Mitochondrial ATP-Sensitive K+ Channel Opening Increased the Airway Smooth Muscle Cell Proliferation by Activating the PI3K/AKT Signaling Pathway in a Rat Model of Asthma

Min Gao1, Qinran Sun2, and Qingfa Liu3

1Department of Respiratory, The First Affiliated Hospital of Shandong First Medical University, Jinan, Shandong, China
2Department of Pain Management, The First Affiliated Hospital of Shandong First Medical University, Jinan, Shandong, China
3Department of Respiratory Medicine, Liaocheng People’s Hospital of Shandong Province, Liaocheng 252000, China

Correspondence should be addressed to Min Gao; flyingdreamss@163.com

Received 26 August 2020; Accepted 13 November 2020; Published 12 July 2021

Motivation: Asthma, characterized by airway remodeling, chronic airway inflammation, and airway hyperresponsiveness, posed a major threat to humans worldwide [1, 2]. Airway remodeling is mainly characterized by the epithelial-to-mesenchymal transition, mucous cell metaplasia, mucus hypersecretion, basement membrane thickening, collagen deposition, and hyperplasia of airway smooth muscle cells (ASMCs) [3–6]. When stimulated by extracellular factors, ASMCs perform a contractile function and undergo phenotypic transformation [7]. According to the previous research, ASMC proliferation is involved in airway remodeling and irreversible airway obstruction during severe asthma [8].
ASMCs, in addition to phenotypic transformation and airway reconstruction, may be regulated by the opening of the mitoK$_{\text{ATP}}$ channels and depolarization of Δψm [12, 13]. However, the underlying mechanism remained unclear.

The phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathway mediates various targets (e.g., glycogen synthase kinase-3, mammalian target of rapamycin, phosphodiesterase-3B, and insulin receptor substrate-1) to regulate cellular survival, growth, and proliferation through many mechanisms [14]. This signaling pathway plays a crucial role in inflammatory cell recruitment, regulating the expression and activation of inflammatory mediators, as well as in airway remodeling, immune cell function, and corticosteroid insensitivity in chronic inflammatory respiratory disease [14]. A previous study demonstrated that the PI3K/AKT pathway played a major role in the proliferation of ASMCs isolated from asthma patients [15].

This study aims to determine whether the mitoK$_{\text{ATP}}$ channel plays a functional role in the proliferation ability of ASMC and whether this role is related to the PI3K/AKT signaling pathway, to provide a research basis for finding new targets for the treatment of asthma.

2. Materials and Method

2.1. Generation of the Asthma Rat Model. This study used 48 male Sprague Dawley (SD) rats (weight: 250–350 g) maintained under a 12 h light/dark cycle in a temperature- (22 ± 2°C) and humidity- (45–60%) controlled environment. The animals had access to food and water ad libitum. The rats were equally divided into the asthma group and the control group. The rats in the asthma group were intraperitoneally injected with 10% ovaalbumin (OVA) plus alum on days 0 and 14, whereas the control rats received normal saline [16]. The rats in the asthma group were then challenged with OVA aerosols (1% in PBS; 30 min/d) for 7 consecutive days. As a control, rats in the control group were sensitized with saline. The experiments were approved by the ethics committee of our hospital and conducted according to the guidelines for the care and use of animals.

2.2. ASMC Preparation and Culture. Primary ASMCs were isolated from the tissues of the asthmatic and control rats. Smooth muscle was separated from parenchyma and connective tissue by tissue digestion with the solution consisting of 1 mg/mL of collagenase I, 10 mg/mL of BSA, and 1 mg/mL of papain in D-Hanks solution at 37°C for 15 min. The separated cells were then cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 20% FBS and seeded in cultured flasks with 10% FBS-DMEM. They were passaged when they reached 80–90% confluence [17]. The ASMCs at the 3rd to 6th generations were used for performing the experiments. The primary ASMCs obtained from the asthma rat model were grouped as follows: (i) asthma group; (ii) asthma + diazoxide group, which received an additional treatment of diazoxide (a selective mitoK$_{\text{ATP}}$ channel opener) (diluted in DMSO; 100 μmol/L); and (iii) asthma + 5-HD group, which received an additional treatment of 5-HD (5-hydroxydecanoate, a selective mitoK$_{\text{ATP}}$ channel blocker) (diluted in DMSO; 500 μmol/L).

