Calpain-activated mTORC2/Akt pathway mediates airway smooth muscle remodelling in asthma

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Summary

Background Allergic asthma is characterized by inflammation and airway remodelling. Airway remodelling with excessive deposition of extracellular matrix (ECM) and larger smooth muscle mass are correlated with increased airway responsiveness and asthma severity. Calpain is a family of calcium-dependent endopeptidases, which plays an important role in ECM remodelling. However, the role of calpain in smooth muscle remodelling remains unknown.

Objective To investigate the role of calpain in asthmatic airway remodelling as well as the underlying mechanism.

Methods The mouse asthma model was made by ovalbumin sensitization and challenge. Calpain conditional knockout mice were studied in the model. Airway smooth muscle cells (ASMCs) were isolated from smooth muscle bundles in airway of rats. Cytokines IL-4, IL-5, TNF-α, and TGF-β1, and serum from patients with asthma were selected to treat ASMCs. Collagen-I synthesis, cell proliferation, and phosphorylation of Akt in ASMCs were analysed.

Results Inhibition of calpain using calpain knockout mice attenuated airway smooth muscle remodelling in mouse asthma models. Cytokines IL-4, IL-5, TNF-α, and TGF-β1, and serum from patients with asthma increased collagen-I synthesis, cell proliferation, and phosphorylation of Akt in ASMCs, which were blocked by the calpain inhibitor MDL28170. Moreover, MDL28170 reduced cytokine-induced increases in Rictor protein, which is the most important component of mammalian target of rapamycin complex 2 (mTORC2). Blockage of the mTORC2 signal pathway prevented cytokine-induced phosphorylation of Akt, collagen-I synthesis, and cell proliferation of ASMCs and attenuated airway smooth muscle remodelling in mouse asthma models.

Conclusions and Clinical Relevance Our results indicate that calpain mediates cytokine-induced collagen-I synthesis and proliferation of ASMCs via the mTORC2/Akt signalling pathway, thereby regulating airway smooth muscle remodelling in asthma.

Keywords airway smooth muscle cells, asthma, calpain, PI3K/Akt pathway, remodelling

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**Introduction**

Asthma is a chronic airway disease, which is characterized by persistent airway inflammation, reversible airway obstruction, airway remodelling, and airway hyperresponsiveness [1]. Airway remodelling is described as increased airway wall thickness due to various structural alterations that include epithelial abnormality, subepithelial membrane thickening, excessive deposition of extracellular matrix (ECM), neoangiogenesis, mucus gland hypertrophy, and increased airway smooth muscle (ASM) mass [2, 3]. ASM remodelling is characterized by increased deposition of ECM proteins in and around ASM bundles and increased ASM mass and ASM hypertrophy [4, 5], which are correlated with increased airway responsiveness and asthma severity, contributing to airflow obstruction in asthma [3, 6, 7]. However, the mechanisms underlying ASM remodelling remain to be elucidated.

Many inflammatory mediators and growth factors, including Th2 cytokines IL-4 and IL-5, TNF-α, and TGF-β1, play crucial role in ASM remodelling [8, 9]. For example, IL-4 and IL-5 participate in the proliferation, maturation, migration, and survival of eosinophils as well as some modulatory role in the development and function of basophils and mast cells [10]. TNF-α is increased in the airways of patients with asthma. Increased TNF-α level in the bronchoalveolar lavage (BAL) fluid is associated with airway remodelling in patients with asthma [11]. Increased levels of TGF-β have been reported in BAL fluid and airways of patients with asthma [12, 13] and in the ASM layer [14].

