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RESEARCH PAPER

Tachykinin receptor modulation of cyclooxygenase-2 expression in human polymorphonuclear leucocytes

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Background and purpose: We investigated the ability of natural and synthetic selective NK receptors agonists and antagonists to modulate cyclooxygenase-2 (COX-2) expression in human polymorphonuclear leucocytes (PMNs).
Experimental approach: The presence of all three tachykinin in PMNs was assessed by Western blot and PCR techniques. Natural and synthetic ligands selective for the tachykinin receptors were used to modulate COX-2 protein (measured with Western blotting) and activity [as prostaglandin E_2 (PGE_2) output]. Effects of substance P (SP) on phosphorylation of mitogen-activated protein kinases (MAPKs) and nuclear factor-kappa B (NF-κB) activation were studied to analyse the signalling pathway involved in COX-2 up-regulation mediated by SP.
Key results: Stimulation of NK receptors with the natural ligands SP, neurokinin A (NKA) and neurokinin B, in the pmol·L^{-1}-μmol·L^{-1} concentration range, modulated COX-2 expression and PGE_2 release in a concentration- and time-dependent manner. Experiments with synthetic selective agonists [Sar^9, $Met(O_2)^{11}$]SP, [β-Ala^8] NKA(4-10), senktide or selective antagonists L703,606, SR48,968 or SR142801, confirmed that COX-2 up-regulation was mediated by NK receptors. We found that mainly p38, p42 and p46 MAPKs were phosphorylated by SP and SB202190, PD98059 and SP600125, which are selective inhibitors of these kinases, blocked SP-induced COX-2 expression. SP also induced nuclear translocation of NF-κB concentration-dependently, with a maximum effect at 1 nmol·L^{-1}.
Conclusions and implications: Human PMNs possess functional NK_1, NK_2 and NK_3 receptors, which mediate the induction of COX-2 expression and NF-κB activation by SP.
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Keywords: substance P; neuropeptides; tachykinins; MAPKs; NF-κB

Abbreviations: COX, cyclooxygenase; iNOS, inducible nitric oxide synthase; JNK, c-Jun terminal NH_2 kinase; MAPK, mitogen-activated protein kinase; NF-kB, nuclear factor-kappa B; NKA, neurokinin A; NKB, neurokinin B; PGE_2, Prostaglandin E_2; SP, Substance P

Introduction

The release of neuropeptides from primary sensory nerve terminals produces an inflammatory response defined as neurogenic inflammation (Julius and Basbaum, 2001). The main neuropeptides involved in neurogenic inflammation are substance P (SP) and calcitonin gene-related peptide, which are located in a subset of sensory neurons including the lightly myelinated A delta and unmyelinated C fibres (Holzer, 1988; Richardson and Vasko, 2002). These neuropeptides, which are released from the peripheral terminals, evoke vasodilation and plasma extravasation as well as activation of pro-inflammatory cells, such as mast cells and neutrophils. These cells in turn may contribute to the inflammatory response. SP is an undecapeptide belonging to the tachykinin family, along with neurokinin A (NKA) and neurokinin B (NKB). They share a common carboxyl-terminal sequence that is required for receptor binding and activation. Tachykinins interact with three G-protein-coupled receptors, identified as NK_1, NK_2 and NK_3 receptors (nomenclature follows Alexander *et al.*, 2008). Natural ligands bind all the three receptors, and act as full agonists for each of them. However, they show differences in their affinity: SP is more selective for NK_1, NKA for NK_2, and NKB for NK_3 receptor (Regoli *et al.*, 1994). By comparing mRNA expression of tachykinin receptors in neuronal and non-neuronal cells, Pinto *et al.* (2004) concluded that the NK_1 receptor mRNA was ubiquitously expressed, while the other two receptors, NK_2 and NK_3, were mainly detected in peripheral tissues and the CNS respectively. As for the presence of NK receptors on leucocytes, particularly polymorphonuclear cells (PMNs), which is a cell type extensively involved in neurogenic inflammation, all data from the literature support the presence of NK_1 receptors on human PMNs (Dianzani *et al.*, 2001; 2003), lymphocytes (Lai *et al.*, 1998), and monocytes, as well as alveolar

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macrophages as demonstrated by Bardelli *et al*. (2005). Authors from the same group have previously demonstrated that NKA and the selective NK_2 receptor agonist [β-Ala^8] NKA(4-10) evoked a dose-dependent respiratory burst in alveolar macrophages from healthy smokers (Brunelleschi *et al*., 1996), thus suggesting the presence of functionally active NK_1 and NK_2 receptors on human alveolar macrophages. These data are in keeping with previous data obtained in guinea pig alveolar macrophages (Brunelleschi *et al*., 1990; 1992).

The presence of SP receptors demonstrated by Payan *et al*. (1984) in human peripheral blood lymphocytes was subsequently confirmed by Lai *et al*. (1998) who identified the presence of mRNA of NK_1 receptor in the same cellular types. Goode *et al*. (2000) demonstrated that NK_1 was highly expressed by human mucosal, rather than peripheral, lymphoid cells supporting the notion that SP plays a specific role in mucosal immunoregulation. The NK_2 receptors in human central airways have been identified in inflammatory cells such as T lymphocytes, macrophages and mast cells (Mapp *et al*., 2000). Experiments performed in human PMNs by Brunelleschi *et al*. (1991) and Dianzani *et al*. (2001) described the ability of micromolar concentrations of SP to enhance cell response to a given stimulus, either platelet-activating factor (PAF) or IL-8. In comparison with SP, NKA was less active and NKB did not act at all in either experimental series. In those experiments, the same concentrations of SP, given alone, were inactive. Later, Dianzani *et al*. (2003) tested the ability of SP to affect human neutrophil adhesion to an endothelial lining and discovered that SP promotes neutrophil adhesion to human umbilical vein endothelial cells (HUVEC) in a femtomolar–nanomolar range. NKA acted in the nanomolar range only, while NKB was inactive. Considered together these data suggest a wide-ranging involvement of the NK_1 receptor in regulating neutrophil activity, but they do not exclude the possibility that even NK_2 receptors may participate. It has to be stressed that SP acted differently and at different concentrations according to the functional parameters which were evaluated. Data from Dianzani *et al*. (2003) suggested that tachykinins also acted on endothelial cells. This was confirmed by Gallicchio *et al*. (2006), who showed that nanomolar concentrations of SP induced cyclooxygenase-2 (COX-2) expression in HUVEC. The concentration-dependent response was expressed as a bell-shaped curve with the maximum at 100 $nmol \cdot L^{-1}$ SP. Data obtained with selective agonists allowed us to demonstrate the presence of both NK_1 and NK_2 receptors on HUVEC. This was also confirmed by a molecular approach (mRNA and protein). These experiments suggested an experimental model to evaluate the interaction between SP and PMNs, other than those previously used, to verify whether or not SP might enhance COX-2 expression in human PMNs. This functional interaction could increase the extent of neurogenic inflammation.

