p70 Ribosomal S6 Kinase Is Required for Airway Smooth Muscle Cell Size Enlargement but Not Increased Contractile Protein Expression

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We examined the contribution of p70 ribosomal S6 kinase (p70S6K) to airway smooth muscle hypertrophy, a structural change found in asthma. In human airway smooth muscle cells, transforming growth factor (TGF)- β , endothelin-1, and cardiotrophin-1 each induced phosphorylation of p70S6K and ribosomal protein S6 while increasing cell size, total protein synthesis, and relative protein abundance of α -smooth muscle actin and SM22. Transfection of myocytes with siRNA against either p70S6K or S6, or infection with retrovirus encoding a kinase-dead p70S6K, reduced cell size and protein synthesis but had no effect on contractile protein expression per mg total protein. Infection with a retrovirus encoding a constitutively active, rapamycin-resistant (RR) p70S6K increased cell size but not contractile protein expression. siRNA against S6 decreased cell size in myocytes expressing RR p70S6K. Finally, TGF- β treatment, but not RR p70S6K expression, increased KCl-induced fractional shortening. Together, these data suggest that p70S6K activation is both required and sufficient for airway smooth muscle cell size enlargement but not contractile protein expression. Further, ribosomal protein S6 is required for p70S6K-mediated cell enlargement. Finally, we have shown for the first time in a functional cell system that p70S6K-mediated myocyte enlargement alone, without preferential contractile protein expression, is insufficient for increased cell shortening.

Keywords: asthma; hyperresponsiveness; hypertrophy; remodeling

Increased smooth muscle mass is the most prominent pathologic change observed in the airways of patients with asthma. Clinical studies examining the underlying cellular mechanism are limited, but suggest that increased cell size (1, 2), increased contractile protein expression (2), and hyperplasia (1, 3) each play a role. Despite evidence of increased airway smooth muscle mass in asthma, little is known about the biochemical mechanisms regulating this process. Further, it is unclear whether increased smooth muscle mass is accompanied by relative increases in contractile protein expression, or whether increased smooth muscle mass is sufficient to induce a state of airways hyperresponsiveness, the physiological hallmark of asthma.

A number of peptide growth factors and bronchoconstrictor agonists have been shown to induce elements of airway smooth muscle hypertrophy *in vitro*. We showed that transforming growth factor (TGF)- β , a pro-asthmatic cytokine (4–7), increased human bronchial smooth muscle cell size, protein synthesis,

expression of α -smooth muscle actin and smooth muscle myosin heavy chain, formation of actomyosin filaments, and cell shortening to acetylcholine (8). Cardiotrophin (CT)-1, a member of the IL-6 superfamily present in human lungs, has been shown to induce protein synthesis and cell enlargement in cultured human bronchial smooth muscle cells (9) and guinea pig airway explants (10). Endothelin (ET)-1, a G protein-coupled receptor-activating peptide that is increased in the airway epithelium and bronchoalveolar lavage fluid of patients with asthma (11, 12), increases both airway smooth muscle cell size and contractile protein expression (13).

Translational mechanisms play an important role in regulating cardiac, skeletal muscle, and smooth muscle hypertrophy. For example, we previously showed that inhibition of glycogen synthase kinase (GSK)-3 β increases human airway smooth muscle cell size in part by reducing phosphorylation of eukaryotic initiation factor-2B ϵ (14). In the current study, we focus on ribosomal p70 S6 kinase (p70S6K), a mitogen- and amino acid-sensitive serine-threonine kinase that ubiquitously regulates cell size (15, 16). p70S6K is phosphorylated and activated by mammalian target of rapamycin (mTOR) (17). p70S6K, in turn, phosphorylates the 40S ribosomal protein S6. The precise mechanism by which p70S6K controls translation is unclear. In addition to ribosomal protein S6, eukaryotic elongation factor-2 kinase is a phosphorylation target of p70S6K (18). In addition, p70S6K also mediates assembly of eukaryotic initiation factor-3 translation preinitiation complex (19).

The specific contribution of p70S6K activation to airway smooth muscle hypertrophy has not been established. In serum-deprived cultured canine airway myocytes, *in vitro* assays revealed sustained activation of p70S6K with concomitant accumulation of smooth muscle (SM)-22 and smooth muscle myosin heavy chain (smMHC). Conversely, rapamycin, a chemical inhibitor of the p70S6K kinase mammalian target of rapamycin (mTOR), blocked p70S6K activation, cellular enlargement, and accumulation of contractile proteins SM22 and smMHC, suggesting a requirement for p70S6K (20). However, experiments using rapamycin to infer the role of p70S6 kinase are obscured by the inhibitory effect of rapamycin on another mTOR downstream effector, eukaryotic initiation factor-4E binding protein (4E-BP). We have found that 4E-BP, which regulates cap-dependent mRNA translation, is required for TGF- β -induced cell size enlargement and contractile protein expression human airway smooth muscle cells (8, 21).

