Insulin increases the expression of contractile phenotypic markers in airway smooth muscle

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Chronic inflammation of the airways occurs in patients with chronic obstructive pulmonary disease (COPD) and asthma. Airway remodeling is a key feature of inflammatory diseases of the airways and includes cellular hyperplasia and hypertrophy and increased ECM deposition. The molecular mechanisms involved in the development of airway inflammation and remodeling are not fully understood.

Insulin plays a role in the development of airway inflammation and remodeling. Increased intracellular signaling of insulin is an important feature of chronic obstructive pulmonary disease (COPD) and asthma. The objective of the present study was to determine the role of insulin in the development of airway inflammation and remodeling.

In the present study, we investigated the role of insulin in the development of airway inflammation and remodeling. We found that insulin increased the expression of contractile phenotypic markers in airway smooth muscle. These results suggest that insulin may play a role in the development of airway inflammation and remodeling.

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determined to what extent insulin may contribute directly to ASM maturation via the signaling pathways that are known to be critical in this process.

Long-term exposure (8 days) of bovine tracheal smooth muscle (BTSM) strips to insulin has been shown to induce a functional hypercontractile ASM phenotype characterized by increased contractile response to methacholine and KCl and decreased proliferative response to growth factor (14). Beyond its biological importance, the effects of insulin on ASM function are of clinical relevance, since the use of aerosolized insulin formulations has recently been approved in Europe and the United States for the treatment of diabetes mellitus type 1 and 2 (31).

To elucidate molecular mechanisms by which insulin might underpin maturation of contractile phenotype ASM cells, we investigated the effects of insulin stimulation in serum-free primary cultures on protein accumulation of specific contractile phenotypic markers and on the abundance and stability of mRNA encoding contractile phenotype markers. In addition, we used microscopy or immunoblotting to assess insulin effects on ASM cell morphology, phenotype, and induction of PI 3-kinase and Rho kinase signaling. Because Rho kinase (16, 32) and PI 3-kinase (18) are tightly associated with transcription and translation of contractile phenotypic markers, respectively, we also studied the effects of the selective Rho kinase inhibitors Y-27632 and HA-1100 and the selective PI 3-kinase inhibitor LY-294002 on insulin-induced protein expression and phenotype maturation.

MATERIALS AND METHODS

AASM cell and organ culture. Bovine tracheas were obtained from local slaughterhouses and rapidly transported to the laboratory in Krebs-Henseleit buffer of the following composition (mM) 117.5 NaCl, 5.60 KCl, 128 NaH2PO4, 25.0 NaHCO3, and 5.50 glucose, pregassed with 5% CO2 and 95% O2 (pH 7.4). After the removal of mucosa and connective tissue, tracheal smooth muscle was chopped using a McIlwain tissue chopper three times at a setting of 500 μM and three times at a setting of 100 μM. Tissue particles were washed two times with sterile DMEM supplemented with NaH2PO4 (10 mM), HEPES (20 mM), sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 μg/ml), and 0.5% FBS. Enzymatic digestion was performed using a McIlwain tissue chopper three times at a setting of 500 μM and three times at a setting of 100 μM. Tissue particles were washed two times with sterile DMEM supplemented with NaH2PO4 (10 mM), HEPES (20 mM), sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (1.5 μg/ml), and 0.5% FBS. Enzymatic digestion was performed in the same medium, supplemented with collagenase P (0.75 mg/ml), papain (1 mg/ml), and soybean trypsin inhibitor (1 mg/ml). During digestion, the suspension was incubated in an incubator shaker at 37°C and 55 revolutions/min (rpm) for 20 min, followed by a 10-min period of shaking at 70 rpm. After filtration of the obtained suspension over 50 μm gauze, BTSM cells were washed three times in medium supplemented with 10% FBS (S*). Cells were grown to 70–80% confluence (time point 0) in six-well plates, after which they were kept in DMEM (S0) or DMEM supplemented with insulin (1 μM) in the presence or absence of (+)-trans-4-((1-aminoethyl)-N-(4-pyridyl)cyclohexane carboxamide (Y-27632, 1 μM), 1-[I2-dihydro-(1-oxo-5-isoquinolinyl)sulfonyl]hexahydro-1H-1,4-diazepine [hydroxyfasudil (HA-1100), 10 μM], and/or 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY-294002, 10 μM) for 2, 4, or 8 days. Each condition was performed in triplicate for each experiment. For all protocols, cells in passage 1–3 were used. In separate experiments, organ culture of BTSM strips under serum-deprived or insulin-stimulated conditions was performed as described previously (14).

