

Akt activation induces hypertrophy without contractile phenotypic maturation in airway smooth muscle

Lan Ma,¹ Melanie Brown,² Paul Kogut,¹ Karina Serban,¹ Xiaojing Li,¹ John McConville,¹ Bohao Chen,¹ J. Kelley Bentley,³ Marc B. Hershenson,³ Nickolai Dulin,¹ Julian Solway,¹ and Blanca Camoretti-Mercado¹

Departments of ¹Medicine and ²Pediatrics, University of Chicago, Chicago, Illinois; and Department of Pediatrics³, University of Michigan, Ann Arbor, Michigan

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Ma L, Brown M, Kogut P, Serban K, Li X, McConville J, Chen B, Bentley JK, Hershenson MB, Dulin N, Solway J, Camoretti-Mercado B. Akt activation induces hypertrophy without contractile phenotypic maturation in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 300: L701–L709, 2011. First published March 4, 2011; doi:10.1152/ajplung.00119.2009.—Airway smooth muscle (ASM) hypertrophy is a cardinal feature of severe asthma, but the underlying molecular mechanisms remain uncertain. Forced protein kinase B/Akt 1 activation is known to induce myocyte hypertrophy in other muscle types, and, since a number of mediators present in asthmatic airways can activate Akt signaling, we hypothesized that Akt activation could contribute to ASM hypertrophy in asthma. To test this hypothesis, we evaluated whether Akt activation occurs naturally within airway myocytes in situ, whether Akt1 activation is sufficient to cause hypertrophy of normal airway myocytes, and whether such hypertrophy is accompanied by excessive accumulation of contractile apparatus proteins (contractile phenotype maturation). Immunostains of human airway sections revealed concordant activation of Akt (reflected in Ser⁴⁷³ phosphorylation) and of its downstream effector p70S6Kinase (reflected in Thr³⁸⁹ phosphorylation) within airway muscle bundles, but there was no phosphorylation of the alternative Akt downstream target glycogen synthase kinase (GSK) 3 β . Artificial overexpression of constitutively active Akt1 (by plasmid transduction or lentiviral infection) caused a progressive increase in size and protein content of cultured canine tracheal myocytes and increased p70S6Kinase phosphorylation but not GSK3 β phosphorylation; however, constitutively active Akt1 did not cause disproportionate overaccumulation of smooth muscle (sm) α -actin and SM22. Furthermore, mRNAs encoding sm- α -actin and SM22 were reduced. These results indicate that forced Akt1 signaling causes hypertrophy of cultured airway myocytes without inducing further contractile phenotypic maturation, possibly because of opposing effects on contractile protein gene transcription and translation, and suggest that natural activation of Akt1 plays a similar role in asthmatic ASM.

asthma; airway remodeling; airway hyperresponsiveness; bronchial; proliferation

THE SERINE/THREONINE KINASE protein kinase B (PKB/Akt) is an important regulator of cell growth, survival, and metabolism in many species. Germline deletion of Akt1 (one of three Akt isoforms) in mice leads to reduction in the size of multiple organs (23), and mice deficient in Akt3 exhibit reduced brain size (7). Akt integrates signaling through a number of upstream pathways, including signaling through its upstream activator phosphatidylinositol 3-kinase (PI3-kinase). The PI3-kinase/Akt pathway plays a clear cut role in determining the size of

striated myocytes. For example, targeted deletion of the insulin receptor (insulin binding activates PI3-kinase) from the heart in mice (3) results in small hearts comprised of individually small myocytes (1). Akt may represent a particularly important effector of insulin signaling that regulates cell size, since global deletion of both Akt1 and Akt2 resulted in, among other effects, markedly reduced skeletal muscle mass attributable to reduced myocyte size (24). Conversely, forced overexpression of constitutively active Akt increases overall heart size and individual cardiomyocyte size, and interestingly heart function remains normal initially despite hypertrophy (5, 20). Akt overexpression also rescues the small heart phenotype induced by either cardiac-specific insulin receptor knockout (29) or cardiac expression of a kinase-defective mutant of PI3-kinase (28), and causes hypertrophy in mouse skeletal (16) and vascular (13) muscle as well. PI3-kinase and Akt have been implicated in proliferation of airway smooth muscle (ASM) induced by platelet-derived growth factor and thrombin (33), and recent data from our laboratory and others' implicate activation of PI3-kinase and Akt during hypertrophy of cultured human, canine, and bovine airway myocytes induced by various stimuli (9, 11, 26, 32, 36–37).

Excessive accumulation of ASM is a salient feature of asthmatic airway remodeling (8). In addition to myocyte hyperplasia (35), myocyte hypertrophy (i.e., increased cell size and protein content) is an important contributor to ASM overaccumulation, especially in severe asthma (2, 8); however, relatively little is known about the molecular mechanisms that mediate ASM hypertrophy. In light of its ability to induce hypertrophy of cardiac, skeletal, and vascular myocytes, we wondered whether Akt1 could play a similar role in airway myocytes and whether this mechanism might contribute to the ASM hypertrophy seen in asthma. Our results indicate that Akt1 activation is sufficient to cause hypertrophy but not contractile phenotypic maturation of normal cultured airway myocytes and that Akt is activated in the ASM of asthmatic patients. Together, these results implicate Akt as an important mediator of asthmatic ASM hypertrophy.

MATERIALS AND METHODS

ASM culture. Canine tracheal smooth muscle cell (CTSMC) primary cultures were established with myocytes dissociated from trachealis muscle from adult dogs as we have previously described (10). For all experiments, passage 2 cultures were used. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM)-F-12 (1:1) containing 10% FBS, 50 U/ml penicillin, and 50 μ g/ml streptomycin or were cultured in serum-free DMEM-F-12 medium supplemented with insulin (10 μ g/ml), transferrin (5.5 μ g/ml), and selenium (5 ng/ml) (BD Biosciences) and the above antibiotics. All reagents were from Invitrogen.

Address for reprint requests and other correspondence: B. Camoretti-Mercado, Univ. of Chicago, 5841 S. Maryland Ave, MC6026, Chicago, IL 60637 (e-mail: bamoret@medicine.bsd.uchicago.edu).

Transient transfection. Three plasmids were used. pCMV-myrAkt-HA contains a cDNA encoding mouse Akt1 made constitutively active by the addition of the Src myristoylation sequence (myrAkt), driven by the CMV promoter; this plasmid was a generous gift from Dr. Philip Tschlis. pcDNA3.1 was used as an empty control plasmid; this vector contains the CMV promoter. pCMV-EGFP, which encodes enhanced green fluorescent protein, was cotransfected with pCMV-myrAkt-HA or pcDNA3.1 to label transfected cells. Passage 2 CTSMC were transiently transfected at 70–80% confluence in six-well plates with 2 μ g pCMV-myrAkt-HA (or pcDNA3.1) and 2 μ g pCMV-EGFP with 10 μ l LipofectAMINE and 16 μ l Plus Reagent (Invitrogen) for 4 h in OptiMem medium. The liposome suspension was then removed, and cells were fed with DMEM-F-12 plus 10% FBS for 5 h and then fed continuously with either serum-containing or serum-free medium thereafter.