Among the pro-inflammatory agents involved in this event, prostaglandins (PGs) play a crucial role. Mainly prostaglandin E_2 (PGE_2) and PGI_2 are released into peripheral tissues and onto the spinal cord during tissue injury or inflammation contributing to the development of neurogenic inflammation (Dirig and Yaksh, 1999). Biosynthesis of PGs is mediated by the rate-limiting enzyme, cyclooxygenase (COX). At least two distinct isoforms of COX have been identified, COX-1 that is responsible for PG and thromboxane production in gastric mucosa and platelets and COX-2 that is constitutively expressed in some organs, including the kidneys and brain, but which can be induced in response to several stimuli (Vane *et al*., 1998). Human PMNs are known to possess a basal level of COX-2 protein and their stimulation with different pro-inflammatory agents, such as lipopolysaccharide (LPS), TNF-α or IL-13, up-regulates COX-2 expression in a time- and dose-dependent manner, thereby demonstrating that human PMNs can synthesize COX-2 when suitably stimulated (Maloney *et al*., 1998; Yu *et al*., 1998). COX-2 protein expression induced by SP has been demonstrated in different cell types, including human dental pulp cells (Kojima *et al*., 2006), human colonic epithelial cells (Koon *et al*., 2006) and HUVEC (Gallicchio *et al*., 2006). Induction of COX-2 expression by SP in these cell types was time- and concentration-dependent, with a maximum expression at 1–100 $nmol \cdot L^{-1}$ SP. These data underline that the ability of SP to modulate the expression of COX-2 in different cellular types might be relevant to the physiopathology of different diseases.

It is known that the signalling pathway responsible for COX-2 expression involves different kinases and among them mitogen-activated protein kinases (MAPKs) play a crucial role (Tsatsanis *et al*., 2006). Three major subfamilies of these kinases have been studied in particular: the extracellular signal-regulated kinase ERK-1/ERK-2 or p42/44; the c-Jun NH_2-terminal kinase/stress-activated protein kinases (JNK/SAPK) and the p38 MAPKs. In different cell types, SP activates these kinases which represent important signalling mechanisms involved in the inflammatory process (Kyriakis and Avruch, 2001; Yang *et al*., 2002). For example, SP activates p38 MAPKs in human dental pulp fibroblasts (Tokuda *et al*., 2005), as well as in HUVEC (Gallicchio *et al*., 2006), where p42/44 are also phosphorylated by this tachykinin. Moreover, in peritoneal mast cells, SP activates both p38 and JNK MAPKs, without affecting p42/44 phosphorylation (Azzolina *et al*., 2002). The data reported above thus demonstrate a cell type-specific involvement of these kinases in mediating SP's effects. Another factor importantly involved in the regulation of COX-2 expression is nuclear factor-kappa B (NF-κB), a transcription factor known to control the expression of pro-inflammatory cytokines (Baldwin, 1996; Tsatsanis *et al*., 2006). NF-κB is activated by SP in many different cells, such as human astrocytoma cells, human T lymphocytes and human alveolar macrophages (Lieb *et al*., 1997; Marriott *et al*., 2000; Bardelli *et al*., 2005). However, these results are not reproduced in human dental pulp fibroblasts, where the neuropeptide does not show any effect on this transcription factor. This suggests, once again, that the signalling pathways are cell-dependent (Lieb *et al*., 1997; Guo *et al*., 2002; Tokuda *et al*., 2005).

The aim of this study was to determine the presence of tachykinin receptors on PMNs and to further investigate their functional role. First, the presence of receptors was detected on human PMNs at both mRNA and protein levels. Therefore, the involvement of those receptors on COX-2 expression was investigated in the presence of SP, other endogenous agonists and synthetic receptor-selective ligands. Finally, the signalling pathways involved in SP-mediated COX-2 expression in PMNs were analysed throughout the evaluation of MAPK activation and NF-κB translocation.

Methods

Cell preparation

Polymorphonuclears were isolated from heparinized venous blood obtained from healthy adult volunteers at a local hospital blood bank by using the standard techniques of dextran sedimentation, Hystopaque® 1077 gradient centrifugation and hypotonic lysis of erythrocytes (Brunelleschi *et al.*, 1991). The purity of the final cell suspension averaged 98%. PMNs were resuspended in RPMI 1640 supplemented with 10% BCS at a final concentration of 5×10^6 cells·mL^{-1}. PMNs were incubated at 37°C in a humidified atmosphere with 5% CO_2 up to 8 h. Viability, as assessed by Trypan blue exclusion, was >95% up to 5 h. Based on these results, cells were tested up to 5 h after isolation, throughout all the experiments here reported. Cell viability was not affected by compound treatment.

Cell incubation

In time-course experiments, PMNs (5×10^6 mL^{-1}) were stimulated with SP 1 nmol·L^{-1} from time 0 to 8 h and every hour cells were processed for protein extraction. In dose-response experiments, PMNs were treated with SP, NKA and NKB (1 pmol·L^{-1}–1 μmol·L^{-1}) for 5 h. To analyse agonist and antagonist effects, PMNs were stimulated with [Sar^9, Met(O$_2$)^{11}]SP, [β-Ala^8] NKA(4-10) or senktide 1 pmol·L^{-1}–1 μmol·L^{-1} for 5 h or pre-treated for 30 min with the selective NK_1, NK_2 and NK_3 antagonists, respectively, L703,606 (1-azabicyclo[2.2.]octan-3-amine,2-(diphenylmethyl)-N-[(2-idophenyl)methyl]-, (2S-cis)-, oxalate), SR48,968 (S)-N-methyl-N[4-(4-acetylamino-4-[phenylpiperidino)-2-(3,4-dichlorophenyl)-butyl]benzamide) and SR142801 (S)-(N)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl) piperidin-3-yl) propyl)-4-phenylpiperidin-4-yl)-N-methyl acetamide) at 1 nmol·L^{-1} and then incubated with SP 1 nmol·L^{-1} for 5 h. To examine the MAPK signalling pathways, PMNs were incubated with SP (1 nmol·L^{-1}) for 5, 15, 30, 45 min. In some experiments, PMNs were pre-incubated for 60 min with the irreversible MAPK inhibitors SB202190 and PD98059 (50 μmol·L^{-1}), respectively, for p38 and p42/44 MAPKs. This was followed by two washes and the PMNs were then incubated with SP 1 nmol·L^{-1} for 5 h. To evaluate the role of p46/55 MAPKs, PMNs were incubated for 60 min with SP600125 (20 μmol·L^{-1}), a reversible inhibitor of these kinases, and then for 5 h with SP (1 nmol·L^{-1}). In the 'chronic' exposure experiments, PMNs were treated with SP (1 nmol·L^{-1}), and every 2 h were washed twice and SP was re-added up to 6 h. Following the appropriate treatment, cells were lysed for Western blot analysis as described below.

Reverse transcription (RT)-PCR

Total RNA was extracted from cells using a NucleoSpin® RNA II kit and reverse-transcribed with the RevertAidTM H Minus M-MuLV Reverse Strand cDNA Synthesis kit. After denaturing at 94°C for 5 min, cDNA was subjected to PCR amplification in 50 μL PCR mix. PCR was performed using a Tpersonal 48 Whatman Biometra thermal cycler. The positive and negative strand PCR primers used were: CAAAATGATGATTGTCGTG GTGTGC and GTGAAGAGCAGTTGGAGGTCAGGTC for NK_1 (425 bp); TGCTGGTGGTGCTGACGTTTGCCATCTGCT and TGTTGACTCTCGTGGAGAGGGAGGTCGT for NK_2 (292 bp); GGCTGGCAATGAGCTCAACCATGTACAATCCCA and GGT GAGCTTATGAAACTTGAAGTGGCGGAGGCA for NK_3 (343 bp); TGACGGGGTCACCCACACTGTGCCCATCTA and CTAGAAGCATTTGCGGTGGACGATGGAGGG for β-actin (660 bp). The PCR cycle consisted of 95°C for 30 s, 63°C for 30 s and 72°C for 1 min for NK_1 amplification, 95°C for 30 s, 68°C for 30 s and 72°C for 1 min for NK_2 and NK_3 amplification and 95°C for 45 s, 60°C for 45 s and 72°C for 90 s min for β-actin amplification. The PCR products were separated by gel electrophoresis, stained with ethidium bromide, and visualized and photographed under UV transillumination. Controls containing no reverse transcriptase and no template were included. Amplicon size was verified by comparison with a DNA mass ladder.

