Azithromycin induces apoptosis in airway smooth muscle cells through mitochondrial pathway in a rat asthma model

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Background: The proliferation of airway smooth muscle cells (ASMCs) is a key feature of airway remodeling in asthma. Azithromycin (AZM) has been shown to decrease bronchial hyperresponsiveness and airway inflammation in asthmatics; however, the role of AZM in ASMC proliferation remains unclear. Thus, we investigated the effect of AZM on ASMC proliferation in a rat model of experimental asthma.

Methods: We isolated ASMCs from rats sensitized and challenged by ovabulmin (OVA), and then treated with different concentrations of AZM. Cytotoxicity of ASMC was evaluated by Cell Counting Kit-8 (CCK-8) assay, morphological change was examined with laser confocal microscope after Annexin V/propidium iodide (PI) double staining, mitochondrial membrane potential was determined with JC-1 staining, and the expression of cytochrome C was examined by western blot.

Results: The relative surface areas of airway wall and smooth muscle layers in OVA-sensitized rats were significantly increased compared to those in the control group. Furthermore, in OVA-sensitized rats, the mitochondrial membrane potential of ASMC was higher, while the expression of mitochondria cytochrome C was lower compared to that in control rats. After AZM treatment, ASMC apoptosis was increased, mitochondrial membrane potential reduced, and the protein level of cytosolic cytochrome C was increased.

Conclusions: This study demonstrated that AZM increased the apoptosis of ASMCs through a mitochondrial pathway, which might play an important role in ASMs proliferation during asthmatic remodeling.

Keywords: Azithromycin (AZM); asthma; airway smooth muscle cells (ASMCs); apoptosis; mitochondria

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Introduction

Asthma is a heterogeneous disease which is influenced by both genetic and environmental factors (1). It is characterized as eosinophilic inflammation of the airways and structural changes of bronchial tissues, known as airway remodeling (2). Hours are needed to complete the process of cytokine-induced recruitment of eosinophils from the blood to the airways after acute allergen challenge (3). Airway remodeling refers to a number of processes
including increased epithelial cell disruption and airway smooth muscle mass, and subepithelial fibrosis. Thus, the reduction of airway smooth muscle cell (ASMC) apoptosis might participate in the remodeling process of asthma (4-6).

Our previous study found that macrolides could reduce airway inflammation by inhibiting the PI3K-δ/Akt signaling pathway and upregulating histone deacetylase 2 (HDAC2) expression (7). Several clinical studies have shown that azithromycin (AZM), an antibiotic, has the ability to reduce bronchial hyperresponsiveness and neutrophilic airway inflammation (8). Furthermore, it has been demonstrated that AZM has an antiproliferative and autophagic effect on ASMCs in adult rabbits (9), and that AZM ameliorates airway remodeling via inhibiting airway epithelium apoptosis (10). However, the underlying mechanism of AZM-induced ASMC apoptosis is still not clear.

There are many mechanisms that cause apoptosis, and whether mitochondrial pathway is involved in them is the focus of research. The most important detection indexes of mitochondrial pathway are mitochondrial membrane potential and cytochrome C. Mitochondria play an important role in the apoptotic pathway of respiratory epithelial cells in asthma (11). As an intermembranous mitochondrial protein, cytochrome C triggers the caspase activation cascade by binding to a cytoplasmic scaffolding protein, and then initiates apoptosis in response to cellular stress (12). At present, there are no reports on the prevention and treatment of asthma by AZM through mitochondrial pathway mediated apoptosis. To explore the mechanism of macrolide antibiotics on ASMCS in asthmatic rats can provide new strategies and new ideas for the prevention and treatment of asthma. Furthermore, patients who have high treatment compliance will benefit from effective intervention. Considering mitochondrial activity is essential in cell apoptosis, we hypothesized that AZM might induce ASMC apoptosis through the mitochondrial pathway.

