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Bidirectional Counterregulation of Human Lung Mast Cell and Airway Smooth Muscle β2 Adrenoceptors

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Human lung mast cells (HLMCs) play a central role in asthma pathogenesis through their relocation to the airway smooth muscle (ASM) bundles. β2 adrenoceptor (β2-AR)-agonists are used to relieve bronchoconstriction in asthma, but may reduce asthma control, particularly when used as monotherapy. We hypothesized that HLMC and human ASM cell (HASMC) responsiveness to β2-AR agonists would be attenuated when HLMCs are in contact with HASMCs. Cells were cultured in the presence of the short-acting β2-agonist albuterol, and the long-acting β2-agonists formoterol and olodaterol. Constitutive and FceRI-dependent HLMC histamine release, HASMC contraction, and β2-AR phosphorylation at Tyr590 were assessed. Constitutive HLMC histamine release was increased in HLMC–HASMC coculture and this was enhanced by β2-AR agonists. Inhibition of FceRI-dependent HLMC mediator release by β2-AR agonists was greatly reduced in HLMC–HASMC coculture. These effects were reversed by neutralization of stem cell factor (SCF) or cell adhesion molecule 1 (CADM1). β2-AR agonists did not prevent HASMC contraction when HLMCs were present, but this was reversed by fluticasone. β2-AR phosphorylation at Tyr590 occurred within 5 min in both HLMCs and HASMCs when the cells were cocultured, and was inhibited by neutralizing SCF or CADM1. HLMC interactions with HASMCs via CADM1 and Kit inhibit the potentially beneficial effects of β2-AR agonists on these cells via phosphorylation of the β2-AR. These results may explain the potentially adverse effects of β2-AR agonists when used for asthma therapy. Targeting SCF and CADM1 may enhance β2-AR efficacy, particularly in corticosteroid-resistant patients. The Journal of Immunology, 2016, 196: 55–63.

Asthma is a common, chronic, and persistent disorder that accounts for significant morbidity and mortality (1–4). Approximately 10% of patients have asthma that is resistant to current therapies (2, 3), and this group consumes 50–60% of health care costs attributed to asthma, reflecting a considerable unmet clinical need.

β2-Adrenoceptor (β2-AR) agonists are an important component of asthma therapy. In fast-acting (rapid-onset) form they are used to provide relief from bronchoconstriction, and in long-acting form are used as preventer medication in conjunction with inhaled corticosteroids (ICS) (5). β2-AR agonists principally target the airway smooth muscle (ASM) to induce bronchodilatation, and confer bronchoprotection against bronchoconstrictor stimuli. However, in many patients the bronchodilator response to a β2-AR agonist is poor (6), and there is a loss of bronchodilator activity during acute asthma exacerbations (7). Many studies have also indicated that the regular use of β2-AR agonists in the absence of an ICS, and sometimes in the presence of an ICS, have deleterious effects in patients with asthma. Thus regular β2-AR agonist use may enhance airway hyperresponsiveness (8–11) and eosinophilic airway inflammation (12, 13), accelerate lung function decline (14), reduce asthma control (15–17), and potentially contribute to asthma deaths in both short-acting and long-acting form (15, 17, 18). The mechanism(s) contributing to a poor therapeutic response and potential adverse effects on airflow function are poorly understood (18). Targeting these mechanisms would have the potential to enhance both the efficacy and safety of β2-AR agonists in many patients.

Human lung mast cells (HLMCs) are fundamental to asthma pathogenesis (19). Importantly in asthma, increased mast cell numbers are found in the ASM bundles (20) where they are activated (21) and have the potential to interact intimately with ASM cells (22–25). Human ASM cells (HASMCs) maintain HLMC survival and induce HLMC proliferation through a co-operative interaction between membrane-bound stem cell factor (SCF) on HASMCs, and the SCF receptor Kit and cell adhesion molecule 1 (CADM1) expressed on HLMCs (23). CADM1 is a key molecule that facilitates initial HLMC–HASMC adhesion, and may present Kit to membrane-bound SCF (23, 26). HASMCs increase “constitutive” HLMC degranulation and the release of mediators such as histamine and tryptase (23). Tryptase release induces HASMC
TGF-β1 release, which results in increased HASMC α-smooth muscle actin expression and contractility (24). β2-AR agonists applied acutely inhibit FceRI-dependent HLMC mediator release both in vitro (27, 28) and in vivo (29). However, chronic β2-agonist administration in asthmatic patients results in a loss of the protective effects of β2-agonists on both HLMCs and HASMCs and may actually enhance allergen-induced bronchoconstriction and associated HLMC mediator release (9, 10, 30). Loss of protection may occur in part due to β2-AR desensitization, but the mechanisms that enhance the response to allergen challenge and lead to loss of asthma control with chronic β2-AR dosing are unexplained.

