Original Article
The role of the PI3K/AKT/mTOR protein synthesis pathway in the skeletal muscle atrophy induced by COPD

Yanli Li1*, Yan Li1*, Fengfeng Han1, Haiyang Yu1, Tianyun Yang1, Wenbin Guan2, Xuejun Guo1
Departments of 1Respiratory Medicine, 2Pathology, Xin Hua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, China. *Equal contributors and co-first authors.
Received November 14, 2015; Accepted February 2, 2016; Epub March 15, 2016; Published March 30, 2016

Abstract: Objective: To confirm the role of PI3K/AKT/mTOR protein synthesis pathway in skeletal muscle atrophy induced by chronic obstructive pulmonary disease (COPD), and to explore the possible targets in the signaling pathway inhibiting muscle atrophy. Methods: Rats exposed to chronic cigarette smoke (CS) were selected for our study, divided into two exposure groups sham-exposed and 12 weeks CS. Total RNA and protein were extracted from extensor digitorum longus muscle (EDLM) for Real-time PCR and Western blot analysis to assess the MHC expression. The proteins expression associated with PI3K/AKT/mTOR signaling pathway (PI3K, Akt, mTOR, GSK-3β, p70S6K1, and 4EBP1) were assessed by Western blot analysis. 2% FBS induces L6 cells to differentiate. IGF-1 (PI3K agonist) and 10% CSE exposed L6 myoblasts for 24 hours, then detect the expression of MHC, and the proteins associated with PI3K/AKT/mTOR signaling pathway. Results: Chronic CS exposure decreased the MHC expression. The phosphorylation levels of Akt, mTOR, GSK-3β, p70S6K1 and 4EBP1 were increased significantly after Chronic CS exposure, and no significant changes were observed in the protein levels of PI3K. CSE treatment also decreased MHC expression. PI3K agonist insulin-like growth factor (IGF-1) treatment increased the MHC expression in comparison with simple CSE treatment. Not only the phosphorylation levels of Akt, mTOR, GSK-3β, p70S6K1 and 4EBP1 decreased significantly after CSE treatment, but also PI3K. Moreover, IGF-1 increased the phosphorylation levels of the protein synthesis pathway in comparison with simple CSE treatment. Conclusion: Chronic CS exposure induces muscle atrophy and activates PI3K/AKT/mTOR signaling pathway. Using IGF-1 activates PI3K/AKT/mTOR signaling pathway inhibits L6 myoblasts atrophy induced by CSE.

Keywords: PI3K/AKT/mTOR, signaling pathway, skeletal muscle atrophy, L6 myoblasts, COPD

Introduction
Chronic obstructive pulmonary disease (COPD), a common preventable and treatable disease, is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways [1]. Cigarette smoking is the most common risk factor. The Global Burden of Disease Study projected that COPD will become the third leading cause of death worldwide by 2020; a newer projection estimated COPD will be the fourth leading cause of death in 2030 [2]. COPD is associated with significant economic burden. Not only do COPD causes pulmonary destruction, but also systemic effects, among which muscle atrophy is one of the most important. Muscle atrophy results in skeletal muscle dysfunction and respiratory muscle weakness, directly affecting the progression and prognosis of the disease [3]. So the research, of mechanisms of muscle atrophy in COPD, is vital to prevent muscle atrophy and improve the quality of life in patients with COPD. Multi-factors and multi-molecular mechanisms participate in muscle atrophy, causing imbalance between the rates of muscle protein synthesis and degradation, eventually resulting in muscle atrophy [4]. Most domestic and foreign studies [5-9] focused on the mechanism of protein degradation pathways in COPD muscle atrophy, including the ubiquitin-proteasome system (UPS), lysosomal pathway, peroxisome proliferator-activated receptor (PPAR) and Cal-
PI3K/AKT/mTOR protein synthesis pathway in the skeletal muscle atrophy

cium-dependent pathway, of which the UPS is particularly important. However, the role of protein synthesis pathway in the mechanism of muscle atrophy in COPD is unclear, so we focused on phosphoinositide 3-kinase (PI3K)/AKT/mechanistic target of rapamycin (mTOR) protein synthesis pathway to observe how it acted in COPD muscle atrophy.