We present the following article in accordance with the ARRIVE reporting checklist (available at https://dx.doi.org/10.21037/atm-21-3478).

**Methods**

**Animal model**

Male Sprague Dawley rats [specific pathogen-free (SPF) grade, 6 weeks old] were used in this study. We randomly allocated 20 Sprague Dawley rats to two groups, with 10 in the control group and 10 in the asthma group. The animals had no access to solid food but received free access to water 12 h before the experiments. The experimental protocol (No. wydw2016-0124) was approved by the Committee of Animal Care at Wenzhou Medical University. All animals were handled in accordance with the Guideline for the Care and Use of Laboratory Animals. A protocol was prepared before the study without registration. Rats were sensitized on days 1 and 8 by intraperitoneal (ip) injection of 10 mg of ovalbumin (OVA) (Sigma, St. Louis, MO, USA) mixed with 10% aluminum hydroxide Al(OH)₃ suspended in a total volume of 1 mL (13). Then, from day 15, animals were challenged with 1% OVA aerosol for 30 min every other day for 8 weeks (14). Saline injection (ip) and nebulization were used for the control rats. The rats were anesthetized and humanely killed 24 h after the last challenge.

**Histological examination**

After the rats were sacrificed, the descending aorta was cut, and their lungs were carefully removed from the thoracic cavity. The right middle lobes of the lungs were fixed in 4% paraformaldehyde, and then imbedded in paraffin. After deparaffinization and staining with hematoxylin and eosin (HE), the sections (5 μm thick) were morphometrically analyzed to determine airway wall and smooth muscle content. Basement membrane perimeter (Pbm), bronchial total area (At), lumen area (Ac), the outer edge of the tracheal smooth muscle area (AMe), and the inner edge of the tracheal smooth muscle area (AMI) were quantified by commercially available imaging software (Image pro plus 6.0; Media Cybernetics Inc., Rockville, MD, USA). In addition, bronchial wall thickness (Wat) and smooth muscle thickness (Wam) were calculated using two formulas, as follows: Wat = (At – Ac)/Pbm; Wam = (AMe – AMi)/Pbm (15). For each index, three bronchioles from each section were chosen and measured to determine the mean value.

**Cell culture**

According to the method described by Dai et al. (16), rat airway smooth muscle was separated, and then minced into 1 mm³ fragments. The tissues were incubated in Ca²⁺ free physiological salt solution containing 2 g/L collagenase, 5 g/L papain, and 2 g/L bovine serum albumin (BSA) for 30 min at 37 °C. Then, the isolated ASMCS were cultured in Dulbecco’s modified Eagle’s medium (DMEM), containing...
20% fetal bovine serum (FBS). Specific mouse monoclonal antibodies α-actin (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was used to verify the purity and identification of ASMCs, which were used between passages 3 and 7. At 24 h before drug treatment, ASMCs were transferred to serum-free DMEM for synchronization when reaching nearly 70% confluence, and then cultured with AZM (0.1, 1, 10, and 100 μg/mL). After 24 h, the cells were collected for further analysis.

Cytotoxicity assay

Cell cytotoxicity assay was determined by Cell Counting Kit-8 (CCK-8) assay. A total of 100 μL of cell suspension (5,000 cells/well) was pre-incubated for 24 h (at 37 °C, 5% CO2). After adding 10 μL of various concentrations of AZM for 24 h, we incubated each well of the plates with 10 μL of CCK-8 solution for 1–4 h. The absorbance was measured at 450 nm by a microplate reader (Bio-Tek, Winooski, VT, USA).

The Annexin V-FITC/propidium iodide (PI) assay

After cells were treated with AZM for 24 h, 5 μL Annexin V-FITC and 5 μL PI were added to the culture medium, and then the cells were transferred onto coverslips (KeyGen Biotech., Nanjing, China). Samples were kept in the dark at room temperature for 10 min, and then examined under a laser confocal microscope (Olympus, Tokyo, Japan).

