Antiasthmatic Effects of Resveratrol in Ovalbumin-Induced Asthma Model Mice Involved in the Upregulation of PTEN

Guo Chen, JiHong Tang, Zhenhua Ni, Qingge Chen, Zhao Li, Wei Yang, JianYun Din, Xumin Luo, and Xiongbiao Wang*

Department of Respiratory Medicine, Putuo Hospital, Shanghai University of Traditional Chinese Medicine; Shanghai 200062, China: and Central Lab, Putuo Hospital, Shanghai University of Traditional Chinese Medicine; Shanghai 200062, China.

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Resveratrol, a natural polyphenolic compound known for its antioxidative and antiinflammatory effects, exerts antiasthmatic effects, although the mechanism underlying these effects remains elusive. The phosphatase and tensin homology deleted on chromosome ten gene (PTEN) is involved in the pathogenesis of asthma, and PTEN overexpression in asthmatic mice improved asthma symptoms. To investigate whether the antiasthmatic mechanisms of resveratrol correlated with the upregulation of PTEN expression, an ovalbumin (OVA)-induced murine asthma model was used to determine the effectiveness of resveratrol treatment. PTEN mRNA and protein expression were assessed with real-time polymerase chain reaction (PCR) and immunocytochemistry. To determine whether airway remodeling occurred, the inner airway wall, mucous layer, and smooth muscle areas were each determined using an image analysis system. The lung epithelial cell line 16HBE was used to study the regulation of PTEN expression levels by resveratrol in vitro. Our data demonstrated that resveratrol inhibited OVA-induced airway inflammation and airway remodeling in asthmatic mice. PTEN expression was increased in the murine asthma model, although the expression of PTEN was restored following treatment with resveratrol. Correlation efficiency analysis showed that PTEN expression was associated with the degree of airway remodeling. Further in vitro studies demonstrated that the inhibition of Sirtuin 1 (SIRT1) activity by a SIRT1 inhibitor and RNA interference decreased PTEN protein expression, while resveratrol attenuated the decreases in PTEN expression induced by the SIRT1 inhibitor. These data suggest the mechanism of the antiasthmatic effects of resveratrol in an OVA-induced murine asthma model, which resulted in the upregulation of PTEN via SIRT1 activation.

Key words resveratrol; asthma; phosphatase and tensin homology deleted on chromosome ten; Sirtuin 1 (SIRT1)

Asthma is an inflammatory disease that has been verified in clinical practice. Corticosteroids are unique anti-inflammatory agents and represent an important therapy for controlling asthma symptoms. The prevalence of asthma is currently increasing; more than 300 million individuals are affected worldwide. Most types of asthma can be controlled; however, the disease has not been eradicated using the currently available therapies. The adverse effects of corticosteroid therapy, the occurrence of drug resistance, the recurrence of asthma attacks, and the cost of asthma treatment are among the highly problematic issues currently faced by clinicians.

Phosphatase and tensin homology deleted on chromosome ten gene (PTEN) has been widely studied, particularly by cancer researchers because its encoded protein acts as a tumor suppressor via phosphatase activity. Recently, PTEN was also shown to be involved in the pathogenesis of asthma; its low expression level was found to represent an independent factor in the development of asthma. Kwak et al. demonstrated that the intratracheal administration of adenoviruses expressing PTEN reduced interleukin (IL)-4, IL-5, and eosinophil cationic protein levels in the bronchoalveolar lavage fluid of asthmatic mice. In addition, PTEN overexpression as a result of the administration of adenoviruses expressing PTEN exerted protective effects in asthmatic mice in the setting of toluene diisocyanate (TDI)-induced asthma. Each of these results suggested that PTEN is located at a key point in the various inflammatory signaling pathways involved in asthma.

Resveratrol (trans-3,5,4'-trihydroxystilbene), a polyphenol found in grapes, has exhibited a wide range of biological and pharmacological activities, including anti-carcinogenic, anti-inflammatory and cardiovascular protective effects. Lee et al. investigated the anti-asthmatic effects of resveratrol in a murine asthma model and found that resveratrol significantly inhibited the production of Th2 cytokines (IL-4 and IL-5) in the plasma and bronchoalveolar lavage fluid, which resulted in reduced airway hyperresponsiveness, reduced eosinophilia, and reduced mucus hypersecretion. In addition, Royce et al. demonstrated that compared with vehicle treated mice, resveratrol treatment reduced subepithelial collagen deposition and attenuated airway hyperreactivity. Several other studies have also confirmed that resveratrol exerts anti-asthmatic effects; however, the mechanisms underlying resveratrol activity in the treatment of asthma have not been clearly elucidated, including whether the inhibition of airway inflammation by resveratrol occurs via the regulation of PTEN expression. In addition to the presence of inflammatory cells, airway remodeling and smooth muscle hyperplasia are important and cardinal features of asthma. However, there is only limited data demonstrating that resveratrol inhibits airway smooth muscle hyperplasia, which is the primary pathological change of airway remodeling in asthma.