Lentiviral infection. We constructed a replication-incompetent, HIV1-based lentivirus that expresses myrAkt (pLenti6-myrAkt) under control of the CMV promoter by subcloning the myrAkt-HA cassette into the pLenti6/V5-D-TOPO shuttle vector according to the manufacturer's instructions (ViraPower Lentiviral Expression System; Invitrogen). pLenti6-LacZ virus, which expresses β -galactosidase, was used as a comparator to control for any potential effects of lentivirus infection on CTSMCs. pLenti6-empty, which expresses no cDNA, was used as an alternate comparator for mRNA quantification studies. Lentiviruses were generated in 293FT cells. CTSMCs were infected for 12 h at a multiplicity of infection of 1–10 with 10 μ g/ml polybrene. Virus-containing supernatant was then replaced with serum-containing or serum-free medium as above. Blasticidin (10 μ g/ml) was included to select for lentivirus-infected cells. In preliminary studies, we confirmed that noninfected cells were killed within 3 days of blasticidin treatment at this concentration.

Measurement of cell size. Canine myocytes were trypsinized from their culture plates 1–7 days after transfection and then washed and resuspended in medium for cell size analysis by flow cytometry. Propidium iodide (10 μ g/ml) was added to medium before flow cytometry to allow identification of live cells. Positive EGFP fluorescence marked transfected myocytes. Forward scatter area, indicative of cell size, was determined exclusively in transfected myocytes by flow cytometry by analyzing only EGFP-positive cells. Absolute cell size (diameter) was calculated from a standard curve generated using beads of known sizes (Spherotech).

Cell protein content. CTSMC cotransfected for 5 days with pCMV-myrAkt-HA or pcDNA3.1, and with pCMV-EGFP, were sorted by flow cytometry, and EGFP-positive cells were collected. Lysates were prepared from similar numbers of EGFP-positive cells in each group. CTSMCs infected with one or the other lentivirus were also collected, counted, and lysed. Lysate protein contents were determined by the Bradford method (Bio-Rad protein assay).

Nuclear and cytosolic extracts. CTSMCs were infected with pLenti6-LacZ or pLenti6-myrAkt as described above. Nuclear and cytosolic extracts were prepared with a commercially available kit (Pierce) with protease inhibitors. Samples were kept at -80°C until analysis by Western blotting.

Western blotting. Cells were washed with PBS and lysed on ice in buffer containing protease inhibitors (Roche Applied Science). After 10 min incubation on ice, lysates were spun at 14,000 rpm at 4°C , and the insoluble pellet was discarded. Equivalent amounts of total cell protein or of nuclear or cytosolic extracts were loaded on a 4–20% gradient gel (Bio-Rad). After electrophoresis, proteins were transferred to nitrocellulose, and membranes were blocked with 5% nonfat dry milk and 0.1% Tween 20 in Tris-buffered saline, washed, and incubated overnight with primary antibody. After incubation with the appropriate secondary antibody, bands were visualized using the Supersignal West Pico Chemiluminescent kit (Pierce). Primary antibodies included: anti-Akt1 (2H10), anti-Akt2 (5B5), and anti-Akt3 (all from Cell Signaling); anti-Akt2 (F7), anti-Akt3 (C14), and anti-Akt1/2/3 (H136) (all from Santa Cruz Biotechnology); rabbit monoclonal anti-phospho-Ser⁴⁷³-Akt or anti-phospho-Thr³⁸⁹-p70 S6 kinase

(p70^{S6K}) (both from Cell Signaling); goat polyclonal anti-SM22 α or rabbit polyclonal anti-smooth muscle (sm)- α -actin (both from Abcam); rabbit anti-glycogen synthase kinase (GSK) 3 α/β (catalog no. AF2157; R&D Systems); rabbit anti-phospho-GSK3 α/β (catalog no. 9331; Cell Signaling); rabbit anti-serum response factor (SRF) (G-20; Santa Cruz Biotechnology); and mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) (PC10; Santa Cruz Biotechnology).

RNA isolation and real-time PCR. Total RNA from CTSMC was isolated 5 days after infection with pLenti6-myrAkt or pLenti6-empty using TRIZOL Reagent (Invitrogen) following the manufacturer's protocol. First-strand cDNA was synthesized from 1 μ g total RNA using oligo(dT) and random hexamers and the iScriptTM cDNA synthesis kit (Bio-Rad). The following PCR primers were used to generate TA-cloned (TOPO cloning vector; Invitrogen) plasmids to serve as positive controls and to amplify the cDNA during real-time PCR: SM22 α , 5'-CTGTTGACCTCTTTGAAG-3' (sense) and 5'-GCTCCTCGCTTTCTTCATA-3' (antisense); sm- α -actin, 5'-GCACCACTATGTACCCTGGCA-3' (sense) and 5'-TCCGGAGGGGCAATGATCTTG-3' (antisense); and β -actin, 5'-TTGCTGACAGGATGCAGAAGGAGA-3' (sense) and 5'-ACTCCTGCTTGCTGATCCACATCT-3' (antisense). Serial 10-fold dilutions (5×10^{-1} ng to 5×10^{-8} ng) were used to create a standard curve for plasmids containing cDNAs encoding sm- α -actin, SM22 α , and β -actin. Thirty five cycles of real-time PCR were performed using iQ SYBR Green supermix (Bio-Rad) and the Bio-Rad iCycler iQ system. For quantification, mRNA expression of sm- α -actin or SM22 α was normalized to that of β -actin.

Cell proliferation and cell metabolic activity assay. CTSMC were infected with pLenti-myrAkt or pLenti-LacZ and selected with blasticidin as described above. Cells (3,000–5,000) were seeded in a 96-well plate, grown for 2–3 days, and quiesced for 24 h thereafter. The next day, cells were stimulated with medium containing 10% serum and assayed 1 or 5 days later. The following three methods were used: trypsinized cells were counted in the presence of trypan blue; bromodeoxyuridine (BrdU) incorporation was quantified (Roche proliferation kit) after a 2-h pulse followed by immunological detection; and, for metabolic activity, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was added according to the manufacturer's instructions (CellTiter 96 Aqueous; Promega) for 35 min. Data were generated from two independent experiments using different primary infected cells with 3–4 replicate wells/time point.

Statistical analyses. Statistical analyses were performed using SigmaStat for Windows Version 2.0 (Jandel), as indicated.

