ERJ Express. Published on July 1, 2010 as doi: 10.1183/09031936.00008109

Collagen remodelling by airway smooth muscle is resistant to steroids and β_2-agonists

Jane E. Bourke, Xin Li, Simon R. Foster, Edmund Wee, Hayat Dagher, James Ziogas, Trudi Harris, John V. Bonacci and Alastair G. Stewart.

Department of Pharmacology, University of Melbourne, Victoria, Australia 3010

Author for correspondence/reprint requests:

Dr Jane Bourke

Department of Pharmacology,

University of Melbourne,

Victoria, Australia 3010

Email: janeew@unimelb.edu.au

Telephone: 61 3 8344 5622 Fax: 61 3 8344 0241

Short title: Collagen remodelling by airway muscle

Word count: approx 4200

Copyright 2010 by the European Respiratory Society.

ABSTRACT

Bi-directional interactions between airway smooth muscle (ASM) and the altered extracellular matrix (ECM) may influence airway wall remodelling and ASM function in asthma. We have investigated the capacity of cultured human ASM to reorganize the structure of three-dimensional collagen gels and the effects of endothelin-1 and agents used to treat asthma.

Human ASM cells were cast in type I collagen gels. Reductions in gel area over 72 hr were determined in the absence and presence of endothelin-1 and potential inhibitors, steroids and β_2-adrenoceptor agonists. Changes in gel wet weights and hydroxyproline content were measured, and ASM gel morphology was examined by scanning electron microscopy.

Cell density-dependent reductions in gel area were augmented by endothelin-1, mediated via ET_A receptors. This process was not associated with ASM contraction or proliferation, but was consistent with ASM tractional remodelling and migration leading to collagen condensation rather than collagen degradation within gels. The collagen remodelling by ASM was unaffected by salbutamol and/or budesonide.

This study demonstrates an additional potential role for ASM in ECM regulation and dysregulation in airways disease that is resistant to steroids and β_2-adrenoceptor agonists. Therapy-resistant collagen condensation within ASM bundles may facilitate ECM-ASM interactions and contribute to increased internal airways resistance.

Key words: asthma, airway smooth muscle, collagen, endothelin-1, glucocorticoid, steroid resistance

Abbreviations:

AHR, airways hyperresponsiveness; ASM, airway smooth muscle; AWR, airway wall remodelling; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulphoxide; ECM, extracellular matrix; EDTA, ethylene diamine tetra-acetic acid; ET-1, endothelin-1; FBS, foetal bovine serum; GCS, glucocorticoid; MAPK, mitogen-activated protein kinase; MMP, matrix metalloprotease; PBS, phosphate-buffered saline; PI3K, phosphoinositide 3-kinase; TGFβ, transforming growth factor β

Prominent features of airway wall remodelling (AWR) in asthma include the accumulation of airway smooth muscle (ASM) and an expansion and alteration in the composition of extracellular matrix (ECM), including an increased abundance of collagen type I [1, 2]. Increased bi-directional interactions between ASM and the altered pericellular ECM may occur due to these changes. These interaction may be influenced by endothelin-1 (ET-1), since ET-1 can increase both ASM proliferation and collagen secretion, in addition to its better characterized potent bronchoconstrictor actions [3].

The contribution of ASM to fibrogenic changes in ECM synthesis and turnover in asthma has been regarded as minor compared to its contractile role mediating airways hyperresponsiveness (AHR) [4, 5]. In contrast, the contribution of airway fibroblasts to sub-epithelial fibrosis in AWR is well documented [6, 7], with differentiation to a myofibroblast phentoype associated with increased collagen synthesis [8]. However, phenotypic modulation of ASM cells from a contractile to a synthetic-proliferative state [5, 9] is also associated with release of an array of pro-inflammatory cytokines and chemokines and the secretion of multiple ECM proteins, including collagen [4].

Even if the contribution of ASM to the ECM bulk is modest, ECM can influence critical ASM functions implicated in asthma. *In vitro* evidence suggests that degradation of the pericellular ECM environment of the ASM could reduce muscle load to facilitate increased muscle shortening [10] while an expanded and stiffer ECM has been associated with reduced distensibility of the airways [11].

In addition, the altered ECM surrounding ASM could achieve significance through autocrine/paracrine influences on non-contractile ASM functions including proliferation, migration and synthesis of secretory products. Culture of ASM on

collagen type I has been shown to induce a greater proliferative response to a variety of mitogens and to increase production of eotaxin, RANTES, and GM-CSF [12-15].

The capacity of glucocorticoids (GCS) to modulate ASM-ECM interactions may also be changed in the presence of an altered ECM. GCS can exert antiproliferative, antimigratory and antiinflammatory effects on both fibroblasts and ASM [16, 17], but the steroid sensitivity of these responses is impaired in the presence of denatured (non-fibrillar) type I collagen [13]. In addition, increased ECM production in response to profibrotic mediators is also steroid-resistant [18].

ASM-ECM interactions may also lead to collagen remodelling through cell-mediated reorganization of the surrounding matrix structure. This process has been extensively studied using human foetal lung (HFL-1) and adult bronchial fibroblasts seeded in three-dimensional type I collagen gels [19-21] as a model of both wound healing and tissue remodelling. Fibroblast-induced reductions in gel area are generally described as gel "contraction", and can be accelerated or increased in magnitude by diverse mediators including ET-1 and transforming growth factor β (TGFβ) [19-21]. Of particular relevance is the observation that increase in collagen density mediated by fibroblasts can be further augmented by glucocorticoids (GCS) [20].

Although ASM has also been shown to cause reductions in collagen gel area [19], neither the mechanism for this process nor its regulation by asthma mediators or GCS has been explored in detail. Given the potential influence of ASM-ECM interactions to contribute to changes in ASM synthetic and contractile function implicated in airway disease, we have used type I collagen gels seeded with human ASM to investigate the potential of these cells to contribute to remodelling of the surrounding ECM. The effects of ET-1 and histamine (HA) on this process have been

assessed in the presence of various inhibitors and selective receptor antagonists to explore the mechanisms underlying ASM-dependent gel contraction. The effects of GCS and β_2-adrenoceptor agonists have also been examined to explore potential modulation of collagen remodelling by agents used in the treatment of asthma.

METHODS

Cell culture

Human ASM cultures were generated from bronchi from macroscopically normal airways, resected from lung transplant recipients or donors and from pneumonectomy specimens. ASM was microdissected from the bronchus wall and enzymatically digested with collagenase (1 mg/ml) and elastase (0.5 mg/ml) [22].

