

Resveratrol has protective effects against airway remodeling and airway hyperreactivity in a murine model of allergic airways disease

Simon G. Royce^{1,2*}, William Dang^{1,3}, Gao Yuan^{1,3},
Jenny Tran¹, Assam El Osta^{2,4,5,6}, Tom C. Karagiannis^{2,3}
and Mimi L.K. Tang^{1,7}

¹Allergy and Immune Disorders, Murdoch Children's Research Institute, Melbourne, Vic., Australia;

²Department of Pathology, The University of Melbourne, Parkville, Melbourne, Vic., Australia; ³Epigenomic Medicine Laboratory, Baker IDI Heart and Diabetes Institute, Melbourne, Vic., Australia; ⁴Epigenetics in Human Health and Disease Laboratory, Baker IDI Heart and Diabetes Institute, Melbourne, Vic., Australia; ⁵Epigenomic Profiling Facility, Baker IDI Heart and Diabetes Institute, Melbourne, Vic., Australia; ⁶Department of Medicine, Monash University, Melbourne, Vic., Australia; ⁷Department of Allergy and Immunology, The Royal Children's Hospital, Parkville, Melbourne, Vic., Australia

Background: New therapies for asthma which can address three main interrelated features of the disease, airway inflammation, airway remodeling and airway hyperreactivity, are urgently required. Resveratrol, a well known red wine polyphenol has received much attention due to its potential anti-aging properties. This compound is an agonist of silent information regulator two histone deacetylases and has many effects that are relevant to key aspects of the pathophysiology of asthma including inflammation, cell proliferation and fibrosis. Therefore, resveratrol may offer a novel asthma therapy that simultaneously inhibits airway inflammation, and airway remodeling which are the main contributors to airway hyperreactivity and irreversible lung function loss.

Methods: We evaluated the effects of systemic resveratrol treatment in a murine model of chronic allergic airways disease which displays most of the clinicopathological features of severe human asthma. Wild-type Balb/c mice with allergic airways disease were treated with 12.5 mg/kg resveratrol or vehicle control. Airway inflammation was assessed by bronchoalveolar lavage fluid cell counts and histological examination of lung tissue sections. Further, remodeling was assessed by morphometric analysis and lung function was assessed by invasive plethysmography measurement of airway resistance and dynamic compliance.

Results: Mice treated with resveratrol exhibited reduced tissue inflammation as compared to vehicle treated mice ($p < 0.05$). Additionally, resveratrol treatment resulted in reduced subepithelial collagen deposition as compared to vehicle treated mice ($p < 0.05$) and attenuated airway hyperreactivity ($p < 0.05$).

Conclusions: These novel findings demonstrate that treatment with resveratrol can reduce structural airway remodeling changes and hyperreactivity. This has important implications for the development of new therapeutic approaches to asthma.

Keywords: *resveratrol; asthma; airway remodeling; allergic airways disease; fibrosis*

Received: 26 March 2011; Revised: 19 April 2011; Accepted: 20 April 2011; Published: 1 June 2011

Severe asthma can be a difficult disease to treat as it consists of three interrelated pathologies: airway inflammation, airway remodeling, and airway hyperreactivity (AHR) (1, 2). The mainstay of asthma therapeutics is inhaled corticosteroids. These drugs are very effective at treating airway inflammation;

however, they have a number of important limitations. Firstly, steroid resistance, where a subpopulation of patients with asthma shows poor response to the drugs, is a concern. Secondly corticosteroids cannot be used at the highest and optimal concentrations in young children. Thirdly corticosteroids have only limited efficacy in

preventing and reversing airway remodeling changes (3). These structural changes in the airway wall include goblet cell metaplasia and mucous hypersecretion, subepithelial fibrosis, smooth muscle thickening, and angiogenesis (4, 5). Airway remodeling occurs early in disease pathogenesis, can interact with and promote chronic inflammation, and contributes to AHR resulting in progressive irreversible loss of lung function (2). Therefore, there is a need for identification of novel treatments that can prevent and/or reverse airway remodeling changes and inhibit AHR in addition to abrogating airway inflammation.

Resveratrol (3,4',5-trihydroxystilbene), a polyphenolic agonist of silent information regulator two histone deacetylases (sirtuins, class III HDAC) is a compound found in the skin of red fruits (e.g. red grapes, a constituent of red wine) and medicinal plants (6–8). The anti-cancer, anti-inflammatory, and anti-oxidant effects of this compound have been reported in numerous mouse and rat models (9). The anti-inflammatory effects of resveratrol have been associated with the inhibition of cyclooxygenase (COX)-2 transcription and inhibition of COX-1 activity via a peroxidase-mediated mechanism (10). In the experimental rat carrageenan-induced paw edema model, resveratrol inhibited both acute and chronic inflammation (11). It has also shown to protect cartilage against the progression of inflammatory arthritis in a rabbit arthritis model (12).

The effects of resveratrol have also been attributed, at least in part, to the inhibition of nuclear factor kappa B (NF- κ B) via the inhibition of I κ B kinase (13). The NF- κ B is an important transcription factor involved in the expression of numerous inflammation-associated proteins such as GM-CSF, IL-8, COX-2, and inducible nitric oxide synthase (iNOS). Activation of airway inflammation releases reactive oxygen species (ROS) leading to oxidative stress. The NF- κ B activation is necessary for the expression of various inflammatory proteins such as GM-CSF and IL-8 (released by alveolar macrophages/polymorphonuclear cells from patients with COPD (14), COX-2, and iNOS (15, 16). Hence NF- κ B inhibition, possibly via inhibition of I κ B kinase (17), may reduce the expression of inflammatory genes, a mechanism by which glucocorticosteroids may also function (18). Resveratrol has also been known to mitigate the release of inflammatory mediators whilst having a limited effect on VEGF (protects from development of emphysema) in COPD (19). Furthermore, the anti-inflammatory effects of resveratrol may be due to inhibition of activator protein-1 (AP-1), which is also a potent regulator of oxidative stress related genes (20).

Importantly, airway inflammation has been shown to be suppressed by resveratrol in an acute mouse model of allergic airways disease, producing similar effects to glucocorticoids (dexamethasone) (21). Overall, resveratrol has potent anti-inflammatory and anti-oxidative functions

and, therefore, may be useful in the treatment of airway diseases such as asthma. We aimed to investigate the effects of resveratrol in a murine model of chronic allergic airways disease that displays most of the features of human asthma on airway inflammation, airway remodeling structural changes, and AHR.