Mitochondrial membrane potential assay

JC-1 dye (KeyGen Biotech.) was used to measure mitochondrial membrane potential. ASMCs (1×10⁶ cells/well) were treated with AZM for 24 h. After washing, ASMCs were incubated with pre-warmed JC-1 solution for 15 min at 37 °C, and then examined with flow cytometry (Facsaria, Becton, Dickinson and Co. Biosciences, Franklin Lakes, NJ, USA). Data was analyzed using CellQuest software (BD Biosciences, USA).

Western blot analysis

Cultured ASMCs were frozen and lysed in lysis buffer. Total proteins were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. Block membrane [in 30 mL phosphate buffered saline (PBS) + 5% non-fat dry milk + 0.1% Tween 20 in a small dish on a shaker] was then incubated with cytochrome C antibody (1:1,000; Cell Signaling Technology, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:1,000; Cell Signaling Technology, USA) was used for control.

Statistical analysis

The data were presented as mean ± standard deviation (SD). Student’s t-test for unpaired samples was used for comparison of means between asthmatic and control groups. One-way analysis of variance (ANOVA) was used for multiple comparisons among more than two groups. A P value <0.05 was considered statistically significant.

Results

Effects of AZM on the proliferation of ASMCs

The HE staining of lung tissue showed that there was a large number of inflammatory cell infiltration, the majority of which were eosinophilic cells. There was also evidence of airway epithelial damage and smooth muscle layer thickening in OVA-sensitized rats compared to the lungs of control rats (Figure 1A). The relative area of Wat and Wam in OVA-sensitized rats was significantly increased compared to those in control rats (Table 1, Figure 1B). To evaluate the effects of AZM on ASMC proliferation, different concentrations (0.1, 1, 10, and 100 μg/mL) of AZM were applied in culture medium for 24 h. As shown in Figure 2, treatment with AZM reduced the viability of ASMCs to the levels of 72.71%±13.16% at 0.1 μg/mL, 66.42%±4.90% at 1 μg/mL, 64.92%±2.82% at 10 μg/mL, and 51.45%±2.83% at 100 μg/mL, indicating that AZM has dose-response effects on inhibiting ASMC proliferation.

In order to confirm the antiproliferative effect of AZM, the morphologic features of cell apoptosis were examined under a laser confocal microscope after Annexin V and PI double staining. As shown in Figure 3A, B, there was no FITC-Annexin V/PI positively stained cells in the control and OVA-sensitized groups. In contrast, Figure 3C, D, E shows that the cytoplasm of ASMCs was gradually stained green with Annexin V-FITC after treatment with 0.1, 1, and 10 μg/mL of AZM. Of note, Figure 3F showed that the nucleus of ASMCs appeared red with PI staining after treatment with 100 μg/mL AZM. These results demonstrated that AZM induced early and late phase of ASMC apoptosis in a dose-dependent manner.
Effects of AZM on mitochondrial membrane potential in ASMCs

To determine whether AZM affects mitochondrial Δφm in ASMCs, we performed flow cytometry with JC-1 fluorescent staining to identify two cell populations with different Δφm, namely, one population with high Δφm (gated region P2, stained in green), and the other with low Δφm (gated region P3, stained in blue). As shown in Figure 4A, flow cytometry analysis revealed that 72.4% cells stained in green and 27.3% cells stained in blue were detected in control rat ASMCs. In contrast, a significantly increased percentage of green cells and decreased percentage of blue cells was seen in OVA-sensitized rat ASMCs. As shown in Table 2, Figure 4B,C, the low mitochondrial membrane potential of asthmatic group was significantly reduced (P<0.01) and the high mitochondrial membrane potential was significantly increased (P<0.01) relative to control group; Compared with asthmatic group and control group, the low mitochondrial membrane potential at each concentration of AZM intervention group was significantly increased (P<0.01) and the high mitochondrial membrane potential was significantly increased (P<0.01) vs control group. HE, hematoxylin and eosin; OVA, ovalbumin; Wat, the total bronchial wall area; Pbm, basement membrane perimeter Wam, smooth muscle thickness; SD, standard deviation.