Immunohistochemical analysis. Immunohistochemical detection of phospho-Ser⁴⁷³-Akt and phospho-Thr³⁸⁹-p70^{S6K} was performed using primary antibodies from Cell Signaling Technologies (catalog nos. 3787 and 9234, respectively). Detection of GSK3 β and phospho-Ser⁴⁷³-GSK3 β was performed using primary antibodies from Cell Signaling (catalog nos. 9315 and 9323, respectively). Airways from human donor lungs that could not be transplanted (generously provided by Gift of Hope/Regional Organ Bank of Illinois under University of Chicago Institutional Review Board Protocol no. 11122A) were fixed in formalin and embedded in paraffin. Tissue sections were cut at 5 μ m, placed on positively charged slides, and then deparaffinized and rehydrated. Antigen retrieval was performed with citrate buffer (Target Retrieval Solution; Dako) and microwave heating for 15 min. Endogenous peroxidase was then blocked with 3% hydrogen peroxide for 10 min and with serum-free Protein Blocking Solution (Dako) for 5 min. The slides were rinsed after each blocking procedure with 50 mM Tris-buffered saline containing 0.05% Tween 20 (TBS-T) solution. Slides were incubated with primary antibody (diluted 1:50) overnight at 4°C . Peroxidase-labeled polymer conjugated to goat anti-rabbit immunoglobulins in Tris-HCl buffer containing carrier protein (Dako Envision System) was applied for 30 min at room temperature. Slides were rinsed with TBS-T two times, and DAB + substrate chromogen solution (Dako) was applied for 5 min. Slides were rinsed in distilled water and counterstained with hematoxylin. All tissues stained with each primary antibody were treated identi-

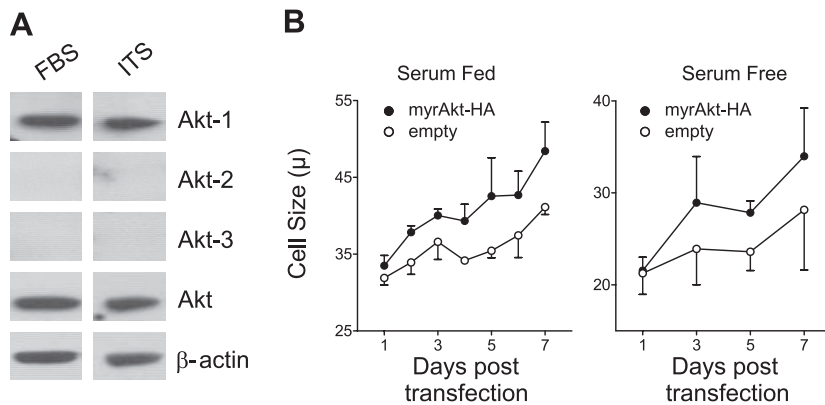


Fig. 1. A: expression of Akt isoforms in cultured canine airway smooth muscle cells. Total cell lysates were prepared from serum-fed (FBS) cells or serum-deprived cells for 7 days maintained in insulin (10 μ g/ml), transferrin (5.5 μ g/ml), and selenium (5 ng/ml) (ITS). Akt1 is the predominant isoform expressed in both serum-fed and serum-deprived cells. Akt, total Akt. B: transfection of cultured canine tracheal myocytes with a plasmid expressing constitutively active Akt1 causes progressive increase in cell size in both serum-fed and serum-free cultures. Cells were cotransfected with a plasmid encoding enhanced green fluorescent protein (EGFP), and only EGFP+ cells were analyzed ($P < 0.0001$ by 2-way ANOVA for each culture condition). myrAkt-HA, cells transfected with pCMV-myrAkt-HA; empty, cells transfected with pCDNA3.1.

cally, and representative photomicrographs at $\times 200$ magnification were taken using identical exposure settings to preserve intersubject differences in apparent immunoreactivity.

RESULTS

Constitutively active Akt1 causes ASM hypertrophy. We first analyzed the pattern of expression of the Akt isoforms in CTSMCs. Figure 1A shows that the Akt1 variant is the predominant isoform expressed in both serum-fed and serum-deprived cells. To determine whether forced Akt1 activation increases airway myocyte size, we cotransfected canine tracheal myocytes with pCMV-myrAkt-HA or pcDNA 3.1 (empty vector as control) plus pCMV-EGFP (to label transfected cells) and assessed the size of transfected cells by flow cytometry. As shown in Fig. 1B, myrAkt-transfected cells were significantly larger in size than were control-transfected cells, suggesting that transient induction of Akt1 activation is sufficient to acutely promote increased cell size. Similar results were obtained from serum-fed and serum-free cells ($P < 0.0001$ by 2-way ANOVA for each culture condition).

Next, we tested whether forced expression of active Akt1 increases cell protein content. The following two approaches were used to overexpress myrAkt in serum-fed CTSMC: plasmid transient cotransfection with pCMV-EGFP to allow for flow cytometric sorting of transfected cells, and infection with lentivirus followed by blasticidin treatment to select for infected cells. Similar results were obtained from these two different methods (Fig. 2). In each case, 5 days overexpression of myrAkt significantly increased CTSMC protein content ($P < 0.006$ by unpaired t -test, each method). Because forced

expression of constitutively active Akt1 in airway myocytes both increased cell size and increased protein content per cell, our results indicate that Akt1 activation is sufficient to cause airway myocyte hypertrophy.

Constitutively active Akt1 does not increase contractile phenotypic maturation in ASM. We sought to determine whether Akt1-induced hypertrophy of cultured ASM cells was associated with augmented contractile phenotypic maturation (12), i.e., whether constitutively active Akt1 overexpression results in a particular increase in contractile protein accumu-

