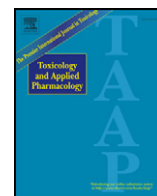




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Losartan attenuates chronic cigarette smoke exposure-induced pulmonary arterial hypertension in rats: Possible involvement of angiotensin-converting enzyme-2

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ABSTRACT

Chronic cigarette smoking induces pulmonary arterial hypertension (PAH) by largely unknown mechanisms. Renin-angiotensin system (RAS) is known to function in the development of PAH. Losartan, a specific angiotensin II receptor antagonist, is a well-known antihypertensive drug with a potential role in regulating angiotensin-converting enzyme-2 (ACE2), a recently found regulator of RAS. To determine the effect of losartan on smoke-induced PAH and its possible mechanism, rats were daily exposed to cigarette smoke for 6 months in the absence and in the presence of losartan. Elevated right ventricular systolic pressure (RVSP), thickened wall of pulmonary arteries with apparent medial hypertrophy along with increased angiotensin II (Ang II) and decreased ACE2 levels were observed in smoke-exposed-only rats. Losartan administration ameliorated pulmonary vascular remodeling, inhibited the smoke-induced RVSP and Ang II elevation and partially reversed the ACE2 decrease in rat lungs. In cultured primary pulmonary artery smooth muscle cells (PASMCs) from 3- and 6-month smoke-exposed rats, ACE2 levels were significantly lower than in those from the control rats. Moreover, PASMCs from 6-month exposed rats proliferated more rapidly than those from 3-month exposed or control rats, and cells grew even more rapidly in the presence of DX600, an ACE2 inhibitor. Consistent with the *in vivo* study, *in vitro* losartan pretreatment also inhibited cigarette smoke extract (CSE)-induced cell proliferation and ACE2 reduction in rat PASMCs. The results suggest that losartan may be therapeutically useful in the chronic smoking-induced pulmonary vascular remodeling and PAH and ACE2 may be involved as part of its mechanism. Our study might provide insight into the development of new therapeutic interventions for PAH smokers.

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Introduction

Pulmonary arterial hypertension (PAH) is a group of diseases characterized by a progressive increase of pulmonary vascular resistance that finally cause right ventricular failure and premature death (Chaouat et al., 2008). It has been generally accepted and acknowledged that alterations in the pulmonary vasculature, commonly marked by vascular proliferation/fibrosis, remodeling, and vessel occlusion, leads to most, if not all, forms of pulmonary arterial hypertension (Humbert et al., 2004). As a key factor blamed for the alterations in the pulmonary vasculature, cigarette smoking has been reported to result in muscularization of pulmonary vessels in the presence or absence of chronic obstructive pulmonary disease (Wright et al., 2003, 2004; Churg et al., 2006; Santos et al., 2002). Moreover, increased expression of vasoactive mediators (e.g., endothelin-1 and vascular endothelial growth factor) have been

detected in the guinea pig lungs after chronic smoke exposure and those mediators are reported to be associated with vascular remodeling and elevated pulmonary arterial pressure (Wright et al., 2006). However, the precise mechanisms by which chronic cigarette smoke exposure produces PAH are still poorly understood.

The renin-angiotensin system (RAS) has been implicated in the pathogenesis of pulmonary vascular remodeling and PAH in a number of studies. For instance, increased expression of angiotensin-converting enzyme (ACE) in pulmonary arteries has been reported in PAH patients (Orte et al., 2000). Increases in angiotensin II (Ang II) and Ang II type 1 (AT1) receptors were both demonstrated in hypoxic and monocrotaline-treated pulmonary hypertensive rats (Chassagne et al., 2000; Ferreira et al., 2009). In the RAS, ACE plays a central role in generating Ang II from angiotensin I (Ang I) (Turner & Hooper, 2002), and Ang II exerts a prominent role in the development of pulmonary vascular remodeling and PAH (Jeffery & Wanstall, 2001), particularly in promoting the growth of pulmonary artery smooth muscle cells (PASMCs) via AT1 receptors (Morrell et al., 1999). Moreover, medication with either ACE inhibitor or AT1 receptor antagonist can attenuate pulmonary arterial remodeling and PAH in

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chronic hypoxia or monocrotaline-treated rats (Nong et al., 1996; Kishi et al., 2006). Additionally, nicotine, an important component of cigarette smoke, and its metabolites can increase both the activity and the expression of ACE in cultured human endothelial cells (Saijonmaa et al., 2005; Ljungberg & Persson, 2008). Furthermore, inhibition of ACE may improve endothelial function in chronic cigarette smokers (Butler et al., 2001). Based on these previous findings, we hypothesized that activated RAS might also contribute to the development of chronic smoking-induced PAH.

Losartan, a specific AT1 receptor blocker, exerts its influence on the RAS via inhibiting the stimulation of the AT1 receptor by Ang II. It has been reported that losartan treatment may attenuate pulmonary vascular remodeling and PAH in hypoxic rats and in piglets with left-to-right shunts (Chassagne et al., 2000; Rondelet et al., 2005). However, it has not been determined whether losartan has a therapeutic effect on chronic cigarette smoking-induced PAH. In addition, losartan has shown a modulatory effect on the expression of angiotensin-converting enzyme-2 (ACE2) in hypertensive and cardiovascular diseases (Ishiyama et al., 2004; Koka et al., 2008). ACE2, with a main role in cleaving Ang II into angiotensin (1–7) [Ang (1–7)] (Ferrario et al., 2005b), is a recently found regulator of the RAS and has been implicated in multiple lung diseases, e.g., severe acute respiratory syndrome-associated coronavirus (SARS-CoV) infection, acute lung injury, pulmonary fibrosis and monocrotaline-induced PAH (Imai et al., 2005; Kuba et al., 2005; Li et al., 2008; Ferreira et al., 2009). However, it has not been elucidated whether ACE2 has a role in chronic smoking-induced PAH. A recent study shows that the antihypertensive action of AT1 antagonists may be partially due to increased Ang II metabolism by ACE2 (Ferrario et al., 2005a). Thus, in the present study, we sought to investigate whether losartan is therapeutically useful in smoke-induced pulmonary arterial remodeling and PAH and the possible participation of ACE2 in the mechanism.

