Transforming Growth Factor- β Induces Airway Smooth Muscle Hypertrophy

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Although smooth muscle hypertrophy is present in asthmatic airways, little is known about the biochemical pathways regulating airway smooth muscle protein synthesis, cell size, or accumulation of contractile apparatus proteins. We sought to develop a model of airway smooth muscle hypertrophy in primary cells using a physiologically relevant stimulus. We hypothesized that transforming growth factor (TGF)- β induces hypertrophy in primary bronchial smooth muscle cells. Primary human bronchial smooth muscle cells isolated from unacceptable lung donor tissue were studied. Cells were seeded on uncoated plastic dishes at 50% confluence and TGF- β was added. Experiments were performed in the absence of serum. TGF- β increased cell size and total protein synthesis, expression of α -smooth muscle actin and smooth muscle myosin heavy chain, formation of actomyosin filaments, and cell shortening to acetylcholine. Further, TGF- β increased airway smooth muscle α -actin synthesis in the presence of the transcriptional inhibitor actinomycin D, evidence that translational control is a physiologically important element of the observed hypertrophy. TGF- β induced the phosphorylation of eukaryotic translation initiation factor-4E-binding protein, a signaling event specifically involved in translational control. Finally, two inhibitors of 4E-binding protein phosphorylation, the phosphoinositol 3-kinase inhibitor LY294002 and a phosphorylation site mutant of 4E-binding protein-1 that dominantly inhibits eukaryotic initiation factor-4E, each blocked TGF- β -induced α -actin expression and cell enlargement. We conclude that TGF- β induces hypertrophy of primary bronchial smooth muscle cells. Further, phosphorylation of 4E-binding protein is required for the observed hypertrophy.

Keywords: 4E-binding protein; α -smooth muscle actin; eukaryotic initiation factor-4E; mammalian target of rapamycin (mTOR); phosphatidylinositol 3-kinase

Increased airway smooth muscle mass is present in asthma. Ebina and coworkers (1) found two asthmatic subtypes, one with airway smooth muscle hypertrophy throughout the airways and another with hyperplasia in central bronchi. Benayoun and colleagues (2) found that patients with severe asthma had increased airway smooth muscle cell diameter and expression of α -smooth muscle actin and myosin light chain kinase (MLCK). On the other hand, Woodruff and coworkers (3) found that patients with mild asthma show no increase in cell size, though cell number was 2-fold higher. While smooth muscle mass increased by 50–83%, contractile protein mRNA expression was not changed, suggesting the importance of post-transcriptional mechanisms.

Little is known about the biochemical pathways regulating airway smooth muscle protein synthesis, cell size, or accumulation of contractile apparatus proteins. We have studied two relevant cell culture models: prolonged serum deprivation of canine tracheal myocytes (4–6) and human bronchial smooth muscle clonal cell lines transduced with a temperature-sensitive large tumor antigen (7). In both, cell cycle arrest induces the formation of large myocytes with increased abundance of contractile proteins but not the corresponding mRNA, suggesting regulation by post-transcriptional mechanisms. However, both models are limited by the absence of a physiologically relevant stimulus for the observed hypertrophy.

We now describe a new model of airway smooth muscle hypertrophy in which primary cells are treated with transforming growth factor (TGF)- β . We selected TGF- β because it is increased in the airways of individuals with severe asthma, some of whom demonstrate airway smooth muscle hypertrophy (2), compared with patients with less severe disease (8–11). In asthmatic airways, TGF- β is localized to airway smooth muscle cells, as well as mucous glands, endothelial cells, and other cells infiltrating the submucosa (12). Together, these data suggest that airway smooth muscle cells may be a physiologic target of TGF- β elaboration in asthma. TGF- β has long been known to induce muscle hypertrophy via the regulation of gene transcription and cell cycle traversal (13–16). In cardiac myocytes, TGF- β induces the transcription of “fetal” contractile proteins, including α -smooth muscle actin (17). TGF- β also induces nonmuscle cells to express contractile proteins (15, 18–20). We hypothesize that, to induce hypertrophy, TGF- β must enhance mRNA translation as well as gene transcription. The effects of TGF- β on contractile protein translation, or on the signaling pathways regulating translation initiation, have not been studied.

