Effects of Mitochondrial ATP-Sensitive Potassium Channels on the Proliferation and Secretion of Human Airway Smooth Muscle Cells

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ABSTRACT

Bronchial asthma is the common chronic inflammatory disease and is characterized by chronic airway inflammation, airway remodeling, and airway hyperreactivity (AHR). Aim of this study was to investigate the effects of mitochondrial ATP-sensitive potassium channels (MitoKATP) on the proliferation and secretion of human airway smooth muscle cells (HASMCs). HASMCs were treated with the serum from asthmatic patients to establish HASMCs asthma model of passive sensitization. Rhodamine 123 (R-123) and 2,7-dichloro-dihydrofluorescein diacetate (DCFH-DA) fluorescence staining were used to detect mitochondrial membrane potential ($\Delta\psi m$) and the content of reactive oxygen species (ROS) in the cells, respectively.

The cell counting was used to detect cell proliferation, and RT-PCR was used to detect the expression of TGF-β1 mRNA.

In the normal + Diazoxide group, the fluorescence intensity of R-123, ROS content, cell proliferation and TGF-β1 expression were enhanced, compared with the normal control group ($p<0.05$). There were no significant differences between the normal + 5-hydroxydecanoate (5-HD) group and the normal control group. In the asthma model control group, the fluorescence intensity of R-123, ROS content, cell proliferation and TGF-β1 expression were enhanced, compared with normal control group, ($p<0.05$). The aforementioned indices were enhanced in the asthma model + Diazoxide group, when compared with the asthma model control group, whereas these indices were attenuated in the asthma model + 5-HD group, when compared with the asthma model control group ($p<0.05$).

In conclusion, asthma could activate MitoKATP channels in HASMCs, promote HASMC proliferation and TGF-β1 expression.

Keywords: Asthma; smooth muscle; Mitochondrial ATP-sensitive potassium channels (MitoKATP); Reactive oxygen species (ROS); Membrane potential

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INTRODUCTION

Bronchial asthma is a common chronic inflammatory disease that seriously threatens human health and is characterized by chronic airway inflammation, airway reconstruction, and airway hyperreactivity (AHR). Chronic airway inflammation is the basis of asthma pathology. Previous studies presumed that airway smooth muscle cells (ASMCs) were merely passive target cells in the pathological process, which generated contractions and spasms through the stimulation of inflammatory mediators. However, recent studies[1-3] demonstrated that ASMCs not only have contractile function but also can generate phenotype transformation when stimulated by extracellular factors. This phenotype transformation, a change from the contraction to proliferation phenotype, enables ASMCs to synthesize and secrete multiple inflammatory factors and cytokines through complex mechanisms.

The ATP-sensitive K-channel is a critical channel that combines cellular energy metabolism and bioelectricity activity. These channels are generally distributed in many cells, such as nerve, heart, smooth muscle, skeletal muscle, and pancreatic β cells. ATP-sensitive K channels (KATP) can be divided into cell membrane KATP channels and mitochondrial inner membrane KATP channels (MitoKATP), according to their different locations.[4] In our previous studies, we found that asthma could induce the opening of MitoKATP (mitochondrial ATP-sensitive potassium channels) channels of ASMCs in rats, promote ASMC proliferation and phenotype transformation, and is involved in the airway reconstruction.[5] However, studies of HASMCs remain rare. Therefore, in this study, we aimed to explore the effects of MitoKATP on the proliferation and secretion of HASMCs to explore the role of MitoKATP in bronchial asthma and to find new approaches for the prevention and cure of asthma.

MATERIALS AND METHODS

The study was approved by the Institutional Review Board of Huazhong University of Science and Technology.

