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Sphingosine-1-phosphate induces pro-remodelling response in airway smooth muscle cells

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Abstract

Background: Increased proliferation of airway smooth muscle (ASM) cells leading to hyperplasia and increased ASM mass is one of the most characteristic features of airway remodelling in asthma. A bioactive lipid, sphingosine-1-phosphate (S1P), has been suggested to affect airway remodelling by stimulation of human ASM cell proliferation.

Objective: To investigate the effect of S1P on signalling and regulation of gene expression in ASM cells from healthy and asthmatic individuals.

Methods: ASM cells grown from bronchial biopsies of healthy and asthmatic individuals were exposed to S1P. Gene expression was analysed using microarray, real-time PCR and western blotting. Receptor signalling and function was determined by mRNA knockdown and intracellular calcium mobilisation experiments.

Results: S1P potently regulated the expression of more than 80 genes in human ASM cells, including several genes known to be involved in the regulation of cell proliferation and airway remodelling (HBEGF, TGFB3, TXNIP, PLAUR, SERPINE1, RGS4). S1P acting through S1P₂ and S1P₃ receptors activated intracellular calcium mobilisation and extracellular signal-regulated and Rho-associated kinases to regulate gene expression. S1P-induced responses were not inhibited by corticosteroids and did not differ significantly between ASM cells from healthy and asthmatic individuals.

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Conclusion: S1P induces a steroid-resistant, pro-remodelling pathway in ASM cells. Targeting S1P or its receptors could be a novel treatment strategy for inhibiting airway remodelling in asthma.

Introduction

Airway remodelling in asthma is characterised by increased airway smooth muscle mass, epithelial and goblet cell hyperplasia, angiogenesis and reticular basement membrane thickening caused by a repeated process of injury and repair (1). Concomitant bronchial mucosal inflammation has been cited as one possible cause of remodelling (2) but the lack of efficacy of corticosteroids in inhibiting remodelling suggests that other pathogenic pathways are also involved (3). Indeed, simple compressive stress resulting from bronchoconstriction has been demonstrated to trigger a pro-fibrotic response reminiscent of remodelling in the absence of eosinophilic inflammation (4). This mechanotransduction pathway has been linked with growth factor shedding and heparin-binding epidermal growth factor-like growth factor (HB-EGF) signalling (5).

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid involved in mediating diverse cellular processes including cell growth, proliferation, survival and migration (6). S1P plays an important role in lymphocyte migration (7) but accumulating evidence implicates the involvement of S1P in pro-fibrotic and pro-remodelling responses in the lungs. Concentrations of S1P were elevated in patients with idiopathic pulmonary fibrosis (IPF) to a degree which correlated with lung function and aberrant epithelial to mesenchymal transition (EMT) (8). S1P has also been found to promote the growth of fibroblasts and production of extracellular matrix proteins (9, 10). All of these processes have been associated with fibrotic lung remodelling.

In the case of asthma, genetic and functional studies have strongly linked genes in the sphingolipid pathway, in which S1P is the most prominent mediator, with the risk of developing the disease (11-13). Increased concentrations of S1P were also found in the bronchoalveolar lavage (BAL) fluid of asthmatics, but not healthy individuals following allergen challenge (14) and S1P has been shown to

be involved in the regulation of allergic inflammation and bronchial hyperresponsiveness in murine “models” of the disease (15, 16).

Airway smooth muscle hyperplasia, driven at least partly by mediators such as transforming growth factor β (TGF- β) and HB-EGF, is considered to be a major contributor to airway remodelling, obstruction and hyperresponsiveness in asthma (17-19). Nevertheless, the capacity for S1P to act directly on human ASM cells and contribute to remodelling has not been extensively studied. One study showed that S1P significantly increased proliferation of human ASM cells and stimulated IL-6 secretion (14). It has also been shown that S1P may regulate ASM contractility *in vitro* (20), but *in vivo* evidence in humans is lacking. The best-characterised activities of S1P have been attributed to signalling through a family of specific G-protein coupled receptor (GPCRs), named S1P₁₋₅ (21). As the role of S1P and its receptors in human ASM functions and asthma remodelling has not been well understood we aimed to investigate the effect of S1P on signalling and regulation of gene expression in ASM cells from healthy and asthmatic individuals.