Western blot

Polymorphonuclears were centrifuged at 259× *g* for 10 min at 4°C; pellets of ice-cold lysis buffer were added (20 mmol·L^{-1} Tris HCl pH 7.5, 150 mmol·L^{-1} NaCl, 0.1% SDS, 1% TritonX-100, 1% sodium deoxycholate, 5 mmol·L^{-1} EDTA, 1 μL·mL^{-1} protease inhibitors, 0.1 mmol·L^{-1} $ZnCl_2$, 1 mmol·L^{-1} phenylmethylsulphonyl fluoride) (Sigma, Chemical Co., St Louis, MO). Following incubation on ice for 30 min, the lysates were centrifuged at 11 093× *g* for 10 min at 4°C and the supernatant represented the total protein lysate.

To analyse NF-κB activation, after stimulation with SP 1 nmol·L^{-1} for 5 h and centrifugation (259× *g*) for 10 min at 4°C, an ice-cold homogenization buffer was added [20 mmol·L^{-1} HEPES-KOH, pH 7.9, 10 mmol·L^{-1} KCl, 1 mmol·L^{-1} EDTA, 0.2% NP-40, 10% glycerol, 1 mmol·L^{-1} dithiothreitol (DTT), 1 mmol·L^{-1} orthovanadate, 1 μL·mL^{-1} protease inhibitors], incubated on ice for 5 min and then centrifuged at 13 201× *g* for 10 min at 4°C. Supernatants consisted of the cytosolic fraction. The pelleted nuclei were resuspended in extraction buffer (20 mmol·L^{-1} HEPES-KOH pH 7.9, 10 mmol·L^{-1} KCl, 450 mmol·L^{-1} NaCl, 1 mmol·L^{-1} EDTA, 20% glycerol, 1 mmol·L^{-1} DTT, 1 mmol·L^{-1} orthovanadate, 1 μL·mL^{-1} protease inhibitors) and incubated on ice for 30 min. They were then centrifuged at 13 201× *g* for 10 min at 4°C. Supernatants consisted of the nuclear fraction. The total, cytosolic and nuclear protein concentrations of cell lysates were determined by using a BCA protein assay following the manufacturer's directions. PMNs lysate samples containing 100 μg of protein were then subjected to SDS-PAGE using an 8% or 10% gel. Proteins were transferred to a polyvinyldenedifluoride (PVDF) membrane which was then incubated with SuperBlock® blocking buffer. COX-1 and COX-2 proteins were detected following incubation with a mouse monoclonal antibody and a rabbit polyclonal antibody, respectively, and diluted at 1:200 in PBS containing 0.1% Tween-20 (PBS-T) for 2 h at room temperature. Inducible nitric oxide synthase (iNOS) protein was detected following incubation with a rabbit polyclonal antibody and diluted at 1:500 in PBS-T overnight at 4°C. Phospho-p38, p42/44 and p46/55 proteins were detected following incubation with a mouse monoclonal antibody diluted at 1:2000 in PBS-T overnight at 4°C. p65 NF-κB activation was detected following incubation with a

mouse monoclonal antibody diluted at 1:400 in PBS-T for 1 h at room temperature. NK_1 tachykinin receptors were detected following incubation with a rabbit polyclonal antibody while for both NK_2 and NK_3 a goat polyclonal antibody was utilized. The secondary antibody for detection of proteins was horseradish peroxidase-conjugated donkey anti-rabbit IgG for COX-2, iNOS and NK_1 receptor, horseradish peroxidase-conjugated donkey anti-goat IgG for NK_2 and NK_3 receptors and horseradish peroxidase-conjugated sheep anti-mouse IgG for all other primary antibodies. Secondary antibodies were diluted at 1:10 000 in PBS-T and incubated for 30 min at room temperature. To confirm the homogeneity of the protein loaded, the membranes were stripped and incubated with β-actin monoclonal antibody (1:5000) and subsequently with horseradish peroxidase-conjugated sheep anti-mouse IgG (1:10 000), both for 30 min at room temperature. The membranes were overlaid with Western Lightning Chemiluminescence Reagent Plus and then exposed to Hyperfilm ECL film. Protein bands were quantified on the films by densitometry, using the software Gel Pro.Analyzer 4.5, 2000. For quantitative evaluation of NK receptors in PMNs, we evaluated the means ± SE of the absolute integrated optical density of each receptor expression measured in all the donors.

Prostaglandin production

Prostaglandin E_2 levels were measured by the PGE_2 EIA Kit following the manufacturer's instructions.

Statistical analysis

Data were expressed as means ± SE. Statistical analysis was performed with Graphpad Prism 3.0 software. One-way analysis of variance (ANOVA) and Student-Newman-Keuls tests were used to determine significant differences between means. $P < 0.05$ was considered significant.

Materials

Dextran was from GE (Healthcare, Bio-Sciences AB, Uppsala, SE). Hystopaque® 1077, heparin, RPMI 1640, Trypan blue, SP, NKA, NKB, [Sar^9, $Met(O_2)^{11}$]SP, [β-Ala^8] NKA(4-10), senktide, L703,606, mouse monoclonal antibody against β-actin, protease inhibitors cocktail were from Sigma (Chemical Co., St Louis, MO). BCS was from GIBCO BRL (Grand Island, NY). SR48,968 and SR142801 were a kind gift from Dr X. Edmonds-Alt, Sanofi Recherche, (Montpellier, FR). The MAPK inhibitors SB202190, PD98059 and SP600125 were from Calbiochem (San Diego, CA). NucleoSpin® RNA II was from Macherey-Nagel (Düren, Germany). RevertAid™ H Minus M-MuLV Reverse Strand cDNA Synthesis kit and Taq DNA Polymerase were from Fermentas (Harrington Court, Burlington, Ontario). All primers were synthesized and purified by MGW-Biotech (Ebersberg, Germany). COX-1 mouse monoclonal antibody, COX-2 rabbit polyclonal antibody and PGE_2 EIA Kit were from Cayman Chemicals (Ann Arbor, MI). The BCA Protein Assay and SuperBlock blocking buffer were from Pierce Biotechnology, Inc. (Rockford, IL); PVDF was from Millipore (Bedford, MA). Rabbit polyclonal antibodies against human iNOS or NK_1, goat polyclonal antibodies against NK_2 or NK_3, anti-mouse, anti-goat or anti-rabbit Ig horseradish peroxidase-linked whole antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against the phosphorylated forms of p38, p42/44 and p46/55 MAPKs were from Cell-Signalling (Beverly, MA). Hyperfilm ECL was from Amersham Biosciences Corp. (Piscataway, NJ) and Western Lightning Chemiluminescence Reagent *Plus* was from PerkinElmer Life Science (Cetus, Norwalk, CT). Gel Pro.Analyser 4.5, 2000 was from Media Cybernetics Inc. (Leiden, the Netherlands), Image Pro Plus Software for microimaging (Media Cybernetics, version 5.0), GraphPad Prism 3.0 software (GraphPad software, San Diego, CA). All the other reagents used were from Sigma.