Materials and methods

Animals. Male Sprague-Dawley (SD) rats weighing 200 to 250 g (supplied by Experimental Animal Center, West China Hospital, Sichuan University) were used. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Sichuan University (Chengdu, China).

Experimental design. Rats were exposed to the whole smoke of 15 commercial, nonfilter cigarettes (Five Oxen, Chengdu Cigarette Factor, China, per cigarette contains nicotine 1 mg and tar 14 mg) in ventilated whole-body smoking chambers (70 cm × 50 cm × 50 cm) for 30 minutes each time, twice per day for up to 6 months as previously described (Ou et al., 2009) with minor modifications. The smoke total particulate matter (TPM) concentration inside the exposure chambers was 250 ± 26 mg/m³, determined by gravimetric analysis of filters at the exhaust port for the duration of the exposure. The control groups were exposed to fresh air under similar conditions. All rats were treated once a day, by oral gavage, with either saline or 10 mg/kg losartan or 30 mg/kg losartan 0.5 hour before the first smoke exposure each day.

Hemodynamic analysis. After an observation of 6 months, rats were anaesthetized with pentobarbitone (50 mg/kg i.p.) and were placed in a supine position, breathing room air. The right ventricular systolic pressure (RVSP) was measured as previously described (Ferreira et al., 2009). The cannula, filled with heparin-saline solution (50 U/ml), was gently introduced through the right external jugular vein down to the right ventricle. The RVSP was recorded using a miniature liquid pressure transducer (Biopac System Inc., USA) and a computerized data acquisition system (MP150, Biopac System Inc., USA). Data was obtained from steady-state waveform for 5 minutes.

Histological analysis and tissue preparation. After the RVSP measurement, all rats were killed by exsanguination via external iliac artery and the lungs were harvested. The right lung was fixed in 4% polyformaldehyde (pH 7.4) overnight for paraffin embedding, sectioning, and histological staining. The left lung was dissected, and snap-frozen in liquid nitrogen, then stored at -80 °C for biochemical analysis.

The paraffin sections (4 μm thick) were stained with hematoxylin and eosin (H&E) and van Gieson's elastic stain. Assessment of vascular morphology was carried out as previously described (Kishi et al., 2006). Briefly, the medial wall thickness (MWT) in fully muscularized arteries with an external diameter of 50 to 100 μm was evaluated by calculating the percentage of medial wall thickness as (medial thickness × 2 / external diameter) × 100% along the shortest curvature. At least 10 muscular arteries per section were examined using Image Plus 5.0 System (Media Cybernetics, Silver Spring, USA) in a blinded fashion by a skilled investigator.

For the immunohistochemical detection of ACE2, the sections were stained with anti-rat ACE2 polyclonal antibody (Santa Cruz). Images were acquired by an optical microscope (H600, Nikon, Tokyo, Japan) fitted with a RT SPOT digital camera (Nikon, Tokyo, Japan).

Western blotting analysis of ACE2 and ACE in lung homogenates. Lung homogenates were prepared in lysis buffer, containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM NaF, 2 mM EDTA, 0.1% SDS, and a protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis, USA) as previously described (Wang et al., 2009). The protein concentration in the lung homogenate was determined by the bicinchoninic acid method (Pierce, Rockford, IL, USA). Equivalent amounts of protein samples (30 μg) were separated on 10% polyacrylamide gels and then transferred onto 0.45 μm polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The membranes were incubated with a 1:500 dilution of ACE2 polyclonal antibody and a 1:1000 dilution of ACE polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). Western blots were developed by enhanced chemiluminescence (Pierce, Rockford, IL) according to the manufacturer's instructions.

Measurement of Ang II levels. Tissue Ang II levels were measured by iodine-125 radioimmunoassay (RIA) using the Ang II RIA kit (Beijing North Institute of Biological Technology, Beijing, China) according to the manufacturer's instructions as previously described (Chen et al., 2002). Briefly, lung tissue was washed with cold saline, minced and heated in 0.1 M HCl at 100 °C for 10 minutes, and then homogenized. After centrifugation at 15,000 × g for 30 minutes, the supernatant was lyophilized and redissolved in 400 μl assay buffer, and the radioactivity was measured by a γ counter.

Measurement of ACE2 levels and cell proliferation in cultured primary pulmonary artery smooth muscle cells from control and smoke-exposed rats. Segments of pulmonary arteries were obtained from lungs of SD rats exposed to fresh air or cigarette smoke for 3 months or 6 months. Pulmonary artery smooth muscle cells (PASMCS) were isolated and cultured as previously described (Ogawa et al., 2005) with minor modifications. The arteries were excised and isolated, cut into small pieces and immersed in 10 ml of phosphate-buffered saline (PBS) with 0.1 mg of collagenase for 15 minutes. The endothelium and adventitia were gently removed. And the remainder of the vascular tissues was placed in culture plates. Cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. And cell immunostaining was performed to confirm that cells were smooth muscle cells with anti- α -smooth muscle actin (α -SMA) monoclonal antibody (NeoMarkers, Lab Vision, Fremont, CA, USA). For the detection of ACE2 in PASMCS, cells were lysed in the lysis buffer as described above in the ACE2 and ACE assay in lung homogenates. Equivalent

amounts of total protein (20 µg/sample) were analyzed by Western blot with ACE2 antibody.