Materials and Methods

Patients

Airway smooth muscle cells from healthy and asthmatic individuals were obtained by deep endobronchial biopsy at fiberoptic bronchoscopy with the approval of the Research Ethics Committees of Guy’s Hospital (10/H0804/66). Samples were obtained from 13 healthy volunteers (8F, 5M) and 5 asthmatic patients (2F, 3M) (3 mild and 2 moderate asthmatics defined according to GINA guidelines and characterised in online supplement).

Cell Culture

Airway smooth muscle cells were grown from bronchial biopsies by explant culture as previously described (22, 23) and characterised as described in online supplement.

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Microarray Analysis

Total RNA was isolated, processed and hybridised to the Affymetrix Human Exon 1.0 ST (Affymetrix) and analysed using the Partek Genomics Suite (Partek) as described in online supplement. Data were submitted to Gene Expression Omnibus database (accession number GSE58657).

Real time PCR

Expression of mRNA encoding selected genes was measured using real time PCR, ABI Prism 7900 (Applied Biosystems) as described in the online supplement.

Western Blot Analysis

Total protein lysates or membrane proteins were isolated as described in the online supplement and proteins detected using primary antibodies against PTGS2 (COX2) (Clone CX229, Cayman Chemical), HBEGF (BioAcademia), TXNIP (Clone JY1, Medical&Biological Laboratories) and control GAPDH (Clone 6C5, GeneTex).

S1P₂ and S1P₃ knockdown

Human ASM cells were transfected as described in the online supplement with 10nM Silencer Select Validated siRNA s4454 (Ambion) and 20nM 27mer siRNA SR306152A (Origene) and respective negative controls using Lipofectamine2000 (Life Technologies) for S1P₃ and S1P₂ knockdown, respectively.

Calcium mobilisation assay

Calcium mobilisation assays were performed using the FLIPR calcium 4 assay kit (Molecular Devices) as previously described (24) and presented in the online supplement.

Results

S1P induces gene expression in human ASM cells.

Microarray analysis identified 88 genes regulated by S1P in ASM cells by 2 fold or more (Fig 1A, online supplement Table 1), including genes involved in cell proliferation and airway remodelling (HBEGF, TGFB3, TXNIP, PLAUR, SERPINE1), intracellular signalling (RGS4, RGS2, DUSP5, MAP2K3, DGKH) and regulation of transcription (NR4A1, NR4A3, EGR3, FOSB). To further investigate S1P-induced effects, mRNA (Fig 1B) and protein expression (Fig 1C) of several significantly modified genes (HBEGF, RGS4, TGFB3, BDKRB1, PLAUR, TXNIP, PTGS2) were analysed over time by real time PCR and Western blotting, confirming the microarray findings. Even though IL-6 has previously been shown to be induced by S1P in human ASM cells (14) it was not significantly up-regulated by S1P in our microarray experiment. This is probably due to different regulation of transcription, with maximum increase observed after 24 hours of S1P stimulation (Fig 1B). Genes, most highly up-regulated (HBEGF, RGS4) and down-regulated (TXNIP) at 4 hour, were selected for further analysis. S1P concentration of 100 nM was chosen for further experiments showing sub maximal responses for calcium signalling and gene expression.

S1P₂ and S1P₃ mediate gene regulation in human ASM cells

Real time PCR identified S1P₂ and S1P₃ as being the most highly expressed, with lower expression of S1P₁, and lack of S1P₄ and S1P₅ (Fig 2A). SEW2871 (selective S1P₁ agonist) did not modify gene expression (Fig 2B) while JTE-013 (selective S1P₂ antagonist) almost completely inhibited S1P-induced HBEGF expression (Fig 2C). S1P₂ down-regulation using siRNA knockdown caused a

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significant reduction of the S1P-induced increase in HBEGF expression (Fig 3A). As with S1P₂ knockdown, S1P₃ knockdown significantly reduced S1P-induced HBEGF mRNA expression compared to control cells (Fig 3B). Similarly, S1P₂ and S1P₃ blockade or knockdown altered the expression of RGS4 and TXNIP (data not shown). Altogether, these data demonstrate that both S1P₂ and S1P₃ are involved in S1P-induced gene regulation in human ASM cells.

