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ARTICLE

PI3K-Akt-mTOR/PFKFB3 pathway mediated lung fibroblast aerobic glycolysis and collagen synthesis in lipopolysaccharide-induced pulmonary fibrosis

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Abstract

Metabolic reprogramming plays a critical role in many diseases. A recent study revealed that aerobic glycolysis in lung tissue is closely related to pulmonary fibrosis, and also occurs during lipopolysaccharide (LPS)-induced sepsis. However, whether LPS induces aerobic glycolysis in lung fibroblasts remains unknown. The present study demonstrated that LPS promotes collagen synthesis in the lung fibroblasts through aerobic glycolysis via the activation of the PI3K-Akt-mTOR/PFKFB3 pathway. Challenging the human lung fibroblast MRC-5 cell line with LPS activated the PI3K-Akt-mTOR pathway, significantly upregulated the expression of 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 3 (PFKFB3), enhanced the aerobic glycolysis, and promoted collagen synthesis. These phenomena could be reversed by the PI3K-Akt inhibitor LY294002, mTOR inhibitor rapamycin, PFKFB3 inhibitor 3PO, or PFKFB3 silencing by specific shRNA, or aerobic glycolysis inhibitor 2-DG. In addition, PFKFB3 expression and aerobic glycolysis were also detected in the mouse model of LPS-induced pulmonary fibrosis, which could be reversed by the intraperitoneal injection of PFKFB3 inhibitor 3PO. Taken together, this study revealed that in LPS-induced pulmonary fibrosis, LPS might mediate lung fibroblast aerobic glycolysis through the activation of the PI3K-Akt-mTOR/PFKFB3 pathway.

Introduction

Pulmonary fibrosis is characterized by abnormal proliferation and activation of lung fibroblasts, accumulation of a large number of extracellular matrix, and the formation of excessive collagen deposition, which is a crucial pathological stage in the development of acute respiratory distress syndrome [1–3].

Lipopolysaccharide (LPS), an endotoxin component of Gram-negative bacilli, is a vital promoter of sepsis caused by Gram-negative bacilli infections as well as sepsis-related pulmonary fibrosis [4–6]. Aerobic glycolysis, which is also indicated as "Warburg effect", oxidizes glucose very quickly than oxidative phosphorylation, contributing to an increased production of lactate [7] and playing a major role in various diseases. Reportedly, aerobic glycolysis in lung tissues also plays a crucial role during pulmonary fibrosis [8–10] and LPS-induced sepsis [11]. However, whether LPS could directly induce aerobic glycolysis and collagen synthesis in lung fibroblasts remains unclear.

Our previous study revealed that LPS induces proliferation and activation of lung fibroblasts via the phosphatidylinositol-3-kinase-protein kinase B-mammalian target of rapamycin (PI3K-Akt-mTOR) pathway [12, 13]. Other studies also showed that PI3K-Akt-mTOR pathway activation was involved in the regulation of cellular aerobic glycolysis [14, 15], which is further associated with the expression and activation of the key enzyme 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 3 (PFKFB3) [16]. Therefore, we hypothesized that LPS might mediate aerobic glycolysis in the lung fibroblasts through activation of the PI3K-Akt-mTOR/

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PFKFB3 pathway, further promoting collagen synthesis and participating in the process of LPS-induced pulmonary fibrosis.

The present study utilized the cellular model of LPS-induced collagen synthesis in the lung fibroblasts and the mouse model of LPS-induced pulmonary fibrosis to verify the above hypothesis through genetic or pharmacological interventions of the PI3K-Akt-mTOR/PFKFB3 pathway.

Materials and methods

Ethics statement and animals

Eight-week-old male C57BL/6 mice weighing 23–25 g, were obtained from the Shanghai SLAC Laboratory Animal, China. All procedures of this study were carried out in accordance with the guidelines for animal care published by the United States' National Institutes of Health (NIH) for animal care (Guide for the Care and Use of Laboratory Animals, Department of Health and Human Services, NIH Publication No. 86–23, revised 1985). All animals were housed in a specific-pathogen-free environment at controlled room temperature (22–24 °C), under 12 h light/dark cycle and unrestricted food and sterilized water.

Reagents and antibodies

LPS (*Escherichia coli* O127:B8) was purchased from Sigma (USA). The primary antibodies used in this study were rabbit anti-PFKFB3 (ab181861, Abcam, USA), rabbit anti-β-actin (#8457 S, Cell Signaling Technology, USA), rabbit anti-p-Akt (#4060, Cell Signaling Technology, USA), rabbit anti-Akt (#4691, Cell Signaling Technology, USA), rabbit anti-p-mTOR (#2971, Cell Signaling Technology, USA), rabbit anti-mTOR (#2983, Cell Signaling Technology, USA), rabbit anti-Collagen I (ab34710, Abcam, USA), rabbit anti-α-SMA (ab32575, Abcam, USA), and mouse anti-TGF-β1 (sc-130348, Santa Cruz, USA). Also, goat anti-rabbit (A0208, Beyotime, China) and goat anti-mouse (A0216, Beyotime, China) secondary antibody was used. The PFKFB3 inhibitor 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO, S7639) and glycolysis inhibitor 2-Deoxy-D-glucose (2-DG, S4701) were purchased from Selleckchem (USA). The PI3K-Akt signaling pathway inhibitor LY294002 (#9901) and mTOR signaling pathway inhibitor rapamycin (#9904) were purchased from Cell Signaling Technology (CST).

Experimental design and treatment

Human lung fibroblasts MRC-5 cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and cultured in Minimum Essential Medium (MEM, Hyclone, USA) containing 10% fetal bovine serum (Gibco, USA), 100 IU/mL penicillin, and 100 IU/mL streptomycin at a 37 °C incubator with fully humidified atmosphere containing 5% CO_2.

