

Angiopietin-1 Protects against Airway Inflammation and Hyperreactivity in Asthma

Davina C. M. Simoes¹, Theodoros Vassilakopoulos¹, Dimitrios Toumpanakis¹, Kalomira Petrochilou¹, Charis Roussos¹, and Andreas Papapetropoulos^{1,2}

¹"G. P. Livanos and M. Simou" Laboratories, Department of Critical Care and Pulmonary Services, Evangelismos Hospital, University of Athens School of Medicine, Athens, Greece; and ²Laboratory of Molecular Pharmacology, Department of Pharmacy, University of Patras, Patras, Greece

Rationale: The angiopoietins (Ang) comprise a family of growth factors mainly known for their role in blood vessel formation and remodeling. The best-studied member, Ang-1, exhibits antiapoptotic and antiinflammatory effects. Although the involvement of Ang-1 in angiogenesis is well recognized, little information exists about its role in respiratory physiology and disease. On the basis of its ability to inhibit vascular permeability, adhesion molecule expression, and cytokine production, we hypothesized that Ang-1 administration might exert a protective role in asthma.

Objectives: To determine changes in the expression of Ang and to assess the ability of Ang-1 to prevent the histologic, biochemical, and functional changes observed in an animal model of asthma.

Methods: To test our hypothesis, a model of allergic airway disease that develops after ovalbumin (OVA) sensitization and challenge was used.

Measurements and Main Results: Ang-1 expression was reduced at the mRNA and protein levels in lung tissue of mice sensitized and challenged with OVA, leading to reduced Tie2 phosphorylation. Intranasal Ang-1 treatment prevented the OVA-induced eosinophilic lung infiltration, attenuated the increase in IL-5 and IL-13, and reduced eotaxin and vascular cell adhesion molecule 1 expression. These antiinflammatory actions of Ang-1 coincided with higher levels of I κ B and decreased nuclear factor- κ B binding activity. More importantly, Ang-1 reversed the OVA-induced increase in tissue resistance and elastance, improving lung function.

Conclusions: We conclude that Ang-1 levels are decreased in asthma and that administration of Ang-1 might be of therapeutic value because it prevents the increased responsiveness of the airways to constrictors and ameliorates inflammation.

Keywords: airway resistance; interleukin 5; eotaxin; VCAM-1; nuclear factor- κ B

Asthma is a chronic inflammatory lung disease with exaggerated T-helper (Th2) cell inflammation characterized by elevated serum IgE levels, mucus hypersecretion, and airway hyperresponsiveness (1, 2). Th2 cells, together with other inflammatory cells, have been proposed to play a critical role in the initiation, development, and chronicity of asthma (3). During the course of the disease, structural changes also occur in the airways, known as remodeling; these include subepithelial fibrosis, increase in the airway smooth muscle mass, and increased vascularization (4).

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Correspondence and requests for reprints should be addressed to Andreas Papapetropoulos, Ph.D., "G. P. Livanos and M. Simou" Laboratories, Evangelismos Hospital, Department of Critical Care and Pulmonary Services, University of Athens School of Medicine, Athens, Greece 10675. E-mail: apapapet@upatras.gr

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AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Angiopietin (Ang)-1 exhibits antiinflammatory properties by inhibiting leukocyte transendothelial migration, cytokine production, and vascular permeability. Ang-1 administration ameliorates endotoxin-induced lung injury and improves survival.

What This Study Adds to the Field

Ang-1 levels are reduced in the lungs of animals with experimental asthma. Local Ang-1 administration prevents the biochemical and functional changes observed in asthma, suggesting that Tie2 agonists might have therapeutic potential in this disease.

Enhanced expression of angiogenic factors has been observed in the lungs of patients with asthma and in animal models of asthma (5-9). Remodeling of the microvasculature in the airways of individuals with bronchial asthma is believed to contribute to airway wall thickness, luminal narrowing, and airway hyperreactivity (10). In some studies, vessel number and vascularity have been positively correlated with the severity of asthma (11, 12).

Angiopietins (Ang) are ligands for the endothelial receptor tyrosine kinase Tie2 with important roles in angiogenesis (13). Ang-1 phosphorylates and activates Tie2; it is perceived as a stabilizing factor that promotes structural and functional integrity of the vasculature (14, 15). In contrast, Ang-2 exerts context-dependent effects and is believed to prime vessel plasticity, allowing for other angiogenic factors to drive the angiogenic response (13, 16). Initially, Tie2 expression was believed to be restricted to the vascular endothelium. However, recent observations have provided evidence for the existence of functional Tie2 receptors on leukocytes (neutrophils and eosinophils) (17, 18). It should be noted that, apart from their role in angiogenesis, the angiopoietins have been shown to modify the inflammatory response *in vivo* (15, 19-21). We and others have shown that Ang-1 inhibits vascular permeability, leukocyte adhesion to the endothelium, cytokine production, and adhesion molecule expression in response to a variety of inflammatory mediators (15, 22, 23). Because mediators that are elevated in asthma, such as transforming growth factor (TGF)- β 1, thrombin, and cytokines, down-regulate Ang-1 expression (16, 24-26), we hypothesized that Ang-1 expression is reduced in the lung in the course of allergic airway inflammation.

Vascular endothelial growth factor (VEGF), a growth factor that plays a key role in angiogenesis (27), has been shown to enhance the Th2 response in animal models of asthma and to be increased in the lungs of patients with asthma (7, 8). Because Ang-1 has been shown to counteract many effects of VEGF linked to inflammation (15, 22), we also hypothesized that

Ang-1 exerts a protective effect in asthma. To this end, the effects of local Ang-1 administration were evaluated in a murine model of allergic airway inflammation. Some of the results of these studies have been previously reported in the form of abstracts (28, 29)

METHODS

Animals and Experimental Protocol

Male BALB/c mice (8–12 wk of age) were sensitized using ovalbumin (OVA) (0.01 mg/mouse) intraperitoneally on Days 0 and 12. Control mice received the same volume of alum. Animals were challenged daily with 5% OVA (aerosolized) from Days 18 to 21. Administration of Ang-1 (1 μ g, intranasally) or vehicle (saline) was performed under light anesthesia (ketamine/xylazine) 30 minutes before each OVA challenge. All measurements were made 24 hours after the final OVA challenge.

Airway Hyperresponsiveness

Mice were anesthetized, tracheostomized, paralyzed, and ventilated with Flexivent (SCIREQ Scientific Respiratory Equipment, Inc., Montreal, PQ, Canada) (*see the online supplement*). After baseline measurements of impedance (Z_{rs}), methacholine (3 or 10 mg/ml) or saline were delivered (Aeroneb; SCIREQ) for 10 seconds. Afterwards, a 2-second forced oscillation perturbation (1–20 Hz) was performed every 10 seconds for 3 minutes. Before measurements and before every aerosol delivery, the volume history of the lung was established with two 6-second deep inflations to a pressure limit of 30 cm H₂O. Measurements of Z_{rs} were fit with the constant phase model (30), where R_n is the Newtonian resistance of the airways, G represents tissue resistance, and H tissue elasticity. After each dose of methacholine, model parameters were expressed as % ratio of the baseline (*see the online supplement*).

Bronchoalveolar Lavage Measurements and Lung Histology

Airways of mice were lavaged, the resulting bronchoalveolar lavage (BAL) fluid was immediately centrifuged (700 $\times$ g, 5 min at 4°C), and supernatants collected. Differential cell counts were performed on Wright-Giemsa-stained cytopins. To evaluate the extent of inflammation and mucus production, lung sections were stained with hematoxylin and eosin and periodic acid Schiff (PAS), respectively.

Inflammatory Mediators and Serum Antibody Concentration

Lung tissue was homogenized in Hanks' balanced salt solution containing protease inhibitors, centrifuged (1,900 rpm for 10 min), and the supernatant collected. Levels of cytokines in the lung tissue and serum levels of total and specific anti-OVA IgE were determined by ELISA (*see the online supplement*).

Nuclear Factor- κ B Binding Assay

Active nuclear factor (NF)- κ B contained in 20 μ g of lung nuclear extracts was bound to an oligonucleotide coating the plate surface of the NF- κ B TransAM kit (Active Motif, Carlsbad, CA). An antibody directed against the p65 subunit was used to determine the presence of this NF- κ B subunit. Optical density at 450 nm was determined using a horseradish peroxidase-conjugated secondary antibody.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from the lungs, quantified, and treated with DNase I. After cDNA synthesis, the levels of Ang-1 and Ang-2 were assessed in a Light Cycler System (Bio-Rad, Hercules, CA) and analyzed. To calculate the relative quantity of the respective subunit, the $\Delta\Delta C_T$ method was used, employing glyceraldehyde-3-phosphate dehydrogenase for normalization.

Western Blot Analysis

One lobe was homogenized in 10 vol (wt/vol) of a lysis buffer (*see the online supplement*). Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by blotting with appropriate primary and secondary antibodies. Immunoblots were visualized using chemiluminescence.

Statistical Analysis

Results are presented as means $\pm$ SEM. Comparisons were made using the *t* test or analysis of variance (ANOVA) followed by the Tukey's post hoc or Kruskal-Wallis ANOVA and Mann-Whitney *U* test, where appropriate (SPSS Version 13.0 software; SPSS, Inc., Chicago, IL). Differences were considered significant when *P* values were less than 0.05.

