Research Article

Epoxyeicosatrienoic Acids Inhibit the Activation of Murine Fibroblasts by Blocking the TGF-β1-Smad2/3 Signaling in a PPARγ-Dependent Manner

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Background. Epoxyeicosatrienoic acids (EETs), the metabolite of arachidonic acid by cytochrome P450 (CYP), reportedly serve as a vital endogenous protective factor in several chronic diseases. EETs are metabolized by soluble epoxide hydrolase (sEH). We have observed that prophylactic blocking sEH alleviates bleomycin- (BLM-) induced pulmonary fibrosis (PF) in mice. However, the underlying mechanism and therapeutic effects of EETs on PF remain elusive.

Objective. In this study, we investigated the effects of CYP2J2/EETs on the activation of murine fibroblasts and their mechanisms.

Results. We found that administration of the sEH inhibitor (TPPU) 7 days after the BLM injection also reversed the morphology changes and collagen deposition in the lungs of BLM-treated mice, attenuating PF. Fibroblast activation is regarded as a critical role of PF. Therefore, we investigated the effects of EETs on the proliferation and differentiation of murine fibroblasts. Results showed that the overexpression of CYP2J2 reduced the cell proliferation and the expressions of α-SMA and PCNA induced by transforming growth factor- (TGF-) β1 in murine fibroblasts. Then, we found that EETs inhibited the proliferation and differentiation of TGF-β1-treated-NIH3T3 cells and primary murine fibroblasts. Mechanistically, we found that 14,15-EET disrupted the phosphorylation of Smad2/3 murine fibroblasts by activating PPARγ, which was completely abolished by a PPARγ inhibitor GW9662.

Conclusion. Our study shows that EETs inhibit the activation of murine fibroblasts by blocking the TGF-β1-Smad2/3 signaling in a PPARγ-dependent manner. Regulating CYP2J2-EET-sEH metabolic pathway may be a potential therapeutic option in PF.

1. Introduction

Pulmonary fibrosis (PF) is the buildup of chronic injury within the mesenchyma, and it represents the common final pathway of nearly all chronic and progressive lung diseases. It is characterized by inflammation, fibroblast proliferation, and differentiation with excess collagen deposition [1]. IPF patients usually have progressive dyspnea and worsening pulmonary function, with a median survival of only 2–4 years after diagnosis [2]. Available treatments to slow PF progression and prevent it are pretty limited. The in-depth investigation of the pathological mechanism of PF and the search for potential therapeutic targets are imminent. The activation of fibroblasts is the central mechanism of PF, and transforming growth factor β1 (TGF-β1) plays a vital role. The TGF-β1/Smad signaling pathway is activated by oxidative stress and inflammation, characterized by an increased expression of TGF-β1 and phosphorylation of Smad2/3 [3]. Inactivation of fibroblast promises to alleviate or reverse PF [4–6].

Arachidonic acid (ARA) is a polyunsaturated fatty acid released by phospholipases. Our previous work has shown that ARA metabolism is disrupted in a bleomycin- (BLM-) induced murine PF model [7]. Epoxyeicosatrienoic acids (EETs) are synthesized from ARA by the cytochrome P450 (CYP), such as CYP2C and 2J families [8]. There are four isomerides, including 5,6-, 8,9-, 11,12-, and 14,15-EET [9]. CYP2J2 is the main
enzyme mediating EET production in humans, mainly 14,15-EET [10]. EETs serve as a vital endogenous protective factor in various diseases [11–14]. EETs have been shown to have various biological effects, such as anti-inflammation, anti-papoptosis, proangiogenesis, antioxidation, and antihypertension [15, 16]. EETs are metabolized by soluble epoxide hydrolase (sEH) rapidly. Inhibiting sEH has been shown to elevate the biological activity of EETs [17]. Our previous works have firstly demonstrated that protective inhibiting EET degradation with an sEH inhibitor [1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU)] prevents BLM-induced PF in mice [18]. Other studies have also reported this phenomenon [19, 20]. However, the exact underlying mechanism remains unknown. Recently, it has been reported that 11,12-EET and sEH inhibition may prevent IPF by inhibiting the activation of patients’ fibroblasts [20], while the effects and specific mechanisms of the CYP2J2-EETs-sEH pathway on the fibroblast activation during PF have not been fully elucidated.