HLMCs cultured in the presence of SCF lost their sensitivity to β2-AR agonists as a result of phosphorylation of the β2-AR at Tyr350 (31). In parallel, there was a dose-dependent increase in HASMC and may actually enhance allergen-induced bronchoconstriction and associated HLMC mediator release (9, 10, 30). Loss of protection may occur in part due to β2-AR desensitization, but the mechanisms that enhance the response to allergen challenge and lead to loss of asthma control with chronic β2-AR dosing are unexplained.

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Materials and Methods

Human subjects

All subjects were recruited from the Glenfield Hospital (Leicester, U.K.) and gave written informed consent. The use of lung resection tissue was approved by the National Research Ethics Service (reference number 07/MRE08/42), and bronchoscopic studies were approved by the Leicester- refere to the previous section.

Materials

The following items were obtained from the sources indicated: sodium bicarbonate (7.5%; Life Technologies); collagen (3 mg/ml; INAMED Biomaterials); sulbutamol hemisulfate salt, formoterol fumarate dihydrate, sodium pyruvate, antibiotic-antimycotic, and DMSO (Sigma-Aldrich, Poole, U.K.); DMEM, 10× DMEM, nonessential amino acids, Dynabeads, and prolong anti-fade mounting medium (Invitrogen, Paisley, U.K.); SCF, IL-6, IL-10, anti-SCF, and normal goat IgG isotype control (R&D Systems, Abingdon, U.K.); FCS (PromoCell, Heidelberg, Germany); PBS (Fisher Scientific, Loughborough, U.K.); anti-FceRI (Merck Millipore, MA); anti-CADM1 (9D2 anti-CADM1); Medical and Biological Laboratories, Nagoya, Japan); normal chicken IgY isotype control and anti-β2-AR (Tyr350) (Santa Cruz Biotechnology, Santa Cruz, CA); purified rabbit IgG control and polyclonal swine anti-rabbit IgG/FITC (Alere, Stockport, U.K.); olodaterol (synthesized at Boehringer Ingelheim International); and Insulin/transferring/selenium (ITS) supplement (Sigma-Aldrich).

β2-AR preparation

Albuterol and olodaterol were prepared as 10 mM stock solutions in distilled water and DMSO, respectively, and formoterol as a 1 mM stock solution in DMSO. Once resuspended, all β2-AR agonists were aliquoted and stored at −80°C.

Human airway smooth muscle isolation and culture

HASMCs were isolated from resected bronchial tissue (n = 1) or bronchoscopic biopsy specimens (n = 8 healthy, n = 14 asthmatic [severity GINA1–5]) and cultured as described previously (32). Briefly, ASM bundles were dissected free of surrounding tissue with the aid of a dissecting microscope. The small smooth muscle bundles were individually seeded onto the wells of 6-well plates and allowed to adhere before the addition of DMEM with GlutaMAX I and 4500 mg/l-glucose (Invitrogen) supplemented with 10% FCS (Invitrogen), 1% antibiotic-antimycotic (containing 100 U/ml penicillin, 100 mg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B; Sigma-Aldrich); 100 mM nonessential amino acids (Invitrogen), and 1 mM sodium pyruvate (Sigma-Aldrich). HASMCs were allowed to grow until 50% confluent and then passaged into T75 flasks. All cultures were examined for the expression of α-smooth muscle actin and myosin using flow cytometry at passage 2 to confirm a smooth muscle phenotype.

Prior to coculture and collagen gel contraction assays, HASMCs were growth arrested for 24 h in ITS medium, where 10% FCS in the medium described above is substituted with ITS supplement.

Mast cell isolation and culture

HLMCs were isolated from macroscopically normal lung tissue obtained within 1 h of resection for malignancy using positive immunomagnetic selection as described previously (33). Isolated HLMCs were immediately cultured in DMEM supplemented with 10% FCS, 100 ng/ml recombinant human SCF, 50 mg/ml IL-6, 10 mg/ml IL-10, 1% antibiotic/antimycotic, and 100 μM nonessential amino acids, as described previously (34). Final mast cell purity was >99%, and viability was >97%.

Epithelial cell isolation and culture

Primary human epithelial monolayers were grown from bronchial epithelial cells obtained at bronchoscopy as described previously (35).