Western analysis. To obtain whole cell lysates, cells were washed one time with ice-cold PBS [composition (mM): 140.0 NaCl, 2.6 KCl, 2.6, 1.4 KH2PO4, and 8.1 Na2HPO4·2H2O; pH 7.4] and then lysed in ice-cold RIPA buffer (composition: 40 mM Tris, 150 mM NaCl, 1% Igepal, 1% deoxycholic acid, 1 mM NaF, 1 mM Na3VO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 7 μg/ml pepstatin A, and 1 μM phenylmethylsulfonyl fluoride, pH 8.0). Equal amounts of protein were subjected to electrophoresis and transferred to nitrocellulose or polyvinylidene difluoride membranes. Membranes were subsequently blocked in blocking buffer (composition: 50.0 mM Tris·HCl, 150.0 mM NaCl, 0.1% Tween 20, and 5% dried milk powder) for 90 min at room temperature. Next, membranes were incubated overnight at 4°C with primary antibodies [sm-myosin (diluted 1:200), calponin (diluted 1:400), and β-actin (diluted 1:2,000); all dilutions in blocking buffer]. After three washes with TBS-Tween 20 (0.1% TBST, containing 0.1% Tween 20) of 10 min each, membranes were incubated with horseradish peroxidase-labeled secondary antibodies (dilution 1:3,000 in blocking buffer) at room temperature for 90 min, followed by an additional three washes with 0.1% TBST. Bands were detected by visual film using enhanced chemiluminescence reagents (Amersham, Buckinghamshire, UK) and analyzed by densitometry (Totallab). All bands were normalized to β-actin expression.

RNA extraction and RT-PCR. Total RNA was extracted using an RNeasy RNA Mini Kit (Qiagen) in accordance with the manufacturer’s instructions. Reverse transcription (first-strand cDNA synthesis) was performed with 2 μg of total RNA (in μl), 1 μl oligo(dT)12-18 primer (500 mg/ml; Invitrogen), and 10 μl double-distilled H2O. After this mixture was heated for 5 min at 65°C, 9 μl of reaction mixture, consisting of 1 μl dNTP PCR mix (10 mM; Amersham), 4 μl 5X first-strand buffer, 2 μl dithiothreitol (0.1 M), 1 μl RNaseOUT (40 U), and 1 μl Moloney murine leukemia virus RT (200 U; Invitrogen), were added. Subsequently, the samples were incubated at 42°C for 120 min, after which the reaction was inactivated by heating the samples at 72°C for 15 min and putting them on ice. cDNA was stored at −20°C until further use.

PCR amplification was performed in a total volume of 50 μl, which included 1 μl RT reaction mixture, 0.5 μM of each forward and reverse oligonucleotide, 1X PCR buffer with 1.5 mM MgCl2, 0.2 mM dNTP PCR mix, and 1.25 units of Platinum Taq Polymerase (Invitrogen). The following primers were used: to amplify a 162-bp fragment of Bos Taurus (bovine) insulin receptor cDNA, 5′-AAA CGG ACG GAT TCT GAC TGT TTT-3′ (forward) and 5′-GTG ATG TTC GAT GCT CGT TTG-3′ (reverse); to amplify a 150-bp fragment of bovine insulin-like growth factor receptor (IGFR)-1 cDNA, 5′-CCG GGA GGT CTC CTC TAC TA-327 (forward) and 5′-TTG TGT CCT GAG TGT CTC TGC-3′ (reverse); and, to amplify a 247-bp fragment of bovine IGFR-2 cDNA, 5′-CGT GTT TGA TCT GAA CCC ACT-3′ (forward) and 5′-CCC GGT CTA GGT CAG GGT TAT-327 (reverse). PCR comprised of a denaturing step of 94°C for 5 min followed by amplification of 10 cycles at 94°C for 1 min, 67°C for 1 min, decreasing 1°C/cycle, and 72°C for 1 min, and then 20 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min. PCR products were separated on a 2% agarose gel.