Cell proliferation was assessed by [³H]-thymidine incorporation as previously reported (Ogawa et al., 2005). When PASMCS were cultured to reach to 90% confluence, cells were detached, seeded and grown for 16 hours at a first density of 10×10^5 cells/ml. Subsequently, cells were incubated in low-serum culture media (DMEM, 0.1% FBS) to be kept quiescent for 24 hours. Then, DX600 (0.1 µM), an ACE2 inhibitor purchased from Phoenix Pharmaceuticals (Belmont, CA, USA) was added to the culture media of the cells from cigarette smoke-exposed rats. In another 24 hours of incubation, cells were labeled with [³H]-thymidine at 1 mCi/ml for the last 3 hours. Then the cells were washed twice with ice-cold PBS, 5% trichloroacetic acid, and 95% ethanol, followed by lysis in 0.5 M NaOH. Finally, aliquots of cell lysates were neutralized with HCl, and the radioactivity was measured by a liquid scintillation counter.

Measurement of cell proliferation and ACE2 levels in cigarette smoke extract-challenged rat PASMCS. An extract of cigarette smoke in DMEM was prepared freshly for each experiment. Commercial, nonfilter cigarettes (the same cigarettes as used in Experimental design) were used. Cigarette smoke extract (CSE) was prepared as previously described (Oltmanns et al., 2005) with a few modifications. Briefly, cigarette smoke derived from one cigarette was drawn slowly into a 50-ml syringe and bubbled through 5 ml of DMEM. And one cigarette yielded 5 draws of 50 ml of the syringe, with each individual draw taking approximately 10 seconds to complete. The resulting solution, which represented “100%” strength, was then adjusted to pH 7.4 with concentrated NaOH and filtered before being diluted in DMEM to the required strength for application to PASMCS cultures.

Cell growth was assessed by Cell Counting Kit-8 (CCK-8, Dojindo, Tokyo, Japan). PASMCS from control rats were plated in 96-well microplates at a first density of 2×10^4 cells/ml per well. Before experiment, cells were incubated in low-serum culture media (DMEM, 0.1% FBS) to be kept quiescent for 24 hours. Then cells were left untreated or pretreated with 1 µM or 10 µM losartan for 30 minutes before exposure to 0%, 0.5%, 1%, 2%, 4%, or 8% CSE for 24 hours, respectively. After addition of 10 µl of the CCK-8 reagent and 2 hours of incubation, the plates were read at 450 nm in a Model 680 microplate reader (Bio-Rad Laboratories, USA).

All experiments were repeated at least three times, and each experimental condition was repeated at least in quintuplicate wells in each experiment. ACE2 levels were measured by Western blotting analysis as mentioned above.

Statistical analysis. Statistical analysis was carried out using either Student's *t* test (for two-group comparison) or one-way ANOVA (for multiple-group comparison) followed by the least significant difference (LSD) test (SPSS for Windows version 13.0, Chicago, USA). A value of $P < 0.05$ was considered statistically significant. Values were expressed as means \pm SD.

Results

Losartan treatment attenuated smoke-induced increases in RVSP and pulmonary artery remodeling in rats

To determine the effect of chronic cigarette smoking and losartan treatment on rat pulmonary arteries, we first examined the histopathology and hemodynamics of rat lungs. The percent medial wall thickness (%MWT) of pulmonary arteries, which is a marker of pulmonary arterial remodeling, was calculated. As presented in Figs. 1A and B, thick-walled pulmonary arteries with apparent medial hypertrophy were observed in smoke-exposed rats as compared to the normal pulmonary vascular structure in control rats. In addition,

hemodynamic assessment shows a notable rise in RVSP after a 6-month smoke exposure in rats ($P < 0.05$, Fig. 2). In contrast, medication with losartan apparently reduced the smoke-induced increase in pulmonary arterial medial thickness and RVSP elevation, suggesting that losartan administration may attenuate pulmonary artery remodeling and PAH induced by chronic cigarette smoke exposure in rats.

Ang II accumulation induced by cigarette smoke exposure and the effect of losartan treatment

Activated RAS and increased Ang II levels have been detected in hypoxic and monocrotaline-treated pulmonary hypertensive rats (Chassagne et al., 2000; Ferreira et al., 2009). To investigate whether Ang II accumulation also exists in smoke-induced PAH, we measured the Ang II concentration in rat lungs after a 6-month smoke exposure using RIA. As illustrated in Fig. 3, chronic smoke exposure induced a marked increase in Ang II levels in the lung, which was significantly inhibited by losartan treatment ($P < 0.05$).

Increased ACE and decreased ACE2 in smoke-induced PAH and the effect of losartan treatment

To further examine the mechanism for Ang II accumulation in smoke-induced PAH in rats, we examined the ACE and ACE2 levels in smoke-exposed rats. Western blotting analysis for ACE and ACE2 protein expression and ACE2 immunohistochemical staining were performed. Immunohistochemistry results showed a notable decrease of ACE2 positive staining in lung sections in the smoke-exposed rats as compared to the control rats (Fig. 4A). Densitometric analysis of Western blotting bands indicates that smoke exposure induced a significant elevation in ACE but a decrease in ACE2 levels in lung tissue (Figs. 4B and C). Losartan administration in rats significantly increased the abundance of the band identified by ACE2 antibody, while no significant difference was found in ACE expression between the smoke-exposed group and the group treated with smoke plus losartan. The results imply that losartan could partially reverse the smoke-induced ACE2 reduction but had no effect on ACE protein expression.

