Evidence for Angiotensin-converting Enzyme 2 as a Therapeutic Target for the Prevention of Pulmonary Hypertension

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***Rationale*: It has been proposed that an activated renin angiotensin system (RAS) causes an imbalance between the vasoconstrictive and vasodilator mechanisms involving the pulmonary circulation leading to the development of pulmonary hypertension (PH). Recent studies have indicated that angiotensin-converting enzyme 2 (ACE2), a member of the vasoprotective axis of the RAS, plays a regulatory role in lung pathophysiology, including pulmonary fibrosis and acute lung disease. Based on these observations, we propose the hypothesis that activation of endogenous ACE2 can shift the balance from the vasoconstrictive, proliferative axis (ACE-Ang II-AT1R) to the vasoprotective axis [ACE2-Ang-(1–7)-Mas] of the RAS, resulting in the prevention of PH.**

***Objectives*: We have taken advantage of a recently discovered synthetic activator of ACE2, XNT (1-[(2-dimethylamino) ethyl-amino]-4-(hydroxymethyl)-7-[(4-methylphenyl) sulfonyl oxy]-9H-xanthene-9-one), to study its effects on monocrotaline-induced PH in rats to support this hypothesis.**

***Methods*: The cardiopulmonary effects of XNT were evaluated in monocrotaline-induced PH rat model.**

***Measurements and Main Results*: A single subcutaneous treatment of monocrotaline in rats resulted in elevated right ventricular systolic pressure, right ventricular hypertrophy, increased pulmonary vessel wall thickness, and interstitial fibrosis. These changes were associated with increases in the mRNA levels of renin, ACE, angiotensinogen, AT1 receptors, and proinflammatory cytokines. All these features of PH were prevented in these monocrotaline-treated rats by chronic treatment with XNT. In addition, XNT caused an increase in the antiinflammatory cytokine, IL-10.**

***Conclusions*: These observations provide conceptual support that activation of ACE2 by a small molecule can be a therapeutically relevant approach for treating and controlling PH.**

Keywords: renin angiotensin system; angiotensin-converting enzyme 2; pulmonary heart disease.

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Currently available therapeutic strategies for pulmonary arterial hypertension are often not successful in preventing significant disease progression or death.

What This Study Adds to the Field

This study shows that activation of pulmonary ACE2 enzyme by a newly discovered small synthetic molecule prevents pulmonary hypertension and vascular remodeling, and suggests that pulmonary ACE2 may be a novel target for the successful control of pulmonary hypertension.

Pulmonary hypertension (PH) presents a diverse etiology and is defined by a mean pulmonary arterial pressure of greater than 25 mm Hg at rest, or greater than 30 mm Hg with exercise (1). The most common causes of PH include chronic obstructive pulmonary disease (often caused by smoking), left heart failure, substance abuse, schistosomiasis, high altitude exposure, drugs, toxins (e.g., chemical warfare), and HIV infection (2, 3). It has been proposed that these risk factors, coupled with predisposing genetic factors, lead to an imbalance between vasoconstrictor and vasodilator mechanisms. This imbalance initiates a cascade of pathophysiological events in the lungs leading to PH (4). These events are suggested to be set in motion by pulmonary vascular endothelial dysfunction causing enhanced proliferation and activation of lung fibroblasts, leading to extracellular matrix formation and fibrosis, infiltration of inflammatory cells, increased production of proinflammatory cytokines, exaggerated pulmonary vascular remodeling, and smooth muscle hypertrophy (5, 6). Vasodilatory therapy has been the mainstay for PH treatment as a result of the imbalance in vasoconstrictor vasodilator hypothesis; however, despite some success, PH remains a fatal disease. Angiotensin-converting enzyme 2 (ACE2) produces angiotensin-(1–7) [Ang-(1–7)] from angiotensin II (AngII) and thus plays a key role in controlling the balance between the vasoconstrictive, proliferative axis (ACE-AngII-AT1 receptor) and the vasoprotective axis [ACE2-Ang-(1–7)-Mas] of the renin angiotensin system (RAS) (7). This observation forms the basis of our proposal: namely, that activation of endogenous ACE2 can affect the balance between the two axes and prevent vascular remodeling and pathological events that lead to PH. Additional evidence confirms that ACE2 is central to pulmonary endothelial function and lung pathophysiology: (*1*) ACE2 is abundantly expressed in the pulmonary endothelium (8–11); (*2*) ACE2 is significantly decreased in lung biopsies of patients with idiopathic pulmonary fibrosis (9); (*3*) bleomycin-induced pulmonary fibrosis and PH are associated with a decrease in ACE2 activity (9); (*4*) patients with PH have an increase in circulating endothelial cells (CEC) (12) due to high pressure in the pulmonary circuits and increased shear stress in the pulmonary vasculature (13), and ACE levels in the CEC of PH are significantly higher (unpublished observation); (*5*) ACE2 knockout mice exhibited severe acute respiratory

