1 **Online Data Supplement**

2 **Comparison of gel contraction mediated by asthmatic and non-asthmatic airway**

3 **smooth muscle cells**

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17 **Online Data Supplement: Methods**

18 **Study population and cell culture**

19 ASM cells were obtained from 9 non-asthmatic and 8 asthmatic patients and were
20 propagated as previously described [1]. Approval for all experiments using human lung
21 cells was provided by the Human Ethics Committees of the University of Sydney and
22 the South West Sydney Area Health Service. Characteristics of ASM cells were
23 determined by immuno-fluorescence and light microscopy (Fig. E1). Cells from
24 passages 3 to 8 were grown to confluence using Dulbecco's modified Eagle's medium
25 (DMEM) supplemented with 5% FBS and were harvested by trypsin digestion and used
26 for experiments.
27

28 **Collagen gel contraction assay using ASM**

29 A collagen-gel contraction assay was used to examine the contractile capacity of ASM
30 cells. Harvested cells were resuspended in DMEM containing 0.1% BSA at a density of
31 5 x 10^5 cells/ml. A collagen solution (3 mg/ml) was made on ice by mixing rat tail
32 collagen type I (BD Biosciences, NSW, Australia) with 1 x PBS, 1N NaOH and 0.02N
33 acetic acid according to the manufacturer's instructions. The collagen solution was
34 mixed with the cell suspension 1:1 to yield a final collagen concentration of 1.5 mg/ml.
35 The collagen suspension containing the ASM cells, 0.6 ml (1.5 x 10^5 cells), was cast in
36 one well of a 24-well culture plate and allowed to polymerize (30 min, 37°C) in a
37 humidified 5% CO_2 incubator. Once polymerized the lateral surface of a gel was
38 carefully detached from the culture well and transferred into a 6-well plate containing 3

39 ml of DMEM with 0.1% BSA. Gels containing ASM cells contracted spontaneously
40 after detachment from the casting plates, approaching a plateau at 12 hours (Fig. E2). To
41 avoid the initial contraction, which was thought to reflect cytoskeletal reorganization or
42 stress fiber formation [2] rather than agonist-induced actomyosin-driven contraction *per*
43 *se*, floating gels were equilibrated overnight in 6-well plates containing 3 ml of DMEM
44 with 0.1% BSA (37°C), and then the medium was changed to modified Krebs solution
45 before being stimulated (method 1, Fig 1A). For a comparison between non-asthmatic
46 and asthmatic ASM cells, gels stimulated immediately after detachment (method 2, Fig
47 1A) were also examined. For method 2, collagen gels, once polymerized, were
48 overlayed with 0.4 ml of DMEM with 0.1% BSA and incubated overnight in 24-well
49 casting plates without being detached. Stimulation was performed no later than 5
50 minutes after detachment from 24-well plates and floated in 6-well plates.

51 During gel contraction the culture plate was placed on a flat-bed scanner
52 (Microtek. Inc. Taiwan) in a non-humidified oven (Thermoline Scientific. Pty. Ltd.
53 NSW, Australia) maintained at 37°C. Gels were scanned automatically every 2 min over
54 20 min. The surface area of each gel was then measured using Image J
55 (http://rsb.info.nih.gov/ij/) and gel contraction was measured at each time point as the
56 ratio of treated gel area to untreated gel area (control) to account for any change in gel
57 area not caused by the treatment.
58

59 **Reagents and antibodies**

60 Modified Krebs Henseleit physiological salt solution containing 58.44 mM NaCl, 74.55
61 mM KCl, 147 mM $CaCl_2.2H_2O$, 203.3 mM $MgCl_2.6H_2O$, 180.16 mM glucose, and
62 238.3 mM HEPES was used during stimulation of the gels. Contraction induced by

63 histamine 100 μM was not altered by carbogenation (5% CO_2 in O_2), therefore all
64 experiments were performed in the absence of carbogenation.
65 Histamine, carbachol, adenosine 5'-triphosphate (ATP), mepyramine,
66 formoterol, prostaglandin E_2 and inhibitors of phospholipase C (PLC) (U73122),
67 MLCK (ML-7), and Rho-associated coiled-coil forming kinase (ROCK) (Y27632) were
68 purchased from Sigma (St Louis, MO). Formoterol, prostaglandin E_2, U73122 and
69 ML-7 were diluted in ethanol: 0.01%, 0.0025%, 1%, and 0.15%, respectively. Other
70 compounds were diluted in distilled water for stock.
71 Mouse monoclonal anti-α-smooth muscle actin FITC conjugated antibody
72 (clone 1A4) and mouse anti-myosin light chain kinase (MLCK) antibody (clone K36)
73 were from Sigma, mouse anti-GAPDH antibody from CHEMICON (Temecula, CA),
74 and goat anti-mouse horseradish peroxidase-conjugated secondary antibody from
75 DakoCytomation (Carpinteria, CA).
76

