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Somatostatin Regulates Intracellular Signaling in Human Bronchial Smooth Muscle Cells

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ABSTRACT

Somatostatin (somatotropin release inhibitory factor; SRIF), is an endogenous peptide family that controls cellular secretion and proliferation. While SRIF's cognate receptors and associated actions have been clearly identified in a range of tissues, such as the endocrine, gastrointestinal and immune systems, a role in human lung physiology remains unknown. Using primary human bronchial smooth muscle cells (hBSMC) as a model for studying SRIF's actions, we have investigated whether SRIF receptors are present and functional in human bronchial smooth muscle. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of hBSMC total mRNA demonstrated the presence of the sst_2 receptor subtype, providing a molecular target for SRIF action. SRIF's functional effects on intracellular signaling were confirmed by Western blotting with phospho-specific antibodies for the extracellular regulated kinases 1/2 (ERK1/2). Using a nonpeptidyl sst_2 selective agonist, L-779,976, we demonstrated an acute inhibition (50 ± 5%) of basal ERK1/2 phosphorylation. In addition, L-779,976-treated hBSMC cell membranes showed a 2.4-fold increase in tyrosine phosphatase activity, which was abolished by the selective tyrosine phosphatase inhibitor sodium vanadate. Furthermore, a 48 h treatment with 100 nM L-779,976 suppressed hBSMC proliferation by 30%. Taken together, our results show that the hBSMC is a direct target of SRIF's antiproliferative activity. We propose that SRIF's actions in controlling lung smooth muscle cell proliferation could prove therapeutically useful in chronic asthma where inappropriate hBSMC proliferation plays a role.

Keywords: somatostatin, somatostatin receptors, inflammation, bronchial smooth muscle cells, extracellular regulated kinase, tyrosine phosphatase.

INTRODUCTION

Asthma, characterized by airway hyperreactivity and airway structure remodeling, is thought to be the consequence of inflammation (Halayko and Amrani, 2003). Among the myriad of changes that take place within the bronchial walls of an asthmatic – subepithelial fibrosis, epithelial detachment, mucus gland hyperplasia, sub-mucosal myofibroblast hypertrophy, increased vascularization, and neuronal differentiation– smooth muscle hypertrophy and hyperplasia play a dominant role in asthma (Holgate, 2003; Jeffery, 2001; Woodruff *et al*, 2004). These changes within the walls of the airway, along with a host of inflammatory cytokines, are implicated in the exacerbation of airway hyperreactivity (Elias, 2000). Clearly any change to the growth or contractile function of airway smooth muscle would be an important contributor to the pathophysiology of asthma.

Since the recognition that the presence of remodeled, non-specific, hyperresponsive airways are critical to the symptomology of asthma, there has been renewed interest in the investigation of bronchial smooth muscles cells to seek answers to fundamental questions. *In vitro* studies of airway myocytes have shown that G protein-coupled receptor (GPCR) signaling and contractile force in human bronchial smooth muscle cells (hBSMC) can be augmented by inflammatory stimuli, both physical and chemical in nature (Amrani *et al*, 2004). In this regard, pro-inflammatory peptides such as Substance P and Neurokinin A, have long been-accepted as contributing to the expression of asthmatic symptoms through their ability to cause bronchoconstriction, neurogenic edema and inflammation. However the airway role of anti-inflammatory

peptides, such as somatostatin (somatotropin release inhibitory factor; SRIF) has not been explored.

SRIF, initially discovered in the hypothalamus, is a potent inhibitor of growth hormone release (Brazeau *et al*, 1973). Since then, SRIF's spectrum of biological activities has grown steadily and today SRIF peptides are known to influence a range of secretory functions, including gastric acid secretion (Larsson *et al*, 1979), thyroid stimulating hormone effects (Singh *et al*, 2000), and insulin and glucagon secretion in the pancreas (Strowski *et al*, 2000). In the immune system, SRIF peptides have anti-inflammatory effects on leukocyte function (Krantic, 2000), namely SRIF inhibition of leukocyte proliferation and cytokine synthesis (Elliot *et al*, 1998).