The ASMCs obtained from the normal rats were also divided into 3 groups as follows: normal group, normal + diazoxide group, and normal + 5-HD group, as described above.

2.3. Identification of ASMCs. Phase-contrast microscopic images were taken to identify the specific morphological features of the ASMCs. The ASMCs were identified by immunohistochemical staining for α-smooth muscle actin.

2.4. Measurement of Rhodamine Fluorescence. The ASMCs were cultured and grown in six-well plates loaded with rhodamine-123 (R-123; 10 μg/mL) and incubated for 30 min at 37°C in the dark. R-123 fluorescence was quenched at resting Δψm and increased when the mitochondrial membrane was depolarized and was selectively taken up by mitochondria, thus revealing a linear correlation between R-123 fluorescence and Δψm. After treatment with diazoxide or 5-HD, unabsorbed R-123 in the ASMCs was washed out by D-Hanks solution to ensure that it did not affect the fluorescence intensity. The fluorescence was stimulated at 488 nm and detected at 530 nm by laser confocal microscopy. The results were analyzed using an HPIAS-1000 image analyzer.

2.5. MTT Assay. The ASMCs were cultured in 96-well plates at a cell density of $1 \times 10^4$ cells/mL at 37°C and 5% CO$_2$, in a culture medium, which was replaced with the fresh medium every 2 days. After adherent cell cultures were established, the ASMCs were treated with diazoxide or 5-HD as previously described. The medium was aspirated from the test wells, and PBS was used to gently wash the cells. MTT solution was added and incubated for 4 h, followed by the addition of DMSO (1.5 mL). Then, the supernatant was carefully aspirated. Absorbance was measured directly by spectrophotometry at 570 nm. LY294002 (a PI3K/AKT pathway inhibitor) with a final concentration of 25 mmol/L was added into the cell culture medium in the asthma group and asthma + diazoxide group, and the absorbance value was measured.

2.6. Western Blotting. After 2 days, the cells were washed with PBS and lysed in lysis buffer containing 0.5 M Tris-HCl (pH 7.4), 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA, and protease inhibitors (Millipore, Bedford, MA) and subsequently incubated on ice for 30 min. The mixtures were then centrifuged (13,000 rpm; 30 min). The protein concentrations were measured with a BCA kit. The protein extracts were electrophoresed on 6% Bis-Tris protein gels and then transferred to PVDF membranes. After incubated in 5% dry milk for 1 h, the membranes were incubated with the following primary antibodies: anti-p-AKT, anti-AKT (BD Biosciences), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Bioworld, USA). Then, the membranes were incubated with the peroxidase AffiniPure...
goat anti-rabbit IgG (H + L chains; Jackson Immuno-
Research Laboratories, West Grove, PA). The enhanced
chemiluminescence (ECL) kit (Amersham, UK) was used to
detect the protein bands. The Image-Pro Plus image analysis
software was used to quantify the band intensity. The mean
grey value was used for further statistical analysis.

2.7. Reverse-Transcription PCR and Quantitative PCR.
Total RNA was extracted from the ASMCs using TRIzol
(Invitrogen, Carlsbad, CA, USA) according to the manu-
facturer’s instructions. Then, the RNA was mixed with the
oligo (dT) primers and reverse-transcribed to cDNA using a
First-Strand cDNA Synthesis Kit (Fermentas, Burlington,
Canada). Afterward, the qPCR was performed using an
SYBR premix Ex Taq kit (TaKaRa, Dalian, China) in an ABI
7900 Imaging System (UVP, LLC Upland, CA, USA). The
PCR conditions were as follows: 94°C for 30 seconds fol-
lowed by 40 cycles of 95°C for 5 seconds, 56°C for 30 seconds,
and 72°C for 60 seconds. Then, the PCR products were
separated on 2% agarose gels. All the reactions were per-
formed in triplicate and the levels of gene expression were
calculated by the relative quantification method. The
GAPDH was used as an endogenous reference gene. All the
primers (Takara Biotechnology Co. Ltd., Dalian, China) are
listed in Table 1.