Smoke exposure decreased ACE2 levels in rat primary PASMCS

To compare the expression levels of ACE2 in rat primary PASMCS from the smoke-exposed group and from the control group, cell lysis samples were used for Western blotting analysis. The results showed that ACE2 protein levels were significantly decreased in the PASMCS from the smoke-exposed rats ($P < 0.05$ vs Con), especially from the 6-month exposed rats (Figs. 5A and B), which was consistent with the findings of ACE2 expression in lung homogenates in our *in vivo* study.

Smoke exposure and ACE2 inhibition promoted rat PASC proliferation

Enhanced proliferation of vascular smooth muscle cells has been reported in PAH patients (Yi et al., 2000). Since PASC proliferation is a major contributor and an important characteristic of PAH, rat primary PASMCS were isolated, and cell proliferation was evaluated by [³H]-thymidine incorporation *in vitro*. As shown in Fig. 5C, the [³H]-thymidine incorporation rates in PASMCS from the smoke-exposed rats, especially from 6-month exposed rats, were significantly higher than that from the control group, implying that smoke exposure may simulate PASC proliferation in rat lungs. Furthermore, cells proliferated even more rapidly in the presence of DX600, an ACE2 inhibitor, implying a possible involvement of ACE2 in the proliferation of PASMCS. There was no significant difference in [³H]-thymidine incorporation between the PASMCS

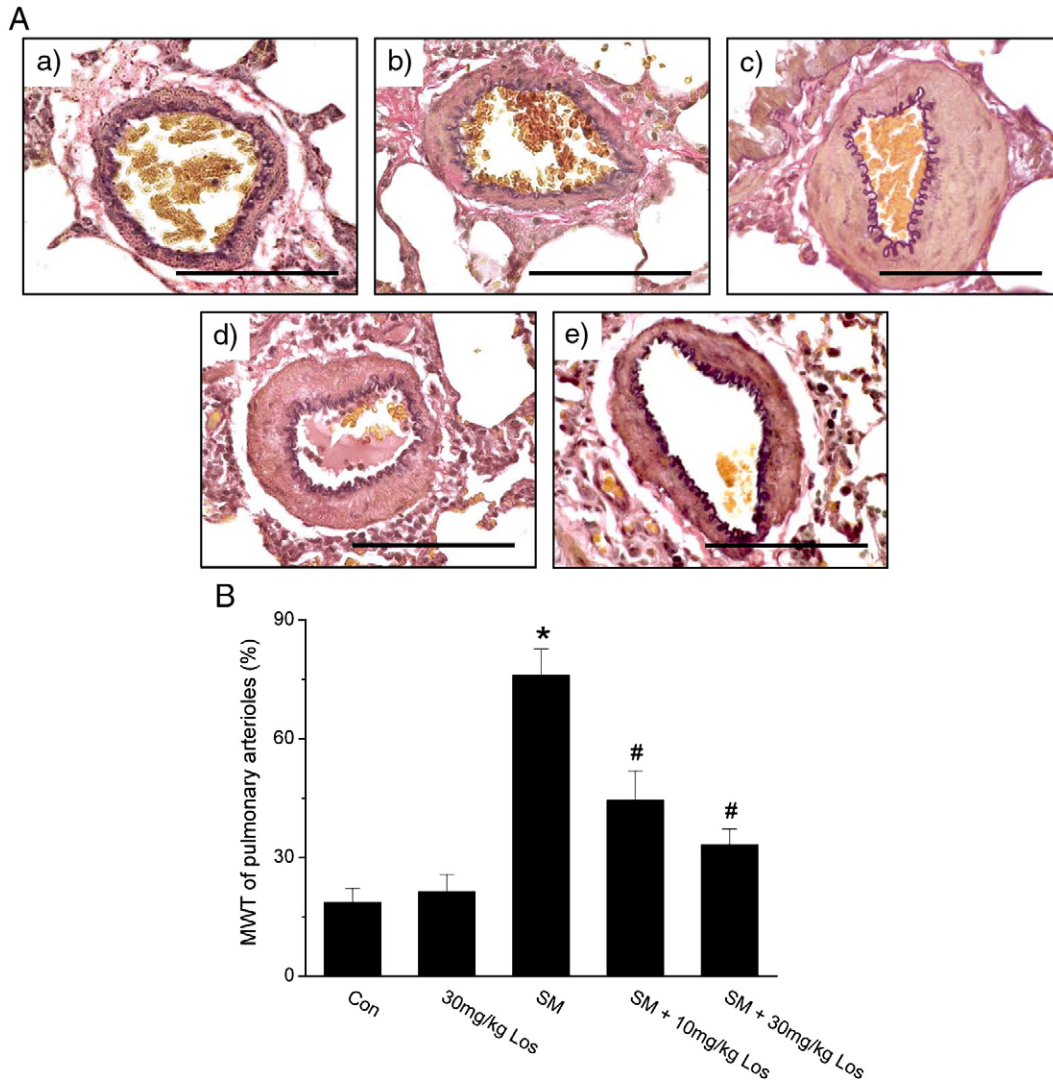


Fig. 1. Light microscopic determinations of pulmonary arterial remodeling in lung sections stained with elastic van Gieson. (A) Representative morphologic images of pulmonary arteries in rats treated with a) control (Con), b) 30 mg/kg losartan-only (30 mg/kg Los), c) smoke-only (SM), d) smoke plus 10 mg/kg losartan (SM + 10 mg/kg Los), and e) smoke plus 30 mg/kg losartan (SM + 30 mg/kg Los). Original magnification, $\times 60$. Scale bars = 100 μm . (B) Medial wall thickness (MWT) of pulmonary arterioles. Values are expressed as mean \pm SD ($n = 4-5$). * $P < 0.05$, as compared with the Con group; # $P < 0.05$, as compared with the SM group.

