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# Substance P induces TNF-α and IL-6 production through NFκB in peritoneal mast cells

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**Abstract**

The neuropeptide Substance P (SP) is an important mediator of neuroimmunomodulatory activity. The aim of this study is to elucidate the mechanism used by SP to promote increased production of pro-inflammatory cytokines in fresh isolated rat peritoneal mast cells (rPMC). We have demonstrated that SP induces production of interleukin-6 (IL-6) in rPMC through the PI-3K, p42/44 and p38 MAP kinase pathways. SP-stimulated rPMC also exhibited an enhanced nuclear translocation of the nuclear factor κ B (NFκB). The tumour necrosis factor-α (TNF-α) and IL-6 production was completely inhibited by using (*E*)-4-hydroxynonenal (HNE) as an inhibitor of IκB-α and -β phosphorylation. Further, TNF-α and IL-6 expression was significantly inhibited by the oligonucleotides (ODNs) containing the NFκB element (NFκB decoy ODNs) but not by the scrambled control ODNs. These findings indicate that the NFκB pathway is involved in the transcriptional regulation of the TNF-α and IL-6 overexpression in primary SP-stimulated mast cells.


*Keywords:* Mast cell; TNF-α; IL-6; SP; MAPK; NFκB; ODN decoy

## 1. Introduction

Mast cells play a central role in both inflammatory and allergic reactions through the release of different biological compounds that are either stored (histamine) or de novo synthesized (prostaglandins and some cytokines) [1,2]. For example, the pro-inflammatory cytokines, tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6), are produced and released by activated mast cells [3]. Mast cell activation occurs via the antigen- or the basic secretagogue-elicited signalling pathways. As a result of antigen activation, FcεRI receptors aggregate and initiate a signalling cascade involving p42/44, p38 and JNK MAP kinases to promote TNF-α and IL-6 production [4,5]. In the second pathway, it has been shown that the neuropeptide Substance P (SP), a basic secretagogue in the family of mast cell agonists, activates the pertussis toxin-sensitive heterotrimeric GTP-binding proteins (G proteins) $G_{i2}$ and $G_{i3}$ [6,7].

In previous studies, we had shown that brief exposure of SP to rat mast cells derived from either uterine, peritoneal (rPMC) or hypothalamic tissues also promotes TNF-α production [8–10]. We also had demonstrated that in mast cells, although only p38 and JNK are involved in TNF-α production, SP is involved in the activation of the three main MAP kinases (p42/44, p38 and JNK) [11]. Since TNF-α and IL-6 promote both beneficial and detrimental effects, their expression is tightly regulated in many cell types, including mast cells. The promoter regions of both the TNF-α and the IL-6 genes contain nuclear factor κ B (NFκB), AP-1, AP-2 and NFAT transcription factor motifs [12,13]. NFκB, the central regulator for the expression of cytokines involved in the inflammatory response, is commonly found as a dimer composed of the RelA (p65) and NFκB1 (p50) or NFκB2 (p52) subunits. The p65 subunit appears to be the subunit that promotes transcription [14]. NFκB activation occurs as a result of phosphorylation, polyubiquitination and proteasome degradation of IκB-α and -β. This leads to the release of activated NFκB, which can then translocate into the nucleus to activate target genes. In mast cells, while NFκB has been shown to be activated during FcεRI aggregation, resulting in the production of IL-6 and TNF-α [15,16], the signalling pathways elicited by SP have not been identified.

*Abbreviations:* SP, Substance P; IL-6, interleukin-6; rPMC, rat peritoneal mast cells; NFκB, nuclear factor κ B; HNE, (*E*)-4-hydroxynonenal; TNF-α, tumour necrosis factor-α; ODN, oligonucleotide; BMMCs, bone marrow-derived mast cells

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In this study, we determined that mast cells produce IL-6 after SP stimulation and we investigated the transcriptional regulatory mechanisms involved in pro-inflammatory cytokine production. We found that in mast cells, SP treatment results in the induction of rPMC leading to the production of TNF-α and IL-6 via NFκB activation. Further, we have shown that treatment of the cells with an NFκB decoy oligonucleotide (ODN) completely abrogates this induction.

## 2. Materials and methods

### 2.1. Reagents

Substance P and wortmannin were obtained from Sigma Aldrich, SB203580 and HNE from Alexis Biochemicals, PD98059 from New England Biolabs. The NFκB ODN decoy [consensus sequence (5′AGTTGAGGGGACTTTCC CAGGC3′)] and the NFκB scrambled ODN decoy [consensus sequence (5′AGTTGAGGCCACTTTCCCAGGC3′)] were acquired from Promega. Antibodies: rabbit anti-rat-IL-6 and -TNF-α antibodies were purchased from Endogen; rabbit anti-rat NFκB p65 was purchased from Santa Cruz Biotechnologies; and rabbit anti-β-actin was purchased from Sigma Aldrich. The antibody against anti-enolase was the generous gift of Dr. A. Giallongo (Istituto di Biomedicina e Immunologia Molecolare ‘‘Alberto Monroy’’, C.N.R.).