PI3K/AKT/mTOR signaling pathway is an important intracellular transduction pathways. PI3K can be activated by extracellular signal (cytokines, growth factors, hormones, etc), then as a second messenger binding of Akt PH area activates Akt [10]. Activated Akt promotes the phosphorylation of mTOR, glycogen synthase kinase-3β (GSK-3β) and other downstream substrate, playing a wide range of biological effects, such as anti-apoptosis, promoting cell survival and other functions [11]. phospho-mTOR initiates the phosphorylated translation of eukaryotic initiation factor 4E binding protein 1 (4EBP1) and 70 kD ribosomal protein S6 kinase (p70S6k1). MTOR, specifically via phosphorylation of p70S6K1 and 4EBP1, plays a central role in regulating the rate of protein synthesis in skeletal muscle [12-16].

Considering the preponderant role of cigarette smoke (CS) as a causative factor and lacking skeletal muscle cells in cellular atrophy models induced by cigarette smoke extract (CSE), which has been used to research the precise molecular mechanisms of the disorders triggered and regulated by CS at the cellular level, rats exposed to chronic CS and L6 myotubes treated with various concentrations of CSE were chosen for our study resemble the previous study [17]. Our research show that in vivo chronic CS and in vitro CSE incubation induced muscle atrophy, which were associated with the activity of PI3K/AKT/mTOR signaling pathway, an agent believed to be critical in the regulation of muscle protein metabolism. Muscle atrophy induced by CSE was associated with activation of PI3K/AKT/mTOR signaling pathway, as specific activator inhibited muscle atrophy.

Materials and methods

Animal cigarette smoke exposure and histological analysis

Adult male Sprague-Dawley (S-D) rats weighing about 150 g were purchased from the Shanghai Laboratory Animal Center and fed in the animal experimental center of Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine. The local institutional animal care committees approved the animal facilities and protocols. All rats were divided into two groups: (a) control group and (b) CS exposure group. All rats were fed adaptively for one week, then the control group was exposed to normal air, while CS exposure group were exposed to CS generated from 24 DaqianMen cigarettes per day (ShangHai, China). CS exposure group were exposed for 12 weeks (Two times of CS exposure a day, each time three cycles, a cycle of four cigarettes). All rats were measured weight every 4 weeks. Rats were given anesthesia by intra-peritoneal injection of 1% sodium pentobarbital.

For histological analysis, firstly, the lungs and extensor digitorum longus muscle (EDLM) were immersed in 4% paraformaldehyde for 24 h. Secondly, they were imbedded in paraffin. Finally, they were stained with hematoxylin and eosin (H&E).

Cell culture and ultrastructural analysis under transmission electron microscope (TEM)

L6 myotubes were purchased from cell bank of Chinese Academy of Science. To preserve the differential characteristics of the L6 myotubes, L6 myotubes were split a maximum of seven times. L6 myotubes were cultured in Dulbecco’s modified Eagle’s medium (DMEM) consisting of 10% fetal bovine serum (FBS), and incubated at 37°C in a humidified 5% CO₂, 95% air atmosphere in 100-mm, 6-well, or 96-well plates. Cells were covered bottom 70% and induced differentiate into multinucleated myotubes by using the 2% FBS for 6 days. Observe characteristics of the differentiated L6 myotubes by an inverted Olympus CK40-F200 microscope.

Differentiated L6 myotubes were fixed with 3% glutaraldehyde for 30 minutes on ice. We collected the fixed cells using cell scrapers and centrifuged the fixed cells. The cell pellets were fixed in 2% glutaraldehyde for 2 hours and in 1% osmium tetroxide 2 hours, dehydrated in an ascending ethanol series and embedded in Epon 618. The ultrathin sections were observed through a PHILIPS CM120 electron microscope (magnifications 3,500 to 15,000).
CSE was prepared according to the method of Liu Qian [17]. Briefly, a DaQianMen cigarette without filters was connected to a channel of three-limb tubes, a 50 ml syringe was connected to another channel and a 50 ml centrifugal tube with 10 ml DMEM was connected to the last channel by a catheter. The smoke was released into the DMEM by pulling the syringe slowly until the unburned butt was less than 1 cm long. This solution represented “100%” strength. Smoked medium was adjusted PH to 7.4 with NaOH and sterilized through a 0.22 μm pore filter. Smoked medium was used for the experiment within 30 min and diluted by DMEM to the required strength.

