Angiotensin II plays a critical role in diabetic pulmonary fibrosis most likely via activation of NADPH oxidase-mediated nitrosative damage

Junling Yang,1,2† Yi Tan,2,3† Fenglian Zhao,1 Zhongsen Ma,1 Yuehui Wang,1 Shirong Zheng,2 Paul N. Epstein,2 Jerry Yu,4 Xia Yin,2,5 Yang Zheng,5 Xiaokun Li,3 Lining Miao,1* and Lu Cai2,3,4*

1Department of Pulmonary Medicine, Second Hospital of Jilin University, Changchun, China; 2Pediatric Diabetes Research at Kosair Children’s Hospital Research Institute, Department of Pediatrics, University of Louisville, Louisville, Kentucky; 3Chinese-American Research Institute for Diabetic Complications, Wenzhou Medical College, Wenzhou, China; 4Department of Medicine, University of Louisville, Louisville, Kentucky; and 5Center of Cardiovascular Diseases, First Hospital of Jilin University, Changchun, China

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Yang J, Tan Y, Zhao F, Ma Z, Wang Y, Zheng S, Epstein PN, Yu J, Yin X, Zheng Y, Li X, Miao L, Cai L. Angiotensin II plays a critical role in diabetic pulmonary fibrosis most likely via activation of NADPH oxidase-mediated nitrosative damage. Am J Physiol Endocrinol Metab 301: E132-E144, 2011. First published April 12, 2011; doi:10.1152/ajpendo.00629.2010.—Diabetic patients have a high risk of pulmonary disorders that are usually associated with restrictive impairment of lung function, suggesting a fibrotic process (van den Borst B, Gosker HR, Zeegers MP, Schols AM. Chest 138: 393–406, 2010; Ehrlich SF, Quesenberry CP Jr, Van Den Eeden SK, Shan J, Ferrara A. Diabetes Care 33: 55–60, 2010). The present study was undertaken to define whether and how diabetes causes lung fibrosis. Lung samples from streptozotocin-induced type 1 diabetic mice, spontaneously developed type 1 diabetic OVE26 mice, and their age-matched controls were investigated with histopathological and biochemical analysis. Signaling mechanism was investigated with cultured normal human lung fibroblasts in vitro. In both diabetes models, histological examination with Sirius red and hematoxylin and eosin stains showed fibrosis along with massive inflammatory cell infiltration. The fibrotic and inflammatory processes were confirmed by real-time PCR and Western blotting assays for the increased fibronectin, CTGF, PAI-1, and TNFα mRNA and protein expressions. Diabetes also significantly increased NADPH oxidase (NOX) expression and protein nitration along with upregulation of angiotensin II (Ang II) and its receptor expression. In cell culture, exposure of lung fibroblasts to Ang II increased CTGF expression in a dose- and time-dependent manner, which could be abolished by inhibition of superoxide, NO, and peroxynitrite accumulation. Furthermore, chronic infusion of Ang II to normal mice at a subpressor dose induced diabetes-like lung fibrosis, and Ang II receptor AT1 blocker (losartan) abolished the lung fibrotic and inflammatory responses in diabetic mice. These results suggest that Ang II plays a critical role in diabetic lung fibrosis, which is most likely caused by NOX activation-mediated nitrosative damage.

nicotinamide adenine dinucleotide phosphate; lung fibrosis; diabetes mellitus; pulmonary nitrosative damage; lung inflammation; connective tissue growth factor

Diabetic complications have threatened diabetic patients by damaging many organs. Currently, the diabetes-targeted organs that have been studied extensively include mainly the kidney, heart, eye, liver, and skin (3, 49). In fact, the other organs are also subjected to similar damage. One such organ is the lung, which has not been well addressed for many years (36, 42). To recognize whether diabetes can induce lung dysfunction is very important since lung dysfunction is an independent cause for fatal cardiovascular diseases (46); therefore, diabetic induction of lung dysfunction may accelerate other diabetic cardiovascular diseases.