### 2.2. Preparation of whole cell and nuclear extracts from rPMC

The purification, stimulation and pretreatment of rPMC with inhibitors were performed as described [11]. Inhibitors were dissolved in dimethylsulfoxide and diluted with Tyrode’s buffer to the final concentrations, as indicated in figure legends. The final concentration of the vehicle in samples was adjusted to 0.1% (v/v); the control buffer contained the same amount of the vehicle. For ODN treatment, purified rPMCs were incubated with 4.3 nM NFκB ODNs or scrambled decoy for different times (15, 30, 60 min) and then stimulated with SP for 30 min.

Nuclear extracts were prepared by lysing $3.5 \times 10^5$ cells in buffer A (20 mM HEPES pH 7.9, 10 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, containing 1.5 μg/ml aprotinin, pepstatin, leupeptin and chymostatin) for 15 min on ice. After the addition of Nonidet P-40 (0.5% final concentration), the cell lysates were centrifuged at $1000 \times g$ for 10 min at 4 °C. The pelleted material was incubated with buffer A supplemented to 550 mM NaCl, for 30 min on ice. Insoluble material was removed by centrifugation at $14\,000 \times g$ for 20 min at 4 °C. The protein concentration of the supernatant was determined using a standard Bio-Rad protein micro-assay.

### 2.3. Western blot analysis

For TNF-α and IL-6 analysis, identical amounts of protein (25 μg) of whole cell extracts were resolved onto 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE); for NFκB analysis, cytoplasmic and nuclear extracts (15 μg) were resolved onto 10% SDS-PAGE. SDS-PAGE gels were electro-transferred, probed with antibodies against IL-6, TNF-α, NFκB p65 and enolase (1:1000 dilution in blocking solution), and detected by enhanced chemiluminescence (ECL plus™ Amersham Pharmacia Biotech). β-Actin immunolabeling and Red-Ponceau staining were performed as loading controls for the cytoplasmic and the nuclear fractions, respectively. Intensities of protein bands were quantified by densitometric scanning (Bio-Rad).

### 2.4. RT-PCR analysis

rPMC ($2.5 \times 10^4$) were lysed and mRNA was recovered by Oligo-dT cellulose (Boehringer Mannheim) as previously reported [8].

Reverse transcription to generate cDNA was performed using Advantage RT-PCR kit as recommended by the supplier (Clontech CA). Competitive-PCR to determine TNF-α levels of expression was performed as described using an exogenous internal standard (the standard size is 249 bp) [9,17]. To analyse IL-6 expression, semi-quantitative PCR was performed using IL-6-specific 5′and 3′primers (5′T A T T G A A A A T C T G C T C T G T T C T 3′and 5′C C A C T C C T T C T G T G A C T C T A A C T T C 3′) which generated a specific band of 233 nucleotides. β-Actin PCR with specific primers was performed as described [11]. Aliquots of the PCR products (10 μl) were fractionated by electrophoresis using 1.6% agarose gel containing ethidium bromide and visualized under UV. The signal intensity was analysed by computerized densitometry using Molecular Analysis Software (Bio-Rad). Relative abundance of target mRNA was estimated after normalization using β-actin cDNA (175 bp) as an internal standard.

### 2.5. Statistical analysis

Data were expressed as mean ± standard error of the mean (S.E.) of repeated experiments. One-way analysis of variance (ANOVA) was used to determine differences among groups. When differences were determined to exist, ANOVA analysis was followed by a two-tailed Student’s test with a $P$ value $< 0.05$ considered significant.

## 3. Results and discussion

### 3.1. SP induces IL-6 production by rPMC

Resting mast cells lack expression of IL-6 but become capable of IL-6 production following IgE-dependent stim-

ulation [18]. In this study we have analysed rPMC production of IL-6 after SP induction. Purified peritoneal mast cells were challenged with different SP concentrations (0.01, 0.1, 1, 10, 100 μM) and the levels of IL-6 mRNA were quantified by comparison with β-actin mRNA. The semi-quantitative analysis of the PCR products showed a dose-dependent curve response for IL-6 mRNA levels reaching the maximum after a challenge with 100 μM SP (Fig. 1A). The time course of IL-6 production by rPMC was also analysed. Mast cells were activated with 100 μM SP for various times (from 5 to 60 min). Fig. 1B shows an enhancement of IL-6 mRNA expression beginning at ~15–30 min and reaching the maximum level of expression after 60 min. In absence of stimulation, very little IL-6 protein is detected within these cells, however, it is expressed at high levels and released after 30 min of SP stimulation (Fig. 1C). These data are in parallel with our previous results, which showed that, when induced by SP, mast cells also express another pro-inflammatory cytokine, TNF-α, in a concentration- and time-dependent manner [9].