Reagents and Instruments

The following reagents were used in this study: diazoxide, 5-hydroxydecanoate (5-HD), methyl thiazolyl tetrazolium (MTT), Rhodamine 123 (R-123), dimethyl sulfoxide (DMSO), propidium iodide (PI), and 2,7-dichloro-dihydrofluorescein diacetate (DCFH-DA). These reagents were purchased from Sigma (Sigma, USA). High-glucose DMEM (Dulbecco's minimum essential medium) culture medium and fetal bovine serum (FBS) were products of Hyclone (Hyclone, USA). Human smooth muscle cell α-actin antibody and SPABC immunohistochemistry kit (Zhongshan Golden Bridge Biotech., China), cDNA reverse transcription kit and PCR-SuperMix were purchased from Toyobo (Toyobo, Japan); all PCR primers were synthesized by Shanghai Sangong (Sangong, China). Other reagents were analytical reagents from China.

Sources of Human Serum

Asthma serum was obtained from 6 cases of acute stage asthma, consisting of 4 male and 2 female patients, who ranged in age from 20 to 45 years. The asthmatic patients were characterized by the following features: the diagnosis was in accordance with Prevention and Cure of Bronchial Asthma Manual;[6,7] patient history of hypersensitivity; the patients had no other respiratory system disease; patients had not used a glucocorticoid in the week prior to serum collection; and patient total serum IgE was >200 IU/mL.

Control serum was obtained from 6 non-asthmatic patients, consisting of 3 male and 3 female patients, who ranged in age from 20 to 45 years. The non-asthmatic patients were characterized by the following features: patients had no history of hypersensitivity and asthma; patients had no other diseases of respiratory system; and patients had taken no drugs within a recent time period prior to the study.

Culture and Identification of HASMC

HASMCs were cultured with the tissue adhesion method as described in a previous study.[8] Normal human bronchial tissues were obtained intraoperatively from 6 patients undergoing pulmonary lobectomy due to pulmonary diseases. These patients consisted of 4 male and 2 female patients, and the mean age was 40 years. Informed consent was given by the patients, who had no history of anaphylactic diseases, including asthma. The normal bronchial tissues of patients with pulmonary cancer were obtained from a location at least 5 cm from the tumor site. The pulmonary tissue was placed into cool D-Hanks solution, containing

penicillin and streptomycin at a dose of 10^5 U/L at 4°C in a germ-free condition. D-Hanks solution was used to carefully wash and separate the tracheal tissue, removing the affiliated connective tissue, blood vessels, and nerve fibers. Subsequently, the inner membrane was cut open and removed, and then the bronchi were sheared into slivers that measured 1 mm×1 mm×1 mm. A small amount of fetal bovine serum (FBS) was added and mixed with the slivers in the culture flask, and then a small amount of high-glucose DMEM, containing 15% FBS, was placed into a 5% CO_2 incubator at 37°C. After 10-16 h, a suitable amount of high-glucose DMEM, containing 15% FBS, was added to avoid the removal of tissue slivers. After this, high-glucose DMEM, containing 15% FBS, was used to change the solution once every 3 days. In 14-21 days, when the cell fusion had achieved 80%, pancreatin (0.125%) was used for the digestion and passage of cells. Human smooth muscle α-actin was used to identify HASMCs by immunocytochemical stain.

Preparation of Asthma Model

The cultured HASMCs were used for passive sensitization for 24 h by DMEM culture medium containing 10% serum of asthma patients (total level of IgE>200 U/ml) to establish the asthma model according to a previous study.[9] The serum of non-asthmatic patients was used for culturing for 24 h in the control group.

Grouping and Medicine Intervention

The high-glucose DMEM culture medium without FBS was used to culture cells to synchronize for 24 h in all groups. HASMCs were divided into 6 different groups: 1) Normal control group, which consisted of normal human ASMCs, with DMSO only added into the culture medium (the high-glucose DMEM, containing 15% FBS) at a final density of 0.1%; 2) Normal + diazoxide group, which consisted of normal ASMCs, with diazoxide (a selective opener of MitoKATP channels) dissolved in DMSO added into the culture medium at a final density of 60 μmol/L, with the final concentration of DMSO in the DMEM culture medium of 0.1%; 3) Normal + 5-HD group, which consisted of normal human ASMCs, with 5-HD (a selective blocker of MitoKATP channel) added into the culture medium at a final density of 300 μmol/L and DMSO added into the solution at a final density of 0.1%; 4) The asthmatic serum treated control group,