In the present study, we examined the requirement of p70S6K for TGF- β -, ET-1-, and CT-1-induced airway smooth muscle cell enlargement and contractile protein expression, as well as the sufficiency of p70S6K activation for these outcomes. By design, we stimulated cells with these mediators under serum-free conditions for 4 days, to obtain a hypertrophic (not hyperplastic) cell phenotype. We found that activation of p70S6K is required and sufficient for airway smooth muscle cell size enlargement but not preferential expression of contractile

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proteins. Further, ribosomal protein S6 is required for p70S6K-mediated cellular enlargement. Finally, we found that p70S6K-mediated myocyte enlargement is insufficient for increased cell shortening.

MATERIALS AND METHODS

Cell Culture

Primary human airway smooth muscle cells were isolated by enzymatic digestion from lung donor tissue unsuitable for transplantation. This protocol was approved by the University of Chicago and University of Michigan Institutional Review Boards. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and penicillin-streptomycin. Cells were seeded on uncoated plastic culture plates at approximately 50% confluence. Experiments were performed on passage 2–5 cells. Prior to experiments, cells were serum deprived for 24 hours. Cells were treated with TGF- β (10 ng/ml), ET-1 (1 μ mol/L), or CT-1 (10 ng/ml) under serum-free conditions for 4 days. Under serum-free culture conditions, there is no change in DNA synthesis or cell number during the course of these experiments (14). Fresh medium and chemicals were added 48 hours after initial treatment. Experiments were performed in the absence of serum.

For selected experiments, mouse airway smooth muscle cells were studied. Cells from BALB/c mice were isolated by dissection of major bronchi from the lung, mincing well, and incubating for 1 hour at 21°C on a rotary mixer in DMEM with 0.1% trypsin and 0.1% collagenase. Proteolysis was stopped by the addition of 9 vols DMEM with 10% FBS. Intact cells and tissue debris were sedimented at $1,000 \times g$ for 5 minutes, dispersed with further mincing, and plated in growth media with antibiotics. Media were changed every day for the next week to remove floating cells, and all tissue fragments were removed at the end of 1 week.

Immunoblotting

Human bronchial smooth muscle cell lysates were matched for protein concentration, resolved by SDS-PAGE, and transferred to nitrocellulose or PVDF membrane. Membranes were blocked in 5% milk for 1 hour and probed with mouse anti- α -smooth muscle actin (Calbiochem, San Diego, CA), mouse anti-SM22 (Abcam, Cambridge, MA), mouse anti-smooth muscle myosin heavy chain (MHC) (clone hSM-V; Sigma-Aldrich, St. Louis, MO), rabbit anti-phospho-threonine 389-p70S6K, rabbit anti-total p70S6K (both from Cell Signaling, Danvers, MA), rabbit anti-phospho-serine 240/244 ribosomal protein S6, and rabbit anti-total ribosomal protein S6 (both from Cell Signaling). Antibody binding was detected with a peroxidase-conjugated anti-rabbit or anti-mouse IgG and chemiluminescence.

Transfection of Cells with siRNA against p70S6K or Ribosomal Protein S6

Nineteen-base pair duplexes of p70S6K siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) were transfected into subconfluent primary human airway smooth muscle cells using Oligofectamine in OptiMEM (Invitrogen, Carlsbad, CA). A pool of double-stranded siRNAs containing equal parts of the following antisense sequences was used to knockdown p70S6K: 1, 5'-CAAGGUCAUGUGAAACUAA-3'; 2, 5'-GAGAGUCAAUGUCAUUACA-3'; 3, 5'-CUCGCGACAU CUUUCUCAA-3'; 4, 5'-PCAAAGAUCAACUCUGGUGCUU-3'. The corresponding nontargeting siRNA sequence was 5'-CGAACU CACUGGUCUGACCGT-3' (sense), 5'-GGUCAGACCAGUGAG UUCGdtdt-3' (antisense). For knockdown of S6, a pool of the following sequences was used: 1, 5'-GAAGCAGCGUACCAAGAAA-3'; 2, 5'-CUGCGAGCUUCUACUUCUA-3'; 3, 5'-GUCUGAAUCCA GUCAGAAA-3'. Six hours later, DMEM and FBS were added. The next morning, cells were incubated in fresh DMEM containing 10% FBS for 24 hours. Finally, cells were treated with the relevant stimulus in serum-free medium for 2 days before harvest.

Cell Size Analysis

Human bronchial smooth muscle cell size was measured by fluorescence-activated cell sorting. Cells were treated with TGF- β , ET-1, or CT-1.