### *3.2. PI-3K, p42/44 and p38 MAPKs are involved in IL-6 production mediated by SP*

We have previously demonstrated that in rPMC, SP activates p42/44, p38 and JNK MAPKs, and that the last two kinases, but not p42/44, are involved in TNF-α production [11]. Here, we have investigated whether these pathways are also involved in SP-induced IL-6 production by using MAPK-inhibitors. rPMC were treated with the pharmacological inhibitors of p42/44 (PD98059), p38 (SB203580) or PI-3K (wortmannin) and stimulated with 100 μM SP for 30 min. Wortmannin was used to investigate the involvement of JNK pathway in IL-6 production, because it has been shown that in rPMC this inhibitor

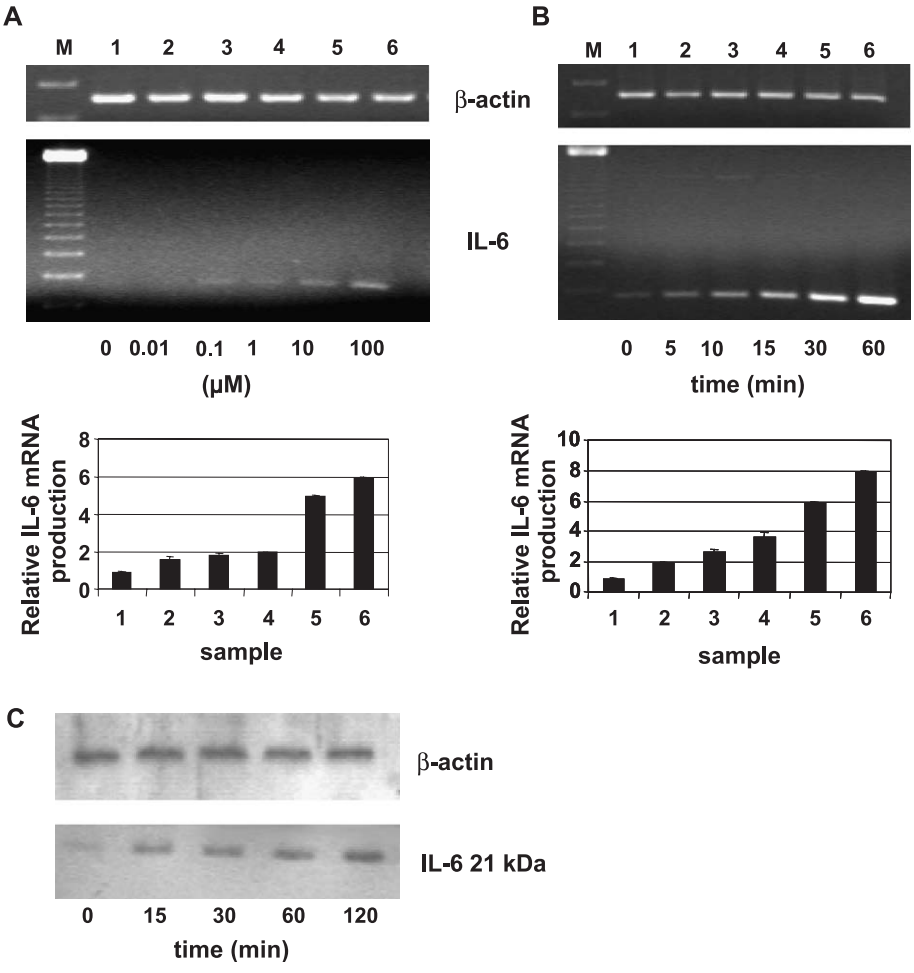


Fig. 1. SP induces IL-6 production in a dose- and time-dependent manner in rPMC. (A) rPMC were stimulated with different concentrations of SP for 30 min, and RT-PCR was performed to generate IL-6 and β-actin cDNAs. The intensity of the resulting bands was evaluated by densitometry analysis and the ratio of IL-6/β-actin reported. Following stimulation of rPMC with 100 μM SP for different times, IL-6 and β-actin expression was evaluated (B) at the mRNA level using agarose gel analysis of RT/PCR products, and (C) at the protein level in whole cell extracts (25 μg) using Western blot analysis. Representative gels and immunonoblots of results obtained from three independent experiments are shown.

abrogated SP-induced phosphorylation of JNK, but not of p38 [11]. Addition of PD98059 (50 μM), SB203580 (4 μM) or wortmannin (100 nM) for 30 min resulted in a partial decrease in the levels of IL-6 mRNA and protein (Fig. 2A and B). A complete inhibition of IL-6 mRNA expression was observed after 60 min of pre-incubation with PD98059, wortmannin and SB203580 inhibitors. The fact that inhibition was obtained with each of these agents (SB203580, PD98059 and wortmannin) suggests that at least three different signalling pathways are involved in IL-6 production following treatment with SP. Similarly, it has been shown that FcεRI aggregation in bone marrow-derived mast cells (BMMCs) involves these three pathways, p42/44, JNK and p38 MAPK, for IL-6 production [5].

### 3.3. SP promotes nuclear translocation of NFκB in mast cells

It has been reported that p38 MAPK and PI-3K are involved in NFκB activation [19,20]. In addition, in antigen presenting cells, NFκB is induced by SP to promote pro-inflammatory cytokine production [21]. Since nuclear translocation of NFκB is associated with NFκB-dependent gene expression, we investigated whether SP induced NFκB translocation to the nucleus of rPMC. Cytoplasmic and nuclear extracts of cells induced by SP over a time course were analysed for the presence of the p65 NFκB subunit. As shown in Fig. 3, mast cells stimulated with 100 μM SP for 30 min showed an increase of NFκB level in the nuclear fraction with a corresponding decrease in the cytoplasm.

