Cellular Signalling 22 (2010) 1727–1733

Contents lists available at ScienceDirect

Cellular Signalling

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

Dual action of sphingosine 1-phosphate in eliciting proinflammatory responses in primary cultured rat intestinal smooth muscle cells

Mihaela Gurgui [a], Roland Broere [a], Jörg C. Kalff [b], Gerhild van Echten-Deckert [a,*]

[a] Kekulé-Institute for Organic Chemistry and Biochemistry, University Bonn, Germany
[b] Dept. of Surgery, University Bonn, Germany

ARTICLE INFO

Article history:
Received 27 January 2010
Received in revised form 23 June 2010
Accepted 26 June 2010
Available online 16 July 2010

Keywords:
Sphingosine 1-phosphate
Inflammation
Intestinal smooth muscle cells
Egr-1
STAT3

ABSTRACT

Sphingosine 1-phosphate (S1P) is involved in the local inflammatory response within the intestinal muscularis, which has been suggested to play a major role in the pathogenesis of postoperative ileus. The aim of the present study was to elucidate the role of S1P and the molecular mechanisms underlying regulation of inflammatory mediators in primary cultured rat intestinal smooth muscle (RISM) cells. Although our experimental data clearly show the mediatory role of sphingosine kinase (SK)-derived S1P in the TNF-α and the LPS induced activation of NF-kB, exogenously added S1P failed to trigger this transcription factor. Instead, exogenous S1P induced early growth response-1 (Egr-1), which was reported to play a proinflammatory role in postoperative ileus. Using RNA interference we found that Egr-1 is required primarily for S1P-induced expression of IL-1 and COX2. Conversely, IL-6 expression following S1P treatment was mediated by STAT3 (signal transducer and activator of transcription-3). In addition our data indicate that the proinflammatory effect of S1P is mediated by its receptors $S1P_{1-3}$ and requires activation of MAP-kinases.
In conclusion, Egr-1 and STAT3 cooperatively mediate S1P-induced inflammatory responses in RISM cells, providing novel targets for attenuation of postoperative ileus.

1. Introduction

Sphingosine 1-phosphate (S1P), which was intensively studied for the past several years, has emerged as an important mediator of a variety of cellular processes including cell growth, survival, angiogenesis and inflammation [1]. There is convincing experimental evidence that S1P is a lipid signalling molecule with dual function. On the one hand, it acts extracellularly by binding to five specific G protein-coupled receptors (GPCRs), designed $S1P_{1-5}$ [2]. On the other hand, it appears to act as an intracellular second messenger independent of its receptors [3]. However, direct intracellular targets of S1P are still elusive. Recent and extensive work has established the involvement of S1P in regulating intracellular events in cytokine signalling and in the induction of inflammatory mediators. In certain cells, such as human umbilical vein endothelial cells [4], hepatocytes [5], and L929 fibroblasts [6] TNF-α has been found to stimulate the production of S1P upon activation of sphingosine kinase (SK). A direct binding between SK1 and TRAF2 has been shown and is necessary for TNF-α induced NF-kB activation, long known to be a key transcription factor in COX2 gene induction [4]. However, the transcription factors that regulate inflammatory gene expression in response to exogenous S1P are poorly defined.

Early growth response-1 (Egr-1) (also known as Zif-268, NGF1-A, Krox-24 or TIS8), a zinc-finger transcription factor, belongs to a large family of early response genes discovered about 15 years ago. Egr-1 is the prototypic member of this family that is induced by a number of stimuli in distinct cell types [7]. Egr-1 was initially identified as an immediate-early gene rapidly activated by serum in fibroblasts [8] and by nerve growth factor in PC12 cells [9]. Subsequently several studies have demonstrated that Egr-1 is a master regulator that plays a key role in a variety of acute and chronic pathological processes such as inflammation, arteriosclerosis, ischemia–reperfusion injury, angiogenesis, smooth muscle hyperplasia and hypertrophy [10]. Egr-1 is rapidly and transiently expressed in response to a variety of inflammatory conditions and in turn induces the expression of other inflammatory mediators including IL-6, TNF-α, ICAM, MCP-1, IL-1β and PAI-1 [10,11]. Recently Egr-1 was reported to play a critical role in the inflammatory responses that accompany postoperative ileus, which occurs after abdominal surgery [12]. We have shown that S1P is involved in postoperative ileus by inducing the expression of some inflammatory mediators such as, COX2, IL-6, and IL-1 and the production of PGE_2 in primary cultured rat intestinal smooth muscle (RISM) cells [13,14].

In the present study, we confirmed that SK/S1P mediates TNF-α-induced NF-kB translocation. However, exogenous S1P failed to activate NF-kB. Conversely, our findings indicate that the proinflammatory

* Corresponding author. Kekulé-Institut für Organische Chemie und Biochemie der Universität Bonn, Gerhard-Domagk-Strasse 1, 53121 Bonn, Germany. Tel.: +49 228 732703; fax: +49 228 734845.
E-mail address: g.echten.deckert@uni-bonn.de (G. van Echten-Deckert).

0898-6568/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.cellsig.2010.06.013

effect of exogenous S1P is receptor dependent and mediated by Egr-1 and STAT3.

2. Materials and methods

2.1. Materials

Male Sprague–Dawley rats (200–250 g) were obtained from Harlan Winkelman, (Borchen, Germany). S1P, PTX, and PD98059 were purchased from Sigma (Taufkirchen, Germany). [γ-^{32}P]-ATP was from Amersham-Buchler (Braunschweig, Germany). D-erythro-N,N dimethylsphingosine (DMS) was obtained from Biomol (Hamburg, Germany). Cucurbitacin I (JSI-124) was provided by Calbiochem (Darmstadt, Germany). Dulbecco's modified Eagle's medium-F12 (DMEM-F12), Minimal Essential Medium, Opti-MEM, trypsin, fetal bovine serum, bovine serum albumin (BSA), Tumor Necrosis Factor-alpha (TNF-α), Hoechst 33342 and goat anti-mouse IgG, goat anti-rabbit IgG both labelled with green fluorescent Alexa Fluor 488 dye, lipofectamineRNAiMAX were obtained from Invitrogen (Karlsruhe, Germany). Primary antibodies against NF-kB, Egr-1 were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Antibodies directed against phosphorylated and unphosphorylated forms of STAT3, respectively, were from NEB (Frankfurt, Germany). IL-6 ELISA kit was purchased from Biosource (Solingen, Germany). RNeasy Mini Kit was purchased from Qiagen (Hilden, Germany). 6 channel μ-slides for immunofluorescence were obtained from Ibidi (Martinsried, Germany).

