

Roxithromycin reduces the viability of cultured airway smooth muscle cells from a rat model of asthma

Y.-R. DAI, H.-Y. WU, L.-Q. WU, H. XU, J. YIN, S.-S. YAN, W.-X. ZENG

Department of Respiratory, the Second Affiliated Hospital of Wenzhou Medical College, Wenzhou, Zhejiang, China

Abstract. – OBJECTIVES: The purpose of this study was to investigate the effect of roxithromycin on apoptosis of airway smooth muscle cells (ASMCs) from a rat model of asthma and uncover signaling pathway underlying the cytotoxicity of roxithromycin.

MATERIALS AND METHODS: ASMCs were isolated from a rat model of asthma and treated with or without roxithromycin for 48 h before parameter detection. Cell viability was assessed by WST-8 assay and flow cytometry after Annexin V/PI double staining. Changes in the mitochondrial membrane potential ($\Delta\Psi_m$) were measured by flow cytometry using JC-1. Cytochrome C (Cyt c), cleaved Caspase-9/3 and P27 were evaluated by Western Blot.

RESULTS: Incubation with roxithromycin reduced ASMCs proliferation and enhanced apoptosis in a dose-dependent manner. Flow cytometry revealed a loss of $\Delta\Psi_m$ and Western Blot displayed Caspase-9/3 activation as well as Cyt c release from mitochondria to the the cytosol after the treatment of roxithromycin. In addition, P27 were more strongly expressed in AMSCs treated with roxithromycin compared with the control group.

CONCLUSIONS: Roxithromycin induced apoptosis of ASMCs derived from a rat model of asthma in a dose-dependent manner via a caspase-3- and caspase-9-dependent mitochondrial pathway, involving the up-regulation of P27.

Key Words:

Roxithromycin, Airway smooth muscle cells (ASMCs), Apoptosis.

panbronchiolitis, chronic bronchitis and bronchial asthma^{1-3,6-8}. A lot of randomised, controlled clinical trials have concluded that the macrolides could improve lung function and symptoms for asthmatics⁹⁻¹¹. However, considering the small number of patients studied, the current International Asthma Guidelines hasn't recommend macrolides as the conventional drugs for asthma treatment¹². Further researches are needed in particular to clarify the pharmacological effects and molecular mechanisms of macrolides used in chronic airway diseases. Ota et al¹³ reported that roxithromycin could prevent airway remodeling in a Guinea pig model with chronic asthma, however the mechanism was obscure. Studies on cultured cells *in vitro* have revealed that roxithromycin could suppress proliferation of human coronary artery smooth muscle cells (CASM)¹⁴, promote lymphocyte apoptosis in Dermatophagoides-sensitive asthma patients¹⁵. Fukui et al¹⁶ found that rokitamycin effectively induced Jurkat cell apoptosis via mitochondrial perturbation. Recently, it has been reported that azithromycin reduced the viability of airway smooth muscle cells (ASMCs) by leading to apoptotic cell death^{17,18}. However, no information is available about the effect of roxithromycin on ASMCs so far.

We hypothesized that roxithromycin may induce mitochondrial dysfunction and increase apoptosis of ASMCs.

Introduction

Macrolides are widely used for the treatment of respiratory infectious disease. In addition to direct antimicrobial activity, macrolides also exert immunomodulating and antiinflammatory effects¹⁻⁵. Low-dose, long-term macrolide therapy has been reported to be very effective in patients with chronic airway diseases, such as diffuse

Materials and Methods

Reagents

Roxithromycin powder was purchased from Sigma-Aldrich (Sigma Aldrich Co., St Louis, MO, USA) and dissolved in dimethylsulfoxide (DMSO: Sigma, USA) at a concentration of 100 $\mu\text{g}/\mu\text{L}$. This solution was sterilized by passing

through a 0.22 mm filter and stored as a stock solution at -20°C . It was diluted with DMSO (final concentration of 0.1%) and complete medium to make different required concentrations (100 $\mu\text{g/mL}$, 50 $\mu\text{g/mL}$, 25 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$). The following reagents were employed: RPMI1640 medium and fetal bovine serum (FBS) (HyClone, Logan, UT, USA); WST-8 assay kit (Dojindo Molecular Technologies, Kumamoto, Japan); Mitochondrial isolation kit for cultured cells (Pierce, Rockford, IL, USA); BCA (bicinchoninic acid) protein assay kit, ECL detection kit, Annexin V-FITC apoptosis detection kit and mitochondrial isolation kit for cultured cells (Pierce, Rockford, IL, USA); Antibodies against cleaved caspase-3, cleaved caspase-9 and P27 (Cell Signaling Technology Inc., Danvers, MA, USA); Antibody against SM-actin (Sigma, USA); Antibodies against VDAC1/Porin and Cyt c (Abcam, San Francisco, CA, USA); JC-1, Antibody against α -Tubulin and all secondary antibodies (Beyotime Biotech., Haimen City, China).

