Biochimica et Biophysica Acta 1823 (2012) 1658–1665

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Sphingosine 1-phosphate induces MKP-1 expression via p38 MAPK- and CREB-mediated pathways in airway smooth muscle cells

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ARTICLE INFO

Article history:
Received 1 February 2012
Received in revised form 17 May 2012
Accepted 12 June 2012
Available online 25 June 2012

Keywords:
Sphingolipid
Cytokine
Adenylate cyclase
CREB
p38 MAPK
DUSP-1

ABSTRACT

Sphingosine 1-phosphate (S1P), a bioactive sphingolipid elevated in asthmatic airways, is increasingly recognized as playing an important role in respiratory disease. S1P activates receptor-mediated signaling to modulate diverse cellular functions and promote airway inflammation. Although many of the stimulatory pathways activated by S1P have been delineated, especially mitogen-activated protein kinases (MAPK), the question of whether S1P exerts negative feedback control on its own signaling cascade via upregulation of phosphatases remains unexplored. We show that S1P rapidly and robustly upregulates mRNA and protein expression of the MAPK deactivator–MAPK phosphatase 1 (MKP-1). Utilizing the pivotal airway structural cell, airway smooth muscle (ASM), we confirm that S1P activates all members of the MAPK family and, in part, S1P upregulates MKP-1 expression in a p38 MAPK-dependent manner. MKP-1 is a cAMP response element binding (CREB) protein-responsive gene and here, we reveal for the first time that an adenylate cyclase/PKA/CREB-mediated pathway also contributes to S1P-induced MKP-1. Thus, by increasing MKP-1 expression via parallel p38 MAPK- and CREB-mediated pathways, S1P temporally regulates MAPK signaling pathways by upregulating the negative feedback controller MKP-1. This limits the extent and duration of pro-inflammatory MAPK signaling and represses cytokine secretion in ASM cells. Taken together, our results demonstrate that S1P stimulates both kinases and the phosphatase MKP-1 to control inflammation in ASM cells and may provide a greater understanding of the molecular mechanisms responsible for the pro-asthmatic functions induced by the potent bioactive sphingolipid S1P in the lung.

1. Introduction

The bioactive sphingolipid sphingosine 1-phosphate (S1P) plays an important role in a number of diseases typified by inflammation, including respiratory disorders such asthma [1,2]. In 2001, we demonstrated that S1P was elevated in human asthma [3] and over the past decade S1P has been shown to promote development of a pro-asthmatic and pro-remodeling phenotype in airway cells *in vitro*, *ex vivo* and *in vivo*. S1P regulates key airway functions such as contraction [4,5], cytokine secretion [3], leukocyte recruitment [6], and airway cell hyperplasia [7]. Moreover, a clear pathophysiologic role has been revealed by recapitulation of asthmatic phenotype by administration of S1P in mouse models of human disease [8]. Thus, S1P has emerged as an important mediator responsible for airway inflammation and hyperresponsiveness in asthma. As S1P is an attractive target for the development of new pharmacotherapeutic strategies, a greater understanding of S1P-mediated signaling in clinically-relevant airway cells is warranted.

S1P regulates myriad cellular functions due to the broad nature of the family of cognate receptors — S1P receptors 1–5 ($S1P_{1-5}$) (reviewed in [9–13]). S1P receptors are G protein-coupled receptors coupled to a range of heterotrimeric G proteins co-ordinating diverse downstream signaling pathways in a species- and cell type-specific manner. Our studies utilize the pivotal airway cell–airway smooth muscle (ASM). ASM cells express $S1P_{1-5}$ [3] and we and others have explored some of the signaling pathways activated in ASM upon exogenous addition of S1P. S1P acts via G_i-coupled S1P receptors to activate members of the mitogen-activated protein kinase (MAPK) family [14,15], and we showed that S1P increases production of the second messenger cAMP in a G_s-coupled manner [3]. To date, whether S1P controls the extent and duration of its own signaling cascade by upregulating the MAPK phosphatase 1 (MKP-1) has not been explored in ASM cells, or any other cell type.

MKP-1 is a potent MAPK deactivator that plays an important negative feedback role in a wide range of inflammatory cells [16,17]. In ASM, we recently reported that stimulation with tumor necrosis factor α (TNFα) increases MKP-1 upregulation via a p38 MAPK manner.

Abbreviation: ASM, airway smooth muscle; CREB, cAMP response element binding; DUSP, dual-specificity phosphatases; ERK, extracellular signal-regulated kinase; IL-6, interleukin 6; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MKP, mitogen-activated protein kinase phosphatase; PKA, protein kinase A; SEM, standard error of the mean; S1P, sphingosine 1-phosphate; TNFα, tumor necrosis factor α

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doi:10.1016/j.bbamcr.2012.06.011

MKP-1 then acts back on the kinases to dephosphorylate them and restrain MAPK-mediated pro-inflammatory signaling pathways and cytokine secretion [18]. Whether S1P activates a comparable negative feedback loop is unknown at present. Therefore, the aim of this study was to examine for the first time whether S1P upregulates MKP-1, the underlying molecular mechanism and the impact on cytokine secretion from ASM cells. We show that S1P-induces MKP-1 expression via activation of parallel p38 MAPK and CREB-mediated pathways and MKP-1 serves to restrain pro-inflammatory MAPK signaling and synthetic function of ASM cells. In this way, MKP-1 acts as a negative feedback effector and allows S1P to control the extent and duration of its own inflammatory signaling cascade.

2. Materials and methods

2.1. Cell culture

Human bronchi were obtained from patients undergoing surgical resection for carcinoma or lung transplant donors in accordance with procedures approved by the Sydney South West Area Health Service and the Human Research Ethics Committee of the University of Sydney. ASM cells were dissected, purified and cultured as previously described by Johnson et al. [19]. A minimum of three different ASM primary cell lines was used for each experiment.

