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Soluble epoxide hydrolase inhibitor 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea attenuates bleomycin-induced pulmonary fibrosis in mice

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Abstract

Epoxyeicosatrienoic acids (EETs), the metabolites of arachidonic acid derived from the cytochrome P450 (CYP450) epoxygenases, are mainly metabolized by soluble epoxide hydrolase (sEH) to their corresponding diols. EETs, but not their diols, have anti-inflammatory properties, and inhibition of sEH might provide protective effects against inflammatory fibrosis. In this study, we tested the effects of a selected sEH inhibitor, 1-trifluoromethoxyphenyl-3- (1-propionylpiperidin-4-yl) urea (TPPU), on bleomycin-induced pulmonary fibrosis (PF) in mice. A mouse model of PF was established by intratracheal injection of bleomycin, and TPPU was administered for 21 days after bleomycin injection. We found TPPU treatment improved the body weight loss and survival rate of bleomycin-stimulated mice. Histological examinations showed that TPPU treatment alleviated bleomycin-induced inflammation, and maintained alveolar structure of pulmonary tissues. TPPU also decreased bleomycin-induced deposition of collagen, and expression of the procollagen I mRNA in lung tissues of mice. TPPU decreased the TGF-β1, IL-1β and IL-6 levels in serum of bleomycin-stimulated mice. Moreover, TPPU inhibited proliferation, collagen synthesis of the mouse fibroblasts, and partially reversed TGF-β1-induced α-SMA expression. Our results indicated that inhibition of sEH attenuates bleomycin-induced inflammation, collagen deposition, and therefore prevents bleomycin-induced PF in mice model.

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Author Disclosure

The authors report no conflict of interest.

Keywords

soluble epoxide hydrolase inhibitor; epoxyeicosatrienoic acids; pulmonary fibrosis; proliferation; mouse

Introduction

Idiopathic pulmonary fibrosis (IPF) is characterized by inexorable, progressive fibrosis, which involves the interstitial space that exists between the vascular endothelium and alveolar epithelium. The pathological processes of IPF include alveolar epithelial cell injury, fibroblast proliferation, and abnormal deposition of extracellular collagen. Transforming growth factor-β1 (TGF-β1), an inflammatory biomarker, is one of the key mediators in the fibrotic process. TGF-β1 is involved in pulmonary inflammation, tissue repair, and fibrosis (Sheppard, 2006). The differentiation of fibroblast to myofibroblast, with high expression of α-smooth muscle actin (α-SMA), contributes to the formation of fibroblastic foci. IPF affects as many as 14–43 people per 100 000 worldwide (Raghu, et al., 2006), and it is lack of useful treatment. Thus, novel therapeutic agents for this unmet medical need are of particular interest.

Epoxyeicosatrienoic acids (EETs) are synthesized from arachidonic acid (AA) by cytochrome P450 (CYP) epoxygenases, and function primarily as autocrine and paracrine effectors. EETs produce vasodilation as well as anti-inflammatory and pro-fibrinolytic effects. EETs are mostly metabolized by soluble epoxide hydrolase (sEH), which coverts EETs to dihydroxyeicosatrienoic acids (DHETs) (Spector, et al., 2004). Inhibition of sEH has been shown to enhance the biological activity of EETs. The lung vasculature is known to be a rich source of lipid mediator biosynthesis from AA, and EETs have been previously described as the predominant eicosanoids in human lungs upon stimulation with the Ca^{2+} ionophore A23187 challenge (Kiss, et al., 2010). EETs in the lung are involved in many pulmonary diseases, such as asthma (Norton, et al., 2012, Yang, et al., 2015), cigarette smoke extract-induced lung injury (Yu, et al., 2015), lung ischemia-reperfusion injury (Chen, et al., 2015), and pulmonary hypertension (Revermann, et al., 2009). A recent study showed that EETs could inhibit the expression of α-SMA and TGF-β1/SMADs, protect the remnant kidney in nephrectomized rat via the inhibition of fibrosis (Zhao, et al., 2012). Furthermore, inhibition of sEH prevents renal interstitial fibrosis and inflammation (Kim, et al., 2014). However, it is unknown whether inhibition of sEH can provide protective effects against pathological processes of pulmonary fibrosis (PF).

Intratracheal administration of bleomycin is the most extensively used experimental model of pulmonary fibrosis because the pathological features produced by this procedure are very similar to IPF (Della Latta, et al., 2015). In the present study, we tested the effect of 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU), a potent sEH inhibitor provided by Prof. Hammock (Sirish, et al., 2013), on bleomycin-induced PF. In addition, we also studied the effect of TPPU on the proliferation, and differentiation of mouse fibrosis, and deposition of extracellular collagen *in vitro*.

Materials and Methods

Animals and treatments

Experimental use of mice in the present study was performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the animal protocol was approved by institutional animal care committee. Adult male C57BL/6 mice were provided by the laboratory animal unit of Central South University. The mice were randomly divided into three groups: (1) intratracheal saline (normal group); (2) intratracheal bleomycin plus vehicle (BLM group); (3) intratracheal BLM plus TPPU treatment (BLM +TPPU group). The mouse model of PF was established by an intratracheal injection of bleomycin (5 mg/kg, Nippon Kayaku, Japan) according to a previous published protocol (Liu, et al., 2013b). All surgeries were performed under anesthesia with an intraperitoneal injection of sodium pentobarbital (30 mg/kg). TPPU (1 mg/kg/day) was administered by gavage dosing after bleomycin injection. TPPU was dissolved in oleic acid-rich triglyceride containing 20% PEG400 (v/v) to give a clear solution. The mice were sacrificed at Day 7, 14, and 21 under anesthesia. The additional 60 mice were used to determine the effect of TPPU on the survival rate of BLM-treatment mice. These mice were treated as described above. The body weight was recorded.

Histopathology analysis

Lung tissue samples were fixed with 4% paraformaldehyde neutral buffer solution for 24 h, dehydrated in a graded ethanol series, embedded in paraffin, and sliced at 5 μm. Paraffin sections were stained with HE and Masson's trichrome stain. The Ashcroft score was used for semi-quantitative assessment of fibrotic changes (Murakami, et al., 2004). Thirty fields in each section were analyzed, and the severity of fibrotic changes in each mouse was assessed as the mean of severity from observed microscopic fields.

Hydroxyproline assay

The hydroxyproline content in the supernatant of cell and lung tissue was calculated according to the manufacturer’s instructions for the hydroxyproline assay kit (Sigma-Aldrich, USA). The sample absorbencies were assessed at 550 nm.

Total RNA extraction and Real-Time PCR

Total RNA from lung tissues was extracted using TRIzol reagent (Invitrogen, USA) and was quantified by spectrophotometric analysis using an ultraviolet spectrophotometer (Bio-Rad, USA). The generation of cDNA from RNA (2 μg) was performed using RevertAid™ Reverse Transcriptase (Thermo-Scientific, USA) and random hexamer primers according to the manufacturer’s instructions. Real-Time PCR of cDNA was performed using the iTaq™ Universal SYBR® Green Supermix. The sequences of the specific primers are shown in Table 1. Relative gene expression was measured by the $2^{-\Delta\Delta CT}$ method and was normalized to β- *actin* mRNA levels.

Measurement of cytokine levels in serum by ELISA

The serum concentrations of pro-fibrogenic cytokine (TGF-β1) and pro-inflammatory cytokines (IL-1β and IL-6) were measured by using the commercially available ELISA kit (Sigma, USA), according to the manufacturer's instructions.

MTT assay

The fibroblast cells (NIH3T3, ATCC Number CRL-1658) were seeded in a 96-well cell culture plate and were incubated with TPPU (0.1, 1, 10 μM) or DMSO (0.1%) for 12, 24 or 36 h at 37°C. 20 μL MTT solution (5 mg/mL, Sigma-Aldrich, USA) was added to each well, followed by incubation at 37°C for an additional 4 h. The supernatant was gently removed and 100 μL DMSO was added. The culture plate was shaken for 10 min at room temperature. The absorbance was measured at 492 nm.