There are three general mechanisms by which translation is regulated. The first two involve steps in the translation initiation pathway. First, translation of the majority of eukaryotic mRNAs is initiated through a 7-methylguanosine cap structure at the 5' end of mRNA. The cap is recognized and “clamped” by the 24-kD eukaryotic initiation factor (eIF)-4E. The scaffolding protein eIF4G binds to and stabilizes eIF4E, poly-A-binding protein, which in turn associates with the 3' mRNA poly-A tail, and eIF4A, an RNA helicase which serves to unwind secondary mRNA structure. In addition to interacting with eIF4G, eIF4E associates with inhibitory proteins termed 4E-binding proteins (4E-BPs). 4E-BP1 undergoes phosphorylation at multiple sites, which results in its release from eIF4E, thereby increasing the availability of eIF4E for binding to eIF4G, eIF4F complex formation, and cap-dependent translation (21, 22). Translation of mRNAs with regions of stable secondary structure within the 5' untranslated region are particularly dependent on eIF4F (23, 24). Second, concurrent with the preparation of mRNA, the pre-initiation complex must be formed. eIF2, a multimer consisting of α , β , and γ subunits, functions to recruit methionyl tRNA and conduct it as a tRNA-eIF2-GTP ternary complex to the 40S ribosomal subunit, to form the 43S pre-initiation complex. eIF2

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GTP loading is determined by the activity of eIF2B, a guanine nucleotide exchange factor. eIF2B phosphorylation by glycogen synthase kinase (GSK)-3 β inhibits its GDP/GTP exchange activity, thereby limiting binding of methionyl tRNA to the 40S ribosomal subunit (25). However, phosphorylation of GSK3 β by Akt inactivates it, leading to eIF2B dephosphorylation and activation and a general enhancement of translation initiation that is independent of the 7-methylguanosine mRNA cap. Finally, the translation of mRNAs with 5' terminal oligopyrimidine (TOP) tracts, many of which encode elongation factors and ribosomal proteins involved in mRNA translation, is upregulated by phosphorylation of the S6 ribosomal protein. S6 ribosomal protein, in turn, is phosphorylated by the mitogen- and amino acid-sensitive serine/threonine kinase p70 ribosomal S6 kinase (S6K)-1, which is in turn phosphorylated by mTOR. In contrast to the first two pathways, which regulate translation efficiency, this pathway regulates translational capacity by increasing the synthesis of ribosomes.

In this report, we show that TGF- β increases airway smooth muscle cell size, total protein synthesis, contractile protein expression, formation of actomyosin filaments, and cell shortening in response to acetylcholine (ACh). Further, TGF- β increased α -smooth muscle actin synthesis in the presence of the transcriptional inhibitor actinomycin D. TGF- β induced the phosphorylation of 4E-BP. Finally, two inhibitors of 4E-BP phosphorylation blocked TGF- β -induced α -smooth muscle actin expression and cell enlargement. While the transcriptional effects of TGF- β on contractile protein gene expression are well-known, these data suggest that translational control is also a physiologically important element of this model system of airway smooth muscle hypertrophy.

MATERIALS AND METHODS

Cell Culture

Primary human bronchial smooth muscle cells were isolated by enzymatic digestion from lung donor tissue unsuitable for transplantation (from Julian Solway, University of Chicago). This protocol was approved by the relevant Institutional Review Boards. Cells were seeded on uncoated plastic dishes at 50% confluence and TGF- β was added. Fresh medium and TGF- β were added 48 h after initial treatment. Experiments were performed without serum.