RESULTS

Ang-1 mRNA and Protein Levels Are Reduced in Allergen-induced Airway Inflammation

To determine whether asthmali-like airway inflammation leads to changes in angiopoietin levels, mice were sensitized and challenged with OVA and Ang-1 and Ang-2 mRNA levels were determined. Ang-1 mRNA levels were reduced by approximately 50% in OVA mice, whereas the levels of Ang-2 were not altered (Figure 1A). Exposing mice to a greater number of OVA challenges (6 instead of 4) led to a more pronounced decrease in Ang-1 levels (data not shown). To test if the changes observed in the mRNA level also translated into changes in protein expression, angiopoietin content in lung homogenates was determined by Western blotting (Figure 1B). In these experiments, we observed that Ang-1 was reduced to $20.8 \pm 8.2\%$ of control in

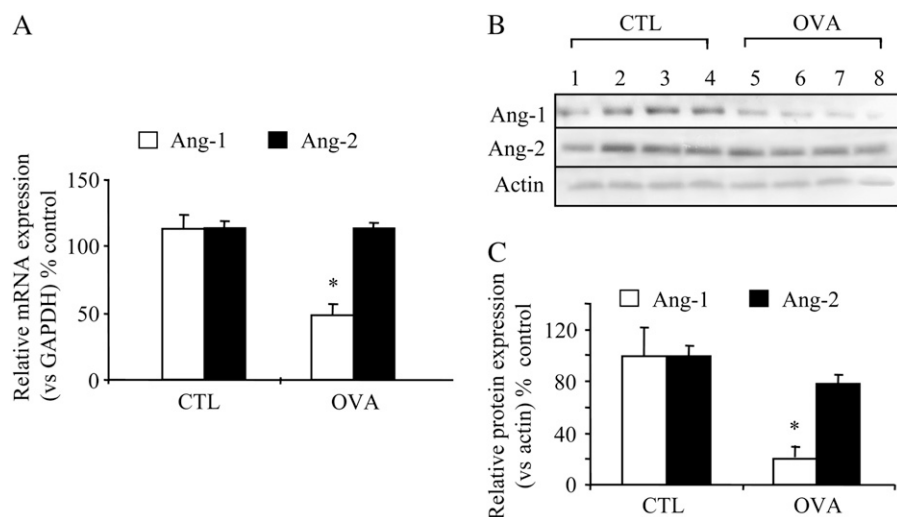


Figure 1. Angiopoietin (Ang)-1 is reduced in mice with asthmali-like airway inflammation. (A) Angiopoietin mRNA levels in lung homogenates were analyzed with real-time reverse transcriptase-polymerase chain reaction as described in METHODS. (B) Representative Western blots depicting Ang-1 and Ang-2 levels in lung homogenates of control (CTL) and ovalbumin-treated (OVA) mice. (C) Quantitation of band intensity from blots shown in (B) by densitometry. Results are presented as means $\pm$ SEM; relative expression of Ang-1 or Ang-2 was set at 100% for control mice after normalization against either GAPDH or actin for mRNA and protein measurements, respectively. *n* = 8–10 mice per group; **P* < 0.05 compared with control.

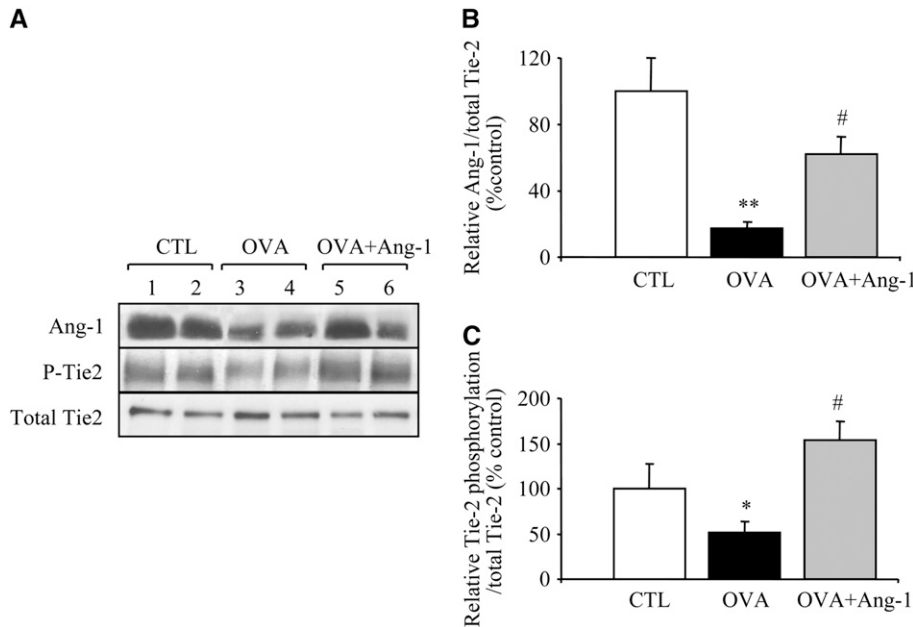


Figure 2. Tie2 phosphorylation in mice sensitized and challenged with ovalbumin (OVA). (A) The Tie2 receptor was immunoprecipitated and samples were processed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Membranes were then blotted using an antibody that recognizes both human and mouse Ang-1 (top panel), a phosphotyrosine antibody (middle panel) or total Tie2 (lower panel). (B, C) Densitometric analysis of blots shown in (A). OVA challenge mice (CTL), mice sensitized and challenged with OVA (OVA), and mice treated with Ang-1 (OVA+Ang-1) before each OVA challenge. Results are presented as means $\pm$ SEM, $n = 5-6$; * $P < 0.05$ from control, # $P < 0.05$ compared with OVA.

OVA-sensitized mice. Ang-2 protein levels were also reduced in the OVA mice; however, this reduction did not reach statistical significance ($P = 0.06$; Figure 1C). Tie2 immunoprecipitation experiments revealed that smaller amounts of Ang-1 were bound to its receptor in mice sensitized and challenged with OVA (Figures 2A and 2B). In addition, Tie2 phosphorylation in OVA-treated mice was reduced compared with control animals (Figures 2A and 2C). Upon administration of exogenous Ang-1, more of this growth factor was found to be bound to Tie2, leading to an increase in phospho-Tie2/total Tie2 ratio (Figures 2A and 2C).

Administration of Ang-1 Reduces Lung Inflammation and Prevents Airway Hyperresponsiveness

We next sought to investigate whether increasing Ang-1 levels through local administration of recombinant protein could prevent the changes observed in the airways of mice with an asthmalike disease. OVA mice exhibited an increase in BAL cellularity; differential cell counts revealed that this was due

primarily to infiltration of eosinophils, and to a smaller extent macrophages and lymphocytes (Figure 3A). Ang-1 administration significantly reduced total cell and eosinophil counts, whereas it did not affect the number of macrophages, lymphocytes, and neutrophils present in the BAL after OVA sensitization and challenge (Figure 3A). It should be noted that administration of Ang-1 in the absence of OVA sensitization had no effect on BAL cell numbers (data not shown). The extent of inflammation in the lungs of OVA mice was also evaluated in histologic sections stained with hematoxylin and eosin (Figure 3B). Large numbers of infiltrating inflammatory cells were present in OVA mice and Ang-1 treatment ameliorated this inflammatory response. In contrast, production of mucus by goblet cells was not different between the OVA and OVA+Ang-1 mice, as suggested by the similar PAS scores obtained in the two groups of animals (Figure 3C).

To determine the effects of Ang-1 on lung function, airway (Newtonian) resistance, tissue resistance (G), and tissue elastance (H) were next determined. Neither OVA immunization

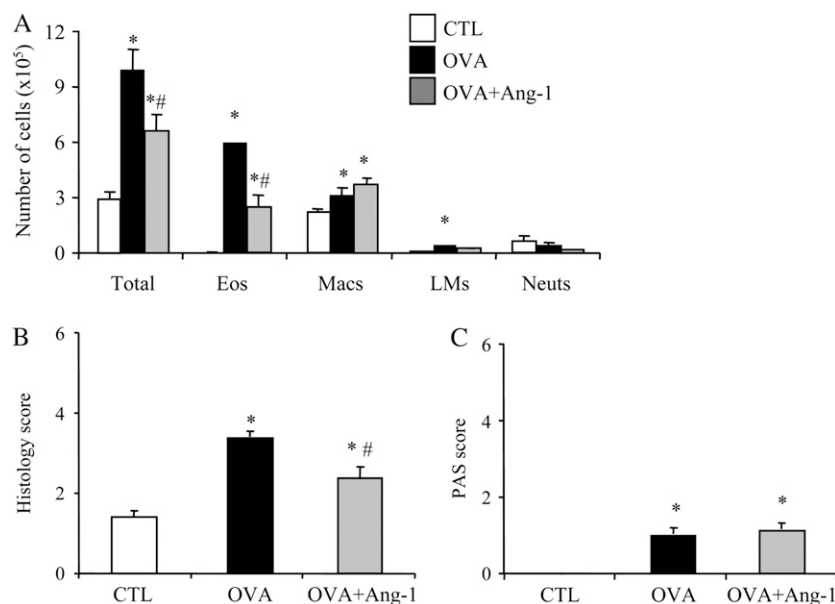


Figure 3. Lung inflammation is reduced in ovalbumin (OVA)-mice treated with Ang-1. (A) Differential cell counts in the bronchoalveolar lavage (BAL) of control animals only challenged with OVA (CTL), mice sensitized and challenged with OVA (OVA), and mice treated with Ang-1 before the OVA challenge (OVA+Ang-1). Animals were killed 24 hours after the final OVA challenge and BAL cells were isolated and counted. In (B) and (C) lungs were prepared for histology, stained with hematoxylin and eosin (B) or periodic acid Schiff (C) and scored by a blinded observer. Values are expressed as means $\pm$ SEM, $n = 10$; * $P < 0.05$ compared with control, # $P < 0.05$ compared with OVA. Eos = eosinophils; LMs = lymphocytes; Macs = macrophages; Neuts = neutrophils.

nor Ang-1 treatment had any effect on baseline lung function, because neither airway resistance nor tissue mechanics differed among the different groups (*see the online supplement*). In OVA-treated mice, all three parameters were increased in a dose-dependent manner after exposure to methacholine (Figure 4). Intranasal treatment with Ang-1, in addition to ameliorating inflammation, significantly decreased tissue resistance and elastance (Figures 4B and 4C), without affecting Newtonian resistance (Figure 4A).

Ang-1 Treatment Reduces the Production of Inflammatory Mediators

To investigate the mechanisms associated with the protective action of Ang-1, we measured the levels of Th2 cytokines implicated in the pathogenesis of asthma, namely IL-4, IL-5, and IL-13. As expected, lung homogenates of mice sensitized and challenged with OVA exhibited increased levels of all the above-mentioned cytokines compared with controls. Interestingly, administration of Ang-1 significantly reduced the levels of IL-5 and IL-13, but not IL-4 (Figures 5A–5C). In addition, Ang-1 prevented the OVA-induced increase in eotaxin expression (Figure 5D), suggesting that reduced eosinophil infiltration could be due to the reduction in this major chemoattractant.