Real-time PCR. cDNA was subjected to real-time PCR, which was performed with a LightCycler (Roche Molecular Biochemicals) and LightCycler FastStart DNA Master SYBR Green I (SYBR-GR; Roche Molecular Biochemicals) in accordance with the manufacturer’s instructions. The following primer sets were used: to amplify a 158-bp fragment of bovine sm-MHC-I cDNA, 5′-ATG TTC CAG TCC ACA ATA GGA GA-3′ (forward) and 5′-TGT GTC AAT GGC AGA ATC AAT AG-3′ (reverse); and, to amplify a 136-bp fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, 5′-AGC AAT GCC TGC ACC ACC AAC-3′ (forward) and 5′-CCG-GAG GGG CCA TCA ACA GT-3′ (reverse). Changes in sm-MHC-I cDNA were calculated relative to GAPDH.
**RESULTS**

**Time-dependent effects of insulin on accumulation of contractile proteins.** The abundance of the contractile proteins sm-myosin and calponin was determined by Western analysis (Fig. 1). To ensure that we were able to effectively study effects of insulin on ASM cell differentiation, the experiments were initiated using subconfluent cultures, since at 100% confluence, likely because of density-dependent mechanisms, ASM cells can start to differentiate spontaneously even in the presence of serum (19). BTSM cells were grown to 70–80% confluence in the presence of serum (S+; day 0), after which they were maintained in serum-free conditions to enable assessment of the direct and exclusive effects of insulin exposure for up to 8 days. Insulin exposure induced a significant and continual accumulation of both sm-myosin (Fig. 1, A and D) and calponin (Fig. 1, B and D) over the 8 days of study. Indeed, protein abundance was increased by approximately nine- and fourfold for sm-myosin and calponin, respectively, compared with day 0. In contrast, in the absence of insulin, contractile protein abundance increased by only approximately onefold (sm-myosin) and twofold (calponin), which was not statistically significant for sm-myosin (P = 0.2 for sm-myosin).

Overall, 8 days after serum withdrawal, insulin exposure induced at least five times more sm-myosin and two times more calponin than was observed for myocytes maintained in serum-free culture without added insulin. Importantly, no difference was detected in myocyte attachment or viability in serum-deprived cultures in the presence and absence of insulin (data not shown).

To be sure that the insulin effects were not an artifact of using primary cultured myocytes, we also measured the effects of 8-day insulin exposure on sm-myosin abundance in organ-cultured BTSM strips (Fig. 1C). As observed for the primary cultured myocytes, prolonged insulin stimulation resulted in a significantly greater abundance of sm-myosin compared with both freshly isolated BTSM strips and BTSM strips cultured in serum-free media without insulin. This strongly suggests that insulin effects on contractile protein abundance are consistent between cultured cells and intact BTSM. Furthermore, the results with organ culture are fully in line with the previously demonstrated development of hypercontractile responses in BTSM strips exposed to insulin in prolonged organ culture (14).

**Time-dependent effects of insulin on accumulation of contractile phenotype mRNA.** sm-MHC is considered to be one of the most stringent markers for mature contractile ASM cells (19). Therefore, we determined the effects of insulin exposure of cultured BTSM cells on the abundance of mRNA for sm-MHC using real-time PCR (Fig. 2A). After 8 days, insulin stimulation had induced a significantly greater increase in sm-MHC mRNA abundance compared with serum-deprived conditions (~1.5-fold). Moreover, sm-MHC mRNA abundance in insulin-exposed cultures was almost threefold greater than that measured on day 0 and mimicked the time-dependent accumulation of sm-myosin protein (Fig. 1). Although there appeared to be a trend for accumulation of sm-MHC mRNA in insulin-deficient cultures, after 8 days exposure, the abundance of this transcript was not significantly different from day 0 (P = 0.2).
In a separate set of experiments, we determined the effects of serum deprivation and insulin stimulation on sm-MHC mRNA stability. Actinomycin D (1 μg/ml), an inhibitor of de novo RNA synthesis, was added to BTSM cells at day 0, when subconfluent cultures were switched to serum-free culture media. Thereafter, we monitored sm-MHC mRNA abundance for up to 24 h in the presence or absence of insulin. As shown in Fig. 2B, no effects of insulin on the rate of sm-MHC mRNA degradation were observed compared with the serum-deprived control. This suggests that the potentiating effects of insulin on sm-MHC mRNA accumulation in prolonged serum-free culture may occur at the level of transcription. Of note, no effects of actinomycin D were observed on GAPDH mRNA expression, which was used to normalize real-time PCR data obtained for sm-MHC.