MRC-5 cells were seeded into six-well culture plates at a density of 3×10^5 cells/well. After the cells adhered and reached 70% confluency, the medium was changed to serum-free MEM to starve the cells overnight. Then the cells were challenged with LPS (1 μg/mL). LY294002, rapamycin, 3PO, and PFKFB3-shRNA lentivirus (PFKFB3 shRNA) were used to inhibit PI3K-Akt, mTOR and PFKFB3 at the protein and gene levels. Cells and supernatants were collected at 6 or 48 h after the treatment.

Also, 2-DG, a glycolysis inhibitor, was added to inhibit LPS-induced aerobic glycolysis.

Animal experiments were carried on male C57BL/6 mice: 5 mg/kg LPS was injected intraperitoneally for 5 consecutive days to establish an LPS-induced pulmonary fibrosis model [17]. PFKFB3 inhibitor 3PO was injected intraperitoneally to inhibit the expression of PFKFB3. Mice were euthanized by overdose pentobarbital injection for the collection of lungs, blood, and bronchoalveolar lavage fluid (BALF) at days 3, 7, 14, and 28 after LPS injection.

Western blot

The levels of PFKFB3, Akt, mTOR, collagen I, α-SMA, TGF-β1 proteins, and the phosphorylation levels of Akt and mTOR were detected by western blot. The total protein from the cells were extracted with RIPA lysis buffer (Beyotime Biotechnology, China) containing 1% phenyl methyl sulfonyl fluoride, protease inhibitor cocktail and phosphatase inhibitor cocktail (KangChen, China). The supernatants were collected by centrifuging of the lysates at 12,000 *g* for 15 min at 4 °C. The protein concentrations were determined using the bicinchoninic acid assay kit (Thermo Scientific, USA) according to the manufacturer's instructions. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinyl fluoride membranes (Millipore, Germany). Then, the membranes were incubated with appropriate primary and secondary antibodies, respectively. The signals of the immunoreactive bands were detected by Image Lab™ software (Bio-Rad, USA) using Enhanced ECL Chemiluminescent Substrate Kit (Yeasen, China).

Real-time cell metabolism assay

XF-96 Extracellular Flux Analyzer (Seahorse Bioscience, Agilent Technologies, USA) was used to analyze real-time extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) according to the manufacturers' instructions. For ECAR, 10 mM glucose, 1 μM oxidative phosphorylation inhibitor oligomycin, and 100 mM

glycolytic inhibitor 2-DG were sequentially injected into each well at indicated time points. For OCR, 1 μM oligomycin, 1 μM reversible inhibitor of oxidative phosphorylation carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), and 1 μM mitochondrial complex I inhibitor rotenone plus the mitochondrial complex III inhibitor antimycin A (Rote/AA) were sequentially injected. Data were analyzed by Seahorse XF-96 Wave software.

Analysis of cell culture supernatants, plasma, and BALF

Cell culture supernatants were collected and centrifuged at 2000 *g* for 5 min. The supernatants were harvested and stored at −80 °C.

Blood samples were collected and placed in anticoagulation tubes, immediately centrifuging at 2500 *g* for 10 min, the supernatants were harvested and stored at −80 °C. Next, BALF samples were collected using a tracheal cannula by washing three times with 300 μL of cold PBS and the recovered BALF was pooled and centrifuged at 2500 *g* for 10 min. The supernatants were stored at −80 °C.

The levels of lactate in the cell culture supernatants, plasma, and BALFs were measured with the Lactate Assay Kit (Nanjing Jiancheng Bioengineering; Nanjing, China) according to the manufacturer's instructions.

Lentivirus transfection

MRC-5 cells were seeded in a six-well plate at a density of 8×10^4 cells/well. Lentivirus (Fubio Biological Technology, China) at a multiplicity of infection of 20 were added to the cells at 50% confluency. At 48 h post transfection, the medium was replaced by conventional culture medium and the green fluorescent protein was observed under a fluorescence microscope to assess the transfection efficiency. Then, the cells were selected by 2 μg/mL puromycin to establish a stable cell line. The level of PFKFB3 protein was measured by western blot.

The primers used were: PFKFB3, F-5′-AGCTGACTC GCTACCTCAAC-3′, and R-5′-GTTGAGGTAGCGAGTC AGCT-3′.

Pulmonary hydroxyproline (HYP) and lactate dehydrogenase (LDH) assays

HYP content, which is an indicator of pulmonary fibrosis based on the estimation of the total collagen deposition, and LDH, which is a key enzyme of the last step of glycolysis were measured using the Hydroxyproline Assay Kit (Nanjing Jiancheng Bioengineering; Nanjing, China) and LDH Assay Kit (Nanjing Jiancheng Bioengineering; Nanjing, China), respectively, according to the manufacturer's instructions.

Lung histopathology

Lung tissue samples were removed and fixed in 4% paraformaldehyde overnight, dehydrated, and embedded in paraffin. 5-μm thick sections were stained with hematoxylin and eosin (H&E) to evaluate lung morphological changes or Masson's trichrome staining and Picrosirius Red Staining to evaluate collagen content and distinguish type I and type III collagen fibers, respectively, as previously described [18, 19]. Aschcroft score was performed to assess the grading of pulmonary fibrosis [20].

Statistical analysis

All data were analyzed using GraphPad Prism 7 Software (USA). The quantitative data were expressed as mean ± standard deviation (SD). One-way ANOVA was used to compare the multiple groups. Two-tailed Student's *t* test was used to compare the two groups. $P < 0.05$ was considered statistically significant.

