Pulmonary Hypertension

Prevention of Pulmonary Hypertension by Angiotensin-Converting Enzyme 2 Gene Transfer

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Abstract—In spite of recent advancements in the treatment of pulmonary hypertension, successful control has yet to be accomplished. The abundant presence of angiotensin-converting enzyme 2 (ACE2) in the lungs and its impressive effect in the prevention of acute lung injury led us to test the hypothesis that pulmonary overexpression of this enzyme could produce beneficial outcomes against pulmonary hypertension. Monocrotaline (MCT) treatment of mice for 8 weeks resulted in significant increases in right ventricular systolic pressure, right ventricle:left ventricle plus septal weight ratio, and muscularization of pulmonary vessels. Administration of a lentiviral vector containing ACE2, 7 days before MCT treatment prevented the increases in right ventricular systolic pressure (control: 25±1 mm Hg; MCT: 44±5 mm Hg; MCT+ACE2: 26±1 mm Hg; n=6; P<0.05) and right ventricle:left ventricle plus septal weight ratio (control: 0.25±0.01; MCT: 0.31±0.01; MCT+ACE2: 0.26±0.01; n=8; P<0.05). A significant attenuation in muscularization of pulmonary vessels induced by MCT was also observed in animals overexpressing ACE2. These beneficial effects were associated with an increase in the angiotensin II type 2 receptor:angiotensin II type 1 receptor mRNA ratio. Also, pulmonary hypertension–induced increases in proinflammatory cytokines were significantly attenuated by lentiviral vector–containing ACE2 treatment. Furthermore, ACE2 gene transfer in mice after 6 weeks of MCT treatment resulted in a significant reversal of right ventricular systolic pressure. These observations demonstrate that ACE2 overexpression prevents and reverses right ventricular systolic pressure and associated pathophysiology in MCT-induced pulmonary hypertension by a mechanism involving a shift from the vasoconstrictive, proliferative, and fibrotic axes to the vasoprotective axis of the renin-angiotensin system and inhibition of proinflammatory cytokines. (Hypertension. 2009;54:365-371.)

Key Words: cardiovascular diseases ■ gene therapy ■ hypertension ■ pulmonary ■ lung ■ remodeling

Pulmonary hypertension (PH) is a refractory disease characterized by a progressive increase in pulmonary artery pressure and resistance. The remodeling in pulmonary arterioles results in PH, increased pulmonary vascular resistance, right ventricular (RV) hypertrophy, and right heart failure. Although the pathogenesis of PH is poorly understood, it has been proposed that endothelial dysfunction or damage could be involved.1,2

Previous studies have implicated the involvement of the renin-angiotensin system (RAS) in the pathogenesis of PH. Evidence for this conclusion includes the following: (1) lungs are the primary site for angiotensin-converting enzyme (ACE) expression3 and are responsible for the generation of high concentrations of circulating and pulmonary angiotensin (Ang) II4; (2) other components of the RAS, including renin, angiotensinogen, and both subtypes of Ang II receptors, are expressed in the lungs4–10; (3) increase in ACE in pulmonary vasculature has been associated with PH in both animal models and in patients11,12; and (4) ACE inhibitors have been shown to attenuate PH in animal models11,13,14. In spite of this, ACE inhibitors and Ang receptor blockers have not proven to be very effective in the management of pulmonary diseases.15,16 This may be primarily because of the fact that these drugs lower basal systemic blood pressure (BP). This would be counterproductive in PH patients, because most of them already exhibit lower BP. In addition, the limited/lack of success of these orally administered therapeutic agents may be related to the differential tissue distribution and drug-specific pharmacodynamics that could limit their therapeutic concentrations in lung tissue.17–19 These observations, taken together
with the well-established hypertrophic actions and emerging role of proinflammatory signaling by Ang II and Ang II type 1 (AT1) receptors,20,21 suggest that the involvement of the RAS in PH should be re-examined. This view takes on an added relevance since the discovery of ACE2 as a new member of the RAS.

