BJP British Journal of Pharmacology

DOI:10.1111/bph.13057
www.brjpharmacol.org

RESEARCH PAPER

Angiotensin-(1-7) attenuates airway remodelling and hyperresponsiveness in a model of chronic allergic lung inflammation

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Received
1 July 2014
Revised
20 November 2014
Accepted
15 December 2014

BACKGROUND AND PURPOSE

A long-term imbalance between pro- and anti-inflammatory mediators leads to airway remodelling, which is strongly correlated to most of the symptoms, severity and progression of chronic lung inflammation. The Angiotensin-(1-7) [Ang-(1-7)]/Mas receptor axis of the renin-angiotensin system is associated with attenuation of acute and chronic inflammatory processes. In this study, we investigated the effects of Ang-(1-7) treatment in a model of chronic allergic lung inflammation.

EXPERIMENTAL APPROACH

Mice were sensitized to ovalbumin (OVA; 4 injections over 42 days, 14 days apart) and were challenged three times per week (days 21–46). These mice received Ang-(1-7) (1 $\mu g \cdot h^{-1}$, s.c.) by osmotic mini-pumps, for the last 28 days. Histology and morphometric analysis were performed in left lung and right ventricle. Airway responsiveness to methacholine, analysis of Ang-(1-7) levels (RIA), collagen I and III (qRT-PCR), ERK1/2 and JNK (Western blotting), IgE (ELISA), cytokines and chemokines (ELISA multiplex), and immunohistochemistry for Mas receptors were performed.

KEY RESULTS

Infusion of Ang-(1-7) in OVA-sensitized and challenged mice decreased inflammatory cell infiltration and collagen deposition in the airways and lung parenchyma, and prevented bronchial hyperresponsiveness. These effects were accompanied by decreased IgE and ERK1/2 phosphorylation, and decreased pro-inflammatory cytokines. Mas receptors were detected in the epithelium and bronchial smooth muscle, suggesting a site in the lung for the beneficial actions of Ang-(1-7).

CONCLUSIONS AND IMPLICATIONS

Ang-(1-7) exerted beneficial attenuation of three major features of chronic asthma: lung inflammation, airway remodelling and hyperresponsiveness. Our results support an important protective role of Ang-(1-7) in lung inflammation.

Abbreviations

Ang II, angiotensin II; Ang-(1-7), angiotensin-(1-7); BALF, bronchoalveolar lavage fluid; CTRL, control; OVA, ovalbumin; RAS, renin-angiotensin system

Tables of Links

TARGETS
GPCR[a]
Mas receptor (MAS1)
Enzymes[b]
ACE2
ERK1/2
JNK

LIGANDS	
Ang II, angiotensin II	IL-5
Ang-(1-7), angiotensin (1-7)	IL-9
CCL2	IL-13
CCL5	IL-25
GM-CSF	TGF-β
IL-4	TNF-α

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander *et al.*, 2013a,b).

Introduction

Epidemiological studies show that asthma is currently the most common chronic disease in children, being the major cause of missed days at school and, in adults, loss of working days. In addition, asthma is associated with significant rate of mortality (Gamble *et al.*, 2009; Rincon and Irvin, 2012), and it is expected that the number of the patients will increase by more than 100 million by 2025 (Rincon and Irvin, 2012).

Asthma is defined as a reversible airway obstructive disease, caused by airway mucosal oedema, inflammation, increased mucus secretion, smooth muscle contraction, and airway hyperreactivity and remodelling. The inflammatory response in allergic asthma is characterized by excess production of IgE, mast cell degranulation, and the infiltration of eosinophils and lymphocytes (Shelhamer *et al.*, 1995; Barnes, 1998; Georas *et al.*, 2005). However, the recruitment and activation of these cells depend on the expression and release of several classes of proteins, such as cytokines, particularly Th2 derived, such as IL-4, IL-5, IL-9, IL-13, IL-25, and chemokines, such as CCL2 and CCL5, which are chemotatic proteins that attract inflammatory cells to the site of injury (Lassalle *et al.*, 1993; Yamamoto *et al.*, 1998; Fahy, 2009; Boyce *et al.*, 2012; Chien *et al.*, 2013).

The activated mast cells also contribute in the production and release of IL-5 and GM-CSF, which, along with other inflammatory mediators, attract and activate other inflammatory cells to the bronchial wall, perpetuating the local inflammatory process (Schuh *et al.*, 2003). TNF-α is another important cytokine, released in the allergic response by mast cells and macrophages via IgE-dependent mechanisms. TNF-α is related to increased bronchial responsiveness and expression of adhesion molecules, facilitating the migration of inflammatory cells into the airways and activation of pro-fibrotic mechanisms in the subepithelial layer of the bronchial mucosa (Lassalle *et al.*, 1993; Thomas *et al.*, 1995).

These inflammatory effects are associated with two signalling pathways that are also involved in asthma, namely phosphorylation of ERK1/2 and of JNK (stress-activated protein kinase; SAPK). Indeed, several asthma-related cytokines have also been shown to signal through the ERK1/2 and JNK-dependent pathways (Alam and Gorska, 2011; Dandekar and Khan, 2012; Zhou *et al.*, 2012). The imbalance between pro- and anti-inflammatory mediators leads to airway remodelling, which has been correlated with most of the symptoms, severity and progression of asthma (Vieira *et al.*, 2007).

There is experimental and clinical evidence indicating that activation of the pulmonary renin-angiotensin system (RAS) is involved in the pathophysiology of allergic pulmonary disease, especially through an inappropriate increase in angiotensin II (Ang II) (Myou *et al.*, 2000; 2002; Wang *et al.*, 2008). However, the Ang-(1-7)/Mas receptor axis, recognized as a counterregulatory peptide system within the RAS, exhibits anti-inflammatory effects and prevents inappropriate remodelling in different pathophysiological states, such as hypertension (Nakamoto *et al.*, 1995; Guimaraes *et al.*, 2012; Bertagnolli *et al.*, 2013), dyslipidemia (Santos *et al.*, 2013) and myocardial infarction (Wang *et al.*, 2010; Marques *et al.*, 2011). Furthermore, in an experimental model of arthritis, Da Silveira *et al.* (2010) showed that treatment with Ang-(1-7) or AVE 0991, a non-peptide mimic of Ang-(1-7), inhibited leukocyte accumulation and the production of inflammatory cytokines. These effects were accompanied by functional improvement of the joint (Da Silveira *et al.*, 2010). In another study, pulmonary overexpression of Ang-(1-7) exerted beneficial cardiopulmonary effects in two experimental models of lung disorders: pulmonary fibrosis and pulmonary hypertension (Shenoy *et al.*, 2010).

There is one published report that Ang-(1-7) presents an anti-inflammatory and anti-fibrotic action in a model of short-term allergic lung inflammation (El-Hashim *et al.*, 2012). More recently, we have shown that AVE 0991 attenuated pulmonary remodelling in a model of long-term allergic lung inflammation (Rodrigues-Machado *et al.*, 2013). In the present study, we sought to extend our previous observation by assessing the effects of treatment with Ang-(1-7) on the three major alterations observed in chronic asthma: lung inflammation, airway remodelling and hyperresponsiveness. Furthermore, we evaluated whether the effects elicited by Ang-(1-7) effect could depend on the ERK1/2 and JNK pathways.

Methods

Animals

All animal care and experimental procedures in this study were approved by the Ethics Committee for Animal Experi-

mentation (CEUA) of the Federal University of Minas Gerais, Brazil (protocol 309/2013). In addition, we have followed the ARRIVE guidelines (Kilkenny *et al.*, 2010, McGrath *et al.*, 2010). A total of 75 animals were used in the experiments described here.