Evaluation of β2-AR-agonist effects on constitutive and FceRI-dependent HLMC histamine release in coculture with HASMCs

HLMCs were seeded onto growth-arrested HASMCs at a ratio of 1:4 and incubated for 16 h in ITS medium with or without albuterol (10−5 M), formoterol (10−6 M), olodaterol (10−6 M), or appropriate vehicle controls (0.1% H2O for albuterol and 0.1% DMSO for formoterol and olodaterol). HLMC monolayers were set up in parallel in ITS medium. No exogenous cytokines were added to either mono- or cocultures. After 16 h, supernatants were collected for measurement of histamine release. For measurement of cell histamine content, cell pellets were resuspended in deionized water and frozen at −20°C. To inhibit SCF and CADM1, neutralizing anti-SCF (0.5 μg/ml) (23), anti-CADM1 (0.1 μg/ml) (23), or appropriate isotype control Abs were included for 16 h. Histamine was measured by radioenzymatic assay as described previously (33).

Assessment of HLMC morphology using electron microscopy

HASMCs and undifferentiated primary bronchial epithelial cells were plated at a density of 100,000 cells on 0.4-μm pore-sized Transwell membranes (Corning) in duplicate. Transwells were immersed in ITS ASM medium or epithelial cell medium (bronchial epithelial cell basal medium [Lonza, Basel, Switzerland]), respectively, and incubated overnight at 37°C.

HLMCs (25,000) were then seeded onto HASMC or epithelial cell monolayers and incubated in ITS or epithelial cell medium, respectively. HLMC monocultures were set up on fibronectin-coated Transwells or epithelial cell medium in duplicate. Cocultures and monocultures were incubated overnight (16 h) at 37°C. The Transwells were then fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate supplemented with 2 mM CaCl2 for 4 h at room temperature. The Transwells were stored at 4°C for 48 h in fixative diluted with sucrose buffer.

The Transwells were fixed in 1% osmium tetroxide for 90 min at room temperature, followed by three successive 15-min washes in distilled, deionized water. Samples were then dehydrated in increasing concentrations of ethanol and processed into Spurr’s resin, followed by polymerization over 16 h at 60°C. Polymerized samples were cut into 5-μm squares and remounted at 90° ready for sectioning. Embedded sections were sectioned transversely using the Leica Ultracut S ultramicrotome. Sections of 90-μm thickness were cut from each sample using a glass knife and collected onto copper mesh. Sections were then counterstained with 2% uranyl acetate for 10 min, followed by incubation in Reynold’s lead citrate for 2 min. Samples were viewed on the JEOL 1400 TEM with an accelerating voltage of 80 kV. Images were captured using the Megaview III digital camera with iTSEM software.

Ten cells from each condition were randomly photographed, and the number of degranulating granules per cell were counted independently by two blinded observers.

Collagen gel contraction assay

Spontaneous HASMC contraction was examined in collagen gels as described previously (24, 25). For each individual collagen gel, 20 μl sodium bicarbonate, 37 μl 10× concentrated DMEM, and 144 μl cell suspension
(2.5 × 10^5 cells resuspended in ITS medium either HASM alone or HLMC–HASMC cocultures incubated together at a 1:4 ratio for 15 min at 37°C before casting of gels) were added to 299 μl collagen. The resulting suspension was then cast in a single well of a 24-well plate, which had been precoated with PBS/2% BSA for 1 h at room temperature. Collagen gels were left to polymerize for 90 min at 37°C.

After polymerization, gels were detached from the well surface to allow free contraction. ITS medium, appropriate vehicle control, salbutamol (10−6 and 10−8 M), formoterol (10−7 M), or olodaterol (10−8 M) was added to appropriate wells and the gels incubated for 16 h at 37°C. Photographs were taken at 0 and 16 h, and percentage gel contraction was assessed by measuring the collagen gel surface area at each time point using ImageJ software (http://rsbweb.nih.gov/ij/, n = 5 independent experiments).

Assessment of β2-AR phosphorylation in cocultured HLMCs
HASMCS were seeded onto wells of a Lab-Tek II 8-well chamber slide (Nunc) at a density of 40,000 cells/well in ASM medium and incubated for 24 h at 37°C. After 24 h, ASM cells were washed with HBSS (Invitrogen) and growth-arrested in ITS medium for an additional 24 h at 37°C. HLMCs were then seeded onto the growth-arrested ASM monolayers at a ratio of 1:4. Cells were cocultured together for 5 min at 37°C before fixation. HLMC monolayers in ITS were set up in parallel on chambers precoated with fibronectin (40 μg/ml; Sigma-Aldrich). HASMC monocultures served as further control. Neutralizing anti-SF, anti-CADM1, and isotype control Abs were added for 15 min prior where appropriate.