2.8. Statistical Analysis. All the data are shown as mean-
± standard deviation and analyzed by the SPSS statistical
software (version 20.0). The t-test and one-way analysis of
variance (ANOVA) test were used for comparisons between
two groups or among multiple groups, respectively. p < 0.05
was considered statistically significant.

3. Results

3.1. Characterization and Identification of ASMCs. The
ASMCs were identified according to the specific morpho-
logical characteristic (i.e., “hill and valley” feature) of these
cells using phase-contrast microscopy. Immunohisto-
chemical staining for α-smooth muscle actin was also
performed to identify ASMCs. The cultured cells were found
to be positive for α-smooth muscle actin. These experiments
demonstrated the identification of ASMCs (Figure 1).

3.2. Δψm in ASMCs in the Asthma Rat Model. Δψm in
ASMCs was detected using rhodamine fluorescence staining.
In the asthma group (Figure 2(a)), the fluorescence intensity
was markedly higher than that in the normal group
(Figure 2(d)). Diazoxide significantly increased the fluo-
rescence intensity of R-123 in the normal + diazoxide
(Figure 2(e)) group as compared with that in the normal
group (Figure 2(d)). The fluorescence intensity in the
asthma + diazoxide group (Figure 2(b)) was also strikingly
higher than that in the asthma group (Figure 2(a)). Con-
versely, the fluorescence intensity of R-123 in the
asthma + 5-HD group (Figure 2(c)) was lower than that in
the asthma group (Figure 2(a)). The Δψm is mainly
produced when there is a proton gradient and the protons
pass through the mitochondrial inner membrane. These
results indicated that diazoxide enhanced the mitoKATP
channel opening in ASMCs in the asthma rat model, whereas 5-HD impeded the channel opening.

3.3. Effect of MitoKATP Channel Opening on the Prolifer-
ation of ASMCs from the Asthma Rat Model. The MTT
method was used to detect the proliferation of ASMCs. As
shown in Figure 3, the absorbance values of ASMCs in the
normal + diazoxide group and asthma control group were
markedly higher than those in the normal group (p < 0.05).
The absorbance value in the asthma + diazoxide group was
also significantly higher than that in the asthma group
(p < 0.05). However, the absorbance value of ASMCs in the
asthma + 5-HD group was lower than that in the asthma
group (p < 0.05). There was no significant difference in the
absorbance value between the normal control group and the
normal + 5-HD group (p > 0.05). These results suggested
that diazoxide increased the proliferation of ASMCs in the
asthma rat model, and 5-HD decreased it.

3.4. Effect of MitoKATP Channel Opening on the PI3K/AKT
Signaling Pathway in ASMCs in the Asthma Rat Model.
As shown in Figure 3, the absorbance value of the
asthma + LY294002 group was lower than that of the asthma
group (p < 0.05) and the absorbance value of the
asthma + diazoxide + LY294002 group was also lower than
that of the asthma + diazoxide group (p < 0.05), indicating
that LY294002 (a PI3K/AKT pathway inhibitor) reversed the
effects of diazoxide on the proliferative ability of ASMCs in
the rat model of asthma. In addition, the expression levels
of AKT and pAKT in the ASMCs treated with diazoxide and 5-
HD in the normal and asthma groups were detected by
Western blot analysis. There was no significant difference in
the protein expression levels of AKT in the different groups
(Figure 4). However, as compared with the normal group,
the expression levels of the p-AKT protein were higher in the
normal + diazoxide group and asthma group (p < 0.05).
Furthermore, the p-AKT protein level was higher in the
asthma + diazoxide group and lower in the asthma + 5-HD
group as compared with that in the asthma group (p < 0.05)
(Figure 4). The results of AKT mRNA expression detected by
the real-time quantitative PCR were consistent with those of
the western blot (Table 2). Therefore, the treatment with
diazoxide induced AKT phosphorylation, and the treatment
with 5-HD decreased AKT phosphorylation in ASMCs in the
rat model of asthma. These results suggested that mitoKATP
channel opening modulated the PI3K/AKT signaling
pathway in asthmatic rats.