Lactate dehydrogenase (LDH) activity assay

Mouse fibroblast cells (NIH3T3) were plated into 12-well plates and serum-starved for 1 day. The cells were divided into three concentrations of TPPU (0.1, 1, 10 μM) treatment groups and DMSO (0.1%) vehicle groups. TPPU or DMSO was added into the four groups respectively, and after 48 h the medium was collected for measurement of LDH activity. LDH activity was determined using a Sigma Tox-7 *in vitro* toxicology kit and reported as the amount of LDH activity in the medium.

Cell cycle analysis

NIH3T3 were seeded in 6-well cell culture plate. The cells were divided into six groups: normal group, DMSO vehicle group, TGF-β1 group, TGF-β1+ DMSO group, and TGF-β1+ TPPU (1 and 10 μM) group. TPPU was added prior to TGF-β1 (10 ng/mL, R&D Systems, USA) treatment for 24 h. The cells were trypsinized and centrifuged at 1000 rpm for 5 min. Then cells were fixed with cold 70% ethanol at 4°C overnight. The cells were resuspended in 1 mL PBS containing 1000 U RNase A. The cells were stained with 1% PI at 4°C for 30 min. The DNA profiles were determined within 2 h using flow cytometer (Beckman Coulter MoFlo™ XDP, USA). The data was acquired and analyzed using Summit v5.2 software (Beckman Coulter).

Western blot

Western blot was used to detect the α-SMA protein expression in mouse fibroblasts (NIH3T3). The cells were lysed with RIPA lysis buffer containing a cocktail of protease inhibitors. Total protein was determined using a modified Bradford protein assay (Sigma-Aldrich, USA). 50 μg proteins were fractionated on a 15% SDS-polyacrylamide gel, and transferred to PVDF membranes. After blocked for 2 hours in 5% non-fat milk in TBST, the membranes was incubated overnight at 4°C with either a mouse monoclonal anti-α-SMA (Sigma-Aldrich, USA), or a rabbit anti-β-actin (Cell Signaling Technology, USA) Ab. The membranes were then washed 3 times, and incubated with the secondary Abs conjugated to horseradish peroxidase (Thermo-Scientific, USA) for two hours at room temperature. The immunostained bands were visualized with SuperSignalH West Pico Chemiluminescent

Substrate kit (Thermo-Scientific, USA). The band intensities on scanned gels were analyzed using the public domain National Institutes of Health ImageJ program.

Lipid mediator analysis and TPPU concentration measurements in blood

The profiles of lipid mediators were measured using the LC/MS/MS method as previously published (Yang, et al., 2009). Briefly, aliquots of plasma (250 μL) were used for the measurements after solid phase extraction protocol. The blood concentrations of TPPU were measured by LC/MS/MS (Liu, et al., 2013a). 10 μL of blood was diluted by 50 μL water with 0.1% EDTA, and the mixtures were extracted using 200 μL ethyl acetate twice and dried by Speedvac. Finally, the residue was reconstituted to 50 μL and measured using LC/MS/MS (ABI, Milford, MA).

Statistical analysis

All values were expressed as the mean ± SEM. Differences between multiple groups were compared using one-way analysis of variance. The SNK test served as the post hoc test for multiple comparisons. Survival rate was evaluated by the Kaplan-Meier test. Significance was established at $P<0.05$.

Results

TPPU attenuated bleomycin-induced body weight loss and mortality

Pulmonary fibrosis is a progressive and highly devastating interstitial lung disease. In this study, we found the body weighty in the bleomycin group was decreased during the day 0~21 post bleomycin administration. TPPU treatment attenuated the body weight loss ($P<0.05$ *vs*. BLM group, Fig. 1a). Furthermore, the mice challenged with bleomycin began dying at day 2 after bleomycin treatment, and the mortality rate was approximately 55 % on day 14. TPPU treatment delayed the onset of death, and improved the mortality rate associated with bleomycin ($P<0.01$ vs. BLM group, Fig. 1b). For example, the mice treated with TPPU began to die on day 5, with a final mortality rate of approximately 25% on day 14.

TPPU attenuated bleomycin-induced morphological changes in mice

The effect of TPPU on bleomycin-induced inflammation and fibrosis was examined on day 7, 14, and 21 after injection of bleomycin (Fig. 2). The HE stain of lung tissues from normal group revealed a well-alveolized pulmonary structure (Fig. 2, left column). Bleomycin induced significantly alveolar wall thickening, and massive infiltration of inflammatory cells in the interstitial (Fig. 2, middle column). Although the lung injury was observed in the bleomycin + TPPU-treated group, both the extent and intensity of the injury were less than those of the bleomycin group (Fig. 2, right column).

TPPU decreased bleomycin-induced collagen deposition in the lung tissue

Masson's trichrome stain was used to detect collagen deposition in lung tissue. Compared with normal group, bleomycin treatment (5 mg/kg) significantly increased collagen deposition in lung tissue on day 14, and collagen deposition was peaked on day 21. TPPU

treatment (1 mg/kg/day) decreased bleomycin-induced collagen deposition at different time (Fig. 3a–i). The Ashcroft score semi-quantitative results demonstrated that the degree of pulmonary fibrosis in the BLM + TPPU group was significantly lower than BLM group at Day 14 and Day 21 (both $P<0.05$, Fig. 3j). In addition, Real-Time PCR (Fig. 4a) showed that bleomycin treatment significantly increased the expression of collagen type I mRNA compared with the control group on day 21 ($P<0.05$). TPPU treatment substantially reduced the expression of collagen type I mRNA as compared with the bleomycin group ($P<0.05$). However, the expression of collagen type III mRNA was unchanged among three groups (data not shown). Furthermore, TPPU treatment reduced amount of the hydroxyproline, a major component of the collagen, compared with the bleomycin group (Fig. 4b, $P<0.05$). These results indicated that TPPU treatment decreased bleomycin-induced deposition of collagen in the lung tissue.

TPPU decreased the serum level of TGF-β1, IL-1β and IL-6 in bleomycin-challenged mice

TGF-β1 is a pro-fibrogenic cytokine. It plays a vital role in proliferation and differentiation of fibroblast, and epithelial-mesenchymal transition (EMT) of pulmonary epithelial cell. We found the concentration of TGF-β1 in bleomycin-challenged mice was increased at day 7 and day 21 after bleomycin treatment ($P<0.05$ *vs*. normal group). TPPU treatment decreased concentration of the serum TGF-β1 ($P<0.05$ *vs*. BLM group, Fig. 5a–b). IL-1β and IL-6 are pro-inflammatory cytokines. We also found bleomycin treatment increased the serum concentration of IL-1β and IL-6 at day 7 and day 21 after bleomycin treatment ($P<0.05$ *vs*. normal group), and TPPU treatment significantly decreased the serum concentration of IL-1β and IL-6 ($P<0.05$ *vs*. BLM group, Fig. 5c–f).

TPPU inhibited proliferation of the fibroblast *in vitro*

The effects of sEH inhibitor on proliferation, differentiation and collagen synthesis of fibroblast were examined. We found that TPPU inhibited proliferation of the fibroblasts in a time- and dose-dependent manner. Lower concentration of TPPU at 0.1 μM did not affect proliferation of mouse fibroblasts as compared with control group ($P>0.05$ vs. DMSO group). However, 1 μM of TPPU treatment for 24 h and 36 h significantly reduced the proliferation of mouse fibroblasts (both $P<0.05$ vs. DMSO group). Higher concentration of TPPU at 10 μM significantly reduced proliferation of mouse fibroblast at various time we tested ($P<0.05$ *vs*. DMSO group, Fig. 6a). LDH is contained in living cells such that the appearance of this enzyme in the medium is an indication that cells have died and released this protein. LDH appearance in the medium was measured and found to be unchanged in cells (Fig. 6b), demonstrating the lack of toxicity of TPPU in these cells. Next, mouse fibroblasts were stained with propidium iodide (PI), and DNA labelling was detected using FCM to analyze the cell cycle. The results showed that 1 and 10 μM TPPU significantly reduced the percentage of S phase ($P<0.05$ vs. DMSO + TGF-β1 group, Fig. 7). These data indicated that TPPU inhibited mouse fibroblast proliferation *via* inhibiting DNA synthesis.