Results

Aerobic glycolysis mediated LPS-induced collagen synthesis in lung fibroblasts

To investigate the effects of aerobic glycolysis on LPS-induced collagen synthesis in lung fibroblasts, 2-DG, an inhibitor of aerobic glycolysis, was applied. MRC-5 cells were treated with LPS (1 μg/mL) for 48 h. We used ECAR to measure lactate production, a representation of the glycolytic rate. Compared with the control group, LPS treatment led to the continuous growth of ECAR in the lung fibroblasts, indicating enhanced glycolysis of the cells. OCR, a surrogate for mitochondrial respiratory activity, decreased after LPS treatment, which indicated a transition from oxidative phosphorylation to aerobic glycolysis in lung fibroblasts, and these effects were inhibited by 2-DG treatment (Fig. 1a, b). Furthermore, lactate, the end product of aerobic glycolysis, also increased after LPS treatment and could be inhibited by 2-DG pretreatment (Fig. 1c). 2-DG pretreatment also prevented the LPS-induced increased expression of collagen I (Fig. 1d). These findings indicated that aerobic glycolysis could mediate LPS-induced collagen synthesis in the lung fibroblasts in vitro.

Inhibition of PFKFB3 precludes LPS-induced aerobic glycolysis and collagen synthesis in lung fibroblasts

PFKFB3 is a key enzyme regulating aerobic glycolysis, and its expression and activation are directly related to the initiation of aerobic glycolysis. To investigate whether

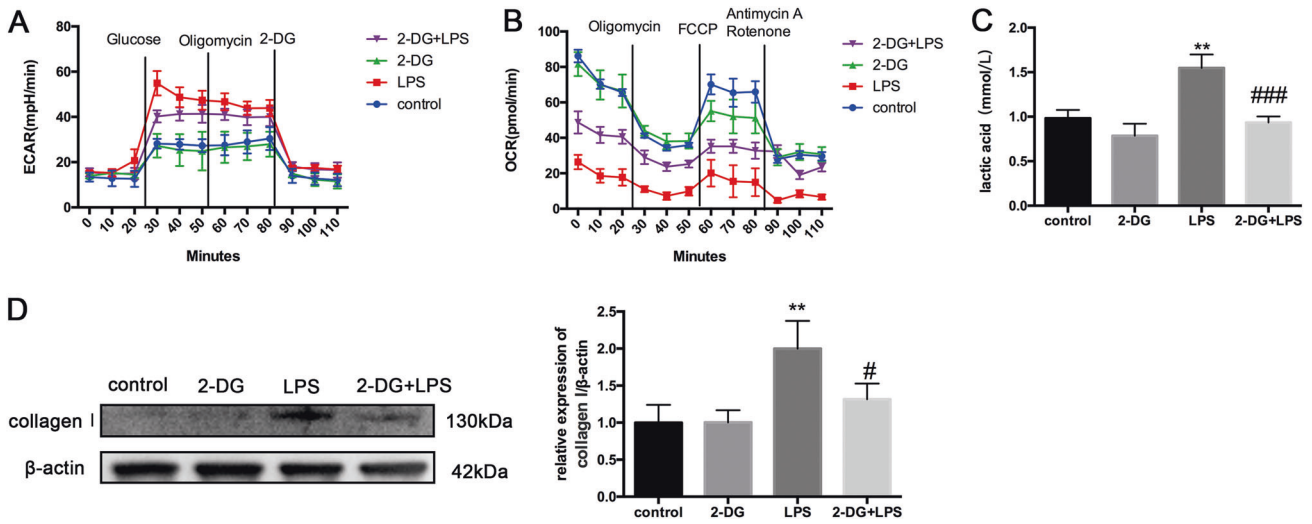


Fig. 1 Aerobic glycolysis mediated LPS-induced collagen synthesis in lung fibroblasts. Human lung fibroblasts MRC-5 cell line was treated with or without 1 μg/mL LPS for 48 h in the absence or presence of the inhibitor 2-DG (0.5 mM for 2 h prior to LPS or PBS administration), followed by sequential treatments with glucose, oligomycin, and 2-DG for measuring ECAR (**a**) and treatment with oligomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), and antimycin A with rotenone for measuring OCR (**b**). The lactate level in the culture medium was measured using the Lactate Assay Kit (**c**). The expression of collagen I after treatment with 1 μg/mL LPS was measured by western bolt (**d**). Values are mean ± SD from triplicate experiments. $^{**}P < 0.01$ vs. control group; $^{\#}P < 0.05$ vs. LPS group; $^{\#\#\#}P < 0.001$ vs. LPS group.

PFKFB3 is essential in LPS-induced collagen synthesis in lung fibroblasts, we first used 3PO, a selective inhibitor of PFKFB3. As shown in Fig. 2a, the pretreatment with 3PO significantly decreased the PFKFB3 level. Furthermore, pretreatment with 3PO inhibited the aerobic glycolysis in the lung fibroblasts as indicated by the lower levels of ECAR, higher levels of OCR, and less production of lactate (Fig. 2b–d), as well as inhibited lung fibroblasts collagen synthesis (Fig. 2e).

To further investigate the role of PFKFB3, PFKFB3-shRNA lentivirus (PFKFB3 shRNA) was used to knock down the expression of PFKFB3 in the lung fibroblasts. As shown in Fig. 3a, the transfection efficiency for MRC-5 cell lines was more than 90%, and the expression of PFKFB3 was significantly decreased in PFKFB3-shRNA group (Fig. 3b) than the empty vector group (control group) with or without LPS stimulation. Similar to the effect of PFKFB3 inhibitor 3PO, PFKFB3 shRNA transfection precluded the LPS-induced lung fibroblasts aerobic glycolysis (Fig. 3c–e) and collagen synthesis (Fig. 3f).These findings suggested that LPS induces lung fibroblasts aerobic glycolysis and collagen synthesis in the lung fibroblasts through PFKFB3.

Inhibition of the PI3K-Akt-mTOR pathway precludes LPS-induced PFKFB3 expression, as well as aerobic glycolysis and collagen synthesis in lung fibroblasts

As shown in Fig. 4a, b, LPS promoted the activation of the PI3K-Akt-mTOR pathway detected by the phosphorylation of Akt (p-Akt) and mTOR (p-mTOR) as well as the expression of PFKFB3 at 6 h after LPS treatment, which could be inhibited by application of PI3K-Akt inhibitor LY294002, and mTOR inhibitor rapamycin, respectively.

