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Activation of AMPK α2 inhibits airway smooth muscle cells proliferation

Lu Liu [a], Yilin Pan [a], Yang Song [a], Xiaofan Su [a], Rui Ke [a], Lan Yang [a], Li Gao [b], Manxiang Li [a,*]

[a] Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi 710061, PR China
[b] Division of Allergy and Clinical Immunology, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21224, USA

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A B S T R A C T

The aims of the present study were to examine the effect of adenosine monophosphate-activated protein kinase (AMPK) activation on airway smooth muscle cells (ASMCs) proliferation and to address its potential mechanisms. Platelet derived growth factor (PDGF) activated phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway, and this in turn up-regulated S-phase kinase-associated protein 2 (Skp2) and consequently reduced cyclin dependent kinase inhibitor 1B (p27) leading to ASMCs proliferation. Pre-incubation of cells with metformin, an AMPK activator, blocked PDGF-induced activation of mTOR and its downstream targets changes of Skp2 and p27 without changing Akt phosphorylation and inhibited ASMCs proliferation. Transfection of ASMCs with AMPK α2-specific small interfering RNA (siRNA) reversed the effect of metformin on mTOR phosphorylation, Skp2 and p27 protein expression and cell proliferation. Our study suggests that activation of AMPK, particularly AMPK α2, negatively regulates mTOR activity to suppress ASMCs proliferation and therefore has a potential value in the prevention and treatment of asthma by negatively modulating airway remodeling.

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1. Introduction

Bronchial asthma is a chronic airway inflammatory disease characterized by airway hyper-responsiveness (AHR) and airway remodeling (Holgate, 2008; Nakagome and Nagata, 2011). Airway remodeling is critical for the development of irreversible airflow obstruction (Park et al., 2012). The pathological changes of airway remodeling include epithelial alterations (shedding of epithelium, loss of ciliated cells and disruption of epithelial tight junction integrity) (Al-Muhsen et al., 2011; Holgate, 2007), subepithelial fibrosis, airway smooth muscle cells (ASMCs) hypertrophy/proliferation/migration, angiogenesis and hypertrophy/hyperplasia of goblet cell and mucous gland (Kudo et al., 2013; Manuyakorn et al., 2013). ASMCs proliferation is convinced to be extremely important in the development of airway remodeling (Bentley and Hershenson, 2008) and AHR (Guedes et al., 2015). Therefore, it is important to explore the molecular mechanisms responsible for ASMCs proliferation to prevent and/or reverse airway remodeling and thus to treat asthma.

Adenosine monophosphate-activated protein kinase (AMPK) is an important cellular energy-sensor, which is a heterotrimeric protein consisting of a catalytic subunit (α) and two regulatory subunits (β and γ). The α subunit exists in two different isoforms (α1 and α2), which have different tissue expression patterns and functions. AMPK is typically activated by physiological or pathological stimuli associated with the increased AMP/ATP ratio, such as heat shock, glucose deprivation, hypoxia, ischemia, and muscle contraction (Hardie, 2003). AMPK is also activated by stimuli independent of energy crisis including hyperosmotic stress, chemical compounds and particular signaling pathways (Hardie, 2004; Jiang et al., 2015; Viollet et al., 2009). It has been shown that activation of AMPK regulates a wide variety of pathophysiological processes such as substrate metabolism, protein synthesis, cell proliferation and apoptosis (Liu et al., 2015; Nagata et al., 2004; Wu et al., 2014; Yu et al., 2015).

Activation of AMPK has been shown to inhibit murine and human ASMCs proliferation and might be a potential target for the treatment of airway remodeling in asthma (Ratnovsky et al., 2007; Zhang et al., 2012). Recent study (Park et al., 2012) has further indicated that lack of AMPK α1 promotes airway remodeling by enhancing mucin secretion, increasing smooth muscle layer thickness and peribronchial fibrosis in a mouse asthma model.