ACE2, a homolog of ACE, shares \( \approx 42\% \) structural identity with the catalytic domain of ACE and cleaves a single residue from Ang I to generate Ang-(1-9). More importantly, ACE2 degrades Ang II into Ang-(1-7) with high efficiency.22,23

Thus, ACE2 is an important player in the vasoprotective axis (ACE2-Ang-[1-7]-Mas) of the RAS and is critical in balancing the activity of the vasoconstrictive, proliferative, and fibrotic axes (ACE-Ang II-AT1 receptor) of the RAS.24 ACE2 is highly expressed in the lungs,25,26 and recent evidence suggest its pivotal role in pulmonary physiology and pathophysiology. This evidence include the following: (2) ACE2 knockout mice develop pulmonary congestion and increased incidence of congestive heart failure27; (2) ACE2 expression is downregulated in both human and experimental lung fibrosis28; (3) alterations in the expression of ACE2 in primary pulmonary hypertensive patients show a direct correlation between Ang II type 2 (AT2) receptors and Ang-(1-7)-forming activity29; (4) ACE2 has been shown to protect lungs from acute respiratory distress syndrome and acute lung injury, which involves increases in vasoprotective members of the RAS, Ang-(1-7) and AT2 receptors25,26; and (5) administration of recombinant ACE2 attenuates lung failure in ACE2 knockout mice.30 Collectively, these observations led us to hypothesize that ACE2 overexpression would produce beneficial effects on PH. Thus, we used gene transfer techniques with a lentiviral vector to obtain long-term expression of ACE2 in the lungs of mice.

Methods

Production of lentiviral-mediated overexpression of ACE2 viral particles, determination of transduction efficiency of the lung by lentivirus, cardiac hypertrophy, immunohistochemical analysis, RNA isolation, and real-time PCR are described in the online Data Supplement (available at http://hyper.ahajournals.org).

Animals

Five- to 6-week–old male C57/BL6 mice were used and housed in a temperature-controlled room (25 ± 1°C). Animals were maintained on a 12:12 hour light:dark cycle with free access to water and food. All of the procedures involving experimental animals were approved by the University of Florida Institutional Animal Care and Use Committee and complied with National Institutes of Health guidelines.

Experimental Design

PH was induced by weekly SC injections of 600 mg/kg of monocrotaline (MCT) (Sigma-Aldrich) for 8 weeks. Control mice received saline (20 µL/g, SC, 8 weeks). Two protocols were used, one to
assess the prevention of PH with ACE2 overexpression and another
to determine the ability of ACE2 gene transfer on the reversal of PH.

In the prevention protocol, 7 days after lenti-green fluorescent
gene delivery of lenti-GFP (control) and lenti-ACE2 (3 × 10^6
transducing units in 30 µL of PBS) was accomplished by injection of
the virus into the trachea of anesthetized mice as described above.

BP Measurements
Seven to 10 days after the last injection of MCT, systemic BP was
measured in conscious mice by the tail-cuff method (n = 6 to 8 in
each group), as described previously.31 RV systolic pressure (RVSP)
was used as an indicator for pulmonary BP. For this measurement,
mice were anesthetized with a mixture of ketamine (100 mg/kg, SC) and
xylazine (15 mg/kg, SC) and were placed in a supine position, breathing
room air. A catheter was inserted into the right descending jugular vein
and forwarded to the RV. The data were recorded after stabilization of
the tracing using a liquid pressure transducer, which was interfaced to
a PowerLab (AD Instruments) unit. The waveform was used to
confirm the positioning of the catheter in the RV. Data were
analyzed by using the Chart program that was supplied with the
PowerLab system. After RVSP measurement, mice were euthanized,
and the hearts and lungs were harvested.

Statistical Analysis
Data are presented as mean ± SEM. Statistical differences were
evaluated by Student’s t test, 1-way ANOVA, or 2-way ANOVA
wherever applicable, followed by the Newman-Keuls posthoc test.

Results

In Vivo Gene Delivery Into the Lungs
A robust, widespread, and random transduction of pulmonary
tissue was observed as early as 7 days after intratracheal gene
transfer with lentiviral vector–containing placental alkaline phosphatase
(PLAP; Figure 1A). The expression persisted for the
duration of experimental protocols (8 to 10 weeks). No
visible transduction was detected in the kidneys and heart
(data not shown). Intratracheal administration of lenti-ACE2
resulted in an indiscriminate expression ACE2 in the pulmonary
region (Figure 1B and 1C). Immunohistochemistry revealed that
ACE2 immunoreactivity was observed in the bronchiolar
epithelial and alveolar cells (Figure 1C). Lentiviral-mediated
ACE2 gene transfer into the lungs resulted in an ≈2 fold
increase in ACE2 mRNA levels compared with control
animals (Figure 1D). Furthermore, Ang-(1-7) immunoreac-
tivity was observed in the arterial epithelial and alveolar cells
of the control lungs (Figure S1A and S1C, available in the
online Data Supplement), which was significantly increased
(≈30%) by lenti-ACE2 injection into the trachea (Figure S1B
and S1D).