Unless otherwise specified, all chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Western blotting

Western blotting was performed using rabbit monoclonal or polyclonal antibodies against phosphorylated (Thr^{180}/Tyr^{182}) and total p38 MAPK, phosphorylated (Thr^{202}/Tyr^{204}) and total extracellular signal-regulated kinase (ERK), phosphorylated (Thr^{183}/Tyr^{185}) and total c-Jun N-terminal kinase (JNK), phosphorylated (Ser^{133}) and total cAMP response element binding (CREB) (all from Cell Signaling Technology, Danvers, MA). MKP-1 was quantified by Western blotting using a rabbit polyclonal antibody against MKP-1 (C-19: Santa Cruz Biotechnology, Santa Cruz, CA), compared to α-tubulin used as the loading control (mouse monoclonal IgG_1, DM1A: Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibodies were detected with goat anti-mouse or anti-rabbit HRP-conjugated secondary antibodies (Cell Signaling Technology) and visualized by enhanced chemiluminescence (PerkinElmer, Wellesley, MA).

2.3. Real-time RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen Australia, Doncaster, VIC, Australia) and reverse transcription was performed by using the RevertAid First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Hanover, MD) as per the manufacturer's protocol.

MKP-1 mRNA levels were measured using real-time RT-PCR on an ABI Prism 7500 (Applied Biosystems, Foster City, CA) with the DUSP1 (Hs00610256_g1) TaqMan® Gene Expression Assay and the eukaryotic 18S rRNA endogenous control probe (Applied Biosystems) subjected to the following cycle parameters: 50 °C for 2 min, 1 cycle; 95 °C for 10 min, 1 cycle; 95 °C for 15 s, 60 °C for 1 min, 40 cycles.

2.4. ELISAs

Cell supernatants were collected and stored at −20 °C for later analysis by ELISA. Interleukin 6 (IL-6) ELISAs were performed according to the manufacturer's instructions (BD Biosciences Pharmingen, San Diego, CA).

2.5. MKP-1 overexpression

ASM cells were transiently transfected with the MKP-1 expression vector pCMV-Flag-MKP-1 [20] generously provided by Andrew R. Clark (Kennedy Institute of Rheumatology, Imperial College London) using the methods established in our previous publication [21]. Cells were stimulated with S1P (1 μM) for 24 h before supernatants were removed for IL-6 protein measurement by ELISA.

2.6. Statistical analysis

Statistical analysis was performed using the Student's unpaired *t* test. *P* values <0.05 were sufficient to reject the null hypothesis for all analyses.

3. Results

3.1. Temporal activation of MAPKs and upregulation of MKP-1 by S1P

To demonstrate the temporal activation of MAPK family members by S1P and the subsequent upregulation of MKP-1 protein, growth-arrested ASM cells were treated with S1P for up to 1 h and MAPK phosphorylation was quantified by Western blotting. As shown in Fig. 1A, S1P stimulates a rapid, but transient, activation of all three MAPK family members within 5 min of stimulation with S1P. Although the temporal kinetics of MAPK phosphorylation can vary slightly between primary cultures, in general MAPK phosphorylation appears to peak at 10–30 min before returning to baseline levels by 60 min. Interestingly, the decline in MAPK activation is mirrored by upregulation of MKP-1 protein. This timing is suggestive of a negative feedback mechanism in human ASM cells, recently identified for TNFα-induced MKP-1 [18] whereby inflammatory stimuli activate kinases (MAPKs) as well as their own phosphatases (MKP-1), in order to regulate the extent and duration of signal transduction.

To determine the MAPK responsible for S1P-induced upregulation of MKP-1 we performed a series of experiments where ASM cells were pretreated with pharmacological inhibitors of p38 MAPK, ERK, and JNK signaling pathways and the resultant effect on S1P-induced MKP-1 upregulation was assessed by Western blotting. As shown in Fig. 1B, pretreatment with the p38 MAPK inhibitor SB203580 inhibited S1P-induced p38 MAPK phosphorylation at 30 min and correspondingly repressed MKP-1 protein upregulation at both 30 and 60 min. Although PD98059 and SP600125 were effective inhibitors of ERK and JNK phosphorylation at 30 min, the protein levels of MKP-1 observed after stimulation with S1P were not repressed by either inhibitor (Fig. 1B). These results suggest that S1P induces upregulation of MKP-1 in a p38 MAPK-mediated manner and this was confirmed by densitometric analysis (Fig. 1C).

3.2. MKP-1 expression is cAMP/CREB-responsive and S1P induces CREB phosphorylation to upregulate MKP-1 expression via an adenylate cyclase/PKA/CREB-mediated pathway

S1P acts on the S1P receptor family to stimulate myriad cell signaling pathways [9–13]. A number of S1P receptors (i.e. $S1P_2$ and $S1P_3$) are coupled to adenylate cyclase [10,11]. In this way, S1P stimulation increases the important second messenger cAMP. In 2001 [3] we demonstrated that S1P acts via S1P receptors to increase cAMP levels in ASM cells. As cAMP activates CREB signaling pathways and MKP-1 is a CREB-responsive gene [22], we now wish to ascertain the involvement of the adenylate cylase/cAMP-mediated cellular signaling pathway in the upregulation of MKP-1 in ASM cells.