Male BALB/C mice, from our animal facility CEBIO, UFMG, were housed under a 12/12 h light-dark cycle (lights on at 06:00 h) with free access to standard chow and tap water. The experimental animals (6–8 weeks of age; weighing 20–25 g) were randomly allocated to three groups: (i) saline-sensitized and saline-challenged, control group (CTRL; n = 25); (ii) ovalbumin (OVA)-sensitized and OVA-challenged group (OVA; n = 25); and (iii) OVA-sensitized and OVA-challenged group treated with Ang-(1-7) [OVA + Ang-(1-7); n = 25].

OVA immunization and challenge

In order to induce chronic allergic lung inflammation, we used the method described by Temelkovski *et al.* (1998). Briefly, sensitization was made by four injections of OVA (20 μg per mouse, i.p.; Sigma, St. Louis, MO, USA) at 14 day intervals (days 0, 14, 28 and 42). Beginning on the 21st day, sensitized mice received OVA challenge by nebulization (1% OVA) during 30 min, three times per week (days 21–46). Challenge was carried out in an acrylic box (30 cm × 15 cm × 20 cm) coupled to an ultrasonic nebulizer, in groups of five animals. The CTRL group was given saline i.p. (0.5 mL per mouse) and challenged with saline (NaCl 0.9%) at the same time points. OVA was diluted in sterile isotonic saline and no adjuvant was used. The experimental model used in the present study replicates many of the features of asthma in patients and facilitates studies of the pathophysiological mechanisms and the development of potential therapeutic drugs.

Ang-(1-7) treatment

On the 21st day of the sensitization period, osmotic mini-pumps (ALZET®, Model 1004, 0.11 μL·h^{-1}; Cupertino, CA, USA) containing Ang-(1-7) (delivering 1 μg·h^{-1}; Millipore, Temecula, CA, USA) were implanted s.c. in OVA-sensitized mice, under anaesthesia with ketamine/xylazine (0.5 and 0.43 mg·kg^{-1}, respectively; i.p.). Ang-(1-7), dissolved in sterile isotonic saline (NaCl 0.9%) immediately before filling the mini-pumps, was continuously infused for 28 days (days 21–49). Animals not treated from CTRL and OVA groups were subjected to a sham surgery procedure consisting of all surgical procedures except pump implantation. Seventy-two hours after the last inhalation (on the 49th day), the animals were separated into smaller groups (usually 5 per group) for different purposes or killed (anaesthetic overdose) to collect blood, heart and lung, as described previously (Rodrigues-Machado *et al.*, 2013).

Airway responsiveness

On the 49th day, in different groups of animals (n = 5–6), mice were anaesthetized, mechanically ventilated and exposed to methacholine to assess airway responsiveness. This method is described in detail in Appendix S1.

Bronchoalveolar lavage fluid (BALF)

CTRL and OVA mice (n = 5 each group) on the 21st day, and CTRL, OVA and OVA + Ang-(1-7) mice (n = 5) on the 49th day were anaesthetized with the mixture of ketamine and xylazine (0.5 and 0.43 mg·kg^{-1}, respectively; i.p.). A midline neck incision was made and the trachea and jugular vein were exposed. After collecting blood from the jugular vein, the trachea was exposed and the head of the mouse was elevated 30° from the horizontal plane. Next, a 16 G cannula was inserted into the trachea and lungs were gently rinsed twice with 0.5 mL of PBS, the BALF collected was centrifuged (600× g for 8 min at 4°C). After centrifugation, the pellet was used for total and differential leukocyte counts.

Morphometric analysis

The lung and right ventricle were fixed in formalin and embedded in paraffin. Sections (4 μm) were stained with haematoxylin and eosin for structural analysis or stained with Gomori's trichrome to evaluate collagen deposition in the lungs. This method is described in detail in Appendix S1. All sections were assessed without knowledge of the treatments.

Immunohistochemistry for Mas receptor

Sections of the lung (5 μm) were incubated with polyclonal anti-Mas receptor antibody (Alomone Labs, Jerusalem, Israel) and processed for DAB staining. This method is described in detail in Appendix S1. All sections were assessed without knowledge of the treatments.

qRT-PCR for collagen I and III mRNA expression

Total RNA from a segment of the lung was extracted using TRIzol reagent (Invitrogen, San Diego, CA, USA), treated with DNAse (RNase-free) (Invitrogen) and reverse transcribed with Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Promega, Madison, WI, USA). The endogenous GAPDH (internal control), collagen I, and collagen III cDNA were amplified using specific primers (Supporting Information Table S1) and SYBR green reagent (Applied Biosystems, Foster City, CA, USA) in ViiA™ 7 System (Applied Biosystems). The relative comparative CT method was applied to compare gene expression levels between groups using the equation $2^{-\Delta\Delta CT}$.

Proteins measured by Western blotting

Total protein was extracted from lung samples (n = 4–6 each group) and 50 mg of total protein was applied into SDS-PAGE 10% and transferred to nitrocellulose membranes. Total and phosphorylated forms of ERK1/2 and phosphorylation of JNK were determined, using primary antibody for total ERK1/2 total [rabbit anti-44/42 Mark (ERK1/2), 1:1000; Cell Signaling, Danvers, MA, USA] or phosphorylated ERK1/2 [rabbit anti-phospho p44/42 Mark (ERK1/2), 1:500; Cell Signaling, Danvers, MA, USA] or phosphorylated JNK (rabbit anti-phospho-SAPK/JNK, 1:500, Cell Signaling), This method is described in detail in Appendix S1.

Cytokine measurements in the lung

The analysis technology kit for multiple cytokines (Multiplex cytokine analysis Technology; Luminex®, Austin, TX, USA) was used for the evaluation of IL-4, IL-5, IL-13, TNF-α, GM-CSF, CCL2 and CCL5, following the instructions of the manufacturer.

Measurement of Ang-(1-7) in the lung

Ang-(1-7) in lung homogenates was measured by RIA, as previously described (Marques *et al.*, 2011; Rodrigues-Machado *et al.*, 2013), using a rabbit polyclonal antibody that cross-reacts less than 0.01% with Ang-(2-7) and Ang-(3-7) and less than 0.08% with Ang-(4-7). The cross-reactivity with Ang I, Ang II, and amino-terminal fragments was less than 0.001%.

Levels of serum IgE antibody

Serum samples were obtained on the 21st day (before the beginning of OVA challenge) in CTRL and OVA mice. Serum samples were also obtained on the 49th day (at the end of the experiment), in CTRL, OVA and OVA + Ang-(1-7) mice. Anti-OVA IgE antibodies were measured by capture-ELISA (IgE Mouse ELISA Kit, Abcam, Cambridge, MA, USA) using plates coated with rat anti-mouse IgE, following the instructions of the manufacturer.

Data analysis

All results are expressed as mean ± SEM. Comparisons among groups were performed by one-way ANOVA followed by Newman–Keuls *post hoc* test or two-way ANOVA followed by the Bonferroni *post hoc* test, as appropriate and indicated in the Results section and figure legends. All analyses and graphics were performed with the GraphPad Prism software (version 5.0, GraphPad Software, Inc., La Jolla, CA, USA). The level of significance was set to $P < 0.05$.

Results

Serum IgE levels and cellular changes in BALF

Serum IgE was increased in sensitized mice (4.7 ± 0.80 ng·mL^{-1}, $n = 5$) in comparison with CTRL mice (2.4 ± 0.44 ng·mL^{-1}, $n = 5$; $P < 0.05$, Student's *t*-test). Further, total leukocytes (19.7 × 10^5 ± 2 cells), in which 6% were neutrophils and 94% were mononuclear cells, in OVA mice were not different from CTRL mice (18 × 10^5 ± 2 total cells, 4% neutrophils and 96% mononuclear cells). This result reproduced those obtained by Temelkovski *et al.* (1998) and showed that mice were immunized but did not initiate the inflammatory response.