4. Discussion

Asthma is a chronic disease associated with hyper-
responsiveness, obstruction, and remodeling of the airways.
Structural changes are related to airway remodeling, in-
cluding epithelial cell shedding, goblet cell metaplasia or
hyperplasia, subepithelial fibrosis, bronchial
neovascularization [18], and smooth muscle cell hyperplasia [19]. ASMCs are effector cells of bronchoconstriction and produce inflammatory mediators and angiogenic factors. Increased ASMC levels may cause airway remodeling and persistent airflow limitation [20]. Previous research showed that ASMCs were strongly associated with the pathogenesis of asthma [21].

MitoKATP channels play vital roles in mitochondrial ion balance in various cells and are involved in various cellular functions [22]. MitoKATP proteins maintain the potassium balance in mitochondria, and opening and closing of mitoKATP channels can change $\Delta \psi_m$. In the present study, diazoxide was used to selectively open and activate mitoKATP channels, and 5-HD was used as a selective blocker to specifically diminish mitoKATP channel opening. The $\Delta \psi_m$ in ASMCs in the different groups was investigated using R-123 fluorescence staining. The results shown in Figure 2 indicated that the diazoxide treatment resulted in mitoKATP channel opening in ASMCs. Conversely, 5-HD significantly reduced mitoKATP channel opening, as evidenced by the decreased fluorescence intensity of R-123.

Previous research demonstrated that mitoKATP channel activation resulted in ASMC proliferation and secretion in asthmatic rats [24]. In this study, we examined the effects of mitoKATP channel opening on the proliferative ability of ASMCs using an MTT assay. According to the MTT assay results, the AMSC proliferation increased in the bronchial asthmatic rats challenged with OVA as compared with that in the normal controls (Figure 3). Furthermore, the absorbance values observed in the asthma + diazoxide group were significantly higher than those in the asthma group, whereas the absorbance values in the asthma + 5-HD group were much lower than those in the asthma group. The results suggested that diazoxide increased ASMC proliferation in the asthma rat model, and 5-HD decreased it. These results pointed to mitoKATP channel opening leading to enhanced ASMC proliferation. These findings were consistent with previously reported results [25]. A recent study indicated that depolarization of the $\Delta \psi_m$ and mitoKATP channel opening might result in the proliferation of human pulmonary arterial smooth muscle cells [12]. Another study reported that depolarization of the $\Delta \psi_m$ and mitoKATP channel opening contributed to the cell proliferative and apoptotic abilities of ASMCs induced by asthma [13].

PI3K/AKT is closely associated with various functions in cell proliferation, cell survival, and cancer progression [26, 27]. AKT, a major downstream target of PI3K, is activated by various stimuli, hormones, and growth factors. AKT phosphorylation can be used as an index of PI3K activity. PI3Ks are a family of proteins involved in cell proliferation, differentiation, apoptosis, and glucose transportation. Proliferation is influenced by several serine/threonine kinase cascades and many cellular signaling pathways, including PI3K/AKT signaling pathways. A previous study showed that inhibition of PI3K/AKT signaling may attenuate ASMC proliferation [28]. Recently, Dai et al. provided evidence that

---

**Table 1: The PCR primer sequences.**

| Gene  | Sequence | Size (bp) | Tm (°C) |
|-------|----------|-----------|---------|
| AKT   | ATGGACTTCCGGTCAGGTTCA GCCCTTGCCCAGTAGCTTCA | 126 | 62 |
| Sense | GGCACAGTCAAGGCTGAAATG ATGGTGTTGAAGACGCCAGTA | 143 | 61.5 |

Note. All sequences are shown in the 5' to 3' orientation. bp: base pair; Tm: temperature; AKT: protein kinase B; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.
Figure 2: The mitochondrial membrane potential $\Delta\psi_m$ in airway smooth muscle cells (ASMCs) in the asthma rat model. (a) Asthma group; (b) asthma + diazoxide group; (c) asthma + 5-HD group; (d) normal group; (e) normal + diazoxide group; (f) normal + 5-HD group; 5-HD, 5-hydroxydecanoate.