2.2. Isolation of cultured rodent intestinal smooth muscle cells

Intestinal muscularis was isolated as described previously [15] and then cut into small pieces, washed twice in Hank's balanced salt solution (HBSS) and incubated with 0.6 mg/ml type II collagenase (Worthington Biochemical Corporation) and 2 U/ml dispase II (LaRoche) in HBSS for 45 min at 37 °C in a shaking water bath. Muscle cells dispersed from the muscularis layer were harvested by filtration using a 500 μm Nitex mesh and centrifuged at 300×*g* for 5 min. Cells were resuspended and washed twice by centrifugation at 300×*g* for 5 min. After resuspension, 3 ml of Accuspin was added carefully prior to density gradient centrifugation (20 min, 800×*g*, 4 °C). Two phases and a pellet containing muscle cells were obtained. The pellet was resuspended in DMEM-F12 containing 10% foetal bovine serum and 1% penicillin/ streptomycin. Cultured cells were incubated in a 5% CO_2 environment at 37 °C. DMEM-F12 medium was replaced every three days until the cells reached confluence. Prior to trypsin/EDTA treatment (0.05%/0.02%, 2 min) cells were washed two times with phosphate buffered saline (PBS). The trypsin activity was neutralized by the addition of excess serum-containing DMEM-F12. The resulting cell suspension was centrifuged at 300×*g* for 5 min. The cell pellet was resuspended in DMEM-F12 and plated in a 1:3–1:5 ratio. Studies were performed in cells after four-six passages.

2.3. Sphingosine kinase activity assay

Sphingosine kinase activity was measured as described previously [16]. Briefly, cells were resuspended in ice-cold 0.1 M phosphate buffer (pH 7.4) containing 20% glycerol, 1 mM mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 15 mM sodium fluoride, 10 μg/ml leupeptin and aprotinin, respectively 1 mM phenylmethylsulfonyl fluorid, and 0.5 mM 4-deoxypyridoxine. Cells were disrupted using a Retsch mixer-mill MM400. Each sample was assayed for sphingosine kinase activity by incubation with 50 μM sphingosine and [γ^{32}P] ATP (1–2 μCi, 20 mM) for 30 min at 37 °C. Reactions were stopped by adding 20 μl of 1 N HCl followed by 0.8 ml of chloroform/methanol/HCl (100:200:1, v/v/v). After vigorous vortexing, 240 μl of chloroform and 240 μl of 2 N KCl were added and the phases were separated by centrifugation. Lipid-containing organic phase was resolved by TLC on silica gel G60 in 1-butanol/methanol/acetic acid/water (80:20:10:20, v/v/v/v). ^{32}P-labelled lipids were visualized by phosphoimaging (bio-imaging analyzer Fujix bas 1000). The radioactive spots corresponding to S1P were identified by their R_F value and quantified using software Tina 2.09.

2.4. ELISA

Secreted IL-6 was assayed using the rat IL-6 ELISA kit according to manufacturer's instructions and measured at 412 nm.

2.5. RNA isolation

Isolation of total RNA from RISM cells was performed using RNeasy mini kit from Qiagen according to manufacturer's protocol. Integrity and size distribution of total RNA were checked by denaturing agarose gel electrophoresis and ethidium bromide staining.

2.6. Quantitative real-time PCR

Total RNA from RISM cells was reversely transcribed using the SuperScript III First-Stand Synthesis System for RT-PCR with Random Primers (Invitrogen) according to manufacturer's instructions and a PTC-200 Thermal Cycler (MJ Research). Quantitative real-time PCR was performed using the Light Cycler TaqMan Master kit (Roche) with a LightCycler 1.5 Instrument (Roche). Detection of amplified cDNA was performed with Universal Probe Library probes (Roche, see below). These probes are pre-designed polynucleotides labelled with fluorescein at the 5′-end and with a dark quencher dye near the 3′-end. Primer design and determination of the corresponding probe was carried out with Probe Finder software (Roche). The following gene specific primers and probes, respectively, were used: IL-6, 5′-GGAACAGCTATGAAGTTTCTCTCC-3′ (forward), 5′-GTGGGTGGTATCCTCTGTGAA-3′ (reverse), probe#55; IL-1, 5′-AGTTTCAATCAGCCCTTTACTGA-3′ (forward), 5′-CTGGGTTGGATGGTCTCTTC-3′ (reverse), probe #85; SK1, 5′-GCTGGCAGCTTCTTTGAACT-3′ (forward), 5′-CAGGAGATCTTCATTAGTGACCTG-3′ (reverse), probe#107; SK2, 5′-GTGGAGAATCGTGCAGAGG-3′ (forward), 5′-GCAGCAATTCAGGGGTGA-3′ (reverse), probe#108; Egr-1, 5′-ATGCGCAAGTACCCCAAC-3′ (forward), 5′-AACAGGGCAAGCATACGG-3′ (reverse), probe#107; COX2, 5′-CTACACCAGGGCCCTTCC-3′ (forward), 5′-TCCAGAACTTCTTTTGAATCAGG-3′ (reverse), probe#5; for normalization of the results 18S rRNA was used as housekeeping gene, 5′-AAATCAGTTATGGTTCCTTTGGTC-3′ (forward), 5′-GCTCTAGAATTACCACAGTTATCCAA-3′ (reverse), probe#55. Calculation of relative expression data was carried out with standard curve method.