Futhermore, this inhibition precluded the LPS-induced aerobic glycolysis and collagen synthesis in the lung fibroblasts as indicated by the decreased levels of ECAR (Fig. 4c), elevated levels of OCR (Fig. 4d), less lactate production (Fig. 4e, f), and downregulation of the expression of collagen I (Fig. 4g, h). Therefore, we speculated that inhibition of the PI3K-Akt-mTOR pathway precludes LPS-induced PFKFB3 expression as well as aerobic glycolysis and collagen synthesis in the lung fibroblasts.

Inhibition of PFKFB3 precludes LPS-induced lung aerobic glycolysis and pulmonary fibrosis in vivo

To investigate whether pharmacological inhibiting PFKFB3 expression in vivo affects LPS-induced aerobic glycolysis and pulmonary fibrosis, mice were intraperitoneally pretreated with 3PO (70 mg/kg) followed by LPS (5 mg/kg) or the same dose of saline injection for 5 consecutive days. We found that the survival rate of each group were 100%, 100%, 75%, and 90% (control, 3PO, LPS, 3PO + LPS), respectively. At 7 days after LPS treatment, we found that PFKFB3 was inhibited in the mouse lung tissue by intraperitoneal injection of 3PO with or without LPS injection (Fig. 5a). In addition, 3PO pretreatment before LPS injection also inhibited the upregulation of LDH activity in lung tissue and the lactate content in the plasma and BALF (Fig. 5b–d), indicating that

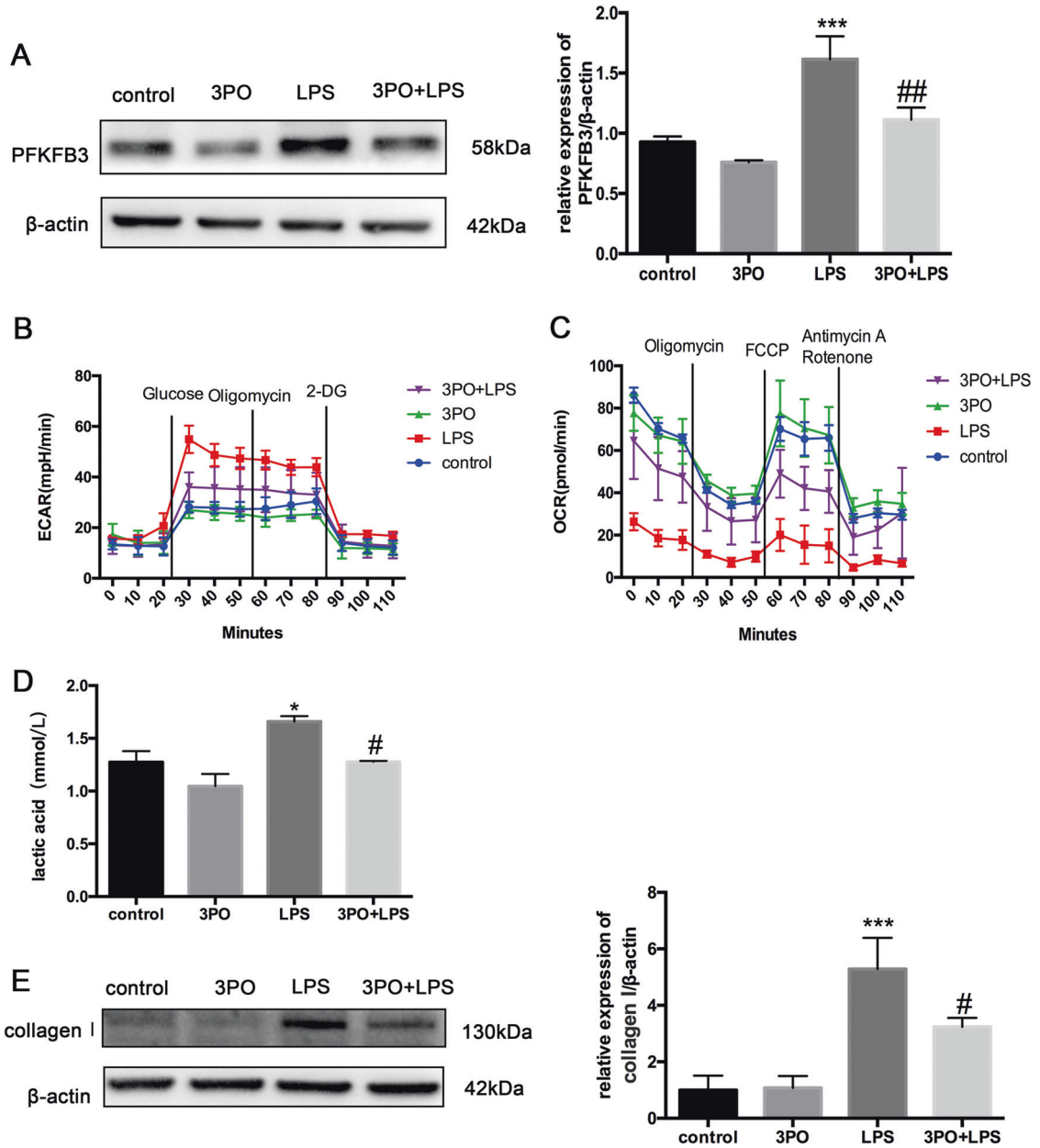


Fig. 2 Pharmacological inhibition of PFKFB3 precludes LPS-induced aerobic glycolysis and collagen synthesis in lung fibroblasts. PFKFB3 in lung fibroblasts stimulated with or without 1 μg/mL LPS for 6 h in the absence or presence of the inhibitor 3PO (10 μM for 1 h prior to LPS or PBS administration) was detected by western blot (**a**). The status of cellular metabolism was measured by ECAR and OCR (**b**, **c**). The lactate level in the culture medium was measured with the Lactate Assay Kit (**d**). The expression of collagen I was measured by western blot (**e**). Values are mean ± SD from triplicate experiments. $^{*}P < 0.05$ vs. control group; $^{***}P < 0.001$ vs. control group; $^{\#}P < 0.05$ vs. LPS group; $^{\#\#}P < 0.01$ vs. LPS group.

pharmacologically inhibition of PFKFB3 precludes LPS-induced lung aerobic glycolysis.