Table 1 Difference of Pbm, Wat, Wam in four groups (x±s, n=6)

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Pbm (μm)</th>
<th>Wat/Pbm (μm²/μm)</th>
<th>Wam/Pbm (μm²/μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>2160±338.71</td>
<td>31.94±5.12</td>
<td>11.30±1.70</td>
</tr>
<tr>
<td>Asthmatic</td>
<td>6</td>
<td>2101±838.95</td>
<td>53.73±20.27*</td>
<td>16.75±5.34*</td>
</tr>
<tr>
<td>F</td>
<td>0.026</td>
<td>6.515</td>
<td>5.668</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>&gt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

*, P<0.05 vs. control group. Pbm, basement membrane perimeter; Wat, the total bronchial wall area; Wam, smooth muscle thickness.

Figure 1 Histologic changes in bronchiole. (A) HE staining of lung tissue from control (a) and OVA-challenged OVA-sensitized (b) rats. Original magnification ×100. (B) The relative area of Wat/Pbm and Wam/Pbm in OVA-sensitized rats was significantly increased compared to those in control. Data are presented as mean ± SD (n=8). **, P<0.01 vs. control group. HE, hematoxylin and eosin; OVA, ovalbumin; Wat, the total bronchial wall area; Pbm, basement membrane perimeter Wam, smooth muscle thickness; SD, standard deviation.
Figure 2 Cytotoxicity of AZM on ASMC. ASMCs were incubated with saline or AZM with various concentrations (0.1, 1, 10, and 100 μg/mL). Data are presented as mean ± SD (n=3). **, P<0.01 vs. saline. AZM, azithromycin; ASMC, airway smooth muscle cell; SD, standard deviation.

Table 2 Effects of AZM on mitochondrial membrane potential in ASMCs

<table>
<thead>
<tr>
<th>Group</th>
<th>High mitochondrial membrane potential (%)</th>
<th>Low mitochondrial membrane potential (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>62.30±7.12</td>
<td>37.45±7.18</td>
</tr>
<tr>
<td>Asthmatic</td>
<td>74.95±6.56**</td>
<td>24.62±7.05**</td>
</tr>
<tr>
<td>0.1 μg/mL</td>
<td>48.23±2.57***</td>
<td>51.45±2.59***</td>
</tr>
<tr>
<td>1 μg/mL</td>
<td>37.43±2.55****</td>
<td>62.28±2.61****</td>
</tr>
<tr>
<td>10 μg/mL</td>
<td>55.67±5.62****</td>
<td>44.05±5.62****</td>
</tr>
<tr>
<td>100 μg/mL</td>
<td>31.83±1.95*****</td>
<td>67.83±1.85*****</td>
</tr>
</tbody>
</table>

**, P<0.01 vs. control group; ***, P<0.01 vs. asthmatic group incubated without AZM treatment. AZM, azithromycin; ASMC, airway smooth muscle cell.

Table 3 Effects of AZM on ASMCs apoptosis

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Control</th>
<th>Asthma</th>
<th>0.1 μg/mL</th>
<th>1 μg/mL</th>
<th>10 μg/mL</th>
<th>100 μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viability (%)</td>
<td>120</td>
<td>100</td>
<td>80</td>
<td>60</td>
<td>40</td>
<td>0</td>
</tr>
</tbody>
</table>