Cells were grown in 96-well plates (2×10^3 cells/well). Cells were exposed to control medium or medium containing various concentrations of CSE (5%, 10%, 20%, 30%). Cell survival was tested by the MTT assay after 24 hours. After removing culture medium we stained cells with 20 ul 5 mg/ml sterile MTT dye (Sigma, USA) at 37°C for 4 hours, then added 200 ul dimethylsulfoxide (DMSO) to each well for 10 minutes. The spectrometric absorbance was measured at 490 nm by automatic multiwell spectrophotometer.
PI3K/AKT/mTOR protein synthesis pathway in the skeletal muscle atrophy

**Immunocytochemistry**

L6 myotubes were seeded in 6-well plates containing sterile cover glasses, incubated overnight, and treated with 2% FBS for 6 days. The cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde 30 minutes, permeabilized with 0.3% TritonX-100 for 30 minutes, and blocked with peroxidase blocking solution (DAKO) and bovine serum albumin (BSA) for 30 minutes respectively. Then cells were incubated with anti-smooth muscle actin (SMA) antibody (1:200) (sigma, USA) at 4°C overnight. After washing in PBS, the cells were incubated with a goat anti-rabbit IgG/HRP antibody for 1 h, incubated with DAB peroxidase substrate, counterstained with hematoxylin. Images were acquired by an inverted Olympus CK40-F200 microscope.

**Western blot**

The rat extensor digitorum longus tissues and L6 myotubes were lysed in lysis buffer (RIPA, 1 mM PMSF and phosphatase inhibitors (Roche, Germany)) on ice for 30 minutes, and centrifuged at 12,000 rpm, 4°C for 30 minutes. The protein concentration was tested using BCA protein assay. Protein extracts (60 ug) were separated by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking for 2 hours at room temperature in 5% non-fat milk, the membranes were incubated with primary antibodies GAPDH (Beyotime, China), MHC (Santa Cruz, USA), PI3K (CST, USA), phospho-Akt (CST, USA), phospho-mTOR (CST, USA), phospho-GSK-3β (CST, USA), phospho-p70S6K1 (CST, USA), phospho-4EBP1 (CST, USA), Akt (CST, USA), mTOR (CST, USA), GSK-3β (CST, USA), p70S6K1 (CST, USA), 4EBP1 (CST, USA) overnight at 4°C. Then the membranes were incubated with the appropriate secondary antibody conjugated to HRP for 1 h at room temperature, and detected by enzyme-linked chemiluminescence (ECL). The protein expression levels were determined by ChemiDoc XRS + Systems and analyzed by Image Lab 2.0 Software. GAPDH was used as internal control.

**Comparative analysis of quantitative real-time polymerase chain reaction PCR (qPCR)**

Total RNA was extracted from the EDLM tissues and L6 myotubes using Trizol reagent, and 1 ug of total RNA was reverse-transcribed into cDNA using the PrimeScript RT Master Mix (Takara, Japan) and RNase Free dH2O in a 20-ml final volume. qPCR was then performed using an ABI Prism 7500 Sequence Detection System and SYBR Premix Ex Taq (Takara, Japan). Sequences of the primers were for GAPDH, forward 5’-AAG GTC GGA GTC AAG GGA TTT-3’ and reverse 5’-AGA TGA TGA GGC ATC GGA CAA-3’; MHC, forward 5’-GGA AGA AGA GTG GCA AGA-3’ and reverse 5’-TGG GAA TGA GGC ATG GGA CAA-3’. Strand cDNA was used as template in 20 ul reactions including 10 ul of 2×SYBRmix Ex TaqTM and 500 nM of each primer. Cycling conditions were performed as follows: Step 1, 30 s at 95°C; step 2, 5 s at 95°C; and step 3, 34 s at 60°C, with 40 repeats of step 2 to step 3. Relative expression of mRNA was calculated using the 2^{-ACt} method with GAPDH as an internal reference gene.