Whether pulmonary dysfunction is related to diabetes was discussed more than 20 years ago (36). The pulmonary dysfunction was often observed in both pediatric and adult diabetic patients and both type 1 and type 2 diabetes (17, 36, 41, 42). The pulmonary function abnormalities observed in these diabetic patients included the reduced forced vital capacity (FVC), total lung capacity (TLC), transfer factor for carbon monoxide (Tlco), and single breath pulmonary diffusion capacity for carbon monoxide (DLco/VA) but not forced expired volume in 1 s (FEV1) (17, 36, 41, 42). In a study by Schnack et al. (37), for instance, the authors performed pulmonary function tests in long-standing type 1 diabetic patients (n = 39) with normal serum creatinine levels (<1.3 mg/dl) and compared these tests with those of healthy controls (n = 44). The diabetic patients were further divided into two groups, patients with a normal urinary albumin excretion rate (AER; <30 mg/day; n = 21) and patients with microalbuminuria (AER = 30–300 mg/day; n = 18). Compared with controls, a significant reduction of the FVC (83.7% predicted, P < 0.001 in diabetic patients with microalbuminuria; and 92.2% predicted, P < 0.03 in patients without microalbuminuria), along with a significant reduction of TLC (83.4% predicted, P < 0.04 only in the patients with microalbuminuria), was observed in diabetic patients. This finding suggested that pulmonary dysfunction and in particular restrictive pulmonary defects were observed predominantly in patients with incipient diabetic nephropathy (37). More recent epidemiological studies from Korea (Refs. 16 and 30) based on 4,001 and 35,456 subjects, respectively (age ≥18 yr), who underwent spirometry analyzed the association of low pulmonary function with metabolic syndrome components or type 2 diabetes. They found that lower pulmonary function and restrictive lung disease were negatively associated with metabolic risk factors or type 2 diabetes in the Korean population. Consistent with the Korean findings, a recent retrospective longitudinal cohort study that used the electronic records of a large health plan in Northern California (n = 1,811,228) also reached the conclusion that the lung restrictive dysfunction and fibrosis were significantly enhanced in the patients with diabetes (9). More importantly, Van den Borst et al. (41) performed
a meta-analysis that included 40 studies with 3,182 diabetic patients and 27,080 controls and showed that restrictive lung dysfunction was the major pattern in either type 1 or type 2 diabetic patients.

From the histopathological view, Vracko et al. (43) reported the increased thickness of the epithelial and capillary basal lamina (BL) of alveoli in diabetic patients compared with age-matched control subjects. The thickness of both types of BL in the lungs correlates significantly with thickness of BL in renal tubules and muscle capillaries, although the BL deposits are five to 10 times greater in muscle capillaries and in renal tubules than that in the lungs. Later, Weynand et al. (48) examined autopsied lung and kidney samples from six diabetic and six control subjects for the thickening of alveolar epithelial BL, endothelial capillary BL, or both fused BL. All alveolar epithelial BL, endothelial capillary BL, and both BL were increased significantly in lungs from diabetic patients compared with those from controls. Unlike the study by Vracko et al. (43), they found the same magnitude of BL thickening between lung and kidney. Through measurements on alveolar capillary walls, the pulmonary arteriolar walls, and the alveolar walls in the autopsied lungs from 35 diabetic patients and 26 nondiabetic groups, increased thickness indicating lung fibrosis was also observed by a Japanese group (22). In addition, Farina et al. (10) examined the histopathological changes in the lung by autopsies of 61 diabetic and 50 nondiabetic patients. They found a type of nodular fibrosis along with the infiltration of inflammatory cell, excessive deposition of collagen and extra-cellular matrix, and impairment of alveolar-capillary barrier basement membrane in the lungs of diabetic patients. It is known that the loss of the alveolar-capillary barrier basement membrane integrity is critical in determining the point of no return, which leads to the promotion of fibrosis (39); therefore, these human data have shown the induction of the increased thickness of alveolar epithelial BL, endothelial capillary BL, pulmonary arteriolar walls, and alveolar walls as well as nodular fibrosis of the lung in diabetic patients. However, whether the lung fibrosis observed in diabetic patients is caused by diabetes or is secondary to other diabetic complications such as cardiac dysfunction remains unknown.