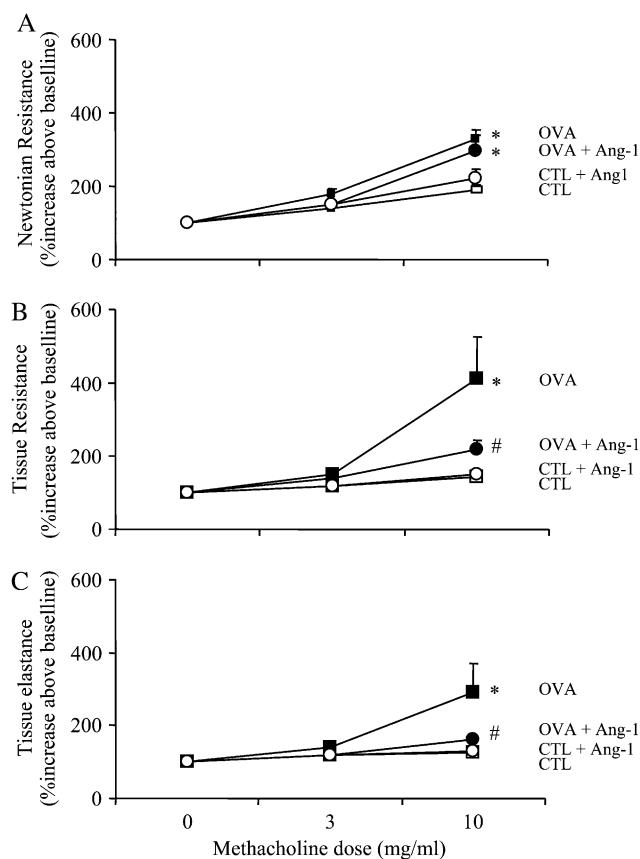


Figure 4. Ang-1 treatment attenuates the ovalbumin (OVA)-induced increase in resistance and elastance. Airway reactivity to increasing amounts of methacholine was measured 24 hours after the final OVA challenge in control mice (CTL), mice receiving Ang-1 only (CTL+Ang-1), mice sensitized and challenged with OVA (OVA), and mice treated with Ang-1 before each OVA challenge (OVA+Ang-1). Newtonian resistance (A), tissue resistance (B), and tissue elastance (C) were determined as described in METHODS. Data are presented as means $\pm$ SEM; $n = 9$ –11; * $P < 0.05$ compared with control, # $P < 0.05$ compared with OVA.

VCAM-1 is an adhesion molecule of major importance for the recruitment of eosinophils into tissues (31). In agreement with current knowledge (24), VCAM-1 was up-regulated in the lungs of OVA-treated mice (Figures 6A and 6B). Ang-1 administration abolished the increase in VCAM-1 expression in mice sensitized and challenged with OVA (Figure 6B).

Apart from its local effects in the lung, intranasal Ang-1 administration exerted systemic effects. Circulating total IgE increased several fold to $18.8 \pm 1.7 \mu\text{g/ml}$ in OVA mice; treatment with Ang-1 reduced these levels to $9.7 \pm 0.98 \mu\text{g/ml}$ (Figure 7A). Similarly, Ang-1 was able to attenuate the production of OVA-specific IgE (639.2 ± 80.8 vs. 389 ± 38 U/ml in OVA vs. OVA+Ang-1 mice, respectively) (Figure 7B). Ang-1 administration also inhibited the number of eosinophils in peripheral blood of mice sensitized and challenged with OVA, suggesting this therapeutic intervention can reduce leukocyte trafficking from the bone marrow (control, $21.5 \pm 6.0 \times 10^3$; OVA, $81.7 \pm 13.6 \times 10^3$; OVA+Ang-1, $49.2 \pm 7.8 \times 10^3$ cells/ml; $n = 14$ –17, $P < 0.05$). It should, however, be noted that when human Ang-1 was measured in plasma at various time points (15 min to 24 h) after intranasal administration of the growth factor, its levels were undetectable.

Protective Effects of Ang-1 Coincide with Reduced NF- κ B Activation

Ang-1 has been proposed to inhibit NF- κ B activation *in vitro* (32). To determine whether it also exerts a negative effect on NF- κ B *in vivo*, we measured the levels of the NF- κ B inhibitor protein I κ B- α . As shown in Figures 8A and 8B, Ang-1 prevented the OVA-induced reduction in I κ B- α . In line with this observation, binding activity of the p65 subunit present in nuclear extracts isolated from lung homogenates of OVA mice was decreased to control levels after Ang-1 administration (Figure 8C), suggesting that Ang-1 might reduce inflammatory gene expression *in vivo* by inhibiting NF- κ B.

DISCUSSION

Ang-1 is known to exert a protective role in the vasculature because it promotes vascular integrity, reduces endothelial transmigration of leukocytes, and reinforces barrier function (13, 15, 23, 33). We hypothesized that, during allergic inflammation of the airways, the levels of this protective growth factor would be reduced. Indeed, mice sensitized and challenged with OVA exhibited reduced mRNA and protein levels of Ang-1, raising the possibility that Ang-1 down-regulation contributes to the pathogenesis of asthma. Under the same conditions, the levels of the context-dependent antagonist of the Tie2 receptor Ang-2 remained unaltered. As the balance between Ang-1 and Ang-2 in the asthmatic lung changes in favor of the latter, the protective action of Tie2 is diminished, as reflected by the reduction in Tie2 phosphorylation. Although the mechanisms responsible for the reduced expression of Ang-1 in asthma were not identified in the present study, several mediators and factors that are increased in asthma could contribute to the inhibition of Ang-1 expression. These include TGF- β 1, thrombin, cytokines, and nitric oxide (NO) (16, 25, 26, 34). Interestingly, the combination of tumor necrosis factor and IFN- γ down-regulates the expression of Ang-1 in an NF- κ B and NO-dependent manner (through up-regulation of inducible NO synthase [iNOS]) (25). This is very similar to the milieu in the asthmatic lung, in which NF- κ B activation coexists with increased production of NO via iNOS up-regulation (3, 24, 35).

Having established that Ang-1 protein levels are reduced in the lungs of mice with asthma, we asked whether administering

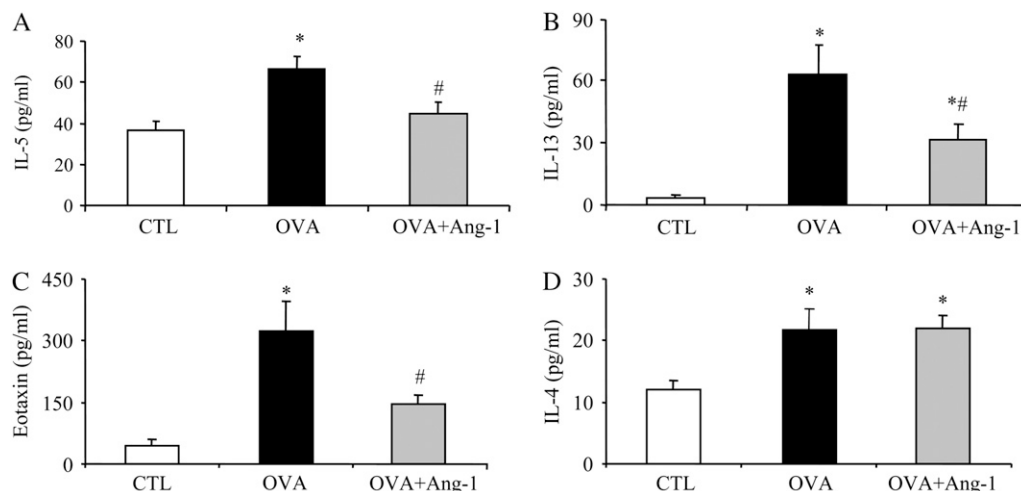


Figure 5. Ang-1 treatment prevents the increase in inflammatory mediators of asthma. Levels of IL-5 (A), IL-13 (B), eotaxin (C), and IL-4 (D) were measured in total lung homogenates by ELISA. The groups of mice used are the same as in Figure 3. Data are expressed as means $\pm$ SEM, $n = 11$ – 15 ; * $P < 0.05$ compared with control, # $P < 0.05$ compared with OVA.

Ang-1 would prevent the changes seen in animals with allergen-induced airway inflammation. Indeed, administration of Ang-1 reduced the OVA-induced increase in the number of eosinophils present in the BAL and ameliorated tissue inflammation in lung sections. In agreement with our observations, McCarter and colleagues (20) showed that Ang-1 improved morphologic and biochemical indices of inflammation in a model of LPS-induced acute lung injury. In another study, adenoviral delivery of Ang-1 improved the histologic appearance of the lung and restricted leukocyte infiltration in mice given endotoxin intraperitoneally (36). Our results showing reduced eosinophil infiltration in OVA mice treated with Ang-1 are in line with a recent report showing that Ang-1, although chemotactic for eosinophils, inhibited VEGF-induced migration (17).

Inhibition of lung eosinophilia by Ang-1 could result either from reduced infiltration or mobilization of eosinophils from the bone marrow. Local treatment with Ang-1 significantly reduced the increase in the number of circulating eosinophils triggered by OVA sensitization and challenge and inhibited the levels of eotaxin, a chemokine that induces eosinophil transendothelial migration of eosinophils *in vivo* and *in vitro* (31, 37). Moreover, Ang-1 blocked the OVA-stimulated expression of vascular cell adhesion molecule 1 (VCAM-1), another molecule that aids in the recruitment of eosinophils into the asthmatic lung, suggesting that Ang-1 inhibits the presence of eosinophils in the lung by acting at multiple levels. In line with our data on the ability of Ang-1 to inhibit VCAM-1 expression, Kim and coworkers (22) reported that Ang-1 reduces the VEGF-stimulated increase in intercellular adhesion molecule (ICAM)-1, VCAM-1, and E-selectin steady-state mRNA levels. Interestingly, Ang-1 attenuates adhesion molecule expression *in vivo* in the lungs of animals challenged with LPS (20, 36).

To further study the ability of Ang-1 to restrict inflammation in the asthmatic lung, we measured the levels of Th2 cytokines with well-established roles in the pathogenesis of asthma. IL-5 is the major stimulus for the differentiation, recruitment, and survival of eosinophils, which plays a key role in the induction of allergen-induced eosinophilic airway inflammation (3, 24). In line with the ability of Ang-1 to inhibit eosinophil infiltration, this growth factor abolished the IL-5 increase seen in OVA mice. Another key mediator in asthma is IL-13, a cytokine that promotes B-cell differentiation, capable of inducing isotype switching leading to increased IgE production; IL-13 has also been shown to contribute to airway hyperresponsiveness (2, 24). Intranasal Ang-1 treatment inhibited the OVA-stimulated increase in IL-13 production. This finding is in line with the ability of Ang-1 to reduce IgE levels and to attenuate airway hyperresponsiveness. Unlike what was observed with IL-5 and IL-13, Ang-1 was ineffective in reducing IL-4, a cytokine that promotes Th2 cell differentiation, mucin gene expression, and goblet cell metaplasia (2, 3, 24). The lack of effect of Ang-1 on IL-4 levels may also explain the fact that mucus production was not reduced in Ang-1-treated mice, as suggested by the similar PAS scores of OVA and OVA+Ang-1 mice. It should be kept in mind that the cellular sources contributing to inflammatory cytokine production were not identified in the course of our experiments; infiltrating leukocytes might contribute to inflammatory cytokine production together with resident lung cells.