2.7. Immunofluorescence

Expressions of NF-kB and Egr-1 were assessed by fluorescence microscopy (Nikon TE-2000 Eclipse, Tokyo, Japan). Cells were grown on 6 channel μ-slides for multiple immunofluorescence staining for up to 24 h. After appropriate stimulation cells were washed twice with PBS, fixed for 10 min at −20 °C with cold methanol and permeabilized for 1 min with 0.1% Triton X-100. Fixed cells were washed three times with PBS and blocked by incubation in PBS with 1% BSA for 30 min at 37 °C. Cells were incubated overnight at 4 °C with primary antibodies (polyclonal rabbit IgG for Egr-1: sc-189 and monoclonal mouse NF-kB p65: sc-8008), diluted in 1%BSA/PBS (1:300; 1:200 respectively). 6 channel μ-slides were washed three times with PBS and then incubated for 1 h at room temperature in dark with secondary antibody conjugated with green fluorescent Alexa Flour 488 dye diluted in 1% BSA/PBS (1:200). Hoechst 33342 dye was used to stain cell nuclei.

2.8. Immunoblot analysis

Western blot analysis following SDS-PAGE was performed as described before [13] using anti-phospho-STAT3 antibody and anti-STAT3 antibody, both diluted 1:1000.

2.9. Inhibition of SK1, SK2, Egr-1 and S1P receptors by small interfering RNA (siRNA)

In order to knock down SK1, SK2, Egr-1 and $S1P_{1-3}$ receptors, the following siRNA duplexes were used: SK1 siRNA se,UGUCACCCAUGAAUCUGCUGUCCCU; as, AGGGACAGCAGAUUCAUGGGUGACA; SK2 siRNA se, UCGAUUCACACUGGGUGCAGUGCUA; as, UAGCACUGCACCCAGUGUGAAUCGA from Invitrogen , (Karlsruhe Germany); Egr-1siRNA se, GUCGAAUCUGCAUGCGUAAdTdT; as, UUACGCAUGCAGAUUCAGCdTdT and Egr-1 siRNA se, GAAUCUGCAUGCGUAAUUUdTdT; as, AAAUUACGCAUGCAGAUUCdTdT; $S1P_1$ siRNA se, GGAAUUUAGCCGCAGCAAAdTdT; as, UUUGCUGCGGCUAAAUUCCdTdT; $S1P_2$ siRNA se CGACAUUUCUGGAGGGUAAdTdT; as, UUACCCUCCAGAAAUGUCGdTdT; $S1P_3$ siRNA se, GUAAGUCAAGCUCCAGUAAdTdT; as, UUACUGGAGCUUGACUUACdTdT from Sigma (Taufkirchen, Germany). RISM cells were transfected with 10 nM Egr-1 or 20 nM $S1P_{1-3}$, SK1 and SK2 siRNA duplexes, respectively, for 24 h using Lipofectamine RNAiMAX and Opti-MEM as recommended by the manufacturer. Then cells were treated as indicated. Knockdown of the target gene transcript was confirmed by quantitative real-time PCR.

2.10. Protein determination

Cell protein was quantified as described by Bradford [17] using BSA as a standard.

2.11. Presentation of data and statistics

All experiments were repeated at least twice with double determinations each. Data are expressed as means ± S.D. and normalized as indicated. Statistical analysis was performed using Student's *t* test.

3. Results

3.1. S1P acts as a second messenger in TNF-α-induced IL-6 production via the NF-kB-pathway

To determine whether SK and hence its catalytic product S1P are involved in TNF-α-induced IL-6 production, primary cultured RISM cells were incubated for 4 h with TNF-α (10 ng/ml) in the absence or presence of 10 μM of N,N dimetylsphingosine (DMS) a potent inhibitor of sphingosine phosphorylation [18]. As shown in Fig. 1A and B, elevated IL-6 expression at transcriptional and translational levels induced by TNF-α was suppressed by DMS, suggesting that SK activation is required for the expression of IL-6 in these cells. Similar results were obtained when cells were treated with LPS (not shown). As illustrated in Fig. 1D, catalytic activity of SK increased by 191% ± 19% in the presence of TNF-α. DMS completely reversed this effect of TNF-α. By contrast, knocking down SK1 and SK2 separately or simultaneously using siRNA did not affect TNF-α-induced IL-6 transcript amount (Fig. 1A). We therefore verified whether TNF-α affects transcription of SK1 and SK2 as previously suggested in a L929 fibroblast model [6]. As shown in Fig. 1C this was not the case, indicating that TNF-α induces elevation of SK activity via a posttranscriptional mechanism in RISM cells. Note that in these cells DMS also blocks TNF-induced NF-kB nuclear translocation (Fig. 1E), confirming previous findings in HEK cells that suggested a role of SK activity for TNF-α-induced nuclear translocation of NF-kB [4].

3.2. Exogenous S1P does not induce nuclear translocation of NF-kB but stimulates Egr-1 expression in RISM cells

The finding that SK activity and hence S1P mediate TNF-α-induced IL-6 expression via NF-kB raised the question whether exogenous S1P, shown to exert a proinflammatory action in intestinal muscle cells [13,14] is able to induce nuclear translocation of NF-kB as well. As depicted in Fig. 2A S1P failed to induce nuclear translocation and thus activation of NF-kB in RISM cells, indicating that the proinflammatory action of exogenous S1P is not mediated by this transcription factor. We therefore examined whether exogenous S1P affects the transcription factor Egr-1, which was recently suggested to be involved in postoperative ileus [12]. As shown in Fig. 2B, S1P induced a transient increase of Egr-1 mRNA level. The time course of S1P-induced Egr-1 expression revealed a maximum after 2 h following S1P addition and returned to basal level after 16 h (Fig. 2B). This effect was dose-dependent and observed already at 100 nM, reaching a maximum at 10 μM of S1P (Fig. 2C). Immunofluorescent staining experiments revealed a nuclear localization of Egr-1 only in the presence of S1P (Fig. 2D). As illustrated in Fig. 2D nuclear fluorescence is already visible after 30 min with heavily fluorescent nuclei after 1 and 2 h of treatment with S1P.

Preincubation of RISM cells with PD 98059, which has been previously shown to specifically inhibit ERK MAP kinase in these cells [13] considerably prevented induction of Egr-1 by S1P (Fig. 2E).