Assessment of β2-AR phosphorylation at Tyr560 was analyzed using immunofluorescent staining. The anti–β2-AR-Tyr560 Ab was previously validated by Western blotting and flow cytometry (31). After incubation at 37°C, all chambers were rinsed with 200 μl PBS (Invitrogen), and the cells were fixed with 200 μl ice-cold methanol (Fisher Scientific) on ice for 20 min. Methanol was then removed from chambers, which were left to air-dry for 10 min at room temperature. Once dry, 200 μl PBS/3% BSA was added to each well for 30 min at room temperature, then removed, and followed by 200 μl appropriate primary anti–β2-AR-Tyr560 Ab (4 μg/ml; Santa Cruz Biotechnology) or isotype control (rabbit IgG, 4 μg/ml; DakoCytomation) for 90 min at room temperature.

Chambers were then rinsed three times with PBS/0.05% Tween-20 before the addition of 200 μl secondary Ab (swine anti-rabbit, 25 μg/ml, Dako) for 90 min at room temperature. Chambers were rinsed three times with PBS/0.05% Tween-20 and then a further three times with PBS. After rinsing, 200 μl DAPI (0.1 μg/ml; Sigma-Aldrich) was added to all chambers for 30 s, followed by six washes with PBS. Chambers were removed from slides, which were mounted with prolong anti-fade medium (Invitrogen) and coverslipped for assessment via fluorescence microscopy.

Original images were captured using an epifluorescent microscope (Olympus BX50, Olympus U.K.) and analyzed using Cell F image analysis software (Olympus). Microscope settings were kept constant throughout and for each experiment matched exposures were used for isotype controls. Images were converted to grayscale and mean grayscale area values indicative of β2-AR phosphorylation at Tyr560 in HLMC monoculture, ASM monoculture or in HLMC–HASMC coculture were obtained for each donor. HLMCs and HASMCs were readily identified by cellular and nuclear morphology. A minimum of 10 HLMCs and 10 HASMCs in two random high-power fields were assessed per condition in five independent experiments.

Immunostaining for trypase and α-smooth muscle actin was included as positive controls.

Statistical analysis
Data are expressed as mean ± SEM unless otherwise stated. Data were tested for normality and compared using paired or unpaired parametric or nonparametric tests with GraphPad version 5.0. All P values are two-tailed. Statistical significance was defined as *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

FIGURE 1. Constitutive histamine release in HLMC–HASMC coculture and its regulation by SCF, CADM1 and β2-AR agonists. (A) Constitutive HLMC histamine release was significantly enhanced in coculture with HASMCs, and this was inhibited marginally by blocking SCF but not CADM1. *P = 0.002 by repeated measures ANOVA, *P < 0.05, **P < 0.01 compared with HLMC monoculture (Dunnett’s multiple comparison test). *P = 0.023 compared with goat isotype control (Sidak’s multiple comparison test [MCT]). (B) Constitutive HLMC histamine release was enhanced further in coculture with HASMCs by β2-AR agonists. *P < 0.0001 compared with respective monoculture (Sidak’s MCT). 

(A–D) The enhancing effect of β2-AR agonists in coculture was attenuated by blocking either SCF (C) or CADM1 (D). *P < 0.05, **P < 0.01 compared with isotype control (Sidak’s MCT). n = 7 independent experiments for all graphs.
nonparametric tests as appropriate. Across group differences were examined using ANOVA with selected post hoc testing for individual comparisons as appropriate. For analysis of β2-AR agonist dose response in monoculture and coculture, two-way ANOVA was used. A p value < 0.05 was taken as statistically significant.

Results

HLMC and HASMC responses were similar irrespective of the source of HASMCs or their disease status. The following experiments contain HASMCs from both asthmatic and healthy donors.

HLMC–HASMC coculture enhances constitutive HLMC mediator release

Constitutive histamine release over 16 h was consistently greater in HLMC–HASMC coculture compared with HLMC monoculture (Fig. 1A, 1B). This was also evident using electron microscopy to study mast cell morphology, which demonstrated significant piecemeal degranulation in HLMC–HASMC coculture compared with HLMC monoculture or HLMC–human airway epithelial cell coculture (Supplemental Fig. 1A). HASMC-dependent HLMC histamine release was inhibited marginally by neutralizing SCF when compared with goat isotype control (p = 0.023; Fig. 1A) but not by neutralizing CADM1 (Fig. 1A).

β2-AR agonists enhance HASMC-dependent HLMC histamine release through an SCF- and CADM1-dependent mechanism

All β2-AR agonists tested significantly increased the enhanced constitutive histamine release seen in HLMC–HASMC coculture when compared with vehicle control (p = 0.0041 albuterol, p ≤ 0.0001 formoterol, p = 0.035 olodaterol) (Fig. 1B). This was particularly evident for formoterol, which increased histamine release in coculture significantly more than olodaterol (p < 0.0001) and which also significantly enhanced constitutive histamine release in HLMC monoculture compared with both DMSO control and olodaterol (p < 0.0001 for both). However, the net formoterol-dependent increase in constitutive histamine release was significantly greater in HLMC–HASMC coculture than HLMC monoculture (p = 0.001). Albuterol and olodaterol did not significantly increase constitutive release by HLMCs in monoculture.