Materials and methods

Animals

Six-week-old female Balb/c mice were housed under specific pathogen-free conditions and maintained on a fixed 12 hour light, 12 hour dark lighting schedule. Balb/c mice demonstrate strong Th2 responses in ovalbumin (OVA)-induced allergic airways disease models (22–24). All experimental procedures were approved by the Murdoch Children's Research Institute Animal Ethics Committee and followed the Australian Guidelines for the Care and Use of Laboratory Animals for Scientific Purposes.

Mouse model of chronic allergic airways disease

An established model of OVA-induced chronic allergic airways disease was used as previously described (25, 26). This model includes many of the pathological features of human asthma including increased allergic responses indicated by increased immunoglobulin E against OVA (OVA-specific IgE), AHR, and remodeling changes such as epithelial remodeling, goblet cell metaplasia, and subepithelial collagen deposition (fibrosis). However, it does not display smooth muscle hyperplasia (25, 26). Briefly, mice were sensitized with 10 μ g of grade V OVA (Sigma Chemical, St. Louis, MO) and 1 mg of aluminum potassium sulfate adjuvant (alum) in 500 μ l saline i.p on day 0 and 14 and then challenged with nebulized 2.5% (w/v) OVA in saline 3 days per week for 6 weeks to establish AAD.

Ovalbumin-exposed mice were treated with resveratrol (OVA-RV, $n = 15$) or vehicle control (OVA-VEH, $n = 15$) intraperitoneally following each OVA nebulization (3 days per week for 6 weeks). A third group of mice, sensitized with saline/alum on days 0 and 14 and nebulized with saline 3 days per week for 6 weeks ($n = 15$), served as additional controls. Resveratrol (Sigma Chemical) was used at a dose of 12.5 mg/kg in 20% ethanol/0.9% saline, which is known to be biologically active and has been used widely in experimental murine models of disease with minimal toxicity (27, 28).

Methacholine-induced AHR

Forty-eight hours after the last OVA nebulization/treatment with resveratrol or vehicle, AHR was measured using a mouse invasive plethysmograph (Buxco Electronics, Troy, NY) as described previously (25). Mice were anesthetized by i.p. injection of ketamine (200 mg/kg) and xylazine (10 mg/kg) and tracheostomized. After

baseline airway resistance had been recorded, increasing methacholine doses (0.25, 0.5, 1, 2, 4, and 8 mg/ml) were delivered by nebulizer (Buxco Electronics) and airway resistance change from baseline level were measured (Finepointe, Buxco Electronics) for 3 min after each dose.

Quantitation of serum OVA-specific IgE levels

Serum was obtained by lethal cardiac puncture of anesthetized mice after AHR measurement and stored frozen at -70°C for measurement of OVA-specific IgE by ELISA (25, 29, 30). OVA-specific IgE levels were expressed as mean optical density (OD) at 490 nm.

Bronchoalveolar lavage (BAL)

Following measurement of airway reactivity, BAL was performed by three washes of 0.5 ml saline containing 5% (v/v) fetal calf serum and total and differential cell counts of inflammatory cells determined (25, 31). Total viable cell counts were determined using a hemocytometer with trypan blue exclusion. Differential counts of eosinophils, neutrophils, lymphocytes, and monocytes/macrophages were determined on cytospin smears of BAL samples (4×10^5 cells) from individual mice stained with Diff-Quick (Life Technologies, Auckland, New Zealand) and identified by standard morphological criteria after counting 300 cells.

Tissue collection

Lung tissues were immediately dissected and weighed (total lung weight) and then separated into individual lobes for histological analysis (25, 29, 32).

Lung histopathology

The right lung was fixed in formalin, routinely processed, and embedded in paraffin (25, 29, 32). Three μm lung tissue sections were stained with Masson trichrome for assessment of epithelial and subepithelial collagen thickness and Alcian blue-periodic acid Schiff (AB-PAS) for assessment of goblet cell numbers (25, 29, 32).

Airway tissue inflammation cell score

The degree of airway inflammatory cell infiltration was scored by two independent blinded investigators. The degree of airway inflammatory cell infiltration around the bronchi was scored as 0 for no inflammatory cell infiltration, 1 for 3 layers of inflammatory cells, 2 for 6 layers, 3 for 10 layers and congestion, or 4 for more than 10 layers of inflammatory cells and severe congestion. For each mouse, five airways were observed and the average scores were taken.

Morphometric analysis of structural changes

Morphometric analysis of lung tissue sections was performed as described previously (25, 29, 33). Images of lung tissue sections were captured using a Digital camera (Q

Imaging, Burnaby, BC, Canada). A minimum of five bronchi measuring 150–350 μm luminal diameter were analyzed per mouse using Image Pro-Discovery software (Media Cybernetics, Silver Spring, MD), which was calibrated with a reference micrometer slide. The thickness of the bronchial epithelial layer was measured by tracing around the basement membrane and the luminal surface of epithelial cells using a digitizer (Aiptek, Irvine, CA) and calculating the mean distance between the lines by Image Pro-Discovery software (Media Cybernetics). Subepithelial collagen thickness was also measured by tracing around the outer extent of the total collagen layer in the submucosal region, around the basement membrane, and the mean distance between these lines calculated. For goblet cell determination, the basement membrane length was measured by tracing in Image Pro-Discovery software (Media Cybernetics) and goblet cells were counted in AB-PAS stained sections and expressed as number of cells per 100 μm of basement membrane.

Immunohistochemistry for TGF- β 1

Sections of lung tissue were stained by immunohistochemistry to detect TGF- β 1 protein expression. The TGF- β 1 was identified using rabbit polyclonal antibodies, sc-146, specific for TGF- β 1 (Santa Cruz Biotechnology, Santa Cruz, CA). Bound primary antibody was detected using anti-rabbit EnVision (Dako, Glostrup, Denmark). The chromagen 3,3-diaminobenzidine was used and sections were counterstained with hematoxylin.