TPPU inhibited the differentiation of mice fibroblasts *in vitro*

Fibroblasts can differentiate into myofibroblasts, and these differentiated myofibroblasts express high level of the α-SMA. We measured expression of the α-SMA mRNA and

protein using Real-Time PCR and western blot respectively. We found that the treatment of TGF-β1 (10 ng/mL) for 24 h significantly increased expression of the α-SMA mRNA and protein in mouse fibroblasts. TPPU significantly reduced expression of the α-SMA mRNA and protein in TGF-β1-treated mouse fibroblasts (Fig. 8a–b). These findings suggested that TPPU could inhibit the differentiation of fibroblasts into myofibroblasts.

TPPU inhibited collagen synthesis of fibroblasts *in vitro*

An additional feature of myofibroblast is collagen synthesis. Therefore, we tested the effect of TPPU on the collagen synthesis of TGF-β1-stimulated mouse fibroblasts. Real-Time PCR results (Fig. 8c) showed that the expression of collagen type I mRNA were significantly increased after treatment with TGF-β1 (10 ng/mL) for 24 h. TPPU (1, 10 μM) inhibited the expression of collagen type I mRNA in a dose-dependent manner. Furthermore, the hydroxyproline content in the supernatant was analyzed. These results (Fig. 8d) demonstrated that treatment with TPPU (1, 10 μM) significantly reduced the TGF-β1-induced hydroxyproline in the supernatant of mouse fibroblasts. These findings indicated that TPPU could inhibit TGF-β1-induced synthesis and secretion of collagen in cultured mouse fibroblasts.

TPPU increased endogenous EETs in plasma

Finally, the serum concentration of TPPU and 14,15-EET, one of the most predominated form of EETs in plasma, were measured at the end of the TPPU treatment. We found that the serum concentration of TPPU in TPPU + bleomycin group (810 ± 88 ng/mL) was significantly higher than that in the normal group (non-detected) and bleomycin-treated group (non-detected) ($P<0.05$, Fig. 9a). Treatment of TPPU significantly increased concentration of the serum 14,15-EET in the TPPU-treated group (4.1 ± 0.9 nM) as compared with bleomycin only (1.8 ± 0.6 nM) or normal group (1.668 ± 0.025 nM) ($P<0.05$, Fig. 9b). These results suggested that TPPU can inhibit EET degradation, and therefore increase the concentration of serum EET.

Discussion

Pulmonary fibrosis (PF) is a pathological process in which normal lung tissues are progressively replaced by fibroblasts and collagen, thereby resulting in an irreversible loss in the ability to transfer oxygen into the bloodstream via pulmonary alveoli. PF is characterized by fibroblastic foci-containing fibroblasts and myofibroblasts which are the primary source of extracellular matrix (ECM) components, such as collagen type I and III and fibronectin (Scotton and Chambers, 2007). Studies on the pathogenesis of pulmonary fibrosis have mainly focused on the mechanisms that regulate the proliferation, activation, and differentiation of collagen-secreting myofibroblasts. The patients with PF show progressive dyspnea, which ultimately result in death (Wynn, 2011). Idiopathic PF is a particularly severe form of pulmonary fibrosis with a life expectancy of 2–6 yr after diagnosis (Chaudhuri, et al., 2014). Currently, lung transplantation is the only effective treatment available for progressive lung fibrosis.

EETs are endogenous bioactive lipid mediators synthesized from AA by a reaction catalyzed by P450 epoxygenase. Four EET regioisomers, 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET are identified, and 11,12-, and 14,15-EET are the predominated form in blood circulation and lung tissue (Spector, Fang, Snyder and Weintraub, 2004). EETs have various beneficial effects against inflammation, platelet aggregation, and migration of vascular smooth muscle cells (Xu, et al., 2011). EETs are involved in pulmonary diseases, such as pulmonary hypertension, allergic asthma, and bronchi (Feng, et al., 2013, Loot and Fleming, 2011, Norton, Wijesinghe, Dellinger, Sturgill, Zhou, Barbour, Chalfant, Conrad and Kepley, 2012, Senouvo, et al., 2011). EETs can be further metabolized by sEH to DHETs, which possess much lesser biological activities than EETs (Lee, et al., 2013). Therefore, inhibition of sEH might potentiate beneficial effects of EETs. TPPU is one of the most recently synthesized, stable, potent sEH inhibitor that has been shown to stabilize EETs *in vivo* (Ulu, et al., 2012). Previous studies showed that TPPU inhibits inflammatory and cardiac fibrosis through inhibition of sEH (Liu, Lin, Qiu, Morisseau, Rose, Hwang, Chiamvimonvat and Hammock, 2013a, Sirish, Li, Liu, Lee, Hwang, Qiu, Zhao, Ma, Lopez, Hammock and Chiamvimonvat, 2013). In this study, we used TPPU to inhibit sEH activity and to determine whether sEH inhibition could provide the protective effects against bleomycin-induced PF in a mouse model and cultured fibroblasts.

The bleomycin challenge is a well-established model of murine pulmonary fibrosis and has been used to investigate the pathophysiology of idiopathic PF. Bleomycin is a chemotherapeutic agent that has been used for the treatment of squamous cell carcinomas, esophageal carcinoma, germ cell ovarian tumors, and gestational trophoblastic neoplasms. It is well recognized that a subset of patients who received bleomycin have developed pulmonary fibrosis in a time- and dose-dependent manner. Thus, many studies have used it to produce pulmonary fibrosis in animal models. A single intratracheal injection of bleomycin is the most prevalent stimulus to cause PF in mice, and its relative simplicity makes this approach very popular. In our study, we established the mouse pulmonary fibrosis model using a single intratracheal injection of bleomycin. Similar to previous observations, the lung tissue structural damage, inflammatory cells infiltration, and obvious edema were observed 7 days after bleomycin injection (Liu, Wan, Han, Li, Feng, Yue, Huang, Chen, Cheng, Li and Luo, 2013b). In addition, the Masson's trichrome stain results showed significant collagen deposition. Inhibition of sEH by TPPU treatment significantly attenuates the bleomycin-induced inflammation and PF illustrated by improvement of the morphological change and a decrease in collagen deposition. Thus, the CYP epoxygenase pathway might be involve in the pathological process of bleomycin-induced PF in mouse. Furthermore, inhibiting degradation of EETs with the sEH inhibitor, TPPU might provide a potential therapeutic approach for the treatment of IPF.

In view of the importance of fibroblast functions in the development of pulmonary fibrosis, we further explored the effect of TPPU on fibroblast function. The results of the MTT assay showed that TPPU inhibited the fibroblast proliferation. The analysis of flow cytometry indicated that TPPU treatment reduced the ratio of the number of cells in S phase, which represents the percentage of cells in DNA synthesis. These results suggest that TPPU inhibited proliferation of the mouse fibroblast via the inhibition of DNA synthesis.

Myofibroblasts differentiated from fibroblasts have contractile functions with histological characteristics of increased expression of the α-SMA and collagen synthesis. TGF-β1 is one of the most potent cytokines that can induce the differentiation of fibroblasts. In our study, we found that TGF-β1 could increase the expression of α-SMA mRNA, collagen type I mRNA, and hydroxyproline content in the cell culture supernatant. Interestingly, TPPU treatment partially reversed the changes induced by hTGF-β1-stimulation. This finding indicated that sEH inhibition reduced the differentiation and collagen synthesis of mouse fibroblasts *in vitro*. Furthermore, TGF-β superfamily ligands bind to the type II receptor, which recruits and phosphorylates the type I receptor. The type I receptor then phosphorylates receptor-regulated Smads (R-Smads), which can now bind to the co-Smad, Smad4. R-Smad/co-Smad complexes accumulate in the nucleus where they act as transcription factors and participate in regulation of the target gene expression. Thus, Smad proteins are essential components of the intracellular signaling pathway activated by TGF-β1 in TGF-β1-induced fibrosis. Chen et al. (Chen, et al., 2011) investigated the overexpression of CYP2J2 epoxygenase on diabetic nephropathy in streptozotocin-induced diabetic mice. They found that overexpression of CYP2J2 attenuates Smad3 phosphorylation, and increases the expression of Smad7. Kim et al. found that loss of sEH (Kim, Imig, Yang, Hammock and Padanilam, 2014) or using of sEH inhibitor (Kim, et al., 2015) promotes anti-inflammatory and fibroprotective effects in unilateral ureteral obstruction kidneys via activation of PPAR isoforms and downregulation of NF-κB, TGF-β1/Smad3 and inflammatory signaling pathways. It is possible that TPPU inhibits TGF-β1-induced functional changes in mouse fibroblast by potentially regulating the TGF-β1/Smad3 and PPAR signaling pathways.

