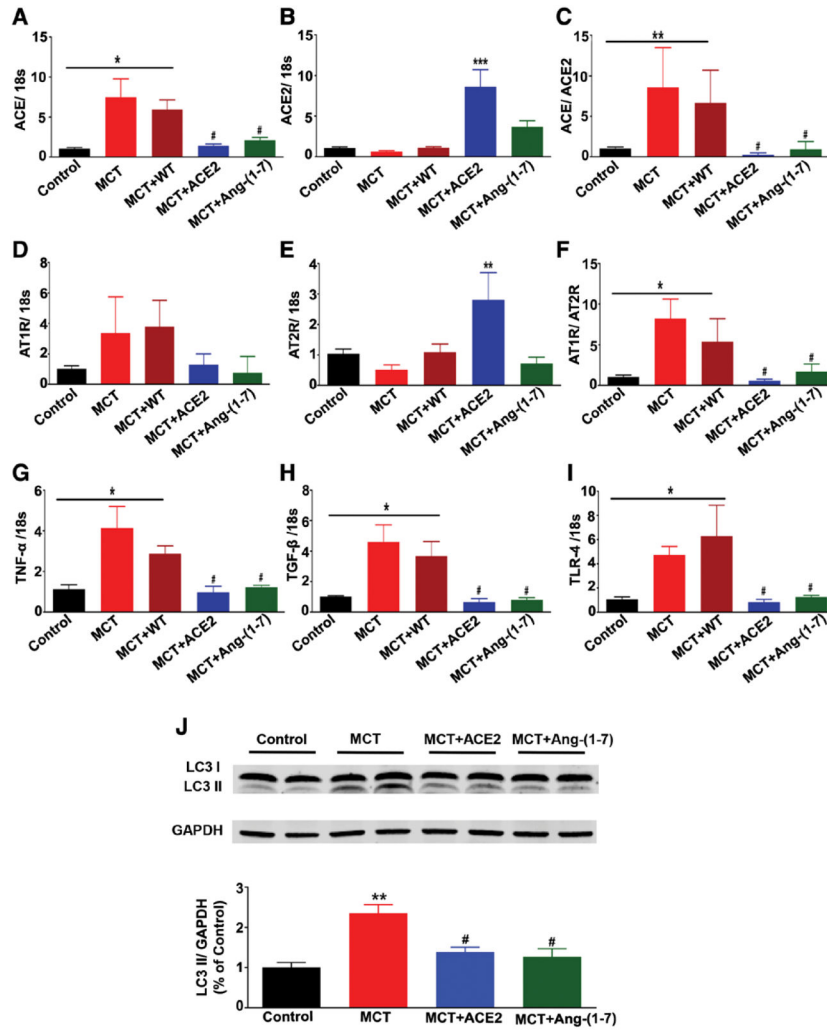


Figure 7. Effects of angiotensin-converting enzyme 2 (ACE2) or angiotensin-(1-7) [Ang(1-7)] treatment on the lung renin–angiotensin system, proinflammatory cytokines, and autophagy (prevention protocol). Relative change in lung mRNA levels of (A) ACE, (B) ACE2, (C) ACE/ACE2 ratio, (D) angiotensin type 1 receptor (AT1R), (E) angiotensin type 2 receptor (AT2R), and (F) AT1R/AT2R receptor. Relative mRNA levels of lung proinflammatory cytokines, G, tumor necrosis factor (TNF)-α, (H) transforming growth factor (TGF)-β, and (I) toll-like receptor-4 (TLR-4) from the monocrotaline (MCT) study. Autophagy marker, LC3-II is increased in the lungs of MCT-exposed animals. J, Immunoblot and densitometry analysis of the lung LC3I/II protein expression. Data are expressed as mean ± SEM. **P*<0.05, ***P*<0.01, and ****P*<0.001 vs control rats. #*P*<0.05 vs MCT group. WT indicates wild type.