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distress syndrome, which is attenuated with administration of recombinant ACE2 (14); (*6*) Ang II up-regulates ACE and down-regulates ACE2 in patients with hypertension (15); and (*7*) Higher ACE2 activity with increased angiotensin-(1–7) formation has been reported in heart ventricles of patients with primary PH (16), suggesting a cardiopulmonary protective role for ACE2 (17). Collectively, these observations led us to propose that activation of endogenous ACE2 could have a protective effect against PH. We have used the monocrotaline (MCT) rat model of PH and a recently identified ACE2 activator (18) to provide evidence in support of this hypothesis. Administration of MCT, a plant-derived toxin, causes pulmonary endothelial cell injury, infiltration of mononuclear cells, and muscularization of pulmonary arteries resulting in PH (19). Although not ideal because of its toxicity to the liver, the MCT-treated rat is used as a standard model for PH, because it mimics several pathological features of human primary PH (20). An exhaustive virtual screening of chemical libraries based on the crystal structure of ACE2 was used in our laboratory to discover compounds that are able to activate this enzyme (18). One such compound is 1-[(2-dimethylamino) ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl) sulfonyl oxy]-9H-xanthene-9-one (XNT). XNT is a small synthetic molecule that can activate ACE2, and cause striking reversal of cardiac hypertrophy, ventricular fibrosis, and renal interstitial fibrosis induced by systemic hypertension (18). Thus, we decided to use XNT to determine its effects on lung pathophysiology associated with PH. Some of the results of these studies have been previously reported in the form of abstracts (21, 22).

METHODS

Materials

MCT and α smooth muscle actin (clone 1A4) were purchased from Sigma-Aldrich (St. Louis, MO). Alzet osmotic pumps (model 2004 and 2ML4) were purchased from Durect Corporation (Cupertino, CA). XNT was synthesized in Castellano's laboratory. The Mas-receptor antagonist, A-779 was purchased from Bachem (Torrance, CA).

Monocrotaline-induced PH

Male Sprague-Dawley rats (12 wk old) were used in this study. Animals were housed in a temperature-controlled room (25 ± 1°C) and maintained on a 12:12 hour light:dark cycle with free access to water and food. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida and complied with National Institutes of Health guidelines. PH was induced by a single injection of MCT (50 mg/kg in 0.5 ml saline, subcutaneous), whereas control rats received vehicle (0.5 ml of saline, subcutaneous). At the same time, osmotic minipumps containing either 10 mg/ml XNT or the vehicle (low pH saline: 2–2.5) were implanted subcutaneously to allow 28 days of infusion of XNT/saline at a rate of 120 μg/day. To evaluate the role of the Ang-(1–7) receptor, Mas, on the effects of XNT, an additional group of rats received an extra osmotic minipump (model 2ML4) containing A-779, a Mas antagonist, at an infusion rate of 120 μg/day for 28 days. Systemic blood pressure was measured indirectly by the tail-cuff method in conscious animals every week for 4 weeks as described previously (23).

Right Ventricular Systolic Pressure Measurement

Right ventricular systolic pressure (RVSP) was measured after 4 weeks of MCT administration. Animals were anesthetized with a mixture of ketamine, xylazine, and acepromazine (30, 6, and 1 mg/kg, respectively) and were placed in a supine position, breathing room air. The RVSP was measured using a silicone elastomer cannula (Helix Medical, Carpinteria, CA). The cannula, filled with heparin-saline solution (40 U/ml), was introduced into the right ventricle through the right descending jugular vein and RVSP was measured with a liquid pressure transducer, which was interfaced to a PowerLab (AD Instruments, Colorado Springs, CO) signal transduction unit. The waveform was used to confirm the positioning of the catheter in the right ventricle. Data were analyzed by using the Chart program that was supplied with the PowerLab system (n = 4–13 in each group).

Histological Analysis

All rats were killed post-RVSP measurement and their hearts and lungs were harvested. The RV was separated from the left ventricle (LV) plus ventricular septum and the wet weights were determined. RV hypertrophy was expressed as the ratio of RV to LV plus ventricular septum (RV/LV+S) (n = 5–10 in each group). The left lung was perfused with phosphate-buffered saline (PBS), pH 7.4, followed by 4% paraformaldehyde in PBS and post fixed with 4% paraformaldehyde for 24 hours by immersion (24). After fixation and paraffin embedding, 5 μm–thick lung sections were cut and stained with anti–α smooth muscle actin (1:600) as described previously (25). The external diameter and medial wall thickness were measured in approximately 15 muscular arterioles per animal for analysis of the medial wall thickness. The medial thickness was calculated as follows: percent wall thickness = [(medial thickness × 2)/external diameter] × 100 (n = 5–8 rats per group) (25). Only vessels with diameter between 30 and 90 μm were analyzed. In addition, Sirius red staining was performed to assess the extent of collagen deposition in lungs. Interstitial fibrosis at 100× magnification was measured by percent area analysis. An Olympus BX 41 microscope was used for imaging, and quantification of collagen density data was performed with ImageJ software from the National Institutes of Health (26).