**Statistical analysis**

All data are presented as the mean ± SD from three independent experiments. SPSS statistics v19.0 software was used for statistical analysis. Statistical analyses were performed using Student’s-t test or one-way ANOVA followed by Dunnett’s tests. P < 0.05 was considered statistically significant.

**Results**

**Chronic CS exposure causes muscle atrophy**

The mean bodyweights of the rats in the 8 weeks and 12 weeks of CS exposure were lower significantly than those in the control groups (Figure 1A, P < 0.01). Twelve weeks of CS exposure to rats led to pulmonary lesions that morphologically resembled human chronic bronchial inflammation and emphysema (Figure 1B). Compared with the control group, skeletal cell numbers per high-power (HP) lens increased after 12 weeks of CS exposure (Figure 1C, P < 0.01), suggesting muscle wasting. Chronic CS exposure decreased the mRNA and MHC protein level of MHC in the 12-week groups (Figure 1D, P < 0.01). Taken together, these data show muscle atrophy.

**Chronic CS exposure activates PI3K/AKT/ mTOR signaling pathway**

Western blot analysis was used to assess the protein levels of PI3K, and phosphorylation lev-
Figure 2. Chronic CS exposure activates PI3K/AKT/mTOR signaling pathway. A. Western blotting for PI3K protein. B. Western blotting for phospho-Akt and Akt protein. C. Western blotting for phospho-mTOR and mTOR protein. D. Western blotting for phospho-GSK-3β and GSK-3β protein. E. Western blotting for phospho-p70S6K1 and p70S6K1 protein. F. Western blotting for phospho-4EBP1 and 4EBP1 protein. GAPDH served as the internal standard. *P < 0.05 compared with control group; **P < 0.01 compared with control group.
PI3K/AKT/mTOR protein synthesis pathway in the skeletal muscle atrophy

Figure 2. Chronic CS exposure activates PI3K/AKT/mTOR signaling pathway. The phosphorylation levels of Akt (Figure 2B, P < 0.01), mTOR (Figure 2C, P < 0.05), GSK-3β (Figure 2D, P < 0.01), p70S6K1 (Figure 2E, P < 0.05) and 4EBP1 (Figure 2F, P < 0.05) were increased significantly after Chronic CS exposure, and no significant changes were observed in the protein levels of PI3K (Figure 2A).

2% FBS induces L6 cells to differentiate into multinucleated myotubes

After cultured with 2% FBS, L6 myotubes exhibited an elongated cell shape, with branched

Figure 3. 2% FBS induces L6 cells to differentiate. A. The morphology of L6 myotubes were evaluated by inverted microscope (×100 magnification). B. The structures of L6 myotubes were presented by magnification of electron micrographs (×3,500-12,000 magnification). C. SMA expression was examined by immunocytochemistry (×100 magnification).
PI3K/AKT/mTOR protein synthesis pathway in the skeletal muscle atrophy

and multi-nucleated, and even displayed fusion (Figure 3A). Moreover, a large number of filaments were longitudinally arranged in L6 myotubes (Figure 3B). The SMA-stained myoblasts were increased obviously in differential L6 myotubes (Figure 3C). Taken together, Figure 3 shows that L6 myotubes differentiate into multinucleated myotubes successfully.

**MTT and L6 myotubes atrophy**

Exposed to various concentrations of CSE, L6 myotubes survival was tested by MTT assay 24 hours later. CSE produced a decrease in cell survival at the concentration of 20% (P < 0.05) and 30% (P < 0.01). Conversely, treated with 5, 10, and 20% CSE, the survival rates of cells had no change (Figure 4A). 10% CSE was selected for subsequent experiments. CSE treatment decreased the mRNA and protein level of MHC 24 hours later (Figure 4B, P < 0.01), suggesting L6 myotubes atrophy. However, CSE and PI3K agonist insulin-like growth factor (IGF-1) treatment increased the MHC expression in comparison with simple CSE treatment (Figure 4B, P < 0.01).

