

Resveratrol induces cell cycle arrest and apoptosis in human eosinophils from asthmatic individuals

XIN HU^{1,2*}, JING WANG^{3*}, YU XIA¹, MIHEREGULI SIMAYI¹, SYED IKRAMULLAH⁴,
YUANBING HE¹, SHIHONG CUI¹, SHUANG LI⁴ and QIMANGULI WUSHOUER¹

¹Department of Respiratory Medicine, The First Affiliated Hospital of Xinjiang Medical University;

²Postgraduate College of Xinjiang Medical University; ³Department of Geriatrics,

The First Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang 830011; ⁴Department of Medicine, The Changji Branch of The First Affiliated Hospital of Xinjiang Medical University, Changji, Xinjiang 831100, P.R. China

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Abstract. Eosinophils exert a number of inflammatory effects through the degranulation and release of intracellular mediators, and are considered to be key effector cells in allergic disorders, including asthma. In order to investigate the regulatory effects of the natural polyphenol, resveratrol, on eosinophils derived from asthmatic individuals, the cell counting Kit-8 assay and flow cytometry analysis were used to determine cell proliferation and cell cycle progression in these cells, respectively. Cellular apoptosis was detected using annexin V-fluorescein isothiocyanate/propidium iodide double-staining. The protein expression levels of p53, p21, cyclin-dependent kinase 2 (CDK2), cyclin A, cyclin E, Bim, B-cell lymphoma (Bcl)-2 and Bcl-2-associated X protein (Bax) were measured by western blot analysis following resveratrol treatment. The results indicated that resveratrol effectively suppressed the proliferation of eosinophils from asthmatic patients in a concentration- and time-dependent manner. In addition, resveratrol was observed to arrest cell cycle progression in G₁/S phase by increasing the protein expression levels of p53 and p21, and concurrently reducing the protein expression levels of CDK2, cyclin A and cyclin E. Furthermore, resveratrol treatment significantly induced apoptosis in eosinophils, likely through the upregulation of Bim and Bax protein expression levels and the downregulation of Bcl-2 protein expression. These findings suggested that resveratrol may be a potential agent for the treatment of asthma by decreasing the number of eosinophils.

Introduction

Asthma is a complex respiratory disease that involves the remodeling of airway tissues and chronic inflammation (1). Eosinophils are considered to be key inflammatory effector cells that are implicated in the pathogenesis of various diseases, including asthma (2). Previous studies have indicated that eosinophils exert numerous proinflammatory effects in allergic disorders through the release of various proinflammatory cytokines, chemokines and cytotoxic molecules, which may ultimately lead to the manifestation of allergic asthma (3,4). Besides their recruitment and activation, an increase in the survival and a reduction in the apoptosis rate of eosinophils has been proposed as a putative mechanism that accounts for the accumulation of inflammatory cells in allergic asthma (5). Currently, inhaled corticosteroids are the mainstay pharmacotherapy for asthma. The broad anti-inflammatory effects of these agents are divided into delayed actions through alterations in protein expression, and rapid actions probably through the membrane-bound glucocorticoid receptor and direct interaction with the airway vasculature. However, a small number of asthmatic patients do not respond well to inhaled corticosteroids. In addition, side-effects of corticosteroids include glaucoma, osteoporosis, growth retardation in children, wound healing and metabolic effects. Therefore, in order to improve the therapeutic strategies for patients with allergic inflammation, including asthma, the development of novel agents that inhibit eosinophil accumulation and survival is required.

Resveratrol (3,5,4'-trans-trihydroxystilbene) is a natural polyphenol and phytoestrogen found in peanuts, mulberries and grapes (Fig. 1A) (6,7). An increasing number of previous studies have indicated that resveratrol possesses a variety of pharmacological properties, including antioxidant (8), anti-platelet (9), anticancer (10) and anti-inflammatory activities (11). A previous study indicated that resveratrol downregulates the production of monocyte chemoattractant protein-1 by endothelial cells, which is a key mediator that stimulates the infiltration of inflammatory cells into the lungs, through the inhibition of p38 mitogen-activated protein kinase activation in a rat model of acute pulmonary thromboembolism (12).