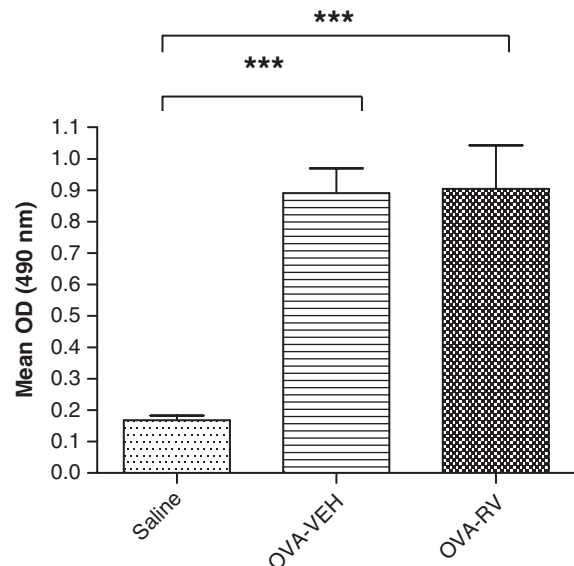


Fig. 1. Serum levels of OVA-specific IgE in the chronic allergic airways disease model. Enzyme-linked immunosorbent assay was performed on mice blood serum samples. OD values were read at 490 nm with a reference wavelength of 650 nm. There was a significant increase in the OVA-VEH and the OVA-RV sensitized groups with respect to saline group ($***p < 0.001$), $n = 15$ per group.

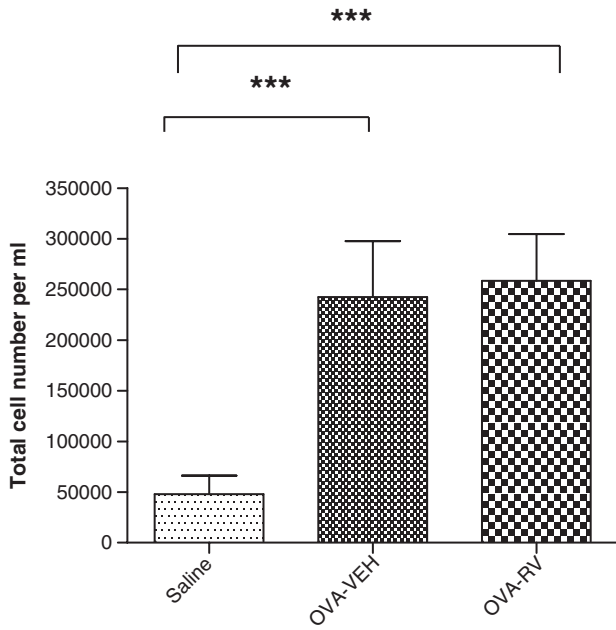


Fig. 2. Total inflammatory cell count in BALF. Following sacrifice, BAL was performed and total inflammatory cell number per ml of BALF determined. The OVA-VEH and OVA-RV groups showed a significant increase in total inflammatory cell numbers compared to the saline mice ($***p < 0.001$), $n = 15$ per group.

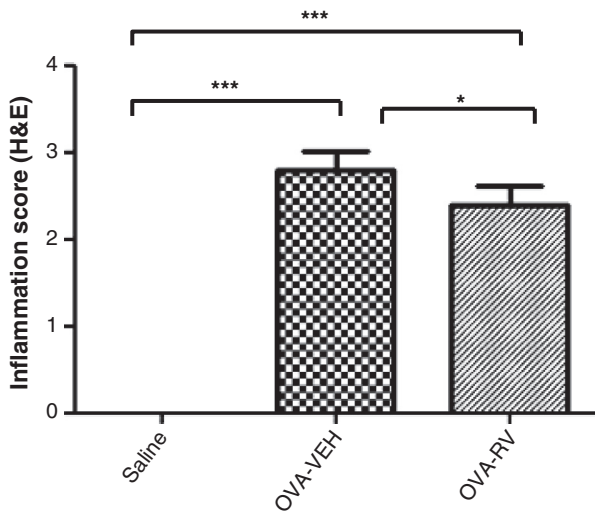


Fig. 3. Inflammation score of hematoxylin and eosin stained sections of mouse airways. Inflammatory cell infiltration was scored by two independent blinded investigators as 0 for no inflammation infiltration, 1 for 3 layers of inflammatory cells, 2 for 6 layers, 3 for 10 layers and congestion, or 4 for more than 10 layers of inflammatory cells and severe congestion. Both OVA-VEH and OVA-RV had elevated inflammation scores as compared to saline mice. OVA-RV was significantly reduced compared to OVA-VEH ($*p < 0.05$; $***p < 0.001$), $n = 15$ per group.

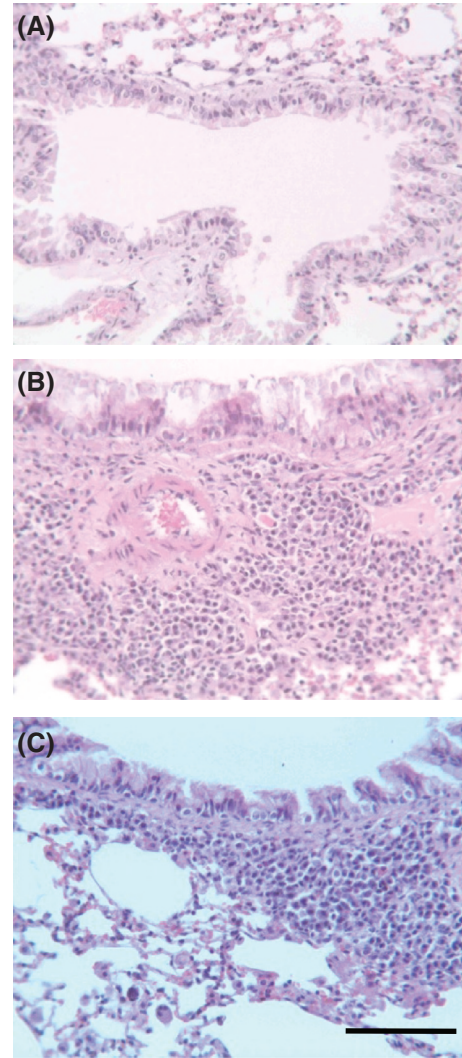


Fig. 4. Representative photomicrographs of hematoxylin and eosin stained sections of mouse airways. Formalin fixed paraffin embedded sections of mouse lung tissue were stained with hematoxylin and eosin. Airway from a saline treated mouse showing little or no peribronchial inflammatory infiltrate (A). Airway from an OVA-VEH mouse shows severe peribronchial inflammatory infiltrate (B). Airway from an OVA-RV mouse showing moderate peribronchial inflammatory infiltrate (C), $n = 15$ per group; Bar = 100 μm .

Statistical analysis

Results were expressed as mean \pm SEM with 95% confidence interval and analyzed using the Mann-Whitney test.