EETs are natural ligands for peroxisome proliferation-activated receptor γ (PPARγ) [21]. PPARγ is a ligand-activated transcription factor, significantly beneficial in a series of chronic diseases, including cardiac remodeling [22] and renal fibrosis [23]. In vascular smooth muscle cells (VSMCs), PPARγ inhibits metabolic and proliferative events by combining Smad3 directly and downregulating the TGF-β1/Smad3 signaling [24]. Nonetheless, the mechanisms of PPARγ in EETs attenuating the activation of lung fibroblasts in PF are unclear.

In this study, we found that EETs therapeutically reversed PF by attenuating fibroblast activation in BLM-treated mice via PPARγ. We showed that inhibition of sEH reversed lung fibrosis in BLM-treated mice. The overexpression of CYP2J2 and EETs could attenuate TGF-β1-induced activation of fibroblasts by suppressing Smad2/3 phosphorylation in PPARγ-dependent manner.

2. Materials and Methods

2.1. Animal. The male C57BL/6 mice (8 to 10 weeks of age, 20-25 g) were provided by Hunan SIA Laboratory Animal Co., Ltd. (Hunan, China). They were feeding under pathogen-free conditions (12 h-dark/light cycle and free access to water and food). The experimental use of mice in the present study was approved by the Ethics Committee of the Institute of Clinical Pharmacology at Central South University (No. 2020ysdw0685) to keep with the guidelines of the National Institutes of Health.

2.2. BLM-Induced PF Model and Animal Treatment. Mice were randomly divided into the following groups using the table of random numbers: (i) the control group: intratra-
Figure 1: Continued.
PBS 3 times, slides were then mounted with 90% glycerol and detected by a fluorescence microscope (Nikon, Japan).

For PPARγ staining, the samples were fixed with PFA and blocked with 5% BSA supplemented with 0.4% Triton-X for 1 h at room temperature. Fixed samples were incubated with the primary PPARγ antibody (Sigma-Aldrich, 1:200) at 4°C overnight. After washing, the samples were incubated with the Goat Anti-Rabbit IgG H&L (1:2000; Abcam) for 30 min and counterstained with DAPI. After mounting, the samples were detected by a fluorescence microscope (Nikon TS2R, emission spectrum 488 nm, absorption spectrum 519 nm).

2.8. Isolation of Primary Murine Lung Fibroblasts. Primary murine lung fibroblasts were isolated from lung tissue derived from 6-8-week-old C57BL/6 mice under sterile conditions. The mice were anesthetized with sodium pentobarbital and sacrificed. A small incision was cut on the right auricle after exposing the thorax. The lungs were operated on with pulmonary artery lavage from ventriculus sinister with sterile PBS (5 mL) three times. Subsequently, the lung tissue was cut into 1 mm diameter pieces by ophthalmic scissors, digested with 1 mg/mL collagenase I (Sigma-Aldrich, USA), and incubated at 37°C for 1 h. Afterward, cells were filtered by a 70 μm cell strainer and grown in Dulbecco’s Modified Eagle Medium/F12 (DMEM/F-12, Gibco, USA) containing 15% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin.