In summary, we found that TPPU attenuated bleomycin-induced pulmonary inflammation and fibrosis in the mouse *in vivo*, and demonstrated that TPPU inhibited the proliferation, differentiation, and collagen synthesis of mouse fibroblasts *in vitro* (Fig. 10). TPPU inhibits conversion of EETs to their diols, and exerts anti-inflammatory and anti-fibrotic effects. Inhibition of sEH might be a novel approach for the treatment of pulmonary fibrosis.

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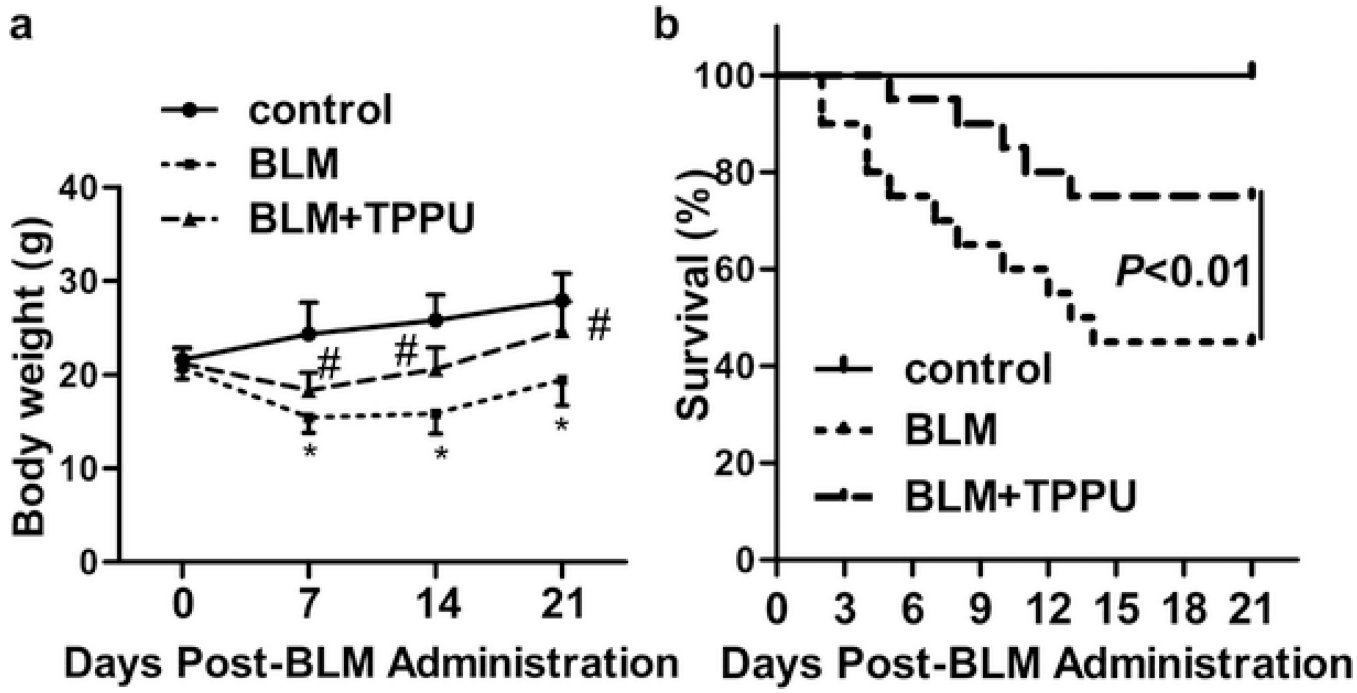

Fig 1. TPPU decreased bleomycin-induced body weight loss and mortality

The mice were treated once daily with TPPU (1 mg/kg) after bleomycin treatment (5 mg/kg). TPPU decreased (a) weight loss, and (b) mortality, 30% reduction at day 14 (n = 20 in each group)

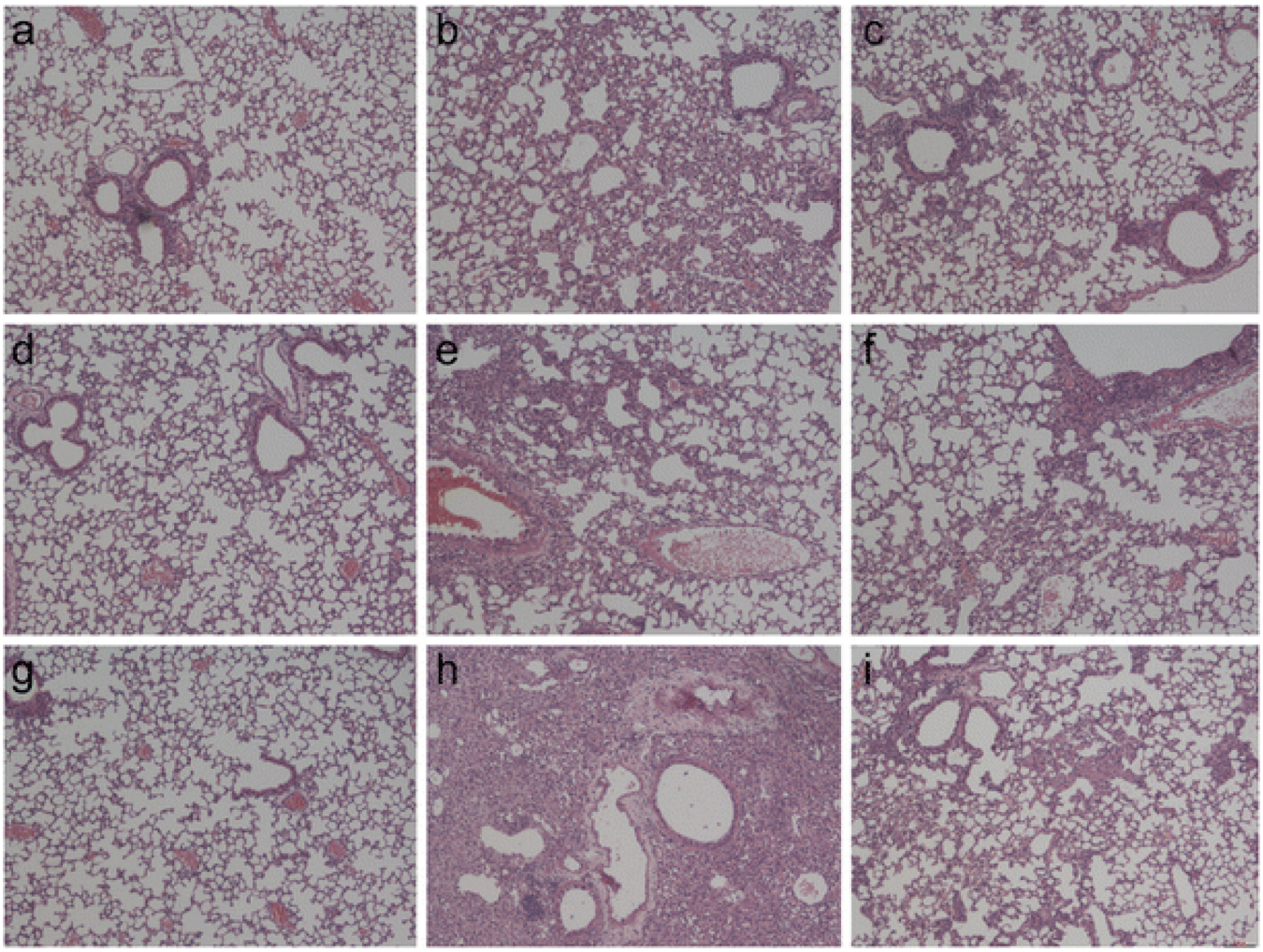

Fig 2. Representative histological lung sections from each group stained with Hematoxylin-Eosin