Analysis of Cell Size

Cell size was determined by fluorescence-activated cell sorting (FACS) (7). Cells were sorted by cell size (high forward versus sidescatter) and cell length (time of flight). Before FACS, fixed cells were washed and incubated at 37°C for 20 min in buffer containing 0.1% Triton X-100 and 250 μ g/ml RNase A to avoid clumping. Doublet discrimination was performed to sort out doublets, triplets, or damaged cells.

Protein Synthesis

Cells were incubated in [3 H]-leucine (100 μ Ci; PerkinElmer, Boston, MA) for 24 h. 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.

Immunoblotting

As described previously (7), cellular proteins were probed with mouse anti- α -smooth muscle actin (Calbiochem, San Diego, CA), mouse hSMv anti-smMHC or mouse anti-MLCK (Sigma Chemical, St. Louis, MO). 4E-BP phosphorylation was assessed using anti-4E-BP antibody (Cell Signaling, Beverly, MA) with inspection for gel shift.

Fluorescence Microscopy

Cells were grown on glass coverslips and fixed in 4% paraformaldehyde. To stain filamentous actin, slides were incubated with Alexa Fluor

488-conjugated phalloidin (Molecular Probes, Eugene, OR). For immunocytochemistry, slides were probed with mouse anti- α -smooth muscle actin-Cy 3 or mouse hSMv anti-smMHC (Sigma Chemical) followed by FITC-conjugated secondary antibody (Molecular Probes).

Cell Contraction

Individual cell length before and after contraction with ACh was measured by computerized image micrometry (26). Cells were seeded in 100-mm dishes and grown to confluence in serum-free medium or medium supplemented with TGF- β (1 ng/ml). 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 then allowed to float freely and relax for 24 h with occasional swirling to prevent settling or sticking to the sides of the tube. During this period, cells regain a spindle shape and extend processes. Aliquots of cultured cell suspension (2.5×10^4 cells/0.5 ml) were stimulated with ACh (10^{-4} M). The reaction was allowed to proceed for 4 min and stopped by the addition of 0.1 ml of acrolein 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 50 cells encountered in successive microscopic fields.

Northern Analysis

α -Smooth muscle actin steady-state mRNA levels were measured as described previously (7). mRNA half-life was assessed by examining the level of α -smooth muscle actin mRNA after incubation with actinomycin D, a transcriptional inhibitor (5 mg/ml for 8–24 h). mRNA loading was assessed by measurement of GAPDH mRNA or staining of 28S rRNA.

Effect of TGF- β on Contractile Protein Synthesis in the Presence of Actinomycin D

Cells were pretreated with TGF- β (1 ng/ml) for 24 h to generate α -smooth muscle actin transcripts and then incubated for an additional 24 h with actinomycin D (5 mg/ml) in the presence or absence of TGF- β . To measure α -actin protein synthesis (27), cells were incubated in leucine- and methionine-free medium and [35 S]-methionine added for second 24 h of the experiment (100 μ Ci; PerkinElmer). Cell lysates were immunoprecipitated for α -smooth muscle actin (BioGenex, San Ramon, CA). Samples were resolved by SDS-PAGE, transferred to nitrocellulose, and exposed to film.

Retroviral Transduction of Human Bronchial Cell Lines

Bronchial smooth muscle cell lines expressing empty vector or hemagglutinin (HA)-AA-4E-BP1 (Thr37/46Ala) were provided by Nahum Sonenberg of McGill University (Montreal, PQ, Canada) (28). cDNA encoding AA-4E-BP-1 was subcloned into the pMSCVpuro retroviral vector (BD Biosciences Clontech, Palo Alto, CA). 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 either pMSCVpuro-AA-4E-BP-1 or pMSCV alone, along with pHCMV-G, which contains the VSV envelope glycoprotein. Viral supernatant was collected, filtered, and supplemented with polybrene (8 μ g/ml). Human bronchial smooth muscle cells were infected with viral supernatant (4 h $\times$ 4). Infected cells were selected with puromycin (2 μ g/ml). After selection, cells were grown to confluence, split into 6-well plates, and incubated in the absence or presence of TGF- β .