Cells were maintained at 37^{o}C in 5% CO_2 in air in Dulbecco's Modified Eagles Medium (DMEM) (with 2 mM L-glutamine, 0.25 % BSA, 100 U/ml penicillin G, 100 μg/ml streptomycin, 2 μg/ml amphotericin B and 10 % v/v foetal bovine serum (FBS)). Cells passaged weekly at a 1:4 split ratio were used between passages 3-14.

Measurement of calcium mobilization

The effects of ET-1 (0.1 – 100 nM) or histamine (0.1 – 100 μM) on intracellular calcium levels were assessed as previously described [23]. ASM were plated at a density of 2x10^4 cells/ml (4000 cells/well) in 96 well plates and when >90% confluent, DMEM was removed and cells washed twice with HBS buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4.7H_2O$, 10 mM D-glucose, 10 mM HEPES, free acid, 2 mM $CaCl_2.2H_2O$, 2.5 mM probenecid, 37^{o}C, pH 7.4). The buffer was then replaced with HBS containing 1 μM Fluo4 for 60 minutes at 35^{o}C, before washing. Changes in intracellular [Ca^{2+}] evoked by increasing concentrations of agonist were measured over a 2 min period using the Flexstation II (Molecular Devices).

Preparation of collagen gels

Flasks of confluent ASM were serum-deprived for 72 hr in incomplete medium (FCS-free DMEM, 0.25 % w/v BSA) to cause growth arrest. Cells were then displaced with

0.5% trypsin, collected and centrifuged (1500 rcf, 5 min), before resuspension at cell densities of up to 3.75 x 10^6 cells/ml in 4X concentrated incomplete DMEM. The cell suspension was immediately mixed thoroughly with fibrillar Type I collagen (1.6 mg/ml dialysed rat tail tendon collagen extracted as previously described [24], at 4^o C) in a 1:3 ratio and gels were cast in 24 well culture plates (0.5 ml gel/well, 37°C, 15 min). Once set, gels were dislodged by adding 1 ml incomplete DMEM, transferred to 6 well plates, suspended freely floating in 3 ml incomplete DMEM and incubated for up to 72 hrs.

Drug treatments and measurement of gel area

The effects of ET-1 (0.1 – 10 nM) or histamine (0.01 – 100 μM) on ASM-mediated changes in gel area were assessed. The ET-1 antagonists BQ123 (ET_A-selective) and BQ788 (ET_B-selective) were used to ascertain whether ET-1 was mediating gel contraction via ET_A or ET_B receptors respectively. The activity of these agents was evaluated at 0.1 μM - 10 μM, as this concentration range spanned the published pA_2 values of 6.9-7.4 for BQ123 and 6.9 for BQ788 [25]. Inhibitors of protein synthesis (cycloheximide, 1μg/ml), phosphoinositide 3-kinase (PI3-K, LY294002, 20 μM), p38 mitogen-activated protein kinase (p38 MAPK, SB203580, 30 μM) and MEK 1/2 (U0126, 50 μM) were added at concentrations known to cause inhibition in airway smooth muscle (reviewed in [5]). The effects of cytochalasin D (100 nM) and latrunculin A (2 μM) were assessed at concentrations shown to inhibit actin polymerization within collagen gels [26]. cAMP-elevating agents, 8-bromo-cAMP (300 μM) and salbutamol (10-1000 nM), and glucocorticooids, dexamethasone (10-1000 nM) and budesonide (100 nM), were also tested at appropriate concentrations [13, 22]. All potential inhibitors were added 30 min prior to ET-1 (10 nM) where applicable.

ASM gel areas were initially determined by the diameter of 24 well culture

plates in which they were cast. Areas were then measured at intervals up to 72 hrs with an image analyzer system (Kodak Image Station 440CF). The coefficient of variation determined by repeated measures of area of the same gel was less than 3%.

Scanning electron microscopy of ASM gels

ASM cells in collagen gels fixed in 2.5% glutaraldehyde in 0.1M PBS for 2 hr were rinsed (3 x 15 min) and postfixed in 1% osmium tetroxide for 1 hr. After rinsing (PBS, 3 x 15 min), specimens were dehydrated in a graded ethanol series (30, 50, 70, 90, 100% ethanol in water, 20 min each), critically point dried in a Bal-Tec 030 CPD, and mounted onto 25 mm aluminium stubs with double-sided carbon tabs. Samples were gold-coated in an Edwards S150B sputter coater and imaged in a Philips XL30 field-emission scanning electron microscope at 2kV.

Measurements of ASM density using MTT assays

ASM-containing gels were incubated with 3-[4, 5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, final concentration 1mg/ml, overnight, 37^{o}C), washed (2 x PBS) and digested with collagenase (0.25 mg/ml, 37^{o}C, 2 hr). After centrifugation (10,000xg, 5 min), 400 μl DMSO was added to the cell pellet to solubilize the blue formazan product, which was then quantified by reading absorbance at 550 nm (method modified from [27]). The linear relationship between increasing cell density and absorbance measured in gels seeded with ASM at densities of up to 3.75 x 10^6 cells/ml was maintained after 72 hr (supplementary fig. A).

Measurements of supernatant MMP-2 activity

Gel supernatants were assayed for proteolytic MMP activity by gelatin zymography, using conditioned media from human cultured ASM as an internal control. Image

capture and densitometry were performed using Kodak 1D software (Eastman Kodak Company, NY, USA) as previously described [28].

Measurements of gel weights and hydroxyproline content

Gels were collected to determine wet weights, and acid lysates of freeze-dried gels were subsequently assayed for total hydroxyproline as an index of collagen content as previously described [29].

Measurement of ASM migration

Migration of ASM was assessed with a wound assay [30]. Cells were grown to confluence in 24 well plates, then serum-starved in incomplete media for 24 hr before treatment with 200nM Mitotracker Green (Molecular Probes for 2h). The cells were washed twice with PBS and fresh incomplete media added. The cell layer was scraped with a pipette tip and cells were then incubated in the absence and presence of ET-1 (10 nM) for 18 hr. The wound edge was viewed and photographed using live cell imaging (Leica DMI 6000B) at 30 minute intervals. Changes in wound area with time were analysed using VideoSavant^{TM} software (IO Industries).

Statistical analysis

All data were expressed as the mean ± SEM of the response from n different primary cultures, each from a different donor, and analysed using Graph Pad Prism^{TM} software version 4.0. Effects of time and either cell density or drug concentration were tested by two-way repeated measures ANOVA on raw area values. Effects of inhibitors were examined by paired t-tests or by one-way repeated measures ANOVA, followed by Bonferroni *post hoc* tests for multiple comparisons. Differences were considered to be statistically significant when $p<0.05$.