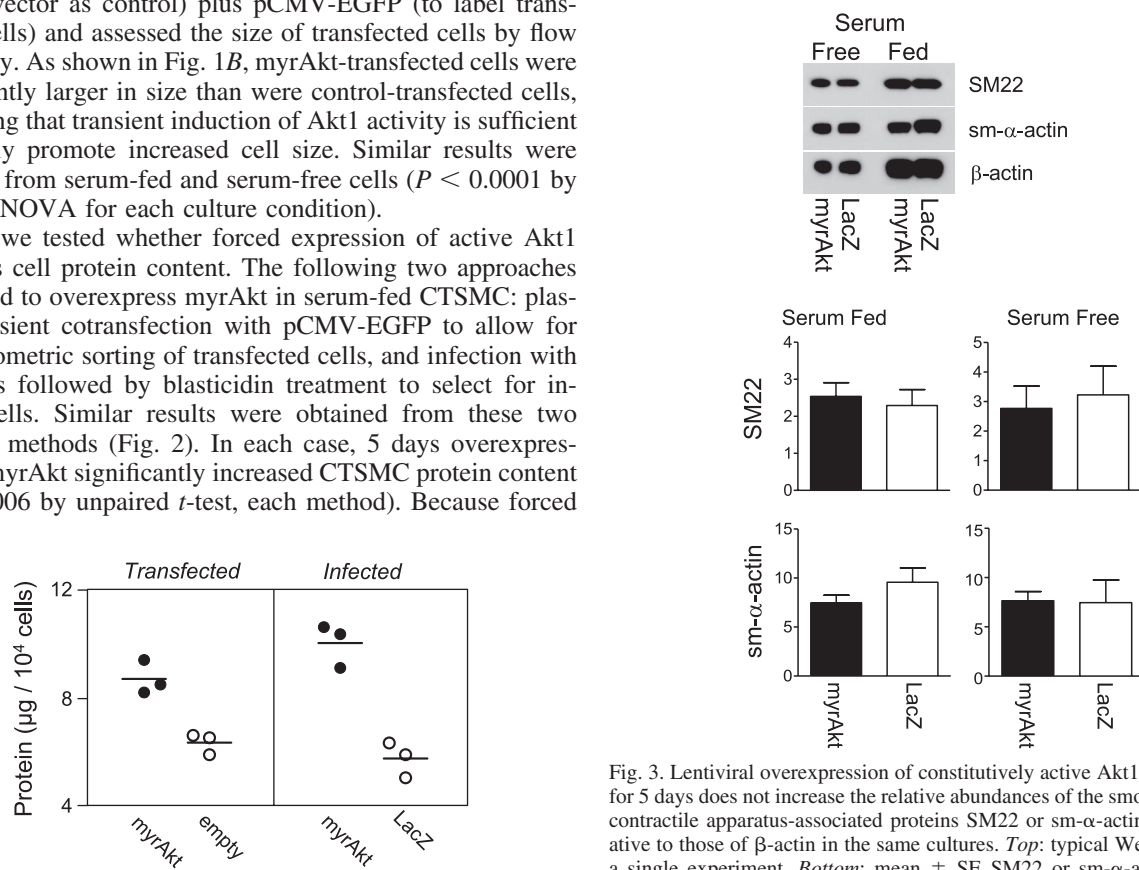


Fig. 3. Lentiviral overexpression of constitutively active Akt1 in cells infected for 5 days does not increase the relative abundances of the smooth muscle (sm) contractile apparatus-associated proteins SM22 or sm- α -actin, calculated relative to those of β -actin in the same cultures. Top: typical Western blots from a single experiment. Bottom: mean $\pm$ SE SM22 or sm- α -actin abundances (relative to that of β -actin in the same cultures) in arbitrary units ($P > 0.25$ by uncorrected unpaired t -test, all comparisons). Similar results were obtained from serum-fed ($n = 4$) and serum-free ($n = 3-4$) cultures. myrAkt, cultures infected with pLenti6-myrAkt; LacZ, cultures infected with pLenti6-LacZ.

Fig. 2. Constitutively active Akt1 significantly increased canine tracheal smooth muscle cell (CTSMC) protein content. Protein content of CTSMCs after 5 days overexpression of myrAkt by transfection or lentiviral infection, as indicated ($P < 0.006$ by unpaired t -test, each method).

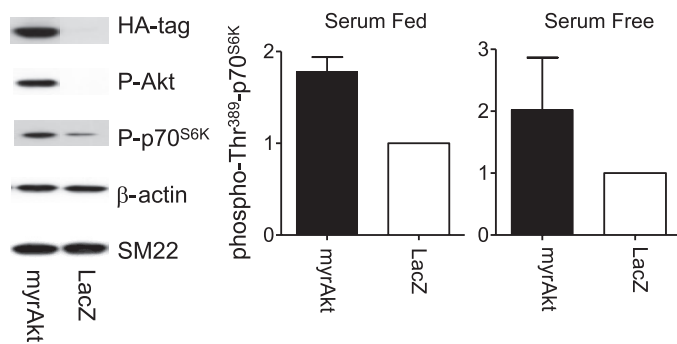


Fig. 4. Lentiviral overexpression of constitutively active Akt1 increases Thr³⁸⁹ phosphorylation of p70 S6 kinase (p70^{S6K}) in cultured CTSM cells. *Left*, typical immunoblots used to quantify phospho-Thr³⁸⁹-p70^{S6K} are shown, along with corresponding immunoblots revealing SM22, phospho-Ser⁴⁷³-Akt, and the HA tag carried on the artificially expressed myrAkt protein. Each immunoblot analyzed (and the example shown) was on a single membrane; in the example shown, irrelevant intervening lanes were removed to facilitate comparison. *Right*, mean $\pm$ SE abundance of phospho-Thr³⁸⁹-p70^{S6K} (relative to that of SM22 in the same culture) in cultures infected with lentivirus expressing constitutively active Akt1 or LacZ. For each experiment, the ratio of phospho-Thr³⁸⁹-p70^{S6K} to SM22 band densities (arbitrary units) for LacZ cultures was adjusted to 1 to facilitate comparison across experiments. Overexpression of myrAkt1 increased phospho-p70^{S6K} abundance [$P < 0.05$ in serum-fed cells, paired t -test ($n = 3$); increase occurred in two experiments in serum-deprived cells].

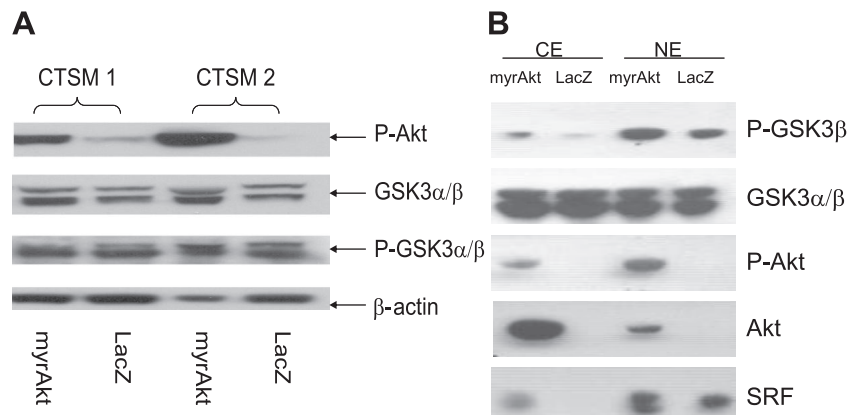
lation. Using cell lysates from CTSMC infected for 5 days with either pLenti6-myrAkt or pLenti6-LacZ, we performed Western analysis to quantify the smooth muscle contractile phenotype markers SM22 and sm- α -actin, as well as β -actin as a "housekeeping" gene; typical Western blots are shown in Fig. 3. Band intensities were quantified by densitometry, and the abundances of SM22 or sm- α -actin calculated relative to those of β -actin in the same cultures; these ratios are shown in arbitrary units in Fig. 3. We found that overexpression of myrAkt did not significantly alter the relative expression of sm- α -actin or SM22 when compared with their relative abundances in pLenti6-LacZ-infected control cells ($P > 0.25$ by uncorrected unpaired t -test, all comparisons). Similar results were obtained from serum-fed and serum-free myocytes. Thus, although expression of constitutively active Akt increases cell size and total protein content, it did not disproportionately further increase contractile protein accumulation in cultured CTSMC. Note that, by virtue of the lentiviral infection and blasticidin selection method employed, all cells in the cultures

analyzed expressed myrAkt (or LacZ as control); as such, our finding that sm- α -actin or SM22 did not accumulate out of proportion to β -actin could not be attributed to incomplete gene transfer into cultured myocytes.