ASMCs Isolation and Culture

Specific pathogen free Sprague-Dawley (SD) rats were used to model asthmatic airway remodeling according to Palmans et al¹⁹. Tracheas were removed from a rat model of asthma killed under chloral hydrate anesthesia and placed into sterile, ice-cold phosphate buffered saline (PBS). Then, epithelium and any unwanted fibrous tissue were removed under a microscope. The tissue was chopped finely (approximately 1 mm³) and digested in 2 ml Roswell Park Memorial Institute (RPMI)-1640 (containing 2 mg/mL bovine serum albumin (BSA), 2 mg/mL collagenase I and 20 U/mL elastase IV) at 37°C for 1 h. The resulting cell suspension was centrifuged (200 g for 5 min) and the pellet was washed twice in supplemented RPMI-1640 containing 10% FBS (fetal bovine serum). Cells were seeded in 25 cm² flasks and maintained in a humidified incubator at 37°C under 5% CO₂. Fresh medium (RPMI-1640 containing 20% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin) was replaced every 72 h. Cells were subcultured with 0.25% trypsin.

The origin of the cells was confirmed by immunocytochemistry techniques with the monoclonal antibody against SM α -actin (Smooth Muscle Actin)²⁰.

Treatment of Cells

All experiments were performed with cells on passages 3-7. Cells were cultured in serum-free

medium for 24 h before roxithromycin stimulation to achieve quiescence. Subsequently, serum-starved cells were exposed to different concentrations of roxithromycin (0, 10, 25, 50, 100 $\mu\text{g/mL}$, along with 0.1% DMSO in each group) for 48 h and then harvested for various detections.

Cell Proliferation Assay

Cells were seeded in 96-well culture plate (2000 cells/well). After incubation with different concentrations of roxithromycin for 48 h, 10 μL 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium (WST-8) was added to each well. After 2 h of incubation, absorbance was measured at 490 nm with a microplate autoreader (Bio-Tek, Winooski, VT, USA).

Quantification of ASMCs Apoptosis by Flow Cytometry

After washing twice with PBS, 5×10^5 cells were resuspended in binding buffer and labelled with Annexin V-FITC (fluorescein isothiocyanate) and PI (propidium iodide) according to the kit instruction manual. After incubation for 5 min in the dark at room temperature, the cells were analyzed on the Becton Dickinson FACS Aria flow cytometer (Bethesda, MD, USA) using cell Quest software program (Aliso Viejo, CA, USA).

Measurement of Mitochondrial Membrane Potential ($\Delta\Psi m$)

Thirty minutes prior to cytometric analysis, JC-1 was added to 1 mL cells (5×10^5) at a final concentration of 10 mM and incubated at 37°C , 5% CO₂ atmosphere for 15 min in the dark. The cells were washed with cold staining buffer twice and resuspended in 500 μL cold staining buffer. 10,000 cells were examined for each sample on a FL-1 (530 nm) versus FL-2 (585 nm) dot plot on a Becton Dickinson FACS Aria.

Preparation of Mitochondria and Cytosol

Mitochondria were isolated by the differential centrifugation method according to the kit instruction manual (Mitochondrial isolation kit for cultured cells, Pierce, Rockford, IL, USA). Briefly, the samples ($2 \times 10^7 - 5 \times 10^7$ cells) mixed with lysis buffer were preprocessed with dounce homogenization, sequentially centrifuged at $800 \times g$ for 10 min at 4°C to remove nuclei and cell debris, and the supernatants were centrifuged again at $12,000 \times g$ for 20 min at 4°C to

recover mitochondria. The supernatants were ultracentrifuged at $12,000 \times g$ for 30 min at 4°C , and the remaining supernatant were used as the cytosol fraction. The mitochondrial pellets were lysed with detergent for Western blot analysis. Both mitochondria and cytosol fractions were used immediately or stored at -80°C until immunodetection of Cyt c.

Western Blot Analysis

Protein concentration was determined using the BCA kit (Pierce, Rockford, IL, USA). Whole cell lysate, mitochondria and cytosol fractions were subjected to electrophoresis on 4%-12% gradient SDS-PAGE gel. Proteins were transferred onto polyvinylidenedifluoride membranes (Millipore, Billerica, MA, USA) and incubated with 5% skim milk for 2 h to block nonspecific binding. Each membrane was then incubated with one of the following primary antibodies at 4°C overnight. Primary antibodies included mouse monoclonal anti-Cyt c (1:250), rabbit monoclonal anti-P27 (1:750), rabbit polyclonal anti-caspase-9 (1:500), rabbit polyclonal anti-caspase-3 (1:500), rabbit polyclonal anti-VDAC1/Porin (1:500), mouse monoclonal anti-Tubulin (1:1000). Further, incubated with respective horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h. Immunoreactivity was detected with enhanced chemoluminescent autoradiography (ECL) ac-

cording to the manufacturer's instructions. The levels of protein expression were quantitatively analyzed with Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA).