2.9. Cell Treatment. Murine fibroblast NIH3T3 cell lines were purchased from the American Type Culture Collection (ATCC; CRL-1658). The cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) supplemented with 10% bovine calf serum (BCS, Gibco) at 37°C and 5% CO2. The cells were seeded in 6-well plates. The next day, fibroblasts were starved (without serum) for 12 h and then treated with different compounds. To elevate the role of EETs in fibroblast activation, we constructed an adenovirus-CYP2J2. Cells were infected with adenovirus-CYP2J2 (1 × 10^9/mL) or vector (1 × 10^9/mL) with the complete growth medium for 48 h and then incubated with or without TGF-β1 (5 or 10 ng/mL, Sino Biological, China). Fibroblasts were exposed to 5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET (0.1, 0.5, 1 μM, Cayman, USA), or ethyl alcohol (1:300) for 5 min and then treated with TGF-β1 (5, 10 ng/mL) or vehicle control for 0.5 h to activate Smad2/3 signaling, for 24 h to activate the p-PPARγ, or for 48 h to induce the proliferation and differentiation. The cells were exposed to GW9662 (a PPARγ inhibitor, 10 μM, Merck, USA) or dimethyl sulfoxide (DMSO, Sigma-Aldrich) for 1 h with the following treatment of 14,15-EET and TGF-β1.

2.10. Cell Growth Assay. Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Japan) was used to determine cell viability. Briefly, the cells (1000 cells/well) were seeded in 96-well plates. The next day, fibroblasts were starved (without serum) for 12 h and then treated with different compounds. Then, fibroblasts were exposed to 5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET (0.1, 0.5, 1 μM, Cayman, USA), or ethyl alcohol (1:300) for 5 min and then treated with TGF-β1 (5, 10 ng/mL) or vehicle control for 48 h. Ten microliters of CCK-8 was added to each well, followed by incubation at 37°C for an additional 1 h. The

**Figure 1:** Inhibition of sEH attenuates the PF induced by BLM in mice. (a) TPPU (1 mg/kg/day, i.p.) was administered daily from the 7th day after BLM injection (1.5 mg/kg, i.t.). (b) Survival curves of mice (n = 10). (c) Bodyweight changes of mice (n = 6). (d) Lung sections of mice were analyzed by H&E staining. (e) The fibrotic area was expressed as a percentage of lung pathology (n = 6). (f) The total protein concentration in BALF (n = 6). (g) The LDH activity in BALF (n = 6). *P < 0.05, **P < 0.01, and ***P < 0.001.
absorbance was measured at 450 nm with an ultraviolet spectrophotometer (Thermo Fisher Scientific).

2.11. Cell Cycle Analysis. Flow cytometry was used to assay the cell cycle of murine fibroblast NIH3T3. Fibroblasts were starved for 24 h. The next day, the cells were maintained in no-serum DMEM with 14,15-EET (1 μM) for 5 min before TGF-β1 (10 ng/mL) treatment for 24 h and harvested. The cells were digested by trypsin and centrifuged at 800 rpm for 5 min. After washing with PBS, the cells were resuspended in 70% ethanol at 4°C gently and fixed at 4°C over-night. The cells were centrifuged at 2000 rpm for 5 min and added to a staining buffer containing RNase A (50 μg/mL, Solarbio, China) and at 4°C for 30 min before adding PI (0.25 mg/mL, Solarbio) to stain at 4°C for 30 min. The DNA profiles were determined by a flow cytometer (BD, USA).

2.12. Total RNA Extraction and PCR Analysis. The total RNA extraction was performed using RNAiso Plus (Takara Bio Inc., Kusatsu, Japan). RNA concentration and purity were determined using an ultraviolet spectrophotometer.
Figure 3: Continued.
Thermo Fisher Scientific, USA) at an absorbance ratio of 260 and 280 nm. cDNA synthesis was carried out using Prime Script RT Reagent Kit with gDNA Eraser (Takara Bio Inc.) according to our previous study [25]. The PCR amplifications were performed on the cDNA (1 μL) in the presence of forward and reversed primers (10 μM) and MIX (Takara Bio Inc.), followed by an initial incubation for 3 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 59°C, and 4 min at 70°C. The sequences of the specific primers are shown in Table 1. Data were digitized and analyzed by the Image Lab Analyzer software (Bio-Rad, CA, USA).