Correspondence to: Dr Qimanguli Wushouer, Department of Respiratory Medicine, The First Affiliated Hospital of Xinjiang Medical University, 137 Liyushan Road, Urumqi, Xinjiang 830011, P.R. China

E-mail: qimanguliwu@tom.com

*Contributed equally

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Previously, it was reported that resveratrol notably inhibited the proliferation of fibroblasts from human hypertrophic scars, and decreased collagen expression in these cells (13). However, the effect of resveratrol on the proliferation and survival of human eosinophils isolated from asthmatic patients remains unclear. Based on the various biological activities of resveratrol, particularly its anti-inflammatory properties, the present study hypothesized that resveratrol may regulate the survival and apoptosis of human eosinophils. The results of the present study demonstrated an inhibitory effect of resveratrol on eosinophils from asthmatic patients through the prevention of cell cycle progression and the induction of apoptosis.

Materials and methods

Chemicals and reagents. Resveratrol was obtained from Sigma-Aldrich (EMD Millipore, Billerica, MA, USA) and was dissolved in dimethylsulfoxide (DMSO). Resveratrol was subsequently diluted with RPMI-1640 medium (Clonetics Corporation, San Diego, CA, USA) when used to treat cells in culture. The final concentration of DMSO was maintained at <0.1% in all cell culture experiments. At this concentration, no obvious effects on cell growth were observed.

Ethical approval. The present study was approved by the Human Research and Ethics Committee of The First Affiliated Hospital of Xinjiang Medical University (Xinjiang, China). Written informed consent was obtained from a total of 10 asthma patients (6 male, aged 25-30 years; 4 female, aged 20-30 years) that were recruited to the study.

Eosinophil purification and culture. Human eosinophils were purified and cultured as described previously (14). Briefly, eosinophils were purified from peripheral blood obtained from the asthmatic patients using Percoll media (Sigma-Aldrich; EMD Millipore) and density-gradient centrifugation was used to separate the mononuclear cells from granulocytes. The lysis buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) containing erythrocytes was subsequently removed, and purification of eosinophils by negative selection was performed with CD16 immunomagnetic microbeads (catalog no. 130-045-701, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was performed. Purified eosinophils were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum at 37°C in 5% (v/v) CO₂. The purity of eosinophils was assessed by staining blood smears with Hemacolor Rapid Staining reagent (Ocon Chemicals Ltd., Cork, Ireland), and was >98%. The viability of eosinophils was evaluated by trypan blue staining and was >98%.

Cell proliferation assay. The effect of resveratrol on the proliferation of eosinophils was determined using the Cell Counting Kit-8 (CCK8; Sigma-Aldrich; EMD Millipore). Briefly, eosinophils at passage 4 were seeded into 96-well plates at a density of 1×10^5 cells/well and incubated at 37°C overnight. The cells were subsequently treated with 0, 20, 40, 80, 160 or 320 μ M resveratrol for 24, 48 and 72 h before 10 μ l CCK8 solution was added to each well. Following incubation for 4 h at 37°C, the plates were read at 570 nm using an automated spectrophotometric plate reader (PerkinElmer,

Inc., Waltham, MA, USA). Experiments were performed three times.

Cell cycle analysis. Purified eosinophils were seeded into 6-well plates at a density of 2×10^5 cells/well. After 12 h, the cells were treated with 0, 80 or 160 μ M resveratrol for 24 and 48 h before they were collected by centrifugation at $1,000 \times g$ for 5 min, at 4°C and washed with phosphate-buffered saline. The cells were subsequently fixed in 75% ethanol for 2 h, resuspended in 1 ml staining buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing 10 μ g/ml RNase A for 30 min and were subsequently stained with 15 μ g/ml propidium iodide (PI) for 30 min. The cells were analyzed using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) to determine cell cycle progression.