S1P signals through intracellular calcium, Erk- and Rho-associated kinases for regulation of gene expression

To investigate downstream signalling of S1P₂ and S1P₃ we studied the effect of S1P on calcium mobilisation in ASM cells. S1P induced a concentration dependent increase in intracellular calcium ($EC_{50}=2.7 \times 10^{-9} M$) (Fig 4A). The S1P₁ agonist SEW2871 and the S1P₂ antagonist JTE-013 did not affect calcium mobilisation, excluding S1P₁ and S1P₂ as receptors signalling through calcium (Fig 4A, B). In contrast, pre-treatment of ASM cells with VPC23019, a S1P₁ and S1P₃ antagonist, prior to S1P stimulation significantly reduced the release of intracellular calcium, suggesting that S1P₃ is a receptor signalling through calcium in human ASM cells (Fig 4C). Furthermore, S1P₃ siRNA treated cells showed reduced calcium mobilisation in response to S1P (Fig 4D) confirming this finding. S1P induced calcium mobilization was mediated in part by G_i since pre-treatment of ASM cells with pertussis toxin (PTX) partially inhibited S1P induced calcium flux (Fig 4E). To further analyse signalling pathways involved in S1P induced gene regulation, inhibitors of calcium (BAPTA-AM and EDTA, intracellular and extracellular calcium chelators, respectively), PTX, inhibitors of extracellular signal-regulated kinase (Erk) and Rho-associated kinase pathways were used (Fig 4F). All reagents inhibited the S1P induced increase in HBEGF mRNA expression (Fig 4F) and expression of other genes regulated by S1P (data not shown). These data are consistent with S1P signalling through calcium, Erk1/2 and ROCK to regulate gene expression in human ASM cells.

S1P induced gene expression is corticosteroid resistant

As several genes regulated by S1P have been shown to be involved in airway remodelling, the effect of corticosteroids on S1P induced gene expression was examined. ASM cells were pre-treated with dexamethasone (Dex) and gene expression was measured following S1P stimulation. There was no significant difference between control and Dex treated samples following S1P stimulation (Fig 5), suggesting that corticosteroids do not target S1P-induced pathways in human ASM cells.

Healthy and asthmatic ASM cells respond similarly to S1P stimulation

To further characterise the role of S1P in inflammatory diseases such as asthma, ASM cells obtained from asthmatic patients were cultured and their responses to S1P were directly compared to ASM cells from healthy volunteers. There was no significant difference in mRNA expression of S1P receptors (Fig 6A) or calcium mobilisation induced by S1P (Fig 6B) between ASM cells from healthy and asthmatic subjects (Fig 6B). S1P increased HBEGF gene expression by more than 3.8 fold (Fig 6C) in ASM cells from asthmatics, but the changes were not significantly different when compared to responses in ASM cells from healthy subjects. Similarly, there was no difference in the regulation of expression of other S1P-regulated genes, e.g. RGS4, TXNIP (Fig 6C) between healthy and asthmatics subjects.

Discussion

Increased proliferation of ASM cells leading to hyperplasia and increased ASM mass is a characteristic feature of airway remodelling in asthma. We have identified a novel S1P- mediated pathway present in human ASM cells from healthy and asthmatic individuals, involved in regulation of pro-remodelling changes. S1P acting through specific S1P₂ and S1P₃ receptors potentially regulated the expression of more than 80 genes in human ASM cells, including several genes known to be involved in the regulation of cell proliferation and airway remodelling (HBEGF, TGFB3, TXNIP,