Statistical Analysis

Signals were quantified using a BioRad gel documentation system (Hercules, CA) or PhosphorImager with ImageQuant software (both from Amersham Biosciences, Piscataway, NJ). 4E-BP phosphorylation was assessed by calculating the ratio of $\beta + \gamma/\alpha + \beta + \gamma$ isoforms (29). Data are expressed as mean $\pm$ SEM. Differences between groups were assessed by one-way ANOVA, with repeated measures where appropriate.

RESULTS

TGF- β Increases Human Airway Smooth Muscle Cell Size and Protein Synthesis

Early passage primary human bronchial smooth muscle cells were treated with serum-free medium containing TGF- β (1–10 ng/ml). TGF- β induced a dramatic phenotypic change within 2–3 d after initial treatment. TGF- β -treated cells appeared thick and spindle-shaped, with ample stress fibers and sharply demarcated borders (Figure 1A; *left panels*, phase contrast; *right*, FITC-phalloidin, magnification $\times 400$). To further assess this, myocytes were sorted according to forward scatter and length (time of flight). Doublet discrimination was used to discriminate hypertrophy from doublets, triplets, or damaged cells. Cells treated with TGF- β (1 ng/ml for 72 h) showed substantial rightward shifts (increases) in forward scatter and time of flight, indicative of hypertrophy (Figure 1B). TGF- β -induced increases in cell size were accompanied by increments in protein synthesis, as assessed by [3 H]-leucine incorporation (Figure 1C).

TGF- β Increases Protein Abundance of α -Smooth Muscle Actin and smMHC, as well as Their Incorporation into Actomyosin Filaments

Cell lysates were resolved by SDS-PAGE and immunoblotted for α -smooth muscle actin, smMHC, or MLCK. Within 2 d, TGF- β induced a concentration-dependent increase in α -smooth

muscle actin protein abundance (Figure 2A). Smaller increases were observed for smMHC (Figure 2A). For MLCK, there was a shift in expression from the long isoform, which has been associated with reduced contractile function (30), to the short isoform (Figure 2A). Nearly all individual cells also showed dramatic increases in α -smooth muscle actin expression, with mature contractile filaments (Figure 2B). Fewer cells showed a dramatic increase in smMHC expression (Figure 2C). These data show that TGF- β treatment increases the incorporation of α -smooth muscle actin and smMHC into contractile filaments. However, while most cells appeared to undergo an increase in cell size and α -smooth muscle actin expression, not all cells increased smMHC expression, suggesting either differential regulation of cell size and contractile protein expression or airway smooth muscle phenotypic heterogeneity, as observed previously (4–6).

TGF- β Increases Cell Shortening in Response to ACh

To determine whether TGF- β treatment alters contractile function, cells were grown to confluence in either serum-free medium or serum-free medium with TGF- β (1 ng/ml). At confluence, cells were then scraped off with a rubber policeman and allowed to float freely for 48 h. Aliquots of cultured cell suspension were stimulated with ACh (10^{-4} M). Individual cell length was measured by computerized image micrometry (26). Relative to untreated cells, TGF- β -treated cells were significantly longer at rest and significantly shorter after stimulation with ACh (Figures 3A and 3B). Taken together with increases in cell size and contractile

Figure 1. TGF- β increases airway smooth muscle cell size and protein synthesis. (A) Primary human bronchial smooth muscle cells cultured without or with TGF- β (1 ng/ml for 48 h). *Left panels*, phase contrast; *right panels*, FITC-phalloidin (magnification $\times 400$). (B) Changes in primary human bronchial smooth muscle cell size after treatment with TGF- β (1 ng/ml for 72 h). Cells were sorted by forward scatter (*left*) and time of flight (*right*). TGF- β (1–10 ng/ml) increased cell size compared with 10% FBS and DMEM. The results shown are representative of three experiments. (C) Overall protein synthesis of human bronchial smooth muscle cells, with and without treatment with TGF- β (1 ng/ml for 24 h), as assessed by [3 H]-leucine incorporation ($n = 3$, mean $\pm$ SEM).