Angiotensin II (Ang II), a key component of the renin-angiotensin system, modulates arterial pressure, regulates blood volume, and promotes growth and proliferation through the activation of multiple signaling mechanisms (1). However, action of Ang II may go beyond its influence on blood pressure. For example, Ang II acts via its receptor angiotensin type 1 (AT1) to activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) and causes a wide range of pathogenic processes related to diabetic complications, including the induction of inflammation and fibrosis in the hearts and kidneys (1, 23, 54). In addition to the system-wide activation, Ang II and AT1 are stimulated in the lung under a variety of conditions, suggesting its importance in these pulmonary pathogenesis (21, 28, 44, 50).

The present study was undertaken to determine whether diabetes directly causes lung fibrosis and to investigate its underlying mechanisms with in vivo and in vitro approaches. To eliminate confounding factors, we used animal models at the time point without significant cardiac dysfunction, i.e., 2 or 3 mo after diabetes onset (5, 19).

**METHODS**

**Animal Models**

FVB and OVE26 mice were housed in the University of Louisville Research Resources Center at 22°C with a 12:12-h light-dark cycle and provided free access to standard rodent chow and tap water. All animal procedures were approved by the Institutional Animal Care and Use Committee at University of Louisville, which is certified by the American Association for Accreditation of Laboratory Animal Care. Total lungs were harvested for protein, mRNA, and histopathology from the following animal studies.

**Streptozotocin-induced diabetic mice.** FVB mice were injected intraperitoneally with multiple low doses of streptozotocin (STZ; Sigma) at 50 mg/kg daily for 5 days to induce type 1 diabetes. Five days after the last injection of STZ, whole blood glucose obtained from mouse tail vein was measured with a SureStep complete blood glucose monitor (LifeScan, Milpitas, CA). The blood glucose levels >250 mg/dl were accepted as diabetes. Since we have demonstrated the lack of significant cardiac dysfunctions at 2–3 mo of diabetes (5, 19), lung samples from diabetic and their age-matched controls were collected at 3 mo after diabetes onset for the histopathological and biochemical analysis. In addition, some of the diabetic mice were immediately treated after diabetes onset by gavage with Ang II receptor AT1 blocker (Losartan; Sigma-Aldrich) at 10 mg/kg body wt daily for 2 mo. OVE26 mice. To exclude direct effect of STZ to induce lung fibrosis, we also examined spontaneously developed type 1 diabetic OVE26 mice. These mice were genetically engineered to carry a calmodulin minigene regulated by the rat insulin II promoter, with a fivefold increase in the content of calmodulin in β-cells (20, 38, 53). These OVE26 mice normally develop severe diabetes around 2 wk after birth, and they develop typical features of diabetic nephropathy and cardiomyopathy at 5 mo or older (20, 38, 53). Animals were euthanized at 3.5 mo after the onset of diabetes.

**Ang II infusion mice.** To determine whether Ang II was able to induce pulmonary fibrosis, Ang II (freshly prepared in 0.9% NaCl; Sigma-Aldrich) was given to 8- to 10-wk-old FVB male mice subcutaneously at suppressor doses (0.5 mg/kg) every other day for 2 mo.

**Echocardiography**

Under sedation with Avertin (2,2,2-tribromoethanol; 240 mg/kg IP), two-dimensional and M-mode echocardiography were performed using a VisualSonics Vevo 770 high-resolution imaging system and a RMV-707B transducer (focal length 12.7 mm, frequency 30.0 MHz). The parasternal long-axis view and parasternal short-axis view were used to measure the following systolic end points: anterior and posterior wall thickness, interventricular septum thickness, left ventricular internal dimension, and left ventricular volume at systole. Left ventricular fractional shortening was assessed, and LV mass was also calculated as described previously (54).

**Cell culture**

Normal human lung fibroblasts CCL-190 were purchased from American Type Cell Collection (Manassas, VA) and cultured in Ham’s F-12K medium with 2 mmol/l l-glutamine adjusted to contain 1.5 g/l sodium bicarbonate supplemented with 15% fetal bovine serum. The fibroblasts in all of the experiments were in passages between 11 and 15. The effects of different doses of Ang II exposure (50, 100, and 200 mmol/l) for 24 h and different Ang II exposure times (8, 16, and 24 h) at 100 nmol/l on connective tissue growth factor (CTGF) expression were examined initially. In the following studies, exposure to Ang II for 24 h at 100 mmol/l was used. Effects of superoxide, nitric oxide, and peroxynitrite accumulation on Ang II-induced CTGF were defined with their corresponding inhibitors or scavengers. Cells were pretreated with 100 μmol/l peroxynitrite
scavenger Urate (Sigma Chemical, St. Louis, MO), 50 μmol/l cell-permeable superoxide dismutase mimetic Mn(II)tetakis(1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP; cat. no: 475872; Calbiochem, San Diego, CA), 100 μmol/l NOX inhibitor apocynin (Calbiochem), and 100 μmol/l nitric oxide synthase inhibitor N-nitro-l-arginine methyl ester (l-NAME; Sigma Chemical) for 1 h and then continually exposed to Ang II for 24 h based on our previous studies (54).