To fully clarify the effect of 3PO on LPS-induced pulmonary fibrosis model, HE and Masson staining were performed at 3, 7, 14, and 28 days after LPS treatment and we found that mild pulmonary fibrosis was observed at 3, 7, and 14 days after LPS treatment, and typical pulmonary fibrosis was detected at 28 days, which could be reversed by 3PO pretreatment (Fig. S1A,B). So we selected 28 days as an appropriate time points to evaluate typically pulmonary fibrosis. Western blot analysis of collagen I, α-SMA and TGF β1 (Fig. 5e), hydroxyproline measurements (Fig. 5f) and pathological analysis by HE, Masson, and Picrosirius Red staining (Fig. 5g, h) at 28 days after LPS injection suggested that pharmacologically inhibition of PFKFB3 prevents LPS-induced pulmonary fibrosis in vivo.

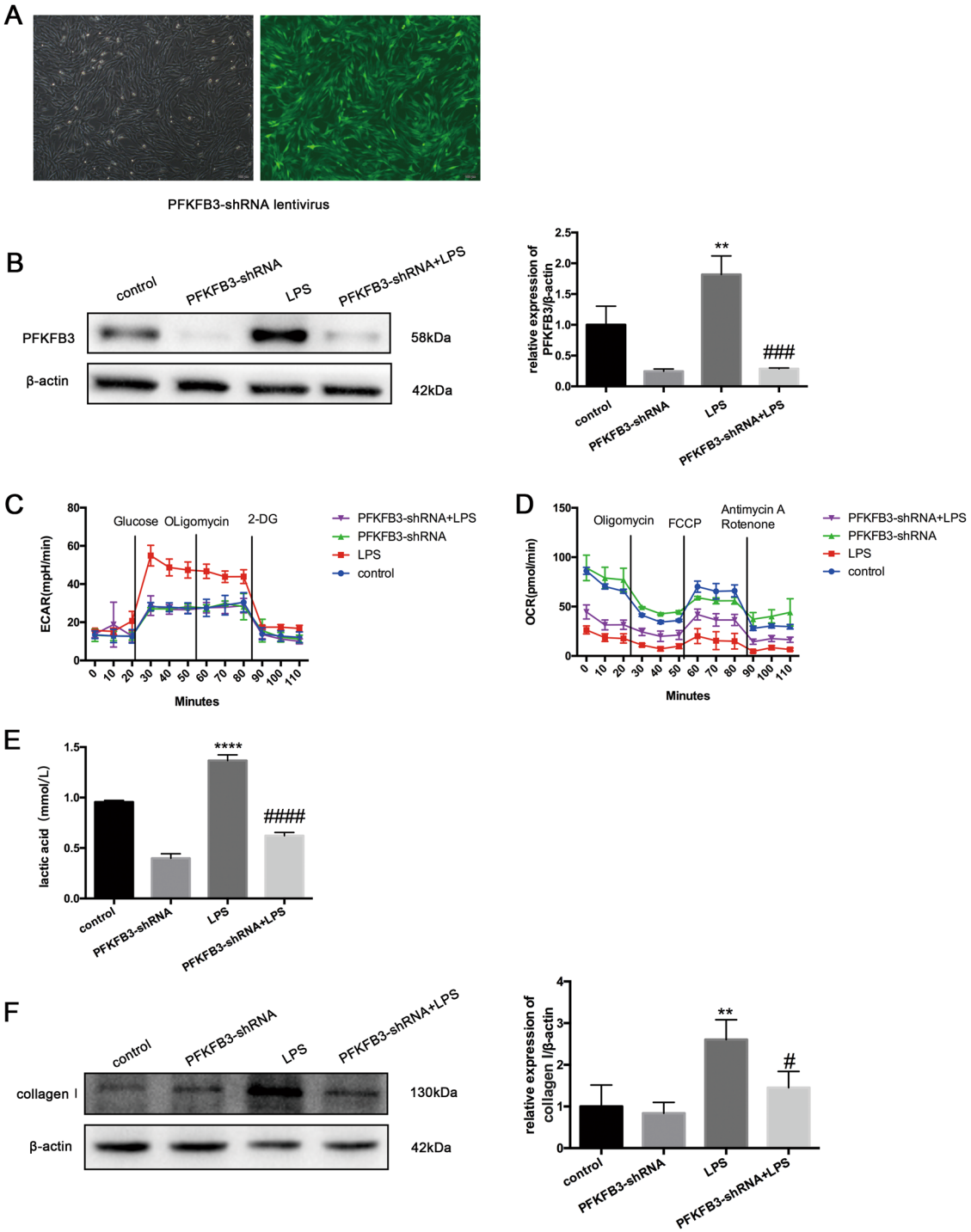


Fig. 3 Genetic inhibition of PFKFB3 precludes LPS-induced aerobic glycolysis and collagen synthesis in lung fibroblasts. Fluorescence labeling indicated the transfection efficiency for MRC-5 transfected by PFKFB3-shRNA lentivirus (**a**). Western blot was used to determine the expression of PFKFB3 in lung fibroblasts at 6 h after treatment in the absence or presence of 1 μg/mL LPS with PFKFB3 shRNA or the empty vector (**b**). PFKFB3 shRNA was applied to determine whether aerobic glycolysis (**c**–**e**) and collagen synthesis (**f**) in the lung fibroblasts were regulated by PFKFB3. Values are mean ± SD from triplicate experiments. $^{**}P < 0.01$ vs. control group; $^{****}P < 0.0001$ vs. control group; $^{\#}P < 0.05$ vs. LPS group; $^{\#\#\#}P < 0.001$ vs. LPS group; $^{\#\#\#\#}P < 0.0001$ vs. LPS group.