Intracellular signaling underlying insulin-induced contractile protein accumulation. In ASM, Rho kinase has emerged to be critically involved in transcription of genes encoding for contractile proteins (16, 32), whereas, at the level of protein translation, a critical role for PI 3-kinase signaling has been identified in ASM cell differentiation (18). To study the involvement of Rho kinase and PI 3-kinase in insulin-stimulated expression of contractile phenotype marker proteins, we coinubated BTSM cells with insulin and selective inhibitors of Rho kinase (Y-27632, 1 μM) and PI 3-kinase (LY-294002, 10 μM; see Refs. 15, 41, and 46) for 8 days (Fig. 3). Inhibition of Rho kinase or PI 3-kinase significantly reduced accumulation of both sm-myosin and calponin in the presence of insulin. In the case of calponin, complete blockade was evident, so accumulation in the presence of each of the inhibitors was similar to that seen in serum-free cultures in the absence of insulin. To address specificity of Rho kinase involvement, the effects of another Rho kinase inhibitor (HA-1100, 10 μM) on expression of calponin were assessed. Similar to our findings using Y-27632, HA-1100 suppressed insulin-induced protein accumulation by ~40%, whereas no effect was observed under serum-deprived conditions in the absence of insulin (data not shown). These data may suggest that the excessive accumulation of sm-myosin and calponin stimulated by insulin is dependent on both Rho kinase and PI 3-kinase signaling. Remarkably, in the presence of insulin, we observed an additive effect of the Rho kinase and PI 3-kinase inhibitors...
in which accumulation of sm-myosin and calponin was virtually abolished. This observation suggests Rho kinase and PI 3-kinase signaling may act in parallel, potentially through different mechanisms, during ASM maturation in the presence of insulin.

Notably, in contrast to our observation using insulin-stimulated cultures, no effect of Rho kinase inhibition by using either Y-27632 (Fig. 3) or HA-1100 (data not shown) was observed on contractile protein expression in insulin-free conditions after 8 days. However, the effects of PI 3-kinase inhibition were similar to that observed in insulin-stimulated cultures (Fig. 3). Moreover, no additive effects of combined treatment with Y-27632 and LY-294002 in insulin-deficient conditions were measured. Collectively, these observations suggest that serum deprivation-induced increases in protein abundance are dependent on PI 3-kinase, but not Rho kinase, although both PI 3-kinase and Rho kinase appear to be necessary for insulin-augmented accumulation of sm-myosin and calponin.

Effects of insulin on ASM cell morphology and phenotype. It has been previously established for canine ASM cells that serum deprivation in the presence of insulin induces the maturation of a select subset of myocytes that are characteristically large and elongate and exhibit abundant accumulation of contractile proteins such as sm-MHC and calponin (17, 33). Thus we assessed myocyte phenotype and morphology resulting from prolonged serum deprivation in the presence and absence of insulin. BTSM cells were grown to 70% confluence on glass cover slips, after which they were serum deprived or stimulated with insulin for up to 8 days. Consistent with previous reports, 1/6 to 1/5 of BTSM cells exhibited dramatic accumulation of contractile phenotype marker proteins in insulin-supplemented, serum-free media (Fig. 4). In the subconfluent state (70%), where both proliferating and nonproliferating cells were present, only low-level labeling of sm-MHC and calponin that was without clear filamentous organization was evident (data not shown).