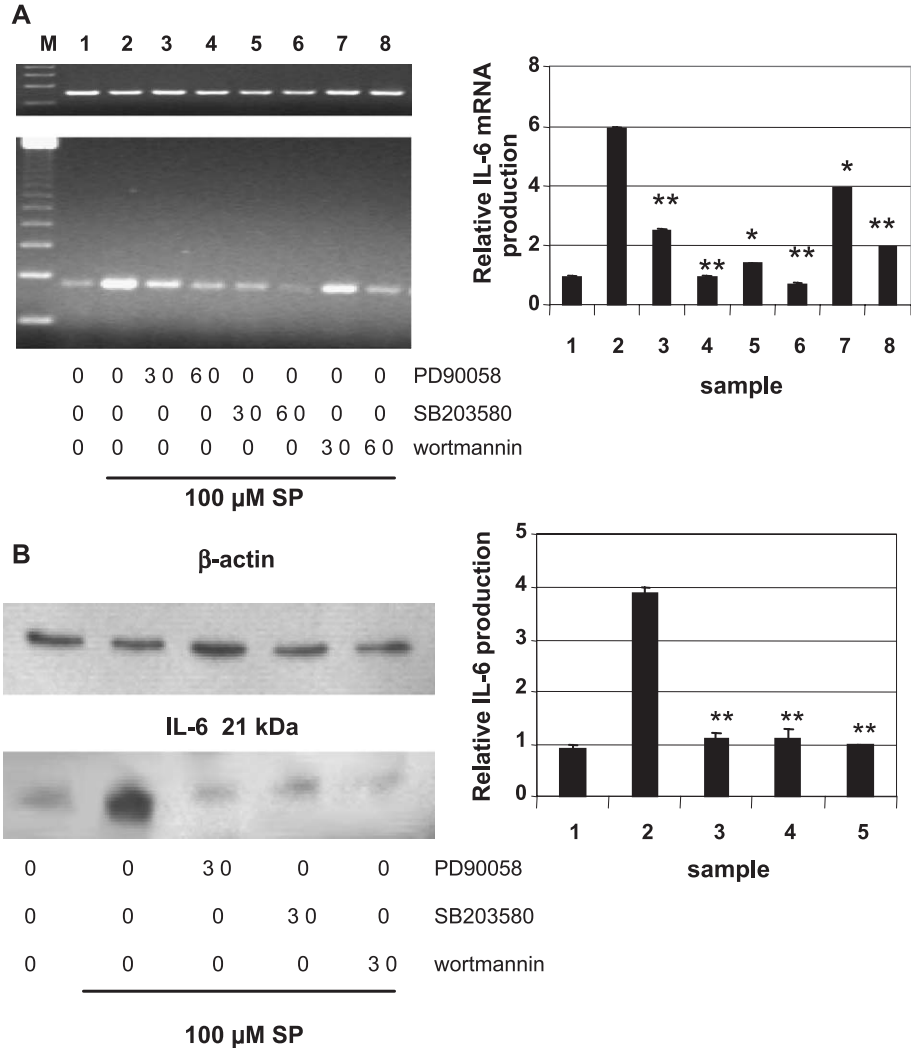


Fig. 2. Inhibition of p42/44, p38 MAPKs or PI-3K reduces IL-6 production. (A) rPMC were stimulated with 100 μM SP for 30 min following 30 or 60 min of pretreatment with 50 μM PD98059 (lanes 3 and 4), with 4 μM SB203580 (lanes 5 and 6) or with 100 nM wortmannin (lanes 7 and 8). To determine IL-6 and β-actin mRNA expression levels, RT/PCR was performed. (B) rPMC were stimulated with 100 μM SP for 30 min following 30 min of pretreatment with 50 μM PD98059 (lane 3), with 4 μM SB203580 (lane 4) or with 100 nM wortmannin (lane 5). β-Actin and IL-6 protein levels in whole cell extracts (25 μg) were determined using Western blot analysis, and representative gel and immunoblots are shown. The mean ± S.E. of values obtained from three independent experiments are shown with $*P<0.05$ and $**P<0.01$ indicating statistically significant differences determined in these experiments.