Statistical Analysis

Data were expressed as mean $\pm$ SD. Statistical analysis was performed using SPSS 11.5 (SPSS Inc., Chicago, IL, USA) via one-way ANOVA test and the results were considered significant at $p \leq 0.05$.

Results

Effects of Roxithromycin on Cell Viability

Immunocytochemical localization confirmed the smooth muscle origin of third-generation cells, because $> 95\%$ expressed the SM contractile protein SM-actin (Figure 1).

Treatment with roxithromycin ($\geq 25 \mu\text{g/mL}$) for 48 h resulted to cell shrink, formation of cytoplasmic vacuoles and particulates, shedding of smaller fragments from the cells, observed under the light microscope. The higher concentration, the more significant changes. Detachment of the whole cell from the substrate was commonly observed at the concentration of $100 \mu\text{g/mL}$, while cell morphologies were similar to the normal after treated with 0 and $10 \mu\text{g/mL}$ roxithromycin (Figure 2). Moreover, the number of ASMCs

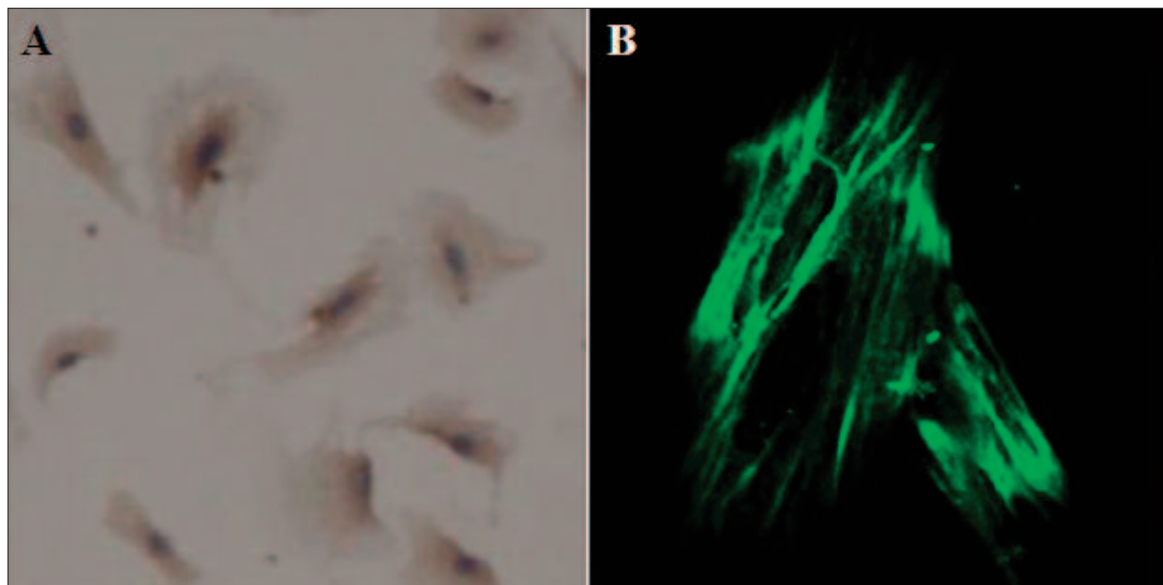


Figure 1. Immunostaining of ASMCs with an anti-SM-actin mouse monoclonal antibody. More than 95% of the cells stained positively for the SM-specific protein by immunocytochemistry (**A**, $\times 200$). Immunofluorescence stain show green parallel myofilament (**B**, $\times 1000$).