PLAUR, SERPINE1, RGS4). Interestingly, several of these genes (HBEGF, RGS4 and PLAUR) have previously been shown to be up-regulated in airways of asthmatic individuals. HBEGF is believed to play a major role in driving remodelling changes in human airways. It has been reported to be expressed both in airway epithelium and in ASM cells of asthmatics and to be responsible for mediating cell proliferation through activation of EGF receptors (19, 25). Its expression also correlated with asthma severity. It has been suggested that HBEGF might be a biomarker for the active stage of ASM remodelling (19). In addition, HBEGF signalling through EGF receptors was identified as a main pathway activated by mechanical stress induced by airway constriction (5). Similarly, Regulator of G protein signalling 4 (RGS4) has been shown recently to be markedly up-regulated in ASM cells from severe asthmatics and its expression correlated significantly with reduced pulmonary function (26). RGS4 was required for ASM hyperplasia and rendered cells poorly contractile, a feature characteristic of more severe, irreversible airway obstruction. Urokinase receptor (PLAUR, uPAR), also up-regulated by S1P in ASM cells, was reported to be increased in patients with asthma and led to attenuated wound repair, a process contributing to development and progression of airway remodelling in asthma (27). Thioredoxin-interacting protein (TXNIP), also called vitamin D3 up-regulated protein (VDUP1), was the gene most potently down-regulated by S1P in our study. Interestingly, TXNIP has marked anti-proliferative effects in smooth muscle cells acting through a suppression of the thioredoxin (TRX) system in response to reactive oxygen species (ROS) and mitogenic factors (28). It was suggested that TXNIP is a critical molecular switch in the transduction of pro-oxidant mitogenic signals upon platelet-derived growth factor (PDGF) and thrombin stimulations. Thus, S1P mediated down-regulation of TXNIP in ASM cells might be expected to have a pro-proliferative effect, acting in an additive or synergistic way with HBEGF or other mitogenic signals as reported previously for S1P and thrombin in ASM cells (14).

S1P binds with low nanomolar affinity to five GPCRs, S1P₁₋₅. We found that three S1P receptors are expressed in ASM cells at the mRNA level, with S1P₂ and S1P₃ being the most predominant. We have also shown that S1P signals through S1P₂ and S1P₃ to regulate gene expression in ASM cells. Intracellular calcium mobilisation and activation of Erk- and Rho- associated kinase pathways are required for transcriptional regulation. Our data suggest that S1P₃, coupled to G_q and G_i, is the major

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S1P receptor signalling through intracellular calcium mobilisation and Erk activation in response to low nanomolar S1P concentrations. A similar signalling pathway for S1P₃ has been described in lung epithelial cells leading to cytosolic phospholipase A2 α activation and arachidonic acid synthesis (29). Nevertheless, S1P₂ activation is also required for S1P-induced gene regulation since the selective S1P₂ antagonist JTE-013 and S1P₂ knockdown potently inhibited gene expression. In contrast to S1P₃, S1P₂ did not signal through calcium, but probably couples to G_{12/13} and activates Rho-associated kinase pathway in ASM cells. Based on our data, it can be suggested that both S1P receptors, S1P₂ and S1P₃, are needed for regulation of gene expression in ASM cells. These findings may be relevant for clinical applications, since it suggests that inhibition of S1P receptors could be potentially effective to prevent pro-remodelling changes.

S1P is a potent lipid mediator regulating diverse biological functions of many cell types, from proliferation and survival to migration and secretion (30). Constitutive concentrations of S1P in most tissues are very low, but high nanomolar concentrations can be found in blood and lymph, creating a sharp circulation-tissue S1P gradient responsible for trafficking of immune cells from secondary lymphoid tissues to blood vessels (21). Upon activation, many immune cells can release S1P, including mast cells, neutrophils, platelets and mononuclear cells. For example, activation of mast cells through the high affinity immunoglobulin E receptor (Fc ϵ RI) induces production and secretion of S1P, important for mast cell degranulation and for increased local tissue S1P concentrations (31). The observations that elevated concentrations of S1P are found in BAL of asthmatic individuals following allergen challenge (14) and that infiltration of airway smooth muscle by mast cells is associated with disordered airway function in asthma (32) suggest that S1P produced locally by activated mast cells may directly activate ASM cells. We have not observed significant differences in S1P signalling and regulation of gene expression between ASM cells from healthy and asthmatic subjects but we cannot exclude possibility that this was due to small number of subjects studied, culture conditions or corticosteroid treatment (asthmatics) rather than lack of real *in vivo* differences. However, based on our data we can speculate that it is repetitive exposure, rather than enhanced

responsiveness, to S1P that determines increased expression of pro-remodelling factors observed in asthma, i.e. HBEGF, RGS4 and PLAUR. This is reminiscent of remodelling changes in human asthma proceeding in “waves” during episodes of disease exacerbation (33). Interestingly, our observed effects of S1P were resistant to corticosteroid inhibition *in vitro*. Again, this is reminiscent of reports of remodelling changes in asthma being resistant to corticosteroid inhibition/reversal (34).