Results

Validation of the chronic allergic airways disease model

It was first necessary to validate that OVA sensitization/challenge had been successfully established in the OVA mice treated with vehicle (OVA-VEH). Allergen-specific

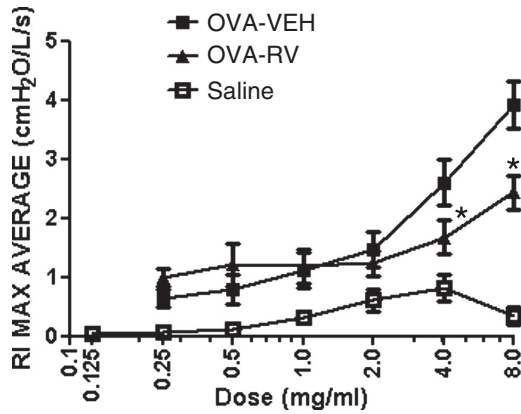


Fig. 5. Airway hyperresponsiveness to methacholine in the chronic AAD model. Plethysmography was performed 48 hours following the final treatment and nebulization. Increasing doses of methacholine was administered and maximal resistance values (cmH₂O/L/sec) after 5 min was recorded. OVA-VEH mice had elevated airway resistance at the highest methacholine dose as compared to saline mice. OVA-RV had significantly lower airway resistance at the highest methacholine dose compared to OVA-VEH, *n* = 10 per group (**p* < 0.05 v OVA-VEH).

immunoglobulin E (OVA-specific IgE), AHR, BAL inflammatory cells, goblet cell hyperplasia, and remodeling changes such as epithelial remodeling and peribronchial fibrosis were assessed. The OVA sensitization was verified by measurement of OVA-specific IgE in serum samples at the end of the 8 week OVA sensitization/challenge protocol. As expected, OVA-VEH mice demonstrated

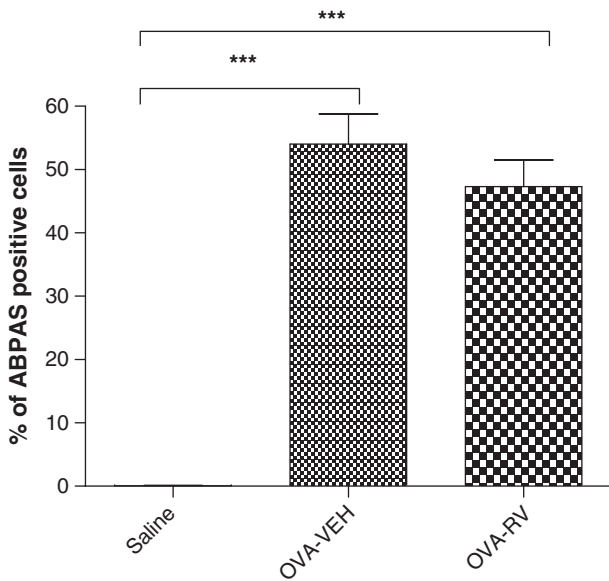


Fig. 6. Percentage of AB-PAS positive goblet cells in mouse airways. Formalin-fixed paraffin-embedded sections of mouse lung tissue were stained with AB-PAS and the percentage of AB-PAS goblet cells determined. Both OVA-VEH and OVA-RV groups had elevated goblet cell percentages as compared to saline mice (***p* < 0.001), *n* = 15 per group.

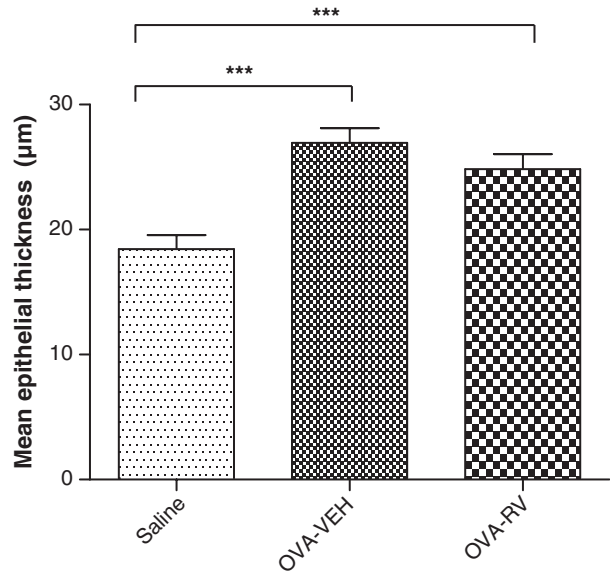


Fig. 7. Morphometric analysis of epithelial thickness in the chronic AAD model. Airway epithelial thickness of Masson trichrome stained lung tissue sections were traced and assessed using morphometry software. The data represent mean epithelial thickness values obtained from mice in their respective treatment group. Both OVA-VEH and OVA-RV had elevated epithelial thickness as compared to saline mice. OVA-RV was not significantly different to OVA-VEH (N.S.) (***p* < 0.001), *n* = 15 per group.

increased levels of OVA-specific serum IgE compared to saline control mice (*p* < 0.001; Fig. 1). Total and differential BAL cell counts were significantly increased in OVA-VEH mice as compared with the saline sensitized/challenged control mice (all *p* < 0.001; Fig. 2). Airway inflammation was significantly increased in OVA sensitized/challenged mice treated with vehicle (OVA-VEH) as compared with the saline control mice (*p* < 0.001; Figs. 3 and 4). The AHR was significantly increased in the OVA-VEH group when compared to the saline control mice (*p* < 0.001; Fig. 5). The OVA-VEH mice also developed airway remodeling changes with increased goblet cell numbers (*p* < 0.001; Fig. 6), epithelial thickness (*p* < 0.001; Fig. 7), and subepithelial collagen deposition (*p* < 0.001; Fig. 8) as compared to the saline control mice. These

results confirmed that the allergic airways disease model was successfully established with development of airway inflammation, airway remodeling, and AHR as previously reported (25, 26, 29, 34).