The lung tissues from Day 7, 14 and 21 after bleomycin administration with/without TPPU treatment were stained with Hematoxylin-Eosin (magnification: ×100, Size bar=100 m). **a, b, c** stained results at Day 7; **d, e, f** stained results at Day 14; **g, h, i** stained results at Day 21; **a, d, g** results of normal group; **b, e, h** results of BLM group; **c, f, i** results of BLM +TPPU group. Bleomycin-induced lung injury such as alveolar wall thickening and infiltration of inflammatory cells at day 7, 14 and 21 are illustrated. TPPU attenuated Bleomycin-induced pathological changes

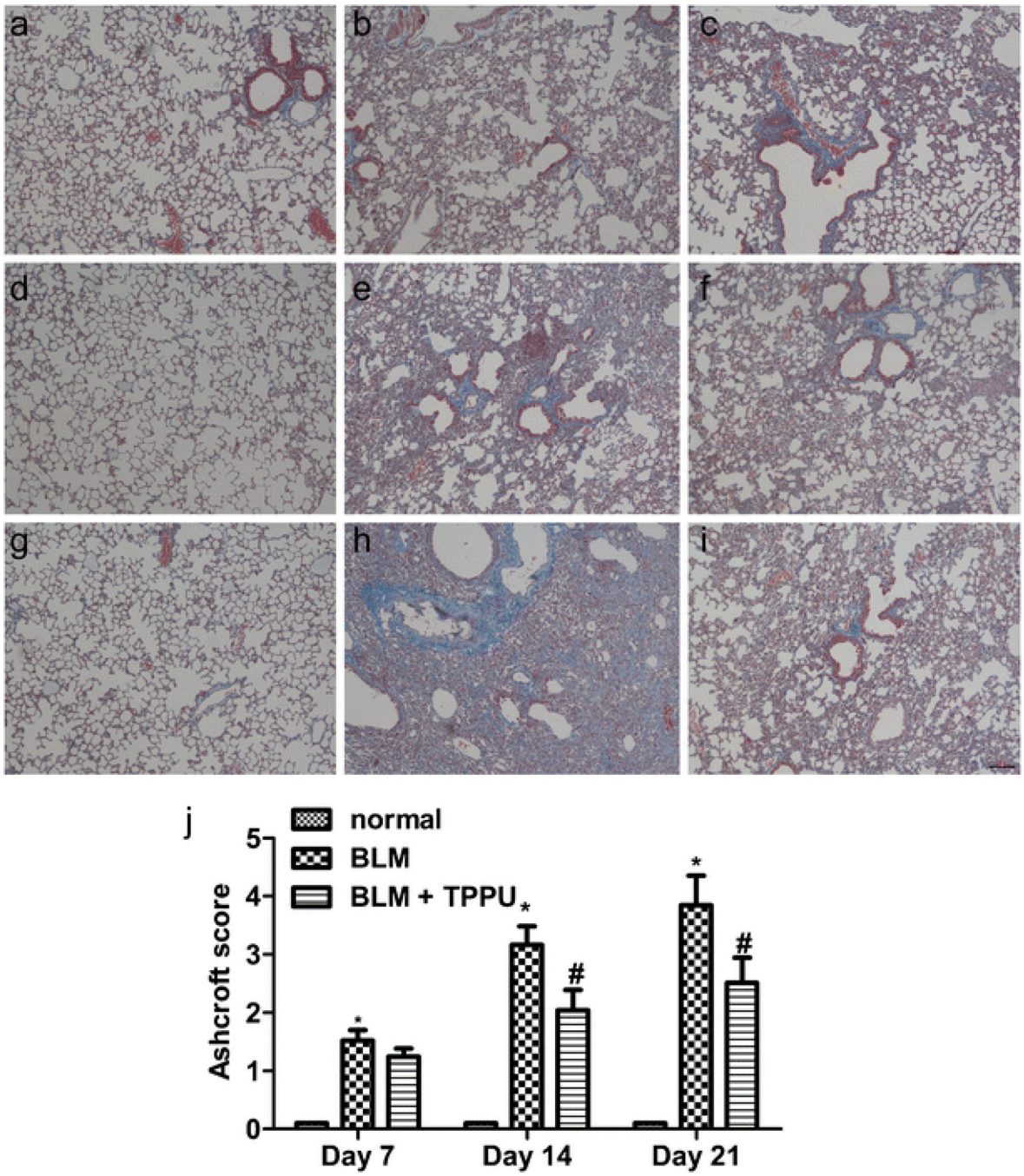

Fig 3. Representative results of lung sections from each group stained with Masson's trichrome stain

The lung tissues from day 7, 14 and 21 after bleomycin administration with/without TPPU treatment were stained with Masson's trichrome stain (magnification: ×100, Size bar=100 m). **a, b, c** stained results at Day 7; **d, e, f** stained results at Day 14; **g, h, i** stained results at Day 21; **a, d, g** results of normal group; **b, e, h** results of BLM group; **c, f, i** results of BLM +TPPU group. Increased collagen deposition is shown in bleomycin treated group at day 14, and peaked at day 21. TPPU treatment attenuated the collagen deposition. **j** semi-

quantitative analyses of lung tissue using the Ashcroft score. TPPU treatment significantly reduced the score at day14 and day21. * $P<0.05$ vs. normal group; # $P<0.05$ vs. BLM group

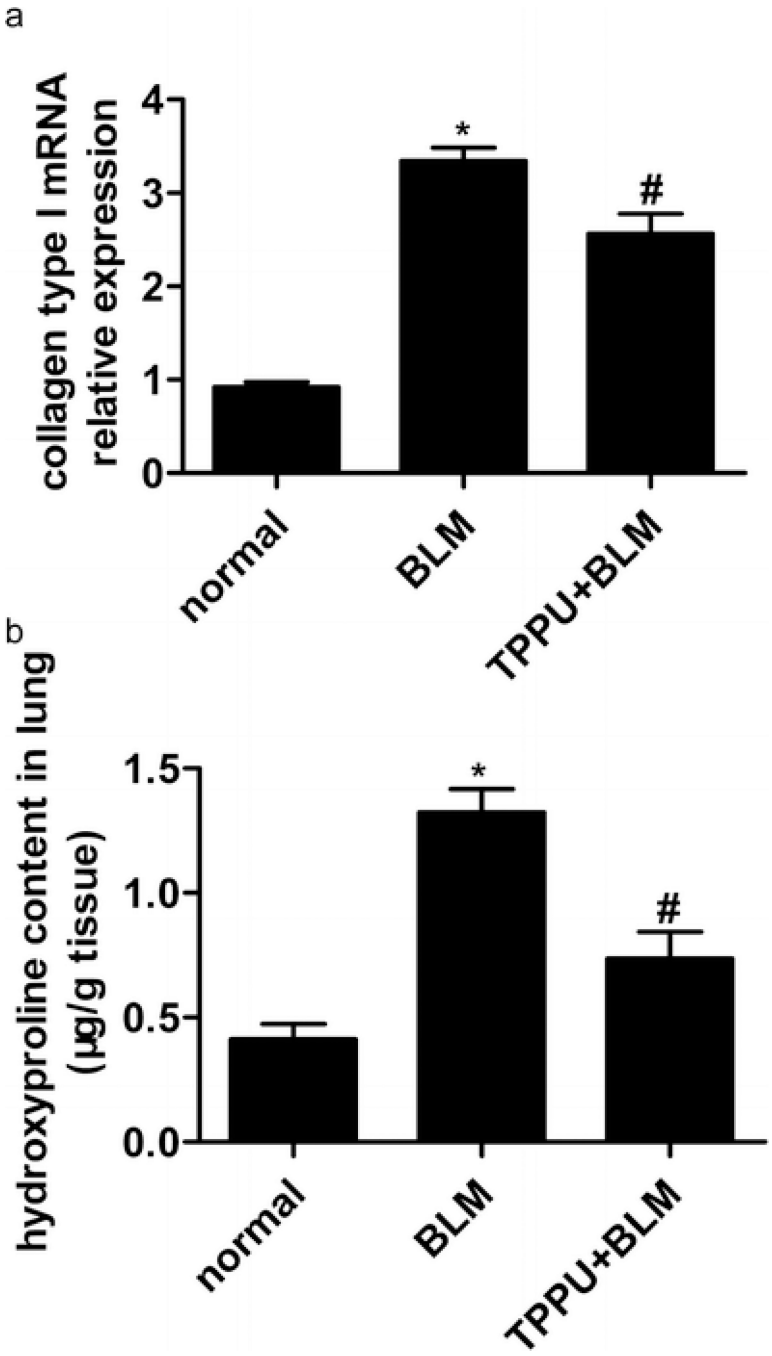

Fig 4. Effect of TPPU on bleomycin-induced collagen expression in the lung *in vivo*

a Collagen type I mRNA in lung of each group was tested by real-time PCR (n=8). **b** Hydroxyproline content was detected to reflect the collagen protein content in lung of each group (n=8). * $P<0.05$ vs. normal group; # $P<0.05$ vs. BLM group