As expected, on the 49th day, serum IgE was much higher in the group of OVA-mice (12.45 ± 1.9 ng·mL^{-1}, $n = 5$) in comparison with CTRL (4.0 ± 0.37 ng·mL^{-1}, $n = 5$). However, treatment with Ang-(1-7) attenuated the alteration in serum IgE (7.0 ± 1.1 ng·mL^{-1}, $n = 5$; ANOVA followed by Newman–Keuls test, $P < 0.05$). Further, OVA + Ang-(1-7) mice did not show the increase in the total number of cells in BALF (18 × 10^5 ± 3 cells, $n = 5$; ANOVA followed by Newman–Keuls test, $P < 0.05$), compared with cell numbers in OVA mice (38 × 10^5 ± 6 cells, $n = 5$). The total number of cells in BALF of OVA + Ang-(1-7) group was not different from that in the CTRL group (17 × 10^5 ± 4 cells, $n = 5$; ANOVA followed by Newman-Keuls test, $P < 0.05$). These data show that Ang-(1-7) prevented the increase in the production of IgE in our model of chronic allergic pulmonary injury, suggesting a decrease in degranulation of mast cells, eosinophils and basophils, which in turn reduced important mediators for the development and maintenance of the allergic inflammatory process.

Cytokine concentrations in the lung

Based on the result that treatment with Ang-(1-7) prevented the increase in plasma IgE of animals chronically challenged with OVA, we evaluated the levels of cytokines and chemokines importantly involved in allergic lung inflammation. Figure 1A–G shows tissue levels of pro-inflammatory cytokines and chemokines in CTRL, OVA and OVA + Ang-(1-7) mice. OVA induced a significant increase in IL-4, IL-5, TNF-α, GM-CSF, CCL5 and CCL2 (Figure 1A, B, D–G). Treatment with Ang-(1-7) prevented the increase in IL-4, IL-5, GM-CSF, CCL5 and CCL2 levels in the lung of OVA-challenged mice (Figure 1A, B, E–G), showing that Ang-(1-7) reduced cytokines and chemokines that are critically involved in the differentiation, proliferation and increase in recruitment and survival of inflammatory cells in allergic inflammation.

Lung tissue inflammation

As expected, OVA sensitization produced a pronounced increase in the density of inflammatory cell infiltrate around the airways (Figure 2A) and blood vessels (Figure 2B) and alveolar parenchyma (Figure 2C) in comparison with the control group. Treatment with Ang-(1-7) attenuated the inflammatory infiltrate in the peribronchial, perivascular and alveolar regions of the lung, so that there was no significant difference in inflammatory cell infiltrates between the OVA + Ang-(1-7) and CTRL groups.

Pulmonary remodelling

Pulmonary remodelling is the most important alteration that leads to the functional abnormalities observed in asthma. Therefore, we next evaluated the degree of remodelling in airways, peribronchial vessels and alveolar parenchyma. Figure 3A–C presents representative images of the lung of all groups showing asthma-induced alterations in alveolar and bronchial wall thickness. OVA mice exhibited significantly greater thickening and inflammation of the alveolar wall than observed in lungs from the CTRL group (Figure 3B and D). OVA-sensitized and challenged animals treated with Ang-(1-7) presented reduced inflammation in the interalveolar space with normal appearance of alveolar lumen (Figure 3C). The epithelial thickness was increased twofold in OVA mice (Figure 3B and E) as compared with CTRL (Figure 3A and E) and OVA + Ang-(1-7) mice (Figure 3C and E). Furthermore, chronic OVA exposure significantly increased collagen in airways and lung parenchyma (Figure 4B and D) compared with CTRL group (Figure 4A and D). OVA-sensitized and challenged mice treated with Ang-(1-7) presented a marked reduction in collagen deposition in airway walls (Figure 4C and D). In addition, OVA induced an increase in the mRNA expression of collagen I (Figure 4E) and collagen III (Figure 4F), which were prevented by Ang-(1-7) treatment (Figure 4E and F).

Vascular remodelling and right ventricle hypertrophy

As can be seen in Figure 5A, OVA-challenged mice exhibit an increase in smooth muscle layer in the peribronchial vessels, while chronic treatment with Ang-(1-7) significantly pre-

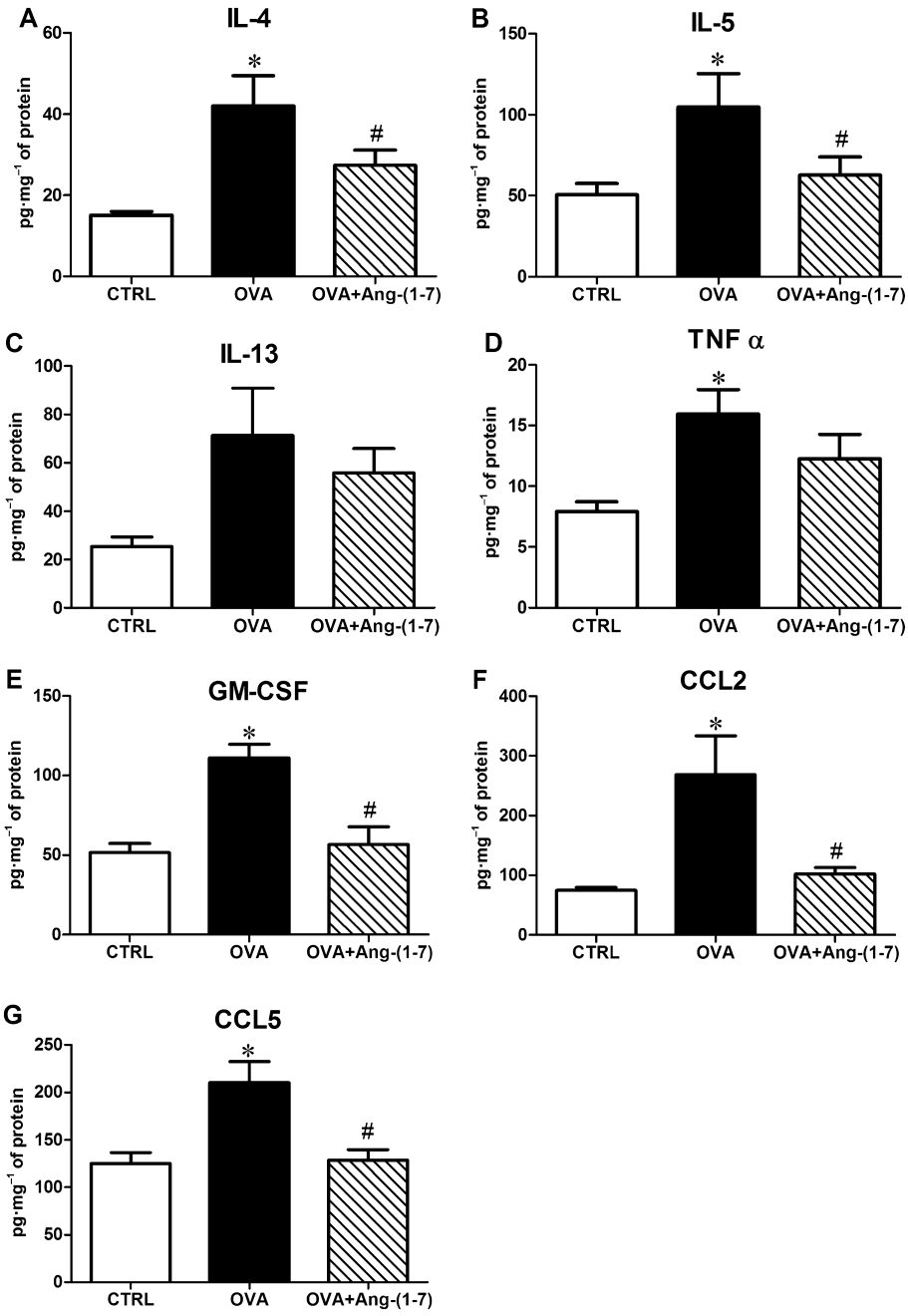

Figure 1

(A–G) Pulmonary levels of IL-4, IL-5, IL-13, TNF-α, GM-CSF, CCL2 and CCL5 in CTRL, OVA and OVA + Ang-(1-7) mice (n = 5–6). *P < 0.05, significantly different from CTRL; #P < 0.05, significant effect of Ang-(1-7); one-way ANOVA followed by Newman–Keuls test.