In summary, the growing perception that S1P is an important pro-inflammatory mediator in asthma and other inflammatory diseases (35-37), its ability to induce pro-fibrotic changes in lung fibroblasts (10) and our present findings that it may facilitate, through up-regulation of HBEGF expression and other mechanisms, the mechanotransduction pathway caused by bronchoconstriction which results in remodelling, all pinpoint S1P as a key molecular target in asthma, particularly in view of its potential insensitivity to corticosteroid inhibition.

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Author contributions:

EF – designed the study, performed experiments, acquired and analysed data, drafted and edited the manuscript; HRF – performed experiments, edited the manuscript; JPTW – supervised smooth muscle culture protocol, edited the manuscript; CJC – supervised patients recruitment and bronchoscopies, edited the manuscript; DJC – assisted with experiments, analysed data, edited the manuscript; GW – designed the study, acquired and analysed data, assisted with experiments, drafted and edited the manuscript. All authors contributed to data discussion and review of the manuscript.

Conflict of interests:

The authors declare no conflict of interests.

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Figure legends

Figure 1. S1P regulates gene expression in human ASM cells. (A) ASM cells from 3 healthy donors were stimulated with S1P (100 nM) for 4 hours and gene expression was analysed using Affymetrix Exon 1.0 ST microarrays. Hierarchical clustering of significantly (ANOVA $p < 0.05$, > 2 fold change) regulated genes is presented as a heatmap. (B) Time course analysis of S1P-induced gene expression using real-time PCR. Data from 3 experiments (mean \pm SEM) from different healthy donors are presented as a fold change in comparison to medium control cells. (C) Protein expression in response to S1P (100 nM). Cells were stimulated with S1P for 6 hours for HBEGF (membrane proteins) and for 8 hours for PTGS2 (COX2) and TXNIP, proteins extracted and analysed by western blotting. Representative blots and densitometry data from 3 different donors presented as fold change (mean \pm SEM) in relation to GAPDH expression.

Figure 2. S1P signals through S1P receptors expressed in human ASM cells. (A) S1P receptor expression (mRNA) in ASM cells analysed by real-time PCR. Data from 7 healthy controls are expressed relative to S1P₃ expression and compared with internal 18S rRNA expression (mean \pm SEM). (B) Cells were stimulated with S1P (100nM), SEW2871 (SEW)

(100nM) or medium control for 4 hours and gene expression was analysed using real-time PCR. Data from 3 healthy donors are expressed as fold change relative to medium control (mean \pm SEM). (C) Regulation of HBEGF expression by S1P₂ inhibitor JTE-013 (JTE). Cells were pre-treated with JTE-013 (1 μ M), stimulated with S1P for 4 hours and analysed using real-time PCR (n=3, mean \pm SEM).

Figure 3. S1P signals through S1P₂ and S1P₃ in ASM cells. S1P₂ (A) and S1P₃ (B) mRNA expression was measured in un-stimulated cells transfected with scrambled siRNA and S1P₂ (A) or S1P₃ (B) siRNA (S1P₂ KD/S1P₃ KD) to confirm knockdown. HBEGF expression was measured after 4 hour stimulation with S1P (100nM) or medium control in control samples and S1P₂ (A) or S1P₃ (B) siRNA treated cells. Data presented from 3 experiments from different healthy donors (mean \pm SEM). *P<0.05, 1-way ANOVA with Bonferroni post test.

Figure 4. S1P signals through calcium, Erk and Rho-associated kinase activation. (A) Concentration response curve of calcium mobilisation in response to S1P and SEW2871 (SEW). Cells were pre-treated with different concentrations of (B) JTE-013 (JTE) or (C) VPC23019 (VPC) prior to stimulation with S1P (100nM) and calcium mobilisation was measured. **P<0.01 and ***P<0.001, 1-way ANOVA with Bonferroni post test. (D) Calcium mobilisation induced by S1P in S1P₃ siRNA treated cells and control cells. (E) ASM cells were pre-treated with pertussis toxin (Ptx) (100ng/ml) for 18 hours followed by stimulation with S1P and calcium mobilisation was measured. All data are expressed as percent of maximum response to S1P (in relative fluorescence units - RFU) from 3-4 experiments with different healthy donors (mean \pm SEM). (F) ASM cells were treated with BAPTA-AM for 30 min. (9 μ M) (BAPTA), EDTA (2.5mM) for 5 min., pertussis toxin (PTX) (100ng/ml) for 18 hours, PD98059 (10 μ M) for 10 min, and Y27632 (1 μ M) for 10 min prior to 4 hour S1P (100nM) stimulation. Regulation of HBEGF expression was analysed by real-time PCR.