We used phase-contrast microscopy to quantify myocyte elongation during prolonged serum deprivation (Fig. 5). No significant morphological changes associated with cell elongation were observed for up to 4 days in insulin-deficient serum-free cultures; rather, a relative homogenous population of BTSM cells was maintained. After 8 days, however, a limited number of aggregations of elongate cells similar in shape to sm-MHC and calponin-rich myocytes observed by immunocytochemistry were evident (Fig. 5, A and E). In contrast, in insulin-supplemented cultures, the formation of large, elongate myocytes was clearly evident within 2 days of serum withdrawal, and this number increased markedly, nearing a plateau in number by 4 days that was only ~20% higher after 8 days (Fig. 5, B and E). Of note, we observed no differences in total cell number in the presence or absence of insulin. These data indicate that insulin had no proproliferative effects. Collectively, our findings reveal a key role for insulin in facilitating ASM maturation in primary culture.

Because Rho kinase and PI 3-kinase were required for contractile protein accumulation in BTSM cells (Fig. 3), we also studied the effects of Y-27632 and LY-294002 on cell morphology under serum-free conditions in the presence and absence of insulin. Consistent with our earlier immunoblot analyses (Fig. 3), Rho kinase inhibition had little effect on development of elongate myocytes in insulin-deficient cultures, whereas inhibition of PI 3-kinase appeared to completely prevent maturation of elongate contractile morphology myocytes in insulin-deficient cultures. Formation of large, elongate contractile ASM cells during 8 days culture in insulin-supplemented serum-free media was, however, largely dependent on both Rho kinase and PI 3-kinase (Fig. 5, C–E). These findings parallel our earlier results using immunoblotting that revealed dependence on both Rho kinase and PI 3-kinase for insulin-augmented accumulation of sm-myosin and calponin. In some experiments, trypan blue was added to the cells to establish cell viability in all conditions. Although LY-294002 induced
~1% loss in cell viability, it was clear that, at the concentrations used, these inhibitors had no significant effect on cell attachment nor did they promote cell death.

Effects of insulin on activation of signaling proteins downstream of PI 3-kinase and Rho kinase. Because our data suggest that PI 3-kinase is required for myocyte maturation in the presence and absence of insulin, we next used fluorescence microscopy to examine whether activation of the downstream signaling effector, p70S6K, was selectively associated with BTSM cell maturation (Fig. 6). After 4 days of serum withdrawal in the presence or absence of insulin, we observed that phospho-Thr421/Ser424-p70S6K was elevated in all myosin-enriched myocytes. Moreover, this pattern was maintained through 8 days of study (data not shown). These data strongly suggest that activation of PI 3-kinase, in parallel with the dramatic augmentation in the number of myocytes induced to a contractile phenotype by insulin, is associated with the accumulation of sm-MHC and calponin during maturation of individual BTSM cells.

To address whether insulin stimulation can induce signaling proteins downstream of Rho kinase, we used immunoblotting to measure the effects of insulin on phosphorylation of MYPT1(Thr850), which is selectively phosphorylated by Rho kinase in different smooth muscle types (1, 29). As shown in Fig. 7, phosphorylation of MYPT1(Thr850) was markedly induced within 15 min of insulin exposure and was sustained for at least 6 h. Importantly, we compared the degree of insulin-induced MYPT1 phosphorylation with that induced by 10% FBS, known to be a potent stimulus of this response; insulin exhibited equal capacity to induce phosphorylation of MYPT1(Thr850).

Insulin receptor identification in ASM. In the present study, we report significant effects of insulin in augmented ASM phenotype maturation. However, to date, insulin receptor expression in ASM has not been described, although expression of the insulin receptor and IGFR are well established in skeletal muscle (9) and vascular smooth muscle (27). We performed RT-PCR, using primers for the bovine insulin receptor and the
bovine IGFR-1 and -2, to determine the profile of receptors expressed by primary cultured BTSM cells. In accordance with previous findings in rabbit ASM (36), we demonstrate the presence of mRNA for IGFR-1 and -2. Moreover, we demonstrate for the first time the presence of mRNA for the insulin receptor in ASM (Fig. 8).

DISCUSSION

Our results demonstrate that prolonged insulin stimulation of ASM cells induces a time-dependent increase in the expression of contractile ASM phenotypic markers in a Rho kinase- and PI 3-kinase-dependent fashion. Moreover, this is associated with...
the formation of large, elongate ASM cells that are selectively enriched in sm-MHC and calponin and are highly reminiscent of previously described (hyper)contractile canine ASM cells (33). In addition, our findings demonstrate the utility of using insulin in smooth muscle cell and organ cultures to promote myocyte maturation and enable studies to assess key aspects of airway remodeling and hyperresponsiveness. Notably, to date, induction of a hypercontractile phenotype in ASM cells has been solely attributed to mechanisms associated with prolonged serum deprivation. Our current studies, however, strongly indicate that insulin has a direct effect that significantly potentiates phenotype maturation to a far greater extent than can be attained in its absence in serum-free conditions.