At the cellular level, SRIF peptides activate a family of five highly homologous, G protein coupled receptors ($sst_1 - sst_5$) that coexist in many tissues (Krantic *et al, 2004;* Reisine, 1995). SRIF modulates intracellular signaling by controlling membrane ion conductance and protein phosphorylation levels, in addition to decreasing cyclic nucleotide levels (Florio *et al*, 1996). Unifying SRIF's cellular actions is the control of cellular secretion and the suppression of cellular proliferation (Florio *et al*, 2003).

Within the body, SRIF can be found in two forms: SRIF-14 and SRIF-28, consisting of 14 and 28 amino acids, respectively. Additionally, cortistatin is a related member of the SRIF peptide family, and may predominate in the immune system (Lichtenauer-Kaligis *et al*, 2004). Currently, SRIF-14 is considered to be the most widely expressed SRIF peptide and its presence can be readily detected in human serum.

While the immunomodulatory effects of SRIF may account, in part, for its anti-inflammatory properties, it is unknown whether SRIF also has direct effects on the

bronchial smooth muscle cell, and therefore a possible role in modulating asthma. SRIF and receptor immunoreactivity observed in a range of lung carcinomas suggest that SRIF may be active in lung physiology (Cattaneo *et al*, 1996). Furthermore, SRIF receptors may be up-regulated in inflammation, suggesting an immunomodulatory role for this peptide family (Krantic, 2000).

Considering the bronchial smooth muscle cell's crucial involvement in asthma and a possible role for SRIF peptides and receptors on bronchial smooth muscle cells, we studied the role of SRIF and SRIF analogs on cultured human bronchial smooth muscle cells (hBSMC). In the current study, we show that SRIF acts through an endogenously expressed SRIF receptor to regulate hBSMC intracellular signaling pathways that are involved in cell proliferation. Our results indicate a possible anti-proliferative role for SRIF in hBSMC monolayers.

MATERIALS AND METHODS

Cell culture

hBSMC were purchased from Clonetics (Walkersville, MD), grown in S Growth Medium plus supplements at 37°C in the presence of 5% CO_2. Cells were maintained in T-75 cm^2 flasks, or multiwell tissue culture dishes and allowed to recover for 72 h before the beginning of the experiment.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

hBSMC mRNA was isolated using the Micro-FastTrack™ 2.0 mRNA Isolation Kit (Invitrogen Life Technologies, CA).

cDNA synthesis and the polymerase chain reaction (PCR) were carried out using the mRNA, the Superscript One-Step RT-PCR system (Invitrogen Life Technologies, CA) with the appropriate oligonucleotide primer pairs ($hsst_{1f\&r-2f\&r}$; Invitrogen Life Technologies, CA & $hsst_{3f\&r-5f\&r}$; Integrated DNA Technologies, Coralville, IA). Human SRIF receptor primer sequences are as follows 5' to 3': $hsst_{1f}$ = GCAACATGCTCATGCC, $hsst_{1r}$ = GCGTTATCCGTCCAGG; $hsst_{2f}$ = AGAGCCGTACTATGACCTGA, $hsst_{2r}$ = AGCCACTCGGATTCCAGAG; $hsst_{3r}$ = TCATCTGCCTCTGCTACCTGC, $hsst_{3f}$ = GAGCCCAAAGAAGGCAGGCT; $hsst_{4f}$ = CATGGTCGCTATCCAGTGCA, $hsst_{4r}$ = CTCAGAAGGTGGTGGTCCTG; $hsst_{5f}$ = GCTCCTGGTGTTCGCGGACGT, $hsst_{5r}$ = GAGGATGACCACGAAGAAGTAGAGG. RT-PCR reactions were performed in 1X Reaction Mix buffer containing RT/Platinum Taq Mix, 10 nM forward primer ($hsst_{1f-5f}$), 10nM reverse primer, ($hsst_{1r-5r}$), template RNA in a final volume 50 µl. RT-PCR was performed using a Perkin Elmer Thermal Cycler 9600 (Perkin Elmer, MA). Samples were incubated at 50°C for 30 min followed

by 94°C for 2 min and the PCR amplification was carried out over 30 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 2 min, with a final extension step of 72°C for 5 min.