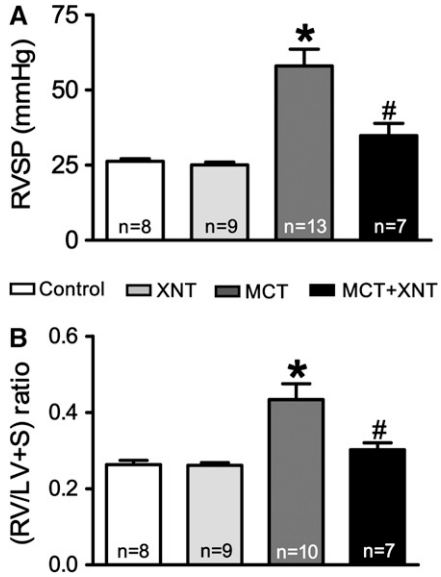

Figure 1. Effect of 1-[(2-dimethylamino) ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl) sulfonyl oxy]-9H-xanthene-9-one (XNT) on pulmonary hypertension induced by a single injection of monocrotaline (MCT). Male Sprague-Dawley rats were injected with 50 mg/kg MCT subcutaneously and fitted with osmotic minipumps to infuse 120 μg/day XNT for 4 weeks. Saline was infused in control rats. (*A*) Effect on right ventricular systolic pressure (RVSP): MCT administration caused a significant increase in the RVSP after 4 weeks, which was significantly blocked by XNT infusion. Data are expressed as mean ± SEM. $^{*}P < 0.05$ versus control group, $^{\#}P < 0.05$ versus MCT group (n = 7–13). (*B*) Effect on right ventricular (RV) hypertrophy: The RV hypertrophy assessed by the ratio between RV and left ventricle plus ventricular septum (LV+S) weights was blunted by XNT treatment. $^{*}P < 0.05$ versus control group, $^{\#}P < 0.05$ versus MCT group (n = 7–10).

Cytokines and RAS mRNA Expression in Lungs

Real-time quantitative polymerase chain reaction was used to measure transforming growth factor β, tumor necrosis factor (TNF), nuclear factor (NF)–κB p50, NF-κB p65, IL-6, IL-10, AT1 receptor, Mas, ACE, and ACE2 mRNA levels in the lungs. Total RNA was extracted with RNaqueous-4RCP Kit (Ambion, Foster City, CA). cDNA samples (2 μl) of reverse transcription reactions were amplified by quantitative real-time polymerase chain reaction using an ABI PRISM 7000 HT Detection system (Applied Biosystems, Foster City, CA). mRNA levels were normalized to 18S RNA from the same samples (n = 3–10 in each group) and the $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression (27).

Statistical Analysis

Data are shown as mean ± SEM. Statistical analyses were performed using one-way analysis of variance followed by the Tukey test. Pulmonary fibrosis was analyzed using the Kruskal-Wallis nonparametric statistical test. *P* values less than 0.05 were considered statistically significant.

RESULTS

XNT Treatment Attenuates MCT-induced Increases in RVSP and Associated Cardiac and Lung Remodeling

A single subcutaneous administration of MCT in rats resulted in a 32 mm Hg increase in RVSP in 4 weeks (26.3 ± 0.9 mm Hg in control vs. 58.0 ± 5.6 mm Hg in MCT, Figure 1A) and a 65% increase in the RV/LV+S ratio (0.26 ± 0.01 in control vs. 0.43 ± 0.04 in MCT, Figure 1B). XNT administration in MCT rats resulted in significant attenuation of RVSP (34.8 ± 4.0 mm Hg), and the RV/LV+S ratio (0.30 ± 0.02). Furthermore, MCT treatment resulted in increase in the medial wall thickness, which also was significantly attenuated by XNT (Figures 2A and 2C). Rats treated with MCT exhibited an increased interstitial fibrosis (5.3 ± 0.6% in control vs. 8.0 ± 0.7% in MCT). This effect was completely blunted by coadministration of XNT (8.0 ± 0.7% in MCT vs. 5.6 ± 1.1 in MCT+XNT). In contrast to RVSP, the systemic blood pressure did not change with MCT treatment or coadministration of XNT with MCT (MAP: control, 120 ± 1 mm Hg, n = 7; MCT, 119 ± 2 mm Hg, n = 8; MCT+XNT, 118 + 2 mm Hg, n = 6). These observations are the first indication that ACE2 activator prevents pathophysiological parameters associated with PH.