Figure 3: Effect of the mitochondrial ATP-sensitive potassium (mitoK$_{ATP}$) channel opening on the proliferation of airway smooth muscle cells (ASMCs) in the asthma rat model. The proliferation of ASMCs in the different groups was measured by the MTT assay. 5-HD, 5-hydroxydecanoate.
inhibition of TGF-β1-induced ASMC proliferation was associated with the PI3K/AKT inactivation [29]. However, the interactions between the mitoK<sub>ATP</sub>-dependent proliferation of ASMCs and the PI3K/AKT signaling pathway remained uncertain. In the present study, LY294002, a PI3K/AKT pathway inhibitor, reversed the effects of diazoxide on the proliferative ability of ASMCs in the asthma rat model, indicating that the PI3K/AKT pathway may be involved in the proliferative ability of ASMCs and the progression of asthma. Our results also indicated that diazoxide treatment induced AKT phosphorylation and that 5-HD treatment decreased AKT phosphorylation in ASMCs in an asthma rat model. These results suggested that the phosphorylation of AKT relies on the mitoK<sub>ATP</sub> channel opening. Taken together, it can be concluded that, in ASMCs, the mitoK<sub>ATP</sub> channel opening activates the PI3K/AKT signaling pathway, thereby increasing cell proliferation and aggravating airway remodeling.

5. Conclusions

The findings of this study provide the evidence that in ASMCs under anaerobic conditions, asthma may lead to activation and opening of mitoK<sub>ATP</sub> channels and partial depolarization of the Δψm, which further promotes the cell proliferation of ASMCs by activating the PI3K/AKT signaling pathway. These findings reveal that mitoK<sub>ATP</sub> channel opening and the PI3K/AKT signaling pathway play important roles in the pathogenesis of asthma. Furthermore, they suggest that mitoK<sub>ATP</sub> channels may represent a new therapeutic target in the treatment of asthma.

Table 2: The mRNA expression of protein kinase B (AKT) in airway smooth muscle cells (ASMCs) in the asthma rat model.

| Group                  | N  | GAPDH-Ct (Ct-G) | AKT-Ct (Ct-AKT) | 2<sup>−ΔΔCt</sup> |
|-----------------------|----|----------------|-----------------|-------------------|
| Normal group          | 9  | 17.87 ± 0.64   | 25.01 ± 0.60    | 1.00              |
| Normal + diazoxide group | 9  | 19.66 ± 0.27   | 26.66 ± 0.16    | 1.14 ± 0.26       |
| Normal + 5-HD group   | 9  | 19.32 ± 1.05   | 27.26 ± 0.56    | 0.98 ± 0.26       |
| Asthma group          | 9  | 18.36 ± 0.26   | 25.49 ± 0.48    | 0.97 ± 0.32       |
| Asthma + diazoxide group | 9  | 15.10 ± 0.53   | 22.11 ± 0.43    | 1.12 ± 0.30       |
| Asthma + 5-HD group   | 9  | 16.66 ± 0.57   | 23.66 ± 0.55    | 1.13 ± 0.27       |

The mRNA expression levels of the AKT in ASMCs were determined by comparative Ct value, and the results were expressed as the mean ± SD.

The Data Availability

The analyzed datasets generated during the study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors’ Contributions

Min Gao and Qinran Sun contributed equally to this work.

References

[1] K. Nakagome and M. Nagata, “Pathogenesis of airway inflammation in bronchial asthma,” *Auris Nasus Larynx*, vol. 38, no. 5, pp. 555–563, 2011.
[2] S. T. Holgate, “Pathogenesis of asthma,” *Clinical & Experimental Allergy*, vol. 38, no. 6, pp. 872–897, 2008.

[3] T. R. Bai, “Evidence for airway remodeling in chronic asthma,” *Current Opinion in Allergy & Clinical Immunology*, vol. 10, no. 1, pp. 82–86, 2010.