Therefore, we used an ovalbumin (OVA)-induced murine...
asthma model and a lung epithelial cell line (16HBE) to examine the effects of resveratrol on airway remodeling and PTEN expression in order to help clarify the anti-asthmatic mechanisms of resveratrol.

MATERIALS AND METHODS

Allergen Immunization and Resveratrol Treatment All experimental procedures complied with the international standards of animal welfare and were approved by the Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine. Female BALB/c mice were purchased from the Shanghai Laboratory Animal Research Center. All mice were kept in well-controlled animal housing facilities and had free access to tap water and food pellets throughout the experimental period. Six-to-eight-week-old BALB/c mice (n=40) were divided into the following four groups: a saline group (saline-induced mice treated with saline), an OVA group (OVA-induced mice treated with saline), an OVA/Dex group (OVA-challenged mice treated with dexamethasone) and an OVA/Res group (OVA-induced mice treated with resveratrol). All OVA induced mice were immunized via intraperitoneal (i.p.) injections of 100 µg of OVA (Grade V; Sigma, St. Louis, MO, U.S.A.) complexed with alum (20 µg) on days 0 and 15 while mice from saline group received saline instead. Then mice from OVA group, OVA/Dex and OVA/Res group received an intranasal dose of 50 µL OVA (100 µg) on days 15, 26, 27, and 28 while mice from control group received saline instead. The OVA/Rev groups received intraperitoneal injections of resveratrol (30 mg/kg, Sigma) once daily between days 29 and 35 according to Lee’s report. The OVA/Dex groups received i.p. injections of dexamethasone (5 mg/kg) once daily between days 29 and 35 according to Brodie’s report. The two other groups were treated with saline. On day 36, 24 h following each experimental group’s final treatment, the mice were sacrificed, and the lungs were dissected out for further analysis.

Lung Histology The mouse lung samples were fixed in 10% formalin solution. Following embedding in paraffin, the tissue sections were stained with hema tolxylin and eosin (H&E). Images were captured using a Leica DM300 microscope. Histopathological scores were awarded according to the Underwood scoring system, on a scale of 0–5 (0, normal; 1, low grade; 2, low to moderate grade; 3, moderate grade; 4, moderate to high grade; and 5, high grade). The thickness of the bronchial wall was scored using the Bai method. A minimum of 3 fields throughout the upper and lower left lung tissue were randomly examined for the morphometric analysis. The histologic and morphometric analyses were performed by individuals blinded to the protocol design. The basement membrane perimeter (Pbm), the area within the outer (Amo) and inner edge (Ami) of the airway smooth muscle layer, the mucous area within the outer (Amuco) and inner edge (Amuci) of the mucous layer, were determined by morphometric analysis (Image-Pro Plus 6.0). The following parameters were calculated based on the measured values: the area of smooth muscle (WAm=Amo−Ami), the area of inner airway wall (WAi=Amo−Ai), the area of mucous glands (Wmuc=Amuco−Amuci). Then we used the ratios of WAi to Pbm (WAi/Pbm) and WAm to Pbm (WAm/Pbm) to evaluate airway remodeling.

Mouse Lung RNA Extraction and Quantification of PTEN mRNA Levels Total RNA was isolated from the lungs using Trizol reagent (Invitrogen, Carlsbad, U.S.A.), and first-strand cDNAs were prepared using a random hexamer primer according to the instructions included with the SuperScript III™ First-Strand Synthesis Kit (Invitrogen). Real-time PCR was performed using SsoFast EvaGreen SuperMix (Biorad, Hercules, U.S.A.), with the primer pairs listed in Table 1. The relative expression levels of the PTEN gene were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and analyzed via the 2ΔCt method $ΔCt=(CΔ_{GAPDH}−C_{PTEN})$.