Effect of resveratrol on airway inflammation in chronic allergic airways disease

The OVA-specific IgE levels in OVA sensitized/challenged mice treated with resveratrol (OVA-RV) were similar to levels in OVA sensitized/challenged mice treated with

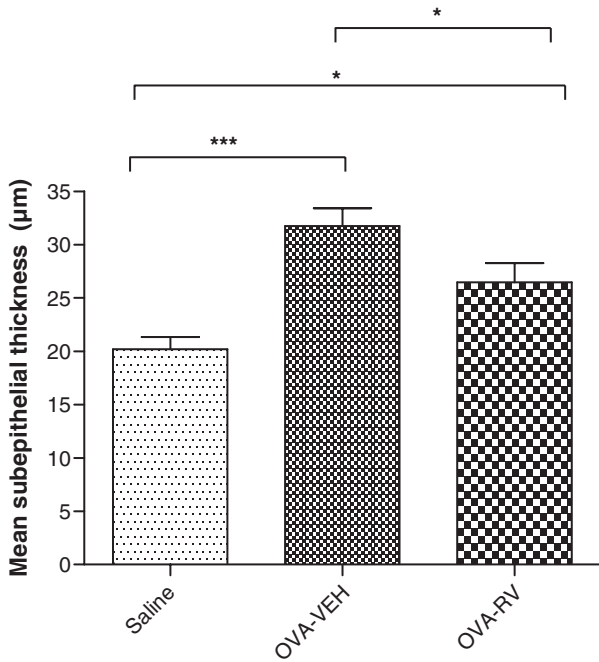


Fig. 8. Morphometric analysis of subepithelial collagen thickness in the chronic AAD model. Airway subepithelial collagen thickness of Masson trichrome stained lung tissue sections were traced and assessed using morphometry software. The data represent mean subepithelial collagen thickness values obtained from mice in their respective treatment group. OVA-VEH mice had elevated subepithelial collagen thickness as compared to saline mice. OVA-RV had significantly less collagen deposition compared to OVA-VEH (* $p < 0.05$; *** $p < 0.001$), $n = 15$ per group.

vehicle (OVA-VEH), and levels in both the OVA-RV and OVA-VEH groups were significantly higher than levels in saline sensitized/challenged control mice confirming successful OVA sensitization in both groups ($p < 0.001$; Fig. 1).

Total and differential BAL cell counts were not significantly different when compared to the vehicle treated mice (OVA-VEH) (Figs. 2 and 9). These results indicate that systemic (intraperitoneal) administration of resveratrol does not significantly inhibit numbers of BAL inflammatory cells following sensitization/challenge with OVA at the completion of the chronic OVA challenge model.

Treatment with resveratrol suppresses airway remodeling changes in subepithelial collagen deposition but not epithelial thickening and goblet cell metaplasia in chronic allergic airways disease

Goblet cell numbers were determined in AB-PAS stained lung tissue sections, while airway epithelial thickness and subepithelial ECM thickness were determined in Masson trichrome stained lung tissue sections. Subepithelial extracellular matrix thickness was significantly lower in resveratrol-treated mice as compared to vehicle treated mice ($p < 0.05$), but remained higher than that observed in the saline sensitized/challenged mice ($p < 0.05$; Figs. 8 and 10).

Percentages of goblet cells in resveratrol treated mice were not significantly different than in vehicle treated

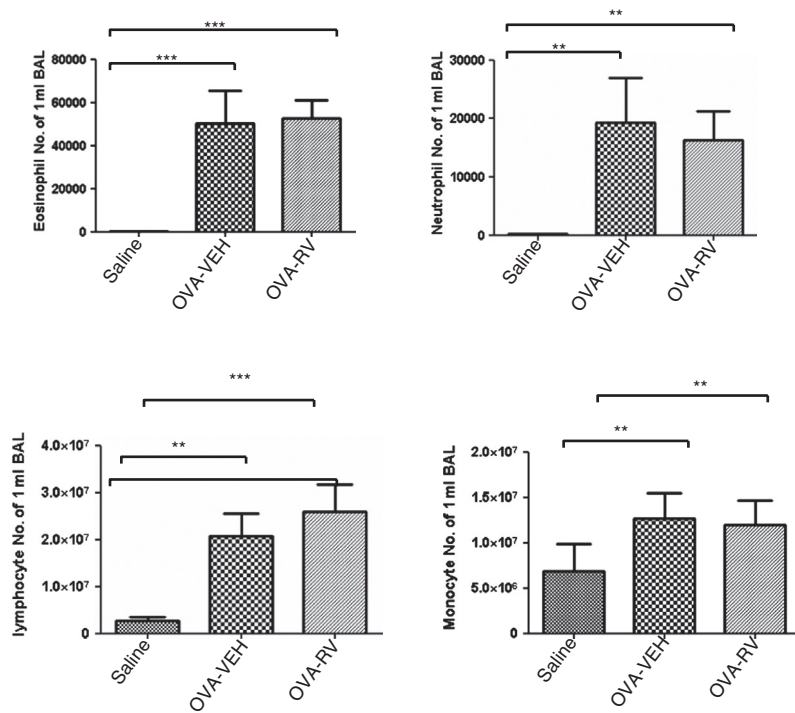


Fig. 9. BALF differential cell counts. Following Diff-quick staining, numbers of differential cells were counted under the light microscope. The value expressed as total number in 1 ml sample. Eosinophils, neutrophils, lymphocytes, and monocytes was significantly increased in OVA-VEH and OVA-RV mice as compared to saline mice (** $p < 0.01$; *** $p < 0.001$), $n = 10$ per group.

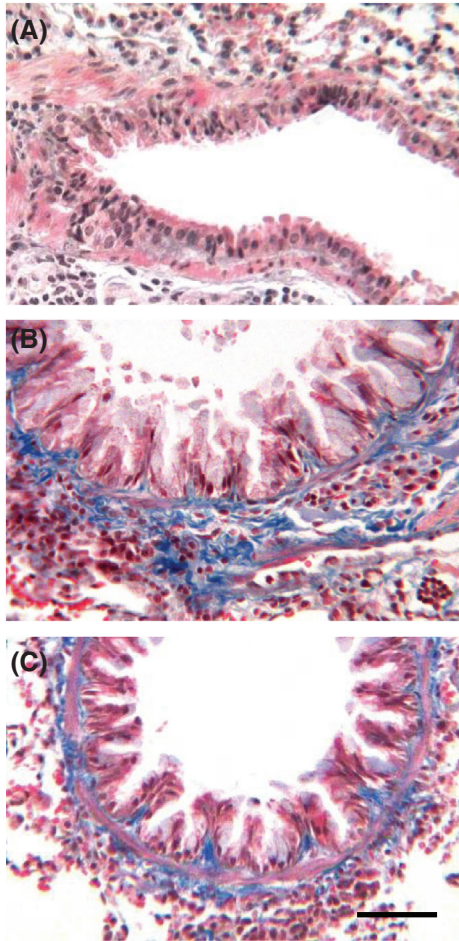


Fig. 10. Representative photomicrographs of Masson trichrome stained sections of mouse airways. Formalin-fixed paraffin-embedded sections of mouse lung tissue were stained with Masson trichrome. Airway from a saline treated mouse showing thin airway wall with thin epithelial layer (arrow) and little subepithelial collagen deposition (arrowhead) (A). Airway from an OVA-VEH mouse with thickened epithelial layer (arrow) and severe subepithelial collagen deposition (arrowhead) (B). Airway from an OVA-RV mouse with thickened epithelial layer (arrow) but little subepithelial collagen deposition (arrowhead) (C), $n = 15$ per group; Bar = 100 μm .