It has been previously reported that Ang-1 inhibits NF- κ B-driven gene expression in a heterologous system (32). Because NF- κ B regulates the expression of several cytokines, chemokines, and cell adhesion molecules involved in the pathogenesis of asthma, we hypothesized that blockade of this transcription factor might contribute to the protective mechanism of Ang-1.

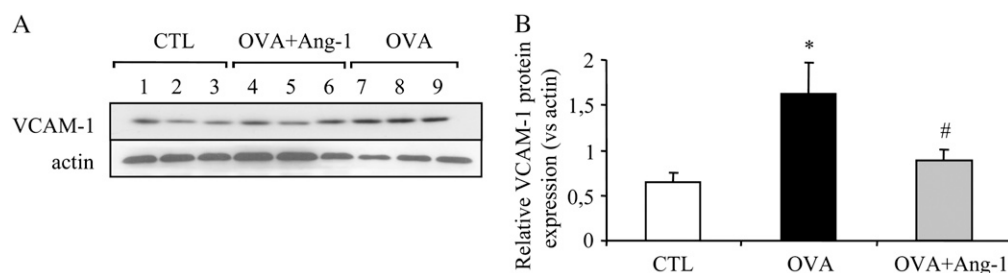


Figure 6. Ovalbumin (OVA)-induced VCAM-1 levels are decreased in mice treated with Ang-1. VCAM-1 protein levels were determined by Western blotting in total lung homogenates of control animals (CTL), mice sensitized and challenged with OVA (OVA), and mice treated with Ang-1 (OVA+Ang-1) before each OVA challenge. (A) Representative Western blot depicting VCAM-1 ex-

pression; (B) densitometric quantitation of blots shown in (A). Relative expression of VCAM-1 was set at 100% for control mice after normalization to actin expression. Results are presented as means $\pm$ SEM; $n = 7$; * $P < 0.05$ compared with control; # $P < 0.05$ compared with OVA.

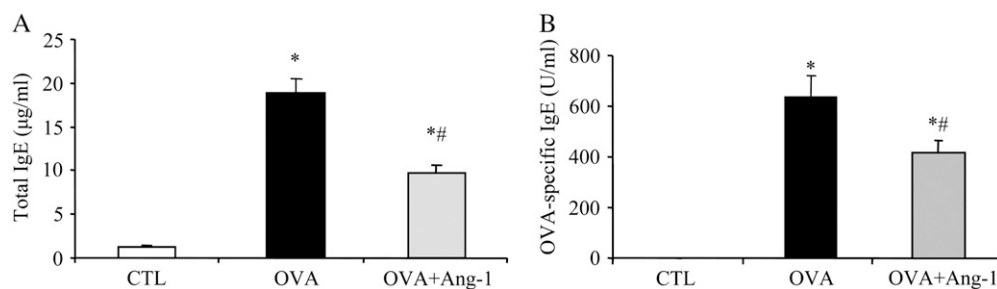


Figure 7. Serum IgE levels in mice sensitized and challenged with ovalbumin (OVA) are reduced after Ang-1 administration. Levels of total IgE (A) and OVA-specific IgE (B) were measured by ELISA in serum samples 24 hours after the final OVA challenge. Specific IgE antibody titers were then related to pooled standards generated in the laboratory and assigned arbitrary units. Data are expressed as means $\pm$ SEM, $n = 11-15$; * $P < 0.05$ compared with control, # $P < 0.05$ compared with OVA.

In our experiments, we observed that Ang-1 prevented the OVA-induced reduction in I κ B- α levels in lung homogenates, indicating that in mice treated with Ang-1 more NF- κ B would be retained in the cytosol, in its inactive form. To prove that in animals exposed to Ang-1 smaller amounts of NF- κ B translocate to the nucleus, nuclear proteins were extracted from lung homogenates and the p65 binding to a NF- κ B consensus sequence was measured. Indeed, Ang-1 treatment reduced the amount of active NF- κ B in mice sensitized and challenged with OVA.

Airway hyperresponsiveness is a hallmark of asthma, but the exact mechanisms underlying it are still unclear. Similar to other studies (38, 39), our OVA mice presented increased Rn, G, and H compared with control mice after methacholine challenge. This hyperresponsiveness of BALB/c mice with allergically inflamed airways can be explained by thickening of the airway mucosa, allowing for excessive narrowing of the airway lumen with some airways proceeding to full closure even when the degree of airway smooth muscle shortening is normal (40, 41). Closure is likely exacerbated by disruption of surfactant function, possibly due to fibrin deposition, which would increase the propensity for the formation of liquid bridges across the lumen of small airways (41) and by the presence of a critical volume of intraluminal liquid due to plasma exudation (42). The greater proportional increase in G relative to H (Figure 3) suggests that this derangement in mechanical function exhibits regional het-

erogeneity (43). Ang-1 administration, apart from its protective effects limiting inflammation, normalized G and H. The reduction of inflammation would reduce airway mucosal thickness as well as the volume of extravasated plasma into the airway lumen. Furthermore, Ang-1 has direct antileakage action in the airway microvasculature (10, 44), which would prevent airway wall edema and intraluminal fluid accumulation, thus reversing the majority of derangements of mechanical lung function induced by allergic airway inflammation. Ang-1 treatment had no significant effect on the peak value of Rn in response to methacholine. However, the area under the curve of the Rn response to methacholine over time tended to normalize in Ang-1-treated OVA mice ($P = 0.06$ vs. OVA-treated mice, $P =$ not significant vs. controls; see the online supplement), suggesting that Ang-1 treatment had some effect on airway smooth muscle contractility or on the clearance of methacholine from the lung, apart from the effect of diminished airway closure on Rn.

Clinical Relevance

Our finding of the protective effect of Ang-1 treatment would be clinically relevant if the animal model used reflected not only molecular mechanisms but also lung function derangements of human asthma. Accordingly, there is substantial evidence to support the importance of airway closure for the airway hyperresponsiveness in humans with asthma. Single-photon emission

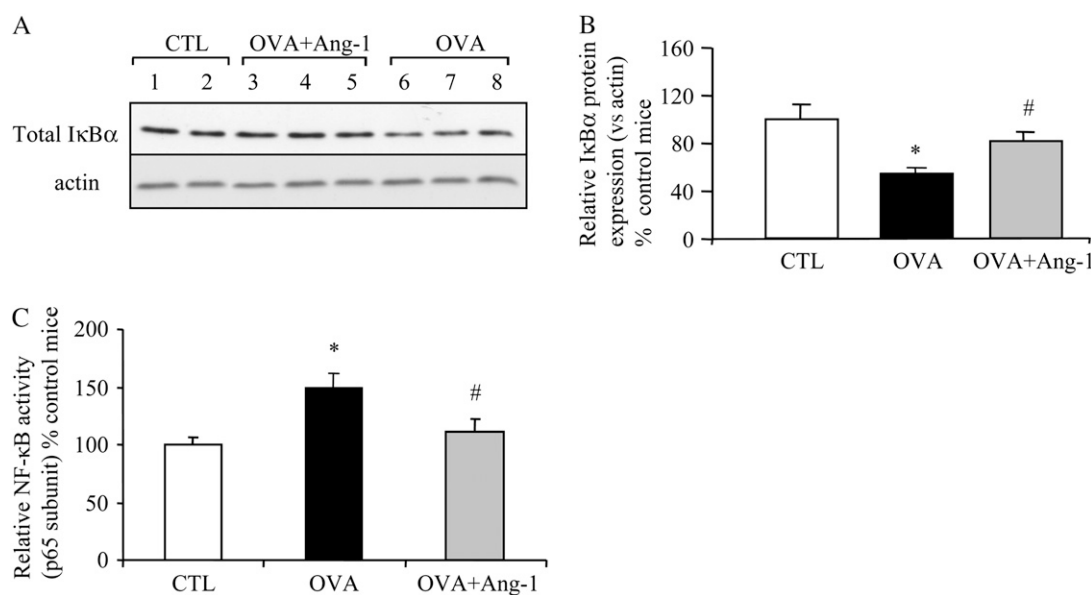


Figure 8. Ang-1 treatment reduces nuclear factor (NF)- κ B activation in the lung of mice sensitized and challenged with ovalbumin (OVA). (A) Representative Western blot of inhibitor of nuclear factor κ B- α (I κ B- α) in lung homogenates from control animals, and animals with inflammatory airway disease in the presence or absence of Ang-1 administration. (B) Densitometric analysis of blots shown in (A). I κ B- α protein expression was set at 100% for control mice after normalization for actin content. (C) Graph depicts the DNA binding of the p65 subunit of NF- κ B in lung cell nuclei in control animals chal-

lenged with OVA (CTL), mice sensitized and challenged with OVA (OVA), and mice treated with Ang-1 (OVA+Ang-1) before each OVA challenge. Results are presented as means $\pm$ SEM, $n = 8-10$; * $P < 0.05$ compared with control, # $P < 0.05$ compared with OVA.

computed tomography with Technegas has revealed that patients with asthma have increased range of closing pressures and a patchy distribution of airway closure (45). Positron emission tomography has shown that bronchoconstricted patients with asthma have large ventilation defects in their lungs, with patchy distribution (46, 47) and clusters of underventilated areas adjacent to normally ventilated areas that can be attributed to clusters of small airway closures (48). Furthermore, the increased amount of air trapped in the asthmatic lung correlates with the reversibility of small airway obstruction (49). Thus, our animal model of allergic airway inflammation reflects significant aspects of deranged mechanical lung function of human asthma.