* Correspondence to: Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital of Xi'an Jiaotong University, No.277, West Yanta Road, Xi'an, Shaanxi 710061, PR China.
E-mail addresses: 443606290@qq.com (L. Liu), 287842481@qq.com (Y. Pan), wyshzl@163.com (Y. Song), 376687378@qq.com (X. Su), 494228013@qq.com (R. Ke), beautiful012@163.com (L. Yang), lgao2@jhmi.edu (L. Gao), manxiangli@hotmail.com (M. Li).

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However, the mechanisms by which AMPK regulates ASMCs proliferation remain to be elucidated. To clarify this, ASMCs were treated with platelet-derived growth factor (PDGF), an effective mitogen, which has been widely shown to contribute to airway remodeling by stimulating ASMCs proliferation (Ning et al., 2013), and the particular effect and mechanisms of activation of AMPK by metformin on ASMCs proliferation were further explored.

2. Materials and methods

2.1. Cell preparation and culture

Primary cultures of ASMCs from Sprague-Dawley rats (70–80 g) were isolated as previously described (Ning et al., 2011). All animal care and experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of Xi'an Jiaotong University Animal Experiment Center. All protocols used in this study were approved by the Laboratory Animal Care Committee of Xi'an Jiaotong University. Briefly, trachea and main bronchi were rapidly removed from killed rats, washed in phosphate-buffered saline (PBS; 4 °C), and then dipped into Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Isle, NY, USA) with 10% fetal bovine serum (FBS; Sijiqing, Hangzhou, China), 100 U/ml penicillin, and 100 μg/ml streptomycin. The epithelium and serosa were carefully stripped off with fine forceps and a surgical blade. Next, the remaining tissue was cut into 0.5-mm pieces and placed into a culture flask and incubated at 37 °C in an atmosphere of 95% air and 5% CO2 till cells reaching 80% confluence. Then cells were passaged using 0.25% trypsin (Sigma, St. Louis, MO, USA). All experiments were performed using cells between passages 4 and 8. The purity of ASMCs was determined by immunostaining with α-smooth muscle actin (α-SMA; Boster, Wuhan, China). Fluorescence microscope images indicated that cells contained more than 90% of ASMCs (data not shown here). Before each experiment, cells were incubated in 1% FBS-DMEM overnight to minimize serum-induced effects. PDGF-BB (PeproTech, Rocky Hill, NJ, USA, distillated water as vehicle) was used to stimulate cell proliferation. LY294002 (Sigma, DMSO as vehicle) was applied to inhibit PI3K activity. Rapamycin (Cell Signaling Technology, Beverly, MA, USA, DMSO as vehicle) was used as an mTOR inhibitor. Metformin was purchased from Bristol-Myers Squibb biopharmaceutical company (DMEM as vehicle).

2.2. Small interfering RNA (siRNA) transfection

To silence expression of AMPK α1 or AMPK α2, ASMCs were transfected with sequence-specific or non-targeting siRNA (GenePharm, Shanghai, China) using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, for each well of a 6-well plate, 10 μl siRNA and 5 μl lipofectamine 2000 were diluted in 250 μl DMEM, separately, and incubated for 5 min at room temperature. The diluted siRNA was combined with the diluted Lipofectamine 2000 and incubated for 20 min at room temperature. Finally, the mixture with the final concentration of 100 nM siRNA was applied to the cells. After incubation for 6 h, the medium was replaced with fresh DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured for an additional 48 h at 37 °C, 5% CO2 in a humidified incubator and effect of siRNA transfection was determined using immunoblotting.