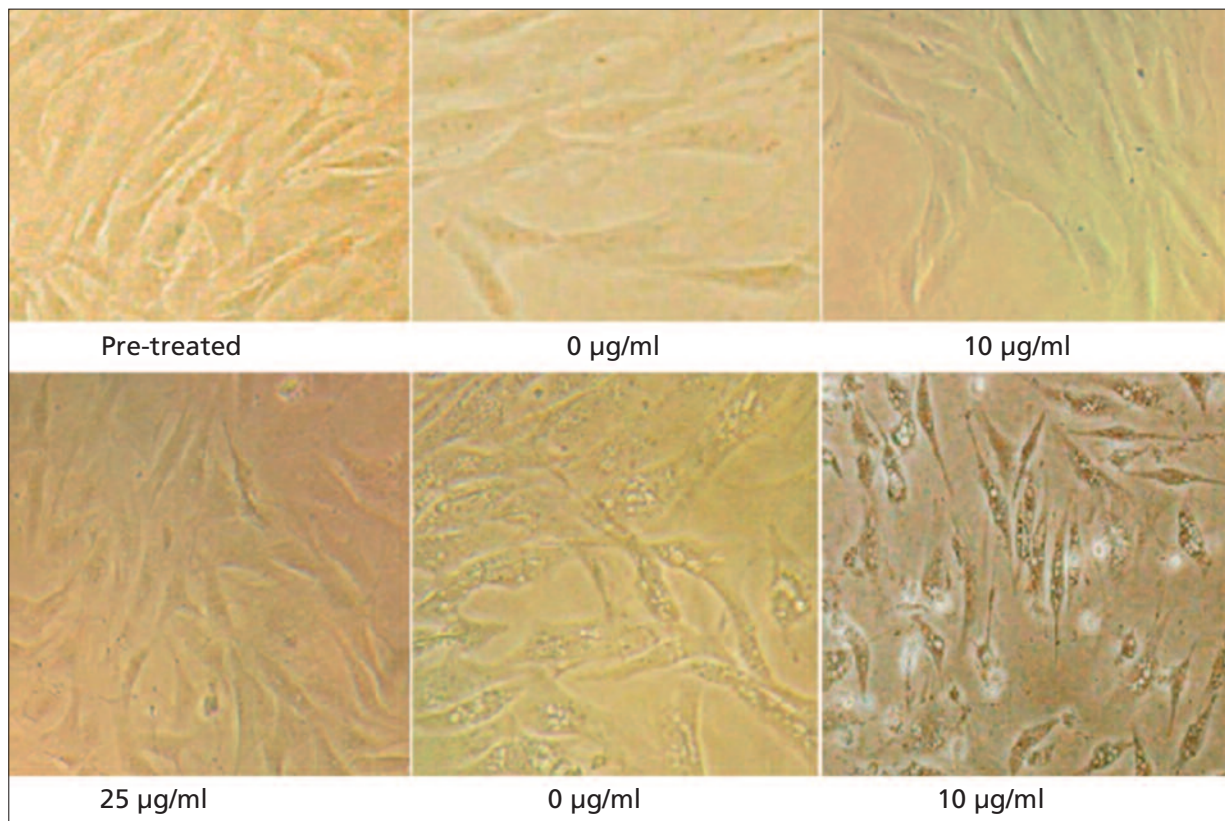


Figure 2. The effect of various concentrations of roxithromycin on the morphology of ASMCs for 48h. Following the exposure to roxithromycin ($\geq 25 \mu\text{g/mL}$), sequentially displayed a reduction in cell size, plasma membrane blebbing, shedding of smaller fragments from the cells, and detaching of the whole cell from the culture dish. 0 and 10 $\mu\text{g/mL}$ roxithromycin didn't significantly altered cell morphologies compared to the pre-treated cells ($\times 100$).

treated with various concentrations of roxithromycin (10, 25, 50, 100 $\mu\text{g/mL}$) was suppressed to $88.1 \pm 6.5\%$, $83.5 \pm 8.5\%$, $72.7 \pm 14.0\%$ and $62.6 \pm 6.5\%$ of control, respectively (Figure 3). On the other hand, roxithromycin increased the percentage of the early apoptotic population in a dose-dependent manner, as seen in Figure 4.

Assessment of Mitochondria Energization

To document the involvement of mitochondrial dysfunction in rat ASMCs apoptosis, we used the lipophilic dye JC-1, which formed red fluorescent aggregates in polarized mitochondria and dispersed into green fluorescent monomers as membrane potential dissipated, to indicate changes in $\Delta\Psi\text{m}$ through flow cytometry analysis. As shown in Figure 5, roxithromycin induced mitochondrial depolarization in ASMCs in a dose-dependent manner, as indicated by a shift from red to green fluorescence.

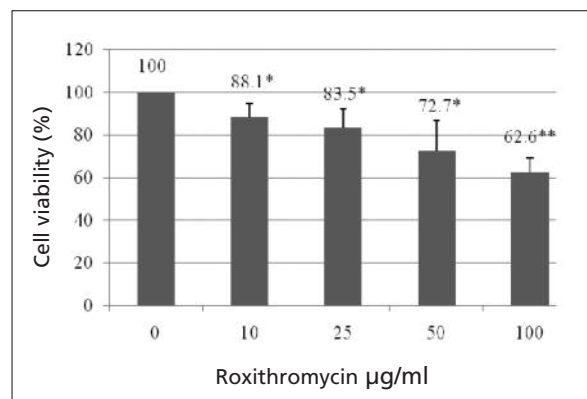


Figure 3. Effects of roxithromycin on the viability of ASMCs. Cells were seeded into 96-well plates and incubated at 37°C for 48 h in fresh medium containing a given concentration of roxithromycin. Absorbance was measured by the WST-8 assay, as described under Materials and Methods. Data shown are expressed as mean $\pm$ SD. Results are representative of six separate experiments. Comparisons among groups were conducted via one-way ANOVA followed by post hoc test with LSD method. * $p < 0.05$ and ** $p < 0.01$ vs control.