vented this alteration. Further, the OVA-group of mice exhibited a significant increase in the cardiomyocyte diameter in the right ventricle in comparison to values from samples of CTRL hearts (Figure 5B), suggesting that alteration in smooth muscle layer may lead to an increase in pulmonary vascular resistance. Ang-(1-7) treatment prevented right ventricular hypertrophy (Figure 5B) probably secondary to attenuation in pulmonary vascular hyperplasia/hypertrophy.

Airway responsiveness

As expected, the airway response to methacholine was significantly higher in OVA mice compared with responses in the CTRL group (Figure 6). However, Ang-(1-7) treatment significantly attenuated the airway hyperresponsiveness. The airway response to methacholine in OVA + Ang-(1-7) mice was not different from CTRL group (Figure 6).

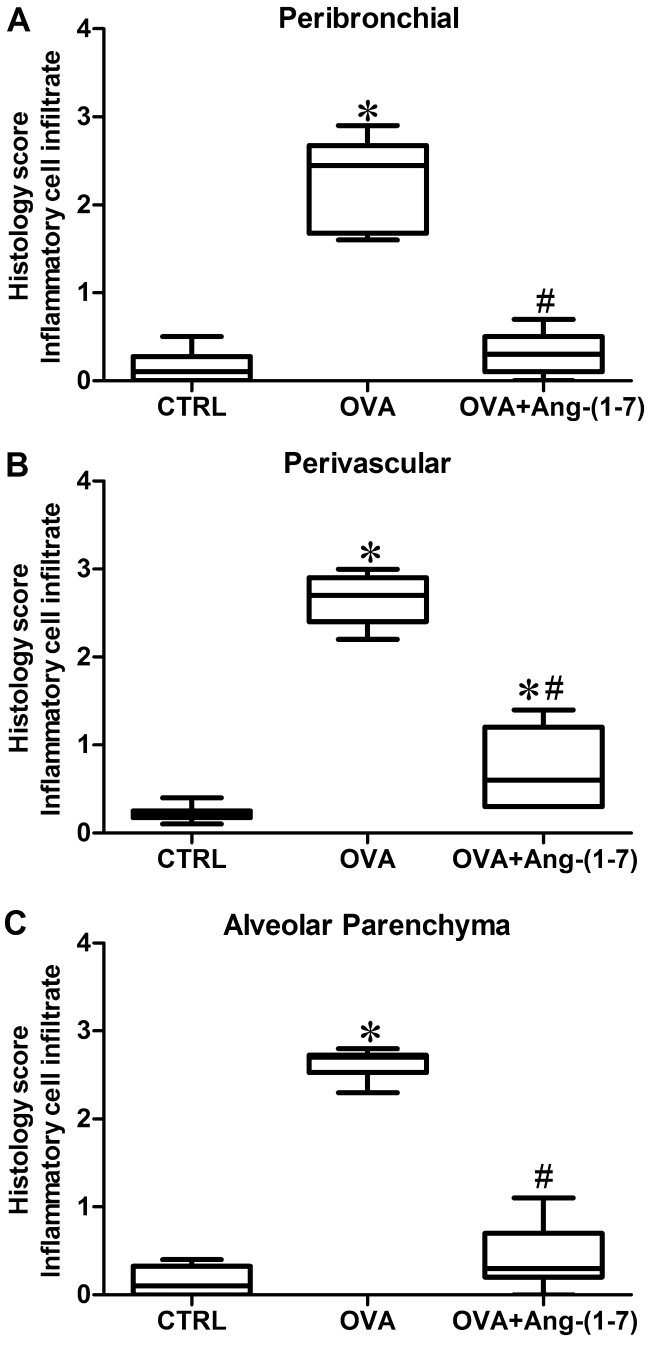

Figure 2

Box plots of histology scores of peribronchial (A), perivascular (B) and parenchymal (C) inflammatory infiltrate in CTRL, OVA and OVA + Ang-(1-7) mice (n = 5–6). Boxes show first and third quartiles, the whiskers show 10% and 90% range, and the horizontal line represents the median value. *P < 0.05, significantly different from CTRL; #P < 0.05, significant effect of Ang-(1-7); one-way ANOVA followed by Newman–Keuls test.

p-ERK1/2 and p-JNK in the lung

OVA challenge significantly increased the levels of total ERK1/2 and p-ERK1/2 compared with control animals (Figure 7A and B). However, there was also an increase in the ratio between phospho and total ERK1/2 in the lung of OVA mice in comparison with CTRL (Figure 7C). Treatment with Ang-(1-7) significantly reduced the level of p-ERK1/2 compared with the OVA-challenged animals (Figure 7B and C). OVA challenge also significantly increased the level of p-JNK compared with saline-challenged control animals (Supporting Information Fig. S1). Treatment with Ang-(1-7) reduced the level of p-JNK in the lungs (Supporting Information Fig. S1). These data suggest that the effects of Ang-(1-7) may involve the ERK1/2 and the JNK signalling pathways.

Pulmonary Ang-(1-7) levels

OVA-sensitized and challenged mice treated with Ang-(1-7) [OVA + Ang-(1-7)] presented approximately a 10-fold higher level of Ang-(1-7) in the lung, in comparison to the lungs from the CTRL group (ANOVA followed by Newman–Keuls test; Figure 8). No significant difference was observed in Ang-(1-7) levels in the untreated OVA group (ANOVA; Figure 8).

Immunohistochemistry for Ang-(1-7) Mas receptor

For the first time, we showed that Mas receptors are present in thin areas of the bronchial epithelium and smooth muscle (Figure 9A and D). Moreover, chronic OVA challenge reduced by 40% the area stained for Mas receptor in the CTRL lungs (P < 0.05; ANOVA followed by Newman–Keuls test; Figure 9E), and interestingly, treatment with Ang-(1-7) prevented this change (Figure 9E).

Discussion

In the present study, in addition to showing that treatment with Ang-(1-7) prevented pulmonary remodelling, bronchial hyperresponsiveness and the release of cytokines IL-4, IL-5 and GM-CSF in chronic OVA-challenged mice, we showed that the attenuation in bronchial remodelling may also be related to a decrease in chemotactic stimulus and to involve two signalling pathways importantly associated with asthma, the ERK1/2 and possibly the JNK pathways. Furthermore, we showed the presence of Mas in the epithelium and smooth muscle of the bronchi, indicating a site in the lung for the beneficial actions of Ang-(1-7) in chronic allergic inflammation.

In keeping with Temelkovski *et al.*'s (1998) study, sensitized mice (on the 21st day) did not present signs of inflammation or pulmonary remodelling. However, Ang-(1-7) protected against the subsequent development of inflammation and pulmonary alterations induced by the long-term OVA challenge. The group of OVA-mice presented increased expression of the Th2 cytokines IL-4, IL-5 and IL-13 in the lung, which are key factors in the recruitment of eosinophil and mast cells, in mucus hypersecretion, and for airway remodelling and hyperresponsiveness (Robinson *et al.*, 1992; Barnes, 1998; Tattersfield *et al.*, 2002; Georas *et al.*, 2005; Rincon and Irvin, 2012). These alterations are recognized as the pathophysiological basis of asthma in patients and experimental models.