2.3. 5-Bromo-2′-deoxyuridine (BrdU) incorporation assay

To measure ASMCs proliferation, the rate of BrdU incorporation was determined using BrdU ELISA Kit (Maibio, Shanghai, China) according to the manufacturer's instructions. Briefly, cells were seeded into 96-well plate at a density of 5×10^3 cells/well, allowing to adhere for at least 24 h, and serum starved overnight (1% FBS in DMEM) before the start of experiments. After different treatments, BrdU labeling reagent was added to the wells and incubated for 2 h at 37 °C. Next, cells were denatured with Fix-Denat solution for 30 min at room temperature, and followed by incubating with anti-BrdU mAbs conjugated to peroxidase for 90 min at room temperature. After removing antibody conjugate, substrate solution was added and allowed to react for 10 min. The absorbance at 370 nm was determined with a microplate reader (Bio-Rad, Richmond, CA, USA). The blank corresponded to 100 μl of culture medium with or without BrdU.

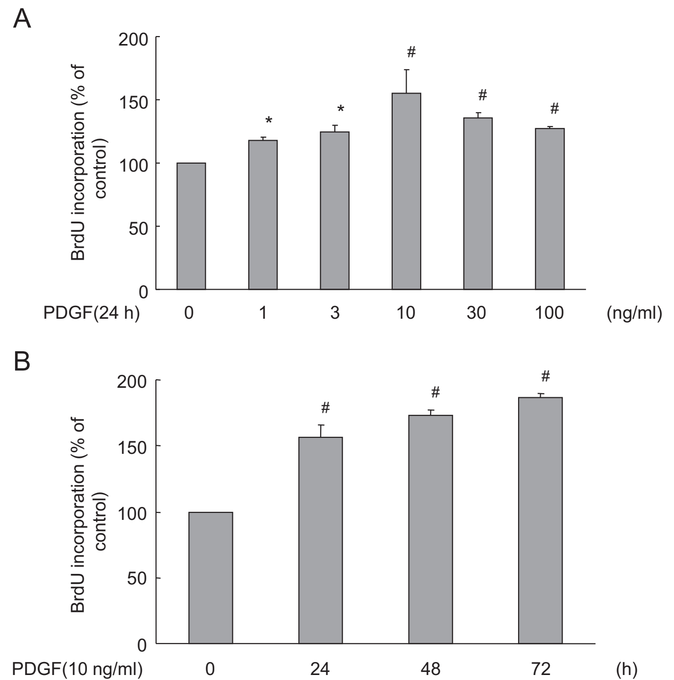

Fig. 1. Effect of PDGF on ASMCs proliferation. (A) ASMCs were stimulated with different concentrations of PDGF ranging from 0 to 100 ng/ml for 24 h, the rate of BrdU incorporation in cells was determined by BrdU ELISA assay Kit (n=3 per group). (B) Cells were exposed to 10 ng/ml PDGF for the indicated times, BrdU incorporation in cells was measured (n=3 per group). *$P < 0.05$ versus control. #$P < 0.01$ versus control.

2.4. Immunoblotting

Cells were lysed in RIPA Lysis Buffer (containing 50 mM Tris pH 7.4, 150 mM NaCl, 1% NP40, 0.5% Sodium-deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 mM NaF and proteinase inhibitors). Lysates were centrifuged at 13000 rpm for 15 min at 4 °C, the supernatant was collected as total protein, and protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). Protein was separated on SDS-PAGE gel and transferred to a Trans-Blot Nitrocellulose membrane (Bio-Rad). Polyclonal antibodies against total-AMPK α1, total-AMPK α2 (Proteintech Group, Chicago, IL, USA, 1:500 dilution), total-mTOR, phosphor-mTOR and S-phase kinase-associated protein 2 (Skp2) (Cell Signaling Technology, 1:1000 dilution) and monoclonal antibodies against total-Akt (protein kinase B), phosphor-Akt, total-AMPK α, phosphor-AMPK α, p27 (cyclin dependent kinase inhibitor 1B) (Cell Signaling Technology, 1:1000 dilution) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Sigma, 1:2000 dilution) were used following manufacturer's protocols. Horseradish peroxidase-conjugated goat anti-rabbit IgG was used as the secondary antibody (Sigma, 1:5000 dilution). Membranes were visualized on a ChemiDoc XRS system and analyzed using Quantity One software (Bio-Rad).

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