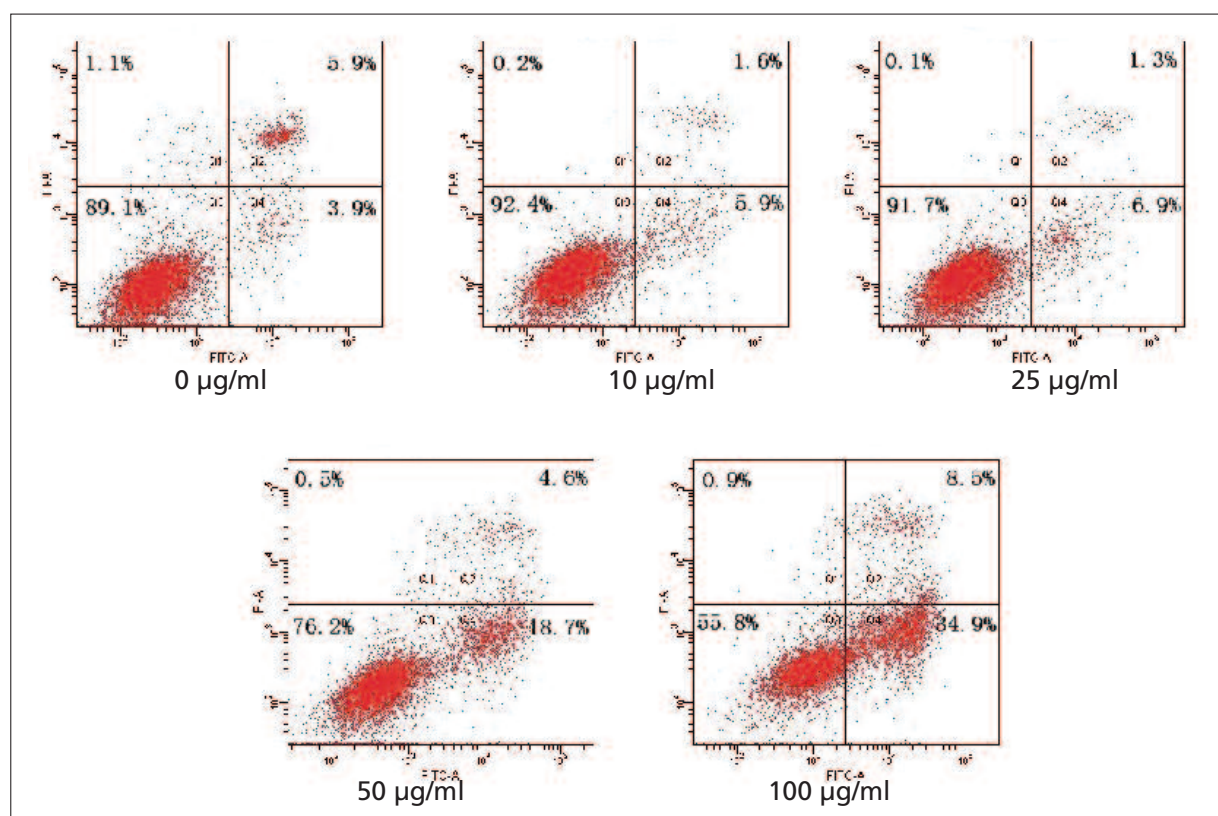


Figure 4. Apoptosis of ASMCs treated with roxithromycin. Q3 quadrants of each panels show the viable cells, which exclude PI and are negative for FITC-Annexin V binding. Q2 quadrants contain the late-phase apoptotic and necrotic cells, positive for FITC-Annexin V binding and for PI uptake. Q4 quadrants represent the early-phase apoptotic cells, FITC-Annexin V positive and PI negative, demonstrating cytoplasmic membrane integrity. After treated with 0, 10, 25, 50, 100 $\mu\text{g/mL}$ roxithromycin, ratio of early-phase apoptotic cells was $(2.3 \pm 0.8)\%$, $(4.4 \pm 1.3)\%$, $(4.5 \pm 0.8)\%$, $(14.9 \pm 1.9)\%$, $(31.3 \pm 4.7)\%$ respectively; ratio of late-phase apoptotic and necrotic cells was $(1.0 \pm 0.2)\%$, $(1.4 \pm 0.1)\%$, $(1.8 \pm 0.1)\%$, $(2.9 \pm 0.9)\%$, $(8.1 \pm 0.4)\%$ respectively. One representative experiment out of three is shown.

Cyt c Release and Caspases Activation

Cyt c is the most prominent pro-apoptotic protein contained in mitochondria and Cyt c exit is an almost universal feature of apoptotic cell death. To further investigate the involvement of mitochondrial dysfunction during roxithromycin-induced apoptosis, Cyt c release was detected by immunoblot analysis. As seen in Figure 6A and B, Mitochondrial Cyt c decreased notably, while cytosolic Cyt c accumulated significantly after treated with roxithromycin, both in a dose-dependent manner. This indicates that there was a substantial release of Cyt c from mitochondria to cytosol on exposure to roxithromycin. Another hallmark of apoptosis is the activation of caspases, especially, the key effector caspase—Caspase-3. This study found a big increase in the activity of Caspase-3 and Caspase-9 by roxithromycin treatment in a dose-dependent manner (Figure 6C, D).