Neutralizing either SCF or CADM1 significantly reduced β2-agonist-dependent increases in histamine release from HLMC-HASMC cocultures for all β2-agonists tested (Fig. 1C, 1D). Blocking SCF or CADM1 did not alter constitutive degranulation in monoculture.

HLMC-HASMC coculture reduces β2-AR responsiveness in FcεRI-activated HLMCs through an SCF- and CADM1-dependent mechanism

The effect of HLMC-HASMC coculture for 16 h on the responsiveness of FcεRI-activated HLMCs to olodaterol, formoterol, and albuterol was assessed (Fig. 2). Net mean ± SEM FcεRI-dependent histamine release by monocultured HLMCs in buffer, 0.1% H2O, and 0.1% DMSO vehicle controls was 17.5 ± 3.6, 16.7 ± 3.7, and 16.1 ± 4.7%, respectively. In HLMC-HASMC coculture, net mean ± SEM FcεRI-dependent histamine release in buffer, 0.1% H2O, and 0.1% DMSO vehicle controls was 14.6 ± 4.1, 16.3 ± 5.2, and 13.1 ± 3.4%, respectively (not significantly different compared with monoculture). Total cell histamine content after overnight culture prior to activation with anti-FcεRIα was 1691 ± 345 ng/10⁶ cells (monoculture) and 1786 ± 335 ng/10⁶ cells (coculture).

In HLMC monoculture, all three β2-agonists tested demonstrated dose-dependent inhibition of FcεRI-dependent histamine release (p = 0.043, two-way ANOVA) (Fig. 2A). However, the ability of albuterol, formoterol, and olodaterol to inhibit FcεRI-dependent histamine release was significantly attenuated when HLMCs were cocultured with HASMCs (p < 0.0001, two-way ANOVA) (Fig. 2A).

In further experiments, we assessed whether neutralizing SCF and CADM1 in coculture could restore the responsiveness of HLMCs to β2-ARs. Following FcεRI-dependent activation, the mean percent inhibition using albuterol (10⁻⁶ M), formoterol (10⁻⁹ M), and olodaterol (10⁻⁹ M) in HLMC monoculture was 70.8 ± 7.6, 81.2 ± 6.2, and 91.0 ± 3.1%, respectively (Fig. 2B). This inhibition was significantly reduced in HLMC–HASMC

FIGURE 2. FcεRI-dependent histamine release in HLMC–HASMC coculture and its regulation by SCF, CADM1, and β2-AR agonists. (A) Inhibition of FcεRI-dependent histamine release by β2-ARs agonists was markedly attenuated in HLMC–HASMC coculture compared with monoculture. *p = 0.043 for drug dose-responses. *p < 0.0001 for monculture versus coculture, two-way ANOVA. (B and C) The loss of HLMC β2-AR responsiveness in HLMC-HASMC coculture was partially restored by neutralizing SCF (B) or CADM1 (C). *p < 0.05, **p < 0.01 compared with coculture. *p < 0.05, ##p < 0.01 compared with isotype control (Sidak’s MCT). n = 5 independent experiments for each figure.
coculture to 6.5 ± 4.2, 2.1 ± 1.8, and 8.1±5.3%, respectively (p < 0.0001 by ANOVA and p = 0.0005, p = 0.0006, p = 0.0007 for monoculture versus coculture for each drug respectively, Sidak’s MCT; Fig. 2B). SCF neutralizing Ab significantly improved the responsiveness of cocultured HLMCs to albuterol, formoterol, and olodaterol, increasing agonist-induced inhibition of FcεRI-dependent histamine release to 47.6 ± 5.6, 60.0±5.4, and 75.4 ± 8.4%, respectively (p = 0.0036, p = 0.0015, p = 0.0023, respectively, compared with isotype control, Sidak’s MCT; Fig. 2B).

In separate CADM1 neutralization experiments, the mean percent inhibition of FcεRI-dependent histamine release by albuterol (10^{-8} M), formoterol (10^{-9} M), and olodaterol (10^{-9} M) in HLMC monoculture was 32.7 ± 14.1, 70.5 ± 10.1, and 81.6 ± 7.5%, respectively (Fig. 2C). Again, the ability of β2-agonists to inhibit FcεRI-mediated histamine release was significantly reduced in HLMC–HASMC coculture (Fig. 2C). CADM1 neutralization improved β2-AR responsiveness to formoterol and olodaterol but not albuterol (Fig. 2C).