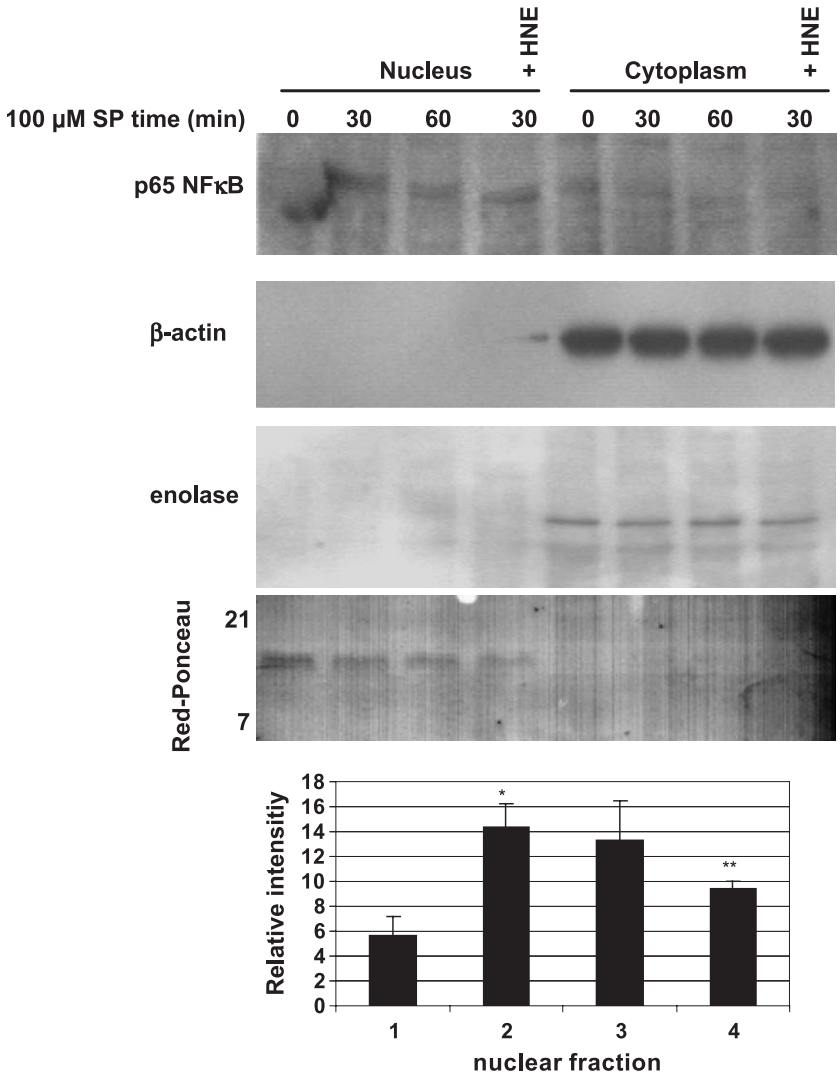


Fig. 3. SP elicits NFκB p65 translocation into the nucleus in rPMC. Cytoplasmic and nuclear extracts (15 μg) from cells stimulated with 100 μM SP at different time points, following treatment with or without 50 nM HNE, were analysed by Western blot. Immunoblotting was performed and followed by anti-NFκB p65, anti-β-actin and anti-enolase antibody detection, with Red-Ponceau staining serving as a loading control for the nuclear fraction. Representative immunoblots are shown. The mean ± S.E. of values obtained from three independent experiments are shown with $*P<0.05$ and $**P<0.01$ indicating significant differences.

Red-Ponceau staining of the PVDF-membrane and immunoblotting with anti-β-actin were performed to confirm equal loading of the protein samples of nuclear and cytoplasmic fractions, respectively. Immunoblotting with anti-enolase antibody served as a marker for cytoplasmic contamination of the nuclear fraction. These results demonstrate that SP promotes the translocation of p65 from the cytoplasm to the nucleus and suggest that NFκB may be a positive regulator of SP-induced cytokines production in rPMC, as has been proposed for murine macrophages [22].

### *3.4. NFκB nuclear translocation is required for SP-induced TNF-α and IL-6 expression in mast cells*

Because NFκB plays a critical role in TNF-α and IL-6 genes expression after FcεRI-stimulation in mast cells [15,16], we investigated to determine if NFκB nuclear translocation is associated with SP-induced TNF-α and IL-6 production in rPMC, using (*E*)-4-hydroxynonenal (HNE). HNE prevents NFκB activation and, in turn, prevents nuclear translocation, through selective blocking of signalling events required for IκB-α and -β stimulus-dependent phosphorylation [23]. We confirmed that in our cell system, HNE inhibits the SP-mediated NFκB nuclear translocation (Fig. 3, lane 4); therefore, we analysed the effects of HNE on TNF-α and IL-6 mRNAs expression by RT-PCR. Treatment with 50 nM HNE, added 15, 30 or 60 min before SP stimulation, resulted in a time-dependent decrease of cytokines production (Figs. 4A and 5A) with maximum inhibition of SP-induced TNF-α and IL-6 expression after 30- or 60-min drug pre-incubation, respectively. These results are consistent with published data which show reduced TNF-α expression in LPS-stimulated human monocytic cells following HNE treatment [23].