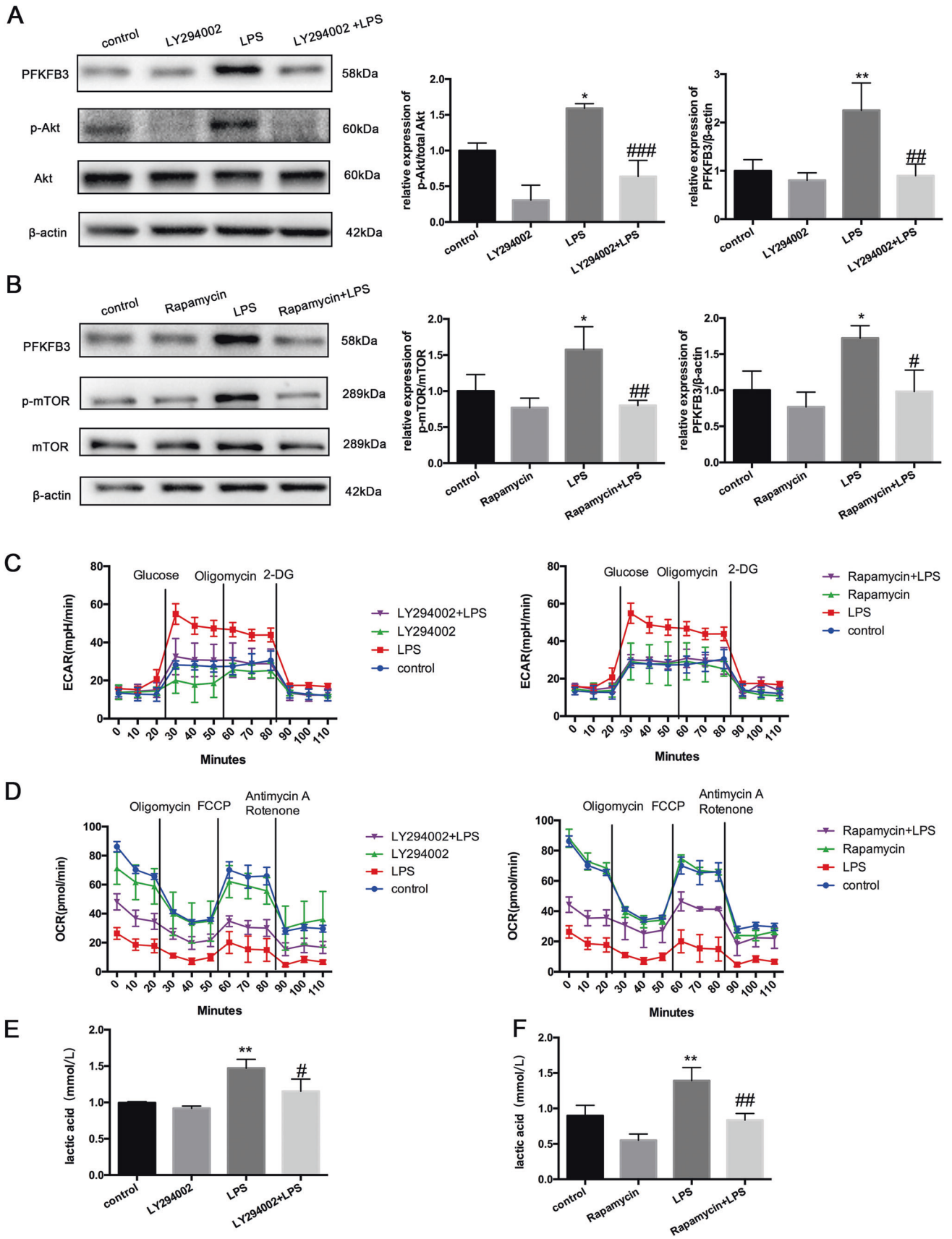


Fig. 4 (Continued)