Calpain is a family of calcium-dependent, non-lysosomal neutral cysteine endopeptidases. There are 15 mammalian isoforms, with the ubiquitously expressed calpain-1 and calpain-2 isoforms consisting of dimmers composed of distinct larger catalytic subunits, encoded by *capn1* and *capn2*, respectively, and a common smaller regulatory subunit, encoded by the *capns1* gene. Genetic disruption of *capns1* abolishes both calpain-1 and calpain-2 activities [15, 16]. Calpastatin functions as a specific endogenous inhibitor for calpain-1 and calpain-2 [17]. Activation of calpain involves calcium, release of calpain from its inhibitor calpastatin, and phosphorylation [18]. Calpain plays an important role in cell proliferation, migration, and differentiation [19–21]. Studies have shown that inhibition of calpain activity improves pulmonary vascular or cardiac function in animal models of pulmonary hypertension, pulmonary fibrosis, hypertension, type-1 diabetes and age-associated aortic wall calcification and fibrosis [17, 22–25]. Calpain activity is increased in murine model of allergic airway inflammation [26, 27]. But the role of calpain in ASM remodelling of asthma is still unknown.

Here, we show that calpain deletion or inhibition reduces cell proliferation and collagen-I synthesis in ASMCs in asthma. Furthermore, we provide evidence that the underlying mechanism of these asthma-associated conditions involves calpain-mediated activation of the mTORC2/Akt signalling pathway.

**Materials and methods**

**Materials and reagents**

Recombinant active TGF-β1, tamoxifen, ovalbumin (OVA), and anticalpastatin antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). MDL28170, calpeptin, LY294002, and the fluorogenic peptide Suc-Leu-Leu-Val-Tyr-AMC were purchased from Calbiochem (La Jolla, CA, USA). Torin 1 was obtained from Tocris (Bristol, UK). Anticollagen-I antibody was purchased from Novus Biologicals (La Jolla, CA, USA). Antibodies against CAPN1, CAPN2, CAPNS1, and α-smooth muscle actin were obtained from Abcam (Cambridge, UK). Antibodies against GAPDH, Rictor, mTOR, phosphorylated-Akt (p-Akt, Ser473), and total-Akt (t-Akt) were obtained from Cell Signaling Technology (Danvers, MA, USA). Cytokines IL-4, IL-5, and TNF-α were obtained from PeproTech (Rocky Hill, NJ, USA). Cell proliferation ELISA assay, BrdU (colorimetric) kit was obtained from Roche (Mannheim, Germany). Rictor siRNA was obtained from Qiagen (Germantown, MD, USA). Lipo-jectamine RNAiMAX Reagent was purchased from Life Technology (Grand Island, NY, USA).

**Serum collection from human subjects**

The study protocol was approved by the Institutional Review Board of the Tongji Medical College, Huazhong University of Science and Technology. Informed consent was obtained from all subjects. Asthma patients with asthmatic history of more than 10 years were recruited in the study. At the time of serum collection, the patients were in acute exacerbation for 24 h. Controls were recruited from healthy subjects who had undergone physical examinations in the hospital. Five millilitres of blood was drawn and placed in ice immediately and was then centrifuged at 1200 g for 5 min. Supernatants were aliquoted and stored at −80°C.

**Conditional knockout of calpain in mice**

The ubiquitously expressed calpain-1 and calpain-2 isoforms consist of *capn1*- and *capn2*-encoded catalytic subunits, CAPN1 and CAPN2, respectively, and an obligate common regulatory unit CAPNS1 encoded by *capn3*1. CAPNS1 is required for the activity of both calpain-1 and calpain-2, and targeted germline
disruption of capns1 is embryonically lethal at midgestation [15]. Therefore, we took advantage of C57/BL6 mice targeted with floxed capns1 alleles (capns1\textsuperscript{lox/lox}) [16] to produce conditional calpain-1 and calpain-2 knockout mice. These capns1\textsuperscript{lox/lox} mice were crossed with B6.Cg-Tg(CAG-cre/Esr1)5Amc/J mice to generate Cre-ER\textsuperscript{+/-}capns1\textsuperscript{lox/lox} mice. PCR was used to genotype the mice. Deletion of capns1 was induced by activation of Cre-ER with tamoxifen administration (20 mg/kg/day, i.p. for 5 days) and was confirmed by Western blotting analysis of CAPNS1, CAPN1, and CAPN2 in lung tissues. Control mice were littermate capns1\textsuperscript{lox/lox} or capns1\textsuperscript{lox/+} mice (negative for the Cre-ER transgene) treated with the same amount of tamoxifen regimen. Mice 8 weeks of age were used for the asthma model.