Immunohistochemical Analysis of PTEN Expression The immunohistochemical detection of PTEN was performed as described previously. Tissue sections from the right lungs were first incubated with a PTEN monoclonal antibody (Clone D4.3, Cell Signaling Technology, Danvers, U.S.A.) overnight at 4°C. Following incubation, tissue sections were then washed with phosphate buffered saline (PBS) and treated with a polyclonal horseradish peroxidase (HRP)–anti-rabbit immunoglobulin G (IgG) (Maixin Bio, Fuzhou, China). Signal detection was performed using diaminobenzidine (DAB). PTEN staining intensity was analyzed by measuring integrated optical density (IOD) values, using Image-Pro Plus 6.0.

Cell Culture The lung epithelial cell line, 16HBE, was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in humidified 5% CO2. The day before the assay, 16HBE cells were seeded into either 96-well plates (1×104 cells per well) or 100 mm dishes (2×105 cells per dish). Following 24 h of incubation, 5 µmol/L salermide (Sigma), 10 µmol/L salermide, 20 µmol/L salermide, 50 µmol/L resveratrol, 20 µmol/L salermide plus 50 µmol/L resveratrol, and 10 µmol/L salermide plus 50 µmol/L resveratrol were added and incubated for 72 h. Following 72 h of incubation, the cells were subjected to further analysis.

Sirtuin 1 (SIRT1) RNA Interference Vector-containing short hairpin RNA (shRNA) interference against the human SIRT1 gene (GeneBank accession number NM_012238) was designed and constructed by Genechem (Genechem, Shanghai, China). To control for off-target effects, three distinct shRNAs were used and the sequence was listed below: s1, 5'-GGG AATCCA AAGGATAAT T-3'; s2, 5'-GGCTTGATG

### Table 1. Sequences of Primers

<table>
<thead>
<tr>
<th>Primers or Probes</th>
<th>GeneID</th>
<th>Amplicon size</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>SYBR-mPTEN-F</td>
<td>19211</td>
<td>74bp</td>
<td>5'-AGGCACAAGAGGCCCCCTAGAT-3'</td>
</tr>
<tr>
<td>SYBR-mPTEN-R</td>
<td></td>
<td></td>
<td>5'-CTGACTGGAATTTGCTGCC-3'</td>
</tr>
<tr>
<td>SYBR-mGAPDH-F</td>
<td>14433</td>
<td>123bp</td>
<td>5'-AGGTGCCTGTAAGCCGATTTG-3'</td>
</tr>
<tr>
<td>SYBR-mGAPDH-R</td>
<td></td>
<td></td>
<td>5'-TGTAGACCATGTAGTGGAGTCA-3'</td>
</tr>
</tbody>
</table>
GTA ATC AGT A-3'; s3, 5'-TCG AAC AAT TCT TAA AGA T-3'. A control shRNA (5'-TTC TCC GAA CGT GTC ACG T-3') was used as a negative control. The shRNA plasmids and the control plasmid were transfected into 16HBE cells using lipofectamine 3000 (Invitrogen) according to standard protocols. After 48 h the expression levels of target proteins in transfected cells were detected by Western blotting with the indicated antibody.

**Immunocytochemical Analysis of PTEN Expression**

Following 72 h of treatment, the cells were fixed using 4% paraformaldehyde. The cells were permeabilized 3 times for 5 min with 0.1% Triton X-100 in PBS and blocked with blocking buffer (10% normal goat serum, 0.1% Triton X-100) for 30 min at room temperature. Following blocking, the cells were washed with PBS and incubated overnight at 4°C with PTEN antibodies (Clone D4.3, Cell Signaling Technology, Danvers, U.S.A.). The following day, the cells were washed 3 times with PBS and incubated with Cy3-labeled goat anti-rabbit IgG for 60 min. They were then washed three times with PBS and observed using a fluorescence microscope.

**Western Blotting**

Following 48 h or 72 h of treatment, the 16HBE cells were harvested, and the total protein was purified using a ProteoJET Mammalian Cell Lysis Kit (Fermentas, Vilnius, Lithuania). The total protein was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked with 5% BSA and washed twice with thio-barbituric acid reactive substance (TBST). The membrane was then incubated with anti-rabbit IgG horseradish peroxidase secondary antibody (Cell Signaling Technology) for 2 h at room temperature. Finally, immunoreactive bands were detected using an ECL reagent (Millipore, Bedford, U.S.A.).

**Statistical Analyses**

All statistical analyses were performed using SPSS, version 16.0, software (SPSS Inc., Chicago, U.S.A.). Results were expressed as means ± standard deviations (S.D.). The one-way ANOVA was used for the comparison of WAmuc/Pbm, WAm/Pbm, WAI/Pbm, PTEN mRNA expression level and IOD values between multiple groups. Kruskal–Wallis test followed by the Mann–Whitney U test was used for the comparison of pathological score between multiple groups. Relationships were estimated through use of the Spearman rank correlation coefficient. Statistical significance was defined at the \( p < 0.05 \).