2.13. Protein Extraction and Western Blot. Total protein lysates were prepared using RIPA buffer (Solarbio, China) according to our previous study [26]. The cells were homogenized in RIPA buffer containing the cocktail (Solarbio) of protease inhibitors and stored at -80°C. The protein concentration was determined with Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Protein samples were dissolved in 2% SDS and denatured at 100°C for 10 min. For SDS-PAGE, protein (30 μg/well) from homogenate fractions was separated in 12% SDS-PAGE gels and transferred to 0.45 μm polyvinylidene difluoride membranes. Membranes were blocked using 5% BSA at room temperature for 1 h. Antibodies against α-SMA (SAB, 1:3000), PCNA (Proteintech, 1:3000), β-tubulin (Proteintech, 1:2000), COL-I (CST, 1:1000), COL-III (SAB, 1:1000), Smad2 (Abcam, 1:2000), Smad3 (Abcam, 1:2000), p-Smad2 (Abcam, 1:2000), p-Smad3 (Abcam, 1:2000), PPARγ (Sigma-Aldrich, 1:1000), and p-PPARγ (Invitrogen, 1:2000) were used at 4°C overnight. After being washed with TBST, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h and revealed by chemiluminescence (Millipore, USA). Images were captured with ChemiDoc XRS+ (Bio-Rad, CA). The protein and phosphorylated protein relative band intensity compared with β-tubulin or total protein was measured with the Image Lab Analyzer software (Bio-Rad, CA).

2.14. Statistical Analysis. Statistical analysis was performed with SPSS 22.0 and Graph Pad Prism software (Version 7.0, CA). Results are expressed as means ± SD. One-way analysis of variance (ANOVA) was used for multiple-group comparisons. Differences between the two groups were determined by a t-test. *P < 0.05, **P < 0.01, and ***P < 0.001.

3. Results

3.1. Inhibition of sEH Attenuates the PF Induced by BLM in Mice. To investigate whether inhibiting EET degradation affected PF, an sEH inhibitor, TPPU, was used on the 7th day after BLM administration (Figure 1(a)). Similar to our previous study in which TPPU was used from the 1st day after BLM injection [18], we found that TPPU treatment significantly increased the survival rate of mice compared with the BLM group (Figure 1(b)). Besides, TPPU reduced the weight loss of mice treated with BLM (Figure 1(c)). H&E stain demonstrated that TPPU therapeutically attenuated the percentage of average lung pathology in the lung after
Figure 4: Continued.
Figure 4: Continued.
BLM exposure (Figures 1(d) and 1(e)). The total protein in BALF reflects pulmonary vascular permeability. Besides, LDH in BALF is positively related to cellular damage. We found that TPPU treatment robustly decreased the total protein content in BALF of BLM-treated mice (Figure 1(f)) and partially reduced the LDH activity (Figure 1(g)). Taken together, these results indicate that therapeutical inhibition of sEH reverses collagen deposition detected with the Masson stain (Figures 2(a) and 2(b)). We also found that TPPU reduced the fluorescence intensity of α-SMA in the lung compared to the BLM group (Figure 2(c)). These data indicate that therapeutically inhibiting sEH reverses collagen deposition in BLM-treated mice.

3.2. Inhibition of sEH Reverses Collagen Deposition in BLM-Treated Mice. The induction of matrix-producing myofibroblasts and excessive extracellular matrix accumulation is the characteristic of PF. We found that TPPU treatment significantly reduced the collagen disposition detected with the Masson stain (Figures 2(a) and 2(b)). We also found that TPPU reduced the fluorescence intensity of α-SMA in the lung compared to the BLM group (Figure 2(c)). These data indicate that therapeutically inhibiting sEH reverses collagen deposition in BLM-treated mice.

3.3. CYP2J2 Overexpression Attenuates TGF-β1-Induced Activation of Murine Fibroblasts. To increase the EETs in fibroblasts, we used adenovirus-CYP2J2 to infect NIH3T3 cells. PCR results showed that CYP2J2 mRNA was successfully overexpressed in NIH3T3 cells (Figures 3(a) and 3(b)). To validate the idea that endogenous elevating EETs decrease the activation of fibroblasts induced by TGF-β1, the proliferation and the differentiation of the cells were measured in the following study. Results showed that the CYP2J2 overexpression resulted in a significant reduction of proliferation induced by TGF-β1 (10 ng/mL) in NIH3T3 cells (Figure 3(c)). Besides, the western blot revealed that the CYP2J2 overexpression downregulated α-SMA and PCNA expression in NIH3T3 cells (Figures 3(d)–3(f)). These data indicate that the CYP2J2 overexpression attenuates the activation of murine fibroblasts induced by TGF-β1.