Firstly, to show that adenylate cyclase stimulation upregulates MKP-1 in ASM cells, we pretreated cells for 30 min with the adenylate cyclase activator – forskolin – and then examined MKP-1 mRNA and protein expression in the absence and presence of S1P treatment

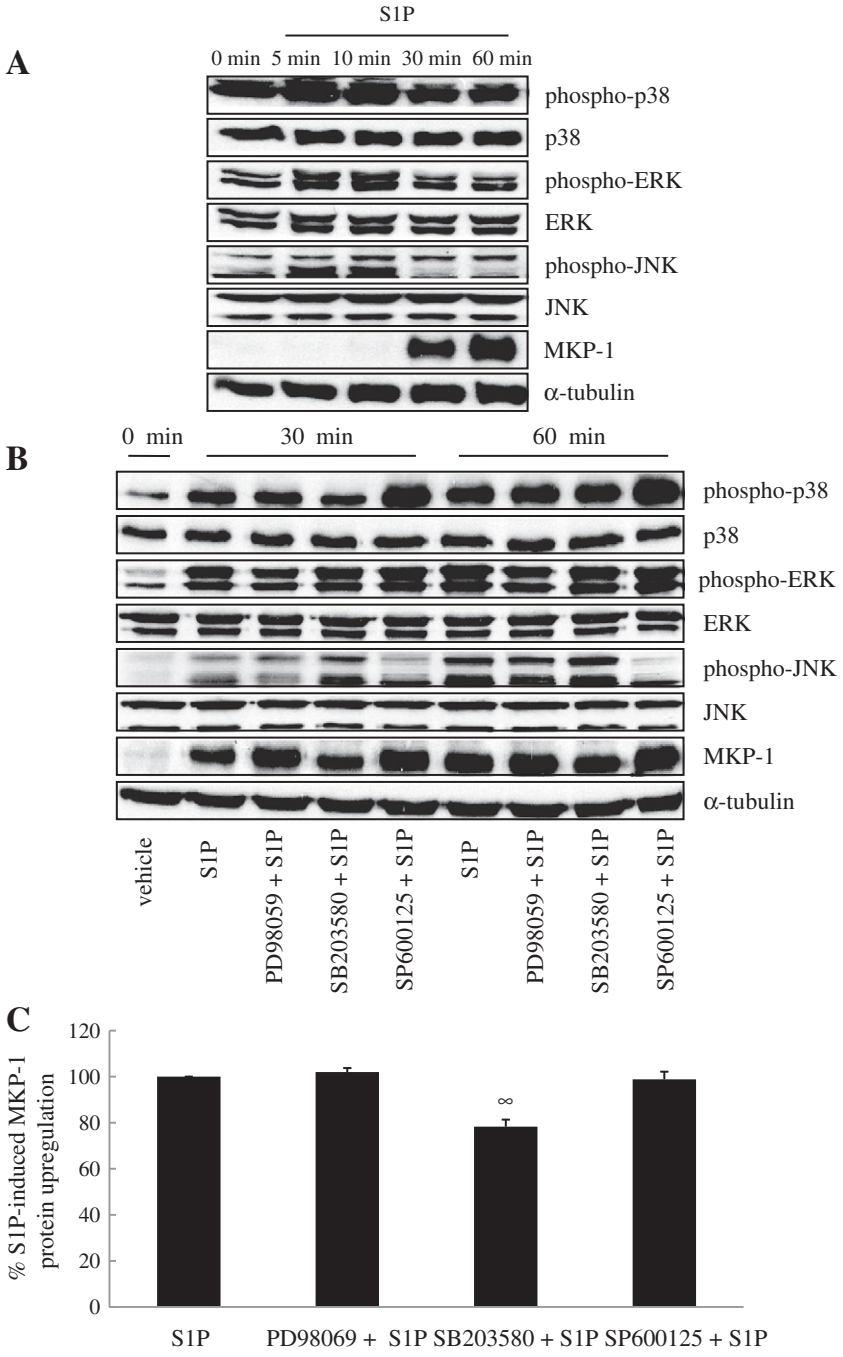

Fig. 1. Temporal activation of MAPKs and upregulation of MKP-1 by S1P. (A) Growth-arrested ASM cells were stimulated with S1P (1 μM) for 0, 5, 10, 30 and 60 min. (B) Growth-arrested ASM cells were pretreated for 30 min with vehicle, or the MAPK inhibitor PD98059 (10 μM), SB203580 (1 μM) or SP600125 (10 μM), then stimulated with S1P for 30 min and 60 min. Western blotting was performed using specific antibodies against phosphorylated (Thr^{180}/Tyr^{182}) and total p38 MAPK, phosphorylated (Thr^{202}/Tyr^{204}) and total ERK, phosphorylated (Thr^{183}/Tyr^{185}) and total JNK, and MKP-1. α-Tubulin was used as the loading control. (A, B) Results are representative Western blots from n = 5 primary ASM cell lines and (C) demonstrates densitometric analysis where results are expressed as % S1P-induced MKP-1 protein upregulation at 60 min. Statistical analysis was performed using the Student's unpaired *t* test where ∞ denotes significant inhibition by SB203580 ($P<0.05$) (mean + SEM values from n = 5 primary ASM cell lines).

for 60 min (Fig. 2A and B). As shown in Fig. 2A, forskolin alone significantly upregulated MKP-1 mRNA expression in ASM cells by 26.8.0 ± 3.0-fold, compared to vehicle-treated cells ($P<0.05$). Interestingly, forskolin and S1P treatment in combination had an additive effect on MKP-1 mRNA expression in ASM cells. As demonstrated in Fig. 2A, forskolin pretreatment significantly increased S1P-induced mRNA expression from 19.6 ± 3.0-fold to 47.6 ± 4.2-fold ($P<0.05$). Adenylate cyclase activation also upregulated MKP-1 protein in similar manner; as shown in Fig. 2B, forskolin treatment alone increased MKP-1 and enhanced S1P-induced upregulation.