In the present study, we showed that chronic s.c. infusion of Ang-(1-7), which induced a 10-fold increase of the peptide

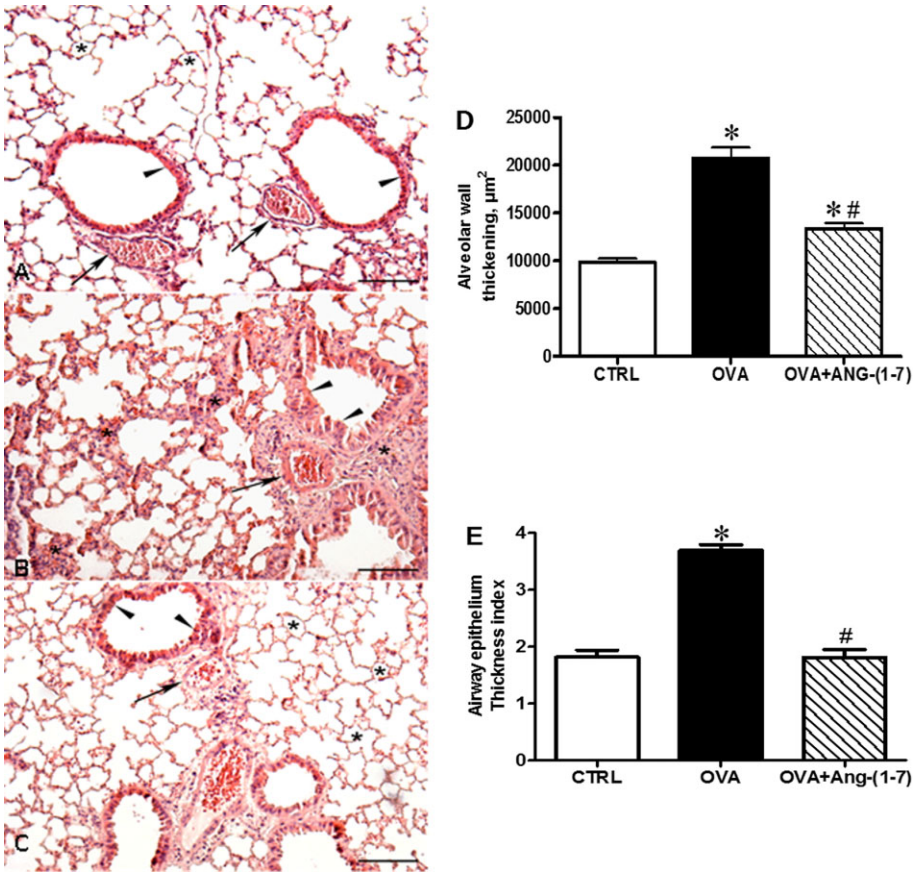

Figure 3

Representative histological images of lung sections stained with H&E from CTRL (A), OVA (B) and OVA + Ang-(1-7) (C) groups (n = 5–7). OVA-challenged mice showed marked inflammatory cell infiltrations in peribronchial, perivascular and alveolar (asterisks in B). Moreover, a large interalveolar interstitial thickening can be seen resulting mainly from the accumulation of inflammatory cells. Bronchiolar wall thickening mainly by hypertrophy and hyperplasia of bronchial epithelium (arrowhead in B) and vascular remodelling (arrows in B) can be also observed. The lung parenchyma of OVA challenged animals treated with Ang-(1-7) (C) presented reduced inflammation in the interalveolar space with normal appearance of alveolar lumen, bronchioles and vessels, similar to CTRL group (A). The effect of Ang-(1-7) treatment in thickening of the alveolar wall can be seen in (D) (n = 5 each) and in the epithelial thickness in (E) (n = 5–7). Bars = 100 µm. *P < 0.05, significantly different from CTRL; #P < 0.05, significant effect of Ang-(1-7); one-way ANOVA followed by Newman–Keuls test.

in the lung, reduced the levels of pro-inflammatory cytokines and chemokines in the site of injury. This result is in keeping with recent findings of our laboratory showing that administration of a synthetic, non-peptide mimetic of Ang-(1-7), AVE 0991, reduced IL-5 and increased IL-10 levels in lung and BALF of mice in a model of chronic asthma (Rodrigues-Machado *et al.*, 2013). In addition, studies of Shenoy *et al.* (2010) showed that a lentiviral-mediated pulmonary overexpression of Ang-(1-7) prevented pulmonary fibrosis after bleomycin administration, resulting in a significant decrease in mRNA levels of TGF-β and some pro-inflammatory cytokines. More recently, Ang-(1-7) attenuated OVA-induced leukocyte influx in airway spaces, perivascular and peribronchial inflammation in a short-term model of allergic asthma (El-Hashim *et al.*, 2012). Altogether, these results suggest that Ang-(1-7) may depress leukocyte migration, cytokine expression and release during the onset of chronic lung allergic inflammation. Further, the present study showed that Ang-(1-7) treatment decreased chemokines and cytokines essential for the initiation and maintenance of the inflammatory process, as well as those important for the migration of eosinophils to the site of injury and reduction of their apoptosis.

There is evidence that lung inflammation and remodelling in both asthmatic patients and in experimental models of asthma are not restricted to the airway and extend into the parenchyma and pulmonary vessels (Singh *et al.*, 2005; Lancas *et al.*, 2006). In our experimental model of chronically OVA-sensitized and challenged mice, we observed an increase in the deposition of collagen fibres in the airway wall, an increase in the expression of collagen I and collagen III in the lung, along with thickening of the alveolar wall and smooth muscle of the arterioles. In addition, the OVA-mice showed right ventricular hypertrophy, probably due to a functional and structural adaptation in response to chronic pulmonary artery pressure overload. The pulmonary circulation is a low-pressure system, with low vascular resistance to right ventricular output, factors that continuously and progressively