Results

Identification of tachykinin (NK) receptors on PMNs

Polymorphonuclears isolated from healthy volunteers were tested to ascertain the presence of NK receptors. All three tachykinin receptors were detected in all the samples tested, both by RT-PCR and Western blot techniques. Receptor proteins were identified by performing Western blot with commercial polyclonal antibodies against NK_1, NK_2 and NK_3 receptors. NK_1 showed a molecular weight of about 46 kDa, NK_2 of about 44 kDa and NK_3 of about 52 kDa, in keeping with the molecular weight predicted by different authors ($n = 6$; Figure 1A,B) (Gerard *et al*., 1990; Takeda *et al*., 1991; Takahashi *et al*., 1992). Quantitative evaluation of NK receptors expression demonstrated that there were no statistical differences among the quantities of each single NK receptor present on PMNs evaluated by means of the densitometric analysis performed in PMNs from all six healthy volunteers (Figure 1C).

Effects of SP on COX-1, COX-2, iNOS expression and PGE_2 release on PMNs

In a study by Maloney *et al*. (1998), a basal level of COX-2 protein was detected in freshly isolated PMNs in all the subjects analysed. Our time-course experiments demonstrated that SP at 1 $nmol \cdot L^{-1}$ increased COX-2 expression, with the maximum expression achieved following 5 h incubation ($n = 6$; Figure 2A). Thereafter, the effects of SP diminished and, after 7–8 h incubation, the COX-2 level was quite close to the basal value. Densitometric analysis of all the autoradiograms showed that SP induced an average of a 1.5-fold increase of COX-2 protein expression over the control ($n = 6$; Figure 2A). To ascertain whether the viability of isolated and suspended PMNs could affect the observed results, the effects of drug treatment on cell viability were matched with those on enzyme level ($n = 6$; Figure 2A). The percentage of cell death was recorded at each point of the time-course experiment and it was found to be less than 5%, up to the fifth hour, with a small increase thereafter ($n = 6$; Figure 2A). SP did not affect expression of COX-1 and iNOS ($n = 6$; Figure 2B). Densitometric analysis of concentration-response experiments, performed with the three natural tachykinins, SP, NKA and NKB, gave a bell-shaped curve in the $pmol \cdot L^{-1}$-$\mu mol \cdot L^{-1}$ range, with the maximum response at 1 $nmol \cdot L^{-1}$. The effect of SP

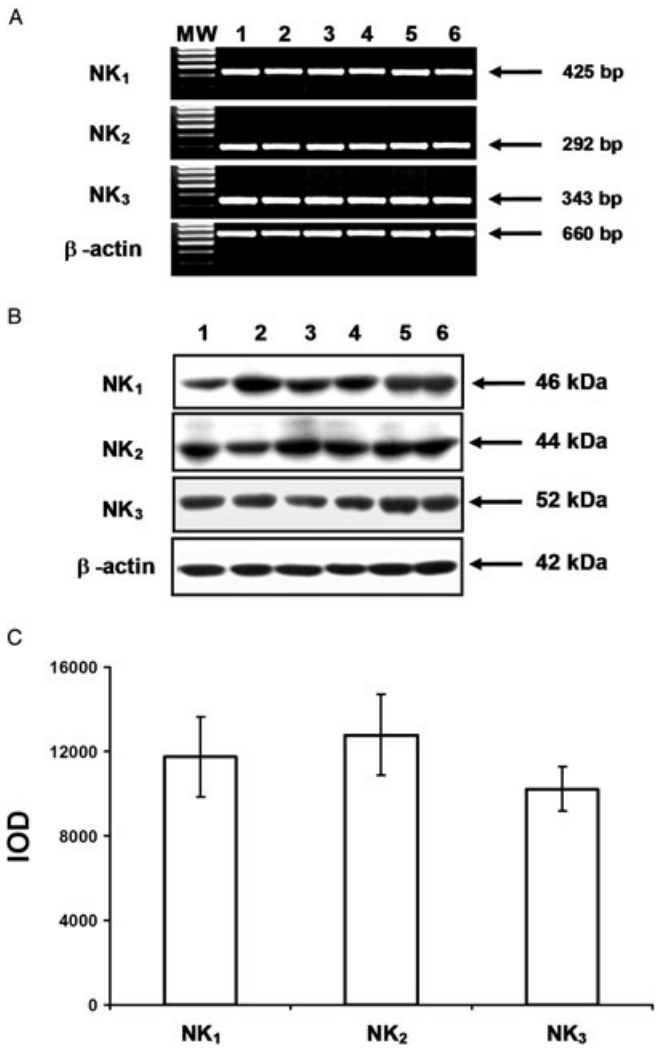

Figure 1 Identification of NK receptors on PMNs. (A) Agarose gel showing products of RT-PCR assay of PMNs isolated from six healthy volunteers. Single transcripts corresponding to the sizes predicted for NK_1, NK_2 and NK_3 receptors were detected. β-actin was used as a housekeeping gene. (B) Western blot analysis of NK_1, NK_2 and NK_3 receptors in PMNs isolated from six healthy volunteers. β-Actin was used to confirm the homogeneity of the protein loaded. (C) Quantitative evaluation of NK receptors expression by densitometry. Results represent the means ± SE of the analysis performed in six healthy volunteers, expressed as integrated optical density (IOD), for each of the NK receptors.