Prevention of PH and Associated Cardiac
and Pulmonary Damages by ACE2
Two-way ANOVA revealed significant interaction between
the pulmonary pressure–lowering effect of lenti-ACE2 and
MCT administration. Weekly injection of MCT for 8 weeks
resulted in an increase in RVSP (MCT: 44 ± 5 mm Hg versus
control: 25 ± 1 mm Hg; P < 0.05; Figure 2A), which was pre-
vented with ACE2 gene transfer (26 ± 1 mm Hg; Figure 2A).
Also, the RV:left ventricle plus septal weight ratio was
significantly increased in MCT-treated mice compared with the control group (MCT: 0.31±0.01 versus control: 0.25±0.01; P<0.05; Figure 2B). This increase was also prevented with ACE2 gene transfer treatment (0.26±0.01; Figure 2B). Lenti-ACE2 administration significantly reduced PH only in the MCT-treated group and not in the control group. No significant differences in systemic BP were observed among any of the groups (systolic BP: control, 127±3 mm Hg; ACE2, 113±4 mm Hg; MCT, 119±3 mm Hg; MCT + ACE2, 123±4 mm Hg).

Immunostaining with an antibody directed to α-smooth muscle actin showed that the medial walls of pulmonary arterioles were markedly thickened by MCT treatment for 8 weeks. This effect was attenuated by ACE2 gene transfer (Figure 3A and 3B). In normal lungs, 77% of the arterioles were nonmuscularized, and 2% were fully muscularized. In contrast, MCT-treated animals showed a substantially greater proportion of small vessels with full muscularization (60%) and a lower proportion with nonmuscularization (10%). ACE2 gene transfer significantly reduced the percentage of small vessels exhibiting muscularization (24% MCT + ACE2 versus 60% MCT; P<0.05; Figure 3C) and increased the percentage of nonmuscularized vessels.

Reversal of PH and Associated Cardiac and Pulmonary Damages by ACE2

ACE2 gene transfer in mice after 6 weeks of pretreatment with MCT also resulted in attenuation of RVSP (28±0.3 mm Hg; P<0.05; Figure 4A) compared with MCT-treated mice. However, the RV:left ventricle plus septal weight ratio was similar in both groups (MCT + ACE2: 0.29±0.01 mg/mg versus MCT: 0.31±0.01 mg/mg; Figure 4B). Immunohistochemical studies revealed that the increase in the wall thickness observed in small pulmonary vessels after MCT treatment was reversed by ~30% in mice overexpressing ACE2 (Figure 5A). In addition, the percentage of fully muscularized intra-acinar vessels in lungs from MCT-treated mice increased, whereas the percentage of nonmuscularized vessels decreased compared with control mice. ACE2 gene transfer reversed the effect of MCT on muscularized vessels demonstrating an ~35% decrease (Figure 5B). No significant difference was observed in the systolic BP of MCT mice treated with lenti-ACE2.

Possible Mechanism of ACE2 Action

mRNA levels of certain members of the RAS and proinflammatory cytokines genes were measured to determine the possible mechanism of ACE2 gene transfer. MCT treatment showed no significant effects on the AT1 receptor, ACE, and ACE2 mRNA levels, whereas renin and AT2 receptor mRNA levels increased by 54% and 100%, respectively, although the increase in the AT2 receptor did not reach significance (Table). However, an interesting pattern emerged when the ratios between the mRNAs of the vasoprotective axis of the RAS (ACE2 and AT2) receptor were compared with the vasoconstrictive, proliferative axis (ACE and AT1) receptor in the lenti-ACE2–
treated group in our prevention protocol. MCT treatment decreased the mRNA ratio of ACE2:ACE, whereas lenti-ACE2 treatment of MCT-treated mice restored this to control levels (Figure 6). In addition, AT2 receptor:AT1 receptor levels increased ~4-fold in the MCT-treated animal and ~8-fold by lenti-ACE2 treatment of MCT-treated mice (Figure 6). In addition, Ang II immunoreactivity was significantly increased in MCT-treated mice, and this was significantly reduced in MCT-treated mice by lenti-ACE2 (Figure S2).