In a recent study, changes in the number of Ang-1-positive vessels in biopsies from patients with asthma were reported to increase, without, however, quantifying the expression of this growth factor (5). Moreover, Ang-1 was found to be increased in induced sputum of patients with asthma (50), whereas Ang-1 levels were inversely correlated with airway vascular permeability index (which lends credence to the protective role of Ang-1 in asthma). Clearly, additional experimentation is needed to comprehend the reasons for the divergent results between the present study and the results published so far with subjects with asthma. One might speculate that initially in the course of asthma development, Ang-1 levels are reduced, allowing for greater vessel plasticity, as has been described for other vascular beds (16). The concomitant rise in VEGF expression at the onset of asthma promotes new blood vessel formation (13). Later on, Ang-1 levels recover and might even be increased as part of a homeostatic response to limit vascular permeability (13).

In summary, we have demonstrated that local administration of recombinant Ang-1 protects against the histologic, biochemical, and functional changes observed in allergic asthma. Ang-1 inhibits NF- κ B activation and reduces inflammatory cytokine production. In addition, Ang-1 inhibits eosinophil infiltration by reducing adhesion molecule expression and chemoattractant production, and by inhibiting eosinophil trafficking. More importantly, Ang-1 improves lung function in the context of asthmatic inflammation. This later beneficial effect of Ang-1 might be related to the barrier-promoting properties of this growth factor. Our results reinforce the notion that Ang-1 exerts a protective action in the setting of inflammation and suggest that exogenous Ang-1 administration could be of therapeutic benefit in asthma.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

- Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma: from bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* 2000;161:1720-1745.
- Cohn L, Elias JA, Chupp GL. Asthma: mechanisms of disease persistence and progression. *Annu Rev Immunol* 2004;22:789-815.
- Elias JA, Lee CG, Zheng T, Ma B, Homer RJ, Zhu Z. New insights into the pathogenesis of asthma. *J Clin Invest* 2003;111:291-297.
- Vignola AM, Mirabella F, Costanzo G, Di Giorgi R, Gjomarkaj M, Bellia V, Bonsignore G. Airway remodeling in asthma. *Chest* 2003;123:417S-422S.
- Feltis BN, Wignarajah D, Zheng L, Ward C, Reid D, Harding R, Walters EH. Increased vascular endothelial growth factor and receptors: relationship to angiogenesis in asthma. *Am J Respir Crit Care Med* 2006;173:1201-1207.
- Hoshino M, Takahashi M, Aoike N. Expression of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin immunoreactivity in asthmatic airways and its relationship to angiogenesis. *J Allergy Clin Immunol* 2001;107:295-301.
- Lee CG, Link H, Baluk P, Homer RJ, Chapoval S, Bhandari V, Kang MJ, Cohn L, Kim YK, McDonald DM, et al. Vascular endothelial growth factor (VEGF) induces remodeling and enhances TH2-mediated sensitization and inflammation in the lung. *Nat Med* 2004;10:1095-1103.
- Lee Y, Lee H. Vascular endothelial growth factor in patients with acute asthma. *J Allergy Clin Immunol* 2001;107:1106-1108.
- Lee YC, Kwak Y-G, Song CH. Contribution of vascular endothelial growth factor to airway hyperresponsiveness and inflammation in a murine model of toluene diisocyanate-induced asthma. *J Immunol* 2002;168:3595-3600.
- Wilson J, Hii S. The importance of the airway microvasculature in asthma. *Curr Opin Allergy Clin Immunol* 2006;6:51-55.
- Hashimoto M, Tanaka H, Abe S. Quantitative analysis of bronchial wall vascularity in the medium and small airways of patients with asthma and COPD. *Chest* 2005;127:965-972.
- Salvato G. Quantitative and morphological analysis of the vascular bed in bronchial biopsy specimens from asthmatic and non-asthmatic subjects. *Thorax* 2001;56:902-906.
- Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. *Nature* 2000;407:242-248.
- Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonpierre PC, et al. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell* 1996;87:1161-1169.
- Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, Glazer N, Holash J, McDonald DM, Yancopoulos GD. Angiopoietin-1 protects the adult vasculature against plasma leakage. *Nat Med* 2000;6:460-463.
- Tsigkos S, Koutsilieris M, Papapetropoulos A. Angiopoietins in angiogenesis and beyond. *Expert Opin Invest Drugs* 2003;12:933-941.
- Feistritz C, Mosheimer BA, Sturn DH, Bijuklic K, Patsch JR, Wiedermann CJ. Expression and function of the angiopoietin receptor Tie-2 in human eosinophils. *J Allergy Clin Immunol* 2004;114:1077-1084.
- Lemieux C, Maliba R, Favier J, Theoret J-F, Merhi Y, Sirois MG. Angiopoietins can directly activate endothelial cells and neutrophils to promote proinflammatory responses. *Blood* 2005;105:1523-1530.
- Bhandari V, Choo-Wing R, Lee CG, Zhu Z, Nedrelov JH, Chupp GL, Zhang X, Matthay MA, Ware LB, Homer RJ, et al. Hyperoxia causes angiopoietin 2-mediated acute lung injury and necrotic cell death. *Nat Med* 2006;12:1286-1293.
- McCarter SD, Mei SHJ, Lai PFH, Zhang QW, Parker CH, Suen RS, Hood RD, Zhao YD, Deng Y, Han RNN, et al. Cell-based angiopoietin-1 gene therapy for acute lung injury. *Am J Respir Crit Care Med* 2007;175:1014-1026.
- Roviozzo F, Tsigkos S, Kotanidou A, Bucci M, Brancialeone V, Cirino G, Papapetropoulos A. Angiopoietin-2 causes inflammation in vivo by promoting vascular leakage. *J Pharmacol Exp Ther* 2005;314:738-744.
- Kim I, Moon S-O, Park SK, Chae SW, Koh GY. Angiopoietin-1 reduces VEGF-stimulated leukocyte adhesion to endothelial cells by reducing ICAM-1, VCAM-1, and E-selectin expression. *Circ Res* 2001;89:477-479.
- Pizurki L, Zhou Z, Glynnos K, Roussos C, Papapetropoulos A. Angiopoietin-1 inhibits endothelial permeability, neutrophil adherence and IL-8 production. *Br J Pharmacol* 2003;139:329-336.
- Busse WW, Lemanske RF. Asthma. *N Engl J Med* 2001;344:350-362.
- Kasama T, Isozaki T, Odai T, Matsunawa M, Wakabayashi K, Takeuchi HT, Matsukura S, Adachi M, Tezuka M, Kobayashi K. Expression of angiopoietin-1 in osteoblasts and its inhibition by tumor necrosis factor-alpha and interferon-gamma. *Transl Res* 2007;149:265-273.
- Nishishita T, Lin P. Angiopoietin 1, PDGF-B, and TGF- β gene regulation in endothelial cell and smooth muscle cell interaction. *J Cell Biochem* 2004;91:584-593.
- Ferrara N, Gerber H-P, LeCouter J. The biology of VEGF and its receptors. *Nat Med* 2003;9:669-676.
- Simoes D, Papapetropoulos A, Roussos C, Gratzou C. Angiopoietin 1 expression is reduced in a mouse model of asthma [abstract]. *Eur Res J* 2006;(Suppl 50):551S.
- Simoes D, Vassilakopoulos T, Petrochilou K, Roussos C, Papapetropoulos A. The anti-inflammatory effects of angiopoietin-1 in mice asthma. *Eur Res J* 2007;S51:680s.
- Hantos Z, Daroczy B, Suki B, Nagy S, Fredberg JJ. Input impedance and peripheral inhomogeneity of dog lungs. *J Appl Physiol* 1992;72:168-178.
- Jia G-Q, Gonzalo J-A, Hidalgo A, Wagner D, Cybulsky M, Gutierrez-Ramos JC. Selective eosinophil transendothelial migration triggered by eotaxin via modulation of Mac-1/ICAM-1 and VLA-4/VCAM-1 interactions. *Int Immunol* 1999;11:1-10.
- Hughes DP, Marron MB, Brindle NPJ. The antiinflammatory endothelial tyrosine kinase Tie2 interacts with a novel nuclear factor- κ B inhibitor ABIN-2. *Circ Res* 2003;92:630-636.

33. Gamble J, Drew J, Tresize L, Underwood A, Parsons M, Kasminkas L, Rudge J, Yancopoulos G, Vadas M. Angiopoietin-1 is an anti-permeability and anti-inflammatory agent in vitro and targets cells junctions. *Circ Res* 2000;87:603–607.
34. Krikun G, Sakkas D, Schatz F, Buchwalder L, Hylton D, Tang C, Lockwood CJ. Endometrial angiopoietin expression and modulation by thrombin and steroid hormones: a mechanism for abnormal angiogenesis following long-term progestin-only contraception. *Am J Pathol* 2004;164:2101–2107.
35. Ricciardolo FLM, Sterk PJ, Gaston B, Folkerts G. Nitric oxide in health and disease of the respiratory system. *Physiol Rev* 2004;84:731–765.
36. Witzembichler B, Westermann D, Kneuppel S, Schultheiss H-P, Tschöpe C. Protective role of angiopoietin-1 in endotoxic shock. *Circulation* 2005;111:97–105.
37. Rothenberg ME, MacLean JA, Pearlman E, Luster AD, Leder P. Targeted disruption of the chemokine eotaxin partially reduces antigen-induced tissue eosinophilia. *J Exp Med* 1997;185:785–790.
38. Tomioka S, Bates JHT, Irvin CG. Airway and tissue mechanics in a murine model of asthma: alveolar capsule vs. forced oscillations. *J Appl Physiol* 2002;93:263–270.
39. Wagers S, Lundblad LKA, Ekman M, Irvin CG, Bates JHT. The allergic mouse model of asthma: normal smooth muscle in an abnormal lung? *J Appl Physiol* 2004;96:2019–2027.
40. Lundblad LKA, Thompson-Figueroa J, Allen GB, Rinaldi L, Norton RJ, Irvin CG, Bates JHT. Airway hyperresponsiveness in allergically inflamed mice: the role of airway closure. *Am J Respir Crit Care Med* 2007;175:768–774.
41. Wagers SS, Norton RJ, Rinaldi LM, Bates JHT, Sobel BE, Irvin CG. Extravascular fibrin, plasminogen activator, plasminogen activator inhibitors, and airway hyperresponsiveness. *J Clin Invest* 2004;114:104–111.
42. Matthay MA, Clements JA. Coagulation-dependent mechanisms and asthma. *J Clin Invest* 2004;114:20–23.
43. Bates JHT, Mitzner W. Point: counterpoint: lung impedance measurements are/are not more useful than simpler measurements of lung function in animal models of pulmonary disease. *J Appl Physiol* 2007;103:1900–1901.
44. McDonald DM. Angiogenesis and remodeling of airway vasculature in chronic inflammation. *Am J Respir Crit Care Med* 2001;164:S39–S45.
45. King GG, Eberl S, Salome CM, Meikle SR, Woolcock AJ. Airway closure measured by a Technegas bolus and SPECT. *Am J Respir Crit Care Med* 1997;155:682–688.
46. Tgavalekos NT, Musch G, Harris RS, Vidal Melo MF, Winkler T, Schroeder T, Callahan R, Lutchen KR, Venegas JG. Relationship between airway narrowing, patchy ventilation and lung mechanics in asthmatics. *Eur Respir J* 2007;29:1174–1181.
47. Venegas JG, Winkler T, Musch G, Vidal Melo MF, Layfield D, Tgavalekos N, Fischman AJ, Callahan RJ, Bellani G, Scott Harris R. Self-organized patchiness in asthma as a prelude to catastrophic shifts. *Nature* 2005;434:777–782.
48. Harris RS, Winkler T, Tgavalekos N, Musch G, Melo MFV, Schroeder T, Chang Y, Venegas JG. Regional pulmonary perfusion, inflation, and ventilation defects in bronchoconstricted patients with asthma. *Am J Respir Crit Care Med* 2006;174:245–253.
49. Laurent F, Latrabe V, Raherison C, Marthan R, Tunon-de-Lara JM. Functional significance of air trapping detected in moderate asthma. *Eur Radiol* 2000;10:1404–1410.
50. Kanazawa H, Nomura S, Asai K. Roles of angiopoietin-1 and angiopoietin-2 on airway microvascular permeability in asthmatic patients. *Chest* 2007;131:1035–1041.