Detection of apoptosis by flow cytometry. Eosinophils were seeded into 6-well plates at a density of 2×10^5 cells/well. After 12 h, the cells were treated with 0, 80 or 160 μ M resveratrol for 48 h, and were collected and washed as before. The cells were subsequently labeled with annexin V-fluorescein isothiocyanate (FITC)/PI and detected using a flow cytometer with CellQuest Pro software version 5.1 (BD Biosciences). The number of apoptotic cells, including those undergoing early apoptosis (annexin V-FITC⁺/PI⁻), late apoptosis (annexin V-FITC⁺/PI⁺) and necrosis (annexin V-FITC⁺/PI⁺), were expressed as a percentage of the total number of cells.

Western blot analysis. After eosinophils were treated with various doses of resveratrol (0, 80 and 160 μ M), the cells were lysed, and the total protein was extracted and collected, as described previously (7). The protein concentration was determined using a bicinchoninic acid assay. A total of 20 μ g protein from each sample was separated using a 10% SDS-PAGE gel. The proteins were subsequently transferred onto a polyvinylidene difluoride membrane. The membrane was then blocked in Tris-buffered saline-Tween-20 (TBST; pH 7.4; 1.5 M NaCl; 20 mM Tris-HCl; 0.05% Tween-20) containing 5% (w/v) non-fat milk for 2 h at room temperature. The membrane was subsequently incubated overnight at 4°C with the following rabbit primary antibodies: Anti-cyclin-dependent kinase 2 (CDK2; 1:1,000; catalog no. ab32147) and anti-cyclin A (1:1,000; catalog no. ab181591), purchased from Abcam (Shanghai, China); and anti-p53 (1:1,000; catalog no. 2527), anti-p21 (1:1,000; catalog no. 2947), anti-cyclin E (1:1,000; catalog no. 20,808), anti-Bim (1:800; catalog no. 2933), anti-Bax (1:800; catalog no. 5023), anti-Bcl-2 (1:800; catalog no. 4223) and anti-GAPDH (1:500; catalog no. 5174), purchased from Cell Signaling Technology, Danvers, USA. After washing three times with TBST, the membranes were incubated with an anti-rabbit peroxidase-conjugated secondary antibody (1:800; catalog no. 611-7302; Rockland Immunochemicals, Inc., Pottstown, PA, USA) at room temperature for 1 h. Protein bands were visualized with an enhanced chemiluminescence reagent (Wuhan Boster Biological Technology, Ltd., Wuhan, China) and detected using a LAS-3000 Luminescent Imaging System (Fujifilm, Tokyo, Japan).

Statistical analysis. The data are expressed as the mean \pm standard deviation, and statistical analysis was performed using

Table I. Effect of resveratrol on the cell cycle distribution of eosinophils.

Resveratrol (μM)	24 h incubation			48 h incubation		
	G ₁ (%)	S (%)	G ₂ /M (%)	G ₁ (%)	S (%)	G ₂ /M (%)
0	43.25 \pm 1.41	35.97 \pm 1.28	20.18 \pm 1.72	49.27 \pm 1.53	33.29 \pm 1.81	17.46 \pm 1.73
80	52.86 \pm 1.65 ^a	30.51 \pm 1.69 ^a	18.34 \pm 1.53	59.63 \pm 2.75 ^a	25.52 \pm 2.08 ^a	14.25 \pm 2.07
160	59.18 \pm 1.49 ^b	25.94 \pm 1.38 ^b	15.76 \pm 1.25 ^a	66.71 \pm 1.29 ^b	20.27 \pm 1.54 ^b	13.27 \pm 1.09 ^a

Eosinophils purified from asthmatic subjects were exposed to 0, 80 or 160 μM resveratrol for 24 and 48 h, and cell cycle progression was subsequently measured using flow cytometry. The data are presented as the mean \pm standard deviation of three experiments. ^aP<0.05 and ^bP<0.01 vs. untreated (0 μM resveratrol) controls at each time point.

SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). Differences between two groups were analyzed using the Student's *t*-test. P<0.05 was considered to indicate a statistically significant difference.