**Figure 3 Effects of AZM on ASMCs apoptosis. Annexin V and PI double staining of ASMCs were examined with a laser confocal microscope from control (A) and asthmatic (B) rats incubated without AZM, and from OVA-sensitized rats incubated with AZM under different concentrations (0.1, 1, 10, and 100 μg/mL) (C,D,E,F). Images are shown at 200× magnification. Green fluorescence indicated Annexin V-FITC staining positive. Red indicated PI staining positive. AZM, azithromycin; ASMC, airway smooth muscle cell; PI, propidium iodide; OVA, ovabulmin.
Figure 4 Effects of AZM on mitochondrial membrane potential in rat ASMCs. (A) ASMCs from control (a) and OVA-sensitized rats incubated without AZM, and from asthmatic (b) rats incubated with AZM under different concentrations (0.1, 1, 10, and 100 μg/mL) (c,d,e,f) for 24 h, stained with fluorescent dye JC-1 for 30 min, and subjected to flow cytometry analysis (ex =488 nm, em =530 nm). (B) The percentage of cells with high Δψm was represented as histograms. (C) The percentage of cells with low Δψm was represented as histograms. Data are presented as mean ± SD. Three independent experiments were performed for each condition. **, P<0.01 vs. control group; ##, P<0.01 vs. asthmatic group incubated without AZM treatment. AZM, azithromycin; ASMC, airway smooth muscle cell; SD, standard deviation.
reduced (P<0.01). As a result, AZM significantly decreased the Δϕm of OVA-sensitized rat ASMCs in a dose-dependent manner. Thus, we concluded that AZM induced apoptosis through alternating the mitochondrial membrane potential of OVA-sensitized rat ASMCs.

Expression of cytochrome C in ASMCs

In order to confirm that AZM induced apoptosis of ASMCs through a mitochondrial pathway, we next examined the expression level of cytochrome C in ASMCs. As shown in Table 3, Figure 5A,B, cytosolic cytochrome C from OVA-sensitized rats was significantly reduced compared to that from control group rats. Upon administration of AZM, cytosolic cytochrome C was markedly increased, while there was no mitochondrial cytochrome C detected. The results indicated that AZM induced the releasing of cytochrome C protein from mitochondria.

Table 3 Western blot

<table>
<thead>
<tr>
<th>Group</th>
<th>Cytosolic cytochrome C (OD)</th>
<th>Mitochondrial cytochrome C (OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.028±0.043</td>
<td>0.087±0.006</td>
</tr>
<tr>
<td>Asthmatic</td>
<td>0.825±0.029*</td>
<td>0.068±0.005**</td>
</tr>
<tr>
<td>0.1 μg/mL</td>
<td>1.622±0.205**</td>
<td></td>
</tr>
<tr>
<td>1 μg/mL</td>
<td>2.174±0.344***</td>
<td></td>
</tr>
<tr>
<td>10 μg/mL</td>
<td>3.175±0.264***</td>
<td></td>
</tr>
<tr>
<td>100 μg/mL</td>
<td>3.445±0.267***</td>
<td></td>
</tr>
</tbody>
</table>

*, P<0.05, **, P<0.01 vs. control group; ***, P<0.01 vs. asthmatic group incubated without AZM treatment. AZM, azithromycin; OD, optical density.

Figure 5 The effects of AZM on the expression of cytochrome C. (A) The expression of cytochrome C was analyzed by western blot. (B) Cytosolic cytochrome C from OVA-sensitized rats was significantly reduced compared to that from control group rats. Data are presented as mean ± SD from at least three independent experiments. *, P<0.05, **, P<0.01 vs. control group; ***, P<0.05 vs. asthmatic group incubated without AZM treatment. AZM, azithromycin; OVA, ovalbumin; SD, standard deviation.

Discussion

ASMCs reversible switching between contractile and proliferative phenotypes, is considered to contribute to proliferative diseases. Airway remodeling is one of the main features of asthma and involves structural changes of airway smooth muscle, epithelia, extracellular matrix, and blood vessels (17,18). Airway smooth muscle remodeling has important effects on airway responsiveness and severity of asthma (18). In the present study, we found that the thickness and relative area of the airway wall and smooth muscle layer were all increased in OVA-sensitized rats, which confirmed that we had successfully established an asthmatic rat model. The drug AZM decreases apoptosis
through mitochondrial pathways in OVA-sensitized rat ASMCs, and thus might play an important role in airway smooth muscle remodeling in asthmatic rats.