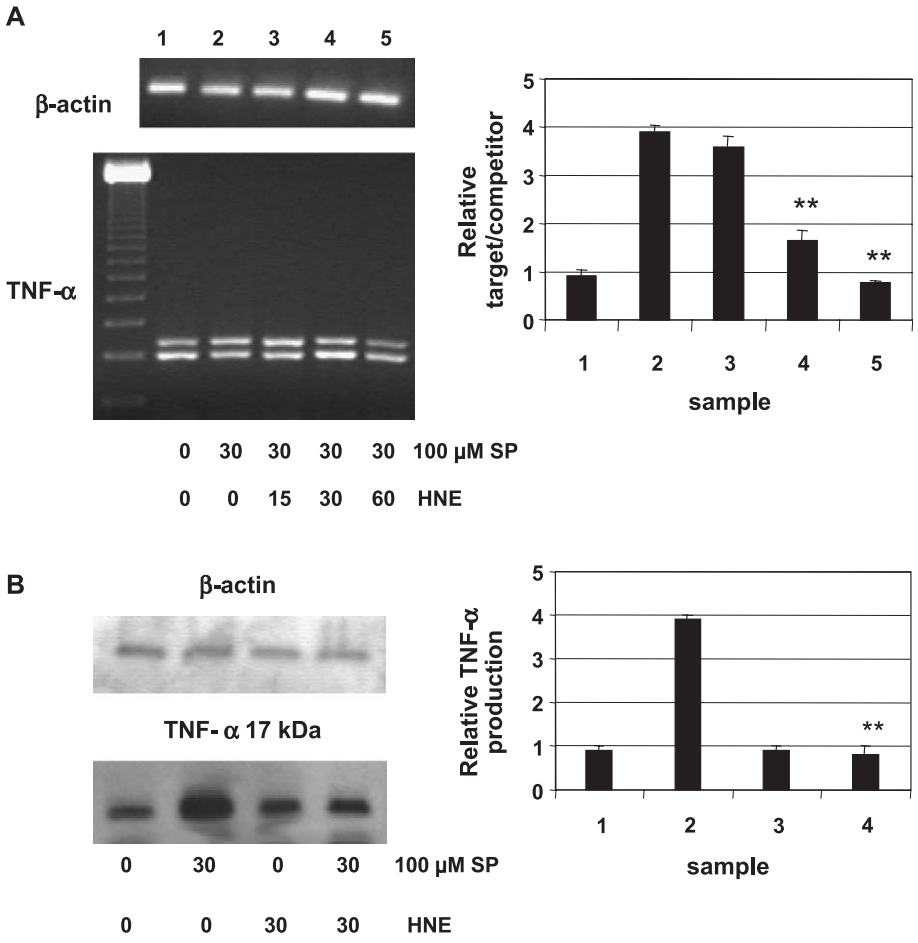


Fig. 4. Inhibition of NFκB nuclear translocation abolishes TNF-α production. (A) rPMC were stimulated with 100 μM SP for 30 min after pretreatment with 50 nM HNE for 15, 30 or 60 min. Competitive RT-PCR was performed to determine the level of TNF-α mRNAs expression in each cDNA samples, which included $2 \times 10^3$ molecules of TNF-α competitor. Following densitometry analysis, resulting products were quantified by calculating the ratio between the target and the competitor, with β-actin cDNAs serving as a control for sample extraction. (B) rPMC were stimulated with 100 μM SP for 30 min and pre-treated with or without 50 nM HNE for 30 min. Western blot analysis of whole cell extracts (25 μg) was performed using anti-TNF-α and anti-β-actin antibodies. A representative gel, and immunoblots are shown. The mean ± S.E. of values obtained from three independent experiments are shown with $^{**}P < 0.01$ indicating significant differences.

Our analysis of protein expression levels confirmed the abolishment of SP-induced TNF-α and IL-6 production following treatment with the NFκB inhibitor, while basal expression of TNF-α and IL-6 proteins was not affected by HNE treatment (Figs. 4B and 5B). As has been proposed for SP-induced antigen presenting cells, the inhibitory effect of HNE on TNF-α and IL-6 production following SP stimulation in rPMC suggests that NFκB nuclear translocation is required for the expression of these cytokines [22]. Similarly, the inhibition of NFκB nuclear translocation by the anti-allergic drug, histaglobin, results in the down-regulation of these pro-inflammatory cytokines in bone marrow-derived macrophage cells [24].

### *3.5. NFκB decoy ODNs inhibit the expression of TNF-α and IL-6 mRNAs induced by SP*

In order to determine the role of the NFκB-DNA binding in SP-activated rPMC, we examined the effect of NFκB decoy ODNs on TNF-α and IL-6 expression induced by SP. Use of ODNs as a therapeutic approach has been investigated recently, because these oligonucleotides, which mimic the consensus sequences of specific transcription factors (such as NFκB), hamper the expression and thereby the functionality of legitimate target genes [25]. Mast cells take up ODN decoy sequences through a pinocytotic mechanism [26] and this strategy has been used here to down-regulate NFκB activity. In this study, treatment of rPMC with an NFκB consensus sequence ODN decoy at 4.3 nM in cellular suspension caused a significant time-course-dependent inhibition of SP-induced TNF-α and IL-6 production. A complete reduction of the SP-induced production of both cytokines was observed after 60 min of treatment (Fig. 6, lanes 5). A control NFκB decoy (scrambled sequence) at 4.3 nM did not affect cytokines production (Fig. 6, lanes 6). Further, the NFκB decoys were non-toxic, in so far that they did not induce histamine release from rPMC (data not shown). These findings demonstrate that NFκB is required for cytokines synthesis in rPMC