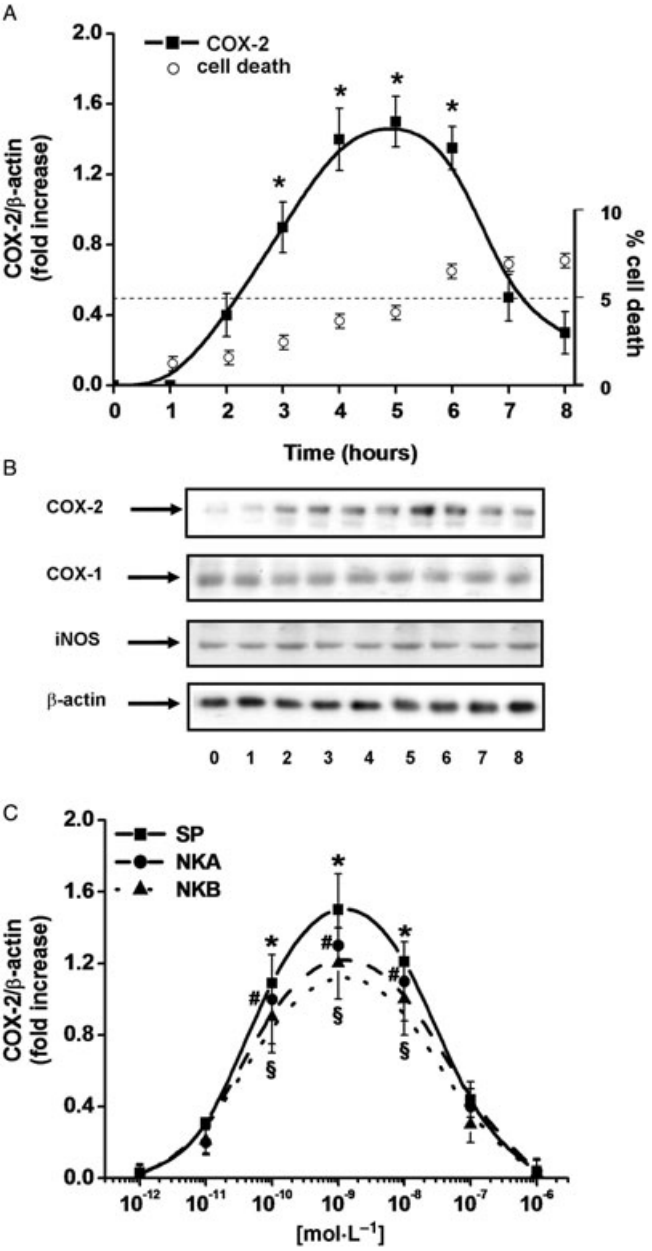

Figure 2 Time course and concentration dependency of COX-2 protein induction by SP. (A) PMNs were stimulated with SP (1 nmol·L^{-1}) from time 0 to 8 h. At the times indicated, cells were processed for Western blot analysis as described in the *Methods* section and analysed by densitometry. Viability of the cells at each hour is shown as % cell death (right hand axis). (B) Immunoblot of one experiment representing modulation of COX-2 protein expression by SP (1 nmol·L^{-1}). In the same experimental conditions COX-1 and iNOS were not affected by SP; β-actin was used to confirm the homogeneity of the protein loaded. (C) PMNs were incubated with SP or NKA or NKB (1 pmol·L^{-1}–1 μmol·L^{-1}) for 5 h, and cells were subjected to Western blot analysis as described in the *Methods* section and analysed by densitometry. Densitometric results, normalized to β-actin, are expressed as fold increases over the control value (=1), which was subtracted from all experimental values (means ± SE of six separate experiments). Data were analysed by one-way analysis of variance (ANOVA) and the Student-Newman-Keuls test (*, #, §$P < 0.05$ vs. control).

was significantly ($P < 0.05$) different from the control, and no statistical differences were found between the potencies of the three endogenous tachykinins ($n = 6$; Figure 2C).

To analyse the functionality of the expressed COX-2, we performed time-course experiments in the same experimental conditions and found that 1 nmol·L^{-1} SP induced PGE_2 release that was detectable, up to 8 h incubation. PGE_2 production did not match COX-2 protein expression perfectly as its release reduced slightly after 7–8 h incubation ($n = 6$; Figure 3A). Concentration-response experiments in the pmol·L^{-1}-μmol·L^{-1} range demonstrated that SP induces PGE_2 release at the same doses that are able to induce COX-2 protein expression ($n = 6$; Figure 3B). As the highest COX-2 protein expression was achieved at 1 nmol·L^{-1} SP and after 5 h

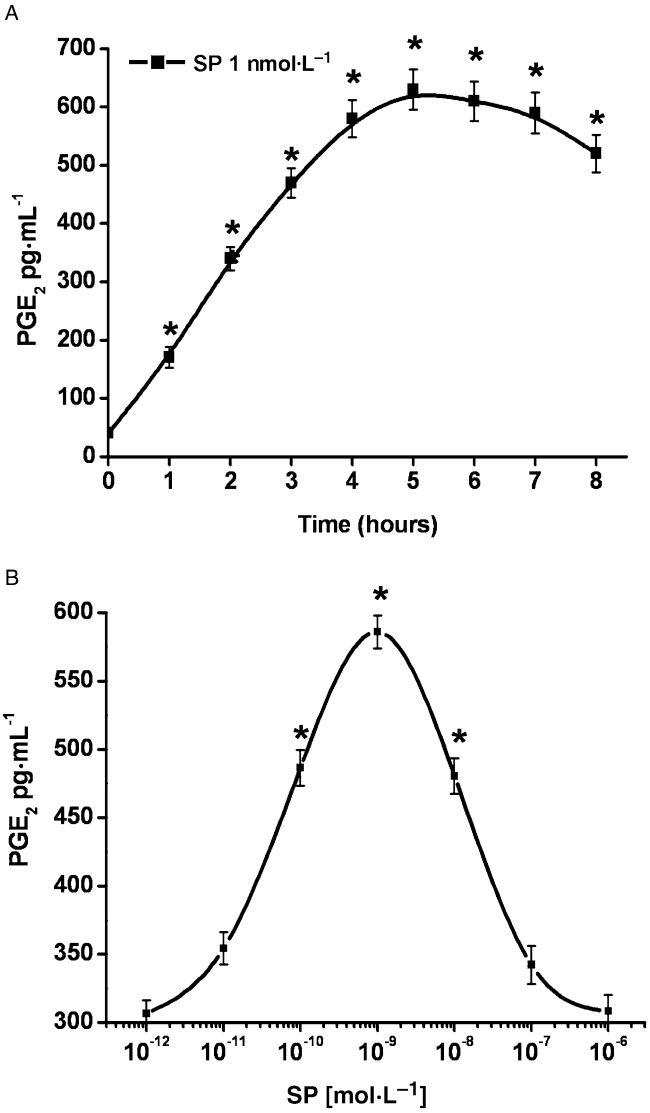

Figure 3 Time course and concentration dependency of PGE_2 release induced by SP. PMNs were stimulated with SP (1 nmol·L^{-1}) from time 0 to 8 h (A) or with SP (1 pmol·L^{-1}–1 µmol·L^{-1}) for 5 h (B) and analysed for PGE_2 release as described in the *Methods* section. The results are expressed as means ± SE of six independent experiments performed in duplicate. Data were analysed by one-way analysis of variance (ANOVA) and the Student-Newman-Keuls test. *$P < 0.05$ versus basal level of PGE_2, which was 40 pg·mL^{-1} for panel (A) and 300 pg·mL^{-1} for panel (B).

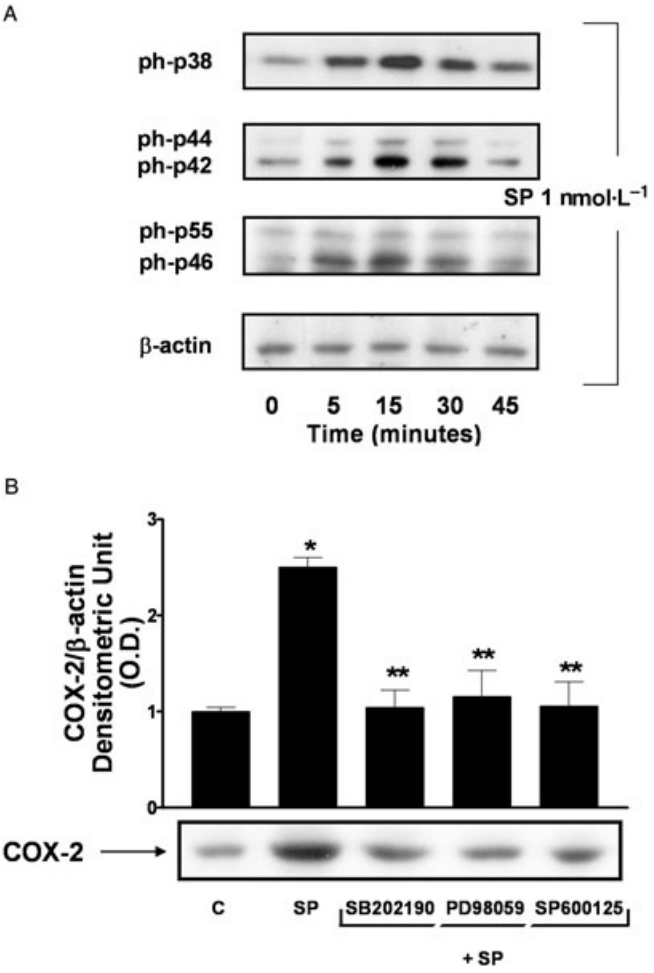

Figure 4 Effect of SP on phosphorylation of p38, p42/44 and p46/55 MAPKs in PMNs. (A) Cells were incubated with SP (1 nmol·L^{-1}) from 0 to 45 min, and processed at the time indicated for Western blot analysis of the phosphorylated forms (ph-) of p38, p42/44 or p46/55 as described in the *Methods* section. Immunoblot of one experiment representative of the six performed. β-actin was used to confirm the homogeneity of the protein loaded. (B) PMNs were pre-incubated for 60 min with the p38 MAPK inhibitor SB202190 (50 µmol·L^{-1}) or the p42/44 MAPK inhibitor PD98059 (50 µmol·L^{-1}) or the p46/55 MAPK inhibitor SP600125 (20 µmol·L^{-1}) followed by the addition of SP (1 nmol·L^{-1}) for 5 h. Cells were then processed for Western blot analysis as described in the *Methods* section. Densitometric results, normalized to β-actin, are expressed in densitometric units (O.D.) with control = 1 (means ± SE of six separate experiments). Data were analysed by one-way analysis of variance (ANOVA) and the Student-Newman-Keuls test. *$P < 0.05$ versus control; **$P < 0.01$, versus SP.

incubation, with cell viability being 95%, these experimental conditions were chosen for all the following experiments.