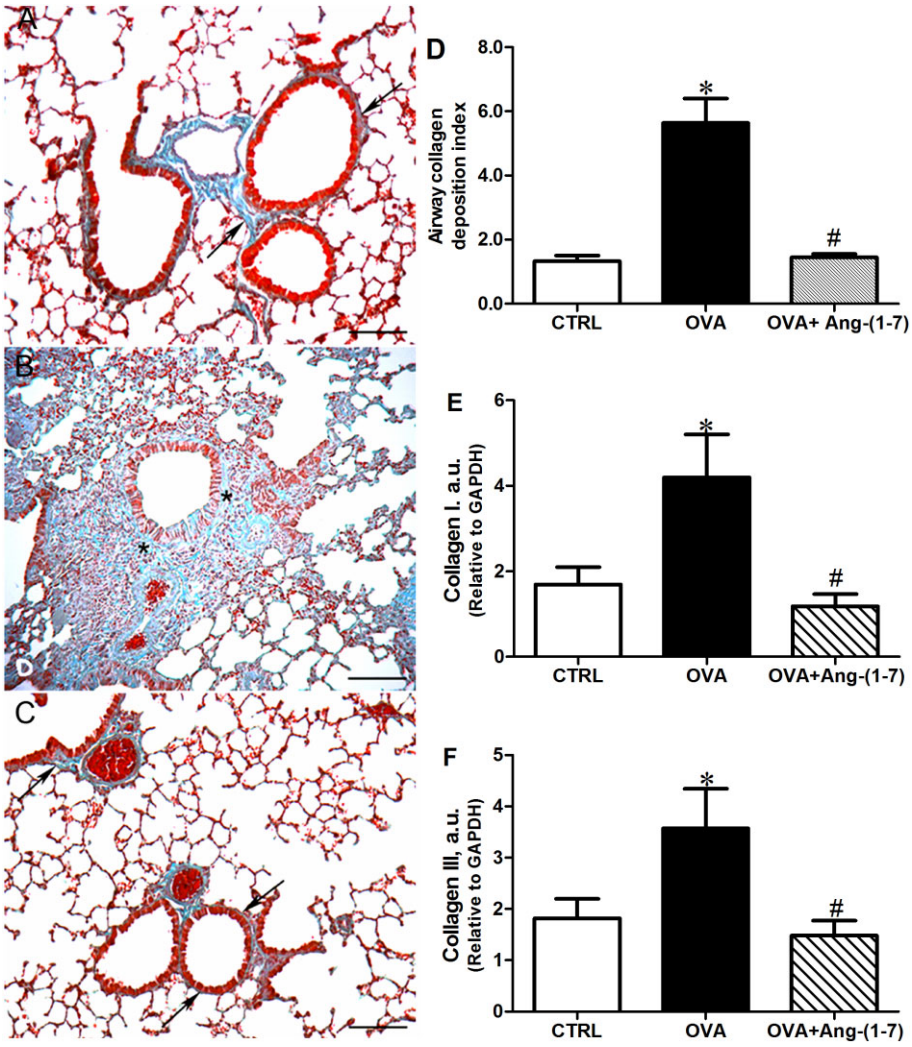

Figure 4

Representative histological images of lung sections stained with Gomori's trichrome from CTRL (A), OVA (B) and OVA + Ang-(1-7) (C) groups. As can be seen, OVA-challenged mice presented marked peribronchial and perivascular fibrosis (asterisks in B) compared with CTRL mice (A), which was prevented by Ang-(1-7) treatment (arrows in C). Panels D–F present total collagen deposition index (D; n = 5–7) and mRNA expression of collagen I (E) and collagen III (F) (n = 4–6) of CRTL, OVA and OVA + Ang-(1-7) mice. Bars = 100 μm. *P < 0.05, significantly different from CTRL; #P < 0.05, significant effect of Ang-(1-7); one-way ANOVA followed by Newman–Keuls test.

induce pulmonary arteriole remodelling and lead to increased afterload and right ventricular hypertrophy, which in turn increases the rate of mortality. Ang-(1-7) treatment prevented these alterations probably due to its anti-inflammatory effects. However, as shown in several studies, Ang-(1-7) also exerts anti-proliferative effects in the vasculature and heart (Santos, 2014). Thus, it is possible that the attenuation in vascular remodelling and right ventricular hypertrophy in OVA + Ang-(1-7) treated mice may be due to a combination of anti-inflammatory and anti-proliferative effects.

The pulmonary remodelling, characterized by the imbalance between synthesis and degradation of the extracellular matrix, has been associated with the degree of severity of the disease and the decrease in lung function (Brown *et al.*, 1984; Wilson *et al.*, 1993; Vieira *et al.*, 2007). Ang-(1-7) treatment in our model of chronic asthma prevented pulmonary remodelling. Shenoy *et al.* (2010) showed that Ang-(1-7) prevented pulmonary fibrosis induced by bleomycin in Sprague-Dawley rats. In addition, we have recently showed that AVE 0991 exerted a protective effect in airway remodelling (Rodrigues-Machado *et al.*, 2013). Moreover, as observed by El-Hashim *et al.* (2012), treatment with Ang-(1-7) resulted in a significant reduction in peribronchial and perivascular fibrosis and inflammation in an short-term model of allergic asthma. Additionally, Klein *et al.* (2013) showed that Ang-(1-7), starting 30 min after oleic acid administration, protected rats from acute lung injury. These data suggest that Ang-(1-7)

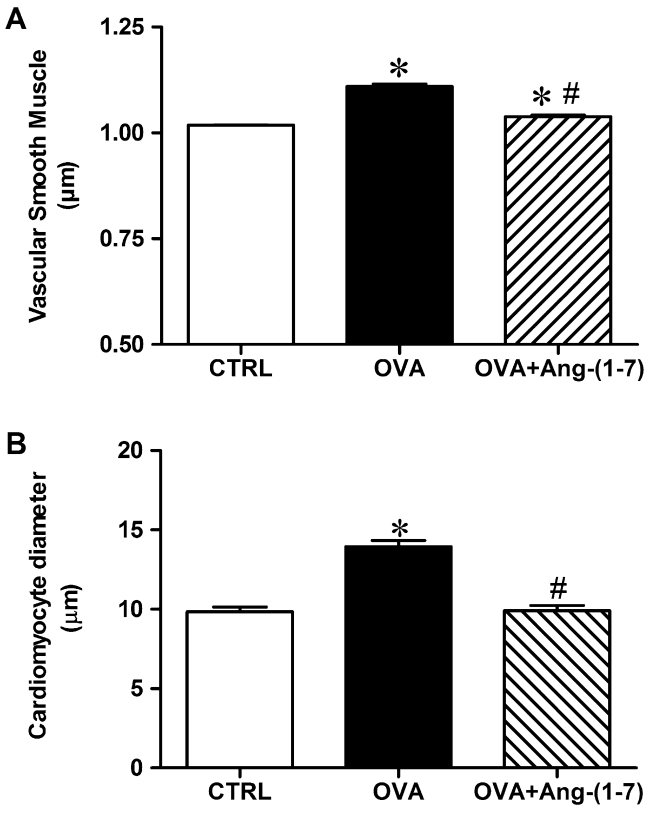

Figure 5

Effect of Ang-(1-7) treatment on vascular remodelling and right ventricle hypertrophy (n = 5–6). Morphometric analysis of the thickness of the vascular muscular layer (A) and transverse measurement of cardiomyocytes (60 cells per animal; B). Only cardiomyocytes with well-defined nuclei and cell boundaries were measured. *P < 0.05, significantly different from CTRL; #P < 0.05, significant effect of Ang-(1-7); one-way ANOVA followed by Newman–Keuls test.

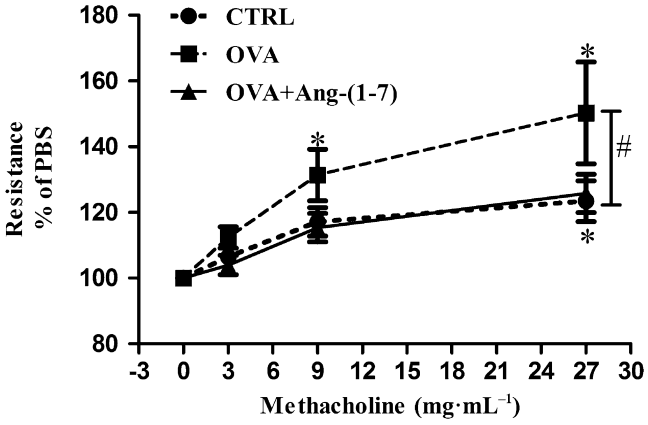

Figure 6

Airway response to methacholine. The methacholine response was expressed as a percentage of the response to PBS. Each response of airway resistance was calculated from the average of the values collected over 5 min after the administration of each concentration of methacholine in the aerosol (n = 5–6). *P < 0.05, significantly different from PBS; #P < 0.05, significantly different from OVA group; two-way ANOVA followed by Bonferroni's test.