Asthma animal model

The mouse asthma model was generated by ovalbumin (OVA) sensitization and challenge as previously described with some modifications [28]. This model demonstrates many features of airway allergy and allergic asthma that are similar to human disease [28]. Cre-ER\textsuperscript{+/-}capns1\textsuperscript{lox/lox}, C57BL/6J, or BALB/c mice were housed in the Experimental Animal Center of Tongji Medical College with ad libitum access to food and water. The study protocol was approved by the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology. After tamoxifen treatment, mice were sensitized with intraperitoneal injection of 10 mg OVA plus 2 mg aluminium hydroxide in 0.5 ml saline on days 1 and 9. Between days 15–60, aerosol challenges containing either 3% OVA or saline were given to mice (for 20 min, three times a week). To investigate the role of calpeptin and torin 1 in asthma, BALB/c and C57BL/6J mice were used, respectively. Calpeptin or torin 1 was injected intraperitoneally at 2 mg/kg body weight, respectively, which was repeated three times weekly 1 h before each OVA challenge. Mice were euthanized at day 60.

Histological analysis

The lungs were removed after thoracotomy. The right lungs were fixed in 4% formaldehyde for 24 h, then sliced mid-sagittally and embedded in paraffin. Masson trichrome staining was performed as an index of collagen deposition. An OLYMPUS DP50-CU digital camera and Image-Pro Plus software were used to analyse the slides. The peribronchial smooth muscle layer thickness was measured in 10 bronchioles (diameter is 200–300 μm) in each lung section. Relative bronchial muscle layer thickness was shown as the ratio of the thickness of the smooth muscle layer to total bronchial wall. The peribronchial collagen deposition was expressed as blue colour in Masson trichrome staining. Relative bronchial collagen area was shown as the ratio of blue colour area to total bronchial area. The parenchymal inflammation around the bronchioles was analysed by counting the number of inflammatory cells. Immunohistochemistry staining was performed to measure p-Akt and Rictor in the lung sections, and the dilution factor of anti-p-Akt and anti-Rictor antibody is 1 : 50. Relative p-Akt or Rictor protein levels were shown as the ratio of positive staining area to total ASM area.

Isolation, primary culture, and treatment of rat ASMCs

ASMCs were isolated from smooth muscle bundles of the large bronchial airways of rats [29]. The bundles were isolated and minced into 1 mm\textsuperscript{3} pieces, then plated onto 25-mL flasks in 20% FBS in Dulbecco’s modified Eagle’s medium (DMEM)/F12 and cultured in a 5% CO\textsubscript{2} 37°C humidified incubator (Thermo Fisher Forma, Waltham, MA, USA). Cells grew from the bundles at day 3 and grew to confluence over 6 days, and then were passaged using 0.25% trypsin with 1 mM EDTA. Smooth muscle cell characteristics were routinely determined by immunofluorescence staining with antibodies against α-smooth muscle actin and microscopy. The cells appear spindle-shaped, with typical hill and valley appearance. Cells of passages 2–4 were used for all experiments.

Measurement of calpain activity in ASMCs and lung tissue homogenates

Calpain activity in ASMCs was measured using the fluorogenic peptide Suc-Leu-Leu-Val-Tyr-AMC as a substrate following a procedure described previously [30] with slight modification. Briefly, cells were cultured in 24-well plates. After being washed twice with PBS, substrate was added to a final concentration of 80 μM in growth factor-free medium. Immediately after the addition of substrate, fluorescence was recorded at 1-min interval for 30 min at excitation 360 nm and emission 460 nm using a Synergy 2 Multi-Mode microplate reader (BioTek Instruments, Winooski, VT, USA). Calpain activity was expressed as the slope of fluorescence units–time curves.