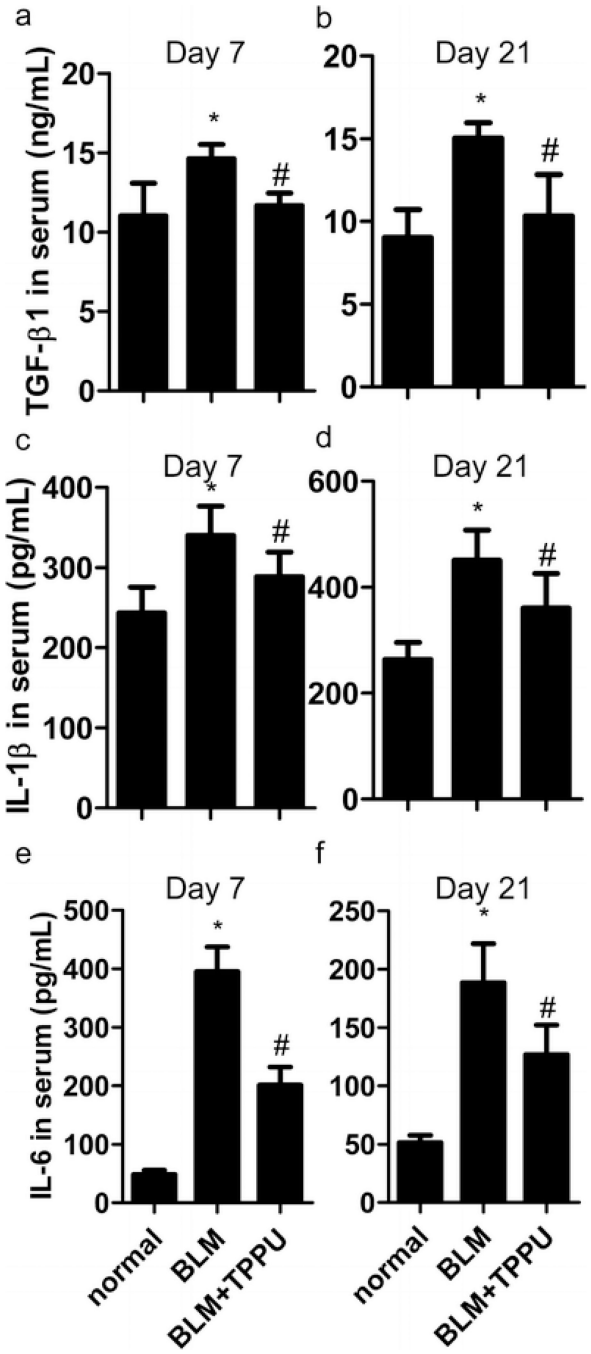

Fig 5. Effect of TPPU on bleomycin-induced production of pro-fibrotic and pro-inflammatory cytokines in serum

A, b the TGF-β1 concentration in serum of mice at day 7 and day 21 respectively. **C, d** the IL-1β concentration in serum of mice at day 7 and day 21. **e, f** the IL-6 concentration in serum of mice at day 7 and day 21. * $P<0.05$ vs. normal group; # $P<0.05$ vs. BLM group

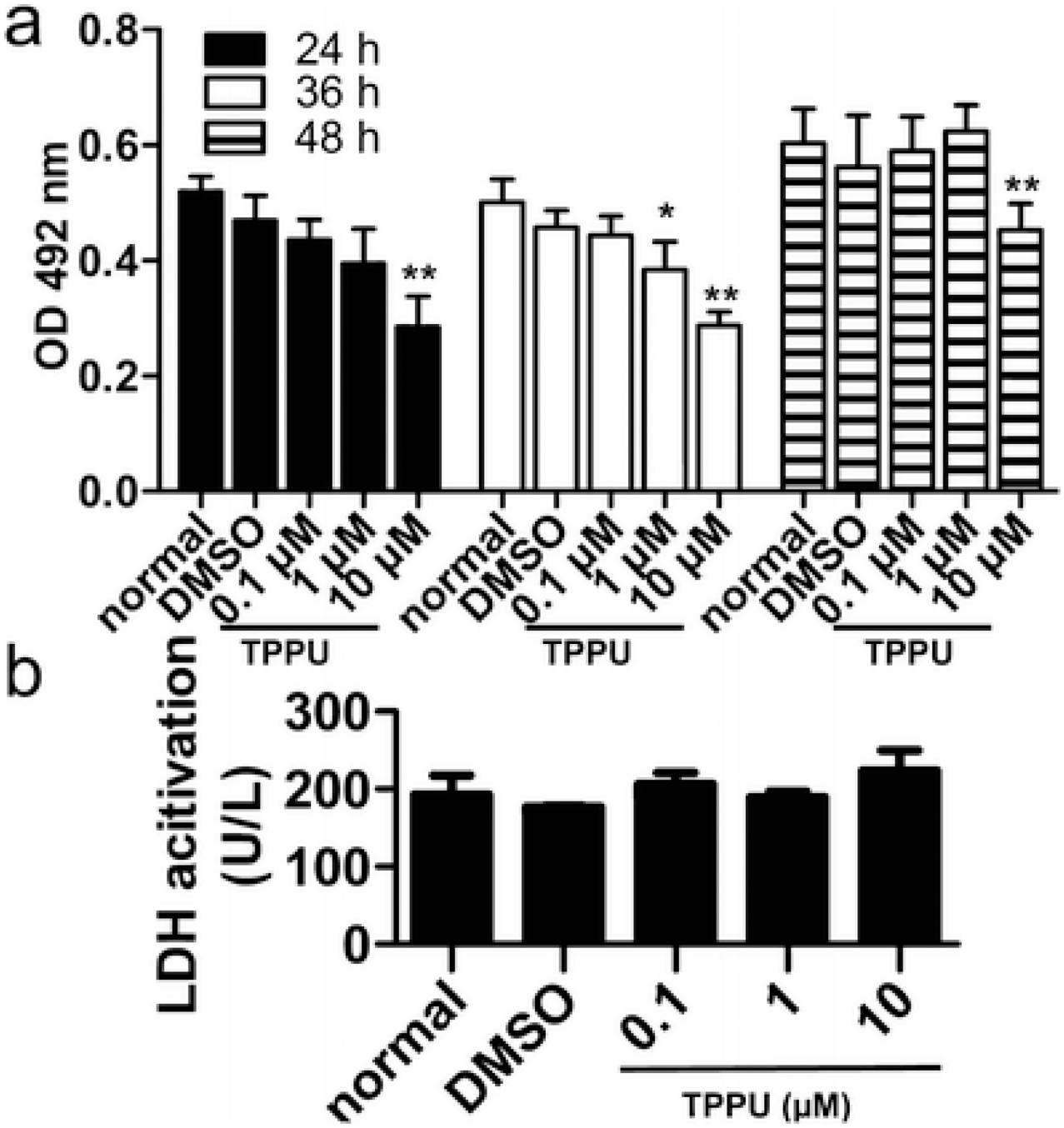

Fig 6. TPPU inhibits the proliferation of mouse fibroblasts without cytotoxicity

a MTT assay was employed to evaluate the effect of different concentrations of TPPU on the fibroblast proliferation. No significant changes on the proliferation of mouse fibroblasts were observed with 0.1 μM TPPU treatment. However, 1 μM TPPU treatment for 24 h and 36 h significantly reduced the OD492. At a concentration of 10 μM, TPPU treatment could significantly reduce the OD492 at three time points (24 h, 36 h, and 48 h). **b** Mouse fibroblasts were seeded in 12-well plate. After achieving confluency, the cells were incubated with TPPU at the indicated concentrations, and LDH release into the medium was

measured as described in *Materials and Methods*. Representative results are shown as above (n=8). * $P<0.05$ vs. DMSO group; ** $P<0.05$ vs. DMSO group