RESULTS

ASM-mediated collagen remodelling is time- and cell-density dependent

To determine whether ASM could mediate collagen remodelling, gels were prepared in the absence and presence of ASM cells at varying numerical density. There was no change in area of cell-free collagen gels over 72 hrs (fig. 1a). Reductions of up to 50% in area were evident in gels prepared using ASM at increasing cell densities ranging from 0.625 – 3.75 x 10^6 cells/ml (fig. 1a).

The morphology of ASM within gels was examined using scanning electron microscopy. Multiple interactions between ASM and surrounding collagen fibres were evident immediately after gels were cast (t=0, fig. 2). Although ASM cells initially appeared rounded, they were clearly elongated after 72 hr (fig. 2). Increased density of collagen fibres was evident within the gels with time (fig. 2, upper panels) and with increasing ASM density (fig. 2, lower panels).

Subsequent studies were performed at an ASM density of 2.5 x 10^6 cells/ml, at which initial gel areas were reduced by 13 ± 3% within the first 4 hr ($n = 19$, $p<0.05$ in comparison with $t = 0$). This density was chosen to permit assessment of augmentation or inhibition of sub-maximal reductions in gel area.

Collagen remodelling is augmented by ET-1 via ET_A receptors, but may be independent of active muscle contraction

To determine the potential contribution of active contraction of ASM to ASM-mediated reductions in gel area, the effects of ET-1 and histamine were assessed. To demonstrate that functional receptors for these contractile agonists were expressed, their effects on calcium mobilization were measured in cultured ASM. Increases in intracellular calcium were evident within minutes in the presence of both ET-1 and

histamine over the concentration ranges tested in the collagen gel assay, with ET-1 being more potent (fig. 3a).

Despite both agonists inducing calcium mobilization, only ET-1 (0.1 – 10 nM) augmented collagen remodelling in a concentration-dependent manner, (fig. 3b, 2-way ANOVA, $p<0.05$) while histamine (0.01- 100 μM) had no effect (fig. 3c).

The receptor-dependence of ET-1 responses were examined, using the selective antagonists, BQ123 and BQ788, which display >10,000 fold selectivity for ET_A and ET_B receptors respectively [25] (fig. 3d). The antagonists did not affect gel area in the absence of ET-1 (data not shown). The response to 10 nM ET-1 (gel area at t = 72 hr: 42 ± 3% initial area, n = 5) was prevented by preincubation with the ET_A antagonist BQ123 (0.1 μM, $p<0.05$ in comparison with ET-1 alone). In contrast, BQ788 was only effective at 10 μM, a concentration 100-fold greater than its pA_2 value at the ET_B receptor.

Collagen remodelling is not associated with ASM proliferation

The potential contribution of ongoing ASM proliferation within gels was assessed, measuring MTT metabolism as a marker of cell density. In gels seeded with ASM at an initial density of 2.5×10^5 cells/ml, absorbance values for conversion of MTT were not altered during collagen remodelling (2.3±0.4 at t=0 in comparison with 2.7±0.6 at t=72 hr, n=8, $p>0.05$, paired t-test). Although the reduction in gel area after 72 hr was greater in the presence of ET-1, absorbance values were not increased (2.0±0.5 for control in comparison with 1.6±0.4 for ET-1, n=8, $p>0.05$, paired t-test).

Collagen remodelling may be associated with ASM migration

Wound assays in the absence and presence of ET-1 were performed to determine

whether ASM migration could contribute to reductions in gel area when ASM were seeded within collagen gels, 1. The reduction in wound area under control conditions over 18 hr was increased by with ET-1 treatment in three of the four ASM cultures tested (fig. 4a).

To examine the dependence of gel contraction on the assembly of monomeric actin into actin filaments, gels were incubated in the presence of cytochalasin D and latrunculin A. Both inhibitors of actin polymerization inhibited gel contraction, with cytochalasin D reducing the change in gel area under control conditions by 42±11% ($n=4$, $p<0.05$). Latrunculin A was more effective, completely abolishing changes in gel area with time in the absence and presence of ET-1 (fig 4b).

The signalling pathways associated with reductions in gel area were also assessed. Inhibition of p38 MAPK by SB203580 and of PI3K by LY294002 partially inhibited reductions in gel area under control conditions by 69±21% and 31±7% respectively ($n=4$, $p<0.05$, paired t-test). The augmented response to ET-1 was still evident in the presence of both inhibitors, while the MEK1/2 inhibitor U0126 had no detectable effect on collagen remodelling in the absence or presence of ET-1 (data not shown).

Collagen remodelling is associated with collagen condensation not degradation

Given the significant reduction in area of untreated ASM gels with time, changes in gel weights were also measured. There was a significant decrease in gel wet weight (gel volume) after 72 hr by 60 ± 11% in control gels ($p<0.01$ in comparison with $t=0$, fig. 5a), This was further decreased to a loss in wet weight of 72 ± 8% with ET-1 treatment ($p<0.01$ in comparison with control, fig. 5a).

To determine if the reduced weight was due to loss of collagen as well as water,

the hydroxyproline content of ASM gels was assayed. Initial hydroxyproline content averaged 7.4 ± 0.4% of the dry gel weight (n = 4). Hydroxyproline could not be detected in the supernatants collected at 72 hrs, and the content within gels was not changed with time or with ET-1 treatment (fig. 5b).

The role of MMPs in regulation of collagen remodelling was then examined. Both latent and active MMP-2 activity, but not MMP-9 activity, was detected in ASM gel supernatants collected at 72 hrs (fig. 5c). There was no detectable increase in MMP activity in gel supernatants in the presence of ET-1 (n = 8, fig. 5c). Reductions in ASM gel area with time were not inhibited by the non-selective MMP inhibitor, ilomastat (fig. 5d).

Collagen remodelling is not modulated by agents used to treat asthma

The potential regulation of gel contraction by agents that relax airway smooth muscle and by glucocorticoids were also assessed. Preincubation with the β_2-adrenoceptor agonist salbutamol did not affect the rate or extent of the reduction in gel area in control or ET-1-treated gels (fig. 6a). The lack of effect of this cAMP-elevating agent was consistent with results obtained using the stable analogue 8-bromo-cAMP (fig. 6b). At concentrations up to 1000 nM, the GCS dexamethasone did not modulate the extent of gel contraction in the absence or presence of ET-1 (table 1). Preincubation with budesonide alone or in combination with salbutamol did not affect the reduction in gel area in control or ET-1-treated gels (fig. 6c).