Effect of SP on MAPK activation

To evaluate the signalling pathways that SP might activate in PMNs, we analysed the effects of the neuropeptide on MAPK activation. In the absence of SP, a basal level of phosphorylation was detectable in all the three MAPKs families analysed. Incubation of PMNs with 1 nmol·L^{-1} SP further increased the phosphorylation of p38 and p46/55 MAPKs, in a time-dependent manner, although differences in the levels of activation were seen ($n = 6$; Figure 4A). Activation of p38 MAPK is the strongest among all the MAPKs evaluated, peaking at 15 min after SP addition and being maintained over 45 min. Both p42/44 MAPKs were phosphorylated with the maximum effect at about 15 min but with a major effect on p42 MAPKs. p46 was the most active JNK kinase after SP stimulation, while p55 JNK was only slightly activated. To further determine the specificity of SP-induced MAPK activation, we utilized selective inhibitors of these three MAPK families. When PMNs were treated with SB202190, PD98059 and SP600125, the SP-induced increase in COX-2 protein expression was consis-

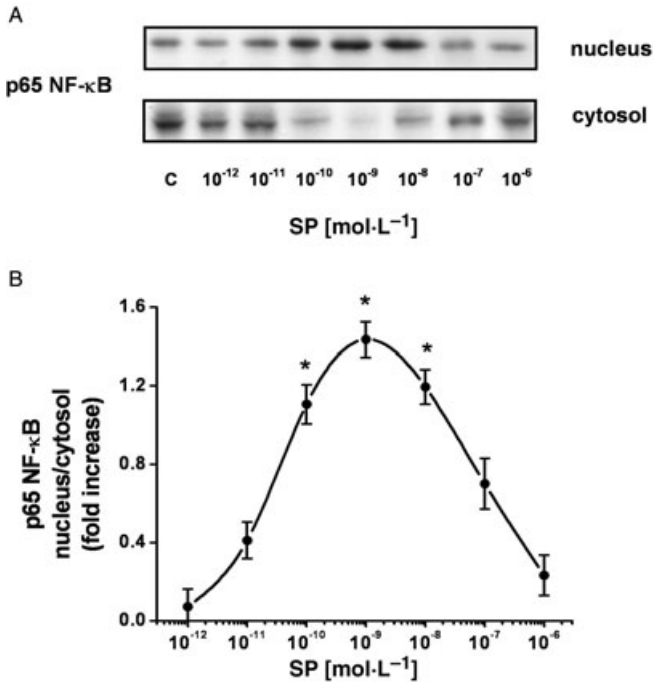

Figure 5 Effect of SP on NF-κB activation. (A) Immunoblot of one experiment representing NF-κB translocation from cytosol to nucleus in PMNs stimulated with SP (1 nmol·L^{-1}). (B) Densitometric analysis of Western blot performed to evaluate NF-κB translocation is expressed as fold increase over the control value (=1), which was subtracted from all experimental values (means ± SE of six separate experiments). Data were analysed by one-way analysis of variance (ANOVA) and the Student-Newman-Keuls test. *$P < 0.05$ versus untreated PMNs.

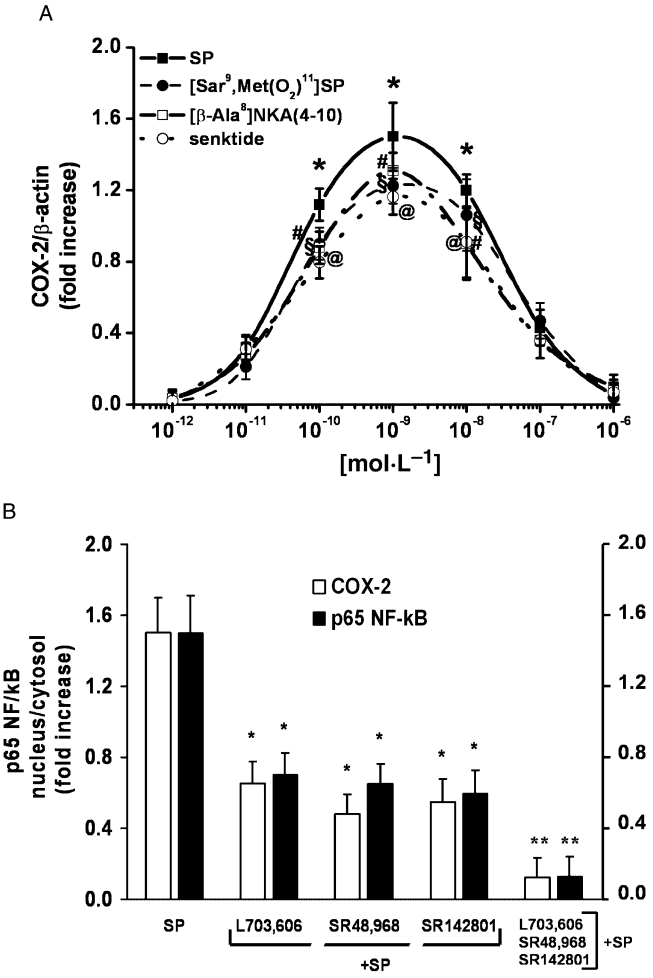

Figure 6 Effect of tachykinin receptor antagonists on COX-2 expression and NF-κB activation. (A) PMNs were stimulated with SP or [Sar^9, Met(O$_2$)^{11}]SP or [β-Ala^8] NKA(4-10) or senktide 1 pmol·L^{-1}–1 μmol·L^{-1} for 5 h and processed for Western blot analysis as described in the *Methods* section. Dentitometric results, normalized to β-actin, are expressed as fold increase over the control value (=1), which was subtracted from all experimental values (means ± SE of six separate experiments). Data were analysed by one-way analysis of variance (ANOVA) and the Student-Newman-Keuls test (*, §, #, @, $P < 0.05$ vs. control). (B) PMNs were stimulated with 1 nmol·L^{-1} SP or with SP plus L703,606 (1 nmol·L^{-1}) or SP plus SR48,968 (1 nmol·L^{-1}) or SP plus SR142801 (1 nmol·L^{-1}) or SP plus all antagonists (all 1 nmol·L^{-1}) for 5 h and processed for Western blot analysis as described in the *Methods* section. Densitometric results, normalized to β-actin, are expressed as fold increase over the control value (=1), which was subtracted from all experimental values (means ± SE of six separate experiments). Data were analysed by one-way analysis of variance (ANOVA) and the Student-Newman-Keuls test (*$P < 0.05$ vs. SP; **$P < 0.01$ vs. SP).

tently inhibited ($n = 6$; Figure 4B). SP thus appears to up-regulate COX-2 expression in PMNs through a mechanism involving all these MAPK pathways.