Blockade of Mas Receptor Abolishes the Protective Effects of XNT

Our next objective was to determine the possible mechanism involved in these effects of XNT on PH. We had hypothesized that the beneficial effects of ACE2 activation would be mediated by an increase in Ang-(1–7) levels to shift the balance from the ACE-AngII-AT1 receptor axis toward the ACE2-Ang-(1–7)-Mas axis of the RAS. This conclusion is supported by the observation that coadministration of XNT with A-779, a Mas antagonist (28), significantly attenuated the improvements in RVSP and RV/LV+S ratio produced by XNT (Figures 3A and 3B). Further support of the shift in RAS axis from the vasoconstrictive-proliferative to the vasoprotective axis is presented in Figure 4. MCT treatment alone caused significant increases in renin and angiotensinogen mRNA, approximately 17-fold and approximately 18-fold, respectively (Figures 4A and 4B). Similarly, approximately fourfold and approximately fivefold increases in the AT1 receptor and ACE mRNA levels were observed by MCT treatment, respectively (Figures 4C and 4D). Mas mRNA levels tended to decrease (Figure 4E). In contrast, MCT treatment resulted in a fourfold increase in ACE2 mRNA (Figure 4F). This effect could be a result of a compensatory protective role of this enzyme in PH, an idea consistent with previous observations (16). Interestingly, XNT treatment resulted in prevention of increases in renin, angiotensinogen, ACE, and AT1 receptor mRNAs (Figures 4A−4D). Furthermore,

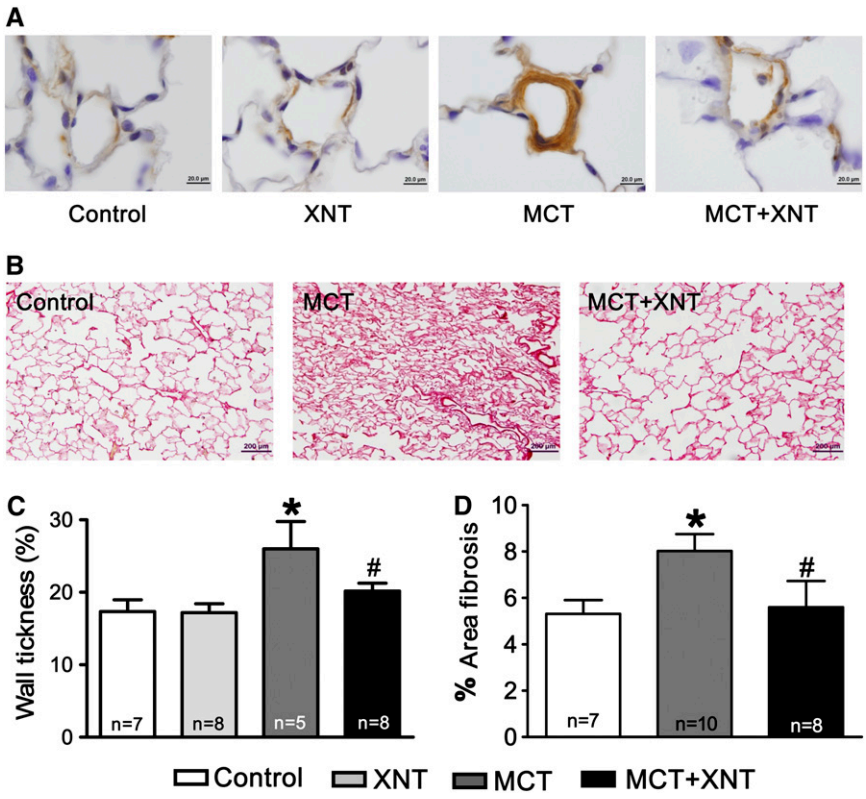

Figure 2. Effect of 1-[(2-dimethylamino) ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl) sulfonyl oxy]-9H-xanthene-9-one (XNT) on monocrotaline (MCT)–induced vessel wall thickness and collagen deposition in the lungs. (*A*) Representative microphotographs of pulmonary vessels from control, XNT, MCT, and MCT+XNT groups (n = 5–8, scale bar = 20 μm). Lung sections were cut and stained with anti-α smooth muscle actin. (*B*) Representative microphotographs of lung sections from control, MCT, and MCT+XNT groups stained with Sirius red (n = 7–10, scale bar = 200 μm). (*C*) For analysis and quantification of the vessel wall thickness, the external diameter and medial wall thickness were measured in approximately 15 muscular arterioles per animal. Only vessels with diameter between 30 and 90 μm were analyzed. MCT treatment induced significant increase in the medial wall thickness of pulmonary vessels, whereas coadministration of XNT attenuated this effect. (*D*) Quantification of interstitial fibrosis was estimated by percent area analysis. Rats treated with MCT exhibited an increased interstitial fibrosis and coadministration of XNT completely blunted this effect. Data are represented as mean ± SEM. $^{*}P < 0.05$ versus control group, $^{\#}P < 0.05$ versus MCT group.