**ANGIOPOIETIN-1 PROTECTS AGAINST AIRWAY INFLAMMATION AND
HYPERREACTIVITY IN ASTHMA**

*Davina C. M. Simoes, Theodoros Vassilakopoulos, Dimitrios Toumpanakis, Kalomira
Petrochilou, Charis Roussos and Andreas Papapetropoulos*

“Online Data Supplement”

Material and Methods

Reagents

Reagents for sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis and western blotting were obtained from Biorad (Hercules, CA). The Supersignal Chemiluminescent Substrate was obtained from Pierce (Rockford, Illinois). X-ray film was obtained from Eastman Kodak (Rochester, NY). Recombinant Ang-1 along with the IL-13 ELISA kit, Eotaxin ELISA kit and the V-CAM antibody were obtained from R&D Systems (Minneapolis, MN) and the IL-5, IL-4 and total IgE ELISA kit from BD Biosciences (San Jose, CA). Nuclear Extract kit and NF- κ B TransAM kits were obtained from Active Motif (Carlsbad, CA). TRIzol, SuperScript First-Strand Synthesis System for RT-PCR, DNase I, dNTPs and platinum Taq Polymearase were purchased from Invitrogen Corporation (Carlsbad, CA). The Ang and Tie2 Abs were from Santa Cruz Biotechnology (Santa Cruz, CA) and the IKB- α Ab was from Cell signalling Technology (Beverly, MA). The Ang-1 Ab used in the immunoprecipitation experiments was obtained from Abcam (Cambridge, UK). The anti-rabbit HRP-labelled secondary antibody for western blotting was purchased from NEN Life Science Products, Inc (Boston, MA). Alum (Au-Gel-S) was obtained from Boehringer-Ingelheim (Ridgefield, CT). All other reagents including ovalbumin, protease inhibitor, actin and phosphotyrosine Abs, methacholine and SYBR Green I, were obtained from Sigma-Aldrich (St Louis, MO).

Animals and experimental protocol

Male BALB/c mice, 8-12 wk of age were obtained from the Hellenic Pasteur Institute (Athens). Mice were housed at the Experimental Surgery Unit in Evangelismos Hospital. All animal protocols were performed according to guidelines of the animal ethics committee of the European Union for animal research.

Mice were sensitized using ovalbumin (OVA), at concentration of 0.01 mg/mouse in 0.2 ml alum, intraperitoneally on days 0 and 12. Control mice received the same volume of alum. On days 18-21 mice were challenged with an aerosolized 5% OVA solution in phosphate-buffered saline for 30 min in a Plexiglas chamber. The aerosol was generated with a jet nebulizer (PARI TurboBOY, Sternberg, Germany) which produced a mean particle diameter of 3.5 μ m. A series of holes, positioned directly opposite the port of entry for the aerosol, allowed the escape of excess air from the exposure chamber. Administration of Ang-1 was carried out under light anesthesia (ketamine/xylazine) by intranasal (i.n.) delivery of 1 μ g of the recombinant protein in saline (50 μ l total volume) 30 min before the aerosolized OVA challenge on days 18 to 21. Control and OVA groups received the same amount of vehicle (saline). In a preliminary set of experiments 3 different doses of Ang-1, i.e. 0.3 μ g, 1 μ g and 3 μ g, were administered and markers of allergic inflammation (IL-5 and eosinophils) were measured to determine the optimal dose for Ang-1. The use of a alum i.p./OVA challenge as a control yields similar results to the OVA i.p./saline challenge as far as inflammatory and protein end-points are concerned (E1). In a separate series of experiments aimed at determining Ang-1 levels, animals were challenged with OVA for six consecutive days (days 18-23). Twenty four hours after the last OVA challenge and after respiratory mechanics and airway hyperresponsiveness had been assessed mice were sacrificed by exsanguination after administration of a lethal dose of anaesthesia (ketamine/xylazine); tissues were then removed and stored at -80°C until used.

Analyses of inflammatory cells in BAL

The day following the last OVA challenge, airways of mice were lavaged via a tracheal cannula with 1.2 (3 times, 0.4ml each time) ml phosphate buffer saline (PBS). The

resulting BAL fluid was immediately centrifuged (700 x g, 5 min at 4°C), supernatants were collected and cells resuspended in 1 ml PBS. Total BAL cell counts were performed, and aliquots (5×10^5 cells/slide) were pelleted on glass slides by cytocentrifugation. Differential counts were performed on Wright-Geimsa-stained cytopins, and percentages of eosinophils lymphocytes, neutrophils and macrophages were determined by counting their number in 400 cells.

Lung Histology

Lung lobes were fixed in 10% formalin, dehydrated, mounted in paraffin and sections (4 μ m) were cut. Tissues were stained with hematoxylin/eosin (H&E) according to standard protocols. Perivascular and peribronchial inflammation were scored on a 1 to 5 scale by a blinded observer, taking into account both the degree of inflammation and its distribution (E2). Inflammatory infiltrate was graded as +1 where > 3 cells in thickness were involved in a single bronchus, +2 where inflammatory infiltrate involved two bronchi, +3 for inflammatory infiltrate around bronchi and blood vessels, +4 inflammatory infiltrate in most of the bronchi and blood vessels and +5 widespread inflammatory infiltrate around the majority of the vessels with alterations on epithelial cell thickening and presence of mucus. Two lung sections were analysed and scored per mouse and a mean value calculated per group of mice. Goblet cells were counted on periodic acid-schiff (PAS)-stained lung sections using an arbitrary scoring system (E2). PAS-stained goblet cells in the airway epithelium were scored in each bronchi individually (0 = <5% goblet cells; 1 = 5-25 %; 2 = 25-50%; 3 = 50-75%; 4>75%). The sum of PAS airway scores from each lung section was divided by the number of bronchioles examined (20-30 per mouse). Two lung sections were examined per mice and final values were taken as a mean of all mice studied per group.

Analyses of inflammatory mediators in lung homogenates and serum antibody concentration

Lung tissue (approximately 100 mg) was homogenized in 1 ml Hanks' balanced salt solution containing protease inhibitor cocktail; it was then centrifuged (1,900 rpm for 10 min) and the supernatant collected. Levels of IL-4, IL-5, IL1-3 and eotaxin were determined in lung homogenates by ELISA, according to the manufacture's instructions. Serum levels of total IgE were measured by ELISA using paired Abs according to the manufacture's instructions. Levels of specific anti-OVA IgE in serum were measured by ELISA and antibody titers were then related to pooled standards generated in the laboratory and assigned the arbitrary value U/ml.

NF- κ B activation assay

Twenty μ g of nuclear proteins were extracted and used for determination of NF- κ B activation using the NF- κ B TransAM kit. The kit provides a 96-well plate to which an oligonucleotide containing an NF- κ B consensus-binding site (5'-GGGATTTC-3') has been immobilized. The active form of NF- κ B contained in nuclear extracts specifically binds to the oligonucleotide. A primary antibody directed against an epitope of the p65 subunit that is accessible only when NF- κ B is activated and bound to its target DNA, was used to bind the NF- κ B complex. Optical density at 450 nm was determined after incubation with an HRP-conjugated secondary antibody.

Quantitative real-time Polymerase Chain Reaction (PCR)

Lungs were homogenized in TRIZOL. Subsequently, total RNA was extracted, photometrically quantified (260 nm) and adjusted to a concentration of 1 μ g/ μ l. RNA quality was determined by running the samples on agarose gels and by determining their OD_{260/280} ratios; only RNA samples with a ratio of 1.85 or higher were used for further analysis. To

eliminate residual genomic DNA, the RNA samples were treated with DNase I. The cDNA was synthesized from 1 µg total RNA using oligo dT₁₅ and SuperScript First-Strand Synthesis System for RT-PCR according to the manufacturer's instructions and diluted 1:5 with water. Two µl of each cDNA sample was used as template for the amplification reaction. Each PCR reaction included 5 µl of 1x PCR buffer with 2.5 mM MgCl₂, 80 nM dNTPs, 0.3 µM of each primer, 2.5 units of Platinum Taq Polymearase, SYBR Green I at final concentration of 0.1x. Primers were designed by using the http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi and <http://biotools.idtdna.com/biotools/mfold/mfold.asp> web sites. The primer specificity was checked using BLAST <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi> and only showed similarity with the specific gene of interest. There was no substantial similarity with any other gene. All PCR products were checked on an agarose gel and gave a single band of the predicted size. The Ang2 primers were the same to those used by Shih et al., Am J Pathol 2002 161: 35-41. The primers used for real time PCR were: GAPDH: 5'-ACC ATC TTC CAG GAG CGA GAC-3' (forward), GAPDH: 5'-GCC TTC TCC ATG GTG GTG AA-3' (reverse), Ang1: 5'-CCA TGC TTG AGA TAG GAA CCA G-3' (forward), Ang1: 5'-TTC AAG TCG GGA TGT TTG ATT T-3' (reverse), Ang2: 5'-TTA GCA CAA AGG ATT CGG ACA AT-3' (forward), Ang2: 5'-TTT TGT GGG TAG TAC TGT CCA TTC A-3' (reverse). PCR amplifications were performed in triplicates in a Light Cycler System (Bio-Rad) and analysed with Light CyclerTM IQ software 3.0. The threshold cycle (C_T value) was chosen as the first amplification cycle giving a signal above background. To calculate the relative quantity of the respective subunit, the $\Delta\Delta C_T$ method was used employing glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for normalization.