RT-PCR products were resolved by agarose gel electrophoresis and stained with ethidium bromide (10μg/mL). DNA products were visualized using a UV transilluminator (UVP, Inc., CA), and photo images were captured using AlphaImager 3.3D (San Leandro, CA) computer software.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Parallel cultures of hBSMC cells were cultured with serum-free basal medium (Opti-MEM, Invitrogen Life Technologies) for 48 h and then incubated in the presence or absence of 100 nM SRIF-14 (Peninsula Labs, CA) for 15 min at 37°C. Cell lysates were prepared, sonicated and heated to 70°C for 10 min., resolved on NuPAGE 10% Bis-Tris gels (Invitrogen Life Technologies, CA) electroblotted onto polyvinyldifluoride (PVDF) membranes and treated for 45 min in a blocking buffer containing 5% (w/v) of non-fat dehydrated milk. ERK1/2 were immunodetected with a 1:1000 dilution of antiphospho ERK1/2 antibodies (Cell Signaling, MA). The membrane associated immunoreactivity was then detected using a 1:2000 dilution of a horseradish peroxidase-conjugated anti-rabbit secondary antibody and detected using ECL chemiluminescence (Amersham, NJ). The resulting autoradiograph was scanned, quantified with NIH Image and expressed as integrated pixel density, as previously described (Badway *et al.*, 2004).

Tyrosine phosphatase assay

hBSMC cultures were incubated in the presence or absence of 100 nM SRIF-14 for 30 min. Cell cultures were washed in PBS and then lysed in a 50mM HEPES buffer

containing 0.5% Triton X-100, 10% glycerol (GibcoBRL), 2.5μM pepstatin A, 12.5μM leupeptin, 12.5μM aprotinin, 12.5μM bacitracin and 200nM polymethylsulfonyl fluoride. Lysates were centrifuged at 13,500 x g for 15 min at 6°C and the supernatant retained.

Protein phosphatase assays were performed in a clear bottom, black sided 96 well dishes (Corning, Corning, NY). hBSMC cell lysate (approximately 65μg) was incubated with 10μM fluorescent phosphatase substrate, 6,8-difluoro-4-methylumberiferyll phosphate (DiF-MUP; Molecular Probes, OR) for 10 min at 37°C with intermittent mixing in a CytoFluor 4000 fluorescent plate reader (Applied Biosystems, CA), with 360nm excitation and 460nm emission filters. Parallel assays were conducted in the presence of 10 μM Na_3VO_4, an inhibitor of tyrosine phosphatase activity. The resulting data was analyzed with GraphPad Prism 4.0 and statistical significance was determined by analysis of variance (ANOVA), followed by a Neuman-Keuls post-test, with statistical significance defined as $p < 0.05$.

Cell viability assay

Cell viability assays were performed in 24-well plates with hBSM cells cultured in serum-free basal medium for 24 h and then incubated in the presence or absence of 100nM L779,976 for 48 hours at 37°C. The samples were incubated with 1 μM calcein-AM for 15 minutes prior to analysis in a CytoFluor 4000 fluorescent plate reader (Applied Biosystems; Blake, 2004). The resulting data was analyzed with GraphPad Prism 4.0 and statistical significance was determined by Student t-test ($p < 0.05$).

RESULTS

Analysis of hsst receptor expression using RT-PCR

RT-PCR using SRIF receptor subtype specific oligonucleotides to analyze hBSMC demonstrated that the human bronchial smooth muscle cells express hsst$_2$ mRNA (Figure 1). The dominant amplified product appears as a 600 base pair (hsst$_2$) band, indicating that the sst2 receptor subtype is present in these primary bronchial smooth muscle cells. While earlier studies have shown that several SRIF receptors are present in human lung, these results are the first to demonstrate a presence of the sst$_2$ receptor subtype in primary bronchial smooth muscle cells (Reubi *et al*, 2001).