There has been considerable investigation on identifying signaling pathways underlying the development of large, elongate contractile-phenotype ASM cells. An important role for PI 3-kinase and its downstream targets has emerged in signaling cascades that promote protein synthesis, differentiation, and hypertrophy in ASM (18) and a variety of other muscle types (5, 21, 22). Recently, it was found that PI 3-kinase is required for the expression of smooth muscle α-actin and the contraction regulatory protein MLCK in a transformed human bronchial smooth muscle cell line (53). Moreover, it was demonstrated that, under insulin-supplemented serum-free conditions, PI 3-kinase/mTOR-induced phosphorylation of PHAS-1, causing dissociation of PHAS-1 from the translation initiation protein eukaryotic initiation factor-4E and subsequent increased protein synthesis (52). Moreover, a direct link between insulin and PI 3-kinase signaling has been reported in C2C12 myoblasts; it was demonstrated that PI 3-kinase and mTOR were required for insulin-induced differentiation of these cells (45). Completely in agreement with these findings, we now demonstrate that PI 3-kinase is required for the accumulation of contractile apparatus proteins and the formation of elongate, contractile phenotype ASM cells, both under serum-free and insulin-stimulated culture conditions. Notably, our studies are the first to show directly that insulin potentiates airway myocyte maturation in serum-free culture.

Fig. 6. The downstream effector of PI 3-kinase, p70S6K, is selectively activated in airway myocytes during maturation to a contractile phenotype. Images shown are from BTSM cells after 4 days in serum-free culture in the absence (A–C) and presence (D–F) of insulin (1 μM). Myocytes were double labeled for both sm-MHC (red) and phospho-Thr421/Ser424-p70S6K (green). Nuclei (blue) are stained with Hoechst 33342. C and F: merged images of sm-MHC and phospho-Thr421/Ser424-p70S6K. Images are typical of those obtained from triplicate wells of 3 different primary cultures.

Fig. 7. Insulin induces phosphorylation of MYPT1, a downstream effector of Rho kinase signaling. Western analysis was performed using whole cell lysates prepared from BTSM cell cultures that were stimulated with insulin (1 μM) or FBS (10%) for 15 min or 6 h. Both insulin and FBS markedly increased phospho-MYPT1(Thr850) after 15 min, an effect that was sustained for at least 6 h (top). No changes in total MYPT1 were observed (bottom). The blots shown are representative of 2 independent experiments.
An essential role for RhoA/Rho kinase signaling in driving the transcription of genes encoding for contractile proteins such as sm-MHC, smooth muscle α-actin, calponin, and SM22 in smooth muscle has become evident (7, 16, 32, 37). Activation of the promoters of these genes is under combinatorial control by a number of transcription factors (37), but virtually all harbor a pair of essential CArG box elements [CC(A/T)6GG] that bind dimers of the MADS transcription regulator family member, SRF (7, 34, 43). Localization and activation of SRF in the nucleus and subsequent induction of smooth muscle-specific genes are chiefly governed by RhoA/Rho kinase signaling in ASM (32). The RhoA/Rho kinase pathway regulates actin filament dynamics that modulate translocation of SRF coactivators to further support transcription of contractile smooth muscle-specific genes (34, 35). Our present findings reveal that Rho kinase is required for the induction of a hypercontractile ASM phenotype in response to insulin; however, the pathway appears to play an inconsequential role in the absence of insulin. Data from the present study reveal that insulin induces Rho kinase-mediated signaling, as evidenced by the rapid and sustained phosphorylation of MYPT1(Thr850) we observed. This suggests that insulin stimulation may activate transcription of genes for contractile proteins. Although insulin-induced gene transcription has been proposed to be Rho/Rho kinase-dependent (8, 47), clearly the specific link to this pathway is complex and requires in-depth study. Previous studies using transient transfection of canine ASM cells have shown that, under serum-free, insulin-supplemented conditions, the activity of promoters for sm-MHC and SM22 is markedly reduced from that measured when cells are grown in the presence of serum (7). However, to date, there are no reports of studies using either luciferase reporter assays or assays of nuclear mRNA synthesis to assess the specific effects of insulin on transcription of other genes that contribute directly or indirectly to the extent of (airway) smooth muscle-specific gene expression. Although such experiments are beyond the scope of the present study, our current findings from studies using actinomycin D indicate that insulin has no effect on the stability of mRNA for contractile apparatus-associated proteins but rather on the gene transcription of these proteins.