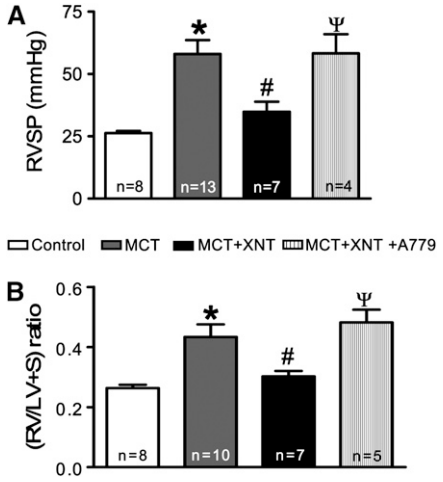

Figure 3. Coadministration of Mas antagonist, A-779, abolishes the protective effects of 1-[(2-dimethylamino) ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl) sulfonyl oxy]-9H-xanthene-9-one (XNT) on pulmonary hypertension. (*A*) Blockade of the Mas-receptor resulted in reversal of antihypertensive effects of XNT, suggesting the participation of the Ang-(1–7)-Mas-receptor axis on the protective effects of XNT. $^{*}P < 0.05$ versus control group, $^{\#}P < 0.05$ versus monocrotaline (MCT) group, $^{\Psi}P < 0.05$ versus MCT+XNT group (n = 4–13). (*B*) A similar mechanism of action of XNT [i.e., activation of the Ang-(1–7)-Mas receptor axis] was observed regarding its antihypertrophic effect because A-779 abolished the prevention of right ventricular hypertrophy by XNT. $^{*}P < 0.05$ versus control group, $^{\#}P < 0.05$ versus MCT group, $^{\Psi}P < 0.05$ versus MCT+XNT group (n = 5–10).

Mas and ACE2 mRNA levels were increased by XNT treatment (Figures 3E and 3F). Thus, XNT treatment resulted in approximately 3.5-fold increase in the ACE2/ACE ratio and approximately 2-fold increase in Mas/AT1 receptor ratio (Figures 4G and 4H).

Protective Actions of XNT Could Be Mediated through Modulation of Lung Proinflammatory Cytokines

Next, we studied the effects of XNT on cytokines because they have been shown to be involved in the pathology of PH (29–31). Table 1 shows quantitation of mRNA levels in MCT- and MCT+XNT–treated rats. It reveals that MCT treatment resulted in increases in mRNA for transforming growth factor β (27-fold), TNF-α (12-fold), IL-1 (6-fold), monocyte chemoattractant protein (MCP)-1 (25-fold), NF-κB p50 (50-fold), and NF-κB p65 (4-fold). In contrast, levels of IL-10, an antiinflammatory cytokine, decreased by 80%. XNT treatment of MCT-treated rats resulted in the reversal of this trend. Thus, increases in the levels of TNF-α, IL-1, IL-6, MCP-1, NF-κB p50, and NF-κB p65 were attenuated by XNT at 80, 80, 63, 84, 86, 93, and 55%, respectively. In contrast there was a significant increase in IL-10 levels (1051%) by XNT in MCT-treated rats. In addition, the Mas receptor antagonist, A-779, which attenuates XNT's effects on RVSP and RV hypertrophy (Figure 3), fails to influence pro- and antiinflammatory cytokines (Figure 5).

DISCUSSION

The most significant observation of this study is that a small molecule ACE2 activator (XNT) attenuates the development of PH. This beneficial effect of XNT is accomplished by tipping the balance of the vasoconstrictive-proliferative axis toward the vasoprotective axis of the RAS.