Secondly, to demonstrate that cAMP increases MKP-1 expression in ASM cells, we treated cells for 1 h with a cell-permeable cAMP analogue — dibutyryl cAMP. As shown in Fig. 2C, dibutyryl cAMP significantly increased MKP-1 mRNA levels by 3.3 ± 0.2-fold, compared to vehicle-treated cells ($P<0.05$). This was associated with CREB activation, as dibutyryl cAMP increased CREB Ser^{133} phosphorylation (at 30 min) with a subsequent increase in MKP-1 protein upregulation after 60 min stimulation with dibutyryl cAMP (Fig. 2D).

Because we demonstrated that MKP-1 expression is adenylate cyclase/cAMP/CREB-responsive in ASM cells we now wished to

examine the temporal kinetics of CREB phosphorylation and subsequent MKP-1 upregulation after stimulation with S1P. As demonstrated in Fig. 2E, S1P robustly and potently induces CREB phosphorylation (at Ser^{133}) as early as 5 min and this activation was sustained for the 60 min time period examined. Fig. 2E also shows S1P-induced MKP-1 upregulation. To link S1P-induced activation of the CREB-signaling pathway with MKP-1 upregulation we pretreated cells with H-89, a pharmacological inhibitor known to attenuate activity of protein kinase A (PKA) — a key kinase responsible for phosphorylation of CREB at Ser^{133}. Although H-89 is relatively non-specific, our results suggest that S1P induces CREB phosphorylation to upregulate MKP-1 expression via a PKA/CREB-mediated pathway. As shown in Fig. 2F, H-89