Effect of SP on NF-κB activation

To gain further insight in the intracellular signalling pathway that might be involved in the COX-2 expression mediated by SP, we analysed NF-κB activation. Concentration-response experiments performed on PMNs stimulated for 5 h with SP in the pmol·L^{-1}-μmol·L^{-1} range, showed a maximum of p65 NF-κB subunit translocation from cytosol to nucleus at 1 nmol·L^{-1} SP ($n = 6$; Figure 5A). Densitometric analysis of the autoradiograms gave a bell-shaped curve, with an average of a 1.5-fold increase in the translocation of p65 NF-κB translocation, as the maximum effect ($n = 6$; Figure 5B).

Effect of synthetic ligands selective for the NK receptors on COX-2 expression and NF-κB activation

Pharmacological evaluation of the three NK receptors on PMNs was performed by using synthetic selective agonists and antagonists. The NK$_1$ receptor agonist [Sar^9, Met(O$_2$)^{11}]SP, NK$_2$ receptor agonist [β-Ala^8] NKA(4-10) and the NK$_3$ receptor agonist senktide increased COX-2 expression as well as SP ($n = 6$; Figure 6A). Each selective agonist, tested at the same concentration range as SP, gave a bell-shaped concentration-response curve, close to the one depicted for SP, with a similar maximum at 1 nmol·L^{-1}. While SP can interact with all the three receptors, the data in Figure 6 indicate that the activation of only one NK receptor by its selective agonist is sufficient to obtain the same response evoked by the natural non-selective ligand. When PMNs were incubated with the most effective SP concentration, 1 nmol·L^{-1}, together with a selective antagonist of NK receptors, L703,606 for NK$_1$, SR48,968 for NK$_2$ and SR142801 for NK$_3$, at the same concentration, the neuropeptide effect on COX-2 expression and

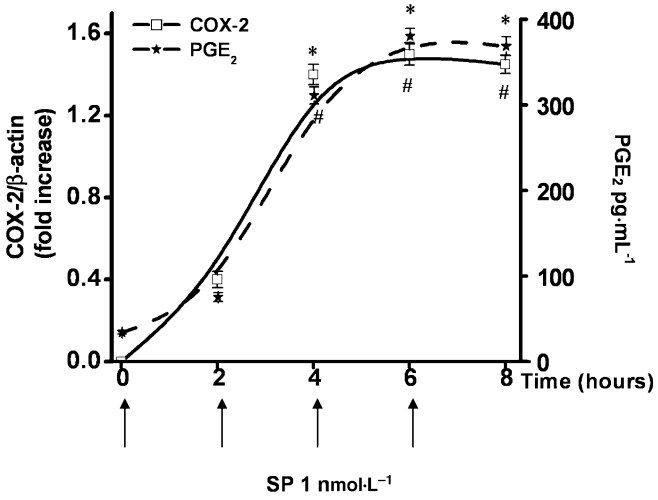

Figure 7 Effect of 'chronic' treatment with SP on COX-2 expression in PMNs. Cells were incubated with SP (1 nmol·L^{-1}), washed twice every 2 h and SP was re-added, for up to 6 h. Every 2 h, cells were processed for Western blot or PGE_2 release analysis as described in the *Methods* section. Densitometric results, normalized to β-actin, are expressed as fold increase over the control value (=1), which was subtracted from all experimental values (means ± SE of six separate experiments). Data were analysed by one-way analysis of variance (ANOVA) and the Student-Newman-Keuls test. $^*P < 0.05$ versus basal expression of COX-2; $^{\#}P < 0.05$ versus basal release of PGE_2.

NF-κB activation was significantly inhibited ($P < 0.05$) ($n = 6$; Figure 6B). Each antagonist almost halved the SP response; when all the antagonists were combined, the effect of the natural non-selective ligand was almost abolished. We performed tests with each selective antagonist demonstrating that, when given alone, they had no effect on COX-2 expression and NF-κB activation on PMNs (data not shown).

Effect of a continuous SP ('chronic') exposure

To evaluate the effect of a continuous exposure to SP on COX-2 expression and PGE_2 release in human PMNs, we replenished SP in the culture medium every 2 h, up to 6 h. Under these experimental conditions, the ability of SP to induce COX-2 expression and PGE_2 release was maintained over the whole time (8 h), until it reached a plateau, demonstrating that SP was able to maintain both COX-2 expression and activity ($n = 6$; Figure 7).

Discussion and conclusion

In this work, we have demonstrated for the first time that PMNs isolated from human healthy volunteers express NK_1, NK_2 and NK_3 tachykinin receptors without showing inter-individual differences in quantities of expression. To evaluate the role of tachykinins and tachykinin receptors on PMNs, we analysed the effects of SP on COX-2 expression. Through Western blot analysis, we demonstrated that SP increased COX-2 expression in all the samples of PMNs tested. The peak of COX-2 expression was achieved by stimulating PMNs with 1 nmol·L^{-1} SP, amounting to a 1.5-fold increase over the control after 4–6 h incubation. In no case was COX-1 affected by SP treatment. In order to evaluate another pro-inflammatory marker, we performed experiments to ascertain if SP was able to modulate iNOS expression and found that the basal level of this enzyme was not modified in PMNs exposed with SP. These results are in keeping with the data of Cedergren *et al.* (2003) who showed that iNOS was present in freshly isolated human PMNs. The lack of effect of SP on iNOS modulation was not surprising, as we found no data in the literature on iNOS expression mediated by SP in PMNs and minimal data about the effect of SP on iNOS expression in other cellular types. In particular, Jeon *et al.* (1999) demonstrated that SP by itself did not affect the basal level of nitrite production in murine macrophages, although it increased nitrite production by LPS-stimulated cells in a concentration-dependent manner (1–1000 nmol·L^{-1}).

Concentration-response experiments, performed with SP, NKA or NKB, displayed a bell-shaped curve, with the maximum COX-2 expression achieved at 1 nmol·L^{-1}. It is known that these natural ligands bind to all the NK receptors, although there are some differences in the affinity for each of them (Regoli *et al.*, 1994). However, in spite of these differences, their respective concentration-response curves were fairly similar. By using synthetic selective NK_1, NK_2 and NK_3 agonists, we were able to reproduce the entire SP concentration-response curve, with the same bell-shape and quite close maximal responses. Experiments performed with SP in the presence of synthetic selective antagonists against each NK receptor demonstrated that each single antagonist almost halved the neuropeptide effect on COX-2 expression. This result is in accordance with SP's ability to bind to each NK receptor, in spite of displaying a different affinity, thereby rendering SP able to induce a receptor activation of about 40–50%. In fact, when incubating SP with all antagonists together, the neuropeptide effect was completely reversed. Considered together, the results obtained with natural and synthetic selective agonists and antagonists suggest that the activation of only one receptor type was enough to induce a maximal COX-2 expression.