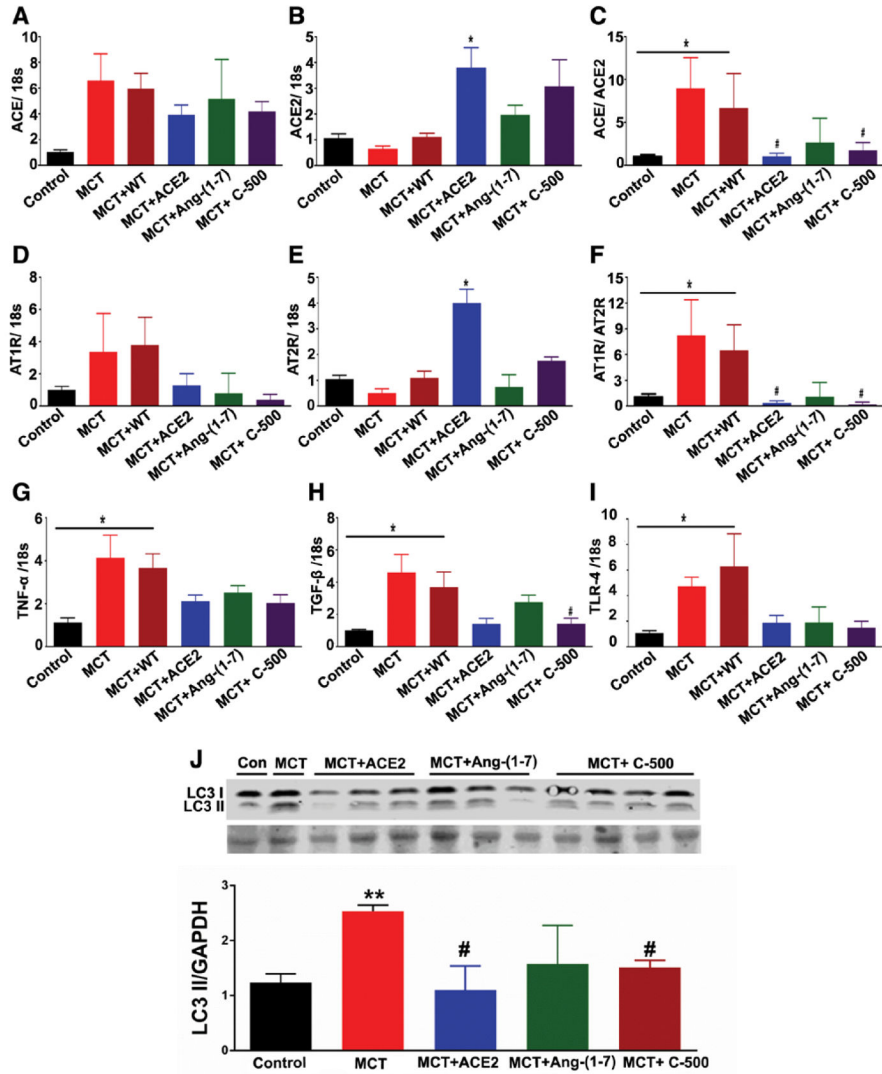


Figure 8. Effects of monotherapy, as well as the combination therapy, on the lung renin–angiotensin system components, proinflammatory cytokines, and autophagy in the reversal protocol. Data represent relative changes in lung mRNA levels of (A) angiotensin-converting enzyme (ACE), (B) ACE2, (C) ACE/ ACE2 ratio, (D) angiotensin type 1 receptor (AT1R), (E) angiotensin type 2 receptor (AT2R), and (F) AT1R/AT2R ratio. Relative mRNA levels of lung proinflammatory cytokines, (G) tumor necrosis factor (TNF)-α, (H) transforming growth factor (TGF)-β, and (I) toll-like receptor-4 (TLR-4) from the same study. J, Immunoblot and densitometry quantification showing lung LC3I/II protein expression. Data are expressed as mean ± SEM. **P*<0.05 and ***P*<0.01 vs control rats, whereas #*P*<0.05 vs monocrotaline (MCT) group. WT indicates wild type.