[4] C. Bergeron, W. Al-Ramli, and Q. Hamid, “Remodeling in asthma,” *Proceedings of the American Thoracic Society*, vol. 6, no. 3, pp. 301–305, 2009.

[5] J. V. Fahy, D. B. Corry, and H. A. Boushey, “Airway inflammation and remodeling in asthma,” *Current Opinion in Pulmonary Medicine*, vol. 6, no. 1, pp. 15–20, 2000.

[6] R. Halwani, S. Al-Muhsen, and Q. Hamid, “Airway remodeling in asthma,” *Current Opinion in Pharmacology*, vol. 10, no. 3, pp. 236–245, 2010.

[7] M. B. Sukkar, A. J. Stanley, A. E. Blake et al., “‘Proliferative’ and ‘synthetic’ airway smooth muscle cells are overlapping populations,” *Immunology & Cell Biology*, vol. 82, no. 5, pp. 471–478, 2004.

[8] B. Yeganeh, C. Xia, H. Movassaghe et al., “Emerging mediators of airway smooth muscle dysfunction in asthma,” *Pulmonary Pharmacology & Therapeutics*, vol. 26, no. 1, pp. 105–111, 2013.

[9] Y. Teshima, M. Akao, R. A. Li et al., “Mitochondrial ATP-sensitive potassium channel activation protects cerebellar granule neurons from apoptosis induced by oxidative stress,” *Stroke*, vol. 34, no. 7, pp. 1796–1802, 2003.

[10] T. Yamauchi, S. Kashi, H. Yasuoyoshi, S. Zhang, Y. Honda, and A. Akaike, “Mitochondrial ATP-sensitive potassium channel: a novel site for neuroprotection,” *Investigative Ophthalmology & Visual Science*, vol. 44, no. 6, pp. 2750–2756, 2003.

[11] P. Korge, H. M. Honda, and J. N. Weiss, “Protection of cardiac mitochondria by diazoxide and protein kinase C: implications for ischemic preconditioning,” *Proceedings of the National Academy of Sciences*, vol. 99, no. 5, pp. 3312–3317, 2002.

[12] H. Hu, T. Wang, Z. Zhang, J. Zhao, and Y. Xu, “The effect of mitochondrial membrane potential on changes of reactive oxygen species and on proliferation of hypoxic human pulmonary arterial smooth muscle cells,” *Chinese Journal of Tuberculosis and Respiratory Diseases*, vol. 29, no. 11, pp. 727–730, 2006.

[13] J.-P. Zhao, M. Gao, Y.-J. Ye, W.-H. Hu, Z.-G. Zhou, and H.-L. Hu, “Regulation of rat airway smooth muscle cell proliferation by mitochondrial ATP-sensitive K+ channel in asthmic rats,” *Sheng Li Xue Bao: Acta Physiologica Sinica*, vol. 61, no. 1, pp. 65–71, 2009.

[14] A. Holfer, T. Nichols, S. Grant et al., “Study of the PDK1/AKT signaling pathway using selective PDK1 inhibitors, HCS, and enhanced biochemical assays,” *Analytical Biochemistry*, vol. 414, no. 2, pp. 179–186, 2011.

[15] J. K. Burgess, J. H. Lee, Q. Ge et al., “Dual ERK and phosphatidylinositol 3-kinase pathways control airway smooth muscle proliferation: differences in asthma,” *Journal of Cellular Physiology*, vol. 216, no. 3, pp. 673–679, 2008.

[16] M. Salmon, D. a. Walsh, H. Koto, P. j. Barnes, and K. f. Chung, “Repeated allergen exposure of sensitized Brown-Norway rats induces airway cell DNA synthesis and remodelling,” *European Respiratory Journal*, vol. 14, no. 3, pp. 633–641, 1999.

[17] P. R. A. Johnson, M. Roth, M. Tam et al., “Airway smooth muscle cell proliferation is increased in asthma,” *American Journal of Respiratory and Critical Care Medicine*, vol. 164, no. 3, pp. 474–477, 2001.