which consisted of the asthma model of HASMCs, with DMSO only added into the culture medium at a final density of 0.1%; 5) Asthma model + diazoxide group, which consisted of the asthma model of HASMCs, with diazoxide at a final density of 60 μmol/L and DMSO at a density of 0.1% added into the culture medium; and 6) Asthma model + 5-HD group, which consisted of the asthma model of HASMCs, with 5-HD at a density of 300 μmol/L and DMSO at a density of 0.1% added into the culture medium. The cells were cultured for 24 h after intervention.

Detection of HASMCs Mitochondrial Membrane Potential (Äøm)

The Rhodamine fluorescence technology was used.[10] The culture grouping and intervention for cells in the 6-well plates were performed as previously described. After intervention with the treatment factors, the cells in each group were washed by D-Hanks solution to avoid affecting the intensity of fluorescence, and the solution was incubated with R-123 at a final density of 10 μg/mL for 30 min at 37°C away from light. D-Hanks solution was used to wash out unabsorbed R-123. R-123 fluorescence was detected by laser confocal microscopy; the fluorescence was stimulated at 488 nm and detected at 530 nm. R-123 fluorescence showed a linear correlation with Äøm, and R-123 fluorescence was gradually enhanced with the depolarization of Äøm. The cells that were incubated by D-Hanks solution were set as controls, and the results were analyzed with the HPIAS-1000 image analyzer.

ROS Level Detected by DCFH-DA Fluorescent Staining

The culture grouping and intervention for cells in the 6-well plates were performed as previously described. After intervention with the treatment factors, the D-Hanks solution with DCFH-DA at a final density of 20 μmol/L was added, incubated for 30 min away from light at 37°C, and observed by fluorescence microscopy. DCFH-DA could enter into the cells for hydrolyzation and oxidation; DCFH-DA formed into DCF with high fluorescence and showed a direct ratio to the ROS level in the cells. Therefore, the mean fluorescence intensity of DCF in the cells could be used to reflect ROS levels directly. DCF emitted green fluorescence, and the results of fluorescence were analyzed with the HPIAS-1000 image analyzer.

Cell Proliferation Detected by Cell Counting

Cells were seeded in 24-well plates (5,000 cells/well) and cultured overnight. Cells were then maintained in serum-free media for 24 h, followed by different treatments. At the end-point, cells were harvested and counted using a hemocytometer.

The Transforming Growth Factor, Beta 1 (TGF-â₁) mRNA Level Detected by RT-PCR

The grouping and intervention methods were performed as previously described. Total RNA was extracted using Trizol with common methods after intervention with the treatment factors, cDNA was obtained by reverse transcription. The gene sequences of the load control β-actin primers were as follows: upstream 5GGAAATC-GTGCGTGACATTA3, and downstream 5GGACTCGTCATATCCTGCTTG3. The length of amplification products was 478 bp. The gene sequences of the TGF-β_1 primers were as follows: upstream 5TGTGACCGGAGTTGTGCG3, and downstream 5GGTCCTTGCGGAAGTCA. The length of the product was 323 bp. The renaturation temperatures in both PCRs were 60°C and 61°C, respectively. The PCR products were analyzed by 2% agarose gel electrophoresis; β-actin was set as positive control. The image was evaluated by a gel image analytical system and band-density scanning. Finally, the ratio of integral optical density between TGFβ-1 and β-actin ($IOD_{TGF-\beta_1}/IOD_{\beta-actin}$) was used for semi-quantitative analysis.

Statistical Analysis

Quantitative data are expressed as the mean ± standard deviation. All of the analyses were conducted using SPSS 13.0 (SPSS Inc., Chicago, IL). One-way ANOVA (analysis of variance) was used for comparisons among the means in multiple groups. The q test was used between the groups. Statistical significance was defined as $p<0.05$.