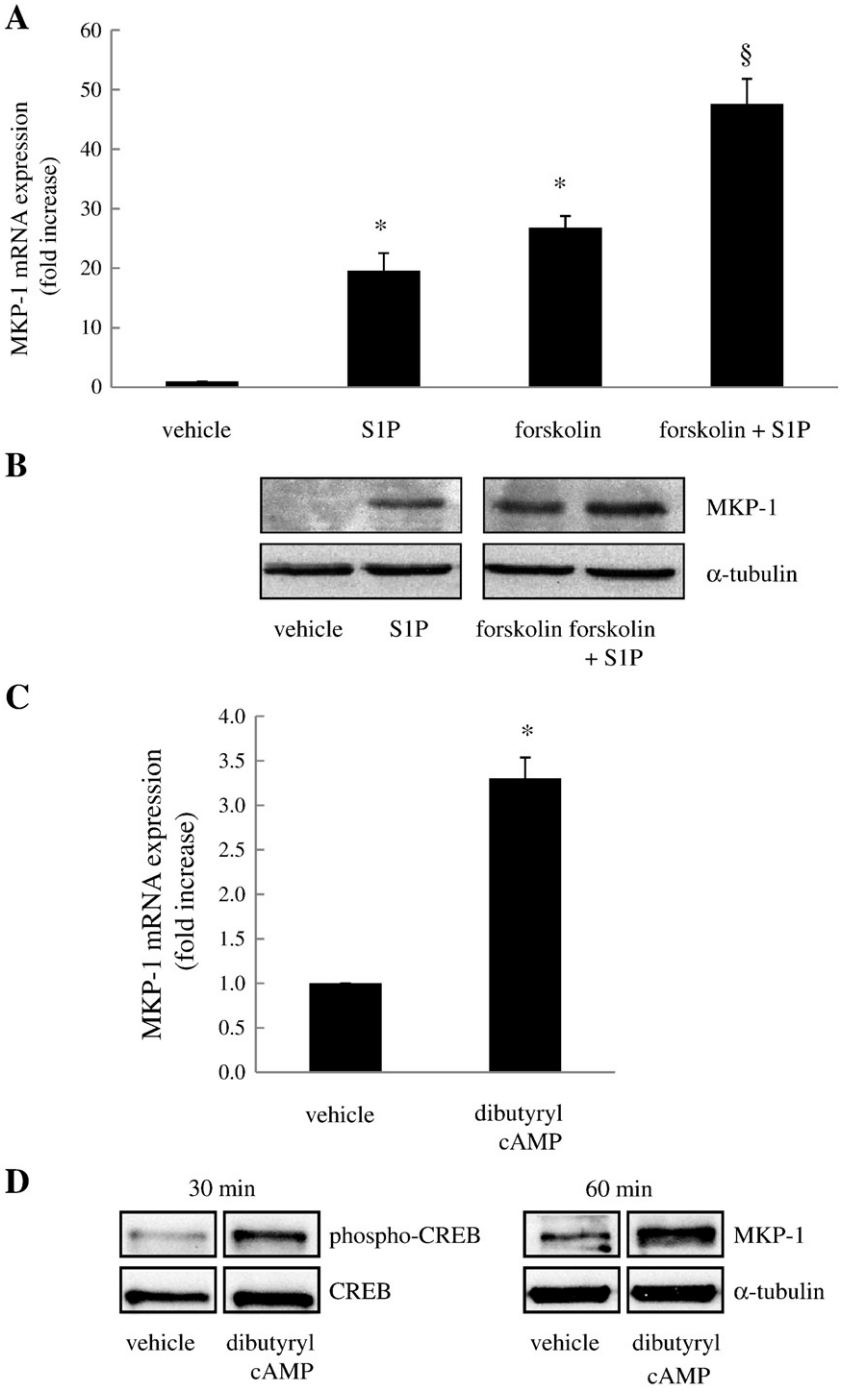

Fig. 2. MKP-1 expression is cAMP/CREB-responsive and S1P induces CREB phosphorylation to upregulate MKP-1 expression via an adenylate cyclase/PKA/CREB-mediated pathway. (A, B) Growth-arrested ASM cells were pretreated for 30 min with vehicle or forskolin (10 μM), then treated with vehicle or S1P for 60 min. (C, D) Growth-arrested ASM cells were treated with vehicle or dibutyryl cAMP (1 mM) for 30 and 60 min. (E) Growth-arrested ASM cells were stimulated with S1P (1 μM) for 0, 5, 10, 30 and 60 min. (F) Growth-arrested ASM cells were pretreated for 60 min with vehicle or H-89 (10 μM), then treated with vehicle or S1P for 60 min. Dibutyryl cAMP was assessed in parallel. (A, C) MKP-1 mRNA expression was quantified by real-time RT-PCR and results were expressed as fold increase compared to vehicle-treated cells at 60 min. Statistical analysis was performed using the Student's unpaired *t* test where * denotes a significant increase in MKP-1 mRNA expression and § denotes significant upregulation of S1P-induced MKP-1 mRNA expression by forskolin ($P<0.05$) (mean + SEM values from n = 3 primary ASM cell lines). (B, D, E, F) CREB phosphorylation at Ser^{133} (compared to total CREB) and/or upregulation of MKP-1 protein (compared to α-tubulin as a loading control) was quantified by Western blotting, where (B, D, E, F) illustrate representative Western blots of n = 3 primary ASM cell lines.

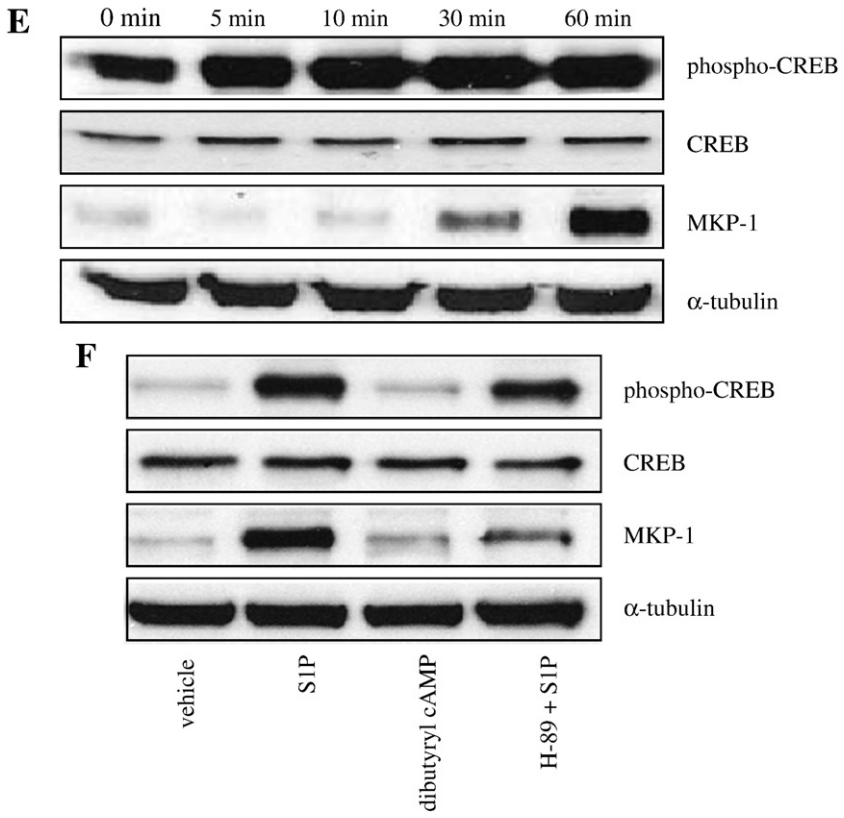

Fig. 2 (*continued*).

suppressed S1P-induced CREB phosphorylation resulting in reduced MKP-1 upregulation at 60 min.

3.3. S1P increases MKP-1 expression via parallel p38 MAPK- and CREB-mediated pathways

Taken together our data shows that S1P-induced MKP-1 upregulation is associated with activation of both p38 MAPK- and CREB-mediated pathways. In cell types apart from ASM, CREB activation has been demonstrated as downstream of p38 MAPK. Thus, two possible explanations exist: (i) S1P-induces CREB phosphorylation in a p38 MAPK-dependent manner; (ii) S1P induces MKP-1 expression via parallel p38 MAPK- and CREB-mediated pathways. To exclude the former possibility we pretreated ASM cells with the p38 MAPK inhibitor SB203580 and examined the effect on S1P-induced p38 MAPK and CREB phosphorylation. As shown in Fig. 3A, SB203580 effectively blocked S1P-induced p38 MAPK phosphorylation. In contrast, S1P-induced CREB activation was unaffected by pretreatment with the p38 MAPK inhibitor. These results are in support of parallel p38 MAPK and CREB-mediated pathways underlying S1P-induced MKP-1 upregulation.

To confirm this assertion, we pretreated ASM cells with inhibitors of the p38 MAPK and PKA/CREB-mediated pathways alone and in combination and measured the effects on S1P-induced MKP-1 mRNA expression and protein upregulation. As shown in Fig. 3B, blocking the p38 MAPK pathway significantly inhibited MKP-1 mRNA expression, reducing the level of MKP-1 stimulated by S1P from 16.8 ± 2.5-fold to 10.3 ± 0.8-fold when cells were pretreated with SB203580 ($P<0.05$). Inhibiting PKA with H-89 also effectively repressed S1P-induced MKP-1 mRNA (to 9.3 ± 0.8-fold: $P<0.05$). Importantly, when both inhibitors were added in combination there was a significantly greater degree of inhibition than achieved with either inhibitor added alone (6.6 ± 0.3-fold; $P<0.05$). The effect of the inhibitors on S1P-induced MKP-1 protein (Fig. 3C and D) was in accord with the mRNA results. Densitometric analysis revealed that the combination of both inhibitors repressed S1P-induced MKP-1 protein upregulation by 44.6 ± 2.3% and had a significantly greater repressive effect than either inhibitor alone (Fig. 3D: $P<0.05$). Taken together our data indicates that S1P activates dual p38 MAPK-and CREB-mediated signaling pathways to induce MKP-1 expression.