**CSE suppresses PI3K/AKT/mTOR signaling pathway**

As shown in Figure 5, CSE suppresses PI3K/AKT/mTOR signaling pathway. After treated with CSE 24 hours later, not only the phosphorylation levels of Akt (Figure 5B, P < 0.05), mTOR (Figure 5C, P < 0.01), GSK-3β (Figure 5D, P < 0.01), p70S6K1 (Figure 5E, P < 0.01), and 4EBP1 (Figure 5F, P < 0.01) decreased significantly, but also PI3K. Moreover, IGF-1 increased the the phosphorylation levels in comparison with simple CSE treatment (Figure 5).

**Discussion**

COPD results in significant systemic effects, among which muscle dysfunction/wasting is one of the most important [17]. Many studies have demonstrated the importance of the mechanism of protein degradation pathways, such as the UPS, lysosomal pathway, PPAR and Calcium-dependent pathway. However, little is known about the role of protein synthesis pathway especially PI3K/AKT/mTOR signaling pathway in this process.

We obtained the evidence that PI3K/AKT/mTOR signaling pathway was activated in animal models of COPD induced by CS. Both 8 and 12 weeks-CS exposure caused significant weight loss, and 12 weeks-CS exposure produced emphysema and decreased in muscle size. Similar to our research, chronic exposure to the smoke of 20 cigarettes per day for 12 weeks...
CSE suppresses PI3K/AKT/mTOR signaling pathway. A. Western blotting for PI3K protein. B. Western blotting for phospho-Akt and Akt protein. C. Western blotting for phospho-mTOR and mTOR protein. D. Western blotting for phospho-GSK-3β and GSK-3β protein. E. Western blotting for phospho-p70S6K1 and p70S6K1 protein. F. Western blotting for phospho-4EBP1 and 4EBP1 protein. GAPDH served as the internal standard. *P < 0.05 compared with control group; **P < 0.01 compared with control group; #P < 0.05 compared with CSE group; ##P < 0.01 compared with CSE group.
induced parenchymal destruction, enlargement of airways, and weight loss in rats [17]. In our study, chronic CS exposure decreased the mRNA and protein level of MHC in the EDLM, suggesting that chronic CS exposure induce muscle wasting. Factors, considered potentially relevant to skeletal muscle atrophy induced by chronic CS exposure, include hypoxia, hypercapnia, systemic or local inflammation, anabolic/catabolic hormone imbalance, nutritional depletion, and oxidative stress [18-20]. PI3K/ AKT/mTOR signaling pathway is involved in regulation of cell growth and apoptosis [11]. All along, Akt is considered to play an important role in rodent skeletal muscle atrophy and hypertrophy. Léger B et al. [21] found that AKT and its downstream signals mTOR and GSK-3β act in the regulation of human skeletal muscle atrophy and hypertrophy for the first time. Knockout PI3K gene caused the weight of mouse skeletal muscle loss and muscle fiber atrophy [22]. PI3K/AKT/mTOR signaling pathway regulates the skeletal muscle protein synthesis. The phosphorylation levels of Akt, 4EBP1, p70S6K1 and total Akt were increased in atrophic denervated muscle. Increased protein degradation, rather than decreased protein synthesis, is likely to be responsible for the loss of muscle mass in denervated atrophic muscles [23]. Our study showed that the phosphorylation levels of Akt, mTOR, GSK-3β, p70S6K1, 4EBP1, total mTOR, and total GSK-3β were increased significantly in chronic CS exposure rats, indicating that PI3K/AKT/mTOR signaling pathway was activated by chronic CS exposure, which increased protein synthesis. Although protein synthesis increased, skeletal muscle still atrophied, suggesting that skeletal muscle protein degradation, rather than protein synthesis, seems to be responsible for the loss of muscle mass in chronic CS exposure rats. Activated PI3K/AKT/mTOR signaling pathway may be the body’s self-protection against skeletal muscle atrophy, and is of great significance to skeletal muscle atrophy in chronic CS exposure rats.