Cells were collected and fixed with 75% ethanol and stored at -20°C before staining. Cells were centrifuged and stained with propidium iodide (50 $\mu\text{g/ml}$)-RNAse (100 $\mu\text{g/ml}$) solution for 1 hour. Cells in G₀/G₁ phase were gated for forward scatter measurement using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Protein Synthesis

Cells were serum starved for 24 hours before experiments. Cells were plated at 5×10^5 cells/well (or 3×10^5 cells/well for experiments involving transfection) and incubated in [^3H]-leucine (0.5 μCi ; PerkinElmer, Boston, MA) for 48 hours. Cells were lysed and proteins precipitated with 10% trichloroacetic acid. After washing with cold ethanol and solubilization with 1% triton-X100 in 0.5 M NaOH, radioactivity was measured by scintillation counter.

Retroviral Transduction of Murine Bronchial Cell Lines

cDNAs encoding a kinase-dead p70S6K (KD; Lys100Arg) and a rapamycin-resistant, constitutively active p70S6K (E₃₈₉ Δ CT) were individually subcloned into the pMSCVpuro retroviral vector (BD Biosciences). The Phoenix-GP retrovirus packaging cell line, a 293-cell derivative line that expresses only the gag-pol viral components (provided by G. Nolan, Stanford University) was transiently transfected with pHCMV-G, which contains the vesicular stomatitis virus envelope glycoprotein, and either pMSCVpuro-HA-KD or HA-E₃₈₉ Δ CT or pMSCVpuro alone. Viral supernatant was collected, filtered, and supplemented with polybrene (8 $\mu\text{g/ml}$). Mouse bronchial smooth muscle cells were infected with viral supernatant (four times for 4 h each). Infected cells were selected with puromycin (1 $\mu\text{g/ml}$). After selection, cells were grown to confluence, split into 6-well plates, and incubated in the absence or presence of TGF- β , ET-1, or CT-1.

Immunofluorescence Microscopy

Mouse bronchial smooth muscle cells were grown on collagen-coated glass slides (BD Biosciences) and fixed in 1% paraformaldehyde. For cells, slides were stained with Hoechst 33342 for nucleic acid and Cy3-conjugated anti- α -actin. For lung sections, slides were stained with Hoechst 33342 for nucleic acid, Cy3-conjugated anti- α -actin, and either anti-phospho-threonine 389 p70S6K, anti-total p70S6K, anti-phospho-serine 240/244 S6 ribosomal protein, or anti-total S6 ribosomal protein (all from Cell Signaling). Binding of antibodies was visualized using AlexaFluor 488 conjugates of either goat or donkey anti-rabbit IgG (Invitrogen).

Cell Contraction

Individual cell length before and after contraction with KCl was measured by computerized image micrometry, as described (14). pMSCV-, pMSCV-AA-KD-, and pMSCV-E₃₈₉ Δ CT-infected murine smooth muscle cells were seeded in 100-mm dishes and grown to confluence in serum-free medium or medium supplemented with TGF- β . At confluence, cells were scraped off with a rubber policeman, triturated, and transferred to polypropylene tubes. At this stage, cells tend to maintain a contracted state due to mechanical stimulation. The cells were treated with 8-bromo-cAMP, then allowed to float freely and relax overnight with occasional swirling to prevent settling or sticking to the sides of the tube. During this period, cells regain a cylindrical shape. Aliquots of cultured cell suspension (2.5×10^4 cells/0.5 ml) were stimulated with KCl (75 mM). The reaction was allowed to proceed for 4 minutes and stopped by the addition of 0.1 ml of glutaraldehyde at a final concentration of 1% (vol/vol). Fixed cells were allowed to settle and then transferred by wide-mouth pipet to a microscope slide for analysis. The average length of cells before or after addition of test agents was obtained from 20 cells encountered in successive microscopical fields.

Ovalbumin Sensitization/Challenge Model and Isolation of Murine Airway Smooth Muscle Cells

Balb/c mice (Charles River Labs, Wilmington, MA) were sensitized and challenged to endotoxin-free ovalbumin (Pierce Chemical, Rockford, IL) as previously described (22). On Day 0, mice were anesthetized and injected intraperitoneally with 200 μl of a suspension of 25% wt/vol alum (Pierce) in either PBS or ovalbumin (5 mg/ml). On Day 11,

animals were given an identical intraperitoneal injection as well as a 50- μ l intranasal instillation of PBS or ovalbumin (20 mg/ml). On Days 18, 21, 22, and 23, intranasal instillations were repeated. Twenty-four hours after the final challenge, mice were killed and the lungs were inflated with 10% formalin to 30 cm H₂O pressure and processed for paraffin embedding and sectioning. Five-micron sections were processed through a xylene/ethanol hydration series, antigens exposed by heat denaturation, and sections processed for immunofluorescence microscopy. Finally, bronchial smooth muscle cells were isolated from selected control and ovalbumin-sensitized and -challenged mice, as described above.