Calpain activity in lung tissue homogenates was measured using the fluorogenic peptide Suc-Leu-Leu-Val-Tyr-AMC as described previously [31]. Freshly dissected lung tissues were homogenized in sample buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol) and frozen until use. The samples (100 μg of total protein) were mixed with 150 μL of assay buffer (10 mM Tris-HCl, pH 7.5, 10 mM CaCl\textsubscript{2},...
0.1% β-mercaptoethanol) containing 100 μM of fluorogenic calpain substrate, and fluorescence was recorded as described above.

Protein analysis in lung tissues and ASMC lysates

The lung tissues or ASMCs were homogenized in RIPA buffer and mixed with Western blot sample buffer. The lysates (10–30 μg protein) were denatured and electrophoresed on SDS-PAGE gel. Separated proteins were electrotransferred to PVDF membranes, blocked with 5% non-fat milk (or 3% BSA) for 1 h, and then incubated with antibodies against CAPN1, CAPN2, CAPNS1, calpastatin, p-Akt, t-Akt, Rictor, GAPDH (dilution 1 : 1000 for each), and collagen-I (dilution 1 : 3000) at 4°C overnight and then washed with TBST, and incubated with horseradish peroxidase-conjugated secondary antibody (ProteinTech, Chicago, IL, USA) at room temperature for 1 h. Enhanced chemiluminescence (Supersignal West Pico, Pierce, Rockford, IL, USA) was used to detect the proteins, and densitometric quantification was performed using Kodak digital Science 1D image analysis software.

Cell proliferation assay

Proliferation of ASMCs was quantified using a kit from Roche that monitors the incorporation of BrdU into newly synthesized DNA. BrdU was detected using anti-BrdU-peroxidase conjugate in accordance with the manufacturer’s instruction. After the reactions were stopped, absorbance at 450 nm was measured using a BioTek Epoch microplate spectrophotometer.

Rictor siRNA transfection

siRNA was transfected using Lipofectamine RNAiMAX Reagent following the manufacturer’s instruction. Lipofectamine RNAiMAX Reagent (17 μL) and Rictor siRNA (17 μL) were diluted with Opti-MEM medium (250 μL), respectively, and then mixed them with 1 : 1 ratio. The mixture was then added to cells. Protein assays and cell treatments were performed 48 h after transfection.

Statistical analysis

Results are shown as the mean ± SEM for n experiments. In animal models, ‘n’ represents the number of mice used in each group. In the in vitro studies, ‘n’ represents the number of rats used to generate ASMCs and the number of cell lines used in the experiments. Differences between groups were analysed using unpaired t-tests or two-way analysis of variance. A P value <0.05 was considered to be statistically significant.

Results

Knockout of calpain attenuates ASM remodelling in a chronic mouse asthma model

To explore the physiologic role of calpain in asthmatic ASM remodelling, we used a conditional capns1 knockout mouse [16, 17, 32]. After systemic treatment with tamoxifen to induce Cre-ER-mediated disruption of floxed capns1 alleles, the levels of CAPNS1, CAPN1, and CAPN2 were lower in the lungs of Cre-ER<sup>+/−</sup>/capns1<sup>−/−</sup> (capns1 KO) mice relative to control mice (Fig. 1a,b). The protein levels of collagen-I and p-Akt were increased after the asthma challenge in control, but not in the capns1 KO lungs (Fig. 1a,b). Asthma challenge was also associated with an increase in measured calpain activity and parenchymal inflammation around the airway in control, but not capns1 KO lungs (Fig. 1f,g). More importantly, excessive bronchial collagen deposition and increases of ASM mass in the asthmatic mice were significantly attenuated in the lungs of capns1 KO mice (Fig. 1c–e). These data indicate that calpain plays a significant role in driving collagen accumulation, Akt phosphorylation, and airway remodelling in the lungs of asthmatic mice.