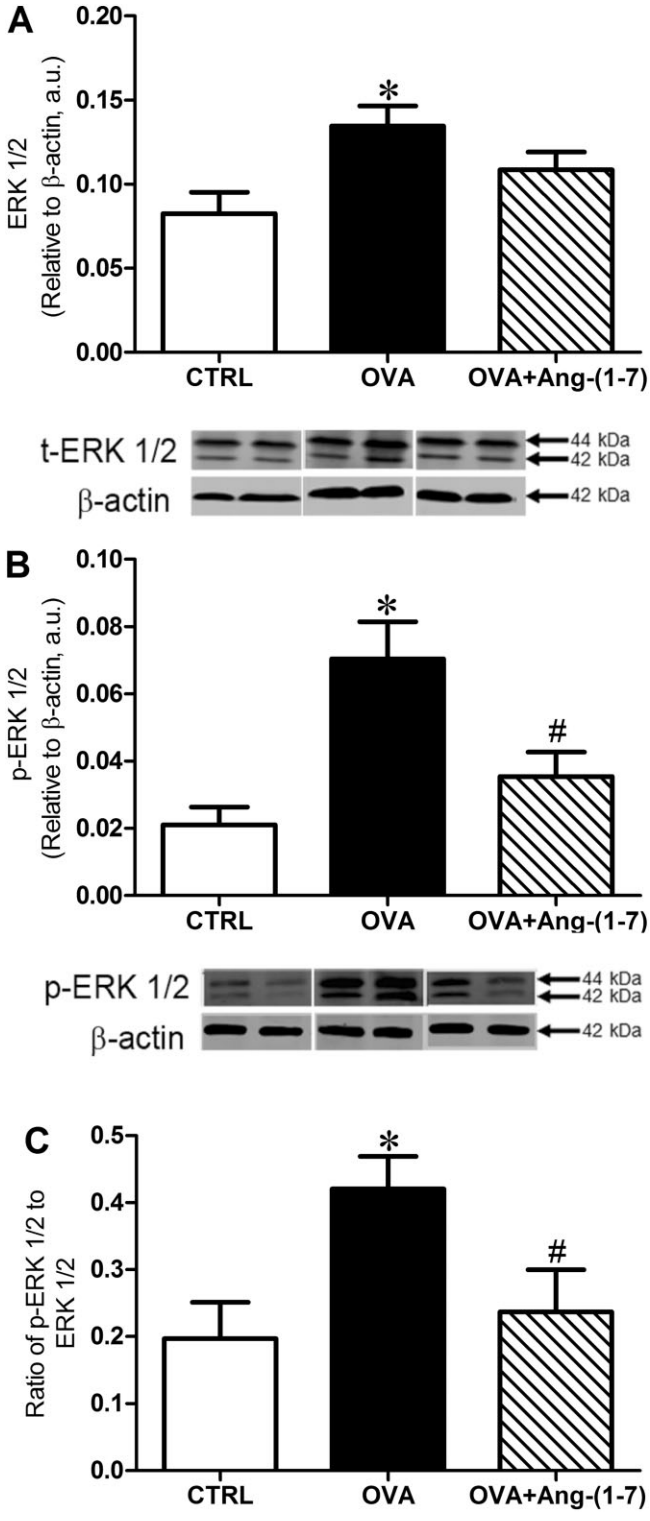

Figure 7

Effect of Ang-(1-7) on the OVA-induced increase in total and p-ERK1/2 in the lungs, as analysed by Western blotting. (A) Relative levels of total-ERK1/2. (B) Relative levels of phospho-ERK1/2. (C) Ratio of phospho-ERK1/2/total-ERK1/2. Values were normalized to β-actin (n = 5–6). *P < 0.05, significantly different from CTRL; #P < 0.05, significant effect of Ang-(1-7); one-way ANOVA followed by Newman–Keuls test.

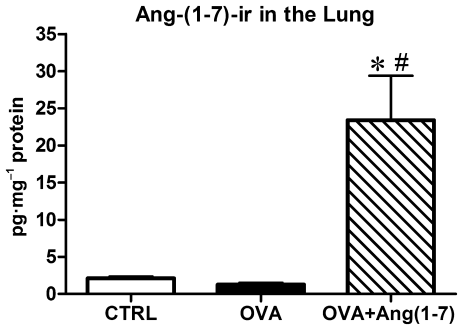

Figure 8

Levels of Ang-(1-7) in lung homogenates, measured by RIA. Data shown are mean (± SEM) values of immunoreactive (ir) Ang-(1-7) expressesd as pg·mg^{-1} of protein (n = 5–6). *P < 0.05, significantly different from CTRL; #P < 0.05, significant effect of Ang-(1-7); one-way ANOVA followed by Newman–Keuls test.

has a protective effect against pulmonary remodelling, probably by reducing pro-inflammatory cytokines in the lung, as shown in the present study.

It is interesting to observe that Ang-(1-7) levels increased 10-fold in the lung of treated mice. Although we have not explored the mechanisms involved, a couple of possibilities can be raised. First, Ang-(1-7) infusion may alter the expression of tissue ACE2 (Mendes *et al.*, 2005; Santiago *et al.*, 2010), which in turn may increase Ang-(1-7) levels in the lung. Studies have shown that with increasing circulating levels of Ang-(1-7) by s.c. infusion (Mendes *et al.*, 2005) or using transgenic rats (Santiago *et al.*, 2010), there is an increase in ACE2 in the heart. The mechanisms underlying this alteration are still not known. On the other hand, Ang-(1-7) may be taken up from the circulation. The Mas receptor was shown to internalize with Ang-(1-7) in cell culture (Gironacci *et al.*, 2011). Further studies will be necessary to show whether the increase in Ang-(1-7) in the lung after chronic s.c. administration is due to stimulation of tissue production of Ang-(1-7) or to its uptake from the circulation or some other mechanisms.

Structural cells, airway epithelium and smooth muscle cells play an important role in the pathogenesis of inflammation and remodelling in asthma (Wong *et al.*, 2006; Lin *et al.*, 2014). Indeed, bronchial epithelium and smooth muscle cells, by secreting a variety of inflammatory mediators and extracellular matrix proteins, can participate in immunomodulation and airway remodelling in asthma (Van Wetering *et al.*, 2007; Lin *et al.*, 2014). In the present study, we showed that airway epithelium and smooth muscle cells were stained positively for Mas receptors. In addition, our data showed that Ang-(1-7) treatment was able to prevent the reduction in Mas receptor expression in bronchial cells of OVA-challenged mice. In keeping with a previous study, we observed here a reduction in Mas receptor protein expression in lung homogenates from OVA mice (Rodrigues-Machado *et al.*, 2013). We can now extend this observation by showing that two structures, epithelium and bronchial smooth muscle, which function as modulators of inflammatory and remodelling in asthma, are important candidate sites in the lung for mediating the beneficial effects of Ang-(1-7).

Asthma is characterized by airway hyperresponsiveness, which involves several factors, such as airway architecture, inflammation and bronchial remodelling (Xisto *et al.*, 2005). A study using IL-4-gene-knockout mice showed that airway hyperresponsiveness was dependent on Th2 cytokines (Komai *et al.*, 2003). Therefore, increased Th2 cytokine production in the lung may subsequently aggravate airway hyperresponsiveness, which was observed in the group of OVA-mice. Our study indicated for the first time that treatment with Ang-(1-7) prevented the three major changes in a model of chronic asthma: lung inflammation and remodelling and airway hyperresponsiveness.