Results

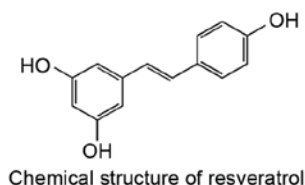
Resveratrol inhibits the proliferation of eosinophils. In order to investigate the effects of resveratrol on the eosinophil cell growth, purified eosinophils were treated with various concentrations of resveratrol (0, 20, 40, 80, 160 or 320 μM) for 24, 48 and 72 h. The results of CCK8 assay demonstrated that resveratrol effectively inhibited the proliferation of eosinophils in a dose- and time-dependent manner (Fig. 1B). The greatest reduction in eosinophil proliferation was observed using 320 μM resveratrol for 72 h.

Resveratrol induces cell cycle arrest in eosinophils. To investigate the effects of resveratrol on cell cycle progression, eosinophils were exposed to 0, 80 or 160 μM of resveratrol for 24 and 48 h, and the cell cycle distribution profiles were determined using a flow cytometer. Compared with untreated controls, the percentage of eosinophils in G₁ phase was increased, while the percentage of cells in S and G₂/M phases was decreased (Table I). These results suggested that resveratrol treatment may arrest the cell cycle progression of eosinophils in G₁ phase.

Previous studies have suggested that the p53 gene serves an important role in the regulation of cell cycle progression (15-17). Therefore, the protein expression levels of p53 and its downstream target gene p21, as well as CDK2, cyclin A and cyclin E cell cycle mediators were investigated following exposure of eosinophils to 0, 80 or 160 μM resveratrol for 48 h. Western blot analysis demonstrated that the protein expression levels of p53 (P=0.0378; P=0.0085), and p21 (P=0.0327; P=0.0054) were significantly increased following treatment with 80 and 160 μM resveratrol when compared with untreated controls (Fig. 2). By contrast, the protein expression levels of CDK2 (P=0.0342; P=0.0034), cyclin A (P=0.0476; P=0.0085), and cyclin E (P=0.0401; P=0.0072) were significantly reduced following exposure to 80 and 160 μM resveratrol compared with the untreated controls.

Resveratrol induces apoptosis in eosinophils. In order to investigate whether the inhibitory effect of resveratrol on

A



B

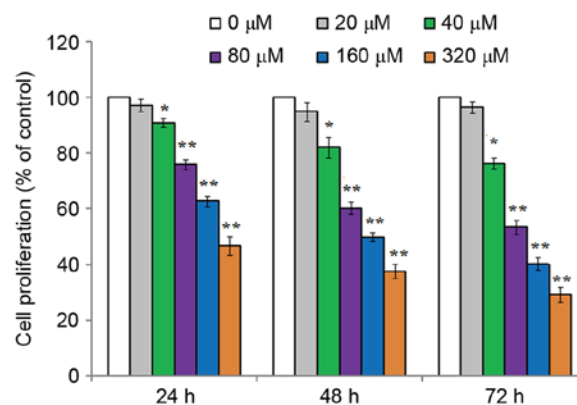


Figure 1. Effect of resveratrol on the proliferation of eosinophils derived from patients with asthma. (A) The chemical structure of resveratrol. (B) The effects of resveratrol on eosinophil proliferation was determined using a cell counting Kit-8 assay. The results indicated that the proliferation of eosinophils was inhibited upon exposure to various doses of resveratrol for 24, 48 and 72 h. The data are presented as the mean \pm standard deviation (n=3; *P<0.05 and **P<0.01 vs. the untreated control at each time point).

eosinophil cell proliferation was associated with induction of apoptosis, eosinophils were treated with 0, 80 or 160 μM resveratrol for 48 h, and the percentage of apoptotic cells was determined using a flow cytometer. The results demonstrated that resveratrol significantly induced apoptosis in eosinophils, in a dose-dependent manner [1.08 \pm 0.67 vs. 23.29 \pm 3.95 (P=0.0091) and 48.51 \pm 5.47% (P=0.0036) following exposure to 80 and 160 μM resveratrol, respectively (Fig. 3)].