3.4. MKP-1 is a negative feedback effector that represses S1P-induced p38 MAPK and ERK-mediated signaling and IL-6 secretion

We have previously reported that S1P induces secretion of the cytokine IL-6 from ASM cells [3]. To determine whether MAPK-mediated signaling pathways contribute to S1P-induced IL-6 secretion ASM cells were pretreated with inhibitors of the p38 MAPK, ERK, and JNK pathways prior to stimulation with S1P, using SB203580, PD98059, and SP600125, respectively. As shown in Fig. 4A, inhibition of p38 MAPK and ERK significantly inhibited S1P-induced IL-6 secretion ($P<0.05$). There was no effect of JNK inhibition. These results suggest that S1P-induced IL-6 secretion from ASM cells is p38 MAPK- and ERK-dependent.

To confirm that S1P temporally regulates MAPK signaling pathways by upregulating the negative feedback controller – MKP-1 – we blocked MKP-1 upregulation with the pharmacological inhibitor, triptolide [18,23,24] and observed the effect on p38 MAPK and ERK phosphorylation. As demonstrated in Fig. 4B, triptolide pretreatment completely repressed S1P-induced MKP-1 protein. Where we had previously demonstrated that MAPK phosphorylation declines at 30 and 60 min (also see Fig. 1A), in the absence of MKP-1 p38 MAPK and ERK phosphorylation continues in an unrestrained manner. In this way, MKP-1 controls the extent and duration of S1P-induced signaling and has a significant impact on cytokine secretion from ASM cells. This is demonstrated in Fig. 4C, where S1P-induced IL-6 secretion from ASM cells was significantly repressed in cells that had been transfected with an MKP-1 overexpression vector, compared to cells transfected with vector control ($P<0.05$). Taken together, our results suggest that S1P-induced MKP-1 serves to restrain