mice ($p < 0.05$; Figs. 6 and 11), but remained significantly higher than in the saline sensitized/challenged mice ($p < 0.001$). The mean epithelial thickness was not significantly different in OVA-RV and OVA-VEH mice, and airway epithelial thickness in both groups was significantly increased as compared to saline sensitized/challenged mice ($p < 0.001$; Figs. 7 and 10). These results suggest that resveratrol can regulate some aspects of airway remodeling (fibrosis) but not epithelial remodeling.

Treatment with resveratrol attenuates AHR in chronic allergic airways disease

Anesthetized tracheotomized mice were challenged with nebulized methacholine and the change in resistance from

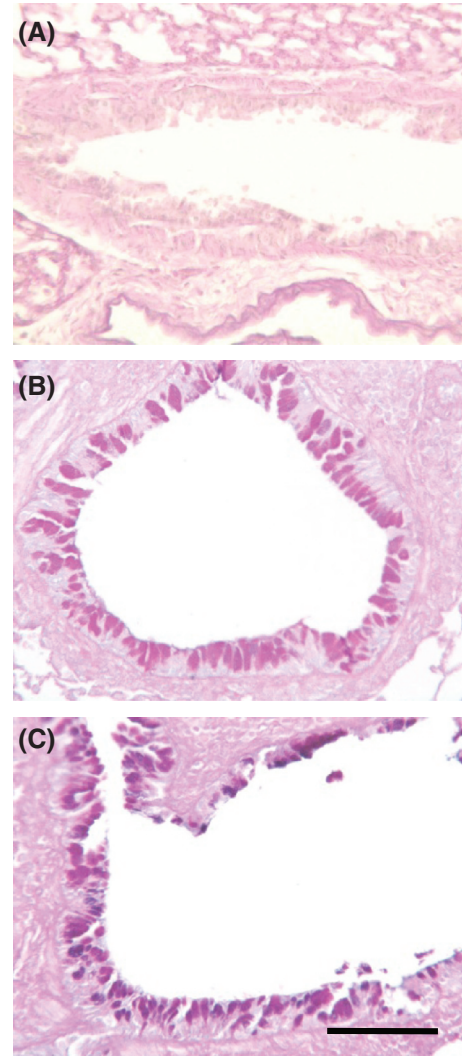


Fig. 11. Representative photomicrographs of AB-PAS stained sections of mouse airways. Formalin-fixed paraffin-embedded sections of mouse lung tissue were stained with AB-PAS. Airway from a saline treated mouse showing little or no goblet cell metaplasia (A). Airway from an OVA-VEH mouse showing goblet cell metaplasia (arrows) (B). Airway from an OVA-RV mouse showing goblet cell metaplasia (arrows) (C), $n = 15$ per group; Bar = 100 μm .

baseline was measured by invasive plethysmography. Methacholine-induced AHR was reduced in the OVA-RV group as compared to the OVA-VEH group, with statistical significance reached at doses of 4 mg/ml and 8 mg/ml of methacholine ($p < 0.05$; Fig. 5). These results show that resveratrol treatment can suppress AHR in the chronic allergic airways disease model.

Immunohistochemical analysis of TGF β 1 expression

The TGF β 1 was investigated by immunohistochemistry in formalin-fixed, paraffin-embedded lung tissue sections (Fig. 12). Little constitutive staining for TGF β 1 was observed in the saline-sensitized and -challenged

control mice. In the lungs of mice receiving chronic OVA sensitization and challenge, staining for TGF β 1 was markedly increased. Strong cytoplasmic staining was present in bronchial epithelial cells, connective tissue cells in the lamina propria and adventitia, and smooth muscle cells. Strong staining was also seen in inflammatory cells. Treatment with resveratrol resulted in markedly reduced staining for TGF β 1 in the lung tissue.

Discussion

We investigated the potential of resveratrol treatment for asthma using a chronic murine model of allergic airways

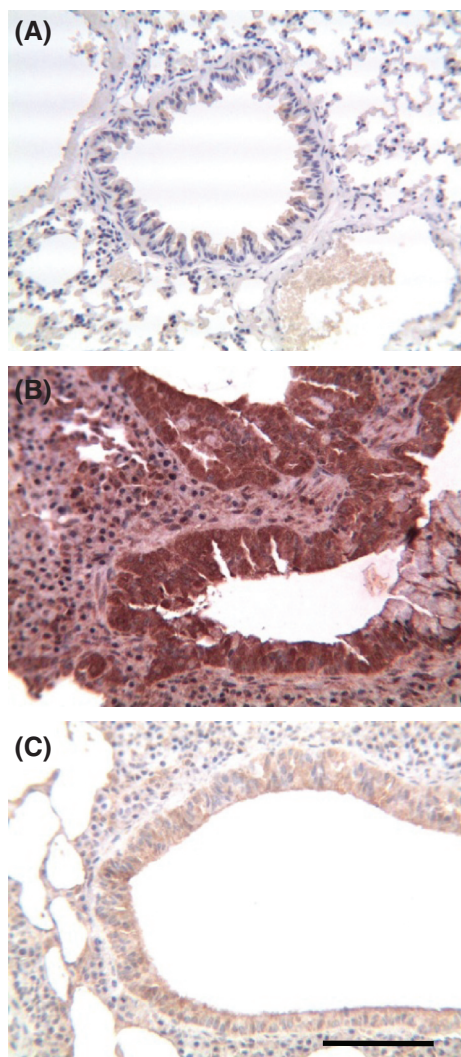


Fig. 12. Immunohistochemical analysis of TGF β 1 expression. TGF β 1 was investigated by immunohistochemistry in formalin-fixed, paraffin-embedded lung tissue sections. Little staining for TGF β 1 was observed in the saline-sensitized and -challenged control mice (A). In the lungs of mice receiving chronic OVA sensitization and challenge, staining for TGF β 1 was markedly increased (B). Treatment with resveratrol resulted in markedly reduced staining for TGF β 1 in the lung tissue (C), $n = 10$ per group; Bar = 100 μ m.