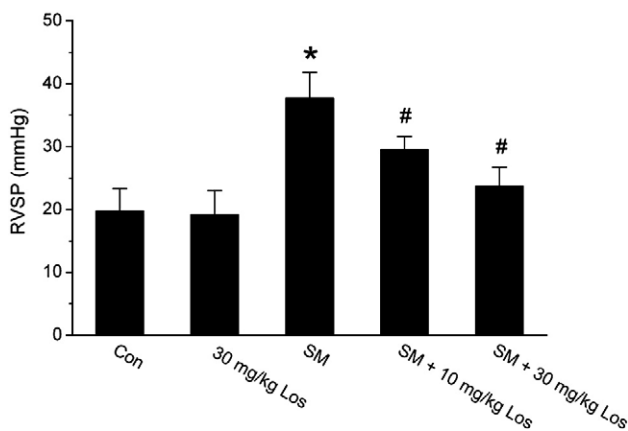


Fig. 2. Losartan inhibits the smoke-induced elevation of right ventricular systolic pressure (RVSP) in rats. Values are expressed as mean \pm SD ($n = 4-5$). * $P < 0.05$, as compared with the Con group; # $P < 0.05$, as compared with the SM group.

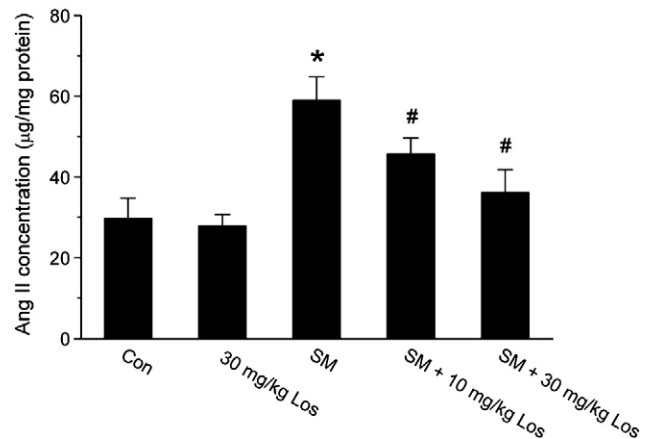


Fig. 3. Losartan reduced smoke-induced Ang II increase in rat lungs. Ang II levels were measured by iodine-125 radioimmunoassay as described in Materials and methods. Values are expressed as mean \pm SD ($n = 4-5$). * $P < 0.05$ vs Con; # $P < 0.005$ vs SM.