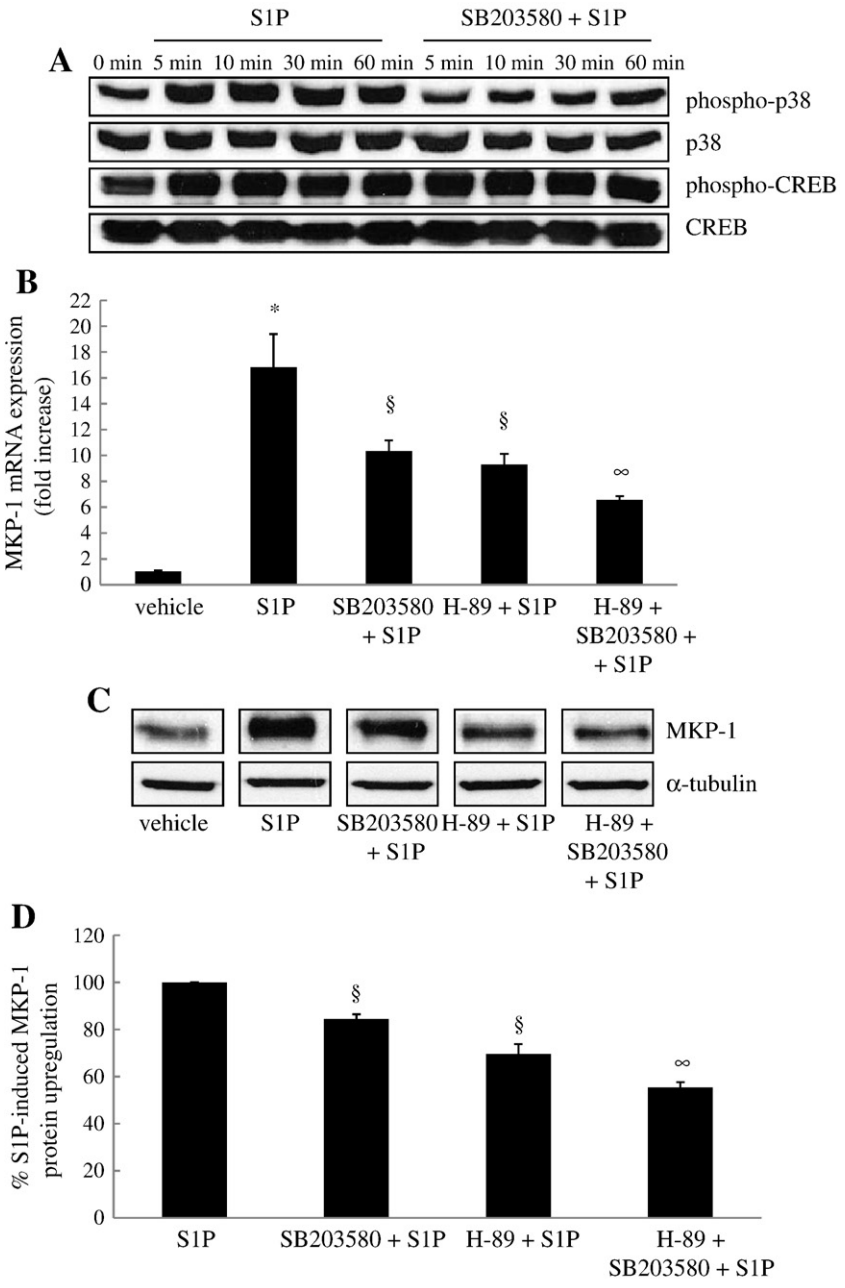

Fig. 3. S1P increases MKP-1 expression via parallel p38 MAPK- and CREB-mediated pathways. (A) Growth-arrested ASM cells were pretreated for 30 min with vehicle or 1 μM SB203580, prior to stimulation with S1P (1 μM) for 0, 5, 10, 30 and 60 min. (B, C, D) Growth-arrested ASM cells were pretreated for 60 min with vehicle or H-89 (10 μM), or for 30 min with 1 μM SB203580, prior to stimulation with S1P (1 μM) for 60 min, compared to vehicle control. (A, C, D) p38 phosphorylation at Thr^{180}/Tyr^{182} (compared to total p38 MAPK), CREB phosphorylation at Ser^{133} (compared to total CREB), or upregulation of MKP-1 protein (compared to α-tubulin as a loading control) was quantified by Western blotting, where (A, C) illustrate representative Western blots and (D) demonstrates densitometric analysis where results are expressed as % S1P-induced MKP-1 protein upregulation at 60 min (mean + SEM values from n = 4 primary ASM cell lines). (B) MKP-1 mRNA expression was quantified by real-time RT-PCR and results were expressed as fold increase compared to vehicle-treated cells at 0 min (mean + SEM values from n = 3 primary ASM cell lines). Statistical analysis was performed using the Student's unpaired *t* test where * denotes a significant increase in MKP-1 mRNA expression, § denotes significant inhibition by SB203580 or H-89, and ∞ indicates a significant effect of H89 and SB203580 in combination, compared to either inhibitor added alone ($P<0.05$).

pro-inflammatory MAPK signaling and synthetic function of ASM cells.

4. Discussion

S1P is an important pathophysiological mediator found elevated in human asthma that drives the development of a pro-inflammatory and pro-remodeling phenotype in the airway. Recent research has highlighted the prospect of targeting S1P as potential therapy and in the current study we illustrate the endogenous anti-inflammatory mechanisms that are activated by S1P itself to exert regulatory control over cellular signaling. We discover that S1P, like other stimuli, upregulates the phosphatase MKP-1 that acts in a negative feedback manner to repress MAPK-mediated pathways and cytokine secretion in ASM cells. In this way, MKP-1 serves as an important negative feedback mechanism to limit the pro-inflammatory response to S1P in airway cells.

In 2001 [3] we demonstrated that S1P modulates ASM functions that promote inflammation and remodeling in asthma. We demonstrated that cAMP levels are increased via activation of the G_s-coupled