Figure 2. TGF- β alters airway smooth muscle contractile protein expression. (A) Representative immunoblots for α -smooth muscle actin, smMHC, and MLCK. TGF- β (1 ng/ml, 72 h) induced dose-dependent increases in α -smooth muscle actin expression. Total smMHC increased slightly, and there was shift in MLCK expression from the long to the short isoform. (B) Primary human bronchial smooth muscle cells were stained with Cy3-tagged anti- α -actin after treatment without (left panel, magnification $\times 400$) or with TGF- β (1 ng/ml for 72 h, center and right panels, magnification $\times 400$ and $\times 100$, respectively). Most TGF- β -treated cells are thick with ample α -smooth muscle actin filaments. (C) Cells were stained with anti-smMHC plus FITC-labeled 2 $^{\circ}$ antibody. After TGF- β treatment, a minority of cells contain smMHC filaments.

protein expression, these data show that TGF- β induces airway smooth muscle hypertrophy which is functionally significant.

TGF- β Increases α -Smooth Muscle Actin Synthesis in the Presence of Actinomycin D

In light of TGF- β 's well-known effect on the transcription of contractile protein genes such as α -smooth muscle actin, we asked whether transcriptional control is a physiologically important effect of TGF- β in this cell system. First, we verified that TGF- β increases steady-state α -actin mRNA levels in human airway smooth muscle cells (Figure 4A). We also examined the effect of TGF- β on α -smooth muscle actin mRNA stability by measuring mRNA levels after incubation with actinomycin D. TGF- β had no effect on effect on α -actin mRNA stability (Figure 4B).

Next, we examined whether TGF- β increases α -smooth muscle actin protein synthesis in the presence of actinomycin D, an inhibitor of gene transcription. Cells were pretreated with TGF- β for 24 h to provide a source of α -actin transcripts and then treated for an additional 24 h with actinomycin D (5 μ g/ml) and [35 S]-methionine in the absence or presence of TGF- β . Immunoprecipitated α -smooth muscle actin was resolved by SDS-PAGE, proteins were transferred to nitrocellulose, and the membranes exposed to film. After TGF- β pretreatment, TGF- β increased α -actin protein synthesis in the presence of actinomycin D (Figure 5). Thus, while TGF- β likely induces airway smooth muscle hypertrophy in part by increasing the transcription of

Figure 3. TGF- β induces a contractile phenotype in primary bronchial smooth muscle cells. (A) Compared with untreated cells, TGF- β increases cell shortening in response to ACh ($n = 50$ for each group, mean $\pm$ SEM, *different from untreated cells, $P < 0.05$, ANOVA). (B) Phase contrast images of TGF- β -treated cells before (left) and after acetylcholine stimulation (right). Contracted cells round up in response to contractile agonists (magnification, $\times 100$).

genes encoding the contractile apparatus, translational control also appears to play an important physiologic role.

A Chemical Inhibitor of Phosphatidylinositol 3-Kinase, LY294002, Blocks TGF- β -Induced 4E-BP Phosphorylation and Cell Phenotypic Change

Translation of the majority of eukaryotic mRNAs is initiated through a 7-methylguanosine cap structure at the 5' end of mRNA. The cap is recognized and "clamped" by the 24-kD eukaryotic initiation factor (eIF)-4E. eIF4E associates with inhibitory proteins termed 4E-BPs. 4E-BP1 undergoes phosphorylation at multiple sites, which results in its release from eIF4E, thereby increasing the availability of eIF4E for cap-dependent translation (21, 22). 4E-BP is typically phosphorylated by the 290-kD kinase mammalian target of rapamycin (mTOR), though other 4E-BP kinases exist. For example, phosphatidylinositol (PI) 3-kinase, which serves as an upstream activator of mTOR, may also phosphorylate 4E-BP directly (31).

