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Comparison of gel contraction mediated by asthmatic and non-asthmatic airway smooth muscle cells

Hisako Matsumoto, Lyn M Moir, Brian G G Oliver, Janette K Burgess, Michael Roth, Judith L Black, Brent E McParland

Correspondence;
Hisako Matsumoto

Discipline of Pharmacology, University of Sydney, NSW 2006, Australia
Tel: 61-2-935-16735
Fax: 61-2-935-13868

Present address
Department of Respiratory Medicine, Kyoto University, Kyoto, Japan

e-mail: hmatsumo@kuhp.kyoto-u.ac.jp, BrentMcParland@med.usyd.edu.au
Online Data Supplement: Methods

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

Collagen gel contraction assay using ASM
A collagen-gel contraction assay was used to examine the contractile capacity of ASM cells. Harvested cells were resuspended in DMEM containing 0.1% BSA at a density of 5 x 10⁵ cells/ml. A collagen solution (3 mg/ml) was made on ice by mixing rat tail collagen type I (BD Biosciences, NSW, Australia) with 1 x PBS, 1N NaOH and 0.02N acetic acid according to the manufacturer’s instructions. The collagen solution was mixed with the cell suspension 1:1 to yield a final collagen concentration of 1.5 mg/ml. The collagen suspension containing the ASM cells, 0.6 ml (1.5 x 10⁵ cells), was cast in one well of a 24-well culture plate and allowed to polymerize (30 min, 37°C) in a humidified 5% CO₂ incubator. Once polymerized the lateral surface of a gel was carefully detached from the culture well and transferred into a 6-well plate containing 3
ml of DMEM with 0.1% BSA. Gels containing ASM cells contracted spontaneously after detachment from the casting plates, approaching a plateau at 12 hours (Fig. E2). To avoid the initial contraction, which was thought to reflect cytoskeletal reorganization or stress fiber formation [2] rather than agonist-induced actomyosin-driven contraction per se, floating gels were equilibrated overnight in 6-well plates containing 3 ml of DMEM with 0.1% BSA (37°C), and then the medium was changed to modified Krebs solution before being stimulated (method 1, Fig 1A). For a comparison between non-asthmatic and asthmatic ASM cells, gels stimulated immediately after detachment (method 2, Fig 1A) were also examined. For method 2, collagen gels, once polymerized, were overlayed with 0.4 ml of DMEM with 0.1% BSA and incubated overnight in 24-well casting plates without being detached. Stimulation was performed no later than 5 minutes after detachment from 24-well plates and floated in 6-well plates.

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

**Reagents and antibodies**

Modified Krebs Henseleit physiological salt solution containing 58.44 mM NaCl, 74.55 mM KCl, 147 mM CaCl₂·2H₂O, 203.3 mM MgCl₂·6H₂O, 180.16 mM glucose, and 238.3 mM HEPES was used during stimulation of the gels. Contraction induced by
Histamine 100 µM was not altered by carbogenation (5% CO$_2$ in O$_2$), therefore all experiments were performed in the absence of carbogenation.

Histamine, carbachol, adenosine 5’-triphosphate (ATP), mepyramine, formoterol, prostaglandin E$_2$ and inhibitors of phospholipase C (PLC) (U73122), MLCK (ML-7), and Rho-associated coiled-coil forming kinase (ROCK) (Y27632) were purchased from Sigma (St Louis, MO). Formoterol, prostaglandin E$_2$, U73122 and ML-7 were diluted in ethanol: 0.01%, 0.0025%, 1%, and 0.15%, respectively. Other compounds were diluted in distilled water for stock.

Mouse monoclonal anti-α-smooth muscle actin FITC conjugated antibody (clone 1A4) and mouse anti-myosin light chain kinase (MLCK) antibody (clone K36) were from Sigma, mouse anti-GAPDH antibody from CHEMICON (Temecula, CA), and goat anti-mouse horseradish peroxidase-conjugated secondary antibody from DakoCytomation (Carpinteria, CA).

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

Protein expression of smooth muscle MLCK (smMLCK) was analyzed by Western blotting and enhanced chemiluminescence. To obtain protein from airway smooth muscle (ASM) cells embedded in collagen gels the cells were harvested by digesting the collagen with collagenase (600 units/ml, Sigma) for 30 min at 37°C. However, this process of cell extraction resulted in degradation of smMLCK. Therefore, smMLCK protein content was measured for ASM cells seeded (2 x 10^4/cm^2) onto collagen-coated 6-well plates (5 μg/cm^2) and left overnight before being lysed in a buffer containing 1 mM EDTA, 20 mM Tris, 10% proteinase inhibitor (Calbiochem Inc., San Diego, CA), 0.5% Triton X-100. After centrifugation (10,000 g, 2 min), the supernatant was collected. Protein concentration was determined using a bicinchoninic acid protein assay kit (Sigma) and 10 μg of protein/lane was applied to an 8% SDS-polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membranes and blocked in PBS containing 0.05% Tween-20 and 1% skimmed-milk powder. Membranes were incubated with mouse anti-MLCK antibody (1:20,000) in blocking solution for 2 h at room temperature. Secondary goat anti-mouse antibody conjugated with horseradish peroxidase (1:4,000) was incubated for 1 h at room temperature and visualized by enhanced chemiluminescence (SuperSignal® West Dura Extended Duration Substrate; Pierce Biotechnology Inc., Rockford, IL). As an internal control, the expression of GAPDH was examined after stripping the membrane with 0.1 M 2-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl (pH 6.7) for 20 min at 50°C. The stripped membranes were again blocked and incubated with mouse anti-GAPDH (1:6,000), followed by incubation with secondary antibody (1:16,000) and visualized by enhanced chemiluminescence. Experiments were done in duplicate.
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Figure E1. Representative non-asthmatic and asthmatic airway smooth muscle cells stained with FITC conjugated anti-α-smooth muscle actin antibody.

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

Figure E3. A) Gel contraction to histamine, 1, 10 and 100 µM (n = 6 different patients). Contraction curves to histamine 1, 10 and 100 µM were significantly different from that of untreated gels (p < 0.0001). A significant difference was also observed between contraction curves to 1 µM and 100 µM histamine (p = 0.003). Results are shown as contraction (% decrease in gel area). B) Inhibition of histamine (100 µM)-induced gel contraction by mepyramine (1 µM; n = 6). C) Relaxation of histamine (100 µM)-induced gel contraction by formoterol (1 µM; n = 4). Contraction curves to histamine treated with mepyramine or formoterol were significantly different from that generated to histamine alone (p ≤ 0.05). D) Inhibition of histamine (100 µM)-induced gel contraction by prostaglandin E₂ (100 nM, 1 µM, 10 µM; n = 4). Curves to histamine treated with each concentration of prostaglandin E₂ were all significantly different from that of histamine alone (p < 0.001). Results are shown as % decrease in gel area relative to control untreated gels (FigE3ABD). For Fig E3C, gel area at 20 min was set as 100%.