RESULTS

TGF- β , ET-1, and CT-1 Increase Phosphorylation of p70S6K and Ribosomal Protein S6 in Human Airway Smooth Muscle Cells

To examine the contribution of p70S6K to TGF- β -, ET-1-, and CT-1-induced hypertrophic responses, we first analyzed if TGF- β , ET-1, and CT-1 increase the phosphorylation of this kinase in cultured human airway smooth muscle cells. Early passage human cells were treated with TGF- β (10 ng/ml), ET-1 (1 μ mol/L), and CT-1 (10 ng/ml) for 4 days, and p70 S6 kinase phosphorylation was assessed by immunoblotting with a phosphospecific antibody. TGF- β , ET-1, and CT-1 each enhanced the phosphorylation of p70 S6 kinase without affecting that of total p70 S6 kinase (Figure 1A). We also assessed changes in the phosphorylation of ribosomal protein S6, a downstream target of p70S6K that regulates protein translation. TGF- β , ET-1, and CT-1 each conferred an increase in the abundance of phospho-ribosomal S6 protein, though the level of S6 phosphorylation did not necessarily correlate with the level of p70S6K phosphorylation (Figure 1B).

TGF- β , ET-1, and CT-1 Each Increase Human Airway Smooth Muscle Cell Size and Contractile Protein Expression

Cell size was analyzed by flow cytometry. TGF- β , ET-1, and CT-1 treatment each caused a rightward shift in forward scatter (Figure 2A). Increases in cell size were accompanied by protein synthesis, as evidenced by [³H]-leucine incorporation (Figure 2B). Contractile protein expression was assessed by immunoblotting. TGF- β , ET-1, and CT-1 each increased α -actin, MHC, and SM22 expression relative to β -actin (Figure 2C).

Figure 1. Transforming growth factor (TGF)- β , endothelin (ET)-1, and cardiotrophin (CT)-1 increase phosphorylation of p70S6K and ribosomal protein S6. (A) Human airway smooth muscle cells were treated with TGF- β (10 ng/ml), ET-1 (1 μ mol/L), or CT-1 (10 ng/ml). Representative immunoblots for phospho- and total p70S6K are shown. TGF- β , ET-1, and CT-1 increased the level of phospho-p70S6K without changing that of total p70S6K. (B) Immunoblots of phospho- and total ribosomal protein S6. TGF- β , ET-1, and CT-1 increased phospho-ribosomal protein S6 without affecting that of total ribosomal protein. The results shown are representative of three experiments.

p70 S6 Kinase Is Required for TGF- β -, ET-1-, and CT-1-Induced Cell Size Enlargement but Not Contractile Protein Expression

To determine the requirement of p70S6K for airway smooth muscle enlargement and contractile protein expression, we knocked down total p70S6K expression using specific siRNA. Immunoblotting showed that cells treated with p70S6K siRNA displayed a marked decrease in p70S6K protein abundance compared with cells treated with nontargeting siRNA (Figure 3A). In cells pretreated with nontargeting siRNA, each stimulus increased cell size, as evidenced by forward scatter, compared with untreated cells (Figure 3B). Treatment with p70S6K siRNA significantly decreased TGF- β -, ET-1-, and CT-1-induced changes in cell size. p70S6K siRNA also significantly decreased

Figure 2. TGF- β , ET-1, and CT-1 increase human airway smooth muscle cell size and contractile protein expression. (A) Effect of TGF- β , ET-1, or CT-1 on human airway smooth muscle cell size. Cell size was determined by flow cytometry. (B) Protein synthesis of human airway smooth muscle cells treated with TGF- β , ET-1, or CT-1. Protein synthesis was assessed by [³H]-leucine incorporation (cpm/well, mean $\pm$ SD, *different from control, $P < 0.05$ relative to control, one-way ANOVA). (C) Representative immunoblots displaying the expression of α -smooth muscle actin, myosin heavy chain (MHC), and SM22 in cells treated with TGF- β , ET-1, or CT-1.

Figure 3. p70S6K is required for TGF- β -, ET-1-, and CT-1-induced increases in human airway smooth muscle cell size but not contractile protein expression. (A) Immunoblots demonstrating expression of total p70S6K in human airway smooth muscle cells treated with nontargeting or p70S6K siRNA. Transfected cells were untreated or stimulated with TGF- β , ET-1, or CT-1. (B and C) Effects of nontargeting and p70S6K siRNA on cell size, as assessed by flow cytometry, and protein synthesis, as assessed by [3 H]-leucine incorporation. Cells were treated with TGF- β , ET-1, or CT-1 ($n = 3-5$, mean $\pm$ SD, *different from unstimulated cells, $P < 0.05$, ANOVA; †different from corresponding TGF- β , ET-1, or CT-1 treated cells, $P < 0.05$, ANOVA). (C) Immunoblots of α -smooth muscle actin, MHC and SM22 expression from cells transfected with non-targeting or p70S6K siRNA.

protein synthesis, though the effect was incomplete (Figure 3B). These data suggest that activation of p70S6K pathway is required for TGF- β -, ET-1-, and CT-1-induced cell size enlargement and maximal protein synthesis. We then measured contractile protein expression in response to these stimuli. In cells pretreated with nontargeting siRNA, TGF- β , ET-1, and CT-1 each increased α -actin, MHC, and SM22 expression per mg protein without affecting β -actin expression. However, there was no effect of p70S6K siRNA on α -smooth muscle actin, MHC, or SM22 expression relative to β -actin (Figure 3C). These data suggest that activation of p70S6K is not required for TGF- β -, ET-1-, and CT-1-induced contractile protein expression.