RESULTS

Characterization of HASMCs

HASMCs showed spindle cell type under phase contrast microscopy; the orbicular-ovate nucleus appeared in the center of the cells. The typical 'hill and valley' appearance of ASMCs was present in the cell growth. The cultured cells were positive for human α-smooth muscle actin by immunocytochemistry. These results demonstrated that the cultured cells were smooth muscle cells (Figure 1).

Variation of Äøm

The fluorescence intensity of R-123 in the normal + Diazoxide group was significantly enhanced compared with that in the normal control group ($p<0.05$). There was no significant difference in the fluorescence intensity of R-123 between the normal + 5-HD group and the normal control group. Nevertheless, both the asthma model control group and the asthma model + Diazoxide group could induce enhancement of R-123 fluorescence intensity in smooth muscle cells ($p<0.05$); the latter group showed more significant effects than the former group ($p<0.05$). The asthma model + 5-HD group could partially reverse the effects of asthma showing enhancement of R-123 fluorescence in smooth muscle cells ($p<0.05$) (Table 1, Figure 2).
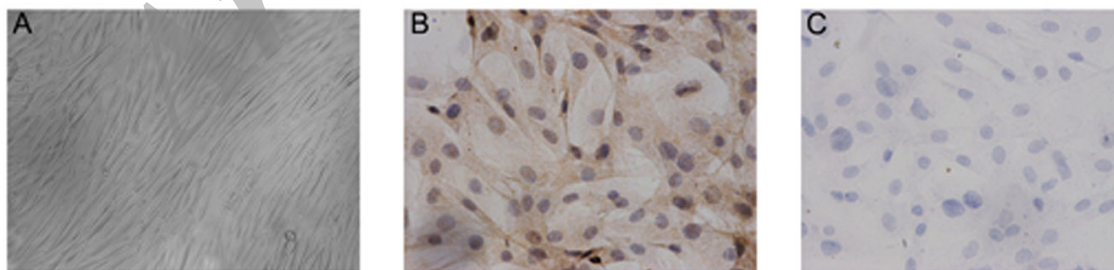

Figure 1. Appearance and identification of HASMCs. A: Typical 'hill and valley' appearance of ASMCs under phase contrast microscopy. B: Cultured cells stained positive for α-smooth muscle actin by immunohistochemical staining. C: Cells stained negative for α-smooth muscle actin by immunocytochemical staining.

Effects of Mitochondrial ATP-sensitive K⁺ Channel on the ROS Level Reflected by DCF Fluorescence in HASMCs

ROS levels in the normal + Diazoxide group were significantly enhanced when compared with the normal control group ($p<0.05$). ROS production in the normal +5-HD group was not markedly changed. ROS production was evidently increased in the asthma model control group compared with normal control group ($p<0.05$). ROS levels were decreased in the asthma model + 5-HD group when compared with the asthma model control group ($p<0.05$). However, ROS levels were significantly enhanced in the asthma model +diazoxide group compared with the asthma model control group ($p<0.05$) (Table 1, Figure 3).

Effects of MitoKATP **Channels on HASMCs Proliferation**

The cell number in the normal + Diazoxide group was significantly increased, compared with the normal control group ($p<0.05$). The cell number in the normal +5-HD group showed no significant change, whereas the cell number in the asthma model control group was increased ($p<0.05$). Likewise, the cell number in the asthma model + diazoxide group was larger than that in the asthma model control group ($p<0.05$). The cell number in the asthma model + 5-HD group was smaller than that in asthma model control group ($p<0.05$) (Table 2).