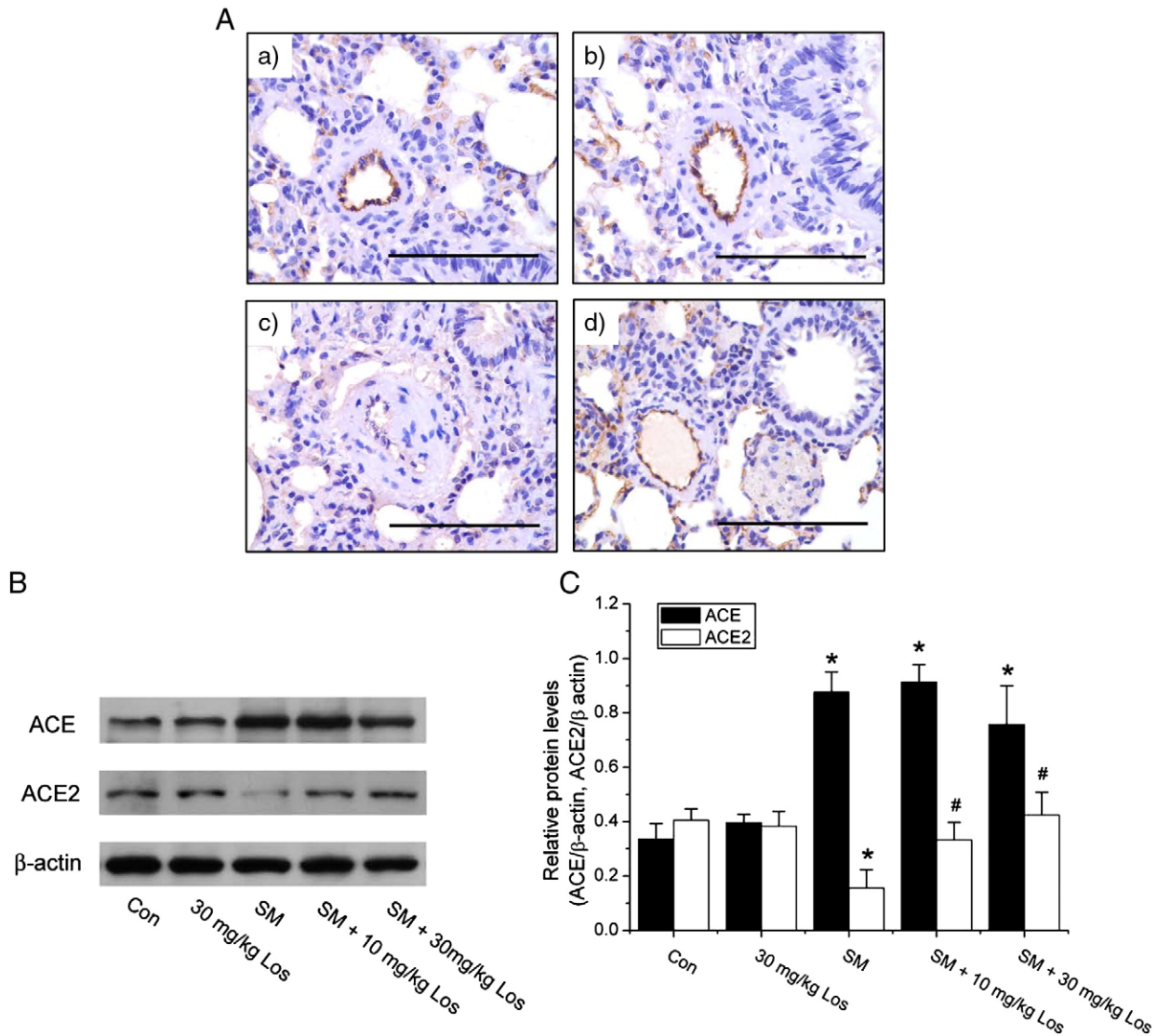


Fig. 4. Measurement of ACE2 and ACE protein levels in rat lungs ($n=4-5$). (A) Immunohistochemistry for ACE2 in rat lung sections from a) Con, b) 30 mg/kg Los, c) SM, and d) SM + 30 mg/kg Los groups (original magnification, $\times 60$). Scale bars = 100 μm . (B) Western blotting analysis for ACE2 and ACE protein in lung tissue. Images are representative of three independent experiments. (C) Semiquantification of ACE (solid bars) and ACE2 (open bars) levels by densitometry. * $P<0.05$, as compared with the Con group; # $P<0.05$, as compared with the SM group.

from 3-month air-exposed rats and those from 6-month air-exposed rats.

Effect of losartan treatment on CSE-induced cell proliferation and ACE2 reduction in rat PSMCs

To investigate the effect of CSE on cell proliferation of PSMCs in vitro, PSMCs from control rats were challenged with 0%, 0.5%, 1%, 2%, 4%, or 8% CSE and cell proliferation was evaluated. As shown in Fig. 6A, CSE at concentrations of 0.5%, 1%, and 2% caused a significant increase in cell number compared with the control samples ($P<0.05$), and the peak increase in PASM cell viability was observed at a concentration of 2% CSE. In contrast, CSE at higher concentrations ($>4\%$) appeared to be toxic to PASM cell.

Based on the cell proliferation results, the effects of losartan on cell proliferation and ACE2 expression in 2% CSE-challenged PSMCs were evaluated. As shown in Fig. 6B, 10 μM losartan pretreatment significantly inhibited 2% CSE-induced PASM cell proliferation. In addition, 2% CSE also reduced ACE2 protein levels in PSMCs, which could be partially reversed by 10 μM losartan pretreatment ($P<0.05$ vs. CSE; Figs. 6C and D). These results were consistent with our

findings in the in vivo study that losartan treatment may attenuate chronic smoking-induced pulmonary artery remodeling and ACE2 reduction in rat lungs.

Discussion

The present study mainly demonstrated that chronic cigarette smoke exposure significantly increased rat pulmonary arterial wall thickness and RVSP, along with an increase in Ang II and a decrease in ACE2 levels. Losartan treatment markedly reduced the pulmonary artery remodeling and RVSP elevation caused by cigarette smoke exposure and partially reversed the smoke-induced ACE2 decrease and Ang II elevation in rat lung. Consistent with our in vivo study results, in PSMCs from smoke-exposed rats, ACE2 levels were significantly lower than in those from the control rats in an exposure time-dependent manner. Moreover, PSMCs from 6-month exposed rats proliferated more rapidly than those from 3-month exposed or control rats, and cells grew even more rapidly by the ACE2 inhibition with DX600. In cultured PSMCs from control rats, in vitro CSE challenge could also stimulate cell proliferation and reduce ACE2 levels, which could be significantly inhibited by losartan pretreatment.