We also knocked down total ribosomal protein S6 using specific siRNA (Figure 4). Similar to siRNA against p70S6K, siRNA against S6 decreased cell size (Figure 4A) but not α -smooth muscle actin or MHC protein expression per mg total protein (Figure 4B).

Activation of p70S6K Is Sufficient for Airway Smooth Muscle Cell Enlargement but Not Contractile Protein Expression

We infected mouse airway smooth muscle cells with retrovirus encoding either empty vector (pMSCV), a constitutively active form of rapamycin-resistant (RR) p70S6K (E389 Δ CT), or kinase dead (KD) p70S6K. Immunoblots of RR and KD p70S6K cell lysates stained positively for the hemagglutinin tag (Figure 5A). Untreated cells expressing RR p70S6K displayed an enhancement of ribosomal protein S6 phosphorylation but not total ribosomal protein S6 expression, indicating that the RR p70S6K is constitutively active (Figure 5B).

In contrast, phosphorylation of ribosomal protein S6 in KD p70S6K cells was reduced. Further, these data confirm that S6 phosphorylation is regulated by p70S6K.

As expected, TGF- β , ET-1, and CT-1 each increased the size of cells infected with empty vector alone (MSCV, Figure 5C). Infection with RR p70S6K increased forward scatter compared with cells infected with empty vector alone, suggesting that activation of p70S6K is sufficient for airway smooth muscle cell size enlargement. Treatment with TGF- β , ET-1, and CT-1 did not further increase cell size in cells infected with RR p70S6K. Overexpression of KD p70S6K blocked TGF- β -, ET-1-, and CT-1-induced increases in cell size, indicating that activation of p70S6K is required for TGF- β -, ET-1-, and CT-1-induced cell size enlargement.

Next, we examined the effects of selective activation and inhibition of p70S6K on contractile protein expression. TGF- β treatment increased α -actin, MHC, and SM22 expression in pMSCV, pMSCV-RR p70SK and pMSCV KD p70S6K cells (Figure 5D). RR p70S6K by itself did not increase α -actin or SM22 expression, and expression of KD p70S6K failed to attenuate TGF- β -induced contractile protein expression. Together, these data suggest that activation of p70S6K is neither sufficient nor required for contractile protein expression.

The precise mechanism by which p70S6K controls translation is unclear. In addition to ribosomal protein S6, eukaryotic elongation factor-2 kinase is also a phosphorylation target of p70S6K (18). p70S6K also mediates assembly of eukaryotic initiation factor-3 translation preinitiation complex (19). siRNA against S6 decreased cell size in myocytes expressing RR

Figure 4. Ribosomal protein S6 is required for TGF- β -, ET-1-, and CT-1-induced increases in human airway smooth muscle cell size but not contractile protein expression. (A) Immunoblot demonstrating significant reduction in S6 protein expression after treatment with siRNA against S6 (*upper panel*). TGF- β -, ET-1-, and CT-1-induced α -actin expression and MHC were unaffected. (B) Effect of siRNA against S6 on cell size, as assessed by forward scatter (*different from control, $P < 0.05$; †different from nontargeting siRNA, $P < 0.05$).

p70S6K (Figure 5E), consistent with the notion that S6 is required for p70S6K-mediated cell size enlargement.

The selective effects of p70S6K inhibition and activation on cell size and contractile protein expression were also examined by fluorescence immunocytochemistry (Figure 6). Untreated mouse cells infected with empty vector (pMSCV) show minimal α -smooth muscle actin staining. After TGF- β treatment, cells increase in size and express abundant α -actin staining. Unstimulated cells infected with RR p70S6K enlarge, but continue to show modest staining for α -actin. Finally, cells infected with KD p70S6K fail to enlarge upon TGF- β treatment, but show ample expression of α -actin. Together, these results confirm that p70S6K is required and sufficient for cell enlargement but not contractile protein expression.