MCT treatment also resulted in ~12.0-fold, ~25.0-fold, and 2.5-fold increases in the inflammatory cytokine interleukin (IL) 6, monocyte chemoattractant protein (MCP) 1, and tumor necrosis factor (TNF)-α mRNA levels, respectively (Figure 7). Treatment with lenti-ACE2 in the prevention protocol caused a 65% decrease in MCP-1, 60% decrease in IL-6, and 90% decrease in TNF-α mRNA levels when compared with their levels in MCT-treated mice. Immunohistochemical data supported the mRNA changes. Figures S3 and S4 show that MCT treatment resulted in significant increases in the intensities of MCP-1 and TNF immunoreactivity in the lungs. This was significantly reduced in lenti-ACE2 lungs of MCT-treated mice. These observations suggest that the shifting of the RAS to a vasoprotective axis is associated with attenuation of the increase in proinflammatory cytokines. This may be linked to the beneficial effects of lenti-ACE2 on PH.

Discussion

In this study we provide evidence of the following: (1) lentiviral vector is extremely efficient in transducing a wide variety of cells in pulmonary tissue on a long-term basis; (2) overexpression of ACE2 results in almost complete attenuation of PH induced by MCT; and (3) this strategy is also successful in a significant reversal of PH-induced lung damage. Thus, these observations provide supportive evidence that ACE2 overexpression or endogenous pulmonary ACE2 activation may have important implications in the development of a novel therapeutic strategy in the treatment and possibly reversal of PH and its associated complications.

Lenti-ACE2 treatment, before the induction of PH, resulted in an almost complete prevention of increases in RVSP, RV hypertrophy, and attenuation of thickening of pulmonary vessels. This was associated with a significant inhibition of muscularization of arterioles. Despite the increase in RVSP and RV hypertrophy, we did not observe significant differences in RV end-diastolic pressure or dP/dt in the right ventricle among the groups. It is conceivable that the animals were still in an adaptive phase at this time and the pathophysiological aspects would be manifested at a later time point. Hessel et al., using a rat model of MCT, also noted normal RV function with regard to dP/dt values in animals treated with a high and a low dose of MCT for 4 weeks despite the increase in RVSP and RV hypertrophy.

In addition, lenti-ACE2 treatment at 6 weeks after MCT administration caused partial reversal of PH-linked pathophysologies. The reason for a partial reversal may be related to the time course and levels of transgenic ACE2, because ACE2 overexpression was performed for only 2 weeks in animals that already exhibited long-standing PH in the reversal study. However, the possibility that the complete reversal could be accomplished by longer overexpression of ACE2 cannot be ruled out at the present time.

Targeting of ACE2 in the lungs appears to be a better strategy than the use of systemic administration of AT1 receptor antagonists and ACE inhibitors, which have been found to have limited or no success in the prevention of PH. The precise mechanism of this success will await further investigation. However, it is tempting to suggest that ACE2 shifts the balance of the vasoconstrictive, proliferative, and fibrotic axes of the RAS (ACE-Ang II-AT1 receptor) toward the vasoprotective axis (ACE2-Ang-[1-7] and AT2 receptor). This contention is supported by our observation that ACE2 gene transfer increases Ang-(1-7) staining, decreases Ang II immunoreactivity, and increases the ratio of the AT2 receptor:AT1 receptor in the MCT-treated mice. Further support for our hypothesis is our recent finding that an ACE2 activator inhibits fibrosis and has similar alterations in cytokines in a rat MCT model of PH. Likewise, we have shown recently that overexpression of ACE2 or Ang-(1-7) provided protective pulmonary and cardiac effects in a bleomycin-induced model of pulmonary fibrosis. Others have also suggested a similar shift in the vasoprotective axis by ACE2 for acute lung injury.
the apparent increase in the AT2R expression observed in our study is consistent with the finding of an increase in AT2R expression observed in other cardiovascular diseases. Zisman et al have previously demonstrated a direct correlation between AT2R expression and Ang-(1-7) forming activity (ACE2) in failing human heart ventricles from patients with primary PH. In addition, AT2 receptors have been shown to suppress myocardial hypertrophy and fibroblast proliferation. Thus, the beneficial effect of ACE2 overexpression may, in part, be attributable to an increase in the ratio of AT2 receptor:AT1 receptor. Furthermore, ACE2 overexpression has been shown to exert a negative influence on AT1 receptors.