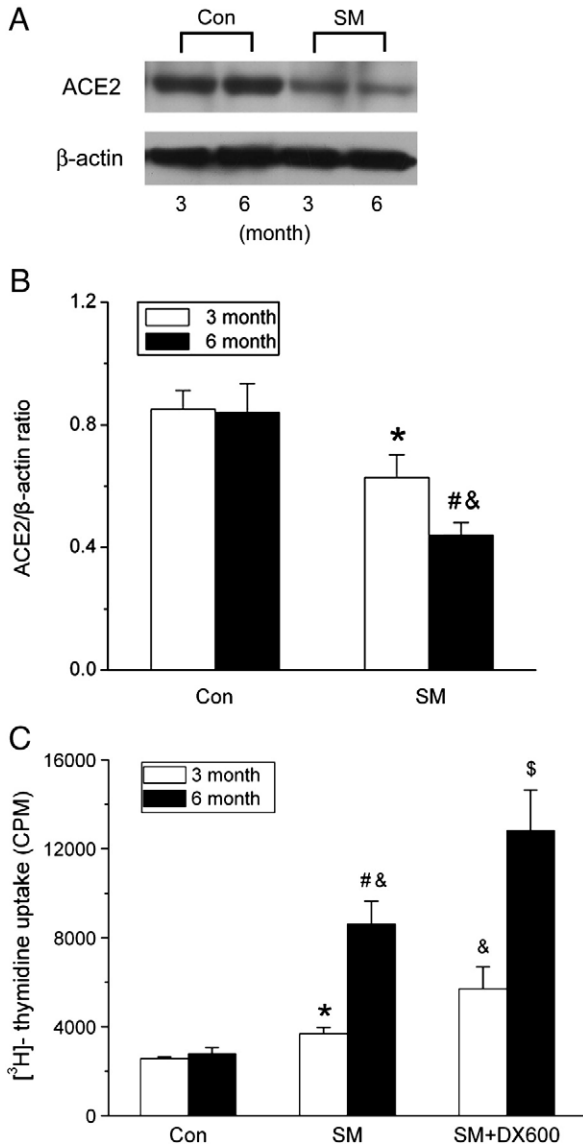


Fig. 5. ACE2 levels in cultured rat PASMCs and measurement of PASMC proliferation in vitro ($n=3$). (A) Western blotting analysis for ACE2 in PASMCs from control rats (Con), 3-month smoke-exposed rats (SM, 3 month), and 6-month smoke-exposed rats (SM, 6 month) rats. Images are representative of three independent experiments. (B) Semiquantification of ACE2 levels by densitometry (ACE2/ β -actin ratio). (C) Measurement of cell proliferation rate by [3 H]-thymidine incorporation. Values are presented as mean counts per minute (cpm) of triplicate samples. DX600 (0.1 μ M), an ACE2 inhibitor was added to the culture media of the cells from SM rats. Open bars: PASMCs from rats exposed to fresh air or cigarette smoke for three months (3 month); solid bars: PASMCs from rats exposed to fresh air or cigarette smoke for 6 months (6 month). * $P<0.05$, as compared with the Con group (3 month); # $P<0.05$, as compared with the Con group (6 month); & $P<0.05$, as compared with SM group (3 month); \$ $P<0.05$, as compared with the SM group (6 month).

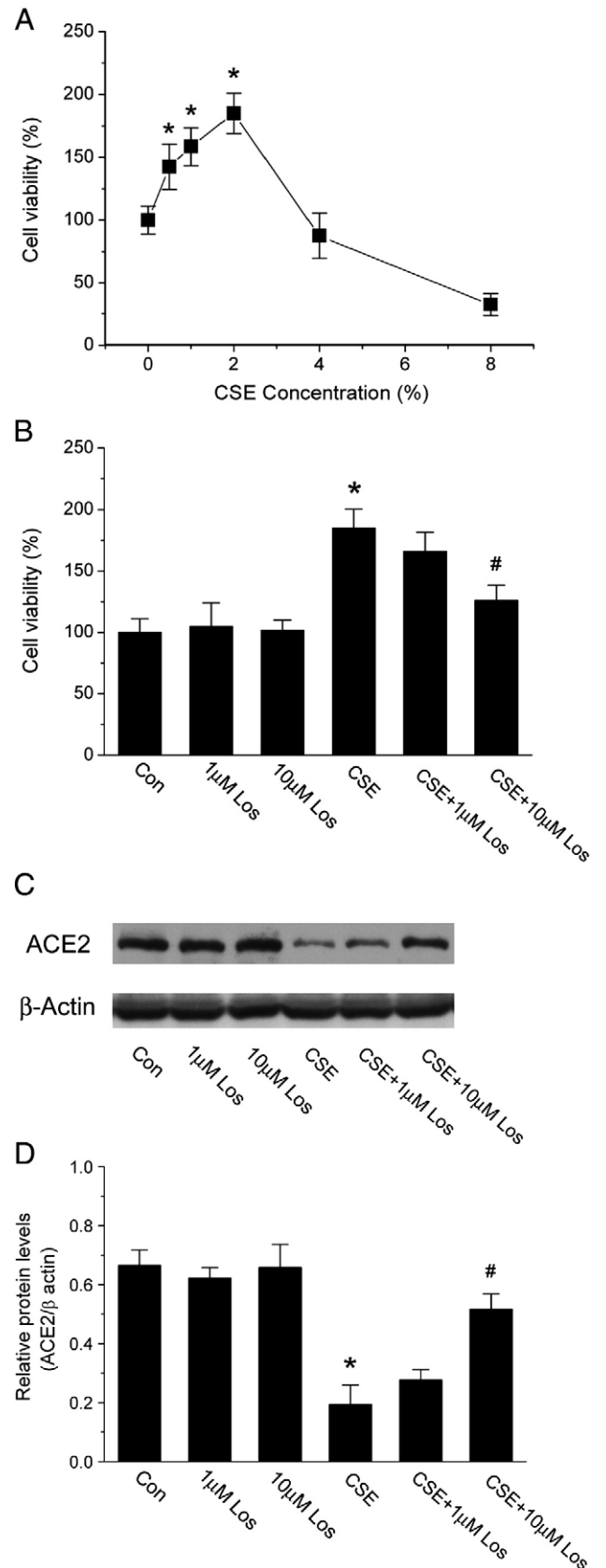


Fig. 6. Inhibitory effect of losartan treatment on cigarette smoke extract (CSE)-induced cell proliferation and ACE2 reduction in rat PASMCs. (A) For determination of cell number by CCK-8, PASMCs were treated with different concentration of CSE (0%, 0.5%, 1%, 2%, 4%, and 8%). Each experimental condition was repeated at least in quintuplicate wells in each experiment. Values are expressed as mean \pm SD. (B) Inhibitory effect of losartan pretreatment on 2% CSE-induced rat PASMC proliferation. (C) Western blotting analysis for ACE2 in 2% CSE-challenged PASMCs. Images are representative of three independent experiments. (D) Semi-quantification of ACE2 levels by densitometry (ACE2/ β -actin ratio). Con: control; Los: losartan; CSE: 2% cigarette smoke extract. * $P<0.05$, as compared with the Con; # $P<0.05$, as compared with the CSE.

Thus, our results suggest that losartan may have a therapeutic role in the smoking-induced pulmonary artery remodeling and PAH, in which ACE2 may serve as part of the mechanism.