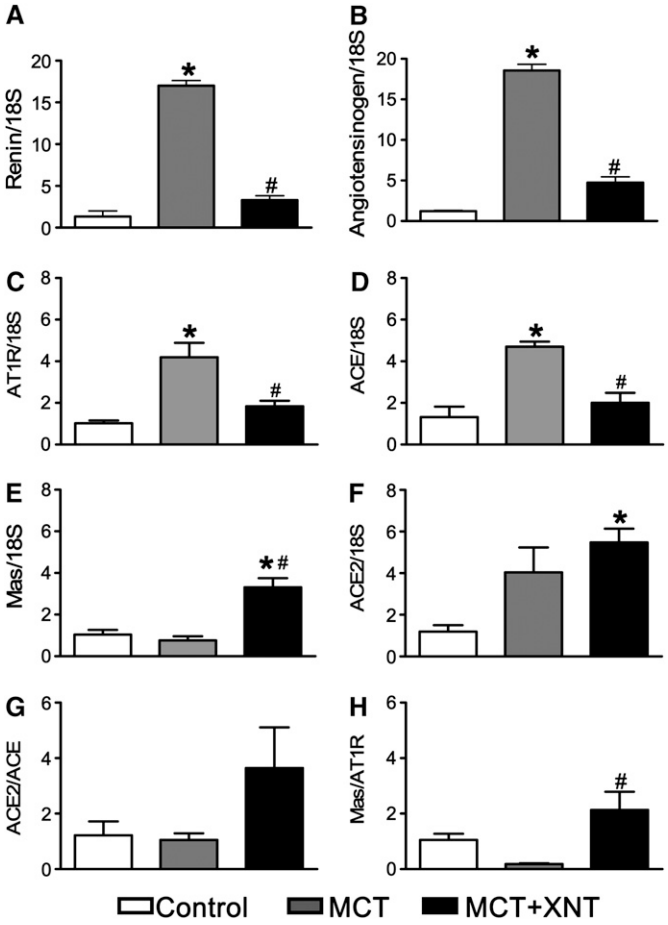

Figure 4. Effect of 1-[(2-dimethylamino) ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl) sulfonyl oxy]-9H-xanthene-9-one (XNT) on mRNA levels of the renin-angiotensin system components in the lungs of monocrotaline (MCT)–treated rats. Real-time quantitative polymerase chain reaction was used to measure (*A*) angiotensinogen (*B*) renin, (*C*) AT_1 receptor, (*D*) angiotensin-converting enzyme (ACE), (*E*) Mas receptor, and (*F*) ACE2 mRNA levels in the lungs. cDNA samples (2 μl) of reverse transcription reactions were amplified and normalized to 18S RNA. MCT treatment resulted in approximately 17-fold and approximately 18-fold increases in the renin and angiotensinogen mRNA, respectively, and approximately 4-fold increase in the AT_1 receptor, ACE, and ACE2 mRNA levels. XNT infusion prevented increases in renin, angiotensinogen, ACE, and AT_1 receptor mRNAs, whereas the levels of Mas and ACE2 mRNA were increased. XNT treatment increased the ratio of lung (*G*) ACE2/ACE, and (*H*) Mas/AT_1 mRNA levels. Data are expressed as mean ± SEM. $^{*}P < 0.05$ versus control group, $^{\#}P < 0.05$ versus MCT group (n = 3–10).

MCT treatment in rats results in severe PH and is associated with increases in the activity of the ACE-Ang II-AT_1R axis of the RAS as represented by increases in mRNA levels of renin, angiotensinogen, ACE, and AT_1R. XNT treatment prevents the increases in RVSP and RV hypertrophy, and attenuates the vascular wall thickening and pulmonary fibrosis in the MCT-treated rats. These protective effects of XNT were associated with the inhibition of mRNA levels for renin, AT_1R, ACE, and angiotensinogen, and an increase in mRNA levels for Mas and ACE2. Thus, we believe that increase in the ACE-Ang II-AT_1R (vasoconstrictive-proliferative) axis of the RAS is associated with PH and XNT is able to shift this toward the ACE2-Ang-(1–7)-Mas axis. This conclusion is supported by the observation

TABLE 1. CYTOKINE mRNA LEVELS IN LUNGS OF MONOCROTALINE-TREATED AND MONOCROTALINE+XNT–TREATED RATS

Cytokine	Control	MCT	MCT+XNT
TGF-β	1.23 ± 0.49 (4)	33.73 ± 2.96* (3)	6.18 ± 1.68# (4)
TNF-α	0.91 ± 0.16 (7)	11.46 ± 1.70* (7)	2.23 ± 0.34# (9)
IL-6	1.26 ± 0.25 (6)	55.56 ± 10.25* (6)	8.88 ± 1.59# (6)
IL-1	1.01 ± 0.01 (6)	6.76 ± 1.6* (6)	2.45 ± 0.38# (6)
NF-κB-50	0.90 ± 0.15 (8)	45.72 ± 11.9* (6)	3.01 ± 0.95# (7)
NF-κB-65	1.05 ± 0.17 (8)	4.72 ± 0.51* (8)	2.10 ± 0.35# (10)
MCP-1	1.26 ± 0.44 (6)	32.08 ± 2.63* (6)	4.22 ± 0.73# (6)
IL-10	1.42 ± 0.21 (8)	0.29 ± 0.06* (8)	3.34 ± 05.86# (10)

Definition of abbreviations: NF-κB = nuclear factor-κB; MCP = monocyte chemoattractant protein; MCT = monocrotaline; TGF = transforming growth factor; TNF = tumor necrosis factor; XNT = 1-[(2-dimethylamino) ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl) sulfonyl oxy]-9H-xanthene-9-one.