Effect of Ovalbumin Sensitization and Challenge on Airway Smooth Muscle and p70 S6 Kinase Phosphorylation

Ovalbumin sensitization and challenge is a commonly employed model of asthma. After a 3-week sensitization and challenge protocol, airways from ovalbumin-treated mice (Figures 7A and 7B) exhibited increased α -actin immunostaining (*red channel*) compared with PBS-treated controls (Figures 7C and 7D), as previously reported (14, 22, 23). In this model, increased airway smooth muscle mass is a consequence of both airway smooth muscle hypertrophy and hyperplasia (24). Ovalbumin treatment also induced an increase in airway smooth muscle layer p70S6K phosphorylation (Figure 7A, *green channel*) and total p70S6K expression (Figure 7B, *green channel*). Airway smooth muscle cells isolated from ovalbumin-treated mice showed similar changes in phospho- and total p70S6K (Figure 7E). In contrast, airway smooth muscle cells from ovalbumin-treated mice showed increased phospho-ribosomal protein S6 content but no increase in the expression of ribosomal protein S6. Together, these data are consistent with the notion that phosphorylation and activation of p70S6K contributes to airway remodeling *in vivo*.

p70S6K-Induced Airway Smooth Muscle Enlargement Does Not Increase Cell Shortening in Response to KCl

To determine whether p70S6K-mediated cellular enlargement is accompanied by increased contractile function, pMSCV-, RR p70S6K-, and KD p70S6K-mouse airway smooth muscle cells were grown to confluence in either serum-free medium or that containing TGF- β . At confluence, cells were gently scraped off the plate, transferred to polypropylene tubes, and relaxed with 8-bromo cAMP. Aliquots were treated with 75 mM KCl and fixed with glutaraldehyde, and cell length was measured by computerized image micrometry. As previously shown (14), cells treated with TGF- β were longer at rest and demonstrated increased fractional shortening compared with untreated cells (Figure 8). Consistent with the flow cytometry data (above), unstimulated KD p70S6K cells were shorter, and RR p70S6K cells longer, than pMSCV cells. However, RR cells failed to shorten upon KCl stimulation.

DISCUSSION

In the present study, we examined the contribution of p70S6K to cellular hypertrophy. We found that treatment with TGF- β , ET-1, and CT-1, each of which have been implicated in asthmatic airway remodeling and the induction of airway smooth muscle hypertrophy (4–13), increased phosphorylation of both p70S6K and its downstream target ribosomal protein S6. Further, TGF- β -, ET-1-, and CT-1-mediated increases in airway smooth muscle cell size and protein synthesis were blocked by specific siRNA against p70S6K and S6, as well as overexpression of kinase-dead p70S6K. However, TGF- β -, ET-1-, and CT-1-induced increases in α -smooth muscle actin, MHC and SM22 expression relative to β -actin were not affected by these interventions. Moreover, overexpression of a constitutively active form of p70S6K elicited an increase in airway smooth muscle cell size but not contractile protein expression or cell shortening. Together, these data suggest that activation of

Figure 5. Activation of p70S6K is required and sufficient for airway smooth muscle hypertrophy but not contractile protein expression. Empty vector (pMSCV), kinase-dead (KD), and constitutively active rapamycin-resistant (RR) p70S6K were expressed in mouse airway smooth muscle cells by retroviral gene transfer. (A) Expression of the hemagglutinin (HA) tag in KD and RR p70S6K-transduced cells. (B) Effects of KD and RR p70S6K overexpression on phosphorylation of ribosomal protein S6. Infected cells were untreated or stimulated with TGF- β , ET-1, or CT-1. Immunoblots representing phospho- and total ribosomal S6 protein are shown. (C) Effects of KD and RR p70S6K overexpression on cell size, as assessed by flow cytometry. pMSCV, KD, and RR p70S6K-transduced cells were untreated or stimulated with TGF- β , ET-1, or CT-1 ($n = 3-5$, mean $\pm$ SD, *different from pMSCV, $P < 0.05$, ANOVA). (D) Contractile protein expression in pMSCV, KD, and RR p70S6K cells untreated or stimulated with TGF- β . (E) siRNA against ribosomal protein S6 attenuates RR p70S6K- and TGF- β -mediated cell enlargement.

p70S6K is required and sufficient for myocyte enlargement in cultured airway smooth muscle cells. Further, they demonstrate that p70S6K-mediated cell size enlargement is due to a global increase in cellular protein, including cytoskeletal proteins such

as β -actin, rather than a preferential increase in contractile proteins. Finally, these data show for the first time in a functional cell system that myocyte enlargement is insufficient for increased cell shortening.

Figure 6. Immunofluorescence studies confirm that p70S6K is required and sufficient for airway smooth muscle hypertrophy but not contractile protein expression. Mouse airway smooth muscle cells infected with pMSCV, RR p70S6K, or KD p70S6K were plated on collagen-coated glass slides, serum deprived for 24 hours, and treated for 6 days without or with 10 ng/ml TGF- β . Cells were fixed and stained with Hoescht 33342 for nucleic acid (blue) and Cy3-conjugated anti- α -smooth muscle actin (red). In the upper panels, phase contrast views of unstimulated cells are superimposed on the immunofluorescence signals to show cell size.