Real-time PCR. Total RNA was extracted from lung tissues using the RNeasy Micro Scale RNA Isolation Kit (Applied Biosystems). RNA concentration and purity were quantified using a Nanodrop ND-1000 spectrophotometer (Biolab). First-strand complimentary DNA (cDNA) was synthesized from total RNA according to the RNA PCR kit (Promega, Madison, WI), following the manufacturer’s protocol. Reverse transcription was performed using 1 μg of total RNA in 12.5 μl of the following solution: 4 μl 10 MgCl₂, 4 μl 10 RT buffer, 2 μl dNTP, 0.5 μl RNase inhibitor, 1 μl of AMV reverse transcriptase, 1 μl of oligo dT primer, and the addition of nuclear-free water to a final volume of 20 μl. Reaction system was run at 42°C for 50 min and 95°C for 5 min. Primers [CTGF: Mmu192933_g1; tumor necrosis factor-α (TNFα): Mmu00443258_m1; AT1: Mmu00616371_m1; plasminogen activator inhibitor-1 (PAI-1): Mmu00435858_m1; β-actin: Mmu0607939_s1] for PCR were performed with Applied Biosystems. The housekeeping gene β-actin was used as a reference. Real-time RT-PCR [quantitative PCR (qPCR)] was carried out in a 20-μl reaction buffer that included 10 μl of TaqMan Universal PCR Master Mix, 1 μl of primer, 6 μl of cDNA, and 3 μl ddH₂O and performed in triplicate for each sample in the ABI 7300 Real-Time PCR system. The fluorescence intensity of each sample was measured at each temperature change to monitor amplification of the target gene. The comparative cycle time (CT) method was used to determine fold differences between samples. The comparative CT method determined the amount of target normalized to an endogenous reference (β-actin) and relative to a calibrator (2^(-ΔΔCT)).

Histopathology

Lung tissue was fixed in 10% formalin, dehydrated in graded alcohol series, cleared with methyl benzoate, embedded in paraffin, and sectioned at 4–5 μm. Slides were stained with hematoxylin and eosin (H & E) and the Naphthol AS-D Chloroacetate Esterase Kit (Sigma Chemical) for inflammation. Lung fibrosis was evaluated by Sirius red staining for collagen accumulation, as described in previous studies (54).

Immunological Staining

Slides were incubated with working proteinase K (20 μg/ml) solution in a humidified chamber for 7 min at 37°C for antigen retrieval, and then they were incubated in 3% hydrogen peroxide for 30 min at room temperature and in 10% animal serum for 1–2 h for blocking. These slides were incubated with primary anti-fibronectin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in a dilution of 1:100 at 4°C overnight, washed three times with PBS, and then incubated with secondary antibody at 1:1,000 dilution in room temperature for 30 min. Color was developed by incubating with diamino-