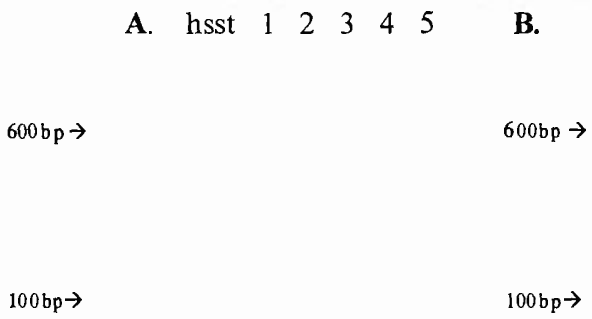

Figure 1. A. PCR amplification was performed on the sst1-5 receptor cDNAs. B. RT-PCR amplification of hBSMC receptor mRNA. The results are from a representative agarose gel of hBSMC total mRNA amplified with SRIF receptor subtype specific oligonucleotides. Molecular weight markers (far left lane) appear in lanes in A and B as a 100 base pair DNA ladder.

Western blot detection of phosphorylated ERK1/2 in hBSMC

Mitogen-activated protein (MAP) kinases play a central role in mitogenesis and differentiation, controlling both activation magnitude and duration in bronchial smooth muscle cellular responses (Zhou and Hershenson, 2003). We examined the activation status of one of these protein kinases, the extracellular regulated kinases (ERK1/2) by Western blotting experiments, using a phospho-specific antibody against ERK1/2. In

parallel cultures maintained under static conditions, we showed that an acute treatment with 100 nM L-779,976 significantly decreased hBSMC basal levels of ERK1/2 phosphorylation (Figure 2). L-779,976 inhibited basal ERK1/2 phosphorylation in hBSMC by 50 ± 5% when compared to parallel, untreated controls (Figure 2, $p<0.05$ control versus L-779,976 treated), indicating a sst_2 receptor-mediated regulation of this enzyme.

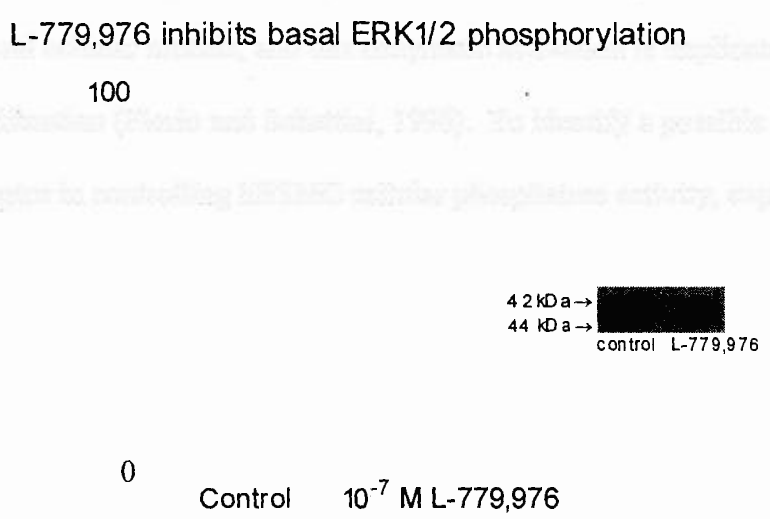

Figure 2. The sst_2 receptor agonist L-779,976 inhibits basal ERK1/2 phosphorylation in hBSMC monolayers. Parallel cultures of hBSMC were incubated in the presence or absence of L-779,976. Cell lysates were resolved by gel electrophoresis, electroblotted and incubated with antiphospho-ERK1/2 antibodies. Autoradiographs were quantified using NIH Image. The results represent the mean + SEM of three separate experiments ($p< 0.05$ between control and L-779,976 treated cells; Students t-test). The inset shows a representative autoradiograph.