Calpain inhibitor calpeptin prevents the progression of ASM remodelling in a mouse model of chronic asthma

We further examined the effect of the specific calpain inhibitor calpeptin on the progression of asthmatic ASM remodelling in mice. As shown in Fig. 2, the collagen area and the thickness of bronchial walls were much larger in OVA-challenged asthmatic mice than in unchallenged control mice, indicating the establishment of ASM remodelling. In the immunohistochemistry staining, the expressions of p-Akt were also increased in the asthmatic ASM. When asthmatic mice were given calpeptin at the beginning of OVA challenge, we found that the collagen area, the thickness of bronchial walls, p-Akt expressions, and measured calpain activity were reduced relative to asthmatic mice treated with vehicle (Fig. 2). These results suggest that calpeptin-mediated calpain inhibition prevents the progression of ASM remodelling.

IL-4-, IL-5-, TNF-α-, and TGF-β1-induced increases in collagen-I synthesis and cell proliferation are associated with activation of calpain in ASMCs

To explore mechanisms that might link calpain to ASM remodelling, we examined the effects of inflammatory mediators and growth factors which have been associated with this process [8–14] on calpain activity, collagen-I synthesis, and cell proliferation in cultured
ASMCs. We found that incubation of ASMCs with IL-4, IL-5, TNF-α, and TGF-β1 for 24 h induced increases in measured calpain activity (Fig. 3a). Moreover, IL-4, IL-5, TNF-α, and TGF-β1 increased intracellular collagen-I protein levels (Fig. 3b–d) and cell proliferation (Fig. 3f); these effects were attenuated with the calpain inhibitor MDL28170. These data suggest that calpain is involved in collagen-I synthesis and proliferation of ASMCs induced by cytokine IL-4, IL-5, TNF-α, and TGF-β1.

Sera from patients with asthma induce increases in collagen-I synthesis and cell proliferation via activation of calpain in ASMCs

To address the clinical relevance of these observations in the animal model and cell experiments, we collected sera from patients with asthma in acute exacerbation and used it to challenge cultured ASMCs. The clinical information of patients with asthma and healthy control subjects is shown in Table S1. Similar to cytokines, the asthmatic sera induced increases in measured calpain activity (Fig. 3g), intracellular collagen-I proteins level (Fig. 3h), and cell proliferation (Fig. 3i). Moreover, the calpain inhibitor MDL28170 blocked asthmatic serum-induced increases in collagen-I protein levels and cell proliferation, suggesting that calpain promotes asthmatic inflammation-induced increases in collagen-I synthesis and proliferation in ASMCs.

Calpain mediates cytokine-induced collagen-I synthesis and cell proliferation through PI3K/Akt signalling

To investigate downstream signalling in calpain-mediated asthmatic ASM remodelling, we examined the effects of cytokines on phosphorylation of Akt. Incubation of ASMCs with IL-4, IL-5, TNF-α, and TGF-β1 induced phosphorylation of Akt, and this was attenuated by MDL28170 (Fig. 4a–d). To further study the role of PI3K/Akt signalling in airway smooth muscle remodelling, we examined the effects of the PI3K inhibitor LY294002 on cytokine-induced increases in intracellular collagen-I protein levels and cell proliferation. Cytokine-induced increases in collagen-I (Fig. 4e–h) and cell proliferation (Fig. 4i–l) were blocked by LY294002.
which suggests that calpain mediates collagen-I synthesis and proliferation of ASMCs via activation of Akt.

**mTORC inhibitor torin 1 attenuates cytokine-induced collagen-I synthesis, cell proliferation, and phosphorylation of Akt in ASMCs**

To determine whether mTORC2 is acting upstream of Akt in cytokine-challenged ASMCs, we examined the effects of the mTORC1/2 inhibitor torin 1 on IL-4- and TGF-β1-induced collagen-I synthesis (Fig. 5a,d), phosphorylation of Akt (Fig. 5b,e), and cell proliferation of ASMCs (Fig. 5c,f). The mTORC1/2 inhibitor torin 1 blocked cytokine-induced increases in phosphorylation of Akt, collagen-I protein level, and cell proliferation in ASMCs (Fig. 5).