To investigate the potential molecular mechanisms underlying resveratrol-induced apoptosis, the protein expression levels of pro-apoptotic Bim and Bax, and anti-apoptotic Bcl-2, were measured by western blot analysis. Compared with the untreated controls, the protein expression levels of Bim (P=0.0355; P=0.0074) and Bax (P=0.0406; P=0.0081) were

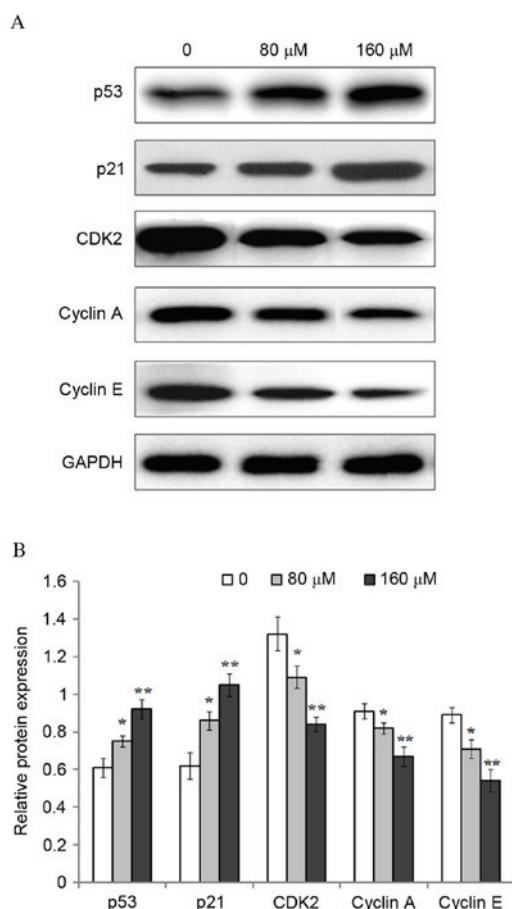


Figure 2. Effect of resveratrol on the protein expression levels of p53, p21, CDK2, cyclin A and cyclin E in eosinophils. The cells were treated with 0, 80 or 160 μ M resveratrol for 48 h prior to total protein extraction and western blot analysis. (A) Representative immunoblotting images and (B) densitometric analysis of the relative protein expression levels of p53, p21, CDK2, cyclin A and cyclin E. GAPDH was used as a loading control. The data are presented as the mean \pm standard deviation ($n=3$; * $P<0.05$ and ** $P<0.01$ vs. untreated controls). CDK2, cyclin-dependent kinase-2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

increased significantly following exposure of eosinophils to 80 and 160 μ M resveratrol for 48 h, whereas Bcl-2 protein expression levels were significantly downregulated ($P=0.0373$; $P=0.0056$; Fig. 4).

Discussion

Asthma is a common chronic inflammatory disease, which has increased in incidence in the United States over the past two decades, and accounts for significant healthcare costs (18). Eosinophils are considered to serve a critical role in the pathogenesis of allergic asthma, since they release a variety of factors that maintain and exacerbate proinflammatory effects (4). The accumulation of eosinophils in the lungs is commonly observed in 50% of patients with allergic asthma. Eosinophil survival is considered to be prolonged in these patients due to the production of proinflammatory factors, which may explain the lack of eosinophil apoptosis in inflamed airways for several days (19). Therefore, the induction of eosinophil apoptosis may present a possible

strategy for the resolution of eosinophil-induced inflammation in the treatment of allergic asthma. In the present study, resveratrol effectively inhibited the proliferation of eosinophils isolated from asthmatic patients in a dose- and time-dependent manner. In addition, resveratrol significantly induced eosinophil apoptosis, likely through the upregulation of Bim and Bax protein expression and the downregulation of Bcl-2 expression.