To ensure that CTSMC infected with pLenti6-myrAkt did in fact express active Akt1, we assessed the phosphorylation status of p70^{S6K} on Thr³⁸⁹, which is known to reflect Akt activity and is required for full p70^{S6K} activation. Typical immunoblots used to quantify phospho-Thr³⁸⁹-p70^{S6K} are shown in Fig. 4, along with corresponding immunoblots for SM22, phospho-Ser⁴⁷³-Akt, and the HA tag carried on the recombinant myrAkt protein. Because β -actin was not quantified in every experiment in which p70^{S6K} phosphorylation was assessed, we normalized phospho-Thr³⁸⁹-p70^{S6K} to SM22 abundance instead, which, as shown in Fig. 3, was similar in cells expressing myrAkt or control LacZ. Overexpression of myrAkt did indeed increase phospho-p70^{S6K} abundance [$P < 0.05$ in serum-fed cells, paired t -test ($n = 3$); increase also occurred in two experiments in serum-deprived cells], confirming that forced activation of Akt1 was accomplished by lentiviral transfer of myrAkt. We next assessed the phosphorylation of another Akt1 target known to regulate protein translation, GSK3 β . As shown in Fig. 5A, constitutively active Akt1 overexpression increased the abundance of total GSK3 β (lower band) but not GSK3 α (upper band) in whole cell lysates with no concomitant increase in phosphorylation of GSK3 β (or of GSK3 α). However, analysis of nuclear and cytosolic fractions revealed a slight increase of phospho-GSK3 β abundance in the nuclear compartment of pLenti-myrAkt infected cells compared with pLenti-LacZ infected cells (Fig. 5B). Thus selective constitutive activation of Akt1, which increases cellular protein content, does so by signaling to p70^{S6K} rather than to GSK3 β . To the contrary, the increased abundance of nonphosphorylated (and presumably active) GSK3 β in myrAkt-overexpressing cells may represent an additional regulatory mechanism that partially mitigates the hypertrophy observed.

SRF is critically required for transcription of many smooth muscle contractile protein-encoding genes (30), and activated Akt causes extranuclear redistribution of SRF in smooth muscle cells (15). Thus, we examined the subcellular localization of SRF in CTSMCs infected with pLenti-myrAkt or pLenti-LacZ. Figure 5B shows that, while SRF is predominantly found

Fig. 5. A: lentiviral overexpression of constitutively active Akt1 (myrAkt) increases the abundance of glycogen synthase kinase (GSK) 3 β but does not increase the phosphorylation of GSK3 β . [In GSK3 α / β and phospho (P)-GSK3 α / β blots, GSK3 α is the band on top and GSK3 β is the band on bottom.] Similar results were obtained in two different cultures of CTSMCs (CTSM 1 and CTSM 2). B: cytosolic (CE) and nuclear (NE) extracts of infected CTSMCs demonstrate low abundance of phosphorylated (P)-GSK3 β in the NE.



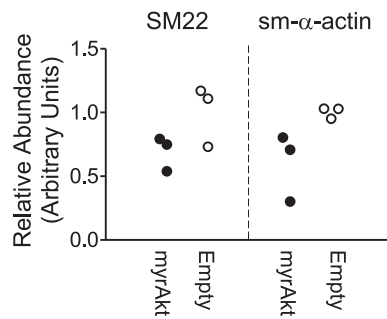


Fig. 6. Abundances of mRNA encoding SM22 and sm- α -actin are reduced in canine tracheal myocytes infected with pLenti6-myrAkt ($P = 0.05$ for myrAkt vs. empty, 2-way ANOVA). Each datum represents a single culture well and indicates the ratio of SM22 or sm- α -actin mRNA to β -actin mRNA, expressed in arbitrary units. The mean relative abundances of these two contractile protein-encoding mRNAs in cells infected with pLenti6-empty were set to 1 and do not imply equivalent mRNA abundances for SM22 and sm- α -actin in those cells. Empty, cultures infected with pLenti6-empty.

in the nuclei of cells infected with either lentivirus, SRF is also present in the cytosolic compartment of pLenti-myrAkt infected cells but not of control pLentiLacZ infected cells. Two additional experiments confirmed increased abundance of SRF within the cytoplasm in Akt1 infected cells compared with LacZ infected cells. This indicates that myrAkt1 overexpression promotes cytoplasmic redistribution of SRF. Because extranuclear trafficking of SRF reduces its transcription-promoting activity (4), we wondered whether forced Akt1 signaling might reduce the abundance of mRNAs of two SRF-dependent genes, SM22 and sm- α -actin. So we quantified these

mRNAs in airway myocytes infected with pLenti6-myrAkt or pLenti6-empty using real-time PCR. As shown in Fig. 6, myrAkt1 expression reduced SM22 and sm- α -actin mRNA expression ($P = 0.05$ for myrAkt vs. empty, 2-way ANOVA). It seems likely that reduced mRNA abundance contributes to the lack of further contractile phenotypic maturation in cultured ASM cells with forced Akt1 activation.

Overexpression of Akt1 results in increased cell proliferation and metabolic activity. Infection of CTSMC with pLenti6-myrAkt virus significantly increased the expression of PCNA compared with control pLenti6-LacZ [$P < 0.05$ in serum-fed cells ($n = 3$); $P < 0.05$ in serum-free cultures ($n = 3$); paired t -tests] (Fig. 7A). Similar results were obtained from serum-fed and serum-free cells. This suggests that, besides causing ASM hypertrophy as evidenced above, Akt1 activation could promote airway myocyte proliferation as well. Because it was shown that Akt1 enhanced proliferation of vascular smooth muscle myocytes, we investigated the effect of active Akt1 expression on ASM proliferation. We used three different methods: cell numeration, BrdU incorporation, and measure of cell metabolism as an indicator of cell abundance (Fig. 7B). From two independent infections, we found that cells expressing constitutively active Akt1 exhibit a higher increase in cell number (3.8- to 5.9-fold) and BrdU uptake (2.2- to 2.8-fold) from day 1 to day 5 than control cells (0.9- to 1.9-fold change in cell number and 0.3- to 0.9-fold change in BrdU incorporation). Conversion of MTS into formazan from day 1 to day 5 resulted in a 2.9- to 3.6-fold increase in myrAkt-infected cells and 1.7- to 1.9-fold change in control cells. Thus, forced expression of active Akt1 causes ASM