Regulation of P27 in the Cultured ASMCs

We examined the effects of roxithromycin on the expression of P27 in the cultured ASMCs. The results showed that roxithromycin induced a significant increase in the level of P27 in a dose-dependent manner (Figure 7). Compared to the control group, the expression in 100, 50, 25, 10 $\mu\text{g/mL}$ group was 3.9, 2.8, 2.6, 2.2 times, respectively.

Discussion

Airway smooth muscle remodeling, including smooth muscle hypertrophy and hyperplasia^{21,22} has been recognized as one of the most important factors of airway remodeling, the pathological basis of airway hyperresponsiveness, present not only in adult asthmatics but also in early childhood²³, leading to progressive airway inflamma-

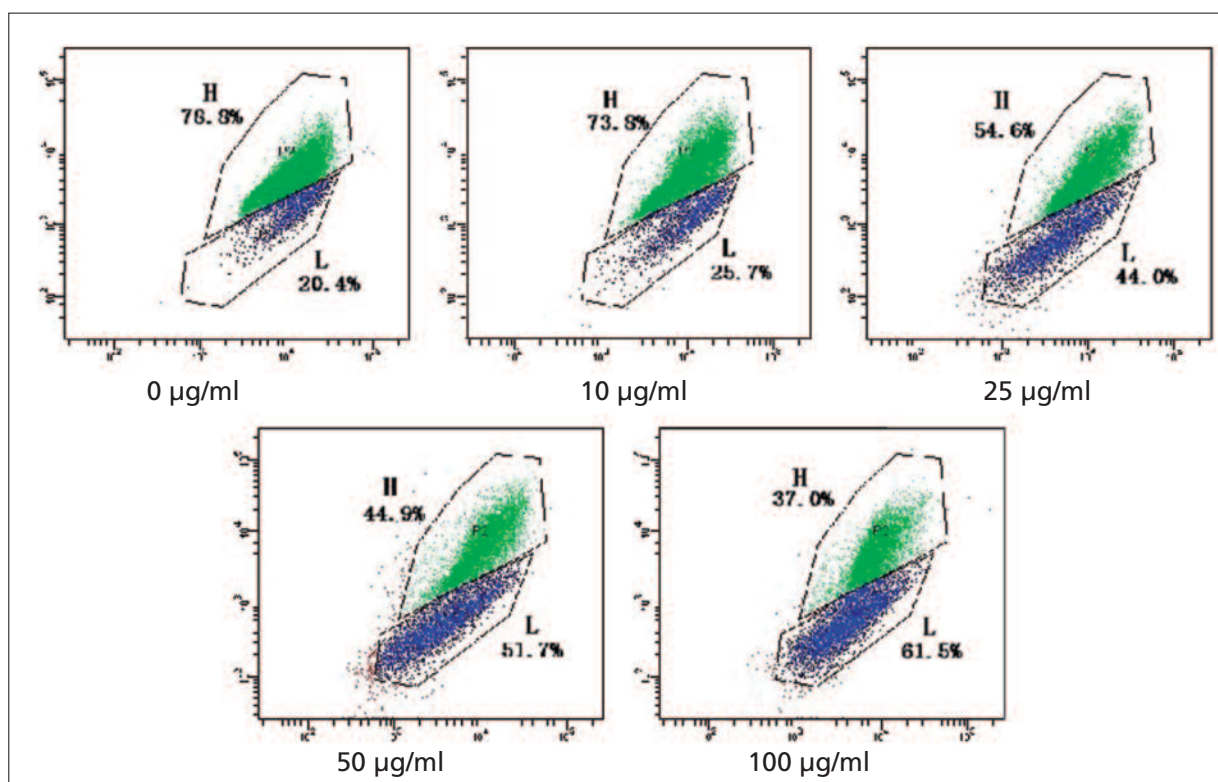


Figure 5. Loss of $\Delta\Psi_m$ in ASMCs with roxithromycin treatment. H gate (top left), cells containing JC-1 aggregates resulting from intact mitochondria, for 0, 10, 25, 50, 100 $\mu\text{g/mL}$ was $(77.4 \pm 6.4)\%$, $(68.3 \pm 8.2)\%$, $(54.1 \pm 1.5)\%$, $(43 \pm 4.6)\%$, $(40.9 \pm 8.9)\%$ respectively; L gate (bottom right) contains cells with lower collapsed mitochondrial membrane potential, for 0, 10, 25, 50, 100 $\mu\text{g/mL}$ was $(21.7 \pm 6.4)\%$, $(28.6 \pm 9.2)\%$, $(44.8 \pm 1.8)\%$, $(54.3 \pm 5.5)\%$, $(57.8 \pm 8.8)\%$ respectively. One representative experiment out of three is shown.

tion and airflow obstruction. It has been generally agreed that targeting ASMCs has the potential to be an innovative venue of treatment for patients with asthma^{24,25}. Previously, in smooth muscle from asthmatic patients, excessive *in vitro* proliferation of ASMCs has been demonstrated^{26,27}. In addition, decreased apoptosis of ASMCs has also been showed, although this was in a rat model of experimental asthma²⁸. Later, Triantafyllidis et al²⁹ revealed that increased mitochondrial biogenesis of ASMCs from rats with asthma accounted for enhanced cell proliferation. However, there're few researches on the influence of roxithromycin on ASMCs. In the present study, we have for the first time examined the effect of roxithromycin on the proliferation and apoptosis of cultured ASMCs derived from a rat model of asthma.