Cytokines up-regulate Rictor, and Rictor knockdown in ASMCs attenuates cytokine-induced phosphorylation of Akt, collagen-I synthesis, and proliferation

To further assess the role of mTORC2 in the phosphorylation of Akt and smooth muscle remodelling, we measured the effect of cytokine stimulation on Rictor protein expression, which is the most important component of mTORC2 complex. Both IL-4 and TGF-β1 promoted Rictor protein expression, and the calpain inhibitor MDL28170 suppressed this effect (Fig. 6a,b). Moreover, siRNA-mediated knockdown of Rictor in ASMCs prevented cytokine-induced increases in Akt phosphorylation, collagen-I protein levels, and cell proliferation (Fig. 6c–i). These data suggest a role for mTORC2 in asthmatic ASM remodelling.
Fig. 3. IL-4, IL-5, TNF-α, TGF-β1, and serum from patients with asthma induce increases in collagen-I synthesis and cell proliferation via activation of calpain in airway smooth muscle cells (ASMCs). ASMCs were incubated with IL-4 (20 ng/mL), IL-5 (20 ng/mL), TNF-α (20 ng/mL), and TGF-β1 (5 ng/mL) in the presence or absence of MDL28170 (20 μM) for 24 h, after which calpain activity (a), intracellular collagen-I protein content (b, c, d, and e), and cell proliferation (f) were measured as described in the Methods. (a) Changes in calpain activity induced by IL-4 (n = 3), IL-5 (n = 3), TNF-α (n = 6), and TGF-β1 (n = 4); (b, c, d, and e) Representative images and quantitative data of collagen-I protein levels in ASMCs incubated with IL-4 (b, n = 4), IL-5 (c, n = 4), TNF-α (d, n = 3), and TGF-β1 (e, n = 6). Collagen-I levels were normalized to GAPDH and expressed relative to untreated controls (white bars). (f) ASMC proliferation induced by cytokine treatments (n = 8). Results are expressed as mean ± SEM. *P < 0.05 vs. control. #P < 0.05 vs. cytokine treatment. (g, h, and i) ASMCs were incubated with 2% serum from four patients with asthma or healthy control in the presence or absence of MDL28170 (20 μM) for 24 h, after which calpain activity (g), intracellular collagen-I protein level (h), and cell proliferation (i) were measured as described in the Methods. Results are expressed as mean ± SEM. Serum from four patients and four control subjects was used to treat ASMC cell lines generated from four rats, n = 4, *P < 0.05 vs. normal control serum, #P < 0.05 vs. asthmatic serum.
Torin 1 attenuates asthmatic ASM remodelling in a mouse model of chronic asthma

We further investigated the involvement of mTORC2/Akt activation in asthmatic ASM remodelling in vivo. Mice were injected with torin 1 one hour prior to each OVA challenge, and bronchial collagen area and bronchial wall thickness were determined at the end of the 6-week chronic challenge protocol. Torin 1 prevented increases in bronchial collagen area, bronchial wall thickness (Fig. 7a–c), p-Akt (Fig. 7d,e), and Rictor expressions (Fig. S1). These data support that the mTORC2/Akt signalling pathway mediates asthmatic ASM remodelling in vivo.

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Discussion

This study provides substantial evidence linking inflammatory cytokines, calpain, and the mTORC2/Akt signalling pathway in ASM remodelling in asthma. We found for the first time that genetic disruption of calpain or pharmacologic inhibition of its attenuated ASM remodelling in vivo. The specific calpain inhibitor calpeptin prevented the progression of asthmatic ASM remodelling. Moreover, inhibition of calpain using MDL28170 attenuated IL-4-, IL-5-, TNF-α-, and TGF-β1-induced collagen-I synthesis, cell proliferation, phosphorylation of Akt, and Rictor protein expression in ASMCs in vitro. Furthermore, blocking mTORC signalling with torin 1 or Rictor knockdown prevented cytokine-induced collagen-I synthesis and cell proliferation. Administration of torin 1 in vivo attenuated ASM remodelling in this mouse asthma model. Thus, these observations indicate that calpain mediates collagen-I synthesis and cell proliferation induced by cytokines via activation of mTORC2/Akt signalling pathway (Fig. 8).