Total RNAs from control, MCT- and MCT+XNT–treated rats were isolated and subjected to quantitation by real-time reverse transcription–polymerase chain reaction for cytokines as described. Data are expressed as mean ± SEM. Numbers in the parentheses are the number of experiments. *$P < 0.05$ vs. control rats, #$P < 0.05$ versus MCT-treated rats (one-way analysis of variance followed by the Tukey test).

that A-779, a Mas antagonist, is able to reverse the beneficial effects of XNT on RVSP and RV hypertrophy (Figure 3). Preliminary experiments from our lab have demonstrated that overexpression of Ang-(1–7) in lungs by lentiviral-mediated gene transfer of Ang-(1–7) fusion protein is similarly effective in the prevention of MCT-induced increases in RVSP. In addition, we observed that Ang-(1–7) immunoreactivity is increased in lungs of mice treated with lenti-ACE2 (unpublished data).

Previous studies have shown that induction of PH is associated with increased production of proinflammatory cytokines in the lungs (29–31). Our data demonstrating increases in TNF-α, IL-1, IL-6, MCP-1, NF-κB p50, and NF-κB p65 confirm this. Furthermore, a decrease in IL-10, an antiinflammatory cytokine, is consistent with this view. Treatment with XNT attenuates the effects of MCT on both the inflammatory and proinflammatory cytokines. This suggests that attenuation of increases in proinflammatory cytokines in combination with the shift of RAS toward vasoprotective axis may be responsible for the overall beneficial effects of XNT in PH. It remains to be determined if changes in the RAS are responsible for changes in cytokines or they are independently altered in PH. We favor the former situation because the RAS is a potent regulator of cytokines and inflammation (32). However, lack of an effect of Mas receptor antagonist on cytokine mRNA levels argues against this proposal. One of the limitations of our study is that our conclusions on the cytokines and the RAS data are based on mRNA levels and have not been confirmed by protein measurement. Because increased mRNA levels generally result in increased protein expression, we believe that this might be true in our study too. Nevertheless, evaluation of both types of gene product will be desirable in future work.An interesting and important aspect of this study is that the beneficial effects of XNT on PH are associated with no adverse effects on systemic blood pressure. This is extremely relevant because any systemic hypotensive effects could be counterproductive in treating patients with PH as they are already at a high risk of developing hypotension due to right ventricular overload (33). This could also explain the lack of success of ACE inhibitors or AT_1 receptor blockers in the treatment of PH, because their primary effects are to reduce systemic blood pressure. The lack of influence on systemic blood pressure potentially puts XNT in a separate class of drugs that may preferentially target the pulmonary RAS. Figure 6 summarizes our current understanding of the role of the RAS in PH. We believe that MCT shifts the balance of the RAS toward ACE-Ang II-AT_1 receptor resulting in increases in proinflammatory cytokines, vascular remodeling, fibrosis, and right ventricular hypertrophy. XNT treatment reverses these pathophysiologies by shifting the balance of the RAS toward the ACE2-Ang-(1–7)-Mas axis. XNT could be a potent active lead compound for the activation of ACE2 in the treatment of PH. However, this compound still needs to undergo more comprehensive pharmacodynamic profiling to establish if this would be the most ideal compound for the next phase of investigation. Other more efficacious compounds can be easily synthesized based on XNT's structure for testing. Finally, we believe that XNT may influence ACE2 activity. This conclusion is based on the following: (*1*) XNT was