Our data showing that insulin regulates phenotype expression of ASM cells might have important implications relating to asthma pathogenesis. Insulin-like growth factor (IGF)-I is greatly increased in bronchoalveolar lavage from asthmatic subjects and murine models of allergic asthma (26, 50). Because our data reveal ASM cells express receptors for insulin and IGF-I, it is likely that IGF-I can have direct effects on airway myocytes in vivo, and this may be potentiated during episodic asthma-associated inflammation. Interestingly, in transgenic mice in which IGF-I expression is driven by the smooth muscle α-actin promoter, marked hypertrophy of arterial smooth muscle has been reported, but unfortunately no measurements of ASM mass were included (48, 51, 54). Because increased ASM mass is a hallmark feature of airway remodeling in asthma, our current results indicate that the role of IGF-I and PI 3-kinase signaling in this process requires future investigation. Our data also implicate Rho/Rho kinase signaling in insulin-mediated effects on ASM. Induction of this pathway has been linked with allergic inflammation, and a number of reports have revealed inhaled Rho kinase inhibitors to be potent bronchodilators and anti-inflammatory compounds (16, 40). The role of IGF-I in induction of Rho kinase signaling in asthma models has not been tested directly, but our current study suggests future experimentation in this area is warranted.

Because the use of aerosolized insulin formulations has recently been approved in the United States and Europe for the treatment of diabetes mellitus type 1 and 2 (31), our observations showing the effects of insulin on ASM function and phenotype might also be of clinical relevance. We have demonstrated that prolonged insulin exposure of BTSM strips induces a functional hypercontractile phenotype (14). Similarly, we now demonstrate that insulin stimulation results in an increased contractile protein expression in intact muscle strips. Importantly, because of a low biological availability of inhaled insulin, effective dosing for inhaled delivery is ~10-fold higher than that used for subcutaneous delivery to achieve glycemic control (23, 24, 39, 44). It has not been fully determined whether large quantities of inhaled insulin might induce ASM hypercontractility and/or airway inflammation. Notably, inhalation of insulin can be accompanied by cough, dyspnea, sinusitis, and pharyngitis (31), and, in a diabetic rat model, insulin treatment promoted mucociliary M2 receptor dysfunction, AHR, and eosinophilia after allergen challenge (2, 3). We have demonstrated that insulin has acute procontractile effects on guinea pig tracheal smooth muscle (42). Inhaled insulin can cause a small decrease in FEV1 of healthy subjects (31). However, because of poor absorption, diabetic patients with asthma or chronic obstructive pulmonary disorder (COPD)
may require high doses of insulin (24); because these patients are hyperresponsive to inhaled contractile stimuli (12, 28, 38), any reduction in forced expiratory volume in 1 s (FEV₁) caused by insulin could be clinically significant. In light of these observations and those in the current study, further investigation of the acute and chronic effects of insulin inhalation on ASM function and phenotype under specific pathophysiological conditions such as asthma and chronic obstructive pulmonary disease (COPD) are warranted.

In conclusion, we demonstrate that prolonged insulin stimulation induces expression of contractile phenotypic markers in a time-dependent fashion, completely confirming previous findings on ASM contractility (14). Moreover, Rho kinase and PI 3-kinase are required for the insulin-induced effects, both for contractile protein accumulation and formation of large, elongate contractile ASM cells. These findings might be of both pharmacological and pharmacotherapeutical interest and might significantly contribute to the ongoing discussion on the use of aerosolized insulin formulations for the treatment of diabetes mellitus type 1 and 2.

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INSULIN-INDUCED HYPERCONTRACTILE ASM PHENOTYPE


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