Bronchial chronic asthmatic inflammation causes tissue injuries leading to repetitive repair processes, which induces ASM remodelling. BAL fluid from patients with asthma can induce human ASMC proliferation [33]. IL-4 and IL-5 are Th2 cell-derived cytokines, which participate in the pathogenesis of asthma. IL-4 regulates cell growth, survival, and gene expression [34]. PI3K/Akt is the downstream signalling pathway of IL-4 [35]. IL-5 plays key role in the proliferation, maturation, migration, and survival of eosinophils as well as some modulatory role in the development and function of basophils and mast cells [10]. TNF-α is a pro-inflammatory cytokine that increases in the airways of patients with asthma. Increased TNF-α levels in BAL fluid are associated with airway remodelling in patients with asthma. Allergen-challenged TNFp55/p75 receptor-deficient mice have significantly reduced levels of peripheral eosinophils and fibrosis [11]. However, the
The direct effects of IL-4, IL-5, and TNF-α on ASMCs proliferation and collagen-I synthesis are not clear yet. TGF-β is a profibrotic cytokine that is a key airway remodelling mediator in asthma [8]. Increased levels of TGF-β have been reported in BALF and airways of patients with asthma [12, 13], and more specifically, in the ASM layer [14]. TGF-β induces collagen-I deposition in ASMCs [36]. TGF-β has been shown to increase the proliferation of ASMCs via the MAPK pathway [37]. The current study demonstrated that IL-4, IL-5, TNF-α, and TGF-β1 induce ASMC proliferation, collagen-I synthesis, and phosphorylation of Akt, suggesting that IL-4, IL-5, TNF-α, and TGF-β1 signalling contribute to asthmatic ASM remodelling.

To examine the role of calpain in the pathogenesis of airway remodelling, we subjected tamoxifen-inducible

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 conditional capns1 knockout mice to an OVA-sensitized and OVA-challenged model of chronic asthma. As we have previously shown, tamoxifen treatment of these mice was associated with reduction in CAPNS1, the regulatory subunit of calpain-1, and calpain-2, as well as their catalytic subunits, CAPN1 and CAPN2 [16, 17, 32]. Our results indicated that capns1 knockout was associated with attenuation of asthmatic inflammation-induced calpain activation, overdeposition of collagen-I, and thickening of the smooth muscle layer of bronchial wall in the lungs of asthmatic mice. Moreover, administration of the calpain inhibitor calpeptin to asthmatic mice also prevented airway smooth muscle remodelling. These data indicate that calpain promotes ASM remodelling in this OVA-sensitized and OVA-challenged asthma model.

Calpain is an intracellular Ca2+-dependent cysteine protease. While there is no direct evidence that the cytokines IL-4, IL-5, and TNF-α can activate calpain, we have reported previously that IL-4, IL-5, and TNF-α enhance the calcium response of ASMCs to contractile agonists [38], suggesting that these increased intracellular Ca2+ levels may activate calpain. TGF-β1 was reported to up-regulate calpain-2 expression and activity via the ERK/MAP kinase pathway, which is responsible for TGF-β1-induced myoblast migration [39]. In the current study, we demonstrated that IL-4, IL-5, TNF-α, and TGF-β1 increased calpain activity, collagen-I synthesis, and cell proliferation of ASMCs, and this was attenuated by the calpain inhibitor MDL28170. These data provide the first evidence that calpain mediates inflammatory cytokine-induced collagen-I synthesis and cell proliferation in ASMCs.