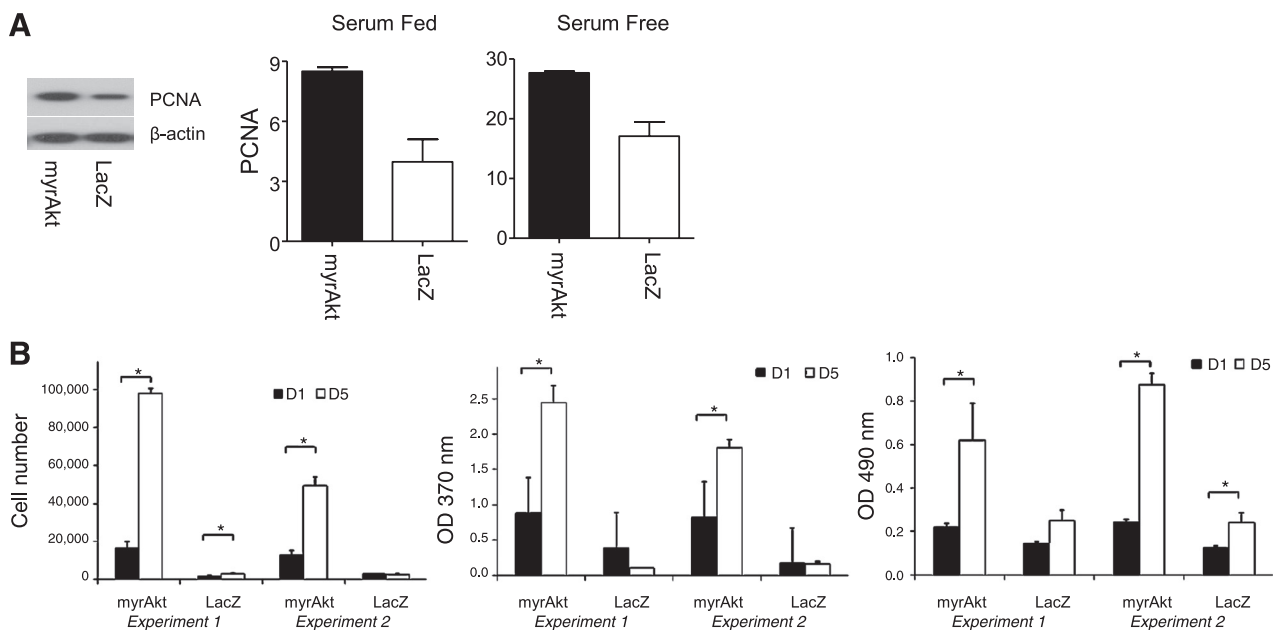


Fig. 7. Lentiviral overexpression of constitutively active Akt1 increases proliferation of cultured CTSMCs. **A:** expression of proliferating cell nuclear antigen (PCNA). *Left*, a typical immunoblot used to quantify PCNA is shown, along with corresponding immunoblots revealing β -actin. *Right*, mean $\pm$ SE abundance of PCNA (relative to that of β -actin in the same culture) in cultures infected with lentivirus expressing constitutively active Akt1 or LacZ. Overexpression of myrAkt increased PCNA abundance [$P < 0.05$ in serum-fed cells ($n = 3$); $P < 0.05$ in serum-free cultures ($n = 3$); paired t -tests]. **B:** CTSMCs were infected with lentivirus encoding either myrAkt1 or LacZ as control. After selection with blasticidin, cells were quiesced and then stimulated with serum for 1 (D1) or 5 (D5) days. Cell proliferation was assayed by cell counting (*left*) or bromodeoxyuridine (BrdU) incorporation (*center*) or estimated by transformation of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) by the cell metabolism (*right*) (mean $\pm$ SD). Cells infected with myrAkt1 show a 4-, 2-, and 3-fold increase in cell number, BrdU incorporation, and MTS conversion, respectively from D1 to D5. Cells infected with LacZ show less than 2-fold increase in cell number and MTS conversion, or no change in BrdU incorporation from D1 to D5. OD, optical density. Results are from two independent infections (experiments 1 and 2) using two different canine cell cultures. Each experiment was performed with 3–4 replicates. * $P < 0.05$, paired t -test.

hypertrophy and promotes airway myocyte proliferation, at least in cultured cells.

Akt signaling is active in ASM of asthmatic lung. To gain insight into whether Akt activation could also contribute to ASM hypertrophy in asthma, we immunostained human airway sections for the activated (Ser⁴⁷³-phosphorylated) form of Akt and also immunostained for (Akt-mediated) phosphorylation of its downstream targets and effectors, p70^{S6K} and GSK3 β . We reasoned that the concordant presence of both phospho-Ser⁴⁷³-Akt and either or both of its effectors, phospho-Thr³⁸⁹-p70^{S6K} or phospho-Ser⁹-GSK3 β , would provide evidence that Akt is activated in situ. Immunohistochemical staining was performed on airways from the lungs of four deceased asthmatic individuals and three nonasthmatic individuals whose lungs had been donated for research; Table 1 shows their available clinical characteristics. As shown in Fig. 8A, the ASM evident in bronchial sections from asthmatic subjects (R47, R68, and R70) appears to have strong immunoreactivity for both phospho-Ser⁴⁷³-Akt and phospho-Thr³⁸⁹-p70^{S6K}, whereas the muscle bands present in the airway section from *asthmatic subject R62* have little to no immunoreactivity for either active enzyme. Like all signaling systems, it is likely that Akt activation varies over time. Thus, the absence of apparent Akt activation in ASM of *asthmatic subject R62* at the time of tissue harvest does not exclude the possibility that such activation might have occurred previously. Although we performed no strict quantification, generally less muscle is apparent in each of the nonasthmatic individuals (R56, R58, and R64), and generally less Akt and p70^{S6K} phosphorylation (activation) is apparent in the ASM of the nonasthmatic subjects. Still, the intensities of Akt and p70^{S6K} activation remain concordant in individual subjects, suggesting that p70^{S6K} phos-

phorylation provides a second indicator of Akt activity. Thus, Akt1 is active in both nonasthmatic and in asthmatic lungs, and it is reasonable to surmise that the increase in Akt1 activity could contribute to increased muscle mass in the asthmatic subjects. In contrast, GSK3 β expression was minimal in the ASM of most subjects, and there was virtually no immunoreactive phospho-GSK3 β in the airway muscle of any subject (Fig. 8B). Taken together, these results indicate that Akt activation does occur in the airways of some (if not all) asthmatic subjects and further suggest that, in ASM tissue, activated Akt signals to p70^{S6K} but not to GSK3 β .