3.3. S1P-induced expression of proinflammatory molecules is mediated by different transcription factors

We showed previously that S1P induces a significant up-regulation of the mRNA content of IL-1, IL-6 and COX2 in RISM cells [14]. An RNAi approach was used to clarify whether Egr-1 mediates transcription of these inflammatory genes in response to S1P. As expected, S1P-induced increase of Egr-1 transcript amount was efficiently blocked in cells preincubated with siRNA directed against Egr-1 (Fig. 3A). As shown in Fig. 3A siRNA-induced Egr-1 knockdown effectively impeded S1P-induced elevation of IL-1 and COX2 transcript amounts, by about 70% and 40%, respectively. By contrast, S1P-induced IL-6 expression was not affected by Egr-1 siRNA. This finding prompted us to examine if STAT3, a transcription factor involved in cytokine signalling mediates the expression of IL-6 in response to S1P. First we examined whether S1P induces STAT3 activation via tyrosine phosphorylation. As shown in Fig. 3B, S1P induced a transient activation of STAT3, which was clearly reduced in the presence of JSI-124 (cucurbitacin), a specific inhibitor of STAT3 (Fig. 3C) [19]. Moreover, we found that S1P-induced IL-6 expression was considerably reduced in the presence of JSI-124 (Fig. 3D). However, JSI also reduced IL-1 and COX2 mRNA levels but to a lesser extent. Taken together these results indicate that Egr-1 and STAT3 might act synergistically in the induction of inflammatory mediators in response to S1P in RISM cells.

3.4. The proinflammatory response elicited by S1P is receptor-mediated in RISM cells

Using RT-PCR we found that in RISM cells $S1P_3$ is abundantly expressed while $S1P_1$ and $S1P_2$ are clearly detectable yet to a lower extent, whereas $S1P_4$ and $S1P_5$ transcripts could not be detected (Fig. 4A). Two approaches were used to clarify the involvement of $S1P_{1-3}$ in the proinflammatory response elicited by S1P in RISM cells. i) Application of PTX, known to inactivate $G_{i/0}$-proteins that might be coupled to $S1P_{1-3,}$ prior to S1P addition abolished S1P-induced expression of IL-6 and IL-1 and considerably reduced that of COX2 (Fig. 4B). This rather unspecific approach suggested an involvement of S1P receptors in the proinflammatory response elicited by S1P. ii) A more specific approach, siRNAs directed against each of the 3 S1P receptors (Fig. 4C) detected in RISM cells confirmed this finding. A

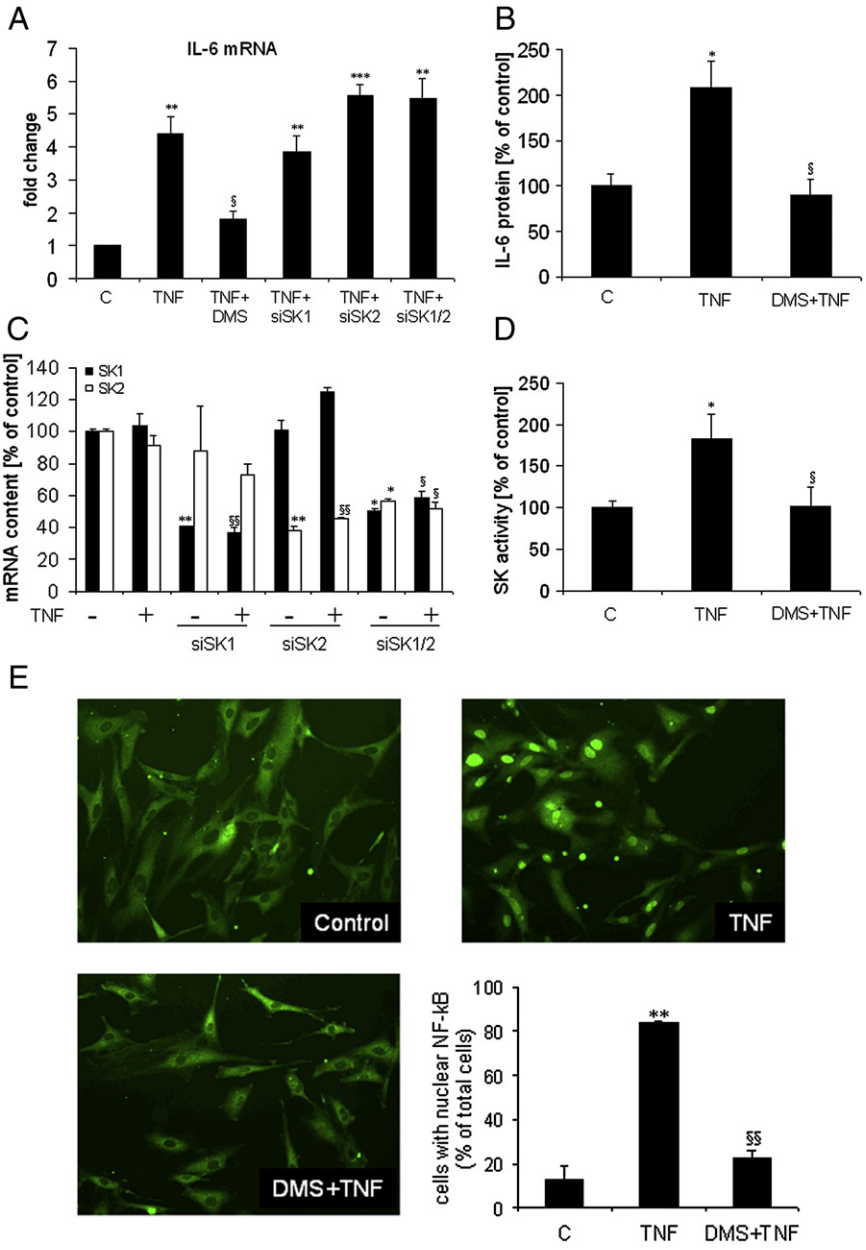

Fig. 1. Sphingosine kinase (SK) activity mediates TNF-induced IL-6 expression via NF-kB pathway in primary cultured RISM cells. Cells were incubated in the absence (C) or presence of TNF-α (10 ng/ml) and DMS (10 μM, 1 h preincubation) or siRNAs (20 nM, 24 h preincubation) for 4 h as indicated. A) and C). Cells were lysed, RNA was extracted and analysed by quantitative RT-PCR as described in Materials and methods. B). Media were collected and subjected to ELISA assay as described in Materials and Methods. D). Cells were harvested and SK activity was measured in cell homogenates as described in Materials and methods. E). Cells were incubated in the absence (control) or presence of TNF-α (10 ng/ml) and DMS (10 μM, 1 h preincubation) for 30 min. Immunofluorescent staining of p65 NF-kB was performed as described in Materials and methods. Immunoreactivity was visualized by fluorescence microscopy (×200). At least 4 independent fields of greater than 50 cells were used for quantitative analysis. Statistical significance is given for TNF-α treated cells vs. control cells, $^{*}p<0.05$; $^{**}p<0.01$ or for cells treated as indicated vs. TNF-α, $^{\S}p<0.05$; $^{\S\S}p<0.01$.