Figure 7. Ovalbumin sensitization and challenge increases airway smooth muscle phospho-p70S6K content and α -smooth muscle actin expression. Immunofluorescence of tissue from ovalbumin-sensitized and -challenged mice (A and B) show greater α -smooth muscle actin expression (red), phospho-p70S6K content (A and C, green), and total p70S6K expression (B and D, green) compared with PBS treated mice (C and D). (E) Immunoblots of phospho- and total p70S6K and ribosomal S6 protein, as well as MHC, from PBS- and ovalbumin-treated animals.

As noted above, TGF- β , ET-1, and CT-1 have each been noted to induce elements of hypertrophy of cultured airway smooth muscle cells. TGF- β augments cell size and contractile protein synthesis through several distinct mechanisms. TGF- β treatment induces phosphorylation of eukaryotic translation initiation factor-4E-binding protein (8) and phosphorylation of GSK-3 β (14). Phosphorylation and inhibition of GSK-3 β , in turn, derepresses transactivation of nuclear factors of activated T cells (NFAT), and serum response factor, transcription factors involved in muscle-specific gene expression. Inhibition of GSK-3 β also reduces phosphorylation of eukaryotic initiation factor-2B ϵ , thereby promoting translation of contractile protein-encoding mRNAs. ET-1 increases airway smooth muscle protein synthesis and α -actin, SM22, and calponin expression in an extracellular signal regulated kinase-dependent manner (13). CT-1 increases airway smooth muscle cell size, protein synthesis (9, 10), contractile protein expression, and GSK-3 β phosphorylation (14). Finally, inhibition of the GSK-3 β

Figure 8. Expression of RR p70S6K in mouse airway smooth muscle cells does not increase cell shortening in response to 75 mM KCl. Compared with unstimulated cells, treatment with TGF- β increased resting length and fractional shortening of pMSCV and KD p70S6K cells. However, hypertrophic RR p70S6K cells failed to respond to KCl. ($n = 20$ for each group, mean $\pm$ SD, * $P < 0.05$ relative to control cells without KCl treatment, ANOVA).

pathway is required for CT-1- but not TGF- β -induced hypertrophy (14).

The precise contribution of p70S6K activation to airway smooth muscle hypertrophy has not been established. Further, the functional consequences of p70S6K-mediated hypertrophy have not been studied. In C2C12 mouse skeletal myoblasts, selective activation of p70S6K was sufficient for myotube hypertrophy (increased cell size) (25). Rapamycin, a chemical inhibitor of mTOR, fails to attenuate myotube formation or myosin heavy chain expression in rat L6E9, mouse Sol8 and primary human myoblasts (26). Cardiac-specific transgenic mice overexpressing a rapamycin-resistant mutant of p70S6K with higher basal activity show cardiac hypertrophy, as assessed by heart weight, though deletion of p70S6K does not attenuate insulin-like growth factor-I or phosphatidylinositol 3-kinase-mediated cardiac hypertrophy (27). The effects of RR p70S6K expression on cardiac function were not examined. In vascular smooth muscle, chemical inhibitors of p70S6K (tosylphenylalanine chloromethyl ketone and tosyllysine chloromethyl ketone) had no effect on angiotensin II-induced protein synthesis (28). Together, these results suggest that p70S6K is sufficient but not required for muscle hypertrophy.

In the present study, using conditions optimized for the induction of cellular hypertrophy, we obtained different results. First, we found that inhibition of p70S6K with either specific siRNA or kinase-dead p70S6K blocked TGF- β -, ET-1-, and CT-1-induced airway smooth muscle protein synthesis and cell enlargement, whereas expression of rapamycin-resistant p70S6K was sufficient to induce these effects, demonstrating that p70S6K is both required and sufficient for airway smooth muscle cell enlargement. Second, when we examined the full range of hypertrophic responses, a more complex picture emerged. Neither specific siRNA nor KD blocked expression of α -smooth muscle actin, MHC, or SM22 relative to β -actin. Moreover, RR p70S6K failed to selectively increase contractile protein expression. When contractile responses to KCl were examined, expression of KD p70S6K failed to inhibit TGF- β -induced shortening. Finally, longer RR p70S6K cells failed to shorten in response to KCl in the absence of TGF- β . Together, these data suggest that increases in cell size alone, without concurrent preferential increases in contractile protein expression, are insufficient to alter airway smooth muscle function. The failure of p70S6K to regulate contractile protein expression is analogous to recent

results we have obtained in canine tracheal smooth muscle cells expressing a constitutively-active form of the serine-threonine kinase Akt1 (L. Ma, J. Solway, unpublished data). Akt activation induced cellular enlargement without increasing expression of α -smooth muscle actin or SM22. Together, these studies hold relevance for the functional significance of airway smooth muscle remodeling in asthma. While increased smooth muscle mass is the most prominent pathologic change observed in the airways of patients with asthma, it is unclear whether cell enlargement is accompanied by specific increases in contractile protein expression. There are relatively few studies examining smooth muscle contractile protein expression in asthma. Two reports showed increased myosin light chain kinase expression (2, 29), while another study showed no increase in the mRNA expression of genes encoding contractile proteins (3). It also remains unclear whether hypertrophy is sufficient to induce a state of airways hyperresponsiveness, the physiological hallmark of asthma. In the present study, we show for the first time that enlarged airway smooth muscle cells do not develop increased shortening in the absence of concurrent changes in contractile protein expression.