DISCUSSION

In the present study, reductions in the area of three-dimensional type I collagen gels seeded with ASM were cell-density dependent and increased by ET-1 acting via ET_A receptors. This process did not appear to involve myocyte contraction, since it occurred over a much longer time-course than is usually associated with smooth muscle shortening and force development. In addition, responses were not mimicked by histamine or prevented by agents that relax airway smooth muscle. Interactions between ASM and the surrounding matrix were associated with increasing collagen fibre density rather than collagen degradation, consistent with tractional remodelling as ASM migrate through the collagen gel. This collagen remodelling was resistant to treatment with glucocorticoids, and could contribute to increased collagen density in bundles of ASM *in situ* in asthmatic airways. This process has the potential to influence diverse airway smooth muscle functions, including proliferation, migration and cytokine production, in addition to changing the physical properties of the cells to oppose both muscle shortening and lengthening.

Cell-mediated collagen gel contraction has been widely used as an *in vitro* model to study tissue remodelling and wound healing by fibroblasts in the context of fibrotic diseases [31, 32]. Very few studies have examined ASM-mediated remodelling of collagen gels [19, 33]. Given the potential for changes in ASM-ECM interactions to influence ASM contractile and synthetic function in airways disease, we sought to explore this *in vitro* process and the potential mechanisms involved in its regulation.

The area of floating collagen gels seeded with ASM spontaneously reduced within several hours in the absence of an exogenous contractile stimulus. In studies using fibroblast gels, this area reduction has been attributed to the isometric tension

applied to the collagen fibrils as the fibroblasts develop a myocyte-like shortening [34]. However, in the current study, the progressive reduction in ASM gel area occurring over several days in the absence of exogenous stimuli was not consistent with acute ASM shortening. Although this apparent "contraction" increased with ASM density, scanning electron microsopy in contracted gels showed that the appearance of cells within gels remained elongated irrespective of the extent of the reductions in gel area.

Despite evidence that ET-1 and histamine, both contractile agonists of ASM, could elicit calcium mobilization in cultured ASM within minutes, the reduction in ASM gel area was augmented by ET-1, but not by histamine. These data suggest that the ET-1-induced collagen remodelling may also be independent of active contraction of ASM within collagen gels. In support of this, the response to ET-1 was mediated via the ET_A receptor, rather than the ET_B receptor responsible for ASM contraction [35]. In addition, neither the stable cAMP analogue 8-bromo cAMP nor the β-adrenoceptor agonist salbutamol that act as functional antagonists to oppose ASM contraction were able to inhibit the response. Limits to diffusion of these agents into the gels do not appear to explain this lack of effect, since reductions in gel area were shown to be inhibited by preincubation with various chemically dissimilar agents, and the response to ET-1 could be inhibited by the selective ET_A antagonist BQ123.

The finding that histamine did not elicit a reduction in ASM gel area was contrary to a previous report [36] where a 10-20% maximal reduction in gel area in response to 100 μM histamine was evident within 20 minutes. However, in the protocol used by Matsumoto and colleagues, gels were either left attached in the casting plate overnight (so-called "pre-stressed" gels) or had already achieved the maximum ASM-mediated reduction prior to drug addition. In the current study, the initial gel area was

not stabilised before histamine was added so that a small acute response to histamine may have not been detected if it was occurring simultaneously upon release of the newly cast gels. Nevertheless, the ongoing progressive reductions in gel area either in the absence or presence of ET-1 of up to 75% over several days provide evidence that active muscle contraction is not the principle mechanism for the long-term remodelling of the collagen matrix observed.

As described here with ASM gels and in previous studies using fibroblasts [31], increased cell density resulted in greater collagen remodelling. A contribution of ASM proliferation is possible, since denatured (non-fibrillar) type I collagen has been shown to enhance ASM proliferation [13, 14]. However, significant reductions in ASM gel area occurred within 24 hrs, much earlier the population doubling time of ASM of 36-48 hr [27]. Furthermore, we have shown that the fibrillar type I collagen matrix used in these gel contraction experiments is anti-mitogenic [37].

ET-1 has been reported to elicit a small increase in DNA synthesis in ASM via ET_A receptors, but appears to require co-mitogens for a significant increase in cell number [22, 38]. In the current study, the gel remodelling response to ET-1 was not sensitive to inhibition of the PI3K or ERK pathways that are required for ASM proliferation [5]. Moreover, cell numbers and viability were stable over the duration of the experiments regardless of the presence of ET-1. Therefore, it appears that collagen remodelling within gels is independent of ASM proliferation.

Alternative mechanisms for reductions in the area of collagen gels include gel dehydration and/or collagen breakdown [31, 39]. In the current study, scanning electron micrographs of contracted ASM gels showed condensation of collagen fibrils around the cells. Since the reductions in gel wet weights with time were not accompanied by

reductions in hydroxyproline content, it is unlikely that the collagen fibrils were undergoing degradation. A previous study also using ASM seeded in collagen gels did not detect ECM degradation unless ASM were co cultured with monocytes or incubated with neutrophil elastase [33].

An alternative mechanism suggested for cell-mediated contraction of gels is the tractional remodelling of the collagen lattices occurring as a consequence of cell migration through the matrix, as has been described for fibroblasts [32, 40]. Consistent with this, we have demonstrated the capacity of ASM cultures used in the gel experiments to migrate in a wound assay. The potential contribution of migration to ASM-mediated collagen remodelling is supported by the finding that the spontaneous reduction in gel area in this study was partially inhibited by SB203580, since the p38 MAPK pathway has previously been implicated in ASM migration [41]. Critically, gel contraction was also prevented by inhibitors of actin polymerization that disrupt the cytoskeletal elements required for ASM traction and migration [42].

Remodelling of ASM collagen gels may be dependent on MMP activity to release cells from their matrix connections, as has been reported for fibroblast gels [43]. Although MMP-2 activity was detected in supernatants from ASM gels, the non-selective MMP inhibitor ilomastat did not inhibit ASM-mediated reductions in gel area. Although ET-1 has been shown to upregulate the expression of MMPs in association with vascular remodelling [44], enhanced collagen remodelling of ASM gels with ET-1 was not associated with an increase in MMP-2 activity. The findings suggest that MMP activity is not a requirement for collagen remodelling by ASM.

Given the potential influence that ASM-mediated collagen remodelling may have on diverse ASM cell functions in the airways, it was of interest to determine

whether ASM gel contraction is modulated by agents used to treat asthma. The finding that salbutamol, which causes airway smooth muscle relaxation, was ineffective was consistent with the evidence already presented that reductions in ASM gel area were due to collagen remodelling rather than acute ASM shortening. However, if the condensation of the collagen gels does require tractional remodelling and migration by ASM, an inhibitory effect of salbutamol may have been anticipated, since it has previously been reported that agents that mobilize cAMP inhibit migration of human ASM [45]. The lack of effect on reductions in ASM gel area requires further investigation to determine whether signalling by β_2-adrenoceptor agonists is impaired in ASM embedded in a 3-dimensional collagen matrix, as has been reported for GCS signalling when ASM were grown on collagen [13].