Previous studies have shown that induction of PH is associated with increased production of proinflammatory cytokines. Our data showing increases in MCP-1, IL-6, and TNF-α confirm this. Furthermore, lenti-ACE2 treatment prevents increases in these proinflammatory cytokines. This suggests that the attenuation of proinflammatory cytokines, in combination with the shift toward the vasoprotective axis, may be responsible for the overall beneficial effects of ACE2 gene transfer in PH. It remains to be determined whether changes in the RAS are responsible for the changes in proinflammatory cytokines or if they are independently altered in PH. However, we favor the former situation, because RAS is a potent regulator of proinflammatory cytokines. A limitation of the study is that the mRNA data are not confirmed by protein measurement. Nevertheless, evaluation of both types of gene product will be desirable in future work.

An interesting aspect of this study is that pulmonary overexpression of ACE2 did not influence systemic BP. This observation is supported by our previous study in which cardiac overexpression of ACE2, which causes significant attenuation of hypertension-induced cardiac hypertrophy, has little effect on high BP. This may turn out to be an important benefit if this strategy could be translated for therapeutics. Patients suffering from severe PH already express lower systemic BP as a result of RV overload, and treatment with ACE inhibitors, AT1 receptor blockers, or other currently available therapy would exacerbate systemic hypotension. However, it appears that pulmonary ACE2 overexpression circumvents influences on systemic hemodynamics and only influences pulmonary pathophysiology.

Finally, the most significant aspect of our observation is that it provides evidence that ACE2 overexpression/activation is an innovative strategy against PH. However, further evidence confirming the safety issues associated with lentiviral vector and validation with other animal models will be needed before the translation of this observation into preclinical strategy. Nonetheless, our data provide evidence that pulmonary ACE2 represents a novel target for therapeutic intervention aiming at the prevention and restoration of lung vascular remodeling and subsequent right heart hypertrophy.

**Perspectives**

Current therapeutic strategies for the control and treatment of PH are primarily based on pharmacological agents with limited efficacy. In spite of their relative success in PH treatment, frequently these pharmacological agents are associated with serious adverse effects. Clearly, there is an urgent need to develop new strategies (eg, new drug targets, novel therapeutic molecule delivery methods, cell-based therapies) to successfully control this disease. The discovery of ACE2, with its potential to shift the adverse effects of RAS hyperactivity toward beneficial outcomes in the cardiovascular system, holds this promise. Our study is timely because it presents evidence that overexpression of ACE2 prevents and reverses PH. It provides conceptual in vivo support for ACE2 as a viable target for the future development of pharmacological and genetic upregulating strategies for the treatment of this disease.

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**Disclosures**

None.

**References**


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SUPPLEMENTAL DATA

Prevention of Pulmonary Hypertension by Angiotensin Converting Enzyme 2 Gene Transfer

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Methods:

Production of Lentiviral-Mediated Overexpression of ACE2 Viral Particles

Lentiviral particles containing reporter genes, human placental alkaline phosphatase (EF1α-IRES-PLAP, lenti-PLAP) or enhanced green fluorescent protein (GFP; EF1α-IRES-EGFP, lenti-GFP) and murine ACE2 (EF1α-ACE2-IRES-EGFP, lenti-ACE2) were prepared by methods described previously.\(^1\) Viral medium containing lenti-GFP or lenti-ACE2 was collected, concentrated, and titrated. Concentration of viral particles was determined with the use of HIV-1 p24 antigen ELISA assay (Beckman Coulter) following the manufacturer’s instructions. Efficacy of lenti-ACE2 in producing active ACE2 enzyme has been established previously.\(^2\)

Determination of transduction efficiency of lung by lentivirus

Mice were anesthetized with isoflurane and the trachea was exposed through a midline incision. Lenti- PLAP particles (3x10\(^6\) transducing units (TU) in 30 μl of PBS) were injected into the trachea followed by air injection. Seven days following gene transfer, animals were sacrificed; lungs were perfused first with PBS, pH 7.4, followed by 4% paraformaldehyde (PFA) in PBS and postfixed with 4% PFA for 1 h by immersion.\(^3\) Tissues were incubated at 72 °C for 3 h, cooled, and subjected to PLAP staining as described previously.\(^1\)