Treatment with TGF- β increased phosphorylation of the β form of 4E-BP (Figure 6A). To determine the requirement of 4E-BP phosphorylation for TGF- β -induced airway smooth muscle hypertrophy, cells were pretreated with a chemical inhibitor of PI 3-kinase, LY294002. LY294002 (10 μ M) attenuated phosphorylation of 4E-BP and α -smooth muscle actin expression (Figure 6A) while decreasing cell number, total protein synthesis, and protein synthesis per cell (Figure 6B). Interestingly, rapamycin (25 nM) had only modest effects on 4E-BP phosphorylation, α -smooth muscle actin expression (Figure 6A), and protein synthesis (Figure 6B). Concentrations ranging from 25–250 nM had no effect on TGF- β -induced phenotypic change (Figure 7A). Twenty-five nanomoles of rapamycin was sufficient to block phosphorylation of p70 ribosomal S6 kinase (Figure 6C). Together, these data suggest that TGF- β -induced increases in protein synthesis and cell size occur in a PI 3-kinase-dependent, mTOR-independent manner.

Figure 4. Effects of TGF- β on α -smooth muscle actin steady-state mRNA level and stability. (A) Northern analysis of α -smooth muscle actin mRNA levels (TGF- β , 1 ng/ml for 48 h). (B) mRNA half-life was assessed by examining the level of α -smooth muscle actin mRNA after incubation with actinomycin D, a transcriptional inhibitor (5 mg/ml for 8–24 h). Group mean data are also shown (mean $\pm$ SEM).

Figure 5. Effects of TGF- β on α -smooth muscle actin protein synthesis. Cells were treated with TGF- β (1 ng/ml) for 24 h and then incubated an additional 24 h with actinomycin D (5 mg/ml) and [35 S]methionine in the presence or absence of TGF- β . α -smooth muscle actin immunoprecipitates were resolved by SDS-PAGE and exposed to film. Group mean data ($n = 3$, mean $\pm$ SEM, *different from no TGF- β treatment, $P < 0.001$, ANOVA).

Cells Transduced with a 4E-BP-1 Double Phosphorylation Mutant Fail to Increase Cell Size or α -Smooth Muscle Actin Expression after TGF- β Treatment

To determine the precise requirement of 4E-BP for TGF- β -induced airway smooth muscle hypertrophy, we infected primary human bronchial smooth muscle cells with retrovirus encoding either empty vector (pMSCV) or a mutant of 4E-BP1 that dominantly binds to and constitutively inhibits eIF4E and therefore cap-dependent translation (HA-AA-4E-BP-1). Immunoblots of AA-4E-BP1 cell lysates stained positively for the HA tag (data not shown). Cells expressing AA-4E-BP1 failed to undergo TGF- β -induced phenotypic change (Figure 7B). Since 4E-BP phosphorylation frees eIF4E for binding with eIF4G, these data imply that unbound eIF4E is required for the development of TGF- β -induced airway smooth muscle hypertrophy.

DISCUSSION

We have developed a cell model of airway smooth muscle hypertrophy in which primary human bronchial smooth cells are treated with TGF- β . We have found that, within 48 h of treatment, TGF- β increases cell size and protein synthesis, protein abundance of α -smooth muscle actin and smMHC, formation of actomyosin filaments, and shortening in response to ACh. This model holds advantages over two previous models of airway smooth muscle hypertrophy, prolonged serum deprivation of canine tracheal myocytes (4–6) and human bronchial smooth muscle cell lines transduced with a temperature-sensitive large T antigen (7). In both models, cell cycle arrest, induced either by serum withdrawal or degradation of large T with consequent release of p53 and induction of the cyclin-dependent kinase

inhibitor p21^{Cip1}, induces the formation of large myocytes with increased abundance of contractile proteins. However, since airway smooth muscle cells are apparently quiescent *in vivo*, as evidenced by the absence of Ki67 staining (2), the physiologic relevance of these models is unclear. Also, in both models, the precise stimulus for airway smooth muscle growth is not known. In contrast, TGF- β is not only known to be increased in the airways of patients with asthma, but expression is relatively higher in those with severe asthma compared with those with less severe disease (8–11), consistent with recent reports demonstrating airway smooth muscle hypertrophy in individuals with severe (2) but not mild asthma (3).