CS induces muscle wasting, which is obtained from the studies in animals. Moreover, CSE has direct effects within cells. To investigate the mechanism of the disorders caused by CS at the cellular level, we checked the effect of CSE on L6 myotubes. We differentiated L6 myotubes with 2% FBS for six days prior to experimentations. The differential L6 myotubes exhibit an elongated cell shape, with branched and multi-nucleated, and even display fusion. Moreover, a large number of filaments are longitudinally arranged in L6 myotubes. The SMA-stained myotubes increased obviously in differential L6 myotubes. SMA is a marker of skeletal muscle cell differentiation [24]. All the above results indicated that L6 myotubes differentiated successfully. The mRNA and protein level of MHC was significantly lower after 10% CSE treatments, suggesting muscle wasting. The phosphorylation levels of Akt, mTOR, GSK-3β, p70S6K1 and 4EBP1 were decreased significantly after 10% CSE treatments; suggesting that CSE could inhibit the phosphorylation of PI3K/AKT/mTOR protein synthesis pathway and thus make skeletal muscle atrophy, which is an attractive possibility for future investigation. However, we noted that the result of L6 myotubes experiment was inconsistent with the animal experiment. The possible reasons were as follows. To begin with, the different preparations of CS and CSE, which was prepared only contained the hydrophilic components of cigarette smoke, were used respectively in animal experiment and L6 myotubes experiment. Secondly, the concentration of CSE used may be too high and the treatment time may be too short, leading that L6 myotubes had no time to compensate and PI3K/AKT/mTOR signaling pathway wasn’t activated; furthermore, it was possible that in early CS exposure protein synthesis was indeed reduced, and then some kind of compensatory mechanisms acted in the body, activating the PI3K/AKT/mTOR signaling pathway. Finally, in vitro CSE exposure bypasses the airways and precludes the filtering action of the lungs [17], while in vivo cigarette smoking might stimulate the body producing a variety of inflammatory cytokines, hormones, cytokines, which activate the immune and endocrine system, and then activate the PI3K/AKT/mTOR signaling pathway through some unknown mechanism.

To further verify the role of PI3K/AKT/mTOR signaling pathway in skeletal muscle atrophy, we used PI3K agonist IGF-1 treating CSE exposure-L6 myotubes for 24 hours, and then detected the protein of PI3K/AKT/mTOR signaling pathway. IGF-1, a survival factor to the smooth muscle cells, fibroblasts, and macrophages, involved in protein synthesis, cell...
migration, and mitosis [25, 26]. IGF-1R, activated by a variety of signaling pathways, induces the growth, differentiation, and migration of smooth muscle cells. IGF-1 is a commonly activator of PI3K pathway [27, 28]. Compared with simple CSE exposure, the phosphorylation levels of Akt, mTOR, GSK-3β, p70S6K1 and 4EBP1 were increased significantly after IGF-1 and CSE treatment. Moreover, the mRNA and protein level of MHC increased significantly when we used IGF-1, indicating the degree of muscle atrophy reduced. Our study showed that activating PI3K/AKT/mTOR signaling pathway inhibited skeletal muscle atrophy induced by CSE.

In summary, PI3K/AKT/mTOR signaling pathway is activated in chronic CS exposure rats. Activating PI3K/AKT/mTOR signaling pathway inhibited skeletal muscle atrophy, induced by CSE. Selecting the activators of PI3K/AKT/mTOR signaling pathway is crucial for the development of new and individualized therapeutic strategies to prevent skeletal muscle atrophy in COPD patients. Since studies on PI3K/AKT/mTOR signaling pathway targets are still fewer, we need further study on these targets.

Acknowledgements

This study was supported by Foundation of Shanghai Science and Technology Commission (No. 14ZR1426800).

Disclosure of conflict of interest

None.

Address correspondence to: Fengfeng Han, Department of Respiratory Medicine, Xin Hua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, 1665 Kong Jiangan Road, Shanghai 200092, China. Tel: 86-13816386372; Fax: 86-21-65153984; E-mail: fengfh86@sina.com

References