significant reduction of S1P-induced IL-6 expression was observed in cells transfected with siRNA directed against $S1P_1$ and $S1P_2$, respectively (Fig. 4D). On the other hand S1P-induced IL-1 expression was considerably suppressed by siRNA against $S1P_2$ and $S1P_3$ whereas COX2 expression was slightly decreased only by siRNA directed against $S1P_2$ (Fig. 4D). Thus, in these cells S1P-induced IL-6 expression is mediated by $S1P_1$ and $S1P_2$ receptors while IL-1 expression is mediated by $S1P_2$ and $S1P_3$.

4. Discussion

We have reported before that S1P content is elevated in the intestinal muscularis, which was proposed to play a major role in the pathogenesis of postoperative ileus [13]. We also showed that in RISM cells S1P induces the expression of proinflammatory molecules including IL-1, IL-6 and COX2 [14]. The molecular mechanism implying the role of S1P in inflammatory processes is, however, not known in detail. There is experimental evidence that S1P acts as second messenger in TNF-α-induced inflammation [4,20]. Our results in RISM cells confirm that SK activation is required for TNF-α-induced expression of IL-6 via NF-kB pathway. The findings in RISM cells clearly show that SK activity is essential for the translocation of NF-kB into the nucleus in response to TNF-α. The role of SK for NF-kB activation in TNF-α signalling has been previously suggested in different cell types [4,20,21]. Moreover, exogenous S1P was reported to mimic the effect of TNF on NF-kB activation in endothelial cells [21]. However, in RISM cells exogenously added S1P failed to induce translocation of NF-kB even though it stimulated transcription of