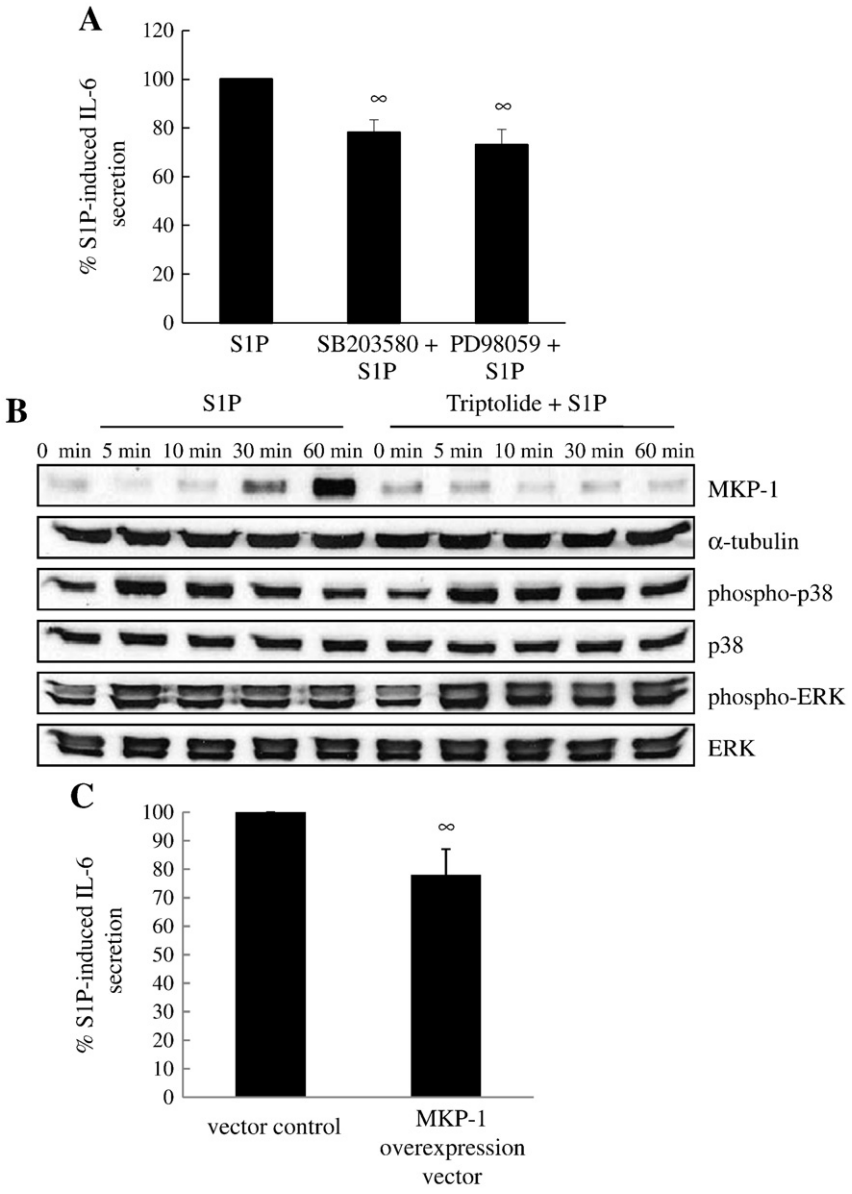

Fig. 4. MKP-1 is a negative feedback effector that represses S1P-induced p38 MAPK and ERK-mediated signaling and IL-6 secretion. (A) Growth-arrested ASM cells were pretreated for 30 min with vehicle, 1 μM SB203580, or 10 μM PD98059 to inhibit p38 MAPK and ERK, respectively, then stimulated with 1 μM S1P for 24 h. IL-6 secretion was measured by ELISA. Data are expressed as % S1P-induced IL-6 secretion (where IL-6 protein secreted in response to 1 μM S1P was 1711.7 ± 193.6 pg/ml (data are mean ± SEM)). Statistical analysis was performed using the Student's unpaired *t* test, where ∞ denotes significant inhibition ($P<0.05$). Data are mean + SEM values from n = 11 primary ASM cell lines. (B) Growth-arrested ASM cells were pretreated for 30 min with vehicle or the MKP-1 inhibitor triptolide (1 μM) prior to stimulation with S1P (1 μM) for 0, 5, 10, 30 and 60 min. Western blotting was performed using specific antibodies against MKP-1 (compared to α-tubulin used as the loading control), phosphorylated (Thr^{180}/Tyr^{182}) and total p38 MAPK, phosphorylated (Thr^{202}/Tyr^{204}) and total ERK. Results are representative Western blots from n = 3 primary ASM cell lines. (C) ASM cells were transiently transfected with a MKP-1 expression vector, or empty vector control, stimulated for 24 h with S1P (1 μM), before IL-6 secretion was measured by ELISA. Data are expressed as % S1P-induced IL-6 secretion in cell transfected with empty vector alone and statistical analysis was performed using the Student's unpaired *t* test, where ∞ denotes significant inhibition ($P<0.05$). Data are mean + SEM values from n = 6 primary ASM cell lines.

S1P receptors and downstream adenylate cyclase and that S1P induces IL-6 secretion. We now extend these studies to show that S1P rapidly and robustly stimulates CREB phosphorylation at Ser^{133}. Moreover, all members of the MAPK family are activated by S1P in a temporally distinct manner, but only p38 MAPK and ERK in particular contribute to S1P-induced IL-6 secretion. In cell types apart from ASM, the MAPK deactivator MKP-1 has been reported to be induced by CREB- and MAPK-mediated pathways [25–27]. Thus, in this paper we report that S1P upregulates MKP-1, illustrate the underlying molecular mechanisms responsible and highlight the impact on cytokine secretion from ASM cells.

MKP-1 is the archetypal member of the MKP family, also known as DUSPs (dual-specificity phosphatases). MKPs direct dual dephosphorylation of MAPKs in a cell-type specific manner. MAPK-mediated cellular function is regulated by both strength and duration of MAPK phosphorylation. This signaling must be strictly controlled to modulate functional outcome. This crucial negative feedback control is achieved by the MKPs, as they are immediate-early genes [28] known to be capable of rapid expression and upregulation. We show that S1P rapidly and robustly upregulates both MKP-1 mRNA expression and protein upregulation within 1 h after stimulation; although the resultant MKP-1 upregulation is transient and returns to baseline levels by 2 h (data not shown). Dual pathways are responsible for S1P-induced MKP-1 expression in ASM cells. Upregulation was shown, in part, to be cAMP-mediated and occurred via the PKA/CREB pathway. Notably, the human MKP-1 promoter is

known to contain two *cis*-acting cAMP response elements [25,26]. CREB-mediated upregulation occurred in parallel with p38 MAPK-mediated expression, as pharmacological inhibition of the p38 MAPK-mediated pathway with SB203580 attenuated S1P-iduced MKP-1 gene expression and protein expression; confirming earlier studies in alveolar macrophages [29] and our recent study in ASM cells [18].

Therefore, in this paper we have underscored the important negative feedback role played by MKP-1 and determined that S1P concomitantly stimulates both inflammatory (CREB and MAPKs) and the anti-inflammatory (phosphatase MKP-1) to control synthetic function in ASM cells. Thus, S1P, like other inflammatory stimuli in ASM cells (e.g. TNFα: (20)), induces MKP-1 to serve as a negative feedback effector. We show for the first time that S1P-induced IL-6 secretion is mediated by p38 MAPK- and ERK-dependent pathways and observe that p38 MAPK and ERK phosphorylation continues in an unrestrained fashion when MKP-1 is not present to "switch them off" at the right time. Moreover, MKP-1 overexpression restrains S1P-induced cytokine secretion. In summary, in this manuscript we have demonstrated that the MAPK responsible for S1P-induced MKP-1 upregulation is p38 MAPK. Once upregulated, MKP-1 can then serve as a MAPK deactivator and can dephosphorylate members of the MAPK family in a cell-type and species-specific manner. Thus, in this way, the identity of the MAPKs responsible for MKP-1 upregulation and those MAPKs that can be dephosphorylated can differ.

Collectively, our results provide further support for the important negative feedback role played by the endogenous anti-inflammatory protein – MKP-1 – in human ASM cells. By inducing MKP-1 expression via parallel p38 MAPK- and CREB-mediated pathways, the biologically active sphingolipid S1P controls the extent and duration of pro-inflammatory cellular signaling and regulates ASM synthetic function. This raises the possibility that this inherent feedback loop via MKP-1 activation might be exploited to restrict S1P action in asthma in the future.

Acknowledgements

This research was supported by PhD scholarships to MM (Senglet Stiftung — Switzerland) and to MMR (Endeavour Postgraduate Award – Australia), and funded by a project grant (570856) from the National Health and Medical Research Council of Australia and the University of Sydney Thompson Fellowship to AJA. The authors wish to thank Andrew R. Clark (Kennedy Institute of Rheumatology, London) for the MKP-1 expression vector and our colleagues in the Respiratory Research Group at the University of Sydney. We acknowledge the collaborative effort of the cardiopulmonary transplant team and the pathologists at St Vincent's Hospital, Sydney, and the thoracic physicians and pathologists at Royal Prince Alfred Hospital, Concord Repatriation Hospital and Strathfield Private Hospital and Healthscope Pathology, Sydney.

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