3.4. EETs Attenuate TGF-β1-Induced Activation of Murine Fibroblasts. To gain further insights into whether EETs reduced the activation of murine fibroblasts, we treated NIH3T3 cells with 5,6-EET, 8,9-EET, 11,12-EET, or 14,15-EET (0.1, 0.5, or 1 μM) for 48 h. We found that EETs did not affect the proliferation of NIH3T3 cells without TGF-β1 stimulation (Figure 4(a)). While four regioisomers of EETs (1 μM) observably inhibited the proliferation of NIH3T3 cells induced by TGF-β1, 14,15-EET was the most effective type compared to the other three types of EETs (Figure 4(b)). Therefore, we used 14,15-EET in the following experiments. CCK8 assay showed that 14,15-EET (0.5 or 1 μM) inhibited the TGF-β1-induced proliferation of both NIH3T3 cells (Figure 4(c)) and primary murine lung fibroblasts (Figure S1A). Two types of cells were treated with 14,15-EET (1 μM) and stimulated by TGF-β1 for 48 h. We found that 14,15-EET decreased the expressions of collagen, α-SMA, and PCNA protein of NIH3T3 cells (Figures 4(d)–4(g)) and primary murine lung fibroblasts induced by TGF-β1 (Figure S1B-E). Next, we use flow cytometry to assay the cell cycle of murine fibroblasts. The results showed that 14,15-EET significantly reduced the G0/G1 phase of the TGF-β1-treated NIH3T3 cells (Figures 4(h)–4(j)). These results suggest that EETs depress the activation and collagen synthesis of murine fibroblasts induced by TGF-β1.

3.5. 14,15-EET Disrupts the TGF-β1-Smad2/3 Signaling in Murine Fibroblasts by Activating PPARγ. TGF-β1 induces fibroblast proliferation and differentiation by activating the
Figure 5: Continued.
Smad2/3 pathway. We further investigated the mechanism of EETs in inhibiting proliferation and differentiation of TGF-β1-treated murine fibroblasts, where 14,15-EET substantially repressed Smad2/3 phosphorylation both of NIH3T3 cells (Figures 5(a)–5(c)) and murine fibroblasts induced by TGF-β1 (Figure S2A, B, C). PPARγ is a receptor of EETs [27]. To assess whether EETs downregulated TGF-β1 induced the activation of fibroblasts via PPARγ, we quantified PPARγ expression and phosphorylation in NIH3T3 cells and primary murine lung fibroblasts. Western blot showed 14,15-EET restored PPARγ expression of two cell lines stimulated by TGF-β1 (Figures 5(d)–5(f), Figure S2D-F). Moreover, treatment with 14,15-EET led to increased phosphorylation of PPARγ in two cell lines compared to the TGF-β1 group (Figures 5(d) and 5(e), Figure S2D-E). The immunofluorescence showed that TGF-β1 inhibited the fluorescence intensity of nuclear PPARγ of NIH3T3 cells while 14,15-EET significantly increased nuclear PPARγ of the cells treated by TGF-β1 (Figure 5(g)). Collectively, these results suggest that 14,15-EET disrupts the TGF-β1-Smad2/3 signal in murine fibroblasts by activating PPARγ.

3.6. Inhibition of PPARγ Abolishes the Inhibitory Effects of 14,15-EET on the Activation of Murine Fibroblasts. Then, we used GW9962, a PPARγ inhibitor, to investigate the role of PPARγ in the inhibitory effects of EETs on fibroblast activation. We found that the inhibitory effects of 14,15-EET were blocked by GW9962 (Figures 6(a)–6(c)). Importantly, GW9962 abolished the reduction of p-Smad2/3 induced by 14,15-EET of TGF-β1-treated NIH3T3 cells (Figures 6(d)–6(f)). These data indicate that 14,15-EET inhibits the proliferation and differentiation of fibroblasts via a PPARγ-dependent manner.