Effects of MitoKATP Channels on TGFβ₁ Expression in HASMCs

The expression of TGFβ₁ mRNA in the normal+ Diazoxide group was enhanced compared with the normal control group ($p<0.05$), whereas the expression showed no significant changes in the normal+5-HD group. The expression of TGFβ₁ mRNA in the asthma model control group was increased compared with the normal control group ($p<0.05$). The expression of

Table 1. Effects of diazoxide, 5-HD and asthma on the mitochondrial membrane potential and ROS content in HASMCs. The mitochondrial membrane potential was detected using Rhodamine 123 (R-123) fluorescence. The level of reactive oxygen species (ROS) was detected by DCF fluorescence. Mean±SD. N=6. *$P<0.05$ vs. Normal control group; #$P<0.05$ vs. Asthma model control group

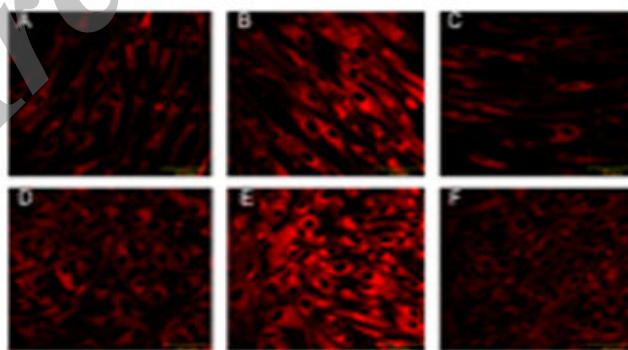| Group	R-123 fluorescence	DCF fluorescence
Normal control	87.40±10.21	98.24±13.02
Normal+Diazoxide	152.34±21.77*	126.42±18.51*
Normal+5-HD	65.71±17.42	69.94±10.87
Asthma model control	195.26±27.65*	185.12±27.06*
Asthma model + diazoxide	237.03±17.92*#	228.91±13.62*#
Asthma model + 5-HD	103.79±26.81#	93.47±15.44#

Figure 2. Effects of mitochondrial ATP-sensitive K+ channel on mitochondrial membrane potential as reflected by R-123 fluorescence in HASMCs. A: Normal control group. B: Normal+Diazoxide group. C: Normal+5-HD group. D: Asthma model control group. E: Asthma model + diazoxide group. F: Asthma model + 5-HD group. Scale bar, 50 μm.

Table 2. Effects of diazoxide, 5-HD and asthma on the expression of TGF-β₁ mRNA in HASMCs. Mean±SD. N=6. *P<0.05 vs. Normal control group; #P<0.05 vs. Asthma model control group

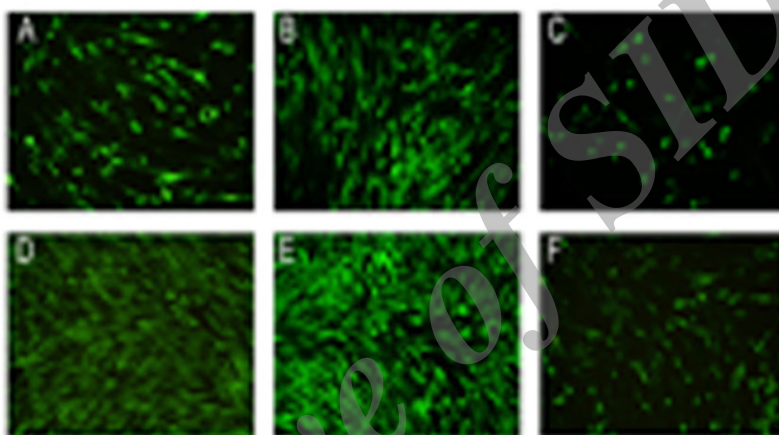| Group	Cell proliferation (cell counting) ×10³	TGF-β₁mRNA (IOD$_{TGF-β1}$/IOD β-actin)
Normal control	8.25±0.24	0.351±0.017
Normal+Diazoxide	9.41±0.01*	0.592±0.047*
Normal+5-HD	7.31±0.02	0.312±0.053
Asthma model control	9.50±0.03*	0.702±0.076*
Asthma model + diazoxide	11.61±0.03*#	0.998±0.063*#
Asthma model + 5-HD	8.42±0.02#	0.357±0.068#

Figure 3. Effects of mitochondrial ATP-sensitive K+ channel on ROS level as reflected by DCF fluorescence in HASMCs. A: Normal control group. B: Normal+Diazoxide group. C: Normal+5-HD group. D: Asthma model control group. E: Asthma model + diazoxide group. F: Asthma model + 5-HD group.