Enzyme Immunoassay for Ang II

Extraction of peptides from lung tissues or plasma was performed on the basis of instruction provided by the company in the kit (Phoenix Pharmaceuticals, Burlingame, CA). Briefly, lung tissue was boiled in 75% acetic acid for 20 min at 100°C to destroy enzymes and then homogenize the tissues in ice bath. The homogenate was centrifuged at 1,300 g for 30 min at 4°C. The supernatant was collected, and the total protein concentration was determined using Bio-Rad protein assay (Bio-Rad, Hercules, CA). Appropriate volume of the supernatant (~1 ml) or plasma (~0.5 ml) was mixed with buffer A at the ratio of 1:3 (cat. no. RR-BA-1; Phoenix Pharmaceuticals) and centrifuged at 3,500 rpm for 15 min at 4°C. Sep-column containing 200 mg of C18 (cat. no. RR-SEPCOL-1; Phoenix Pharmaceuticals) was equilibrated by washing once with 1 ml of buffer A (cat. no. RR-BB-1; Phoenix Pharmaceuticals) and three times with 3 ml of buffer A for each time. The supernatants were loaded onto the pretreated C18 sep-column and washed slowly with buffer A (3 ml, twice) without collection of the washouts. The peptides were slowly eluted with buffer B (3 ml, once). Eluant was collected in a polystyrene tube to be dried with centrifugal concentrator for 4 h, and then the dried extract was kept at −20°C. The extracts from lung tissue or plasma were reconstituted with 1× assay buffer and assayed with the Ang II Enzyme Immunoassay Kit (SPI Bio, Massy, France). The concentrations of Ang II are expressed as Ang II picograms per milligram protein for lung tissue or milliliters for plasma.

Statistical Analysis

Data were collected from repeated experiments and presented as means ± SD. Comparisons were performed by one-way ANOVA for the different groups, followed by post hoc pairwise repetitive comparisons using Tukey’s test with Origin 7.5 Lab data analysis and graphing software. Statistical significance was considered as P < 0.05.

RESULTS

Diabetes-Induced Lung Fibrosis and Inflammation

Lung tissues from control and diabetic mice at 3 mo of diabetes were histopathologically examined with H & E staining, which showed that diabetes induced significant increases in the lung’s interstitial thickness and infiltrated inflammatory cells in the interstitium (Fig. 1A). Histological examination showed no sign of pulmonary edema, suggesting no sign of cardiac systolic dysfunction. However, in the diabetic lung there was evidence of fibrosis localized predominantly in the interstitium, defined by the increased accumulation of the collagen by Sirius red staining (Fig. 1B). Lung interstitial inflammation was also defined by the increased infiltration of inflammatory cells, which were examined with Naphthol AS-D Chloroacetate Esterase staining specifically for macrophages (Fig. 1C).

Since CTGF plays a critical role in lung fibrosis (2, 7, 32), to confirm diabetic lung fibrosis we examined its expression at mRNA and protein levels by qPCR and Western blotting assay (Fig. 2, A and B). Compared with age-matched control, CTGF

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mRNA and protein expression increased significantly, and so did the fibronectin level in the diabetic lung (Fig. 2C). In addition, there was a significant increase in PAI-1 mRNA and protein expression in diabetic lungs (Fig. 2, D and E).

PAI-1 is known as both a profibrotic and inflammatory factor. Since the diabetic lung was inflammatory (Fig. 1C), we further examined TNFα and found that TNFα expression at both the mRNA and protein levels increased significantly in diabetic lungs (Fig. 2, F and G). These results clearly show that diabetes induces remarkable inflammatory and fibrotic response in the lung.

The above findings are from STZ-induced mice. To ensure that the lung fibrosis is a general feature of diabetes and is also not induced by STZ, we examined another type 1 diabetic mouse model, the OVE26 mouse. These mice, at the age of 3.5 mo, did not show any significant cardiac systolic dysfunction, examined by echocardiograph (Supplemental Table S1; Supplemental Material for this article is available online at the AJP-Endocrinology and Metabolism website), which is consistent with our previous study that showed no significant cardiac dysfunction at basic echocardiographic examination except for a reduced response to isoproterenol, a β-adrenergic agonist in the 5-mo-old OVE26 diabetic mice (38). Lung tissues were collected from these 3.5-mo-old OVE26 mice and age-matched wild-type (FVB) mice. Similar to what was observed in STZ-diabetic mice, histological examination showed that, in the diabetic lung, there was no sign of pulmonary edema (Fig. 3A), but fibrosis localized predominantly in the interstitium, defined by the increased accumulation of collagen by Sirius red staining (Fig. 3B) along with interstitial inflammation, showed increased infiltration of inflammatory cells (Fig. 3C).

Figure 4 shows that diabetes significantly upregulated expressions of CTGF (Fig. 4A), PAI-1 (Fig. 4B), and TNFα (Fig. 4C) at both mRNA and protein levels, measured by qPCR and Western blotting assays, respectively. Clearly, lung fibrotic and inflammatory processes exist in both STZ-induced and spontaneously developed type 1 diabetic mice.