4. Discussion

EETs have recently been recognized as critical protective lipid molecules in vivo. The metabolic pathway of CYP2J2-EET-sEH plays an essential role in the pathomechanism of fibrosis, while the therapeutic effects of EETs against PF and their specific mechanism are still unclear. In this study, we identified that therapeutic inhibition of EET degradation reversed lung fibrosis in the BLM-induced murine model. Furthermore, we elucidated that the CYP2J2 overexpression and elevating EETs suppressed TGF-β1-induced activation of murine fibroblasts, including fibroblast proliferation, differentiation, and collagen secretion by blocking the TGF-β1-Smad2/3 signal in a PPARγ-dependent manner (Figure 7). This study suggests that regulating the metabolic pathway of CYP2J2-EETs-sEH is a potential therapeutic strategy in PF.

This study indicates that regulating the metabolic pathway of CYP2J2-EET-sEH attenuates PF by inhibiting the fibroblast activation. A series of changes occur in enzymes involved in EETs production in PF. We have reported that Cyp2j6 and Cyp2j9 genes are highly expressed in the lungs of wild-type mice. Both Cyp2j6 and Cyp2j9 gene expressions are downregulated in the lungs of BLM-treated mice, while the sEH expression is upregulated [7]. It has been reported that sEH is upregulated in IPF patients [20]. Although our laboratory has shown the prophylactical inhibition of sEH prevented BLM-induced PF in mice [18], neither the therapeutic effect nor the specific mechanism of EETs on PF was elucidated. Our results generally illustrate the potential therapeutic role of the CYP2J2-EET-sEH pathway in chronic lung disease. We further showed the therapeutic effect of EETs on PF. The treatment with TPPU on the 7th day reversed lung fibrosis in BLM-treated mice by inhibiting...
the activation of fibroblasts. He et al. found that 11,12-EET attenuates fibroblast activation in Ang II-induced cardiac fibrotic response in mice by targeting Gα12/13 [28]. Moreover, we found the role of EETs on the activation of fibroblasts is PPARγ-dependent.

As the primary effector cells of fibrosis, myofibroblasts actively interact with surrounding cells in the fibrotic microenvironment. On the one hand, myofibroblasts lead to epithelial cell damage in PF, which results in the activation of fibroblasts. On the other hand, myofibroblasts could be activated by changing the microenvironment of fibrosis [29]. We demonstrated that inhibition of sEH reduced α-SMA in the lungs of BLM-induced mice. In vitro, we also confirmed that CYP2J2 overexpression and EETs inhibited

![Figure 6: PPARγ inhibitor abolishes the effects of 14,15-EET on the activation of murine fibroblasts. NIH3T3 cells were treated with the GW9662 (10 μM) for 1 h, followed by treatment with 14,15-EET (1 μM) for 5 min and TGF-β1 (10 ng/mL) for 48 h. α-SMA and PCNA in NIH3T3 cells were detected by western blot ((a)–(c), n = 3). Cells were treated with the GW9662 (10 μM) for 1 h, followed by treatment with 14,15-EET (1 μM) for 5 min and TGF-β1 (10 ng/mL) for 30 min. p-Smad2 and p-Smad3 were detected by western blot ((d)–(f), n = 3). * P < 0.05, ** P < 0.01, and *** P < 0.001.](image)
proliferation and differentiation of murine fibroblasts induced by TGF-β1. A similar effect of EETs has been observed in cardiac fibrosis [28], which confirms our findings. Myofibroblasts secrete various cytokines to promote epithelial cell damage, then aggravate fibroblast-myofibroblast transformation, and eventually produce a large number of myofibroblasts. The interaction between myofibroblasts and the fibrotic microenvironment leads to a vicious loop, eventually forming nonfunctional fibrotic tissue [29]. The overexpression of CYP2J2, elevating EETs, and inhibition of sEH successfully attenuate TGF-β1-induced activation of murine fibroblasts.