Determination of soluble tyrosine phosphatase activity

SRIF receptors can couple to the activation of phosphotyrosine phosphatases in several cellular models, and this enzymatic activation is implicated in controlling cell proliferation (Florio and Schettini, 1996). To identify a possible role of the identified sst_2 receptor in controlling hBSMC cellular phosphatase activity, experiments using the

fluorescent substrate DiF-MUP were performed with hBSMC lysates prepared from untreated and treated (100 nM SRIF-14) cell monolayers. DiF-MUP provides a fluorescent measure of protein phosphatase activity, which when combined with an appropriate inhibitor can define the type of phosphatase activity being studied (Gee *et al*, 1999). As shown in Figure 3, 100nM L-779,976 increases membrane phosphatase activity in hBSMC monolayers by 2.4 fold over the untreated control group ($p < 0.05$; control versus L-779,976), while 100nM SRIF-14 increases membrane phosphatase activity by 2.8 fold over the untreated control group ($p < 0.05$; control versus SRIF). In addition, co-incubation of the cell membranes with 10 μM sodium vanadate (Na_3VO_4), a potent tyrosine phosphatase inhibitor, abolished greater than 90% of the enzymatic activity derived from both control and SRIF-14 treated cells (Figure3; $p < 0.05$ Na_3VO_4 versus SRIF-14 + Na_3VO_4). The DiF-MUP phosphatase activity proved to be sensitive to Na_3VO_4 inhibition, with both control and SRIF-14 treated cell membranes demonstrating an IC_{50} of inhibition in the low nanomolar concentration range (data not shown).

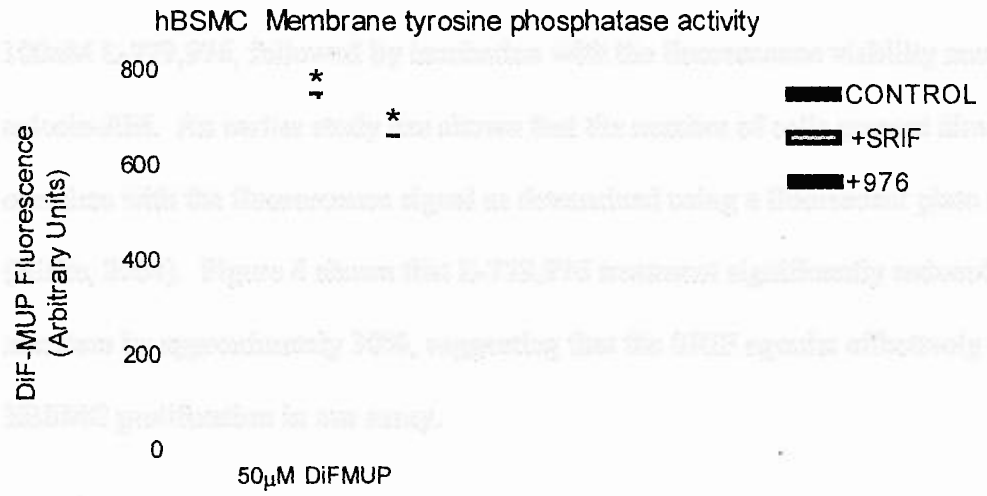

Figure 3. SRIF ligands activate tyrosine phosphatase activity in hBSMC. hBSMC monolayers were either control or pretreated with 100 nM SRIF-14 or L-779.976 for 15 min at 37°C. Phosphatase activity was measured with 50μM DiF-MUP for 10 min at 37°C in a CytoFluor 4000 fluorescent plate reader. Parallel incubations were carried out with 10 μM sodium vanadate. The results were analyzed using GraphPad Prism 3.0 and the data presented represent the mean + SEM of three separate experiments, each performed in triplicate.