To our knowledge, the presence of NK receptors on PMNs has been previously demonstrated using a pharmacological approach. However, this has never been demonstrated at mRNA and protein level. Marazziti *et al.* (2004) failed to detect the presence of high-affinity and saturable binding sites for NK_1 in human blood lymphocytes and granulocytes, whereas the presence of NK_1 tachykinin receptors on PMNs was previously proposed by Dianzani *et al.* (2001) who provided pharmacological evidence that SP primed IL-8 activated PMNs: selective NK_1 receptor agonists and antagonists were able to reproduce or to block, respectively, the effect of SP. Moreover, employing the same experimental approach, Dianzani *et al.* (2003) demonstrated that SP mediated human PMNs adhesion to HUVEC by both NK_1 and NK_2 receptors on these cell types. Recently, and in contrast, Klassert *et al.* (2008) failed to demonstrate the presence of the mRNA for all NK receptors in PMNs, without analysing for expression of the proteins. Interestingly, these authors demonstrated the presence of NKB protein in PMNs without detecting transcription of the gene encoding for this protein, and they suggested that NKB may exert some of the biological actions attributed to SP in the inflammatory process.

According to the data obtained in this study, we can suggest that the tachykinin system in PMNs represents an unusual situation, not only for the simultaneous presence of all the three receptors on the same cell type but also for their functional responses, in terms of the induction of COX-2 protein by tachykinins. The three tachykinin receptors originate from duplication of a common ancestral gene and they show a more than 65% amino acid identity. This finding is closer to that found among subtypes of receptors that bind the same ligand than among distinct receptors binding different ligands (Regoli *et al.*, 1994; Maggi and Schwartz, 1997; Pennefather *et al.*, 2004). Gene duplication can provide a basis for developmental stability and conservation of function as a loss of one gene might be overcome by a functional substitution by a redundant gene. The system of tachykinins and tachykinin receptors constitute a typical example of duplication: all tachykinins can act as full agonists on the three corresponding receptors, which are heterogeneously distributed within each tissue with species-specific differences, suggesting that different receptors can exert similar function (Maggi, 2000; Lecci and Maggi, 2001). This evaluation could explain our data, which showed that each tachykinin receptor was able to induce a fairly similar maximal response when challenged with a specific selective agonist. The simultaneous presence of all three NK receptors has been demonstrated only in a few human tissues such as the foetal brain, prostate, skeletal muscle, gastrointestinal tract and lung (Pinto *et al.*, 2004). In the lung, all the three tachykinin receptors contributed to the onset of some respiratory diseases and to antagonise only one tachykinin receptor subtype may not be enough for the treatment of respiratory diseases (Tsuchida *et al.*, 2008). Moreover, in the gut, all the three tachykinin receptors are expressed in a cell- and region-specific manner, and particular clinical importance has been attributed to changes in expression of more than one tachykinin receptor during gastrointestinal inflammatory diseases (Improta and Broccardo, 2006). Based on these observations, a therapeutic approach currently in use is to employ an antagonist of all three tachykinin receptors and this seems to achieve a better therapeutic effect than each selective tachykinin antagonist individually (Tsuchida *et al.*, 2008). Our data are in keeping with this approach, as we have demonstrated that to completely turn off the tachykinin signal in PMNs, a single selective antagonist is not sufficient. Instead, it is necessary to expose PMNs to a combination of antagonists selective for all three tachykinin receptors at the same time.

The demonstration that 'chronic' exposure to SP was able to maintain up-regulation of COX-2 is an intriguing finding as it underlines the possible role of SP in the maintenance of the pro-inflammatory stimulus in physiopathological conditions, where a continuous SP release could participate in the prolongation of the stimulus.

We evaluated whether COX-2 expression induced by SP might translate into increased secretion of PGE_2 by PMNs. In our experimental model, induction of COX-2 following exposure to SP resulted in an increased release of PGE_2, that was time- and concentration-dependent and was maintained after COX-2 expression had fallen, reaching a plateau after 4 h. This discrepancy may be explained by the non-enzymic formation of PGE_2, as it is known that PGH_2 is an unstable endoperoxide that can be rapidly converted to PGE_2 in an aqueous solution in the absence of any PGE synthases (Nugteren and Christ-Hazelhof, 1980). This hypothesis is also supported by the 'chronic' experiments, where all the PGE_2 measured may be considered to be enzymically produced, as the washing of PMNs before adding fresh SP would limit the amounts of non-enzymically produced PGE_2 and its accumulation in the medium.

As MAPKs are known to represent a crucial step in the regulation of COX-2 expression, we performed experiments to ascertain if SP also utilized this method to induce COX-2 expression in PMNs. All MAPKs tested were activated in PMNs challenged with 1 $nmol \cdot L^{-1}$ SP. However, they showed different degrees of phosphorylation: p38, p42 and p46 JNK MAPKs were the kinases mainly activated, while p44 and p55 were only slightly modulated. The involvement of these MAPKs was further supported by the results of experiments performed with selective inhibitors. We have demonstrated the ability of all three MAPK's families inhibitors (see *Results*) to reverse SP effects on COX-2 expression. This suggests that, in our experimental model, the activation of each kinase is involved in the induction of COX-2 protein expression. To our knowledge this is the first demonstration of SP's ability to activate the MAPK pathway in PMNs, although many other pro-inflammatory stimuli are able to induce MAPKs in the same cell type. For example, LPS induced p38 activation in human PMNs (Nagano *et al.*, 2002), while TNF-α (5–50 $ng \cdot mL^{-1}$) activated p38, and JNK MAPKs in PMNs (McLeish *et al.*, 1998; Kato and Kitagawa, 2006).

It is well accepted that the expression of COX-2 is regulated by several transcription factors including NF-κB (Tsatsanis *et al.*, 2006). SP had previously been reported to activate NF-κB at nanomolar concentrations in human astrocytoma cells, murine macrophages and dendritic cells (Lieb *et al.*, 1997; Marriott *et al.*, 2000), while in another experimental model, Bardelli *et al.* (2005) showed that SP was able to induce NF-κB translocation also at 1 $\mu mol \cdot L^{-1}$ in human alveolar macrophages, demonstrating that SP acts in a cell-type and species-dependent way. Based on this observation, we decided to further investigate the signalling pathways utilized by SP by performing experiments designed to evaluate activation of NF-κB by SP. In fact, we found that SP induced NF-κB translocation in PMNs in a concentration-dependent way with a maximum at 1 $nmol \cdot L^{-1}$ concentration. As described above for COX-2, each single NK receptor antagonist was able to almost halve NF-κB activation in the presence of SP. This confirmed that the activation of only one receptor type was enough to induce a response. Notably, the concentration-response curves we obtained by measuring COX-2 induction and NF-κB activation in SP-stimulated PMNs, showed a very similar shape, thus suggesting a functional relationship between these two events.

Substance P induces different effects on PMNs, such as cell priming in the presence of different pro-inflammatory stimuli (e.g. PAF or IL-8) (Brunelleschi *et al.*, 1991; Dianzani *et al.*, 2001) or cell-to-cell interaction (Dianzani *et al.*, 2003). Many of these effects on PMNs were demonstrated or hypothesized to be mediated by NK receptors, so the demonstration of their presence and functionality is an important step in enhancing our knowledge about the tachykinin system. Modulation of

COX-2 by SP in PMNs adds to the already known action of this neuropeptide to induce COX-2 in HUVEC, stressing once again that cross-talk among peripheral sensory fibres, leukocytes and endothelial cells at the site of injury is crucial in the development of neurogenic inflammation. The ability of SP to induce COX-2 up-regulation can be correlated with the release of mediators such as PGs, allowing not only the amplification, but also a prolongation of a pro-inflammatory signal, as has been well demonstrated by our 'chronic' experiments.

In conclusion, in this paper we have demonstrated, by both a molecular and a pharmacological approach, that PMNs possess all three NK receptors, and that these receptors are functionally active as they are able to mediate COX-2 up-regulation, through the activation of MAPKs and NF-κB.

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Conflicts of interest

The authors state no conflict of interest.

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