**FIGURE 3.** HLMC–HASMC coculture increases phosphorylation of the β2-AR at Tyr^350. (A) Representative immunofluorescent staining for p-β2-AR (Tyr^350) in HLMC monoculture, HASMC monoculture, and HLMC–HASMC coculture. (B) Quantification of p-β2-AR (Tyr^350) expression in HLMCs and HASMCs in monoculture and coculture. n = 5 independent experiments. *p < 0.05, **p < 0.01 compared with respective monoculture (Sidak’s MCT). ##p < 0.01 compared with HASMCs in coculture (Sidak’s MCT).

**FIGURE 4.** SCF and CADM1 neutralization prevents phosphorylation of the β2-AR at Tyr^350. (A) Representative immunofluorescent staining for p-β2-AR (Tyr^350) in HLMCs and HASMCs in coculture and its attenuation by SCF and CADM1 neutralization. (B and C) Quantification of p-β2-AR (Tyr^350) expression in HLMCs and HASMCs in coculture and the effect of SCF (B) and CADM1 (C) neutralization. n = 5 independent experiments. *p < 0.05, **p < 0.01 compared with respective isotype control (Sidak’s MCT).
The $\beta_2$-AR is phosphorylated at Tyr$^{350}$ in HLMC–HASMC coculture

Phosphorylation of the $\beta_2$-AR at Tyr$^{350}$ by receptor tyrosine kinases (RTKs) including Kit results in receptor uncoupling and internalization (31, 36). We therefore examined whether $\beta_2$-AR phosphorylation at Tyr$^{350}$ occurs in HLMC-HASMC coculture using immunofluorescent staining.

Minimal p-$\beta_2$-AR (Tyr$^{350}$) expression was detectable in HLMC and HASMC monocultures (Fig. 3A, 3B). However, p-$\beta_2$-AR (Tyr$^{350}$) expression was significantly enhanced in both HASMCs and HLMCs following 5 min of coculture (Fig. 3A, 3B). However, p-$\beta_2$-AR (Tyr$^{350}$) expression was significantly greater in cocultured HLMCs than in cocultured HASMCs (Fig. 3B). Isotype control staining was negative.

SCF and CADM1 neutralization inhibits phosphorylation of the $\beta_2$-AR at Tyr$^{350}$ in HLMC-HASMC coculture

Because neutralizing SCF or CADM1 restored $\beta_2$-AR function in HLMCs cultured with HASMCs, we examined the effect of SCF and CADM1 neutralization on phosphorylation of the $\beta_2$-AR at Tyr$^{350}$ in HLMC-HASMC coculture (Fig. 4). $\beta_2$-AR phosphorylation at Tyr$^{350}$ was again evident in both HLMCs and HASMCs following 5-min coculture (Fig. 4). Phosphorylation was also evident in HLMC–HASMC cocultures incubated for 5 min with appropriate isotype control Abs (Fig. 4). However, incubation of HLMC–HASMC cocultures with either anti-SCF or anti-CADM1 Ab significantly reduced $\beta_2$-AR phosphorylation at Tyr$^{350}$ in both HLMCs and HASMCs (Fig. 4).

HLMC–HASMC coculture reduces $\beta_2$-AR responsiveness in primary HASMCs

Because HASMCs in coculture with HLMCs also demonstrated phosphorylation of the $\beta_2$-AR at Tyr$^{350}$, we assessed whether HLMC–HASMC coculture also impairs $\beta_2$-AR responsiveness in HASMCs. For this, we used the collagen gel contraction assay, a recognized in vitro model that is useful for examining the mechanisms of cellular cytoskeletal reorganization and stress fiber formation (24).