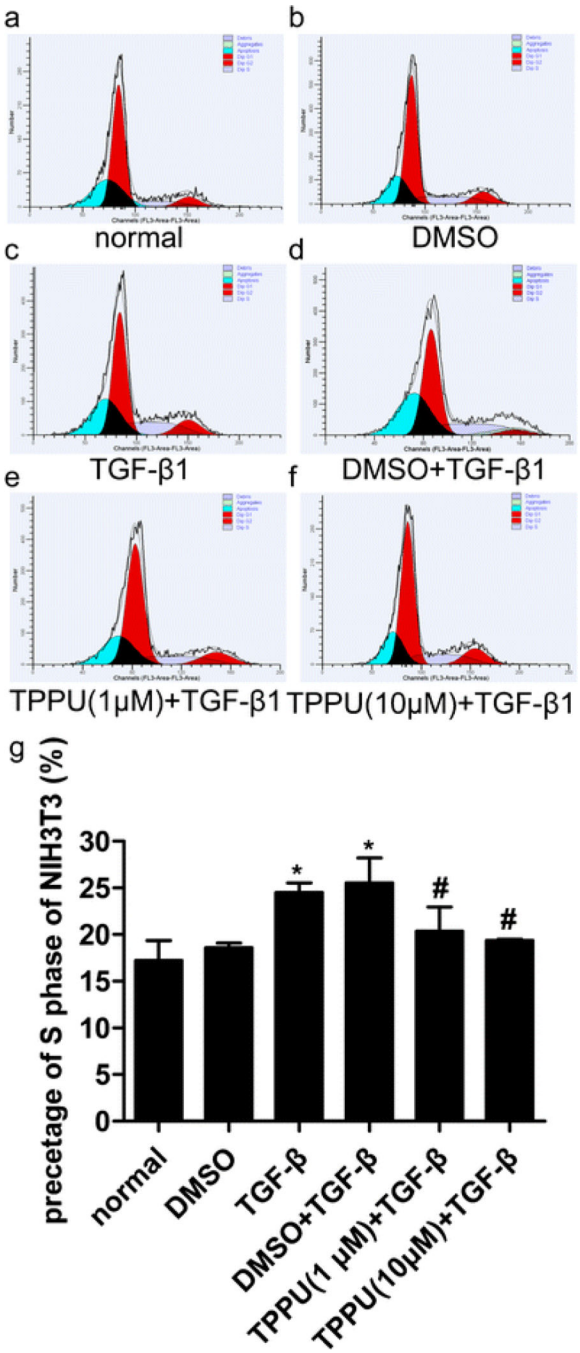

Fig 7. TPPU treatment reduces the percentage of S phase cells in TGF-β1-stimulated mouse fibroblasts

Mouse fibroblasts were treated with TPPU (1, 10 μM) followed by TGF-β1 stimulation (10 ng/mL) for 24 h. Cell cycles were detected using flow cytometry. Representative results are shown (n=3). * P<0.05 vs. normal/DMSO group; # P<0.05 vs. TGF-β1/ TGF-β1+DMSO group

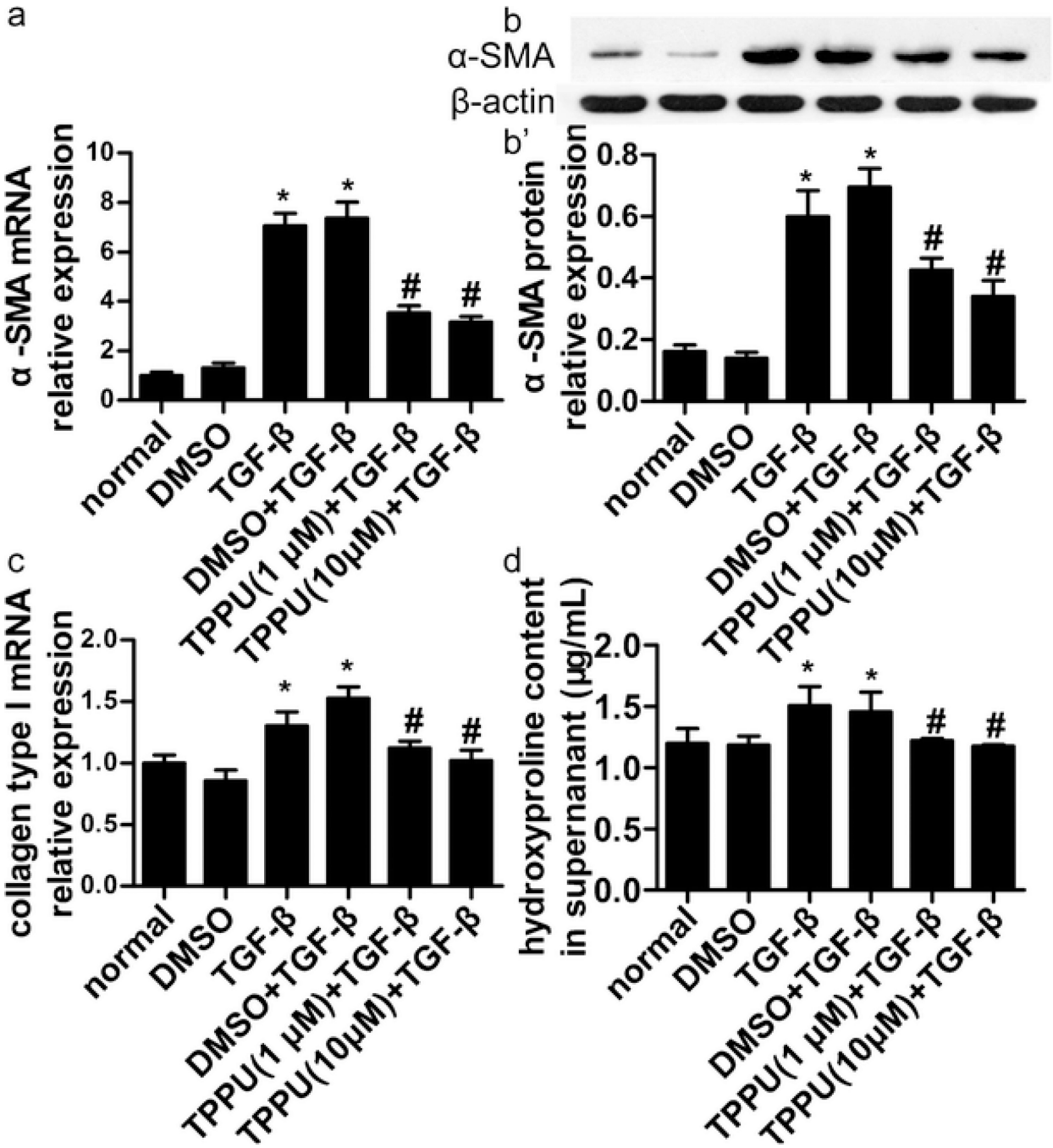

Fig 8. Effect of TPPU on TGF-β1-induced activation of mouse fibroblast

a, b TPPU treatment inhibited TGF-β1-induced α-SMA mRNA and protein expression in mouse fibroblasts. Mouse fibroblasts were treated with TPPU (1, 10 μM) followed by TGF-β1 stimulation (10 ng/mL). α-SMA mRNA and protein expression were detected by real-time PCR and western blot respectively. Representative results are shown. **c** TPPU inhibited TGF-β1-induced collagen type I mRNA expression. Collagen type I mRNA expression in mouse fibroblast was detected by real-time PCR. **d** TPPU inhibited TGF-β1-induced

hydroxyproline content in the supernatant of mouse fibroblast. (n=3). * $P<0.05$ vs. normal/ DMSO group; # $P<0.05$ vs. TGF-β1/ TGF-β1+DMSO group