DISCUSSION

Excessive accumulation of ASM is a cardinal pathological feature of asthmatic airways, and hypertrophy of individual myocytes represents one mechanism by which this occurs, at least in more severe (2, 8) if not all (35) asthmatic individuals. Previous studies from our laboratory and others' suggested that activation of Akt (or of its upstream activator, PI3-kinase) is required for airway myocyte hypertrophy (9, 11, 26–27, 36–37) and for the disproportionately large accumulation of contractile apparatus proteins that accompanies cellular hypertrophy as airway myocytes acquire the contractile phenotype (9, 11, 26–27, 32). Furthermore, artificial overexpression of Akt induced hypertrophy of cardiac (5, 20), skeletal (16), and vascular (13) myocytes. Our current results show for the first time that expression of active Akt1 is sufficient to induce hypertrophy of ASM cells, and that occurs naturally within ASM in human airways. Overexpression of constitutively active Akt1 caused an acute enlargement of cell size with a progressive increase in both the size (Fig. 1) and protein

Table 1. Available clinical characteristics of subjects whose lungs were immunostained to generate images shown in Fig. 8

Subject	Age	Gender	Race	History
Asthmatic				
R47	37	F	Caucasian	Died from motor vehicle accident Treated with albuterol inhaler Had seasonal allergies Smoked 1 pack cigarettes/day for >5 yr and quit 2 yr before death
R68	50	F	African American	Died from intracranial bleed Nonsmoker Not diabetic
R70	48	F	Caucasian	Died from asthma attack/anoxia Treated with albuterol and albuterol/ipratropium inhalers Aspiration noted during hospitalization Smoked 1 pack/day for 20 yr Not diabetic
R62	45	M	Caucasian	Died from intracranial bleed Treated with zolpidem ARDS noted during hospitalization Nonsmoker
Nonasthmatic				
R56	56	M	Caucasian	Died from motor vehicle accident Nonsmoker Not diabetic
R58	25	M	African American	Died from head trauma Smoked cigarettes, 1 pack/wk for 5 yr, quit 1 mo prior Smoked marijuana daily Not diabetic
R64	59	M	Caucasian	Asthmatic as a child—outgrew later Died from intracranial bleed Nonsmoker

M, male; F, female; ARDS, acute respiratory distress syndrome.

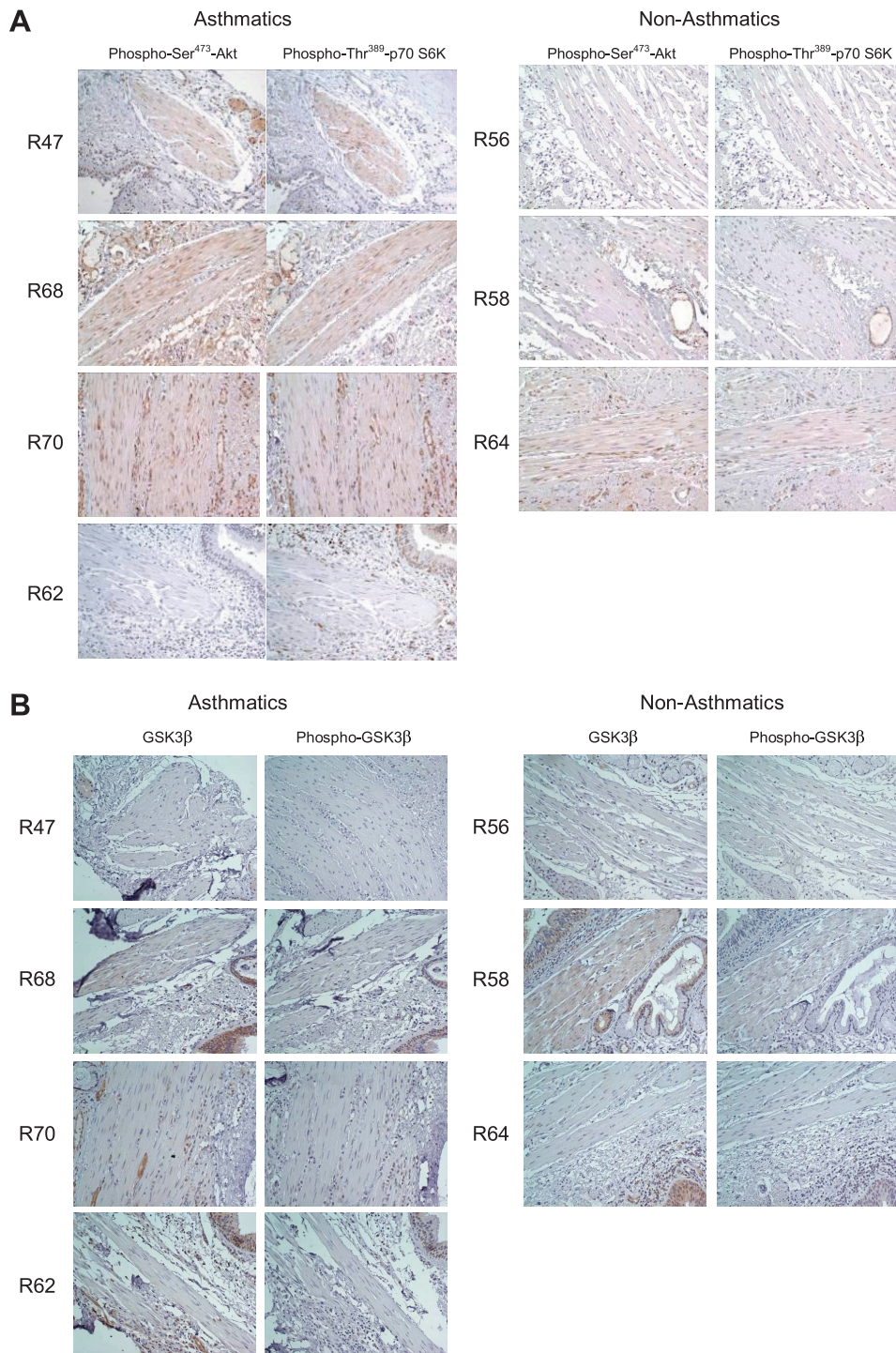


Fig. 8. A: concordant activation of Akt and of p70^{S6K} in airway smooth muscle, as reflected in their concordant phosphorylation detected immunohistochemically. Airway smooth muscle in bronchial sections from asthmatic subjects (R47, R68, and R70) has strong immunoreactivity (brown color) for both phospho-Akt and phospho-p70^{S6K}, whereas the muscle bands present in the airway section from *asthmatic subject* R62 have little to no immunoreactivity for either active enzyme. Generally, less muscle is apparent in each of the nonasthmatic individuals (R56, R58, R64), and generally less Akt and p70^{S6K} activation is apparent in the airway muscle bundles of the nonasthmatic subjects than in those of the asthmatic subjects. Nonetheless, Akt and p70^{S6K} still appear to remain concordant in airway muscle of nonasthmatic subjects. B: immunohistochemical stains for GSK3β and phosphorylated GSK3β in airways from the same subjects shown in A. There is little immunoreactivity for total GSK3β and almost no immunoreactivity for phosphorylated GSK3β in ASM; staining of non-ASM compartments in these immunostains serve as internal positive controls. Magnification, ×200.