Our work highlights the importance of the EETs-PPARγ axis to downregulate detrimental TGF-β1 pathways by inactivating the proliferation and differentiation of lung fibroblasts. PPARγ, a member of the ligand-activated transcription factor superfamily, expresses in many organs, such as the lung. PPARγ involves gene expression, glucose metabolism, lipid metabolism, fibrosis, inflammation, and oxidative stress [30, 31]. PPARγ could be activated by phosphorylating to exert biological effects [32]. It has been reported that the activation of PPARγ plays a protective role by inhibiting TGF-β1-target gene expression in mice [33]. We observed that 14,15-EET promoted PPARγ expression and phosphorylation in murine fibroblasts treated by TGF-β1. Besides, 14,15-EET restored nuclear translocation of PPARγ in the fibroblasts stimulated by TGF-β1. TGF-β1-Smad2/3 signaling is a classic pathway in the process of PF. Our study shows that EETs inhibited the phosphorylation of Smad2/3 by activating PPARγ at the molecular level. In line with our results, activating the PPARγ inhibited TGF-β1-induced Smad2/3 phosphorylation [34, 35]. PPARγ directly binding to Smad3 in VSMCs, reversing PAH [24], which confirms our findings. Moreover, we showed that all types of EETs could inhibit the activation of murine fibroblasts induced by TGF-β1, but only 14,15-EET was used in the following experiments. Because 14,15-EET abounds in the lungs [36], CCK8 assay showed the most significant inhibitory effect on proliferation compared to the other three types of EETs.

In conclusion, our study shows the essential therapeutically antifibrotic role of EETs in PF. We reveal that EETs block fibroblast activation through the PPARγ pathway, contributing to the therapeutic effect of EETs against PF in mice. Regulating CYP2J2-EET-sEH metabolic pathway may be a potential therapeutic option for PF and chronic lung diseases.

**Data Availability**

All data included in this study are available upon request by contact with the corresponding authors.
Ethical Approval

All applicable international, national, and institutional guidelines for the care and use of animals were followed.

Conflicts of Interest

The authors declared no conflict of interest.

Authors’ Contributions

Jia-Hao Tao, Tian Liu, Chen-Yu Zhang, and Cheng Zu performed the experiments. Yu-Biao Liu and Jin-Tong Yang analyzed the data. Jia-Hao Tao and Hui-Hui Yang wrote the paper. Yong Zhou and Cha-Xiang Guan conceived, designed the experiments, and critically reviewed the manuscript.

Acknowledgments

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Supplementary Materials

Figure S1: 14,15-EEt attenuates TGF-β1-induced activation of primary murine lung fibroblasts. The primary murine lung fibroblasts were treated with 14,15-EEt (1 μM) for 5 min, followed by treatment with TGF-β1 (5 ng/mL) for 48 h. The cell proliferation was examined by CCK-8 ((a), n = 5). Protein levels of COL-I, α-SMA, and PCNA were detected by western blot ((b)–(e), n = 3). *P < 0.05, **P < 0.01, and ***P < 0.001.

Figure S2: 14,15-EEt upregulates PPARγ and downregulates the Smad2/3 signaling of TGF-β1-induced primary murine lung fibroblasts. The primary murine lung fibroblasts were treated with 14,15-EEt (1 μM) for 5 min, followed by treatment with TGF-β1 (5 ng/mL) for 0.5 h. p-Smad3 and p-Smad2 were detected by western blot ((a)–(c), n = 3). The primary murine lung fibroblasts were treated with 14,15-EEt (1 μM) for 5 min, followed by treatment with TGF-β1 (5 ng/mL) for 24 h or 48 h. The p-PPARγ and PPARγ were detected by western blot ((d)–(f), n = 3). *P < 0.05, **P < 0.01, and ***P < 0.001. (Supplementary Materials)

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