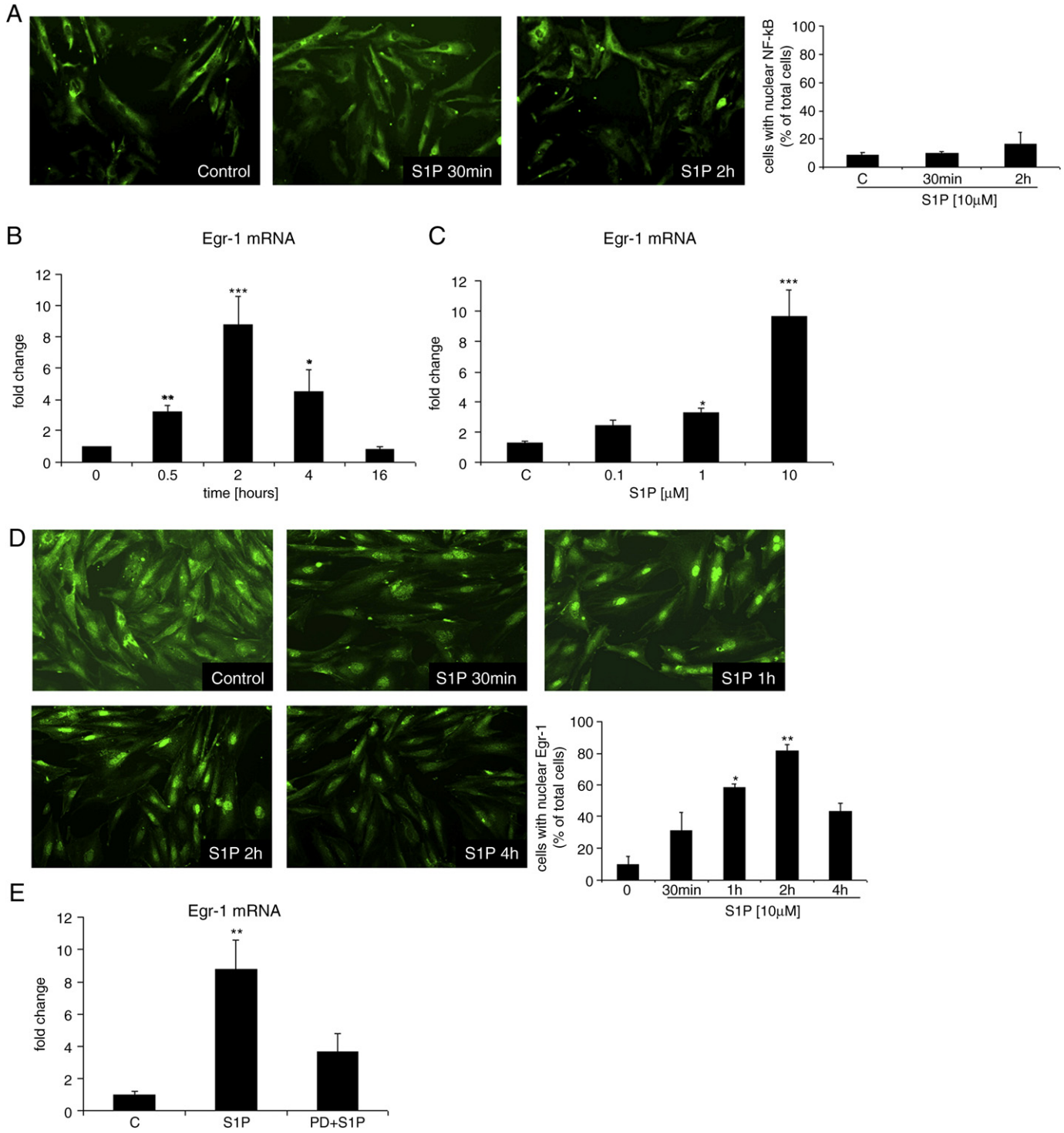

Fig. 2. Exogenous S1P does not induce nuclear translocation of NF-kB but stimulates Egr-1 expression in an ERK1/2-dependent manner in RISM cells. A). Immunofluorescent staining of NF-kB localizes the protein exclusively in the cytoplasm of both, untreated RISM cells (controls) and in cells treated with 10 μM S1P as indicated. B). Cells were incubated with 10 μM of S1P for the indicated times or C) for 2 h with the indicated concentrations of S1P. Control cells (C) were always treated accordingly in the presence of vehicle. After the indicated times cells were lysed, RNA was extracted and analysed by quantitative RT-PCR. D). Immunofluorescent staining of Egr-1 was performed in untreated RISM cells (control) and in cells incubated with 10 μM S1P for the indicated time. Immunoreactivity was visualized by fluorescence microscopy (×200; A, D). E). Cells were incubated with vehicle (C) or with 30 μM of PD98059 (PD) for 30 min, prior to addition of 10 μM S1P. After 2 h cells were lysed, RNA was extracted and analysed by quantitative RT-PCR as described in Materials and methods. At least 4 independent fields of greater than 50 cells were used for quantitative analysis (A, D). Statistical significance is given for S1P treated cells vs. control cells, $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

several proinflammatory molecules. This finding suggests that S1P forwards as a second messenger inflammatory processes induced by cytokines including TNF and IL-1 [4,20] and on the other hand has the potential to induce elevated transcription of proinflammatory molecules by itself. Obviously the molecular mechanism of the latter is cell type dependent.

Recently the transcription factor Egr-1 was proposed to be essential in intestinal inflammatory processes [12]. Initially Egr-1

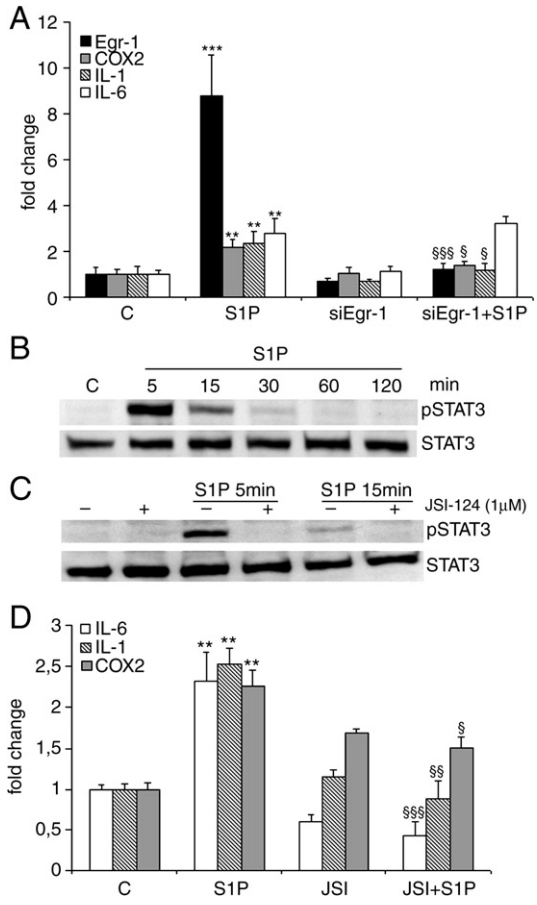

Fig. 3. Egr-1 mediates S1P-induced transcription of COX2 and IL-1 whereas IL-6 expression is dependent on STAT3 activation in RISM cells. A). Cells were transfected with 10nM siRNA duplexes directed against Egr-1 (siEgr-1) or negative control siRNA. After 24 h vehicle (controls, C) or S1P (10 μM) were added. After 2 h cells were lysed, RNA was extracted and mRNA levels were determined by quantitative RT-PCR as described in Materials and methods. B and C). Cells were incubated with S1P (10 μM) for the indicated times in the absence or presence of JSI-124 (1 h preincubation). Phosphorylated STAT3 was determined by immunoblot analysis using antiphospho-STAT3 antibody. Blots were stripped and re-probed with a STAT3 specific antibody to visualize equal loading of protein. D). Cells were incubated with vehicle (C) or 1 μM of JSI-124 (JSI) for 1 h, prior to addition of S1P (10 μM). After 2 h cell were lysed, RNA was extracted and subjected to quantitative RT-PCR. Statistical significance is given for S1P treated cells vs. control cells, $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ or for cells treated as indicated vs. S1P, $^{§}p<0.05$; $^{§§}p<0.01$ §§§ $p<0.001$.