Diabetes-Induced Lung’s Nitrosative Damage and Increases in Lung Ang II and Its Receptor Expression

In previous studies, we have demonstrated extensively the critical role of nitrosative damage in the pathogenesis of
diabetic cardiomyopathy (4, 5, 54). In the present study, to determine whether there is nitrosative damage in diabetic lung, we examined the protein nitration with 3-NT as the index of nitrosative damage. Results showed that diabetes significantly induced nitration in multiple groups (22–50 kDa) of proteins (Fig. 5A).

Since the cardiac nitrosative damage is derived mainly from the products of NOX activation and associated accumulation of peroxynitrite, we repeated the experiments in the lungs of diabetic mice. We found an increase in expression of NOX, shown by increased p47phox in the lungs of multiple low-dose-STZ-induced diabetic mice (Fig. 5B).

**Fig. 2.** Diabetes-induced upregulation of fibrotic mediators and inflammatory cytokines. Lung tissues were collected as described in Fig. 1. As an important profibrotic mediator, expression of connective tissue growth factor (CTGF) at mRNA (A) and protein levels (B) was examined by quantitative PCR (qPCR) and Western blotting, respectively. C: fibronectin protein level was also examined by Western blotting. As inflammatory mediator and cytokines, expression of plasminogen activator inhibitor-1 (PAI-1; D and E) and TNFα (F and G) at mRNA and protein levels was examined by qPCR and Western blotting, respectively (n ≥ 6). *P < 0.05 vs. control.
In our previous studies, we proved that Ang II plays an important role in diabetes-induced cardiac oxidative damage via NOX-activation related nitrosative damage (54); we thus examined whether Ang II receptor expression was changed in the diabetic lung. qPCR analysis showed a significant increase of AT1 expression in the lungs of diabetic mice compared with age-matched control mice (Fig. 5). Diabetes increased plasma and lung Ang II levels (Fig. 5, D and E).

Ang II-Induced Upregulation of CTGF Probably via NOX Activation-Associated Nitrosative Damage

The above studies indicate the induction of lung fibrosis by diabetes and that the lung fibrosis is associated with the increase in lung nitrosative damage that may be caused by increased Ang II and its receptor AT1 via activation of NOX. Since CTGF plays a critical role in fibrosis (32), to define the direct effect of Ang II on fibrotic response, we examined the effects of Ang II on cellular CTGF protein level in normal human lung fibroblast CCL-190 cells in vitro. Exposure to Ang II significantly increased CTGF protein levels in a dose- and time-dependent manner (Fig. 6, A and B).

To define NOX activation-associated nitrosative damage as a cause of the increased CTGF, we examined the effect of urate (peroxynitrite scavenger) applied at 60 min prior to Ang II exposure in CCL-190 cells. Urate significantly attenuated the induction of cellular CTGF protein expression after Ang II exposure at 100 nM for 24 h (Fig. 6C). This result suggests that Ang II-induced CTGF expression is related to peroxynitrite formation, i.e., nitrosative stress. To further define whether the peroxynitrite accumulation and CTGF expression are associated with NOX activation, Ang II-treated CCL-190 cells were pretreated with nitric oxide synthase (NOS) inhibitor (L-NAME; Fig. 6D), superoxide dismutase mimic (MnTMPyP; Fig. 6E), or NOX inhibitor (apocynin; Fig. 6F). All these pretreatments abolished Ang II-induced CTGF expression. This study confirms that Ang II-induced CTGF expression in

Fig. 3. Diabetes-induced lung fibrosis and inflammation in OVE26 mice. Diabetes was developed spontaneously in 2-wk-old OVE26 mice. Lung tissues were collected from both 3-mo-old OVE26 and their age-matched FVB mice and subjected to H & E staining for morphological examination (A), Sirius red staining for collagen (B), and staining by Naphthol AS-D Chloroacetate Esterase Kit for macrophage infiltration (C). Presented images are representative of each group containing ≥6 mice.
human lung CCL-190 cells is caused by nitrosative damage that is most likely mediated by NOX activation.