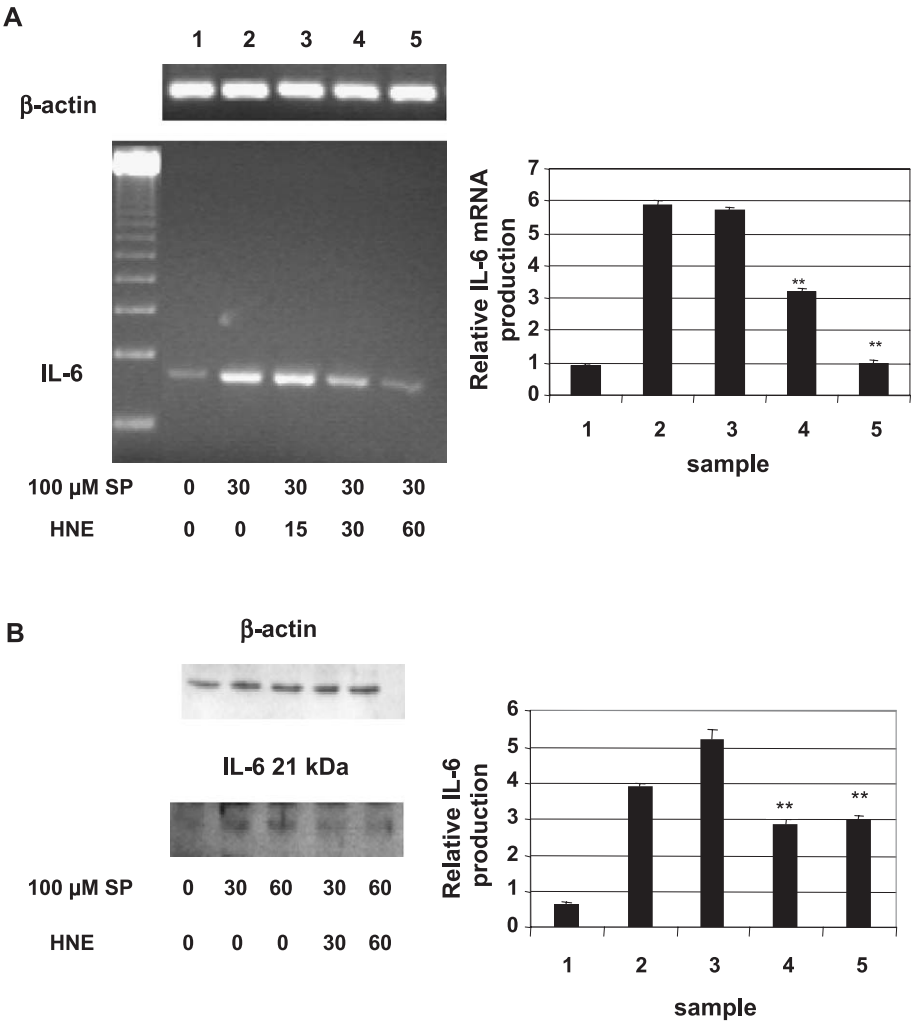


Fig. 5. Inhibition of NFκB nuclear translocation abolishes IL-6 production. (A) rPMC were exposed to 50 nM HNE for different lengths of time and stimulated with 100 μM SP for 30 min. RT-PCR analysis was performed to determine IL-6 and β-actin mRNAs expression levels. (B) rPMC were stimulated with 100 μM SP for different times after treatment with 50 nM HNE for 30 or 60 min. Western blot analysis of whole cell extracts (25 μg) was performed using anti-IL-6 and anti-β-actin antibodies. A representative gel and immunoblots are shown. The mean ± S.E. of values obtained from three independent experiments are shown with $^{**}P<0.01$ indicating significant differences.

stimulated by SP, since an inhibition of NFκB-DNA binding abolished their production.

### 3.6. Concluding remarks

In the present study, we demonstrate that SP induces IL-6 expression in rPMC in a concentration- and time-dependent manner as we have already reported for TNF-α [9]. The signalling pathway activated in mast cells and leading to the production of cytokines depends on the stimulus and on the cell type used. By using PD98059, SB203580 and wortmannin as p42/44, p38 and PI-3K inhibitors, respectively, we demonstrate that these pathways are involved in SP-stimulated IL-6 production. Since we have previously demonstrated a link between PI-3K and JNK pathway [11], we conclude that SP induces IL-6 production by activation of p42/44, p38 and JNK MAPK pathways, similar to FcεRI aggregation in BMMCs [5].

TNF-α and IL-6 gene promoters have consensus sequences targeted by different transcription factors, which are activated by MAPKs. However, little is known about the transcription factors activated by MAPKs in SP-challenged peritoneal mast cells. Studies with FcεRI-stimulated RBL-2H3 mast cell lines and BMMCs showed that TNF-α [15] and IL-6 [16] are produced through NFκB. Likewise our results demonstrate that SP can induce NFκB activation-translocation in peritoneal mast cells. In our cellular system, HNE impairs NFκB nuclear translocation, and consequently we observed a complete block of TNF-α and IL-6 mRNAs, and proteins increase upon SP stimulation. Moreover, after blocking of NFκB-DNA binding by ODN decoys, we confirmed a pivotal role for this transcription factor in peritoneal mast cells production of SP-stimulated cytokines. Recent studies have suggested that the regulation of NFκB activity can occur not only as a result of IκB degradation and the subsequent nuclear translocation of NFκB, but also due