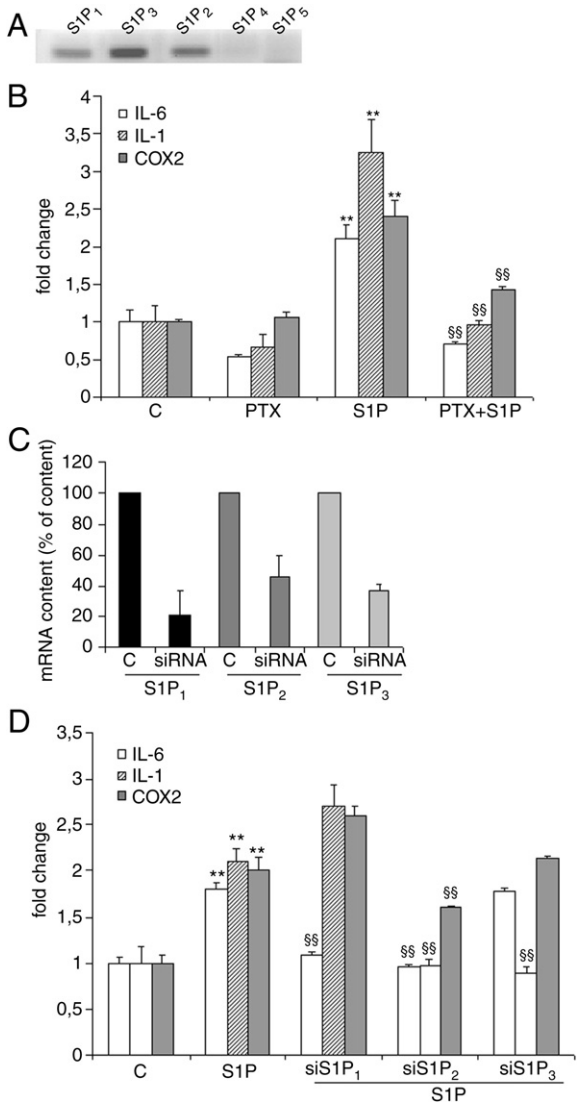

Fig. 4. S1P-induced inflammatory responses are receptor dependent. A). RT-PCR showing the expression of S1P 1–5 receptors in RISM cells. B). Cells were incubated for 2 h in medium containing 100 ng/ml PTX. Then S1P was added for 2 h. Controls (C) were treated accordingly with vehicle. C). Cells were incubated in the absence (C) or presence of siRNAs specific to $S1P_{1-3}$ receptors for 24 h. D). Cells were incubated for 24 h with 20 nM siRNA against $S1P_1$, $S1P_2$ or $S1P_3$ receptors prior to addition of S1P (10 μM) for 2 h. After the indicated times cells were lysed, RNA was extracted and mRNA levels were determined by quantitative RT-PCR as described in Materials and methods. Statistical significance is given for S1P treated cells vs. control cells, $^{**}p<0.01$, or for cells treated as indicated vs. S1P, $^{§§}p<0.01$.

was associated with growth and differentiation [22,23] but recently its importance as an inflammatory transcription factor has been reported [11,24,25]. Intriguingly more than a decade ago the role of S1P as a cell growth-signalling molecule was shown to be mediated by Egr-1 [26]. Our results indicate that the role of S1P as an inflammation-signalling molecule is also mediated at least in part by Egr-1. Thus in RISM cells S1P induces Egr-1 transcription in a time and concentration dependent manner. Moreover it rapidly induces its translocation and accumulation in the nucleus. As reported for endothelial cells [27], Egr-1 could be detected in the cytoplasm but not in the nucleus of untreated RISM cells. Apparently low amounts of Egr-1 are continuously formed in RISM cells in culture. Note that the mechanisms and factors that participate in the retention of Egr-1 within the cytoplasm are not yet fully understood. Egr-1 expression was reported to be regulated by ERK phosphorylation [26,28]. We have recently shown that S1P induced a transient activation of ERK in primary cultured RISM cells [13]. Moreover COX2 expression induced by S1P was inhibited by PD 98059, a specific inhibitor of ERK MAP kinase [13]. In the present study we show that inhibition of ERK MAP kinase with PD 98059 prevents elevation of Egr-1 transcription in response to S1P. This data indicate that Egr-1 might mediate S1P induced COX2 transcription. Indeed experiments with siRNA directed against Egr-1 confirmed that this transcription factor is involved in S1P-induced formation of COX2 and IL-1 transcripts in RISM cells. Surprisingly transcription of IL-6 appeared to be independent of Egr-1 expression in these cells.

The production of IL-6 was reported to be linked to STAT3 activation [29,30]. Apparently there is a complex relation between STAT3 and inflammatory cytokines. Thus STAT3 not only mediates the expression of IL-6 and other cytokines but also responds to several inflammatory cytokines, especially to IL-6 [29–32]. Moreover, STAT3 has been shown to be highly activated in the intestinal muscularis following surgical manipulation and thus proposed to be causative for inflammatory processes known to occur in postoperative ileus [33]. In this study we used a specific STAT3 inhibitor to assess a possible role of this transcription factor in S1P-induced inflammation in RISM cells.

It became clear that S1P-induced STAT3 activation is essential especially for IL-6 transcription. However, IL-1 and COX2 transcripts were also reduced in the presence of this inhibitor, pointing to the complexity of inflammatory processes. Note that induction of STAT3 by S1P has been shown before in ventricular cardiomyocytes [34].

We have reported previously that in RISM cells S1P-induced COX2 expression is PTX sensitive implying the involvement of Gi/o-coupled S1P receptors [13]. Since all five members of the S1P-receptor family can be coupled to $G_{i/0}$-proteins [35] PTX sensitivity does not give any information about S1P receptor subtype involved in the proinflammatory effect of S1P. In the present study we confirmed our previous finding concerning S1P-induced expression of COX2 and in addition found that S1P-induced expression of two inflammatory cytokines IL-1, IL-6 is also PTX sensitive. This result is, however, contradictory to findings in smooth muscle cells isolated from the human trachea, in which modulation of cytokines by S1P was PTX independent [36]. Obviously, the involvement of S1P receptors in S1P-induced inflammatory responses is cell type specific. Apparently all the three S1P receptors, namely $S1P_1$, $S1P_2$ and $S1P_3$ expressed in RISM cells are involved, yet to a different extent, in S1P-induced inflammation. Thus the siRNA approach indicates that $S1P_2$ mediates expression of COX2, IL-1 and IL-6, whereas $S1P_1$ is involved only in the expression of IL-6 and finally $S1P_3$ is implicated in the expression of IL-1. Other studies using siRNA or S1P receptor antagonists also revealed that S1P regulates inflammation-related genes through different S1P receptors in human endothelial cells and human synoviocytes [37,38].

In conclusion, our findings reveal the dual action of S1P in the inflammatory response of RISM cells. On the one hand, it mediates as a second messenger cytokine-induced activation of NF-kB. Exogenous S1P, on the other hand, elicits a receptor-dependent inflammatory response. The latter is independent of NF-kB activation but involves Egr-1 and STAT3. Thus targeting of S1P-induced inflammatory pathways at different steps might represent novel therapeutic modalities for limiting inflammation in postoperative ileus.

Acknowledgements

We wish to thank Mariola Lysson for excellent technical assistance. This study was supported by the Deutsche Forschungsgemeinschaft (KFO 115).