Resveratrol is a polyphenolic natural phytoalexin found in various food products, and has antiproliferative, anti-inflammatory and immunomodulatory properties. For these reasons, resveratrol may be a potential agent for the treatment of inflammatory disorders (20). It has been reported that resveratrol attenuated oxidative stress and suppressed the inflammatory response in the diethylnitrosamine-initiated rat hepatocarcinogenesis (21). An additional study demonstrated that resveratrol inhibited the proliferation of human aortic and pulmonary aortic endothelial cells in a dose- and time-dependent manner, as well as disrupted cell cycle progression (22). These findings are consistent with the results of the present study demonstrating that resveratrol decreased the proliferation of eosinophils in a dose- and time-dependent manner. In addition, the results of the present study suggested that the antiproliferative effect of resveratrol on eosinophils may be associated with cell cycle inhibition. Following exposure of eosinophils to 80 and 160 μ M resveratrol, a greater percentage of cells in G_1 phase, and a reduced percentage of cells in S and G_2/M phases were observed when compared with the untreated controls, which is similar to a previous report involving human hypertrophic scar fibroblasts (13). In order to investigate the mechanism of resveratrol-mediated cell cycle arrest in eosinophils further, the protein expression levels of p53, p21, CDK2, cyclin A and cyclin E were determined by western blotting. The results demonstrated that the protein expression levels of p53 and p21 were significantly decreased following resveratrol treatment. Previous studies have indicated that p53 serves an important role in determining the response of cells to different types and levels of stressful stimuli, and regulates downstream cell cycle, cell metabolism, DNA repair and apoptotic signaling pathways (23–25). In addition, p53 regulates the expression of downstream targets, including p21, which contributes to the modulation of cell cycle progression (26). CDK2 is a catalytic subunit of the cyclin-dependent protein kinase complex, and is essential for the G_1/S phase transition of the cell cycle (27). In the absence of cyclin A and CDK2, apoptotic signaling pathways are induced. The results of the present study demonstrated that resveratrol treatment of eosinophils significantly downregulated the protein expression levels of CDK2, cyclin A and cyclin E. The authors hypothesized that the increase in p53 and p21, and the concurrent reduction in CDK2, cyclin A and cyclin E protein expression levels in eosinophils, may provide an explanation for the resveratrol-mediated cell cycle arrest in G_1/S phase.

It is possible that a defect in apoptosis may contribute to the chronic tissue eosinophilia associated with asthma (28). Eosinophil apoptosis is delayed in asthma, which may be partly explained by production of granulocyte macrophage colony-stimulating factor (28). In a previous study, the apoptotic index of peripheral blood eosinophils from asthmatic