We revealed, using the WST-8 assay, that roxithromycin inhibited the proliferation of ASMCs in a dose-dependent manner. Cell proliferation was suppressed by 25-100 $\mu\text{g/mL}$ roxithromycin, however, was not markedly reduced by 10 $\mu\text{g/mL}$

roxithromycin. Furthermore, we discovered, using flow cytometry, that roxithromycin augmented the early phase of apoptosis of ASMCs in a dose-dependent manner.

There are two major cell-intrinsic pathways for inducing apoptosis, one that begins with ligation of cell surface death receptors and the other that involves mitochondrial release of Cyt c^{30,31}. The latter is characterized by Cyt c release and $\Delta\Psi_m$ loss³⁰. Mitochondria are not only the source of cellular energy, but also play an essential role in cell death mechanisms. Research aimed at elucidating the role of mitochondria in cell death has become one of the fastest growing disciplines in biomedicine³².

In healthy cells, Cyt c is located in the mitochondrial intermembrane space, where it functions as an electron shuttle in the respiratory chain. Several proapoptotic stimuli induce the permeabilization of the outer membrane and the collapse of $\Delta\Psi_m$, allowing for Cyt c release. After translocation into the cytoplasm, cytochrome c stimulates the assembly of a multiprotein com-

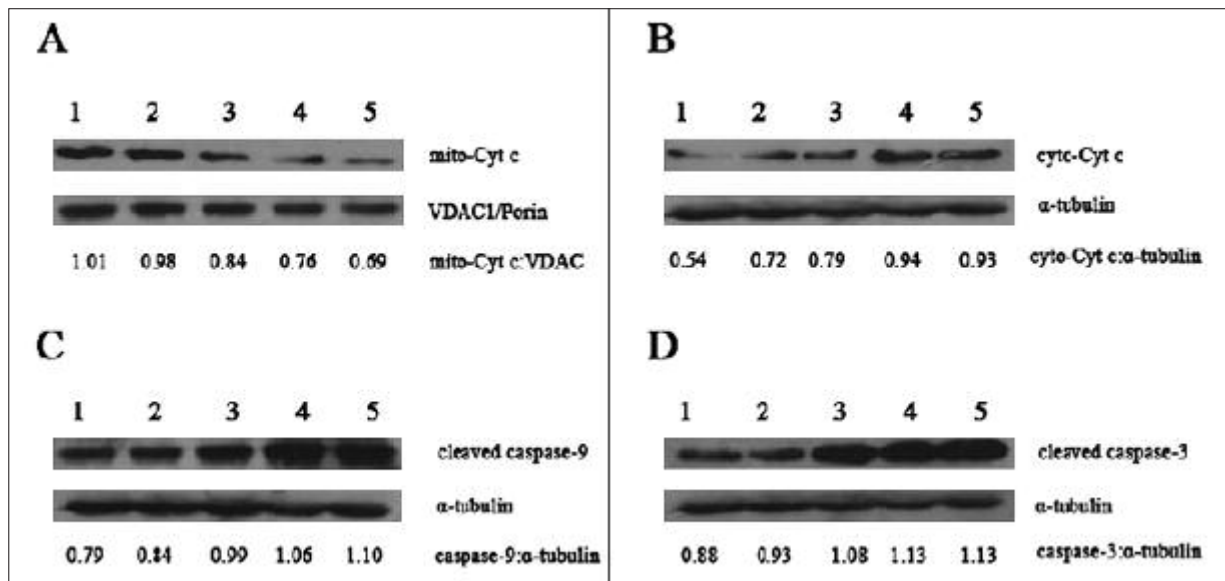


Figure 6. Western blot analysis for mitochondrial Cyt c (**A**), cytosolic Cyt c (**B**), cleaved caspase-9 (**C**) and caspase-3 (**D**) expression in roxithromycin treated ASMCs. (**A**) Dose profile for mitochondrial Cyt c expression [Lane 1-5: cells treated with 0, 10, 25, 50, 100 µg/mL roxithromycin, was (0.69 ± 0.16), (0.69 ± 0.15), (0.60 ± 0.12), (0.54 ± 0.11), (0.49 ± 0.11), respectively]. (**B**) Dose profile for cytosolic Cyt c expression [Lane 1-5: cells treated with 0, 10, 25, 50, 100 µg/mL roxithromycin, was (0.48 ± 0.08), (0.60 ± 0.08), (0.62 ± 0.09), (0.69 ± 0.12), (0.70 ± 0.12), respectively]. (**C**) Dose profile for cleaved caspase-9 expression [Lane 1-5: cells treated with 0, 10, 25, 50, 100 µg/mL roxithromycin, was (0.67 ± 0.08), (0.70 ± 0.08), (0.77 ± 0.11), (0.80 ± 0.13), (0.80 ± 0.15), respectively]. (**D**) Dose profile for cleaved caspase-3 expression [Lane 1-5: cells treated with 0, 10, 25, 50, 100 µg/mL roxithromycin, was (0.69 ± 0.10), (0.70 ± 0.11), (0.77 ± 0.15), (0.80 ± 0.16), (0.80 ± 0.17), respectively]. Data are representative of 3 independent experiments.