References

[1] S. Spiegel, S. Milstien, Nat. Rev. Mol. Cell Biol. 4 (5) (2003) 397.
[2] B. Anliker, J. Chun, J. Biol. Chem. 279 (20) (2004) 20555.
[3] A. Olivera, H.M. Rosenfeldt, M. Bektas, F. Wang, I. Ishii, J. Chun, S. Milstien, S. Spiegel, J. Biol. Chem. 278 (47) (2003) 46452.
[4] P. Xia, L. Wang, P.A. Moretti, N. Albanese, F. Chai, S.M. Pitson, R.J. D'Andrea, J.R. Gamble, M.A. Vadas, J. Biol. Chem. 277 (10) (2002) 7996.
[5] Y. Osawa, Y. Banno, M. Nagaki, D.A. Brenner, T. Naiki, Y. Nozawa, S. Nakashima, H. Moriwaki, J. Immunol. 167 (1) (2001) 173.
[6] B.J. Pettus, J. Bielawski, A.M. Porcelli, D.L. Reames, K.R. Johnson, J. Morrow, C.E. Chalfant, L.M. Obeid, Y.A. Hannun, FASEB J. 17 (11) (2003) 1411.
[7] E. Hoffmann, J. Ashouri, S. Wolter, A. Doerrie, O. Dittrich-Breiholz, H. Schneider, E.F. Wagner, J. Troppmair, N. Mackman, M. Kracht, J. Biol. Chem. 283 (18) (2008) 12120.
[8] V.P. Sukhatme, S. Kartha, F.G. Toback, R. Taub, R.G. Hoover, C.H. Tsai-Morris, Oncogene Res. 1 (4) (1987) 343.
[9] J. Milbrandt, Science 238 (4828) (1987) 797.
[10] L.M. Khachigian, Circ. Res. 98 (2) (2006) 186.
[11] R. Pawlinski, B. Pedersen, B. Kehrle, W.C. Aird, R.D. Frank, M. Guha, N. Mackman, Blood 101 (10) (2003) 3940.
[12] J. Schmidt, B. Stoffels, B.A. Moore, R.S. Chanthaphavong, A.R. Mazie, B.M. Buchholz, A.J. Bauer, Gastroenterology 135 (3) (2008) 926 936 e921.
[13] M. Dragusin, S. Wehner, S. Kelly, E. Wang, A.H. Merrill Jr., J.C. Kalff, G. van Echten-Deckert, FASEB J. 20 (11) (2006) 1930.
[14] R. Broere, M. Gurgui, G. Van Echten-Deckert, Chim. Oggi-Chem. Today 25 (5) (2007) 28.
[15] J.C. Kalff, W.H. Schraut, R.L. Simmons, A.J. Bauer, Ann. Surg. 228 (5) (1998) 652.
[16] G. van Echten-Deckert, A. Zschoche, T. Bar, R.R. Schmidt, A. Raths, T. Heinemann, K. Sandhoff, J. Biol. Chem. 272 (25) (1997) 15825.
[17] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[18] K. Endo, Y. Igarashi, M. Nisar, Q.H. Zhou, S. Hakomori, Cancer Res. 51 (6) (1991) 1613.
[19] M.A. Blaskovich, J. Sun, A. Cantor, J. Turkson, R. Jove, S.M. Sebti, Cancer Res. 63 (6) (2003) 1270.
[20] A. Billich, F. Bornancin, D. Mechtcheriakova, F. Natt, D. Huesken, T. Baumruker, Cell. Signal. 17 (10) (2005) 1203.
[21] P. Xia, J.R. Gamble, K.A. Rye, L. Wang, C.S. Hii, P. Cockerill, Y. Khew-Goodall, A.G. Bert, P.J. Barter, M.A. Vadas, Proc. Natl. Acad. Sci. U. S. A. 95 (24) (1998) 14196.
[22] G. Thiel, G. Cibelli, J. Cell. Physiol. 193 (3) (2002) 287.
[23] V.P. Sukhatme, J. Am. Soc. Nephrol. 1 (6) (1990) 859.
[24] B. Li, M.R. Power, T.J. Lin, Blood 107 (7) (2006) 2814.
[25] S. Mishra, T. Fujita, V.N. Lama, D. Nam, H. Liao, M. Okada, K. Minamoto, Y. Yoshikawa, H. Harada, D.J. Pinsky, Proc. Natl. Acad. Sci. U. S. A. 103 (13) (2006) 5191.
[26] K. Sato, K. Ishikawa, M. Ui, F. Okajima, Brain Res. Mol. Brain Res. 74 (1–2) (1999) 182.
[27] D. Mechtcheriakova, A. Wlachos, H. Holzmuller, B.R. Binder, E. Hofer, Blood 93 (11) (1999) 3811.
[28] M. Guha, M.A. O'Connell, R. Pawlinski, A. Hollis, P. McGovern, S.F. Yan, D. Stern, N. Mackman, Blood 98 (5) (2001) 1429.
[29] L.T. Lam, G. Wright, R.E. Davis, G. Lenz, P. Farinha, L. Dang, J.W. Chan, A. Rosenwald, R.D. Gascoyne, L.M. Staudt, Blood 111 (7) (2008) 3701.
[30] E. Beurel, R.S. Jope, J. Neuroinflammation 6 (2009) 9.
[31] J. Bromberg, T.C. Wang, Cancer Cell 15 (2) (2009) 79.
[32] S. Grivennikov, E. Karin, J. Terzic, D. Mucida, G.Y. Yu, S. Vallabhapurapu, J. Scheller, S. Rose-John, H. Cheroutre, L. Eckmann, M. Karin, Cancer Cell 15 (2) (2009) 103.
[33] S. Wehner, N.T. Schwarz, R. Hundsdoerfer, C. Hierholzer, D.J. Tweardy, T.R. Billiar, A.J. Bauer, J.C. Kalff, Surgery 137 (4) (2005) 436.
[34] M.A. Frias, R.W. James, C. Gerber-Wicht, U. Lang, Cardiovasc. Res. 82 (2) (2009) 313.
[35] I. Ishii, N. Fukushima, X. Ye, J. Chun, Annu. Rev. Biochem. 73 (2004) 321.
[36] A.J. Ammit, A.T. Hastie, L.C. Edsall, R.K. Hoffman, Y. Amrani, V.P. Krymskaya, S.A. Kane, S.P. Peters, R.B. Penn, S. Spiegel, R.A. Panettieri Jr., FASEB J. 15 (7) (2001) 1212.
[37] C.I. Lin, C.N. Chen, P.W. Lin, H. Lee, Biochem. Biophys. Res. Commun. 355 (4) (2007) 895.
[38] C. Zhao, M.J. Fernandes, M. Turgeon, S. Tancrede, J. Di Battista, P.E. Poubelle, S.G. Bourgoin, J. Lipid Res. 49 (11) (2008) 2323.