**Ang II Infusion-Induced Lung Fibrosis**

To ensure that the in vitro finding applies to the in vivo situation, FVB mice were infused with Ang II at a subpressor dose (0.5 mg/kg) for 2 mo, as used in our previous study (54). This treatment did not change blood pressure (Supplemental Table S2), but caused significant pathological changes in the lung (Fig. 7A). Lung histology was similar to that in diabetic mice (Fig. 1A). Lung fibrosis was verified by Sirius red staining for collagen (Fig. 7B), CTGF protein expression (Fig. 7C), and PAI-1 expression (Fig. 7D). Lung inflammation could be demonstrated by increased PAI-1 and TNFα levels (Fig. 7E), and lung nitrosative damage could be detected by increased 3-NT accumulation (Fig. 7F). All these findings point to an important role for Ang II in diabetic lung fibrosis.

**Prevention of Diabetic Lung Fibrosis by Ang II Antagonist (AT1 Receptor Blocker) Losartan**

To further verify that Ang II plays a critical role in diabetic lung fibrosis, following diabetes onset STZ-induced diabetic mice were treated with and without losartan (10 mg·kg⁻¹·day⁻¹) for 2 mo. In these diabetic mice, both diastolic and systolic blood pressures were not different from controls (Supplemental Table S2). Two-month treatment with losartan prevented diabetes-induced lung pathological damages (examined by H & E staining; Fig. 8A) and fibrosis [reflected by Sirius red staining (Fig. 8B), CTGF protein levels (Fig. 8C), and PAI-1 expression (Fig. 8D)]. In addition, losartan also prevented lung inflammation (reversed
PAI-1 and TNFα expression; Fig. 8E) and nitrosative damage (reversed 3-NT accumulation; Fig. 8F). This study clearly indicates the critical role of Ang II in the development of diabetic lung fibrosis.

**DISCUSSION**

The present study demonstrated for the first time that diabetes can induce the pulmonary fibrotic process in the animal models; the diabetic induction of the pulmonary fibrotic process is most likely mediated by NOX activation-mediated nitrosative damage, in which Ang II plays a critical role. This statement was indicated by our results that the levels of serum and cardiac Ang II were increased along with AT1 receptor expression in diabetic mouse lung that showed a significant fibrosis. In lung fibroblast cell culture, Ang II induced CTGF expression in a time- and dose-dependent manner, which was most likely mediated by NOX activation and nitrosative damage. In normal mice, low-dose infusion of Ang II induced diabetes-like lung fibrosis. In diabetic mice, treatment with an AT1 receptor blocker prevented nitrosative stress and lung fibrosis and inflammation.

Diabetes affects various organs, such as the kidneys, retina, nerves, and cardiovascular system. In recent years, the pulmonary dysfunction in diabetes has also become a subject of interest (13, 36, 42). Considering its large vascular network and richness in collagen and elastin, the pulmonary system is prone to microvascular damage and nonenzymatic glycation in diabetes. Many authors have investigated the pulmonary function and diffusion capacity in diabetic patients, but the results have been inconsistent (13, 36, 42). As discussed in the INTRODUCTION, a recent epidemiological study has shown that individuals with diabetes are at increased risk for several pulmonary disorders, including fibrosis (9). A meta-analysis based on 40 published independent articles with 3,182 diabetic and 27,080 control subjects showed that, in the absence of overt pulmonary disease, diabetes is associated with statistically significant, albeit modest, impaired pulmonary function. The diabetic effect presents a restrictive pattern (41). The results were obtained irrespective of body mass index, smoking, diabetes duration, and Hb A1c levels. These two studies provide evidence for an association between lung dysfunction and diabetes. However, it remains unclear whether the pulmo-
nary dysfunction is caused by diabetes or precedes diabetes (46). Our study adds evidence for the direct induction of the lung fibrotic process by diabetes in two mouse models of type 1 diabetes (Figs. 1–4) and supports a previous pilot report that STZ-induced diabetic mice had more severe lung fibrosis, evaluated by morphological changes, than mice treated with bleomycin alone (40). These studies demonstrate a causative role of diabetes in lung fibrosis.