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time PCR and data from 3 experiments from different healthy donors are presented as percentage of changes compared with S1P stimulated samples (mean \pm SEM).

Figure 5. Corticosteroids do not inhibit S1P-induced gene expression. ASM cells were treated with dexamethasone (Dex) (100nM) prior to 4 hour stimulation with S1P (100nM) and expression of (A) HBEGF and (B) RGS4 were measured using real-time PCR. Data from 3 different healthy donors are presented as fold change in comparison to controls (mean \pm SEM). ns=non statistically significant; 1-way ANOVA with Bonferroni post test.

Figure 6. S1P signals similarly in healthy and asthmatic patients. (A) S1P receptor expression in ASM cells from healthy and asthmatic subjects analysed by real-time PCR. Data are expressed relative to 18S rRNA and normalized to S1P₃ expression in healthy subjects (mean \pm SEM). (B) ASM cells from healthy and asthmatic donors were stimulated with different concentrations of S1P and calcium mobilisation was measured. Data are expressed as percentage of the response to calcium ionophore (1 μ M) (n = 3, mean \pm SEM). (C) Cells from healthy and asthmatic donors were stimulated with S1P (100nM) for 4 hours and gene expression was analysed using real-time PCR. Data are expressed as fold change (mean \pm SEM). All data presented from experiments from 3-4 different healthy or asthmatic subjects. ns=non statistically significant; 1-way ANOVA with Bonferroni post test.

Figure 1

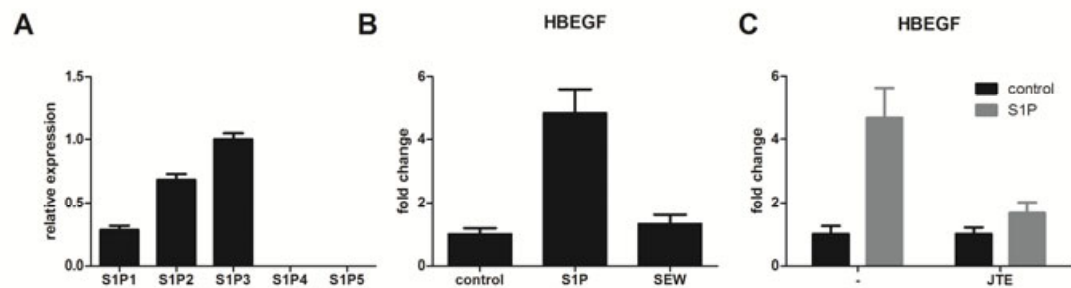
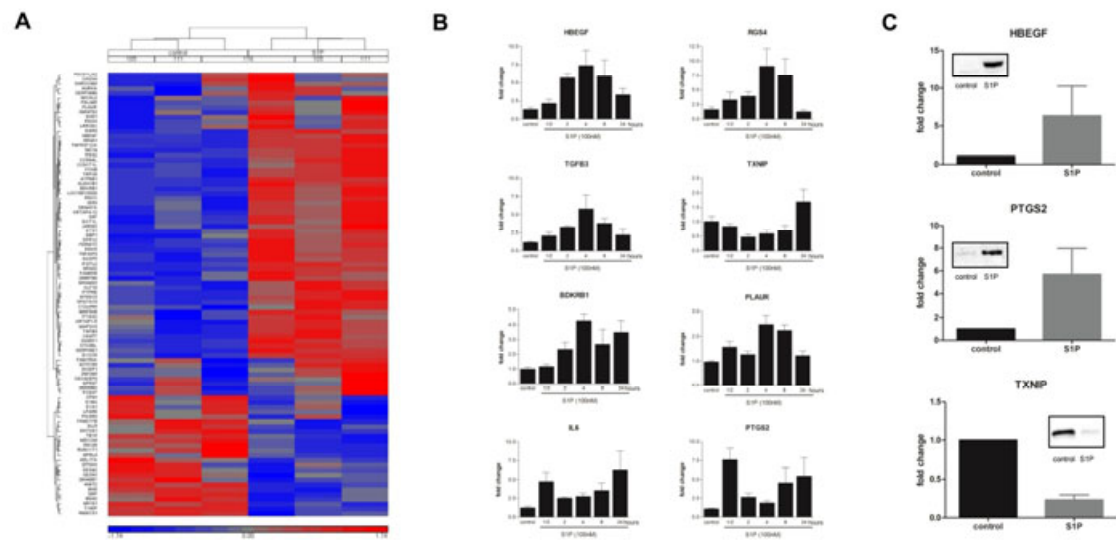