Hypertrophy and histological analysis

The right ventricle (RV) was separated from the left ventricle (LV) plus ventricular septum (S) and the wet weights were determined. RV hypertrophy was expressed as the ratio of RV to LV plus ventricular septum (RV/LV+S) (n=6-8 in each group). Left lungs were perfused and fixed as described above. After fixation and paraffin embedding, 5 μm-thick lung sections were cut and stained with anti-α smooth muscle actin (SMA) (1:600, clone 1A4, Sigma, St Louis, Mo), as described previously.\(^3\) Sixty to eighty intra-acinar vessels with diameter between 20 to 80 μm, accompanying either alveolar ducts or alveoli were analyzed in each mouse. Each vessel was categorized as nonmuscularized (i.e. no apparent muscle), partially muscularized (i.e. with only a crescent of muscle), or fully muscularized (i.e. with a complete medial coat of muscle).\(^4\) A population of vessels was expressed as a percentage of the total vessel numbers counted from the section. The external diameter and medial wall thickness were measured in 30 muscular arteries per lung section for analysis of the medial wall thickness of the pulmonary arterioles. The medial thickness was calculated as follows: percent wall thickness = [(medial thickness x 2)/external diameter] x100 (n=4-5 mice per group).\(^5\)
**Immunohistochemical analysis**

Paraffin embedded lung sections were first incubated with 0.3% H$_2$O$_2$ in PBS for 15 min followed by incubation with 1.5% goat serum in PBS containing 0.3% Triton X100 for 1 h. Sections were incubated overnight at 4°C with one of the following antibodies diluted in PBS containing 0.3% Triton X100 and 0.3% BSA: rabbit polyclonal anti-ACE2 (1:500, GTX15348, GeneTex), rabbit polyclonal anti-Ang-(1-7) (1:600) or rabbit polyclonal anti-Ang II (1:100, Abcam, Cambridge, MA), rabbit polyclonal anti-MCP-1 (1:100, Santa Cruz, CA) or rabbit polyclonal anti-TNF-alpha (1:100, Santa Cruz, CA). After 4-5 rinses in PBS, biotinylated goat anti-rabbit IgG secondary antibody was added for 1 h followed by incubation with avidin-biotin-peroxidase complex reagents for 1 h, stained with diaminobenzidine solution for 4 min (Vector Laboratories), and analyzed using an Olympus BX 41 microscope. Each step was followed by washing the sections with PBS containing 0.3% Triton X100. Sections incubated without primary antibodies were used as negative controls.

**RNA Isolation and Real-time PCR**

Total RNA was extracted from frozen lung tissues and real-time RT-PCR (qRT-PCR) was performed as described previously using Bio-Rad PCR Master Mix to determine the expression levels of AT$_1$ receptor, AT$_2$ receptor, ACE, ACE$_2$, renin, MCP-1, TNF-α, and IL-6 by using ABI Prism 7900 sequence detection system. mRNA levels were normalized to 18s RNA from the same samples (n=3-10 in each group).

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**Disclosures:**

None
REFERENCES


Supplemental Figure 1

Representative photomicrographs of Ang-(1-7) immunoreactivity in lungs of lenti-ACE2 treated mice:

Mice were injected with 3x10^6 TU of lenti-GFP or lenti-ACE2. Eight weeks following transduction, lungs were inflated, fixed and subjected to Ang-(1-7) immunohistochemistry as described in the Methods section. Ang-(1-7) immunoreactivity was significantly higher in (b and d) lenti-ACE2-treated lungs compared to (a and c) control lenti-GFP-treated lungs.
Effects of lenti-ACE2 on Ang II immunoreactivity in the lungs of MCT-treated mice:

Sections of lungs from control, MCT and MCT+ lenti-ACE2 mice from the reversal protocol were fixed and incubated with anti-Ang II antibody. This was followed by incubation with FITC-labeled secondary antibody as described in the Methods section.
Supplemental Figure 3

Supplemental Figure 4

Representative photomicrographs of MCP-1 and TNF-α immunostaining in lungs of lenti-ACE2-treated mice:

Sections of lungs from control, MCT-treated and MCT+lenti-ACE2-treated mice from the prevention study were prepared as described in the Methods section. They were subjected to immunohistochemistry with the use of antibodies specific for MCP-1(Figure 3) and TNF-α (Figure 4) and rhodamine and FITC labeled secondary antibody, respectively. ACE2 overexpression attenuates levels of MCP-1 and TNF-α immunostaining induced by MCT treatment.