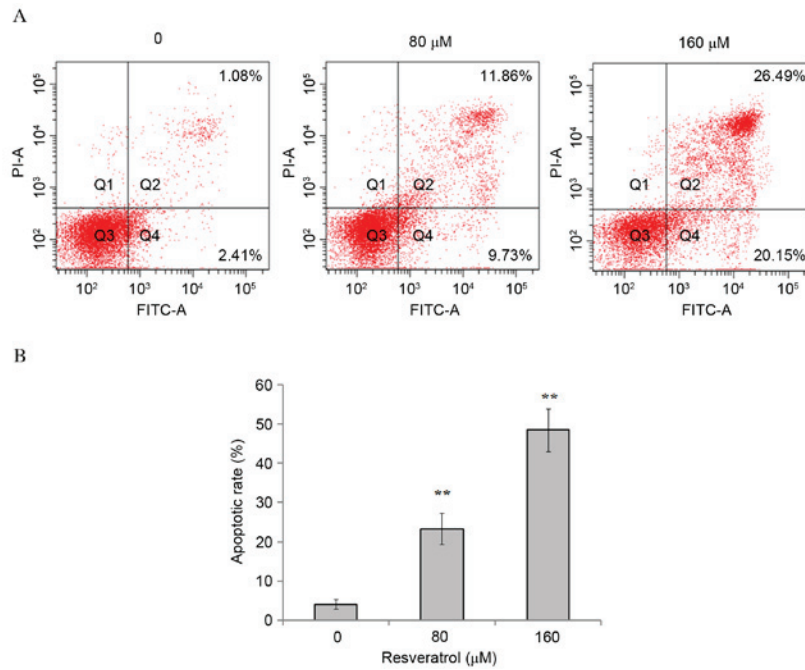


Figure 3. Effect of resveratrol on the induction of apoptosis in eosinophils. The cells were treated with 0, 80 or 160 μ M resveratrol for 48 h prior to staining with annexin V-FITC and PI, and were analyzed using a flow cytometer. (A) Representative flow cytometry plots showing the percentage of cells in early apoptosis (annexin V-FITC⁺/PI⁻), late apoptosis (annexin V-FITC⁺/PI⁺) and necrosis (annexin V-FITC⁻/PI⁺). (B) The mean percentage of apoptotic eosinophils following resveratrol treatment from three individual experiments was plotted. The data are presented as the mean \pm standard deviation (n=3; **P<0.01 vs. the untreated control). FITC, fluorescein isothiocyanate; PI, propidium iodide.

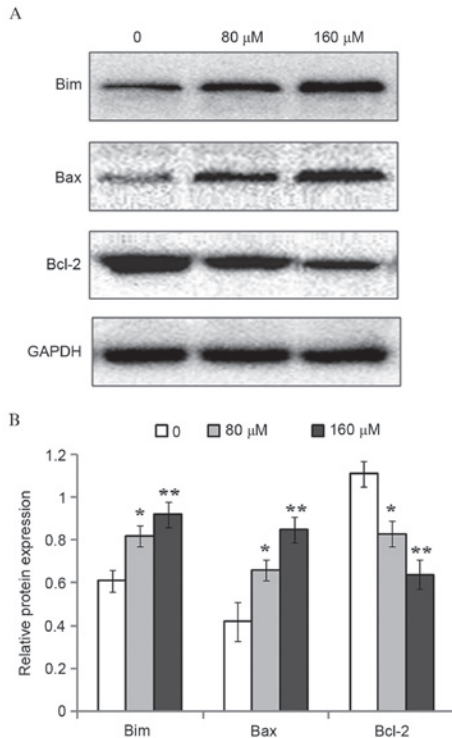


Figure 4. Effect of resveratrol on the protein expression levels of Bim, Bax and Bcl-2 in eosinophils. The cells were exposed to 0, 80 or 160 μ M resveratrol for 48 h, and the protein expression levels were analyzed by western blotting. (A) Representative immunoblotting images and (B) densitometric analysis of the relative protein expression levels of Bim, Bax and Bcl-2 in eosinophils, demonstrating that resveratrol upregulated the expression of pro-apoptotic Bim and Bax mediators, and downregulated anti-apoptotic Bcl-2 expression. GAPDH was used as a loading control. The data are presented as the mean \pm standard deviation (n=3; *P<0.05 and **P<0.01 vs. untreated controls). Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein.

patients and healthy volunteers was observed to be different. Eosinophils from asthmatic patients not taking steroid medication exhibited a lower apoptosis index (0.25) than those from healthy control subjects (0.40) (28). In addition, eosinophils isolated from asthmatic patients not undergoing treatment with steroid medication survived for a longer period of time compared with those from healthy control subjects. The induction of eosinophil apoptosis is a current strategy used for the treatment of allergic asthma, and forms the basis of a number of available anti-asthmatic agents, including glucocorticoids, theophylline and leukotriene modifiers (19). The results of the present study demonstrated that the treatment of eosinophils with resveratrol induced apoptosis in a dose-dependent manner. Further investigation demonstrated that the expression levels of proapoptotic Bim and Bax proteins were upregulated, while the expression of the antiapoptotic protein Bcl-2 was reduced following exposure to resveratrol. This may explain the observed increase in the proportion of apoptotic eosinophils following exposure to resveratrol.

In conclusion, the results of the present study demonstrated that resveratrol treatment effectively suppressed the proliferation of eosinophils from asthmatic patients. In addition, resveratrol exposure increased the percentage of eosinophils in G₁ phase, and reduced the percentage of cells in S and G₂/M phases, potentially through the upregulation of p53 and p21 and the downregulation of CDK2, cyclin A and cyclin E protein expression levels. Furthermore, resveratrol induced apoptosis in eosinophils, likely through increasing Bim and Bax protein expression and reducing Bcl-2 protein expression levels. These findings suggested that resveratrol may be a potential candidate for the treatment of asthma.

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