The precise mechanism by which p70S6K controls translation is unclear. Other targets of p70S6K exist besides ribosomal protein S6, including the transcription factor cAMP-responsive element modulator (30), the 80-kD subunit of the nuclear RNA cap-binding complex, CBP80 (31), eukaryotic elongation factor-2 kinase (18), and the pro-apoptotic protein Bcl-xL/Bcl-2-associated death promoter (BAD) (32). p70S6K also mediates assembly of eukaryotic initiation factor-3 translation preinitiation complex (19). In our study, we found discrepancies between the level of p70S6K and S6 phosphorylation following mediator stimulation, consistent with the notion that p70S6K has multiple downstream phosphorylation targets. However, we also found that phosphorylation of ribosomal protein S6 was increased in RR p70S6K cells and decreased in KD p70S6K cells, demonstrating that p70S6K regulates S6 phosphorylation. However, it is unclear from these data alone whether S6 is responsible for p70S6K-mediated airway smooth muscle hypertrophy. To test this, we examined the effects of S6 knockdown on myocyte size and contractile protein. Like p70S6K knockdown, siRNA directed against S6 blocked TGF- β -induced cell size enlargement. Further, S6 knockdown blocked RR p70S6K-mediated myocyte hypertrophy. Together these results suggest that, in airway smooth muscle, p70S6K-mediated hypertrophy is mediated by ribosomal protein S6.

We would like to mention some potential limitations of our study. First, as noted in the Introduction, we studied cells in serum-free medium supplemented with TGF- β , ET-1, or CT-1 for 4 days to obtain a hypertrophic cell phenotype. Because hypertrophy depends in part on post-transcriptional mechanisms, and because the turnover of contractile proteins is relatively slow, the hypertrophic phenotype takes some days to develop. Under these conditions, it is possible that p70S6K was stimulated not only by these stimuli, but also by a second autocrine factor. Nevertheless, we felt that this experimental strategy was justified by our interest in the common downstream signal transduction mechanisms regulating hypertrophy, rather than the specific stimuli *per se*. On this basis, we also chose not to examine the specific concentrations of TGF- β , ET-1, and CT-1 necessary for the hypertrophic phenotype. Finally, in our study, we measured the shortening of unloaded dispersed airway smooth muscle cells to KCl stimulation. Measuring unloaded shortening capacity can be complicated by the possibility that the degree of activation and starting length may vary from cell to cell due to the preparation procedure. To address

this, cells were treated with a cell permeable cyclic AMP analog, 8-bromo-cAMP, and allowed to "relax" overnight prior to study. Nevertheless, we acknowledge that results could be different in living, excised strips of airway smooth muscle.

In conclusion, TGF- β , ET-1, and CT-1 cause airway smooth muscle cell size enlargement and protein synthesis, which require activation of p70S6K. However, selective increases in contractile protein synthesis caused by these factors are not dependent on p70S6K. Further, activation of p70S6K is sufficient for cellular enlargement, but not for contractile protein expression. Finally, hypertrophic RR p70S6K-expressing cells fail to demonstrate enhanced shortening. On this basis, we speculate that increases in airway smooth muscle mass must be accompanied by preferential increases in contractile protein expression if they are to induce airways hyperresponsiveness in asthma. Further studies examining the biology of airway smooth muscle hypertrophy may provide insight into the pathogenesis and functional significance of airway remodeling.

Conflict of Interest Statement: J.S. has received or will receive consultant fees of \$1,000 in 2006 from Tanox, \$1,600 in 2006 from Merck, \$2,700 in 2007 from AstraZeneca, \$3,500 in 2007 from Genentech, \$6,000 in 2008, and \$6,000 in 2009 from Cytokinetics, and \$2,500 in 2008 from Sepracor; he has received a \$1,017 honorarium from Eisai for delivering a lecture on research unrelated to Eisai's products; he has also served as principal investigator on a grant of \$125,031 from AstraZeneca to the University of Chicago in 2008–2009, with similar additional funding anticipated during 2009–2010. M.B.H. has received sponsored grants from the NIH for more than \$100,001, and received a grant from GlaxoSmithKline for a Collaborative Research Trial 2004–2006 for \$50,001 to \$100,000. D.C.F. has received sponsored grants from the NIH and the American Diabetes Association (both for more than \$100,001). J.K.B. has received a sponsored grant from the NIH for more than \$100,001. None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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