Neither dexamethasone nor budesonide regulated reductions in ASM gel area. The lack of effect of this drug class on ASM-mediated collagen remodelling is consistent with other studies examining steroid modulation of diverse ASM functions. GCS do not regulate ECM production by ASM in response to profibrotic mediators [18], and their inhibitory effects on ASM proliferation and migration are impaired in the presence of collagen, due to increased alpha2beta1 integrin signalling [13]. However, the findings in ASM collagen gels are in contrast with studies showing that HFL-1 gel contraction is augmented by budesonide or hydrocortisone [20, 46]. Although fibroblasts and ASM have a common action in mediating collagen remodelling, it is possible that differential regulation of this process in lung mesenchymal cells may determine their relative contributions to ongoing remodelling in health and disease.

In conclusion, this study demonstrates an additional potential role for ASM in the dynamic regulation of ECM and its dysregulation in airways disease that is resistant

to the effects of both glucocorticoids and β_2-adrenoceptor agonists. This cell-dependent process could increase the collagen density in bundles of ASM *in situ*, and further facilitate ASM-ECM interactions, with the potential for profound effects on airway function. Ultimately, ASM-mediated gel contraction may provide a useful screen for identification of selective agents to prevent cell-mediated collagen remodelling in lung diseases including asthma.

Acknowledgements

We thank Professor John Wilson and the anatomical pathologists at the Alfred Hospital for facilitating the provision of human lung samples, Shenna Langenbach for preliminary assistance with hydroxyproline measurements and Dr Simon Crawford for assistance with preparation of samples for electron microscopy. This work was supported by the National Health and Medical Research Council [Grants 299823, 509001, 509239]; Contributing to Australian Scholarship and Science (CASS) Foundation; ANZ Medical Research and Technology in Victoria Fund. Australian Academy of Science and Asthma Foundation of Victoria.

References

1. Stewart AG, Tomlinson PR, Wilson J. Airway wall remodelling in asthma: a novel target for the development of anti-asthma drugs. *Trends Pharmacol Sci* 1993; 14: 275-9.
2. James A. Remodelling of airway smooth muscle in asthma: what sort do you have? *Clin Exp Allergy* 2005; 35: 703-7.
3. Hay DW. Putative mediator role of endothelin-1 in asthma and other lung diseases. *Clin Exp Pharmacol Physiol* 1999; 26: 168-71.
4. Howarth PH, Knox AJ, Amrani Y*, et al.* Synthetic responses in airway smooth muscle. *J Allergy Clin Immunol* 2004; 114: S32-50.
5. Hirst SJ, Martin JG, Bonacci JV*, et al.* Proliferative aspects of airway smooth muscle. *J Allergy Clin Immunol* 2004; 114: S2-17.
6. Roche WR, Beasley R, Williams JH, Holgate ST. Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* 1989; 1: 520-4.
7. Brewster CE, Howarth PH, Djukanovic R*, et al.* Myofibroblasts and subepithelial fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol* 1990; 3: 507-11.
8. Schmitt-Graff A, Desmouliere A, Gabbiani G. Heterogeneity of myofibroblast phenotypic features: an example of fibroblastic cell plasticity. *Virchows Arch* 1994; 425: 3-24.
9. Halayko AJ, Solway J. Molecular mechanisms of phenotypic plasticity in smooth muscle cells. *J Appl Physiol* 2001; 90: 358-68.
10. Bramley AM, Roberts CR, Schellenberg RR. Collagenase increases shortening of human bronchial smooth muscle in vitro. *Am J Respir Crit Care Med* 1995; 152: 1513-7.
11. Wilson JW, Li X, Pain MC. The lack of distensibility of asthmatic airways. *Am Rev Respir Dis* 1993; 148: 806-9.
12. Freyer AM, Johnson SR, Hall IP. Effects of growth factors and extracellular matrix on survival of human airway smooth muscle cells. *Am J Respir Cell Mol Biol* 2001; 25: 569-76.
13. Bonacci JV, Schuliga M, Harris T, Stewart AG. Collagen impairs glucocorticoid actions in airway smooth muscle through integrin signalling. *Br J Pharmacol* 2006; 149: 365-73.
14. Nguyen TT, Ward JP, Hirst SJ. beta1-Integrins mediate enhancement of airway smooth muscle proliferation by collagen and fibronectin. *Am J Respir Crit Care Med* 2005; 171: 217-23.
15. Peng Q, Lai D, Nguyen TT*, et al.* Multiple beta 1 integrins mediate enhancement of human airway smooth muscle cytokine secretion by fibronectin and type I collagen. *J Immunol* 2005; 174: 2258-64.
16. Stewart AG, Fernandes D, Tomlinson PR. The effect of glucocorticoids on proliferation of human cultured airway smooth muscle. *Br J Pharmacol* 1995; 116: 3219-26.
17. Ward JE, Harris T, Bamford T*, et al.* Proliferation is not increased in airway myofibroblasts isolated from asthmatics. *Eur Respir J* 2008; 32: 362-71.
18. Burgess JK, Oliver BG, Poniris MH*, et al.* A phosphodiesterase 4 inhibitor inhibits matrix protein deposition in airways in vitro. *J Allergy Clin Immunol* 2006; 118: 649-57.