Determination of cell viability

To further establish SRIF-14's role in dampening cell proliferation, assays were carried out in which hBSMC monolayers were incubated in the presence or absence of 100nM L-779,976, followed by incubation with the fluorescence viability marker, calcein-AM. An earlier study has shown that the number of cells present directly correlate with the fluorescence signal as determined using a fluorescent plate reader (Blake, 2004). Figure 4 shows that L-779,976 treatment significantly reduced cell numbers by approximately 30%, suggesting that the SRIF agonist effectively suppressed hBSMC proliferation in our assay.

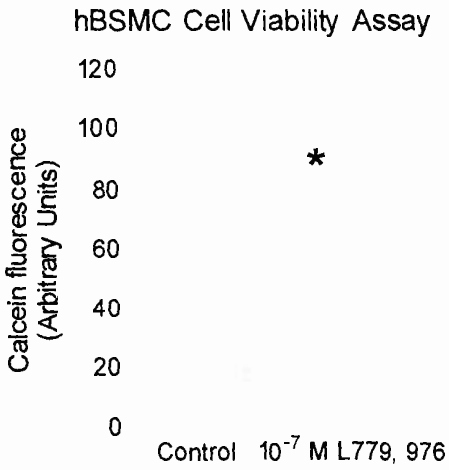

Figure 4. L779, 976 decreases BSMC cell number. hBSM cells were cultured in serum-free basal medium in 24-well plates for 24 hand then incubated in the presence or absence of 100 nM L779, 976 for 48 hours at 37°C. The samples were incubated with calcein-AM for 15 minutes prior to analysis in a CytoFluor 4000 fluorescent plate reader (Applied Biosystems). L779, 976 reduced cell viability by approximately 30%. Student t-test, *$p<0.05$

DISCUSSION

SRIF regulates intracellular signaling through actions on intracellular ion concentrations, cyclic nucleotides and protein phosphorylation levels (reviewed in Badway and Blake, 2005). In our experiments we used primary human bronchial smooth muscle cells (hBSMC) as a model system to assess SRIF-14 analogue actions, as these cells are thought to play a significant role in asthmatic airway remodeling, a significant component of chronic asthma (Halayko and Amrani, 2003].

We first established the SRIF receptor subtypes present in hBSMC, using RT-PCR and oligonucleotides specific to $hsst_{1-5}$. The results of our RT-PCR amplification of total hBSMC RNA clearly revealed the presence of sst_2. This finding is of considerable interest, since our study presents the first identification of the SRIF receptor subtype present in normal, isolated bronchial smooth muscles cells.

Although SRIF receptors can couple to multiple intracellular signaling pathways (Badway and Blake, 2005), we were interested in examining whether the sst_2 receptor could control the growth of the hBSMC. MAP kinase activation plays a critical role in many cellular responses including proliferation and cell cycle progression (Taille *et al*, 2003). Previous studies have demonstrated a role for SRIF and SRIF analogues in regulating MAP kinase pathways in a range of primary cells and cultured cell lines (reviewed in Dasgupta, 2004; Badway and Blake, 2005). In addition, it has been demonstrated that up-regulation and therefore activation of the MAP kinase ERK1/2 pathway can lead to the promotion of cell proliferation and cell cycle progression, and increased cell survival thereby protecting cells from apoptosis (Chang and Karin, 2001; Werry *et al*, 2005). After discovering SRIF receptor expression in hBSMC, we carried

out western blot experiments using ERK1/2 phospho-specific antibodies to determine SRIF's ability to modulate the MAP kinase pathway in hBSMC. Since the active kinase is phosphorylated our experiments showed that treatment with 100nM L-779,976, the sst2 agonist, effectively reduces the ERK phosphorylation state within hBSMCs (Figure 2). By inference, it is possible that SRIF functions in an anti-proliferative action to inhibit airway smooth cell expansion. This may be an important counter regulator of the hypertrophic response found in asthmatic airways (Zhou *et al*, 2004)