**FIGURE 5.** $\beta_2$-AR agonists do not inhibit spontaneous HASMC contraction in HLMC–HASMC coculture. (A) $\beta_2$-AR agonists attenuated the spontaneous contraction of collagen gels containing HASMCs alone. *p < 0.05, **p < 0.01 compared with respective controls (paired t tests). (B) $\beta_2$-AR agonists increased the contraction of collagen gels containing both HLMCs and HASMCs. *p < 0.05, **p < 0.01 compared with respective controls (paired t tests). (C) The net change (drug minus vehicle control) in gel contraction with $\beta_2$-AR agonist exposure for monoculture compared with coculture. *p = 0.01, **p = 0.006, ***p = 0.0006 compared with monoculture (paired t tests). (D) Initial experiments (n = 2) investigating the effects of cetirizine and leupeptin on HASMC contraction in coculture showed that in the presence of formoterol, both reduced coculture contraction to values seen in monoculture. (E) Further experiments (n = 4) showed that there was similar inhibition with cetirizine and leupeptin alone, with no interaction between formoterol and either cetirizine or leupeptin.
Incubation of HASMC-embedded collagen gels for 16 h with 10⁻⁸ M albuterol, 10⁻⁷ M formoterol, and 10⁻⁹ M olodaterol reduced spontaneous gel contraction compared with vehicle controls (p = 0.041 [log transformed], p = 0.002, p = 0.011, respectively; Fig. 5A, Supplemental Fig. 2). In contrast, incubation of HLMC–HASMC-embedded gels with β₂-AR agonists failed to inhibit spontaneous gel contraction compared with vehicle controls. In the experiments involving all three β₂-AR agonists studied in parallel, there was some enhancement of spontaneous gel contraction (Fig. 5B). In this study, for all β₂-AR agonists tested, there was a significant difference in the change in spontaneous gel contraction (compared with relevant vehicle control) between gels embedded with HASMCs alone and those embedded with HLMCs plus HASMCs (Fig. 5C). In further experiments using formoterol to study the effects of the H1 receptor blocker cetirizine and the tryptase inhibitor leupeptin on coculture contraction, formoterol failed to inhibit contraction (Fig. 5D, 5E). Both cetirizine and leupeptin markedly inhibited background contraction in coculture (Fig. 5D, 5E), but not monoculture (not shown), and no additive effect of formoterol in coculture was evident.

Corticosteroids restore β₂-AR responsiveness in HASMCs in the presence of HLMCs

The corticosteroid fluticasone propionate did not affect HASMC contraction in collagen gels on its own, but restored the ability of β₂-AR agonists to attenuate spontaneous HASMC contraction in the presence of HLMCs, and to values similar to those seen in HASMC monoculture (Fig. 6, Supplemental Fig. 3).

Discussion

Infiltration of ASM bundles by activated mast cells is a key pathological feature of asthma (20, 21, 37), occurring across inflammatory phenotypes and the spectrum of asthma severity (38–40). This study demonstrates that when HLMCs and HASMCs are cultured together in vitro, HASMCs induce mast cell degranulation [as described previously (23)], and this is enhanced in the presence of β₂-AR agonists. Furthermore, HLMCs are less susceptible to inhibition of degranulation by β₂-AR agonists following FcεRI-dependent activation. These changes in β₂-AR agonist responsiveness in HLMCs were associated with phosphorylation of the β₂-AR at Tyr³⁵⁰, a process which uncouples the β₂-AR and promotes its internalization (36, 41). Surprisingly, HLMC–HASMC coculture also induced phosphorylation of the β₂-AR at Tyr³⁵⁰ in HASMCs, with loss of protection by β₂-ARs on HASMC contraction (summarized in Supplemental Fig. 4).

Cross-talk between GPCRs such as the β₂-AR and RTKs is well described, particularly for insulin, which acts via its receptor to rapidly inhibit β₂-AR function (36, 41). This occurs through phosphorylation at Tyr³⁵⁰, which uncouples the β₂-AR and also promotes β₂-AR sequestration through the creation of an SH2 binding site to which molecules such as Src and Grb2 bind to induce β₂-AR internalization (41). SCF, acting via its RTK Kit, exerted similar effects on the β₂-AR in monocultured HASMCs previously (31). In consequence, β₂-AR agonists no longer inhibited FcεRI-dependent HASMC degranulation or leukotriene C₄ production (31) and actually enhanced HLMC histamine release in the presence of SCF and monomeric IgE (31).

The effects of SCF on HLMC β₂-AR agonist responses described previously are very similar those found here when HLMCs were cocultured with HASMCs. Indeed, the loss of β₂-AR responsiveness, the enhancement of histamine release by β₂-AR agonists and the Tyr³⁵⁰ phosphorylation induced by HASMCs was reversed largely by neutralizing either SCF or CADM1. CADM1 interacts cooperatively with Kit and likely orientates it with membrane-bound SCF at points of cell–cell adhesion (23, 26). Thus, both Kit and CADM1 or their ligands are potential targets that might enhance β₂-AR responsiveness in HLMCs and HASMCs and attenuate the potential prossecretory effects of β₂-AR agonists.

Perhaps surprisingly, HLMC–HASMC coculture also lead to rapid phosphorylation of the β₂-AR at Tyr³⁵⁰ in HASMCs. This was also dependent on SCF and CADM1, suggesting that release of a mast cell-derived product(s) stimulated by cell contact is involved. This would probably need to be present preformed and could include one of a number of mediators including histamine, tryptase, TGF-β1, basicFGF and adenosine nucleotides. Alternatively, if Kit docking is also important for CADM1 function, then a signal delivered via the CADM1 counterreceptor on HASMCs, most likely nectin-3, might also play a role (42). Additional work is required to elucidate the mechanism(s) at work. However, the consequences of this β₂-AR phosphorylation in HASMCs appear functionally important in that β₂-AR agonists are no longer able to prevent HASMC contraction in the presence of cocultured HLMCs.