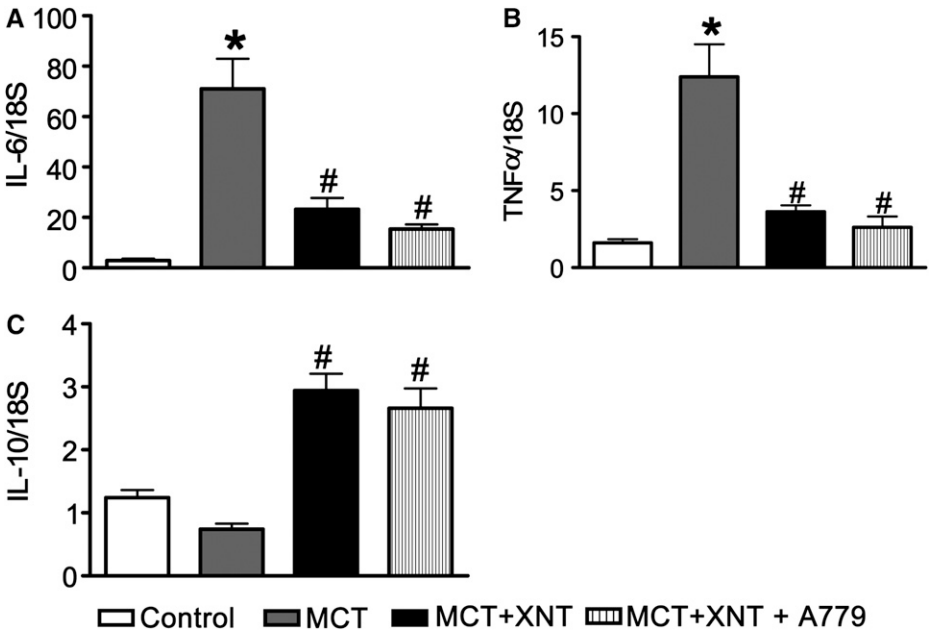

Figure 5. Effect of coadministration of A-779 with 1-[(2-dimethylamino) ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl) sulfonyl oxy]-9H-xanthene-9-one (XNT) on cytokine mRNA levels in monocrotaline (MCT)–treated rats. Mas-receptor antagonist, A-779, fails to influence pro- and antiinflammatory cytokines (*A*, *B*, *C*) modulated by XNT in MCT-treated rats. Data are represented as mean ± SEM. *$P < 0.05$ versus control group, #$P < 0.05$ versus MCT group (n = 3–10).

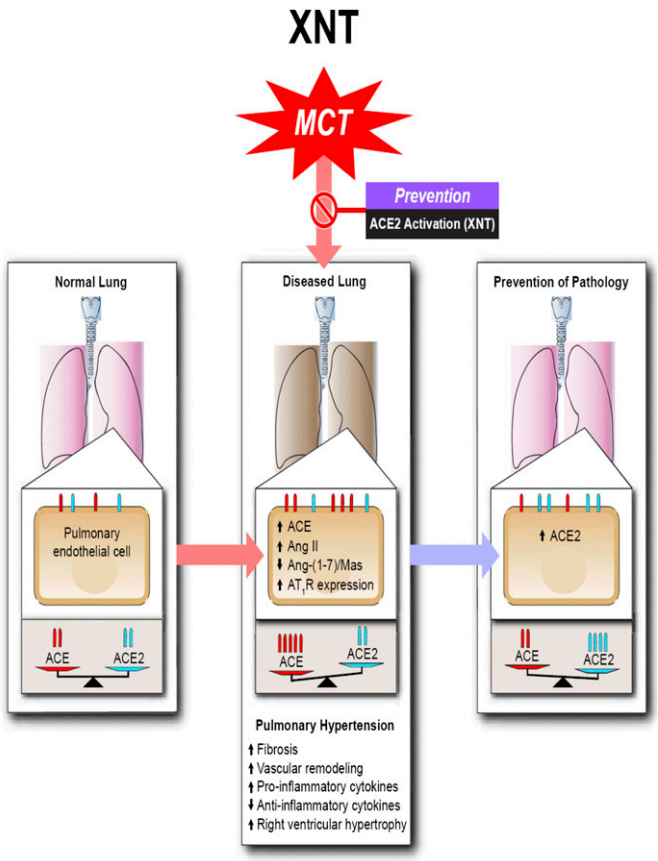

Figure 6. Diagrammatic representation of the hypothesis of the role of ACE2 in monocrotaline (MCT)–induced pulmonary hypertension (PH). Normal pulmonary endothelial functions are maintained by a critical balance between angiotensin-converting enzyme (ACE) and ACE2. MCT treatment impairs this balance by decreasing the ratio of ACE2/ACE leading to an increase in proinflammatory cytokines and decrease in antiinflammatory cytokines, resulting in fibrosis and vascular remodeling. This leads to PH and right ventricular hypertrophy. 1-[(2-Dimethylamino) ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl) sulfonyl oxy]-9H-xanthene-9-one (XNT) treatment reverses this imbalance by increasing the ratio of ACE2/ACE, thus preventing PH and lung remodeling.

discovered by virtual screening technology based on the crystal structure of ACE2 (18), and (*2*) XNT increases ACE2 activity (18). However, other effects of XNT, such as a direct antiinflammatory action, cannot be ruled out at the present time. Nonetheless, our observations provide conceptual support that pulmonary ACE2 presents an interesting target for lung vascular remodeling and PH.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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