In vitro fluorescence assays using a synthetic substrate (DiF-MUP) explored SRIF's intracellular signaling mechanism in hBSMC. Treatment of hBSMC with 100 nM L-779,976 or SRIF-14 increased DiF-MUP fluorescence in prepared cell membranes. Parallel experiments carried out in the presence of sodium vanadate, a potent tyrosine phosphatase inhibitor, abolished the DiF-MUP activity. These results suggest that the measured DiF-MUP activity results from activation of soluble tyrosine phosphatases. ERK1/2 is phosphorylated on tyrosine and threonine residues, it is possible that the increase in tyrosine phosphatase activity as a result of sst_2 receptor stimulation by SRIF-14 or L-779,976 treatments plays a role in the decrease in phospho-ERK1/2 levels. MAP kinase phosphatases (MKP), a group of dual specificity phosphatases, are members of a superfamily of protein tyrosine phosphatases (Tonks *et al*, 2001), and our data raise the possibility that some members of these hBSMC enzymes may be under SRIF-14 control.

Since these results suggested that SRIF may function to inhibit proliferation and hinder cell survival in hBSMC, we performed a cell proliferation assay using the fluorescent marker calcein-AM (Blake, 2004). After treatment with 100nM L-779,976 for 48 hours and subsequent calcein-AM staining, a 30% decrease in cell retained

fluorescence was measured as compared to control, untreated cells. Since calcein AM retention in the cellular monolayer directly correlates with viable cell number (Blake, 2004), our results suggest that L-779,976-mediated inhibition of ERK1/2 may have a direct effect on hBSMC growth and proliferation.

Chronic inflammation, leading to airway remodeling and airway hyperreactivity, is the hallmark of asthma. The structural changes, including airway smooth muscle hypertrophy and hyperplasia, disrupt a number of homeostatic mechanisms in the lung. This includes disrupted homeostasis in the airway epithelium, subepithelial fibrosis, bronchial vascular remodeling, as well as the noted increase in airway smooth muscle mass (McKay *et al* 2002). While SRIF-14 and its analogues have been shown to regulate cellular homeostasis and exert anti-inflammatory effects in a number of cells and tissues, the current study provides the first evidence for possible SRIF inflammatory control in bronchial smooth muscle.

Our results have shown that a SRIF analogue is capable of regulating hBSMC intracellular pathways that may be therapeutically important to inhibition of the structural remodeling found in asthma. Our results demonstrate for the first time the presence of an sst2 receptor subtype in hBSMC, implicated in controlling distinct intracellular signaling pathways, thereby implicating the body's endogenous SRIF peptide family in direct actions on bronchial smooth muscle.

In order to further elucidate SRIF's anti-inflammatory effects on bronchial smooth muscle, it would be advantageous to measure SRIF's effects on ERK1/2 phosphorylation, tyrosine phosphatase activation, and cell viability in human bronchial smooth muscle cells obtained from asthmatic subjects. In addition, other intracellular

signaling pathways, including p38 and JAK-STAT have been studied in hBSMC (Simon *et al*, 2002) however, the effect of somatostatin on these kinase cascades remains to be seen. Such a parallel experiment would extend our knowledge of SRIF's ability to modulate inflammation *in vitro*. Ultimately, demonstration of the physiological results of SRIF administration would provide greater insight into the future therapeutic value of SRIF in asthma.

CONCLUSIONS

In the current study, we establish the presence of a functionally active sst_2 receptor in human bronchial smooth muscle cells. Our novel results show that L-779,976, an sst_2 selective analogue, is capable of regulating the ERK1/2 pathway, resulting in the deactivation of phosphorylated ERK1/2. Our data further postulates that SRIF-treatment causes an increase in protein tyrosine phosphatase activation, present in the membranes of hBSMC, which may be responsible for the dephosphorylation of ERK1/2. Finally, we observed that treatment with a SRIF analogue could suppress cell viability by approximately 30%, suggesting a downregulation of hBSMC proliferation, a downstream target of ERK1/2. These results suggest that SRIF and its analogues may play a role in preventing the hypertrophy of the airway via intracellular signaling in human bronchial smooth muscle cells.

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