**RESULTS**

**Resveratrol Inhibited Airway Remodeling in OVA-Induced Mice**

A histological examination of the lung samples demonstrated the marked infiltration of inflammatory cells...
into the perivascular and peribronchial regions of the OVA-induced mice, and most of the infiltrated cells were eosinophils (Fig. 1). By contrast, in the groups of mice receiving either resveratrol or dexamethasone treatment, inflammation was reduced (Table 2).

More attention was paid to the effects of resveratrol on airway remodeling. The WAm/Pbm and WAm/Pbm ratios were used to evaluate airway remodeling. An image analysis revealed that OVA-induced mice (OVA group) manifested thicker airway walls (WAm/Pbm, Table 3) than did the control group, following correction for the airway basement perimeter. Resveratrol and dexamethasone were equally effective in reducing airway wall thickening, as no significant difference between the resveratrol and dexamethasone groups was noted. Furthermore, the OVA-induced mice exhibited significantly increased airway smooth muscle thickness (WAm/Pbm, compared with the PBS control animals), which was significantly reduced by resveratrol (Res vs. OVA, Table 3). Additionally, no significant difference in airway smooth muscle thickness was observed between dexamethasone-treated mice and resveratrol-treated mice. In summary, airway remodeling was markedly inhibited by resveratrol treatment.

**Table 3. Effect of Resveratrol on Airway Remodeling (Mean±S.D.)**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>WAmuc/Pbm</th>
<th>WAm/Pbm</th>
<th>WAi/Pbm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>10</td>
<td>30.85±9.95</td>
<td>7.02±3.83</td>
<td>19.32±2.80</td>
</tr>
<tr>
<td>Asthma</td>
<td>10</td>
<td>63.69±15.88*</td>
<td>27.01±6.99*</td>
<td>52.24±9.94*</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>10</td>
<td>50.99±5.54**</td>
<td>14.77±2.41***</td>
<td>29.15±3.32***</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>10</td>
<td>34.23±7.78***</td>
<td>14.29±5.96***</td>
<td>25.58±5.83***</td>
</tr>
</tbody>
</table>

* p<0.001, vs. normal group, ** p<0.01 vs. asthma group, *** p<0.001 vs. asthma group. WAmuc: the area of mucous glands; WAm: the area of smooth muscle; WAi: the area of inner airway wall; Pbm: the basement membrane perimeter.

**PTEN Expression Was Up-Regulated by Resveratrol Treatment**
PTEN signaling is central to the pathogenesis of asthma; therefore, we studied the effects of resveratrol on PTEN expression. The IOD values which showed the intensity of the PTEN staining was measured by computer. We found that the IOD value was lower in OVA-induced mice compared with the control group, indicating PTEN expression was inhibited in OVA-induced mice; however, dexamethasone reversed this inhibition (Fig. 2), which is consistent with the findings of a previous study.6) When the OVA induced mice were treated with resveratrol, the IOD value became elevated to the same level as that observed in mice treated with dexamethasone (Fig. 2E), which demonstrated the expression of PTEN protein was restored following treatment with resveratrol.

In order to demonstrate that the upregulation of PTEN expression was the result of enhanced transcription, PTEN mRNA levels from lung samples were quantified via real-time PCR. We observed that PTEN mRNA expression was significantly decreased in the OVA mice compared with the control group, whereas PTEN mRNA expression was significantly increased in the resveratrol and dexamethasone groups compared with the OVA mice (Fig. 2F). There was no significant difference in PTEN mRNA expression between mice treated with either resveratrol or dexamethasone (Fig. 2F).

**Correlation of PTEN Expression with Airway Remodeling**
We then examined the relationships between PTEN expression and clinicopathologic features associated with airway remodeling among 4 groups. As shown in Fig. 3, the IOD value of PTEN was significantly and negatively correlated with the area of mucous glands (WAmuc/Pbm), the area of smooth muscle (WAm/Pbm) and the area of inner airway wall (WAi/Pbm). These findings indicate that PTEN expression was associated with the degree of airway remodeling.