disease. We assessed the effect of the drug in preventing airway remodeling and suppressing AHR. Our findings indicate that resveratrol suppressed AHR and inhibited the development of subepithelial bronchial fibrosis. This effect on fibrosis involved reduced TGF β protein expression in the airways of mice treated with resveratrol. In our study we did not see significant reduction in BAL fluid inflammatory cell numbers although we did see modest reduction in lung tissue inflammation infiltrate in the chronic allergic airways disease mice treated with resveratrol. This likely reflects the treatment regimen used and the chronic ovalbumin model that exhibits only moderate inflammation and eosinophilia compared to the acute and subacute models of allergic airways disease (29).

One of the major features of the chronic allergic airways disease model is the relatively high AHR to methacholine. In the current study we found that resveratrol resulted in lower AHR at high concentrations of methacholine. The AHR is the most important clinical endpoint in asthma; increased AHR is not only symptomatic of asthma but is a correlate of disease severity (GINA guidelines) and can be used for diagnosis and monitoring the disease. Whilst we only demonstrate modest evidence of action on airway inflammation, resveratrol had a powerful anti-remodeling activity, particularly in airway subepithelial fibrosis. Airway fibrosis is an important pathological process in many airway diseases including chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, and scleroderma. Fibrotic remodeling of the basement membrane, the formation of the lamina reticularis, is pathognomonic of asthma and correlates with severity in endobronchial biopsies from asthma sufferers (35). Fibrosis in the lamina reticularis, lamina propria, and serosa contributes to the overall thickness of the airway wall that impacts AHR (36, 37). Furthermore, extracellular matrix is deposited between airway smooth muscle cells in asthma (38). Extracellular matrix not only contributes to increased muscle mass for AHR but matrix components such as collagen I have been shown *in vitro* to promote airway smooth muscle cell proliferation (39).

Airway fibrosis in asthma is due to extracellular matrix deposition by fibroblasts and is accompanied by differentiation of myofibroblasts, activated fibroblasts with contractile properties, and α smooth muscle actin expression. These processes are regulated by TGF β , a potent profibrotic cytokine. The TGF β is hypersecreted by epithelial cells and fibroblasts in the airway as well as the influx of inflammatory cells including eosinophils in asthma (40). We found significantly less TGF β protein in mice treated with resveratrol than in ovalbumin control mice.

In the current study resveratrol was administered intraperitoneally over a 6 week period. The dose used was efficacious without any side effect in the rodent model as assessed by histopathology of representative

organ samples, animal welfare scorings, and bodyweight (data not shown). However, the current study has the caveat that resveratrol was used to prevent disease rather than reverse established disease. Nevertheless, we have provided *in vivo* evidence for an anti-fibrotic action of resveratrol in inflammatory obstructive lung disease and suppression of AHR in a model of chronic allergic airways disease. Our findings add to the recent literature establishing the anti-fibrotic effects of resveratrol in a wide range of organ systems, providing evidence of key anti-aging properties. Overall, the findings highlight that a dietary and pharmacological approach using resveratrol may represent safe and timely treatment for asthma and lung disease that target a central aspect of airway remodeling and may subvert steroid resistance.

Acknowledgements

The authors would like to acknowledge the assistance of Dr. Aloka Seneviratne in manuscript preparation.

Conflict of interest and funding

Studies were funded by an Australian National Health and Medical Research Council (NHMRC) project grants (546428, 566559, 566559) and a Murdoch Children's Research Institute Infection, Environment and Immunity Grant. Further, the authors acknowledge grant and fellowship support from the Australian Institute of Nuclear Science and Engineering (AINSE) and the CRC for Biomedical Imaging Development (CRC-BID).