content (Fig. 2) of individual myocytes, and this occurred under both serum-fed and serum-free culture conditions. Because Akt is activated in the ASM of at least some asthmatic subjects (Fig. 8), it is reasonable to surmise that Akt activation can contribute to the airway myocyte hypertrophy seen in some but not all asthmatic subjects (2, 8). Akt activation results in more abundant PCNA expression and cell proliferation (Fig. 7) and hence could also promote airway myocyte proliferation similar to its influence on vascular myocytes (31); thus, Akt

activation in airway muscle bundles (Fig. 8) might contribute to ASM hyperplasia (8, 35) as well.

Although the total abundances of two contractile apparatus-related proteins, sm-α-actin and SM22, were increased in hypertrophic, constitutively active Akt1-overexpressing cells, these increases occurred only in proportion to the overall elevation in cellular protein content (Fig. 3), that is, Akt1 activation did not induce relative hyperaccumulation of contractile apparatus proteins in either serum-fed or insulin-sup-

plemented serum-free myocyte cultures. Maintenance in insulin-supplemented serum-free medium for 5–7 days causes about one-sixth of cultured canine tracheal myocytes to acquire a contractile phenotype, characterized by substantial cellular enlargement (primarily elongation) and dramatic accumulation of contractile apparatus proteins (SM22, sm- α -actin, and smooth muscle myosin heavy chain) relative to β -actin (10). One distinguishing feature of myocytes that acquire this contractile phenotype is that they, but not surrounding cells that do not acquire the contractile phenotype, contained activated Akt as well as activated p70^{S6K} (11, 26). Furthermore, pharmacological inhibition of PI3-kinase (an upstream activator of Akt) with LY-294002 or of mammalian target of rapamycin (mTOR) (a downstream target of Akt and upstream activator of p70^{S6K}) with rapamycin blocked the accumulation of contractile apparatus proteins during serum-free culture, suggesting that signaling through the PI3-kinase/Akt/mTOR/p70^{S6K} pathway was required for contractile apparatus protein hyperaccumulation (11). These findings are consistent with those of other investigators using other culture systems (9, 26–27), although other PI3-kinase or Akt-activated protein translation control mechanisms [including phosphorylation of 4E-binding protein (36) and phosphorylation of GSK3 β (34)] probably also play important roles. Our present results extend current knowledge by demonstrating that Akt1 activation is not sufficient to cause exuberant accumulation of contractile proteins [termed contractile phenotypic maturation (12)], even when accompanied by downstream p70^{S6K} activation (Fig. 4) in the absence of enhanced GSK3 β phosphorylation (Fig. 5). The lack of enhanced phosphorylation of GSK3 β by active Akt1 has been reported previously by others (14, 20, 28). In addition, we recently reported that overexpression of a constitutively active p70^{S6K} mutant in cultured human airway myocytes increased cell size and protein content but did not cause a disproportionate increase in contractile apparatus protein accumulation (6). These results are consistent with our present data, which also suggest that activation of p70^{S6K} by Akt with no contribution of GSK3 β can be sufficient for cell enlargement. The inability of Akt signaling alone to enhance contractile phenotypic maturation may also stem from another effect of Akt1 activation, reduced abundance of mRNAs encoding contractile proteins (Fig. 6), an effect that likely stems from Akt-induced redistribution of SRF out of the myocyte nucleus (Fig. 5), as occurs in vascular smooth muscle (15). We speculate that activation of Akt in ASM in asthma might play a similar role in blunting an increase in contractile protein mRNAs that might otherwise have been expected as a result of inflammatory extracellular stimuli that could increase RhoA signaling and so increase SRF-dependent contractile protein-encoding gene expression (12, 18–19, 22, 25). In fact, the abundances of mRNAs encoding contractile apparatus proteins in ASM laser microdissected from asthmatic subjects' airways have been reported as similar to (not increased over) levels found in normal ASM (35). A recent report (17) that abundances of contractile protein-encoding mRNAs are increased in whole biopsies of asthmatic subjects' airways might simply reflect greater muscle mass within those biopsies, rather than increased mRNA concentration within ASM itself, and so might not contradict Woodruff et al.'s finding (35).

Although the relative hyperaccumulation of contractile apparatus proteins during contractile phenotypic maturation of

cultured ASM cells can be striking (9–10, 21), we know of no evidence that there is a similarly disproportionate accumulation of most contractile apparatus proteins in ASM tissue in asthma. To the contrary, Benayoun and colleagues (2) found no significant increases (vs. normal control subjects) in the intensity of immunostaining for sm- α -actin or smooth muscle myosin heavy chain (SM1 and SM2 isoforms, stained individually) in asthmatic subjects, including asthmatic subjects with severe disease who had significantly enlarged ASM cell size. (There was an increase in myosin light chain kinase expression in asthmatic subjects of all severities, however.) In light of the results of our present study, it is important to distinguish a disproportionate increase in contractile apparatus protein expression from a greater total amount of contractile apparatus proteins attributable to a greater muscle mass. For example, Woodruff et al.'s (35) finding of 50–83% more sm- α -actin positive muscle in asthmatic than control subjects' biopsies confirms that asthmatic subjects have more ASM than normal individuals, but this finding does not address whether the contractile protein content is relatively increased within that muscle. In contrast, Benayoun et al.'s study cited above does address the latter possibility and indeed excludes this possibility. Our results show that prolonged activation of Akt1 alone can cause cellular hypertrophy, i.e., increased cell size and protein content, without disproportionate hyperaccumulation of contractile apparatus protein. Furthermore, this hypertrophic effect of Akt1 (Figs. 2 and 3) can occur even in airway myocytes cultured under (serum-free) conditions that themselves induce contractile phenotypic maturation (10–11). As such, it seems likely that Akt1 activation (Fig. 8) contributes to hypertrophy of airway myocytes in the airway wall and that such hypertrophy is accompanied by proportional (but not overexaggerated) contractile protein accumulation. In addition, Akt1 can promote cell proliferation (Fig. 7). Thus, Akt1 may prove to be a useful therapeutic target to prevent or perhaps reverse asthmatic ASM hypertrophy. This possibility remains to be tested.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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