Figure 4. Effects of mitochondrial ATP-sensitive K⁺ channel on the expression of TGF-β₁mRNA. A: Normal control group. B: Normal+Diazoxide group. C: Normal+5-HD group. D: Asthma model control group. E: Asthma model+diazoxide group. F: Asthma model+5-HD group.

TGFβ₁ mRNA in the asthma model+diazoxide group was higher than that in the asthma model control group ($p<0.05$). The expression of TGFβ₁ mRNA in the asthma model+5-HD group was lower than that in the asthma model control group ($p<0.05$) (Table 2, Figure 4).

DISCUSSION

In recent years, many studies have indicated that the airway ion channel plays critical roles in the genesis and development of airway inflammation, airway hyperreactivity, and airway remodeling of asthma.[11] MitoKATP can be activated by diazoxide and inhibited by 5-hychoxyde.[12,13] In this study, diazoxide is a specific opener of MitoKATP, which could specifically open MitoKATP at a low concentration, while 5-HD is a selective blocker of MitoKATP; however, neither showed an effect on MitoKATP in the cell membrane.[14] MitoKATP plays a critical role in the maintenance of potassium balance in mitochondria, and the inhibition of mitochondrial volume changes, which is a critical factor that affects Δψm. Further, MitoKATP is very sensitive to hypoxia. The cellular ATP and NDP contents regulate the activity of the MitoKATP channel.[15] When ATP is increased and NDP is decreased, MitoKATP channel activity, in this state of normoxia, is attenuated and closed. However, with the decrease of ATP and enhancement of NDP in hypoxia, the MitoKATP channel is activated and opens, which induces the depolarization of Δψm. Currently, the effects of the MitoKATP channel in the proliferation and secretion of HASMCs in asthma remains uncertain.

As shown in our data in this study, we presumed that HASMCs in asthma might induce the reduction of ATP and enhancement of NDP in an anaerobic condition, which would induce the activation of MitoKATP channels, inflow of potassium ions, and partial depolarization of Δψm. Meanwhile, there appeared to be a functional decoupling of the electron transport chain and oxidative phosphorylation; reactive oxygen species were generated and converted to hydrogen dioxide by superoxide dismutase in the mitochondria, which further enhanced ROS production and evoked HASMCs hyperplasia. The hyperplasia and proliferation of HASMCs might induce the transformation of HASMCs synthetic phenotype from a contraction type to a proliferation type and increase of synthesis of TGF-β1, which plays multiple roles in the pathogenesis of asthma. TGF-β1 could promote hyperplasia of HASMCs and goblet cells. Further, it can enhance the synthesis of collagen and fibronectin and facilitate their deposition out of the cells, which can induce trachea stenosis and irreversible changes of pulmonary function. Moreover, the expression of TGF-β1 mRNA showed a positive correlation with basilar membrane thickness and asthma severity.[16] Plasmin is a critical serine stretch protein, which can split cellulose and is involved in airway repair and remodeling. TGF-β₁ can reduce the synthesis of plasmin of ASMs and fibroblasts, attenuate the spallation of cellulose, and induce airway fibrosis and remodeling.[17-19]

In conclusion, our results indicate that HASMCs in asthma under anaerobic conditions might induce the activation and opening of MitoK_ATP channels, cause partial depolarization of Δψm, enhance ROS levels, resulting in the remarkable proliferation of HASMCs, induce the transformation of HASMCs' synthetic phenotype from the contraction type to the proliferation type, and enhance synthetic TGF-β₁. From our results, we can presume that the MitoK_ATP channel plays critical roles in the pathogenesis of asthma. The relationship between the MitoK_ATP channel and asthma might pave the way for the identification of pathological mechanisms of asthma airway remodeling and possible future asthma therapies.

ACKNOWLEDGEMENTS

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