We also examined signalling downstream of calpain in ASMCs. We found that p-Akt levels were increased in lung tissues of asthmatic mice, and capns1 knockout attenuated those increases. Consistent with those in vitro observation, the in vivo studies using cultured ASMCs showed that the cytokines IL-4, IL-5, TNF-α, and...
TGF-β1 induced up-regulation of p-Akt levels, and the calpain inhibitor MDL28170 prevented these increases. Furthermore, the PI3K inhibitor LY2940022 attenuated cytokine-induced collagen-I synthesis and cell proliferation of ASMCs. These observations indicate that calpain induces ASMC remodelling via activation of the PI3K/Akt pathway. Moreover, we found enhanced parenchymal inflammation around the airway in asthma challenged lung, and this was attenuated in capns1 knockout mice. Nozaki et al. [40] reported that calpain mediated secondary inflammatory changes in the muscle cells. Thus, calpain-induced secondary inflammation around the airway might also contribute to ASMC remodelling.

We then studied the mechanism of calpain-mediated activation of the PI3K/Akt signalling pathway. Akt is an upstream activator of the mTOR kinase, which in turn activates critical downstream pathways that drive cell growth. Interestingly, the Rictor-associated rapamycin-insensitive mTORC2 kinase phosphorylates Akt on Ser-473, thus also serving an important role upstream of Akt [41]. Although there has been no direct evidence reported on the activation of mTORC2 kinase by calpain, it has been observed that Akt activation is disrupted by calpain knockout [42, 43]. Here, we show that inhibition of calpain reduces cytokine-induced increases in Rictor protein levels. Our additional experiments revealed that calpain inhibitor also prevented the increased expression of Rictor in asthmatic lungs (Fig. S2). Furthermore, the mTORC1/2 inhibitor torin 1 and Rictor knockdown attenuated cytokine-induced phosphorylation of Akt, collagen-I synthesis, and cell proliferation of ASMCs. Moreover, torin 1 attenuated the increased expressions in p-Akt and ASM remodelling in a mouse asthma model. Collectively, these data suggest that there is a regulatory interaction between calpain, mTORC2, and Akt in cytokine-stimulated ASMCs. Calpain exerts its function by limited cleavage of its substrates, including the phosphatase and tensin homolog on chromosome 10 (PTEN) and protein phosphatase 2A (PP2A) [44, 45]. Because PTEN negatively regulates mTORC2 formation [46], degradation PTEN by calpain results in an increase in mTORC2. Thus, we speculate that calpain activates mTORC2/Akt by cleavage of PTEN.

In conclusion, this study presents strong evidence that calpain mediates cytokine-induced collagen-I synthesis and proliferation of ASMCs via the mTORC2/Akt signalling pathway and that calpain therefore plays an important role in asthmatic ASM remodelling (Fig. 8).

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Conflict of interest

The authors declare no conflict of interest.

References

4 Hassan M, Jo T, Risse PA et al. Airway smooth muscle remodeling is a dynamic process in severe long-standing asthma.

5 Johnson PR, Roth M, Tamm M et al. Airway smooth muscle cell proliferation is increased in asthma. Am J Respir Crit Care Med 2001; 164:474–7.
9 Khan MA. Inflammation signals airway smooth muscle cell proliferation in asthma pathogenesis. Multidiscip Respir Med 2013; 8:11.
12 Redington AE, Madden J, Frew AJ et al. Transforming growth factor-beta 1 in asthma. Measurement in


45 Bhattacharya K, Maiti S, Mandal C. PTEN negatively regulates mTORC2 formation and signaling in grade IV glioma via Rictor hyperphosphorylation at Thr1135 and direct the mode of action of an mTORC1/2 inhibitor. *Oncogene* 2016; 5:e227.
Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Torin 1 attenuates Rictor expression in ASM in a mouse model of chronic asthma.
Figure S2. Calpain inhibitor calpeptin attenuates Rictor expression in ASM in a mouse model of chronic asthma.
Table S1. Clinical information of patients with asthma and control subjects.