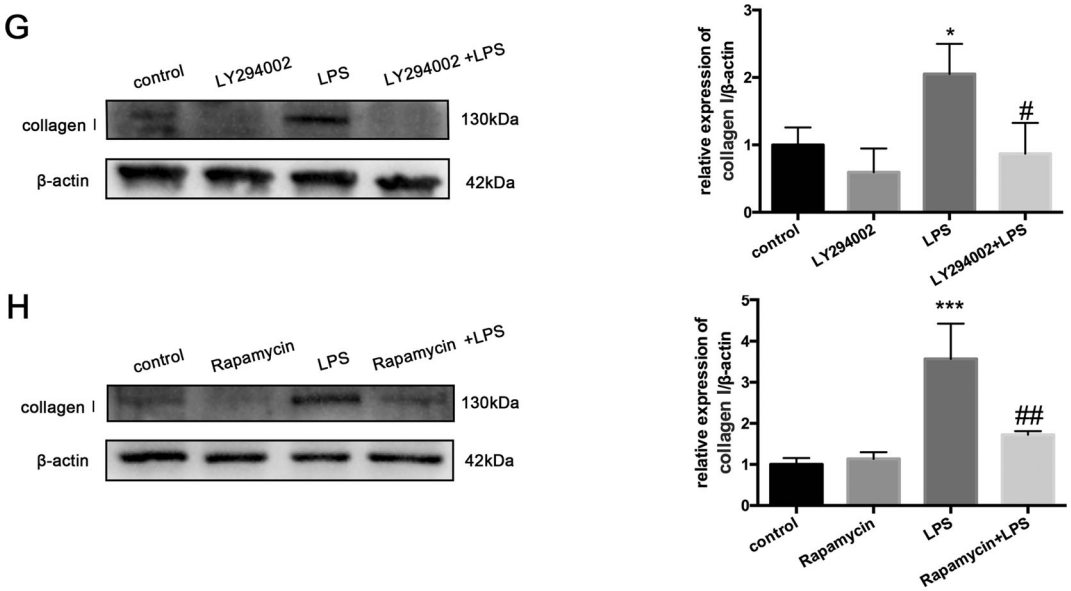


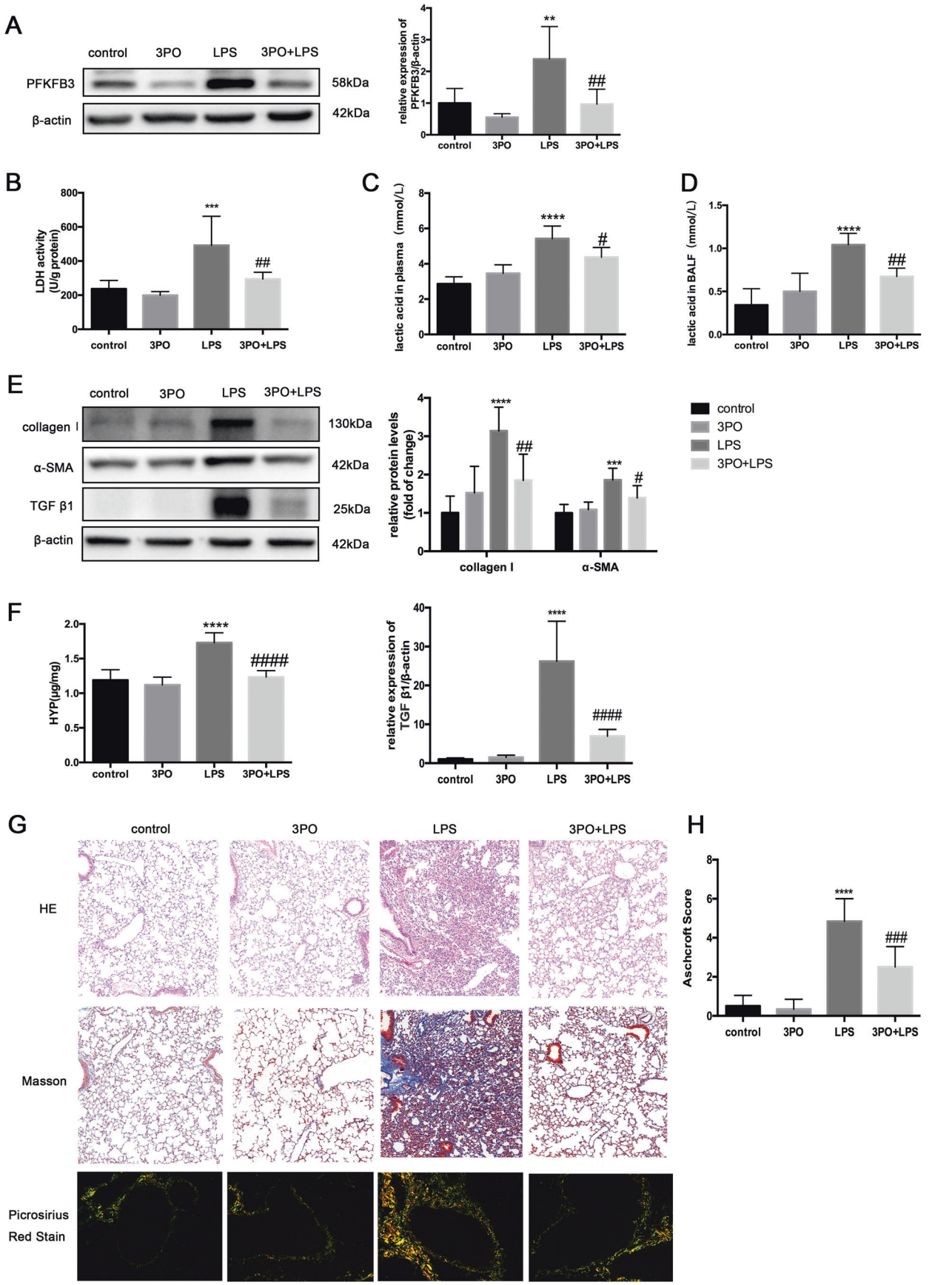
Fig. 4 Inhibition of the PI3K-Akt-mTOR pathway precludes LPS-induced PFKFB3 expression, as well as aerobic glycolysis and collagen synthesis in lung fibroblasts. The phosphorylation of Akt, total Akt, phosphorylation of mTOR, total mTOR, and PFKFB3 in lung fibroblasts at 6 h after treatment in the absence or presence of 1 μg/mL LPS with/without Akt or mTOR pathway inhibitors LY294002 (10 μM for 1 h prior to LPS or PBS administration) (**a**) and rapamycin(10 nM for 1 h prior to LPS or PBS administration) (**b**) were determined by western blot. Values are mean ± SD from triplicate experiments. LY294002 or rapamycin was applied to determine whether aerobic glycolysis (**c**–**f**) and collagen synthesis (**g**, **h**) in the lung fibroblasts were regulated by the PI3K-Akt-mTOR pathway. $^{*}P < 0.05$ vs. control group; $^{**}P < 0.01$ vs. control group; $^{***}P < 0.001$ vs. control group; $^{\#}P < 0.05$ vs. LPS group; $^{\#\#}P < 0.01$ vs. LPS group; $^{\#\#\#}P < 0.001$ vs. LPS group.

Discussion

It was reported that aerobic glycolysis in lung tissues is closely related to pulmonary fibrosis [21], but whether it plays a role in LPS-induced pulmonary fibrosis is largely unknown. The current study firstly confirmed both in vitro and in vivo that LPS directly induces aerobic glycolysis in the lung fibroblasts, which was mediated by the PI3K-Akt-mTOR/PFKFB3 pathway, followed by collagen synthesis in LPS-induced pulmonary fibrosis.