The pathogenic mechanism for diabetic lung fibrosis has not been addressed, although systemic and lung inflammation is proposed as the cause. Since nitrosative damage is critical for diabetes- and Ang II-induced damage in various organs (4, 5, 14, 29, 54), here we propose that Ang II plays a critical role in diabetic lung fibrosis via activation of NOX-mediated nitrosative damage. Ang II plays an important role in fibrogenesis in various organs, including the lung (18, 25, 28, 50). The role of Ang II
or angiotensin-converting enzyme has been implicated in lung fibrosis induced by other pathologies (18, 25, 28, 50). For example, a high concentration of oxygen can cause lung injury and lead to pulmonary fibrosis. Rennin-angiotensin system components and Ang II production were increased significantly after hyperoxic exposure. Hyperoxia induced AT1 receptor but not AT2 receptor expression. Furthermore, silencing of AT1 receptor signaling with small interfering RNA or AT1 receptor blocker (losartan) suppressed hyperoxia-induced collagen synthesis (18). Consistent with the above previous studies on lung fibrosis induced by other diseases, we also found the increase in 3-NT accumulation in the diabetic and Ang II-infused, which could be prevented by infusion of losartan. Therefore, Ang II also plays a critical role in induction of lung nitrosative damage and lung fibrosis by diabetes.

Regarding the mechanism of how Ang II induces the nitrosative damage in the diabetic lung, we found that Ang II-induced 3-NT accumulation in the lung fibroblast cells could be inhibited by pretreatment with either apocynin (a NOX inhibitor), MnTMPyP (a superoxide dismutase mimic), L-NAME (a NOS inhibitor), or urate (peroxynitrite scavenger). We are aware that each of these pharmacological inhibitors used in this study may...
not be fully specific for each target, and more specific definition of the pathways using transgenic mouse model or siRNA approach is required in the future. However, activation of NOX-dependent generation of superoxide and associated peroxynitrite has been extensively approved as a major mechanism for Ang II-induced nitrosative damage in the diabetic hearts and kidneys (11, 15, 34, 51, 54). Induction of NO and superoxide was also defined in the cultured cells exposed to Ang II in vitro via induction of endogenous NOSs and NOX (8, 12, 31, 35, 47, 52). Therefore, we would draw the conclusion that Ang II-induced nitrosative damage in the diabetic lungs is also most likely mediated by activation of NOX-mediated generation of superoxide and associated peroxynitrite.

CTGF is a polypeptide implicated in extracellular matrix synthesis. It regulates the fibrotic phenotype in bleomycin-induced lung fibrosis (32). In the current studies, we showed that Ang II induced CTGF mRNA and protein in lung fibroblast cell culture (Fig. 6) and that the CTGF induction in the lungs could be induced by direct infusion of Ang II into normal mice (Fig. 7) and was preventable by losartan in the diabetic mice (Fig. 8). Therefore, it is speculated that prevention of CTGF results in a prevention of lung fibrosis by Ang II or diabetes.

There may be a concern about whether these mouse models (FVB mice background) used in the present study are particularly susceptible to diabetes-induced pulmonary fibrosis. Although we did not use different strains of mice and species in the present study to directly address this possibility, indirect evidence seems to not support this notion. There were several previous studies that demonstrated the induction of lung fibrosis in STZ-induced diabetes with RAP (33) or ICR (40) mice and rats (26, 27) and also in spontaneously developed cat diabetes (24).
There may be a limitation in the present study. We did not measure lung functions in these two mouse diabetic models since the focus of the present study was to define whether diabetes can directly induce lung fibrosis and its mechanisms. Although lung dysfunction has been reported extensively in patients with diabetes, which have been characterized by the reduced FVC, TLC, TLco, and DLco/VA, whether the fibrotic changes observed in the present study can be directly linked to these lung dysfunctions remains unclear. In future studies, the features of lung dysfunction in the rodent diabetic models such as mouse, rat, and even rabbit will be studied and compared with the observations from diabetic patients. The contributive role of lung fibrosis to the lung dysfunction under diabetic conditions will be further investigated, too.

In summary, our present studies demonstrate for the first time that diabetes induces lung fibrosis, and Ang II plays the critical role in diabetes-induced lung fibrosis most likely via the activation of NADPH oxidase-mediated nitrosative damage. Therefore, targeting AT1 may be a new strategy for prevention or therapy of diabetic lung fibrosis. At least AT1 blocker should be combined with anti-inflammation therapy for the patients with diabetic lung fibrosis.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

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