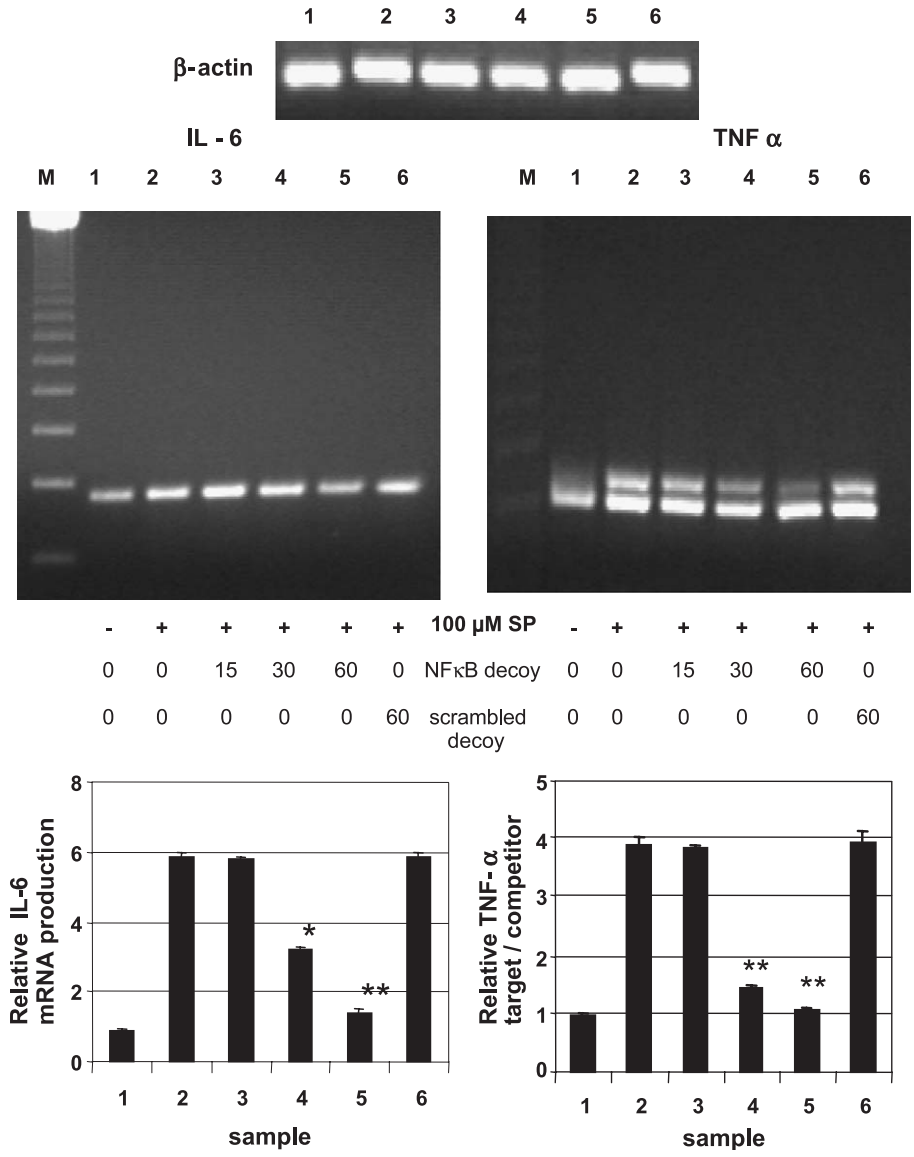


Fig. 6. Effects of NFκB decoy on IL-6 and TNF-α expression induced by SP. rPMC were treated with or without NFκB decoy at a 4.3 nM final concentration for 15, 30 or 60 min and stimulated with 100 μM SP for 30 min. As a control, cells were treated with 4.3 nM scrambled ODN final concentration for 60 min. RT-PCR analysis of the IL-6 and TNF-α mRNAs was performed as described and representative gels are shown. The mean ± S.E. of values obtained from three independent experiments are shown with $^{**}P < 0.01$ indicating significant differences.

to transcriptional activity [27]. In fact, in mast cells NFκB activity can also be regulated at the transactivation step by p38 and p42/44 MAPKs, without affecting IκB degradation, NFκB nuclear translocation or NFκB-DNA binding [5]. Our data show that in rPMC SP induces NFκB nuclear translocation, however, we have not excluded the possibility that regulation at the transactivation step may also occur.

Increased levels of pro-inflammatory cytokines have been associated with a number of neuro-immune disorders, and elevated levels of SP have often been found in these diseases [28]. The role of MAPKs and NFκB in these disorders has been poorly investigated, but it may be speculated that their activation provides a link between SP and the increased levels of cytokines that are observed in these disorders. If this is indeed the case, then drugs targeting these MAPKs and/or the NFκB pathways might afford a new target for therapy in SP-correlated neuro-immune disorders. It is of note that the lack of observed side effects, when using an NFκB decoy strategy in mast cells, suggests it as an attractive tool for new drugs designed to treat neurogenic inflammation.

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