77 **Viability assay**

78 Viability and cell number in the gels was estimated using
79 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazo
80 lium (MTS) (Promega, NSW, Australia). A total of 25 μl of the ASM cell-containing
81 collagen solution was cast into one well of a 96-well plate and 75 μl of DMEM with
82 0.1% BSA was added after gel polymerization, before incubation at 37°C overnight.
83 After one hour incubation with 20 μl of MTS, absorption at 490 nm was measured with
84 spectrophotometer. Experiments were done in duplicate. Trypan blue exclusion test was
85 also used to examine cell viability.
86

87 **Western Blotting**

88 Protein expression of smooth muscle MLCK (smMLCK) was analyzed by Western
89 blotting and enhanced chemiluminescence. To obtain protein from airway smooth
90 muscle (ASM) cells embedded in collagen gels the cells were harvested by digesting the
91 collagen with collagenase (600 units/ml, Sigma) for 30 min at 37°C. However, this
92 process of cell extraction resulted in degradation of smMLCK. Therefore, smMLCK
93 protein content was measured for ASM cells seeded (2 x 10^4/cm^2) onto collagen-coated
94 6-well plates (5 μg/cm^2) and left overnight before being lysed in a buffer containing 1
95 mM EDTA, 20 mM Tris, 10% proteinase inhibitor (Calbiochem Inc., San Diego, CA),
96 0.5% Triton X-100. After centrifugation (10,000 g, 2 min), the supernatant was
97 collected. Protein concentration was determined using a bicinchoninic acid protein
98 assay kit (Sigma) and 10 μg of protein/lane was applied to an 8% SDS-polyacrylamide
99 gel. Proteins were transferred to polyvinylidene difluoride membranes and blocked in
100 PBS containing 0.05% Tween-20 and 1% skimmed-milk powder. Membranes were
101 incubated with mouse anti-MLCK antibody (1:20,000) in blocking solution for 2 h at
102 room temperature. Secondary goat anti-mouse antibody conjugated with horseradish
103 peroxidase (1:4,000) was incubated for 1 h at room temperature and visualized by
104 enhanced chemiluminescence (SuperSignal® West Dura Extended Duration Substrate;
105 Pierce Biotechnology Inc., Rockford, IL). As an internal control, the expression of
106 GAPDH was examined after stripping the membrane with 0.1 M 2-mercaptoethanol,
107 2% SDS and 62.5 mM Tris-HCl (pH 6.7) for 20 min at 50°C. The stripped membranes
108 were again blocked and incubated with mouse anti-GAPDH (1:6,000), followed by
109 incubation with secondary antibody (1:16,000) and visualized by enhanced
110 chemiluminescence. Experiments were done in duplicate.

111 **Online Data Supplement: References**

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113 1. Johnson PR, Roth M, Tamm M, *et al.* Airway smooth muscle cell proliferation is
114 increased in asthma. *Am J Respir Crit Care Med* 2001; **164**:474-7.
115 2. Kuzuya M, Cheng XW, Sasaki T, *et al*. Pitavastatin, a
116 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor, blocks vascular smooth
117 muscle cell populated-collagen lattice contraction. *J Cardiovasc Pharmacol* 2004;
118 **43**:808-14.

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120 **Online Data Supplement: Figure legends**

121 Figure E1. Representative non-asthmatic and asthmatic airway smooth muscle cells
122 stained with FITC conjugated anti-α-smooth muscle actin antibody.

123

124 Figure E2. Time course showing gel contraction without exogenous stimulation. Gels
125 containing airway smooth muscle cells contracted and approached a plateau at 12 hours
126 (n = 3 different patients, solid line), while gels without cells did not contract (n = 3,
127 dotted line).

128

129 Figure E3. A) Gel contraction to histamine, 1, 10 and 100 μM (n = 6 different patients).
130 Contraction curves to histamine 1, 10 and 100μM were significantly different from that
131 of untreated gels ($p < 0.0001$). A significant difference was also observed between
132 contraction curves to 1μM and 100μM histamine ($p = 0.003$). Results are shown as
133 contraction (% decrease in gel area). B) Inhibition of histamine (100 μM)-induced gel
134 contraction by mepyramine (1 μM; n = 6). C) Relaxation of histamine (100
135 μM)-induced gel contraction by formoterol (1 μM; n = 4). Contraction curves to
136 histamine treated with mepyramine or formoterol were significantly different from that
137 generated to histamine alone ($p \leq 0.05$). D) Inhibition of histamine (100 μM)-induced
138 gel contraction by prostaglandin E_2 (100 nM, 1 μM, 10 μM; n = 4). Curves to histamine
139 treated with each concentration of prostaglandin E_2 were all significantly different from
140 that of histamine alone ($p < 0.001$). Results are shown as % decrease in gel area relative
141 to control untreated gels (FigE3ABD). For Fig E3C, gel area at 20 min was set as 100%.

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