The hypertrophic response to TGF- β is not unexpected, as similar results have been shown in vascular smooth muscle cells and cardiac myocytes, and the effects of TGF- β on the transcription of contractile protein genes is well established (13–17). In the present manuscript, we confirmed that TGF- β increases steady state levels of α -smooth muscle actin mRNA. However, we reasoned that, to induce hypertrophy, TGF- β must also enhance the efficiency of mRNA translation. We based this hypothesis on the following information. First, selective alterations in the transcription rates of individual genes or groups of genes are unlikely to be sufficient to account for the global increases in protein synthesis observed during hypertrophy. Second, in the intact heart, the availability of mRNA is not generally limiting for increases in overall cell protein synthesis during growth (32). Finally, translational control mechanisms have been shown to contribute to both cardiac (33, 34) and skeletal muscle hypertrophy *in vivo* (35, 36), not to mention our previous cell culture models of airway smooth muscle hypertrophy (4–7). We found that TGF- β had no effect on α -actin mRNA stability, and pilot pulse-chase studies have shown no degradation of radiolabeled α -actin protein 48 h after withdrawal of hot probe (not shown), suggesting that protein degradation does not play a role. On the other hand, TGF- β increased α -smooth muscle synthesis in the presence of actinomycin D, an inhibitor of gene transcription, demonstrating that TGF- β increases the translation of α -actin mRNA into protein.

Figure 6. Effects of chemical PI 3-kinase and mTOR inhibitors on 4E-BP phosphorylation, α -smooth muscle actin expression, cell proliferation, and protein synthesis. (A) Immunoblots showing effects of LY294002 (10 μ M) and rapamycin (25 nM) on TGF- β -induced phosphorylation of 4E-BP1 and α -smooth muscle actin protein abundance. Group mean data for 4E-BP phosphorylation, as assessed by $\beta + \gamma / \alpha + \beta + \gamma$ ratio, are also shown ($n = 3$, mean $\pm$ SEM, different from TGF- β alone, * $P < 0.001$, ** $P = 0.005$, ANOVA). (B) Effects of LY294002 (10 μ M) and rapamycin (25 nM) on cell number (left), total protein synthesis (middle), and protein synthesis per cell (right). Left panel: $n = 3$, mean $\pm$ SEM, *different from control, $P = 0.044$; middle panel: $n = 3$, *different from control, $P < 0.001$, **different from TGF- β , $P < 0.001$; right panel: $n = 3$, *different from control, $P < 0.001$, **different from TGF- β , $P < 0.001$, ANOVA, ***different from TGF- β , $P = 0.002$. (C) Anti-p70 ribosomal S6 kinase immunoblot shows basal phosphorylation shift which is slightly increased by TGF- β treatment (1 ng/ml for 48 h). Pretreatment with rapamycin abolishes basal and stimulated phosphorylation shift, implying that mTOR is required for p70 ribosomal S6 kinase activation.

As noted above, there are three general mechanisms by which TGF- β might increase translation. First, translation of the majority of eukaryotic mRNAs is initiated through a 7-methylguanosine cap structure at the 5' end of mRNA. The cap is recognized and "clamped" by eIF4E, which in turn associates with inhibitory 4E-BPs. 4E-BP1 undergoes phosphorylation at multiple sites, which results in its release from eIF4E, thereby increasing the availability of eIF4E for binding to eIF4G, eIF4F complex formation, and cap-dependent translation (21, 22). Second, concurrent with the preparation of mRNA, the preinitiation complex must be formed. eIF2, a multimer consisting of α , β , and γ subunits, functions to recruit methionyl tRNA and conduct it as a tRNA-eIF2-GTP ternary complex to the 40S ribosomal subunit, to form the 43S preinitiation complex. Finally, the translation of mRNAs with 5' TOP tracts, many of which encode elongation factors and ribosomal proteins involved in mRNA translation, is upregulated by successive phosphorylation of mTOR, S6K-1, and the S6 ribosomal protein.