19. Liu X, Kohyama T, Wang H*, et al.* Th2 cytokine regulation of type I collagen gel contraction mediated by human lung mesenchymal cells. *Am J Physiol Lung Cell Mol Physiol* 2002; 282: L1049-56.
20. Wen FQ, Skold CM, Liu XD*, et al.* Glucocorticoids and TGF-beta1 synergize in augmenting fibroblast mediated contraction of collagen gels. *Inflammation* 2001; 25: 109-17.
21. Shi-Wen X, Chen Y, Denton CP*, et al.* Endothelin-1 promotes myofibroblast induction through the ETA receptor via a rac/phosphoinositide 3-kinase/Akt-dependent pathway and is essential for the enhanced contractile phenotype of fibrotic fibroblasts. *Mol Biol Cell* 2004; 15: 2707-19.
22. Tomlinson PR, Wilson JW, Stewart AG. Inhibition by salbutamol of the proliferation of human airway smooth muscle cells grown in culture. *Br J Pharmacol* 1994; 111: 641-7.
23. Grange RL, Ziogas J, North AJ*, et al.* Selenosartans: novel selenophene analogues of milfasartan and eprosartan. *Bioorg Med Chem Lett* 2008; 18: 1241-4.
24. Elsdale T, Bard J. Collagen substrata for studies on cell behavior. *J Cell Biol* 1972; 54: 626-37.
25. Davenport AP, Battistini B. Classification of endothelin receptors and antagonists in clinical development. *Clin Sci (Lond)* 2002; 103 Suppl 48: 1S-3S.
26. Wakatsuki T, Schwab B, Thompson NC, Elson EL. Effects of cytochalasin D and latrunculin B on mechanical properties of cells. *J Cell Sci* 2001; 114: 1025-36.
27. Hirst SJ, Barnes PJ, Twort CH. Quantifying proliferation of cultured human and rabbit airway smooth muscle cells in response to serum and platelet-derived growth factor. *Am J Respir Cell Mol Biol* 1992; 7: 574-81.
28. Ward JE, Fernandes DJ, Taylor CC*, et al.* The PPARgamma ligand, rosiglitazone, reduces airways hyperresponsiveness in a murine model of allergen-induced inflammation. *Pulm Pharmacol Ther* 2006; 19: 39-46.
29. Langenbach SY, Wheaton BJ, Fernandes DJ*, et al.* Resistance of fibrogenic responses to glucocorticoid and 2-methoxyestradiol in bleomycin-induced lung fibrosis in mice. *Can J Physiol Pharmacol* 2007; 85: 727-38.
30. Kimura C, Oike M, Koyama T, Ito Y. Alterations of Ca2+ mobilizing properties in migrating endothelial cells. *Am J Physiol Heart Circ Physiol* 2001; 281: H745-54.
31. Bell E, Ivarsson B, Merrill C. Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. *Proc Natl Acad Sci U S A* 1979; 76: 1274-8.
32. Grinnell F. Fibroblast-collagen-matrix contraction: growth-factor signalling and mechanical loading. *Trends Cell Biol* 2000; 10: 362-5.
33. Zhu YK, Liu X, Wang H*, et al.* Interactions between monocytes and smooth-muscle cells can lead to extracellular matrix degradation. *J Allergy Clin Immunol* 2001; 108: 989-96.
34. Roy P, Petroll WM, Chuong CJ*, et al.* Effect of cell migration on the maintenance of tension on a collagen matrix. *Ann Biomed Eng* 1999; 27: 721-30.
35. Goldie RG, Henry PJ, Knott PG*, et al.* Endothelin-1 receptor density, distribution, and function in human isolated asthmatic airways. *Am J Respir Crit Care Med* 1995; 152: 1653-8.

36. Matsumoto H, Moir LM, Oliver BG*, et al.* Comparison of gel contraction mediated by asthmatic and non-asthmatic airway smooth muscle cells. *Thorax* 2007.
37. Schuliga M, See I, Ong S*, et al.* Fibrillar collagen clamps lung mesenchymal cells in a non-proliferative and non-contractile phenotype. *Am J Respir Cell Mol Biol.* 2009: doi:10.1165/rcmb.2008-0361OC.
38. Panettieri RA, Jr., Goldie RG, Rigby PJ*, et al.* Endothelin-1-induced potentiation of human airway smooth muscle proliferation: an ETA receptor-mediated phenomenon. *Br J Pharmacol* 1996; 118: 191-7.
39. Fang Q, Liu X, Al-Mugotir M*, et al.* Thrombin and TNF-alpha/IL-1beta synergistically induce fibroblast-mediated collagen gel degradation. *Am J Respir Cell Mol Biol* 2006; 35: 714-21.
40. Ehrlich HP, Rajaratnam JB. Cell locomotion forces versus cell contraction forces for collagen lattice contraction: an in vitro model of wound contraction. *Tissue Cell* 1990; 22: 407-17.
41. Hedges JC, Dechert MA, Yamboliev IA*, et al.* A role for p38(MAPK)/HSP27 pathway in smooth muscle cell migration. *J Biol Chem* 1999; 274: 24211-9.
42. Gerthoffer WT. Migration of airway smooth muscle cells. *Proc Am Thorac Soc* 2008; 5: 97-105.
43. Phillips JA, Vacanti CA, Bonassar LJ. Fibroblasts regulate contractile force independent of MMP activity in 3D-collagen. *Biochem Biophys Res Commun* 2003; 312: 725-32.
44. Ergul A, Portik-Dobos V, Giulumian AD*, et al.* Stress upregulates arterial matrix metalloproteinase expression and activity via endothelin A receptor activation. *Am J Physiol Heart Circ Physiol* 2003; 285: H2225-32.
45. Goncharova EA, Billington CK, Irani C*, et al.* Cyclic AMP-mobilizing agents and glucocorticoids modulate human smooth muscle cell migration. *Am J Respir Cell Mol Biol* 2003; 29: 19-27.
46. Skold CM, Liu XD, Zhu YK*, et al.* Glucocorticoids augment fibroblast-mediated contraction of collagen gels by inhibition of endogenous PGE production. *Proc Assoc Am Physicians* 1999; 111: 249-58.

Table 1 Effect of dexamethasone on gel areas in control and 10 nM ET-1-treated ASM gels (n=4, 72 hr, mean ± SEM). Initial areas were 206 ± 5 mm^2. *p<0.05 in comparison with control in the absence of dexamethasone.

		Dexamethasone (nM)			
		0	**10**	**100**	**1000**
Gel area (mm^2)	**Control**	90 ± 18	93 ± 17	99 ± 17	95 ± 17
	ET-1	54 ± 5	49 ± 3*	48 ± 5*	49 ± 6*

Figure Legends

FIGURE 1. Collagen gel area is reduced with a), b) time and b) cell density in ASM gels. Gel areas showing effect of ASM cell density (0.625 – 3.75 x 10^5 cells/ml) over 72 hr are expressed as mean ± SEM (n=5) with error bars only shown for selected data sets for clarity. Two-way ANOVA with repeated measures, $p<0.001$ for time, $p<0.05$ for cell density.