In our study, AZM was administrated at a concentration that equaled the lung tissue concentration of 8.93 mg/L (19), which did not upregulate the caspase-3 expression and did not change the percentage of dead cells after AZM treatment for 24 h, indicating that AZM did not induce apoptosis in control rat ASMCs (9). In this study, we investigated the morphologic features of cell apoptosis with Annexin V and PI double staining under laser confocal microscope and revealed the early and late phase of apoptosis of ASMCs upon administration of AZM with dose-response effects in ASMCs isolated from OVA-sensitized rats. The results indicated that AZM might only induce apoptosis of ASMCs isolated from OVA-sensitized rats. Patients with persistent symptomatic asthma experience fewer asthma exacerbations and improved quality of life when treated with AZM (20). Studies have reported that AZM could reduce bronchial hyper responsiveness and airway inflammation in asthmatic patients (8,21).

Recently, it has been shown that AZM inhibits the expression of multiple signaling molecules, such as tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8), IL-6, nuclear factor kappa-B (NF-kB), ERK1/2, and JNK in epithelial cells from a cystic fibrosis model, which suggests that AZM has an immunomodulatory effect (22). Combined treatment of bortezomib and clarithromycin (CAM) or AZM enhances cytotoxicity in several myeloma cell lines, induces the proapoptotic transcription factor C/EBP homologous protein (CHOP), and subsequently upregulates the expression levels of proapoptotic genes including BIM, BAX, DR5, and TRB3 (23). It has been shown that BH3 domain-only molecules regulate the caspase activation cascade, initiating apoptotic response (24,25).

In ASMCs of asthma, the signal transduction pathway of PKCα may be involved in cell proliferation, which is induced by the opening of mitoK<sub>ATP</sub> and the depolarization of Δψm (26). Mitochondrial membrane potential is a sensitive parameter reflecting minor changes of the cellular environment and plays an important role in cell apoptosis (27). Mitochondrial membrane potential is formed when proton transfers from the mitochondrial matrix to the intermembranous space. When membrane potential falls, permeability transition pores will open, which allows the release of small molecules, including apoptosis initiating factors such as cytochrome C, from the mitochondria through the pores. The released cytochrome C binds to apoptotic protease activating factor-1, initiates the caspase activation cascade, and induces cellular apoptosis (27). Based on the cytotoxicity assay and Annexin V and PI double staining experiments, our in vitro experiments showed that a lower membrane potential and higher viability of ASMCs were detected in OVA-sensitized rats, which provided further evidence that mitochondrial membrane potential is altered during asthmatic airway remodeling.

Furthermore, our study showed that the level of cytochrome C in cytoplasm from control group rats was significantly higher than that in the asthmatic rat group. In ASMCs from OVA-sensitized rats, the level of cytochrome C was lower compared to ASMCs from the control group, while AZM increased the protein expression of cytochrome C. These results demonstrated that AZM could increase apoptosis in asthmatic airways through a mitochondrial dependent pathway.

Conclusions

In summary, this study demonstrated that decreased ASMC apoptosis, higher mitochondrial membrane potential, and lower expression levels of mitochondrial cytochrome C may play an important role in ASMC remodeling of asthmatic rats. It was shown that AZM can upregulate cytochrome C protein level, leading to cell apoptosis in asthmatic rat ASMCs. Therefore, our study provides a new insight into the mechanisms of AZM-induced ASMC apoptosis in asthma airways with a potential new target for future asthma treatment.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://dx.doi.org/10.21037/atm-21-3478
The authors have no conflicts of interest to declare.

**Ethical Statement:** The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The experimental protocol (No. wydw2016-0124) was approved by the Committee of Animal Care at Wenzhou Medical University. All animals were handled in accordance with the Guideline for the Care and Use of Laboratory Animals.

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