Previously, glycolysis was considered to be a special metabolic form to meet the energy requirements of cells in the absence of oxygen. However, aerobic glycolysis, first detected by Warburg, suggested that tumor cells also initiate aerobic glycolysis to produce lactate under aerobic conditions, which in turn, promotes the proliferation of the cells [22, 23]. Recent studies have shown that not only in tumor cells, aerobic glycolysis also exhibits and plays an important role in many diseases [24], such as sepsis [25, 26] and pulmonary fibrosis [21]. Also, LPS stimulates aerobic glycolysis in macrophages raises the level of lactate [25]. In addition, enhanced aerobic glycolysis occurs in TGF-β-induced lung fibroblasts activation [27]. However, it is not yet clear whether LPS directly induces aerobic glycolysis in the lung fibroblasts and whether it is associated with lung fibroblasts collagen synthesis. The current study revealed that LPS directly induces aerobic glycolysis and collagen synthesis at the cellular level in the lung fibroblasts, which could be inhibited by 2-DG, an inhibitor of aerobic glycolysis. These data indicated that LPS-induced aerobic glycolysis is a crucial part of collagen synthesis in the lung fibroblasts, but the underlying mechanisms are yet unknown.

PFKFB3 is one of the critical glycolytic enzymes that regulate the synthesis of fructose-2, 6-bisphosphate (Fru-2, 6-BP), which is the potent allosteric activator of glycolytic rate-limiting enzyme 6-phosphate fructose kinase 1 (6-phosphofructo-1-kinase, PFK-1). This enzyme converts fructose-6-phosphate (F6P) into fructose-1, 6-bisphosphate (Fru-1, 6-BP), and plays a key role in glycolysis [28]. Therefore, the expression and activation states of PFKFB3 are directly related to the initiation of aerobic glycolysis. It was reported that in pulmonary fibrosis, the expression of PFKFB3 is upregulated in lung tissues [21]. However, its function has not yet been clarified in LPS-induced pulmonary fibrosis. The current data confirmed that LPS upregulates the expression of PFKFB3 in both lung fibroblasts and fibrotic lungs while inhibiting the level of PFKFB3 with the pretreatment of 3PO or PFKFB3 shRNA precludes the LPS-induced aerobic glycolysis and pulmonary fibrosis. Thus, it can be speculated that PFKFB3 plays a key role in regulating LPS-induced aerobic glycolysis and pulmonary fibrosis.


A
control
3PO
LPS
3PO+LPS
PFKFB3
β-actin
58kDa
42kDa
relative expression of PFKFB3/β-actin
B
LDH activity (U/g protein)
C
lactic acid in plasma (mmol/L)
D
lactic acid in BALF (mmol/L)
E
collagen I
α-SMA
TGF β1
β-actin
130kDa
42kDa
25kDa
42kDa
relative protein levels (fold of change)
collagen I
α-SMA
F
HYP(μg/mg)
relative expression of TGF β1/β-actin
G
HE
Masson
Picrosirius Red Stain
H
Aschcroft Score

◀ **Fig. 5 Inhibition of PFKFB3 precludes LPS-induced lung aerobic glycolysis and pulmonary fibrosis in vivo.** Eight-week-old male C57BL/6 mice were pretreated with 3PO (70 mg/kg) for 2 days, followed by LPS (5 mg/kg) administration for consecutive 5 days. Western blot was performed to detect the expression of PFKFB3 in lung tissues (**a**). LDH activity in lung tissue was measured using the LDH Assay Kit (**b**). Both plasma and BALF lactate content were determined by the Lactate Assay Kit (**c**, **d**). Western blot was performed to detect the expression of collagen I, α-SMA, and TGF β1 (**e**). The severity of collagen deposition was measured by assessing hydroxyproline content (**f**). The severity of pulmonary fibrosis was measured by hematoxylin and eosin (**h**, **e**) staining (magnification, ×200); collagen deposition was assessed by Masson's trichrome staining (magnification, ×200); the type of fibers was distinguished by Picrosirius Red staining (magnification, ×400, orange and green colors indicate type I and III collagen, respectively) (**g**). Values are mean ± SD ($n = 6$). $^{**}P < 0.01$ vs. control group; $^{***}P < 0.001$ vs. control group $^{****}P < 0.0001$ vs. control group; $^{\#}P < 0.05$ vs. LPS group; $^{\#\#}P < 0.01$ vs. LPS group; $^{\#\#\#\#}P < 0.0001$ vs. LPS group.

Our previous study showed that LPS induces the proliferation and activation of lung fibroblasts through the activation of PI3K-Akt-mTOR pathway [12, 29]. As a significant signaling pathway, the activated PI3K-Akt-mTOR pathway plays a crucial role in the initiation of glycolysis [14], activation of cell proliferation [15, 30], as well as the occurrence of pulmonary fibrosis [15, 31]. It was reported that PFKFB3 could be upregulated by mTOR [32], and the PI3K-Akt-mTOR pathway was reported to be involved in the expression of PFKFB3 in liver [33]. This study confirmed that LPS activates the PI3K-Akt-mTOR pathway accompanied by the upregulation of PFKFB3 and aerobic glycolysis in the lung fibroblasts, which could be rescued by using PI3K-Akt-mTOR selective inhibitors, LY294002, and rapamycin, respectively. These results indicated that the PI3K-Akt-mTOR/PFKFB3 pathway plays a crucial role in LPS-induced lung fibroblasts aerobic glycolysis.

Taken together, the current study revealed that the PI3K-Akt-mTOR/PFKFB3 pathway mediates aerobic glycolysis in the lung fibroblasts during LPS-induced pulmonary fibrosis, and intervention of the activation of the PI3K-Akt-mTOR/PFKFB3 pathway to regulate the metabolism of lung fibroblasts could be an effective therapeutic target for LPS-induced pulmonary fibrosis.

In conclusion, this study demonstrated that LPS promotes collagen synthesis in the lung fibroblasts through aerobic glycolysis via activation of the PI3K-Akt-mTOR/PFKFB3 pathway in LPS-induced pulmonary fibrosis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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