FIGURE 1

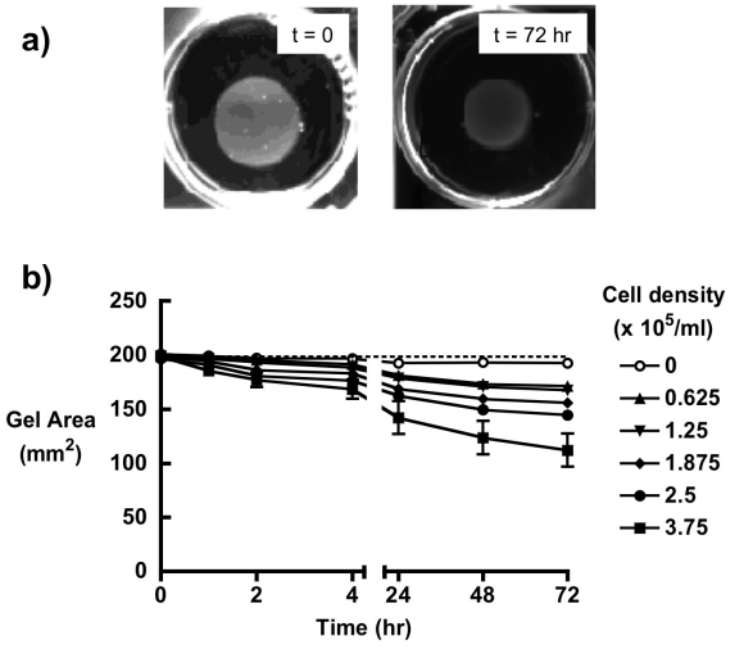

FIGURE 2. Collagen density is increased with time and ASM cell density. Scanning electron micrographs of collagen gels seeded with ASM were prepared as described in Methods. Upper panels have scale bars of 10 μm for gels seeded at an ASM density of

2.5 x 10^5 cells/ml for t = 0 and 72 hr. Lower panels have scale bars of 100 μm for gels seeded at ASM densities of 0.625 x 10^5 cells/ml (low) and 3.75 x 10^5 cells/ml (high), both at t=72 hr.

FIGURE 2

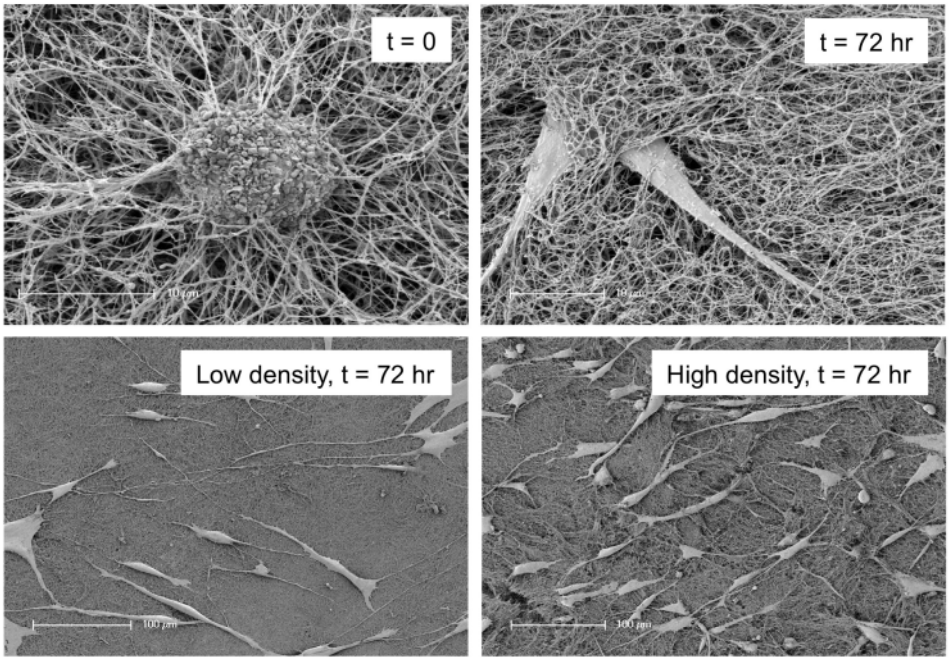

FIGURE 3. ET-1, but not histamine, increases ASM gel contraction. a) Effect of ET-1 (0.1-10 nM) and histamine (0.01-10 μM) on calcium mobilization in cultured ASM. Relative fluorescence units (RFU) in response to 2 minutes incubation with agonists are expressed as mean ± SEM (n=4). b) c) Effect of ET-1 (0.1-10 nM) and histamine (0.01-100 μM) on collagen gels seeded with ASM ($2.5x10^5$ cells/ml). Gel areas over 72 hr are expressed as mean ± SEM (ET-1 n=5, 2-way ANOVA, $p<0.001$; histamine n=4). Error bars only shown for selected data sets for clarity. b) Effect of preincubation with ET-1 antagonists BQ123 (ET_A-selective) or BQ788 (ET_B-selective) (0.1-10μM) on reduction in gel area with ET-1 (10 nM). Changes in gel area after 24 hr are expressed as mean ± SEM (n=5) from initial areas of 203 ± 3 mm^2. *$p<0.05$ in comparison with control, $^{\#}p<0.05$ in comparison with ET-1 alone.

FIGURE 3

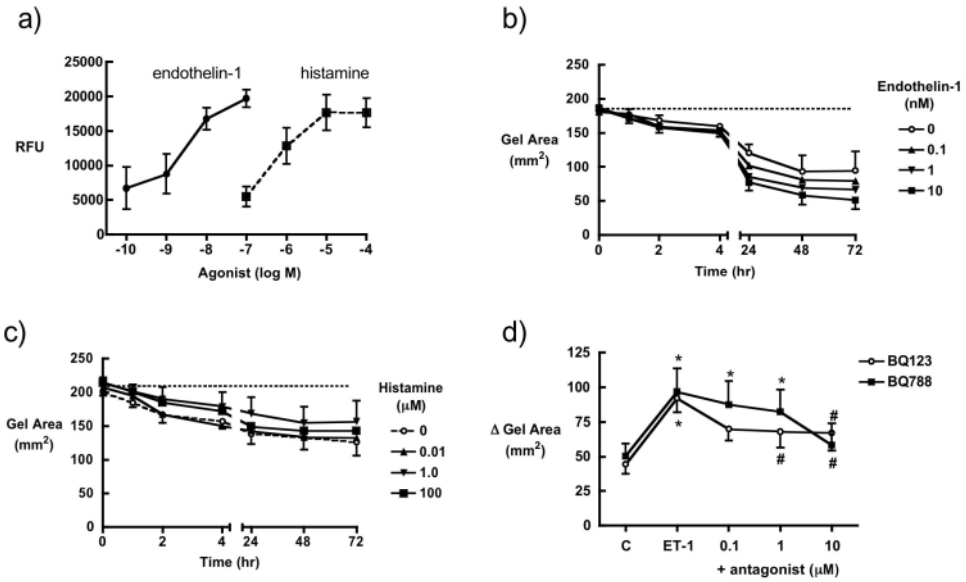

FIGURE 4. Effect of ET-1 on ASM migration, and effects of inhibitors of actin polymerization on ASM gel contraction. a) Using a wound assay, migration of ASM with time were assessed in the absence and presence of 10 nM ET-1. Data are expressed

as % reduction in wound area over 18 hr (mean ± SEM, n=4). b) ASM gel areas were measured in the absence and presence of 10 nM ET-1 following 30 min preincubation with latrunculin A (Lat A, 2 μM, n=4). Initial gel areas were 197 ± 2 mm^2. Gel areas at 24 hrs are expressed as mean ± SEM. *$p<0.05$ in comparison with control, $^{\#}p<0.05$ in comparison with ET-1.