The ability of β₂-AR agonists to enhance constitutive histamine release in coculture is interesting but not easily explained. A po-

![FIGURE 6. Fluticasone restores β₂-AR function in HLMC–HASMC coculture. (A) In HASMC monoculture, fluticasone (10⁻⁷ M) did not alter HASMC contraction, and the fluticasone/β₂-AR(10⁻⁷ M) combination inhibited spontaneous HASMC contraction. *p < 0.05 compared with DMSO control (paired t tests). (B) In HLMC–HASMC coculture, fluticasone (10⁻⁷ M) did not alter spontaneous gel contraction, but β₂-ARs (10⁻⁹ M) in combination with fluticasone were able to reduce spontaneous gel contraction. *p < 0.05. **p < 0.001 compared with DMSO control (paired t tests). (C) The net change in gel contraction (drugs minus 0.1% DMSO control) in HLMC–HASMC coculture for formoterol alone (n = 5), olodaterol alone (n = 4) (data from Fig. 5C), or the combination of formoterol or olodaterol with fluticasone (n = 6 for each, data calculated from B). ***p < 0.001, ****p < 0.0001 compared with respective combination, unpaired t tests.]
ential mechanism whereby β₂-agonists increase constitutive HLMC mediator release in coculture is through interference with HLMC organic cation transporters (43, 44). These transporters are inhibited by albuterol and formoterol, and this may result in reduced histamine reuptake (43). However, perhaps with the exception of formoterol, a second and relatively mild stimulus is also required for the potentiating effects of β₂-AR agonists to become evident. Previously, both SCF and monomeric IgE were required, although in this study, a consistent effect across all β₂-AR agonists was only evident when a stimulus from HASMCs was present. Alternative mechanisms must therefore be considered, and there are potential parallels with the paradoxical effects of β₂-AR agonists in other cell types. For example, in HASMCs, β₂-AR agonists enhance HASMC contraction though the inhibition of regulator of G protein signaling 5 (45). In airway epithelial cells, the β₂-AR agonists salmeterol and albuterol enhanced both rhinovirus- and IL-1β-induced IL-6 production, through a cAMP-dependent mechanism which potentiated rhinovirus-induced IL-6 promoter activation (46). Additional work is clearly required to delineate the potential mechanisms behind the augmentation of HLMC histamine release by β₂-AR agonists.

Caution is always required in extrapolating in vitro findings to the in vivo clinical situation, but we believe that our results have potentially important clinical implications and provide a plausible mechanistic explanation for many of the paradoxical effects of β₂-AR agonists observed in human asthma. Many papers have described potential adverse effects of β₂-AR agonists in asthma, and examples are highlighted in the introduction to this manuscript. Of particular note, when short-acting β₂-agonists (SABAs) or long-acting β₂-agonists (LABAs) are given regularly in the absence of an ICS, mast degranulation and the early-phase bronchoconstrictor response following allergen challenge or exercise are increased, implying that not only is there a loss of protection on mast cell degranulation but also loss of inhibition of ASM contraction (9, 10, 30, 47). These latter observations are similar to those we have observed in HLMC–HASMC coculture. We therefore propose that the regular use of β₂-AR agonists in the absence of an ICS may in some patients enhance HLMC mediator release within the ASM bundles, which in turn enhances airway hyperresponsiveness and uncouples the HASMC β₂-ARs. Thus background asthma control would likely deteriorate, and with a superimposed airway insult, for example during viral infection or allergen exposure, the subsequent exacerbation might be worse.

Treatment guidelines for asthma recommend that LABAs are never used in the absence of an ICS, yet it is still considered reasonable to use SABAs in isolation with perceived mild disease. However, a significant number of patients with so-called mild disease still die of asthma (48). In addition, many patients with asthma of all severities adhere poorly to corticosteroid therapy and are overreliant on SABA for symptom relief (49, 50). Importantly, in this study, we found that the corticosteroid fluticasone propionate restored the protective effects of β₂-AR agonists on HASMC contraction in the presence of HLMCs. We believe this reinforces the view put forward by others (18), that all β₂-AR agonist inhalers used for asthma whether SABA or LABA, should contain an ICS. Secondly, in patients with relative corticosteroid resistance, targeting the pathways identified through the inhibition of SCF-Kit or CADM1-nectin 3, has the potential to reverse this β₂-AR deregulation, and thus enhance both the efficacy and safety of β₂-AR agonists.

Disclosures

The authors have no financial conflicts of interest.

References