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**Fig. 2. The Expression of PTEN Protein and mRNA in the Lungs**
Immunohistochemical staining for PTEN in the lung tissue: A: Saline control group (nontreated). B: Asthma group (OVA group) with chronic allergic airway inflammation. C: Dexamethasone group. D: Resveratrol group. E: Integrated optical density (IOD) values of PTEN staining analyzed by IPP6.0. * p<0.05, vs. normal group, ** p<0.05 vs. asthma group. F: Effect of resveratrol on PTEN mRNA. Lung PTEN mRNAs were analyzed using real-time polymerase chain reaction. # p<0.05, vs. normal group, ## p<0.05, vs. asthma group. Values are expressed as mean±S.D., n=10/group.
Fig. 3. Correlation of PTEN Expression with Airway Remodeling among 4 Groups

Expression of PTEN was analyzed by measuring integrated optimal density (IOD) values using Image-Pro Plus 6.0. Its correlation with WA muc/Pbm (A), WA m/Pbm (B), and WA i/Pbm (C) among 4 groups was analyzed.

Fig. 4. Effect of Resveratrol and Salermide (a SIRT1 Inhibitor) on PTEN Regulation in 16HBE Cells

16HBE cells were treated with 5µmol/L salermide, 10µmol/L salermide, 20µmol/L salermide, 50µmol/L resveratrol, 20µmol/L salermide plus 50µmol/L resveratrol, and 10µmol/L salermide plus 50µmol/L resveratrol for 72h. The protein expression level of PTEN was detected via Western blotting (A and B) and immunocytochemistry (C–I). Saler: salermide, Res: resveratrol.
Effect of SIRT1 Gene Activation on PTEN Expression

It has been reported that SIRT1 is the primary molecular target of resveratrol; we therefore treated pulmonary epithelial 16HBE cells with increasing doses of the SIRT1 inhibitor, salermide, to determine the role of SIRT1 on PTEN expression. We found that salermide decreased PTEN protein expression in a dose-dependent manner (Fig. 4) and SIRT1 knockdown decreased PTEN expression levels (Fig. 5), thereby illustrating the role of SIRT1 in PTEN regulation. Following this, we investigated whether resveratrol regulated PTEN expression through SIRT1 activation in a mouse asthma model and identified one of the anti-asthmatic mechanisms of resveratrol, which correlated with up-regulated PTEN expression.

The mediation of PTEN expression by resveratrol has been described previously. However, these studies were carried out using several types of cancer cells (e.g., prostate cancer cells) and breast cancer cells, and the regulatory mechanisms underlying these processes are not uniform. Wang et al. found that resveratrol exposure decreased AKT phosphorylation and GATA2 protein levels in breast cancer cells, resulting in the upregulation of PTEN. Additionally, Stefanska et al. discovered that resveratrol reduced PTEN promoter methylation, which therefore induced PTEN expression. However, whether resveratrol induces PTEN expression in normal cells, particularly airway epithelial cells, remained unknown.

We also examined the role of SIRT1 in the regulation of PTEN expression on the basis of data that SIRT1 is the primary molecular target of resveratrol. We previously reported that resveratrol bound to the SIRT1 enzyme-peptide substrate complex at an allosteric site at the amino-terminus of the catalytic domain, thereby increasing the affinity of the complex for acetylated substrates. Therefore, resveratrol acts as a SIRT1 activator. Following activation by resveratrol, SIRT1 may exert its effects by controlling several transcription factors (e.g., proliferator activated receptor gamma (PPAR)), p53 or nuclear factor-kappa B (NF-κB) in order to regulate gene expression. Our in vitro results demonstrated that the inhibition of SIRT1 activity decreased PTEN expression, whereas resveratrol attenuated the salermide-induced decrease in PTEN expression, thereby illustrating the role of SIRT1 in PTEN regulation. Following this, we investigated whether resveratrol regulated PTEN expression through SIRT1 activation by treating lung epithelial 16HBE cells with salermide and resveratrol. As shown in Fig. 3, resveratrol attenuated the salermide-induced decrease in PTEN expression, which indicated that the mechanism underlying the regulation of PTEN by resveratrol was mediated by SIRT1 activation.

DISCUSSION

Asthma is a chronic respiratory disorder characterized by airway eosinophilia, airway hyperresponsiveness (AHR), IgE production, increased mucus secretion, and airway remodeling. Resveratrol, a type of polyphenol present in grapes, was shown to inhibit IL-4 and IL-5 and reduce airway remodeling and GATA2 protein levels in breast cancer cells, resulting in the upregulation of PTEN. Additionally, Stefanska et al. discovered that resveratrol reduced PTEN promoter methylation, which therefore induced PTEN expression. However, whether resveratrol induces PTEN expression in normal cells, particularly airway epithelial cells, remained unknown.

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