FIGURE 4

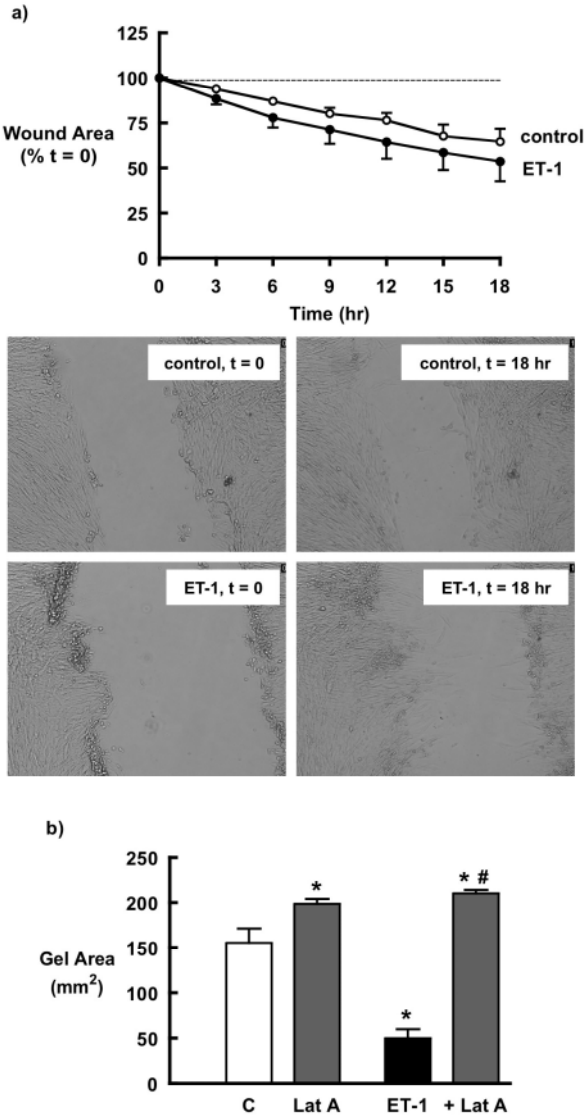

FIGURE 5. Effect of ASM and ET-1 on gel weight and hydroxyproline content, and supernatant MMP activity. Gels and supernatants were collected after 72 hr incubation in the absence and presence of 10 nM ET-1. a) Wet weight and b) hydroxyproline

content were measured at t = 0 (n = 4) or after 72 hr (n = 14). c) Supernatants were assayed for MMP activity by gelatin zymography. Activity is expressed as ratio of densitometry values for either latent or active MMP-2 in the presence and absence of ET-1 (10 nM, n=8). Conditioned media from human cultured ASM was used as an internal control. d) The effect of ilomastat (30 μM) on gel areas (n=6). All data are expressed as mean ± SEM. *$p<0.05$ in comparison with t = 0, $^{\#}p<0.05$ in comparison with control.

FIGURE 5

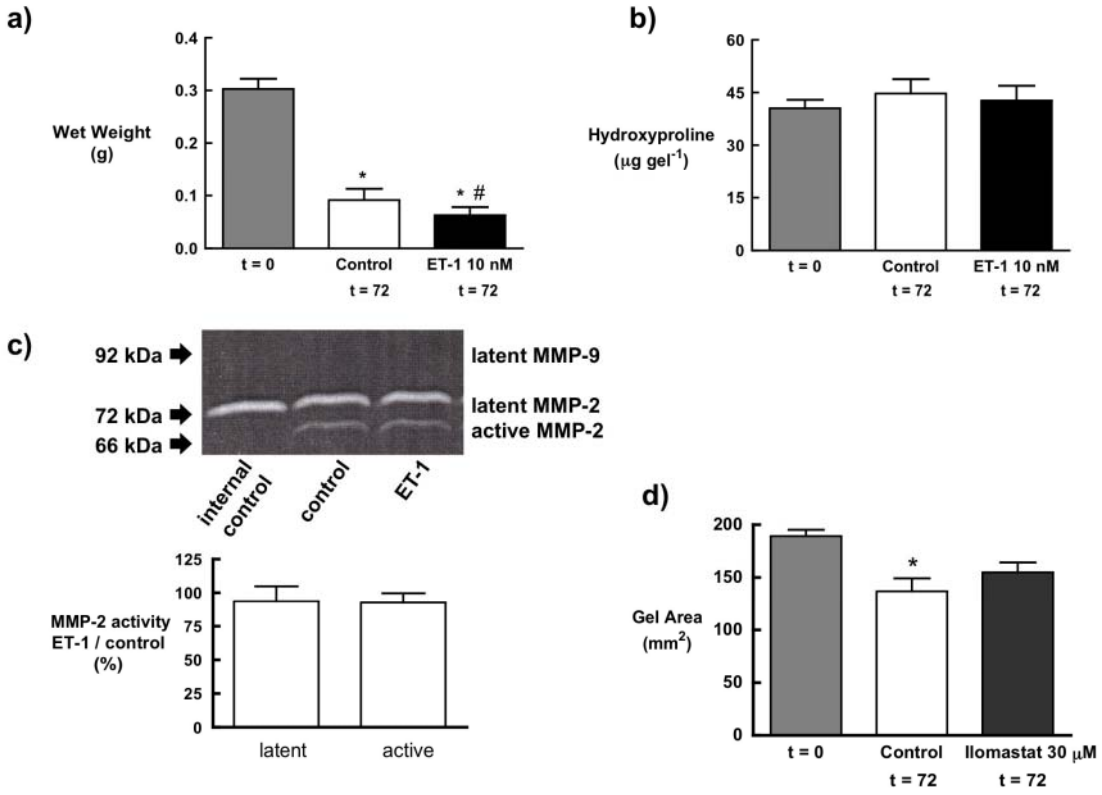

FIGURE 6. The effect of agents used to treat asthma on gel contraction. Collagen gel areas were measured in the absence (control) or presence of a) salbutamol (10-100 nM) (n=3), b) 8-bromo cAMP (300 μM, 24 hr, n = 3) or c) budesonide (100 nM) and/or

salbutamol (100 nM) (24 hr, n = 6). Gels were also preincubated with the same agents for 30 min prior to 10 nM ET-1. Gel areas are expressed as mean ± SEM. *p<0.05 in comparison with t = 0, #p<0.05 in comparison with control.