In the present study, we showed that one possible mechanism underlying the effects of Ang-(1-7) we have observed here may be related to the suppression of the phosphorylation of ERK1/2. ERK1/2, activated in response to stimulation of mast cells through IgE receptors (Tsai *et al.*, 1993), is important for mast cell differentiation,proliferation and survival, and eicosanoid release, eosinophil differentiation and activation (Hirasawa *et al.*, 1995; Kimata *et al.*, 2000). Inhibition of this pathway has been reported to decrease cytokine production by eosinophils. Moreover, studies using pharmacological inhibitors suggest that the release of eicosanoids and the production of GM-CSF depend upon ERK1/2 signalling (Alam and Gorska, 2011). The data of the present study also suggested that Ang-(1-7)-treated OVA mice presented a reduced phosphorylation of JNK in the lung. Indeed, several asthma-related cytokines have also been shown to signal through the ERK1/2 and the JNK-dependent pathways (Alam and Gorska, 2011; Dandekar and Khan, 2012; Zhou *et al.*, 2012). Taken together, these studies indicate the importance of activation of ERK1/2 and JNK pathways in the development of asthma pathophysiology. Our results are also in line with different studies showing the inhibitory effect of Ang-(1-7) on ERK1/2 in different preparations. El-Hashim *et al.* (2012) showed a reduction in phosphorylated ERK1/2 in mice with a short-term model of asthma treated with Ang-(1-7). In addition, Ang-(1-7) significantly reduced Ang II-induced phosphorylation of p38, ERK1/2 and JNK MAPK, and the production of TGF-β in primary cultures of rat of proximal tubular cells (Su *et al.*, 2006). Furthermore, treatment with Ang-(1-7) inhibited the activation of ERK1/2 in the hearts of rats with Ang II-induced cardiac hypertrophy (Giani *et al.*, 2008).

In conclusion, the results of the present study showed that Ang-(1-7) treatment of OVA-sensitized and challenged mice decreased inflammatory cell infiltration, collagen deposition in the airways and lung parenchyma, and prevented airway remodelling and hyperresponsiveness. In addition, Ang-(1-7) inhibited the vascular remodelling and right ventricular hypertrophy. Furthermore, our results suggest that the protective effects of Ang-(1-7) in chronic lung inflammation and remodelling can be mediated by bronchial cells and to depend on a reduction in the phosphorylation of ERK1/2. The findings of the current study reinforce the hypothesis that pharmacological strategies that lead to an increase in Ang-(1-7) in lung may represent an important additional tool for the treatment of chronic lung inflammatory disease, which has been shown to increase in severity and mortality, in spite of all the recent advances in understanding its pathophysiology.

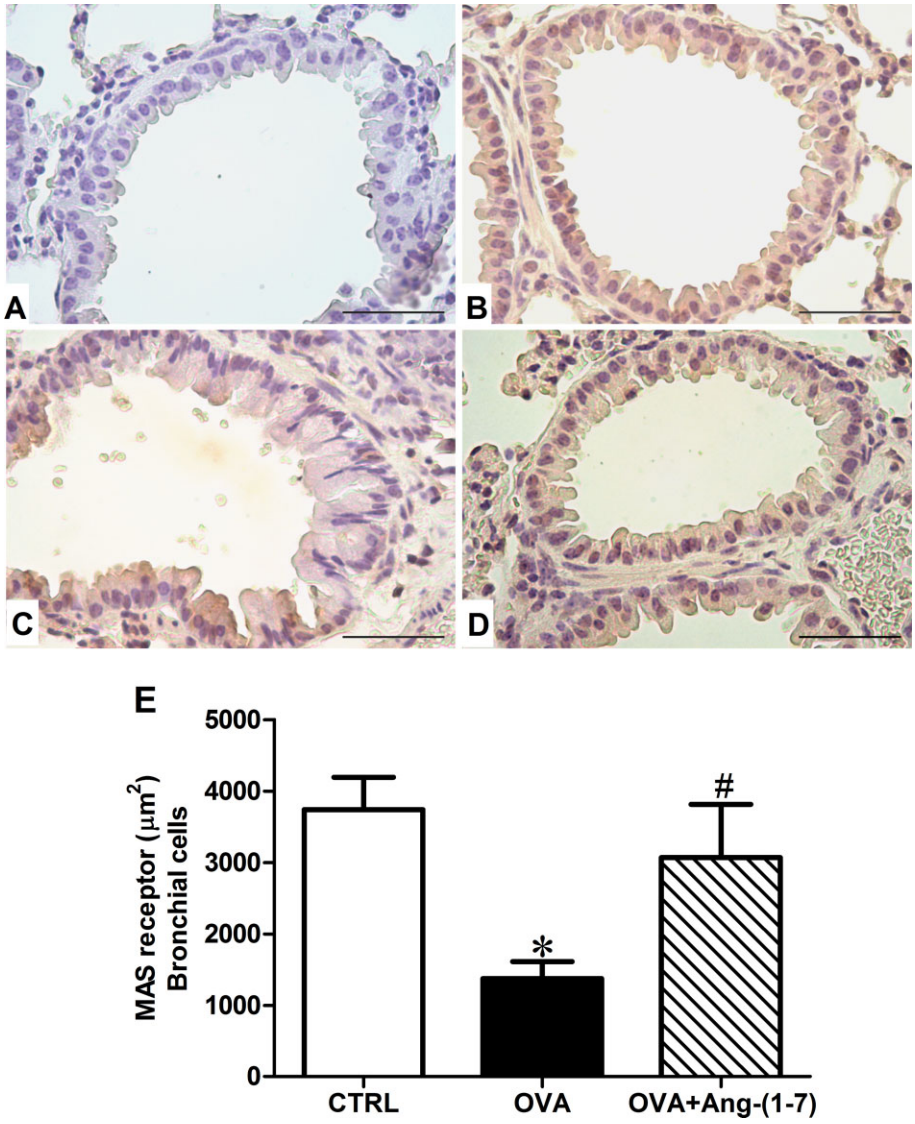

Figure 9

Immunohistochemical staining for Mas receptor in the airway of CTRL (B), OVA (C) and OVA + Ang-(1-7) (D) mice. (A) Negative control. (B) CTRL group showing brown positive staining for Mas receptor in bronchial epithelium and smooth muscle. (C) OVA group showing a reduction in brown staining in bronchial epithelium and smooth muscle. (D) OVA + Ang-(1-7) group showing brown positive staining for Mas receptor in bronchial epithelium and smooth muscle. Counterstaining with haematoxylin. Bar = 40 μm. (E) Quantification of stained area (μm^2). *$P < 0.05$, significantly different from CTRL; #$P < 0.05$, significant effect of Ang-(1-7); one-way ANOVA followed by Newman–Keuls test.

Acknowledgements

The authors are thankful to Jose Roberto da Silva, Marilene Luzia Oliveira and Dr Angelica T Vieira for technical assistance. This study was funded by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) through INCT-Nanobiofar and Programa de Núcleos de Excelência (PRONEX-#CBB-APQ-04758-10); Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). G. S. M. is a recipient of CNPq PhD fellowship at the Post-graduation Program in Biological Sciences: Physiology and Pharmacology, UFMG.

Author contributions

G. S. M.: Conception and design, acquisition of data, analysis and interpretation of data, drafting the article, revised and approved the final version of the manuscript. M. G. R. M. and R. A. S. S.: Analysis and interpretation of data, edited and revised the manuscript, approved the final version of the manuscript. D. M. S., A. R. S., L. P. O. and S. C. A.: Acquisition of data, analysis and interpretation of data and approved the final version of the manuscript. P. R. M. R., M. V. C. and L. S. B.: Analysis and interpretation of data and approved the final version of the manuscript. M. J. C. S.: Conception and design, analysis and interpretation of data, edited and revised

the manuscript, revised and approved the final version of the manuscript.

Conflict of interest

No conflict of interest exists.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.13057

Figure S1 Protein expression of JNK phosphorylated in the lung measured by Western blotting.
Table S1 Sequence of the primers used to perform qRT-PCR.
Appendix S1 Additional methods and results.