Western Blot analysis

After sacrificing the animals, lung tissue was frozen in liquid nitrogen and stored at – 80 °C until used. One lobe was homogenized in 10 volumes (w/v) of a lysis buffer containing 1% Triton-X, 1% SDS, 150mM NaCl, 50mM NaF, 1mM Na₃VO₄, 0.5% sodium deoxycholate, 1mM EDTA, 0,1mM EGTA and protease inhibitors (10 µg/ml aprotinin, 10 µg/ml pepstatin, and 20 mM PMSF). Samples were subjected to SDS-PAGE followed by blotting with appropriate antibody. After incubation with a secondary antibody, bands were visualized using a chemiluminescent substrate.

Assessment of OVA-induced Airway Hyperresponsiveness

Respiratory mechanics was studied 24 h after the final challenge (day 22). Mice were anaesthetised with an intraperitoneal injection of Xylazine (8 mg/kg) and penthodal (100 mg/kg), tracheostomised and then connected to a mechanical ventilator (Flexivent, SCIREQ, Montreal, Canada). During the experiment the heart rate of the animal was monitored to ensure adequate level of anesthesia. Mice were ventilated at 150 breaths/min with a tidal volume of 8 ml/kg and a positive end expiratory-pressure (PEEP) of 3 cm H₂O. Following a 5 min period of ventilation, the animals were paralyzed with succinylcholine (15 mg/kg) to cease spontaneous breathing. After baseline measurements of respiratory system impedance (Z_{rs}) airway hyperresponsiveness was assessed following aerosol delivery via an ultrasonic nebulizer (Aeroneb, Scireq) of either saline (baseline value) or increasing concentrations (3 and 10 mg/ml) of methacholine dissolved in saline. This was achieved by directing the inspiratory flow from the ventilator through the aerolization chamber of the ultrasonic nebulizer. Following this, a 2 sec forced oscillation perturbation (1-20 Hz) was performed every 10 sec for 3 min. Prior to measurements and every aerosol delivery, the volume history of the lung was established with two 6 sec deep inflations to a pressure limit of 30 cm H₂O. The pressure and volume data of the perturbation were used to calculate the input impedance

[Zrs(f)] of the respiratory system and then Zrs(f) was fitted to a constant phase model (E3) $Zrs(f) = R_n + i2\pi fI + (G-iH)/(2\pi f)^a$, where R_n is the Newtonian resistance of the airways, I is the imaginary unit, f is the frequency, I is the inertance of the gas in the airways, G represents tissue resistance and H represents tissue elasticity and a can be calculated through the equation

$$\alpha = (2/\pi) \arctan (H/G)$$

For each dose of methacholine the peak response of R_n , G and H were determined and expressed as % ratio of the respective value following saline (baseline) challenge. Furthermore, the areas under the curve of the R_n , G and H responses to methacholine [(i.e. the respective values ΔR_n , ΔG and ΔH , above baseline (saline))] over time were calculated for each animal, and these areas were compared across treatment groups to have an index of the overall response of these parameters over time to the respective treatments, which is a complementary information to the peak responses. (E4). Data sets were excluded, when fitting to the model resulted in a coefficient to determination lower than 0.85. Thus, approximately 10% (mean value for each treatment group) of forced oscillation data sets were excluded.

Results

Selection of Ang-1 dose

Intranasal administration of 1 μ g of Ang-1 was the lowest dose tested that yields maximal inhibition of markers of allergic inflammation (IL-5 and eosinophils). Increasing the dose did not lead to any further reduction (Figure E1).

Lung histology

Representative photomicrographs of lung sections from control, OVA and OVA+Ang-1 treated animals stained with H&E (A) or PAS (B) are presented in Figure E2

Airway Hyperresponsiveness

The absolute baseline values of the functional parameters Rn, G and H are presented in Table E1.

The areas under the curve of G (ANOVA F 4.83, p 0.009), H (ANOVA F 3.71, p 0.025) and Rn (ANOVA F 4.62, p0.01) were significantly elevated in OVA treated mice (Figures E3 and E4). Angiopoietin treatment tended (p=0.06) to normalize Rn (Figure E3), and normalized the areas under the curve of both G and H (Figure E4).

References

E1 Reinhardt AK, Bottoms SE, Laurent GJ, McAnulty RJ. Quantification of collagen and proteoglycan deposition in a murine model of airway remodeling. *Respir Res* 2005;6:30.

E 2 McMillan SJ, Xanthou G, Lloyd CM. Manipulation of allergen-induced airway remodeling by treatment with anti-TGF- β antibody: Effect on the Smad Signaling Pathway. *J Immunol* 2005; 174: 5774-5780.

E3 Hantos Z, Daróczy B, Suki B, Nagy S, Fredberg JJ. Input impedance and peripheral inhomogeneity of dog lungs. *J Appl Physiol*. 1992; 72:168-78

E4 Held HD and Uhlig S. Basal lung mechanics and airway and pulmonary vascular responsiveness in different inbred mouse strains. *J Appl Physiol* 2000; 88:2192-8.

Figure Legends

Figure E1 Selection of Ang-1 dose. Mice sensitized and challenged with OVA (OVA) were treated intranasally with vehicle (PBS) or the indicated dose of Ang-1 as described in the methods. Animals were sacrificed 24 h after the final OVA challenge and eosinophils were counted in the BAL (A); IL-5 levels were determined in lung homogenates (B). Values are expressed as means $\pm$ SEM, $n = 4$; $*p < 0.05$ from control.

Figure E2 Angiopoietin-1 reduces OVA-induced airway inflammation. Control mice and mice sensitized and challenged with OVA (OVA) with or without Ang-1 treatment were sacrificed 24h after the last OVA challenge. Tissue were then removed and processed as described in the Methods section. In panel A tissues were stained with H&E and in B with PAS and counterstained with hematoxylin. The H&E scores for control, OVA and OVA+Ang-1 for the pictures shown were 1, 4 and 3; The PAS score for control, OVA and OVA+Ang-1 were 0, 1 and 1.

Figure E 3 Angiopoietin-1 treatment tends to normalize overall Rn response to metacholine in OVA-treated mice. The area under the curve (AUC) of the time course of Rn increase above baseline was used as an index of the overall (not just the peak) airway resistance responsiveness to metacholine challenge. Ang-1 treatment in OVA treated mice tended to decrease overall Rn increase following metacholine (10 mg/ml) challenge ($p 0.06$). Values are expressed as means $\pm$ SEM, $n = 7$

Figure E4 Angiopoietin-1 treatment ameliorates overall increase of tissue resistance (G) and elasticity (H) following metacholine challenge in OVA-treated mice. The AUC of G and H increase above baseline over time (after challenge with 10 mg/ml mch) were

significantly lower in mice treated with Ang-1 as compared to untreated asthmatic mice (p 0.01 and p 0.02, respectively). Indeed, the AUC of G and H time course had no significant difference between Ang-1 treated asthmatic mice and naïve mice. Values are expressed as means $\pm$ SEM, n = 7

Tables

Table E1: Baseline values of constant phase model parameters.

	OVA	OVA + Ang-1	Ctl	ctl + Ang-1
Rn (cmH2O.s/mL)	0,33 $\pm$ 0,04	0,27 $\pm$ 0,01	0,36 $\pm$ 0,06	0,32 $\pm$ 0,06
G (cmH2O/mL)	3,61 $\pm$ 0,45	3,51 $\pm$ 0,18	3,36 $\pm$ 0,40	3,62 $\pm$ 0,28
H (cmH2O/mL)	18,05 $\pm$ 0,77	18,25 $\pm$ 0,60	18,23 $\pm$ 1,49	17,53 $\pm$ 0,59

Effect of different doses of Ang-1 on OVA-induced markers of inflammation

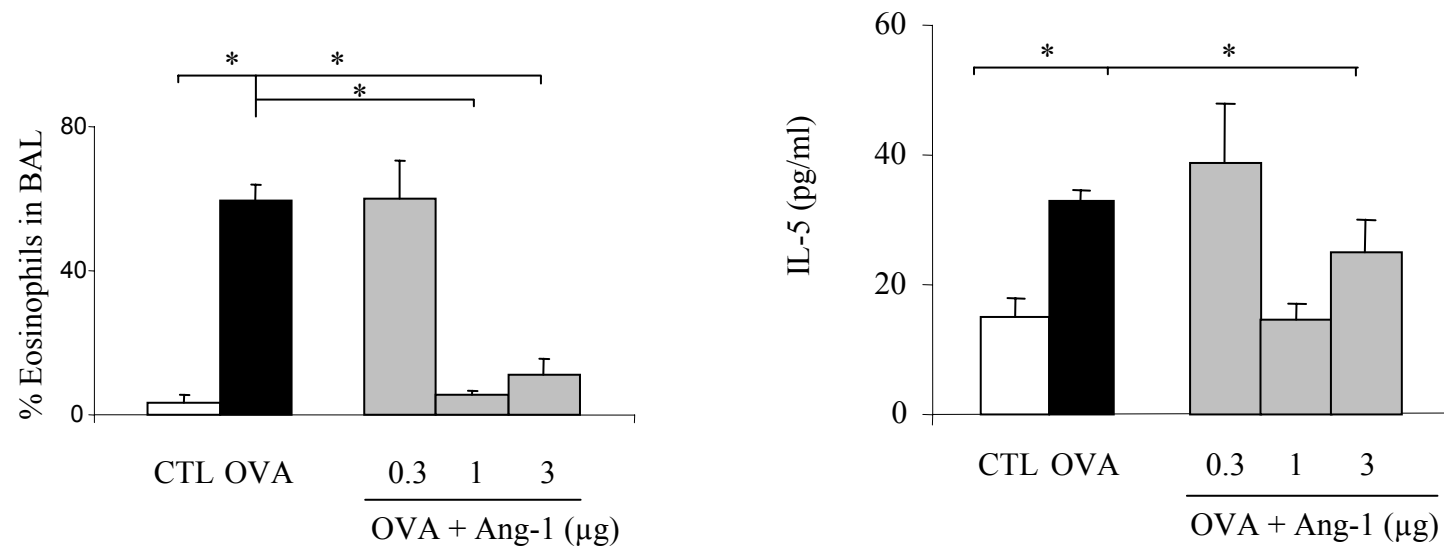


Figure E1

Representative photomicrographs of lung sections from control, OVA and OVA+Ang-1 stained with H&E (A) or PAS (B)