References

- Djukanovic R, Roche WR, Wilson JW, Beasley CR, Twentyman OP, Howarth RH, et al. Mucosal inflammation in asthma. *Am Rev Respir Dis* 1990; 142: 434–57.
- Tang ML, Wilson JW, Stewart AG, Royce SG. Airway remodelling in asthma: current understanding and implications for future therapies. *Pharmacol Ther* 2006; 112: 474–88.
- Leung DY, Szeffler SJ. New insights into steroid resistant asthma. *Pediatr Allergy Immunol* 1998; 9: 3–12.
- Belleguic C, Corbel M, Germain N, Lena H, Boichot E, Delaval PH, et al. Increased release of matrix metalloproteinase-9 in the plasma of acute severe asthmatic patients. *Clin Exp Allergy* 2002; 32: 217–23.
- Davies DE, Holgate ST. Asthma: the importance of epithelial mesenchymal communication in pathogenesis. *Inflammation and the airway epithelium in asthma*. *Int J Biochem Cell Biol* 2002; 34: 1520–6.
- Penumathsa SV, Maulik N. Resveratrol: a promising agent in promoting cardioprotection against coronary heart disease. *Can J Physiol Pharmacol* 2009; 87: 275–86.
- You M, Liang X, Ajmo JM, Ness GC. Involvement of mammalian sirtuin 1 in the action of ethanol in the liver. *Am J Physiol Gastrointest Liver Physiol* 2008; 294: G892–8.
- Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, et al. Small molecule activators of sirtuins extend *saccharomyces cerevisiae* lifespan. *Nature* 2003; 425: 191–6.
- Elliott PJ, Jirousek M. Sirtuins: novel targets for metabolic disease. *Curr Opin Investig Drugs* 2008; 9: 371–8.
- Subbaramaiah K, Chung WJ, Michaluart P, Telang N, Tanabe T, Inoue H, et al. Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. *J Biol Chem* 1998; 273: 21875–82.
- Gentili M, Mazoit JX, Bouaziz H, Fletcher D, Casper RF, Benhamou D, et al. Resveratrol decreases hyperalgesia induced by carrageenan in the rat hind paw. *Life Sci* 2001; 68: 1317–21.
- Elmali N, Baysal O, Harma A, Esenkaya I, Mizrak B. Effects of resveratrol in inflammatory arthritis. *Inflammation* 2007; 30: 1–6.
- Manna SK, Mukhopadhyay A, Aggarwal BB. Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-kappa B, activator protein-1, and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation. *J Immunol* 2000; 164: 6509–19.
- Culpitt SV, Rogers DF, Fenwick PS, Shah P, De Matos C, Russell RE, et al. Inhibition by red wine extract, resveratrol, of cytokine release by alveolar macrophages in COPD. *Thorax* 2003; 58: 942–6.
- Blackwell TS, Christman JW. The role of nuclear factor-kappa B in cytokine gene regulation. *Am J Respir Cell Mol Biol* 1997; 17: 3–9.
- Newton R, Kuitert LM, Bergmann M, Adcock IM, Barnes PJ. Evidence for involvement of NF-kappa B in the transcriptional control of COX-2 gene expression by IL-1beta. *Biochem Biophys Res Commun* 1997; 237: 28–32.
- Holmes-McNary M, Baldwin AS, Jr. Chemopreventive properties of trans-resveratrol are associated with inhibition of activation of the I-kappa B kinase. *Cancer Res* 2000; 60: 3477–83.
- Newton R. Molecular mechanisms of glucocorticoid action: what is important? *Thorax* 2000; 55: 603–13.
- Wood LG, Wark PA, Garg ML. Antioxidant and anti-inflammatory effects of resveratrol in airway disease. *Antioxid Redox Signal* 2010; 13: 1535–48.
- Karin M. The regulation of AP-1 activity by mitogen-activated protein kinases. *Philos Trans R Soc Lond B Biol Sci* 1996; 351: 127–34.
- Lee M, Kim S, Kwon OK, Oh SR, Lee HK, Ahn K. Anti-inflammatory and anti-asthmatic effects of resveratrol, a polyphenolic stilbene, in a mouse model of allergic asthma. *Int Immunopharmacol* 2009; 9: 418–24.
- Melgert BN, Postma DS, Kuipers I, Geerlings M, Luinge MA, van der Strate BW, et al. Female mice are more susceptible to the development of allergic airway inflammation than male mice. *Clin Exp Allergy* 2005; 35: 1496–503.
- Corteling R, Trifilieff A. Gender comparison in a murine model of allergen-driven airway inflammation and the response to budesonide treatment. *BMC Pharmacol* 2004; 4: 4.
- Hayashi T, Adachi Y, Hasegawa K, Morimoto M. Less sensitivity for late airway inflammation in males than females in BALB/c mice. *Scand J Immunol* 2003; 57: 562–7.
- Mookerjee I, Solly NR, Royce SG, Tregear GW, Samuel CS, Tang ML. Endogenous relaxin regulates collagen deposition in an animal model of allergic airway disease. *Endocrinology* 2006; 147: 754–61.
- Temelkovski J, Hogan SP, Shepherd DP, Foster PS, Kumar RK. An improved murine model of asthma: selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolised allergen. *Thorax* 1998; 53: 849–56.
- Gatouillat G, Balasse E, Joseph-Pietras D, Morjani H, Madoulet C. Resveratrol induces cell-cycle disruption and apoptosis in chemoresistant B16 melanoma. *J Cell Biochem* 2010; 110: 893–902.

28. Mokni M, Elkahoui S, Limam F, Amri M, Aouani E. Effect of resveratrol on antioxidant enzyme activities in the brain of healthy rat. *Neurochem Res* 2007; 32: 981–7.
29. Locke NR, Royce SG, Wainwright JS, Samuel CS, Tang ML. Comparison of airway remodeling in acute, subacute, and chronic models of allergic airways disease. *Am J Respir Cell Mol Biol* 2007; 36: 625–32.
30. Keramidas E, Merson TD, Steeber DA, Tedder TF, Tang ML. L-selectin and intercellular adhesion molecule 1 mediate lymphocyte migration to the inflamed airway/lung during an allergic inflammatory response in an animal model of asthma. *J Allergy Clin Immunol* 2001; 107: 734–8.
31. Fiscus LC, Van Herpen J, Steeber DA, Tedder TF, Tang ML. L-selectin is required for the development of airway hyper-responsiveness but not airway inflammation in a murine model of asthma. *J Allergy Clin Immunol* 2001; 107: 1019–24.
32. Samuel CS, Zhao C, Bathgate RA, Bond CP, Burton MD, Parry LJ, et al. Relaxin deficiency in mice is associated with an age-related progression of pulmonary fibrosis. *FASEB J* 2003; 17: 121–3.
33. Samuel CS, Royce SG, Burton MD, Zhao C, Tregear GW, Tang ML. Relaxin plays an important role in the regulation of airway structure and function. *Endocrinology* 2007; 148: 4259–66.
34. Royce SG, Tang ML. The effects of current therapies on airway remodeling in asthma and new possibilities for treatment and prevention. *Curr Mol Pharmacol* 2009; 2: 169–81.
35. Saglani S, Molyneux C, Gong H, Rogers A, Malmstrom K, Pelkonen A, et al. Ultrastructure of the reticular basement membrane in asthmatic adults, children and infants. *Eur Respir J* 2006; 28: 505–12.
36. Milanese M, Crimi E, Scordamaglia A, Riccio A, Pellegrino R, Canonica GW, et al. On the functional consequences of bronchial basement membrane thickening. *J Appl Physiol* 2001; 91: 1035–40.
37. Aoshiba K, Nagai A. Differences in airway remodeling between asthma and chronic obstructive pulmonary disease. *Clin Rev Allergy Immunol* 2004; 27: 35–43.
38. Thomson RJ, Bramley AM, Schellenberg RR. Airway muscle stereology: implications for increased shortening in asthma. *Am J Respir Crit Care Med* 1996; 154: 749–57.
39. Hirst SJ, Martin JG, Bonacci JV, Chan V, Fixman ED, Hamid QA, et al. Proliferative aspects of airway smooth muscle. *J Allergy Clin Immunol* 2004; 114: S2–S17.
40. Minshall EM, Leung DY, Martin RJ, Song YL, Cameron L, Ernst P, et al. Eosinophil-associated TGF-beta1 mRNA expression and airways fibrosis in bronchial asthma. *Am J Respir Cell Molec Biol* 1997; 17: 326–33.

***Simon Royce**

Allergy and Immune Disorders
Murdoch Children's Research Institute
Melbourne, Vic., Australia
and
Department of Pathology
The University of Melbourne
Parkville, Melbourne, Vic., Australia
Tel: (613) 9345 6882
Fax: (613) 9345 6348
Email: simon.royce@mcri.edu.au