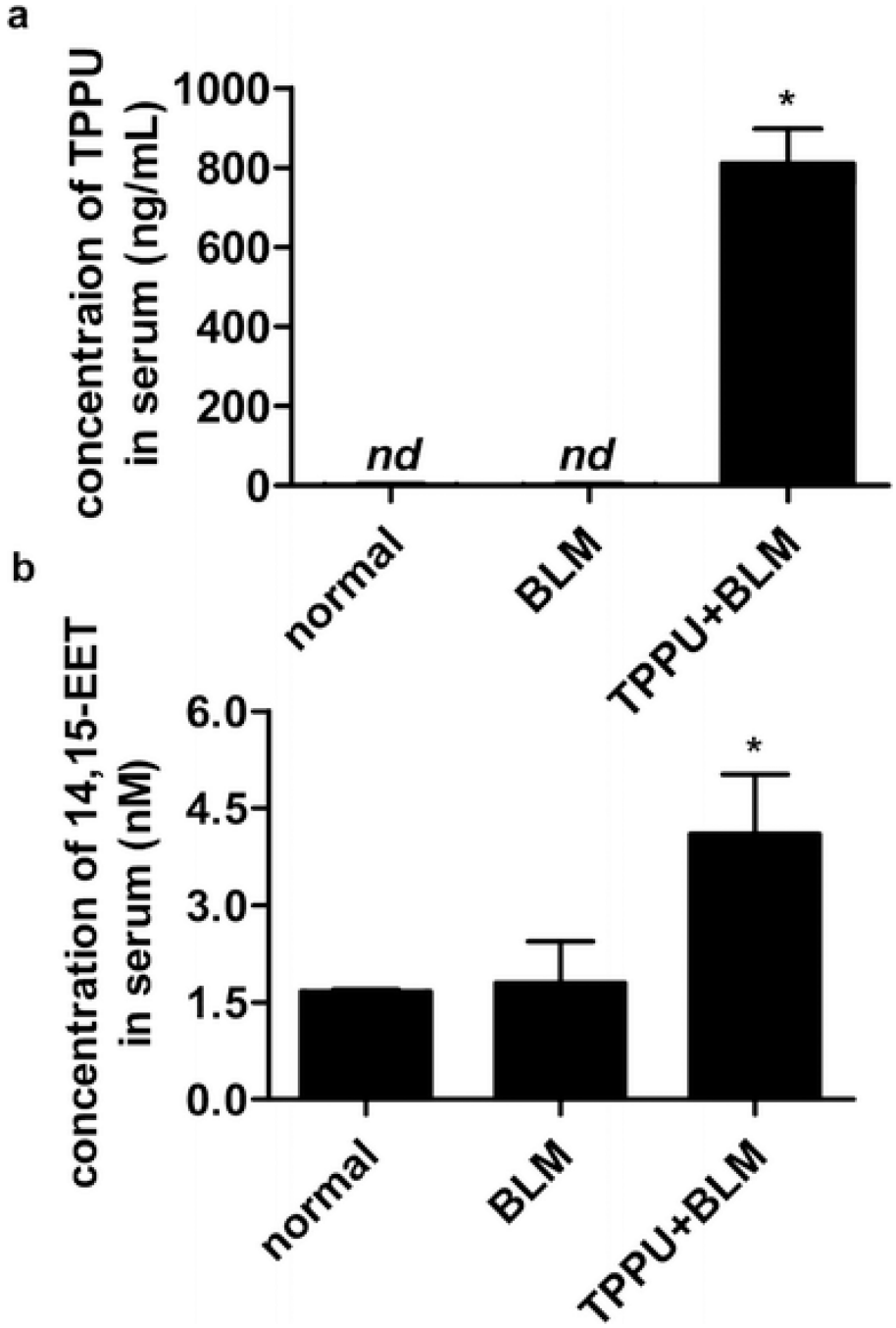

Fig 9. TPPU treatment increases endogenous 14,15-EET in plasma

a The concentration of TPPU was increased in the TPPU treatment group (n=8). * $P<0.05$ vs. normal group. **b** TPPU increased endogenous 14,15-EET in plasma. LC/MS/MS was used to analyze the lipid mediators, and the 14,15-EET of TPPU + bleomycin group was significantly higher than normal and BLM groups (n=8). * $P<0.05$ vs. normal group

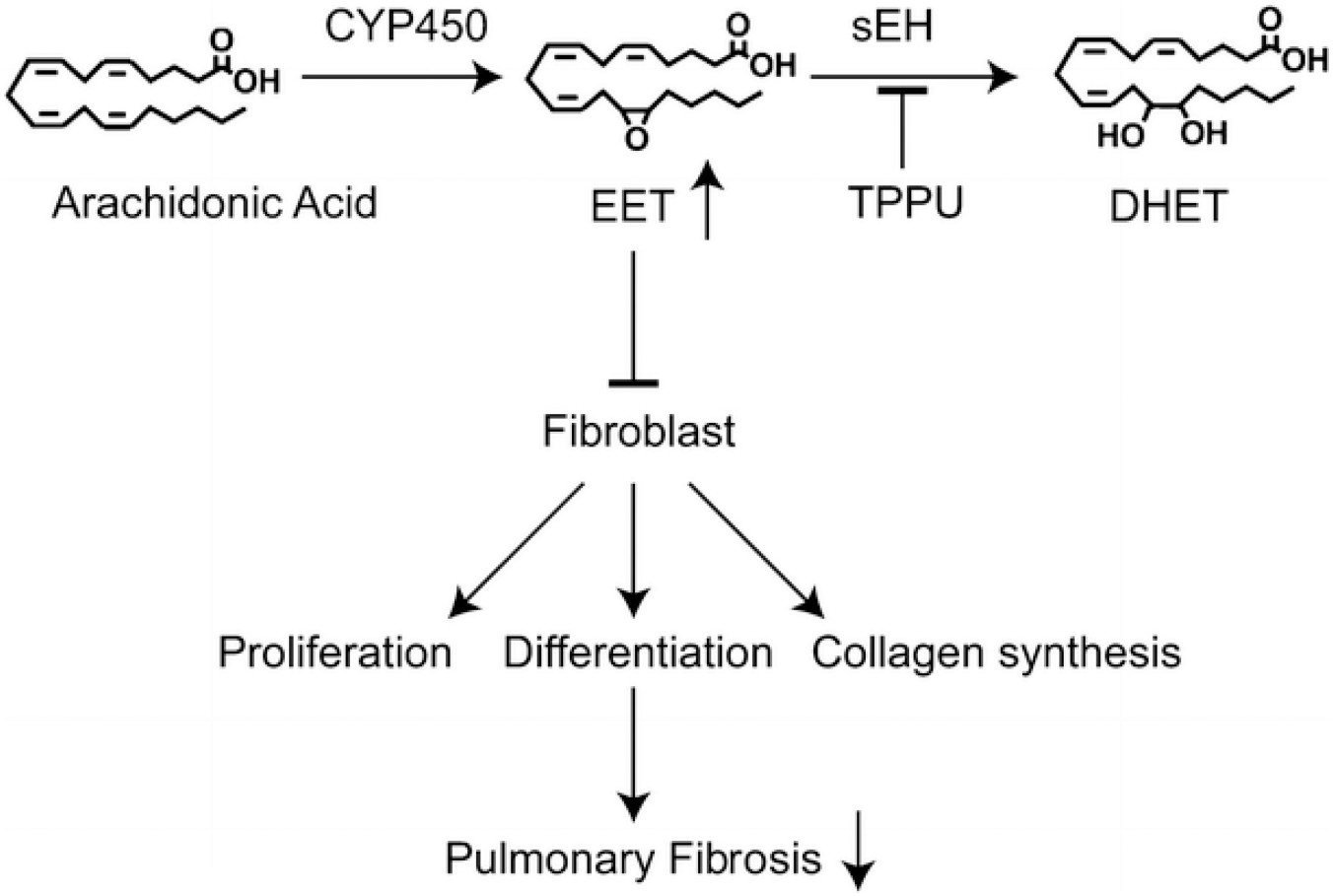

Fig 10. The proposed mechanisms of the protective effect of TPPU on bleomycin-induced pulmonary fibrosis

Table 1

Sequences of specific primers used in this study

Gene name	forward primer (5’ to 3’)	reverse primer (5’ to 3’)
procollagen I	GAGCGGAGAGTACTGGATCG	GCTTCTTTTCCTTGGGGTTC
α-*SMA*	CTGACAGAGGCACCACTGAA	CATCTCCAGAGTCCAGCACA
β-*actin*	TTCCAGCCTTCCTTCTTG	GGAGCCAGAGCAGTAATC