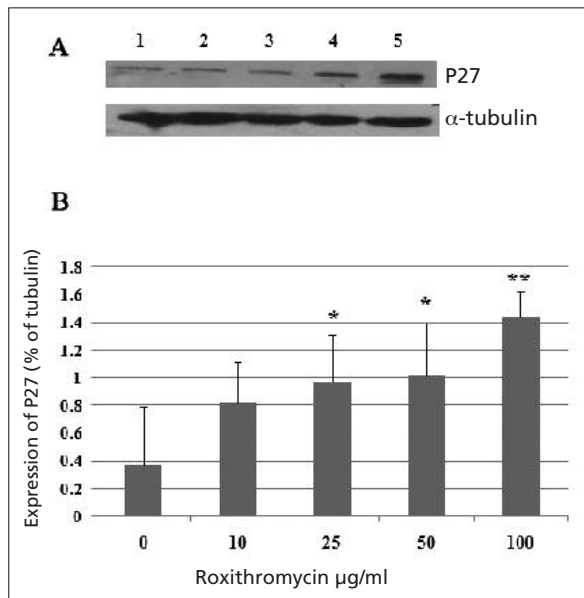


Figure 7. Western blot analysis for P27 in roxithromycin treated acinar cells. (**A**) Dose profile for P27 expression [Lane 1-5: cells treated with 0, 10, 25, 50, 100 µg/mL roxithromycin]. (**B**) Histograms showing P27 expression as % of α-tubulin. Data shown are expressed as mean ± S.D. Results are representative of three separate experiments. Comparisons among groups were conducted via one-way ANOVA followed by post hoc test with LSD method. ** $p < 0.01$ and * $p < 0.05$ vs control.

plex known as the Apaf-1 apoptosome. Caspase-9 is recruited to the apoptosome and activated, initiating a cascade of effector caspase activation. Activated caspases ultimately lead to apoptotic cell dismantling³³. The release of Cyt c from mitochondria is a landmark of the apoptotic program³⁴. Caspases are thought to coordinate the execution phase. In particular, caspase-9 is the crucial upstream activator³⁵ and caspase-3 is the primary downstream executioner of the caspase cascade, necessary for nuclear apoptosis and other morphological changes³⁶.

We examined the expressions of Cyt c in mitochondria and cytosols, respectively. Interestingly, we found that Cyt c accumulated in cytosol after roxithromycin exposure, accompanied with reduction of mitochondrial Cyt c compared to the control, confirming that roxithromycin induced Cyt c efflux. We further demonstrated caspase-3 and caspase-9 were activated by roxithromycin in ASMCs as shown by our Western blots.

A decrease in $\Delta\Psi_m$ has been reported to accompany apoptosis induced by diverse stimuli³⁷⁻³⁹. To investigate whether roxithromycin induced loss of $\Delta\Psi_m$, changes in the $\Delta\Psi_m$ were exam-

ined using the mitochondrial specific dye JC-1. We revealed that roxythromycin directly triggered the reduction of $\Delta\Psi_m$ in a dose-dependent manner.

Taken together with the data reported here, these results suggest that roxythromycin-induced mitochondria perturbation is essential for roxythromycin-induced apoptosis.

P27 is a negative regulator of G₁-phase progression and also acts as a promoter of apoptosis⁴⁰⁻⁴². It is reported that overexpression of p27 increases ceramide-induced apoptotic cell death in HL-60 cells⁴³. Recently, others have demonstrated that TNF- α induced increase in the expression and activity of P27 and caspase-3 to induce cell cycle arrest and apoptosis in smooth muscle cells of human carotid plaques⁴⁴. In this study, we showed that overexpression of P27 enhanced cell death, cytochrome c release and caspase-9/3 activation in roxythromycin treated ASMCs. These observations indicate that P27 is an important regulator in mediating roxythromycin-induced apoptosis which involves mitochondrial signaling. This is consistent with the previous findings performed by Kim et al⁴³.

Conclusions

Roxythromycin enhances apoptosis of ASMCs from rats with asthma associated with increase in the level of P27 through mitochondrial signaling pathway. This is a new approach for the treatment of airway remodeling in asthma. Further studies will be required to identify the mechanism by which roxythromycin induces P27 up-regulation.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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