Figure 3

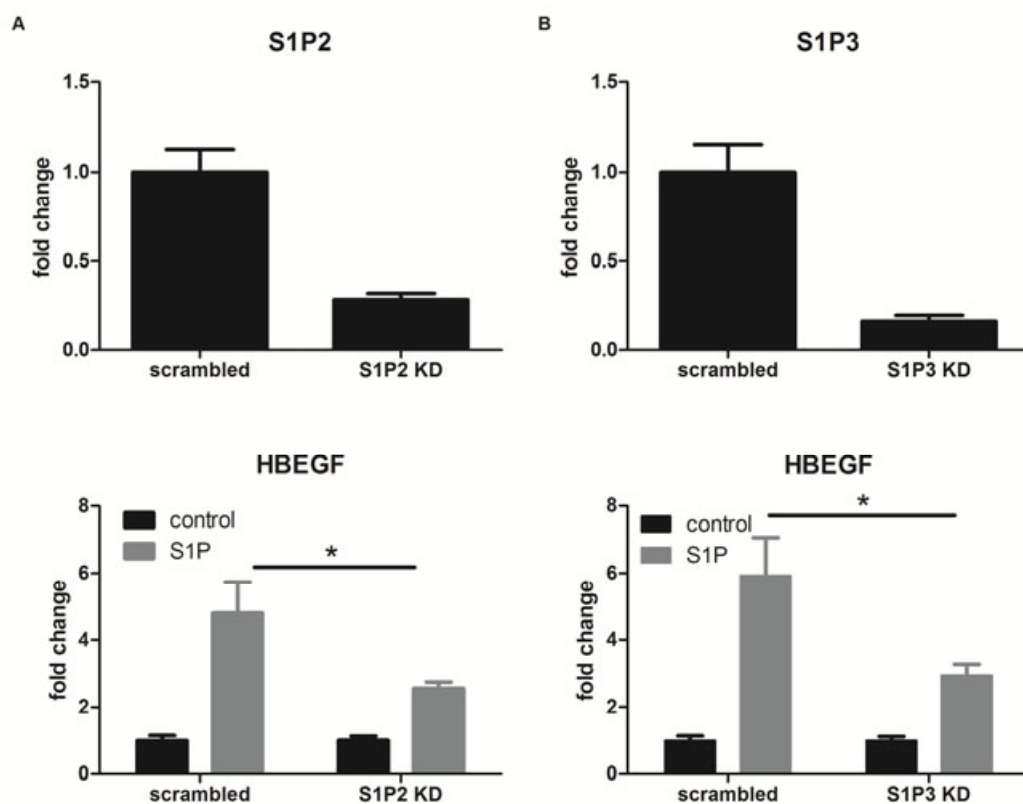


Figure 4

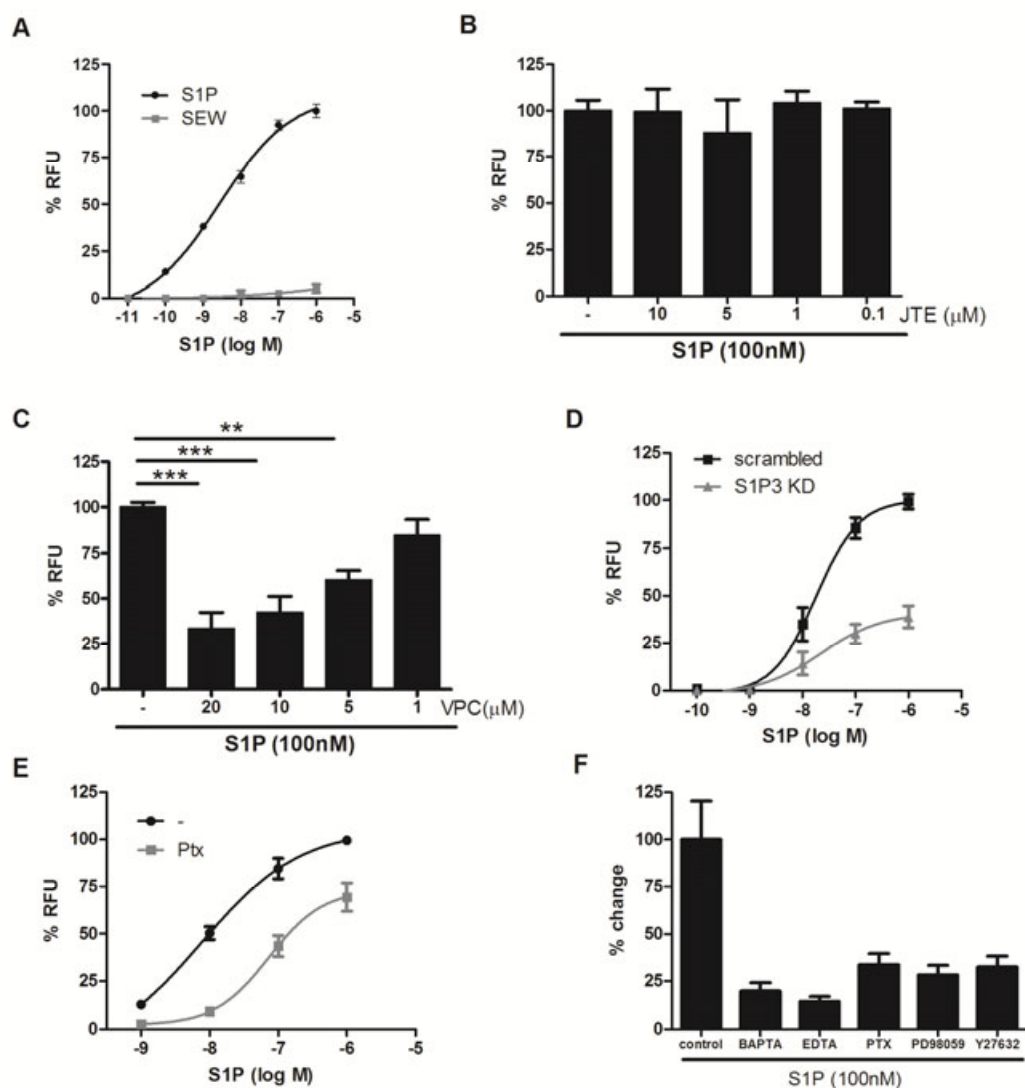