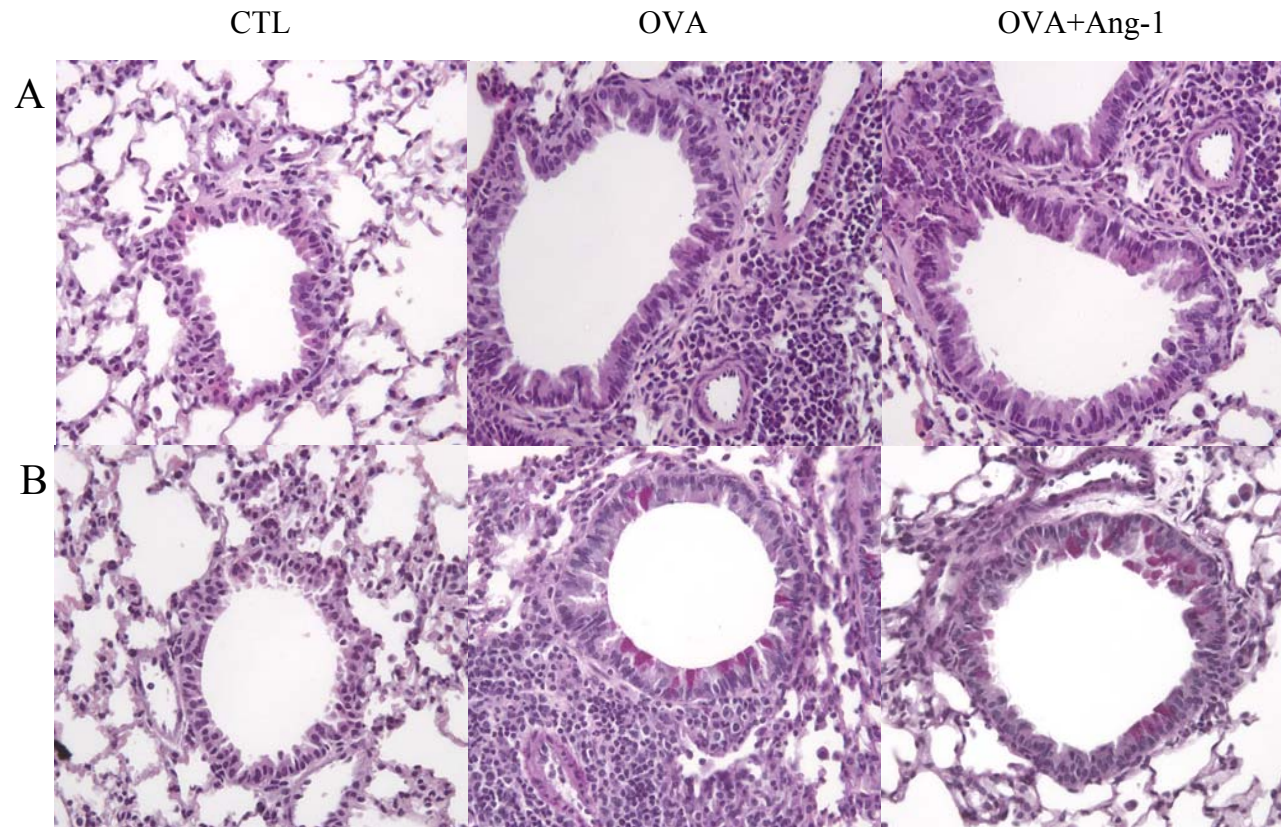


Figure E2

ΔRn time course following 10mg/ml Mch (area under the curve)

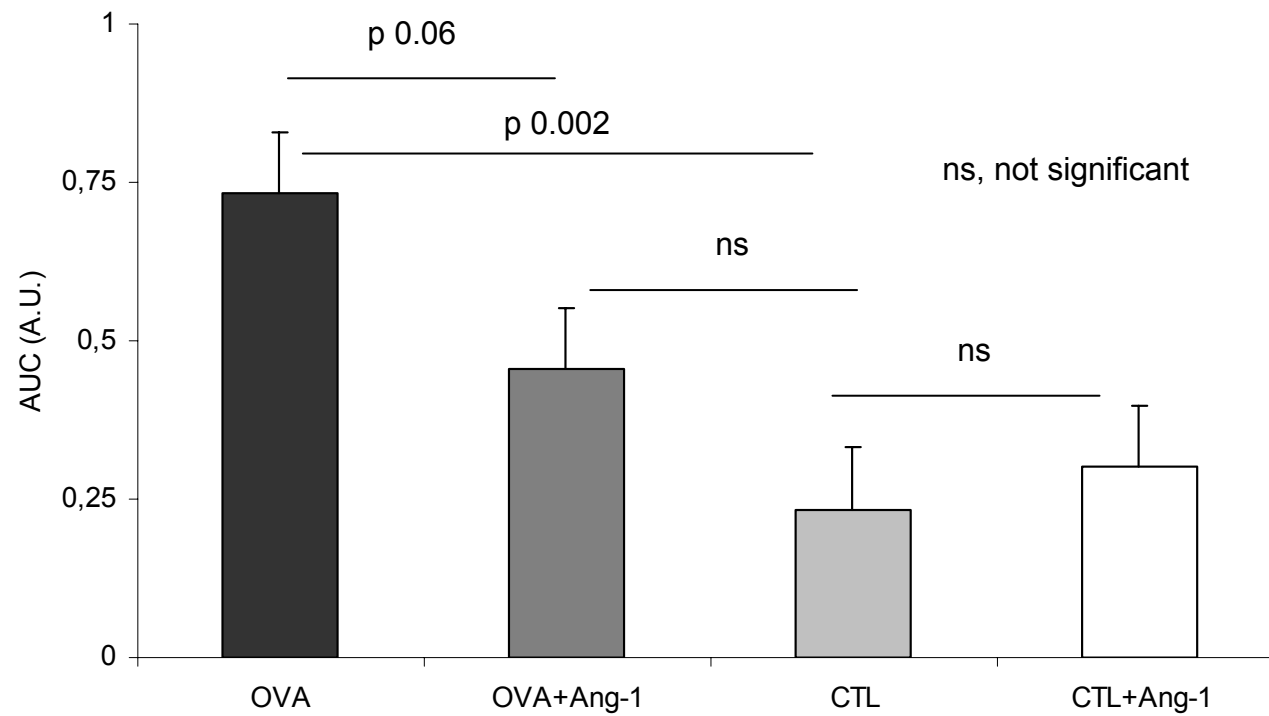


Figure E3

ΔG and ΔH time course following 10mg/ml Mch (area under the curve)

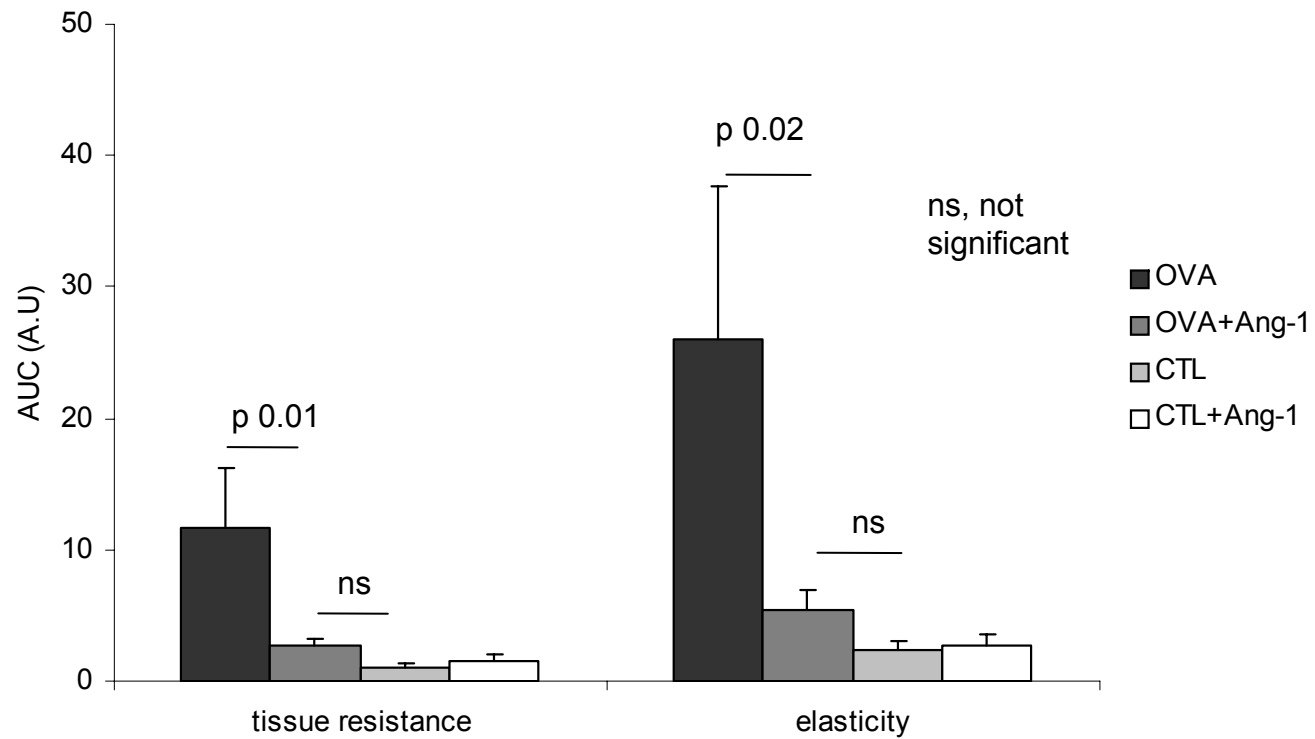


Figure E4