In the present study, we found that treatment with TGF- β induced phosphorylation of 4E-BP. Further, inhibition of 4E-BP phosphorylation by a chemical inhibitor of PI 3-kinase,

LY294002, attenuated TGF- β -induced airway smooth muscle cell enlargement and α -smooth muscle actin expression. While these data suggest that 4E-BP phosphorylation is required for hypertrophy in this context, these experiments must be viewed with caution, not only because of the potential nonspecific effects of a chemical inhibitor, but also because correlations between phenotypic change and dephosphorylation of 4E-BP do not prove causality. Indeed, it is conceivable that LY294002 blocks TGF- β -induced phenotypic change by blocking transcription as well as translation. To directly test the hypothesis that 4E-BP phosphorylation is required for TGF- β -induced hypertrophy, we expressed AA-4E-BP1, a nonphosphorylatable mutant of 4E-BP1 that dominantly binds to eIF4E, in primary human bronchial smooth muscle cells by retroviral infection. AA-4E-BP1 also inhibited TGF- β -induced airway smooth muscle hypertrophy, demonstrating that 4E-BP phosphorylation, a prerequisite for eIF4E release and cap-dependent translation, is required for this process. The requirement of 4E-BP phosphorylation and cap-dependent translation initiation in the regulation of cell size has been demonstrated only once previously, in human U2OS osteosarcoma cells expressing the same phosphorylation site-defective

Figure 7. Effects of chemical inhibitors (A) and mutant 4E-BP (B) on TGF- β -induced changes in cell size and α -smooth muscle actin expression, as assessed by Cy3-labeled anti- α -smooth muscle actin staining (magnification, $\times 200$).

mutant of 4EBP1 (37). In the latter study, the 4E-BP mutant blocked the ability of eIF4E overexpression to increase cell growth.

On the other hand, we found that rapamycin, an inhibitor of mTOR, had only modest effects on TGF- β -induced 4E-BP phosphorylation and phenotypic change, despite inhibiting phosphorylation of p70 S6 kinase. These data suggest that, in TGF- β -treated cells, 4E-BP phosphorylation may occur in a PI 3-kinase-dependent, mTOR-independent manner. As noted above, it has recently been shown that PI 3-kinase may directly phosphorylate 4E-BP *in vitro* (31). Further, since TGF- β -induced hypertrophy occurred in the presence of p70 S6 kinase dephosphorylation,

these data suggest that the upregulation of 5' TOP mRNAs is not required for the observed hypertrophic response. These data vary from those obtained in serum-deprived canine tracheal myocytes, in which rapamycin blocks S6K1 phosphorylation and phenotypic change (6). On the other hand, these data are consistent with a recent report in which deletion of S6 kinase failed to attenuate insulin-like growth factor-1 or PI 3-kinase-mediated cardiac hypertrophy (38).

In conclusion, we have developed a new model of airway smooth muscle hypertrophy in which primary cells are treated with TGF- β . TGF- β increased cell size and total protein synthesis, expression of α -smooth muscle actin and smMHC, formation of actomyosin filaments, and cell shortening to ACh. Further, in addition to transcriptional effects, TGF- β increased the translation of α -actin mRNA into protein. Finally, phosphorylation of 4E-BP is required for the TGF- β -induced hypertrophy, suggesting that eIF4E-, cap-dependent translation is necessary for this process. Further studies examining the translational control pathways regulating airway smooth muscle hypertrophy may provide new insight into the pathogenesis of severe asthma, and lead to new therapeutic interventions.

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