Figure 5

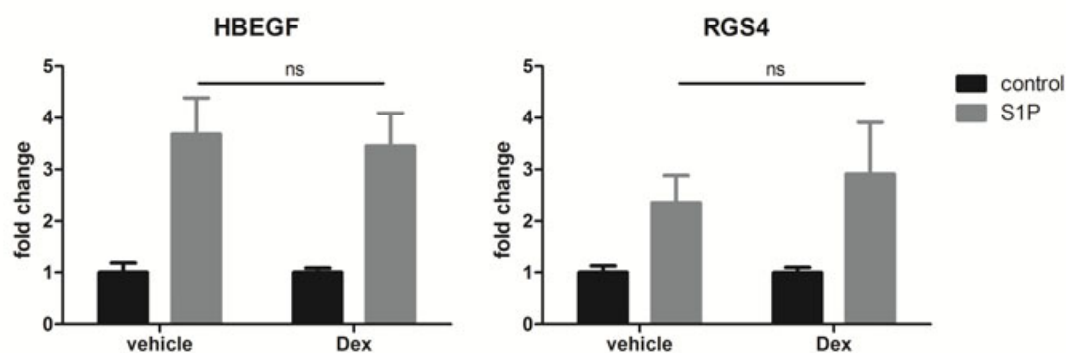


Figure 6