It has been reported that guinea pigs exposed to cigarette smoke for 6 months produced an increase in pulmonary arterial pressure and enhanced muscularization of small pulmonary arteries (Wright et al., 2003, 2006). Similarly, elevated mean pulmonary arterial pressure and increased muscularized pulmonary vessels have also been detected in rats after 16-week cigarette smoke exposure (Lee et al., 2005). Consistent with these findings, in the present study, 6 months of smoke exposure induced a marked medial wall thickening in rat pulmonary arteries and significantly increased RVSP in rats. These results suggest that chronic cigarette smoking may directly lead to pulmonary artery remodeling and PAH.

Recently, it has been found that both the expression and activity of ACE in human endothelial cells can be increased after the exposure to nicotine, an important component of cigarette smoke (Saijonmaa et al., 2005; Ljungberg & Persson, 2008). Since ACE plays a key role in converting Ang I into Ang II in the RAS, we hypothesized that cigarette smoke exposure may increase Ang II production in vivo by activating the RAS. In the present study, we found a significant elevation in ACE expression and an almost two-fold increase in Ang II levels in the lungs from the PAH rats exposed to cigarette smoke for 6 months as compared to those from the control rats. Since Ang II is believed to play an important role in PAH via binding to AT1 receptors (Jeffery & Wanstall, 2001), our results suggest that activated RAS may also be involved in the smoke-induced pulmonary artery remodeling and PAH.

Losartan is a specific Ang II antagonist that directly blocks AngII at the AT1 receptor. Therapeutic effects of losartan on PAH in hypoxia rats and shunted piglets have been reported (Rondelet et al., 2005). Our study showed that losartan treatment effectively suppressed pulmonary artery medial wall thickening and RVSP elevation in smoke-exposed rats as well as the CSE-induced cell proliferation in rat PSMCs. The inhibitory effect of losartan may be due to its property of directly blocking the AT1 receptor, which, as we speculate, might result in the accumulation of Ang II via an undetermined mechanism. But interestingly, in the rats treated with smoke exposure plus losartan administration, we observed a significant decrease in Ang II levels in the lung as compared to the rats exposed to smoke alone, implying the existence of additional potential mechanisms for losartan-attenuated PAH in the smoke-induced rat model. In other words, losartan can not only inhibit the effect of Ang II but may also decrease Ang II levels in cigarette smoking-induced PAH.

In the RAS, ACE cleaves Ang I to generate the potent vasoconstrictor Ang II, whereas ACE2 hydrolyses Ang II to inactive Ang (1–7) and is a negative regulator of the system. In the present study, we found a significant decrease in ACE2 levels in the cigarette smoke-induced PAH rats and in the cultured CSE-challenged rat PSMCs, which could be partially restored by losartan treatment. On the contrary, losartan had no effect on the smoke-induced ACE elevation. In a previous study, Ferrario et al. (2005a) also found that losartan treatment (10 mg/kg) showed no effect on plasma ACE activity in normotensive Lewis rats. Similarly, in another study in Wistar rats with experimental congestive heart failure (CHF), ACE activity in the myocardial homogenate did not differ between the treatment with and without eprosartan (another AT1 receptor antagonist) administration (Karram et al., 2005). Indeed, several studies have demonstrated that losartan can efficiently increase both the expression and activity of ACE2 in rat heart or human kidney (Koka et al., 2008; Xia et al., 2009). Increased ACE2 may augment the conversion of Ang II into Ang (1–7), leading to the decrease in Ang II concentration. Thus, these findings suggest that losartan treatment may have more influence on ACE2 than on ACE, and the reduction of Ang II in the attenuation of losartan-treated PAH is probably due to the losartan-induced elevation in ACE2 levels. As a

result, the stimulation of the AT1 receptor in response to Ang II is reduced, which may also contribute to the attenuation of smoke-induced PAH by losartan treatment.

There is now growing evidence to suggest that ACE2 may play an important part in PAH. It has been found that ACE2 plays a protective role but its expression is decreased in human and experimental pulmonary fibrosis (Li et al., 2008). In a very recent study, chronic treatment with XNT, a synthetic activator of ACE2, prevented the elevation of RVSP, right ventricular hypertrophy, and thickness of pulmonary vessel wall in monocrotaline-induced PAH rat model (Ferreira et al., 2009). In our present study, we found that ACE2 protein expression was significantly decreased in the lung along with apparent pulmonary arterial remodeling and PAH in the 6-month smoke-exposed rats. Moreover, in our in vitro study, Western blotting results also showed that ACE2 was markedly decreased in the PSMCs from the smoke-exposed rats compared with those from the control rats. In addition, ACE2 inhibition with DX600 significantly increased PSMC proliferation rate above the level induced by smoke exposure alone, indicating a possible involvement of ACE2 in the smoke-induced growth of PSMCs. There is recent evidence that Ang II downregulates ACE2 via AT1 receptor-mediated ERK/p38 MAP kinase signaling pathway (Koka et al., 2008), therefore the decrease in ACE2 protein expression induced by chronic cigarette smoke exposure may be mediated by a mechanism dependent on Ang II and its receptors. The detailed mechanism itself warrants further study.

In summary, chronic cigarette smoke exposure significantly induced pulmonary artery wall thickening, RVSP elevation, as well as Ang II accumulation and ACE2 decrease in rat lungs. Losartan administration effectively attenuated the smoke-induced pulmonary arterial remodeling and PAH, reduced Ang II elevation, and reversed ACE2 reduction. ACE2 inhibition with specific inhibitor in vitro further increased rat PSMC proliferation induced by smoke exposure. Similarly, losartan treatment significantly suppressed CSE-induced cell proliferation and ACE2 decrease in cultured rat primary PSMCs. These results suggest that losartan may attenuate the chronic smoking-induced pulmonary vascular remodeling and PAH in which ACE2 may serve as part of the mechanism. This study might provide insight into the development of new therapeutic interventions for PAH smokers.

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