

Tachykinin activation of human monocytes from patients with interstitial lung disease, healthy smokers or healthy volunteers

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Summary Three types of tachykinin receptors, NK₁, NK₂ and NK₃, have been described to preferentially interact with substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) respectively. Experimental evidence indicates that SP and NKA modulate the activity of inflammatory and immune cells, including mononuclear ones, and points to their involvement in lung pathophysiology. We previously reported that NK₁ and NK₂ receptors are present on monocytes (MO) isolated from healthy donors or rheumatoid patients – a greater sensitivity to NK₂ receptor stimulation was observed in the latter condition. This study evaluated the effects of SP and NKA, as well as NK₁ and NK₂ selective agonists and antagonists, on MO obtained from healthy volunteers, healthy smokers or patients with interstitial lung diseases (e.g. sarcoidosis and idiopathic pulmonary fibrosis). Superoxide anion (O₂⁻) production was chosen as a parameter of cell activation. SP and NKA dose-dependently evoked O₂⁻ production from MO in all the conditions evaluated, their effects being competitively antagonized by selective antagonists (CP 96 345 and MEN 10 627, respectively). When selective NK₁ and NK₂ agonists were used, [Sar⁹Met(O₂)¹¹]SP, a selective NK₁ agonist, induced a more than doubled O₂⁻ production in MO obtained from patients with interstitial lung diseases as compared to healthy volunteers, whereas MO isolated from healthy volunteers were more sensitive to NK₂ receptor stimulation. © 2000 Harcourt Publishers Ltd

INTRODUCTION

The mammalian tachykinins substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) are widely distributed throughout the central and peripheral nervous system, where they act as neurotransmitters or neuromodulators. Tachykinins, which are derived from prepro-tachykinins (PTT; there are three different genes, all coding for SP) and are subjected to enzymatic degradation (mainly by angiotensin converting enzyme and by neutral endopeptidase), have been implicated in a large

array of biological actions, e.g. pain transmission, neurogenic inflammation, smooth muscle contraction, vasodilation and activation of the immune system.

Their effects are largely mediated via specific G-protein-coupled receptors and at least three subtypes of tachykinin receptors (NK₁, NK₂ and NK₃) have been characterized by the rank order of potency of agonists, by using selective antagonists and by molecular cloning.¹⁻³

The undecapeptide SP, which is synthesized in primary afferent neurons and released from the terminals in response to different stimuli, is known to largely contribute to the local control of the inflammatory and immune responses. It stimulates lymphocyte proliferation and interacts with specific receptors on human T lymphocytes and cultured IM-9 lymphoblasts; degranulates mast cells and induces TNF- α mRNA and TNF- α release from human skin mast cells; modulates human neutrophil activity (by stimulating phagocytosis and

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priming PMN for an enhanced respiratory burst); induces eosinophil activation and fibroblast proliferation; acts synergistically with insulin-like growth factor-1 to enhance corneal epithelial migration; and stimulates guinea-pig peritoneal macrophages as well as rodent and human alveolar macrophages.⁴⁻¹³

Mononuclear phagocytes, either circulating blood monocytes (MO) or tissue macrophages, participate in host defense responses through their capacity to present antigens, to undergo a respiratory burst, to produce and release cytokines, eicosanoids and other soluble mediators. In 1988, it was demonstrated that human blood MO release inflammatory cytokines (such as IL-1, TNF- α and IL-6) upon challenge in vitro with low concentrations (maximal effects are obtained at 10 nM) of SP, NKA and SP (4-11).¹⁴ SP has been demonstrated to bind human MO/macrophages with high affinity and specificity, K_d value being in the nanomolar range,¹⁵ even if another group reported that a non-neurokinin SP receptor is present on MO and demonstrated that SP acted only at high micromolar concentrations.¹⁶ More recently, it has been demonstrated that NK₁ receptor are expressed in MO¹⁷ and a delta isoform of preprotachykinin mRNA has been identified in human MO and lymphocytes.¹⁸

There is some evidence that tachykinins may be synthesized in non-neural cells: human macrophages and MO express α -preprotachykinin gene (α -PPT) and SP is released by capsaicin from these cells;¹⁷ SP immunoreactivity has also been detected in human peripheral leukocytes and endothelial cells.¹⁹ In rat alveolar macrophages, α -PTT mRNA and SP-like immunoreactivity are observed in response to inflammatory stimuli, thus suggesting a possible increased SP release in inflammatory diseases.²⁰

By using selective agonists and antagonists, we previously reported that NK₂ and NK₁ receptors are present in MO isolated from healthy donors.²¹ NKA, SP and NKB dose-dependently evoked superoxide anion (O₂⁻) production: the NK₂ selective agonist [β -Ala⁸]-NKA(4-10) induced a full response, NK₃ selective agonists were inactive, while the NK₁ selective agonists septide and [Sar⁹Met(O₂)¹¹]SP had a stimulating effect.²¹ We also demonstrated that MO isolated from patients with newly diagnosed rheumatoid arthritis present a greater sensitivity to NK₂ receptor stimulation and that tachykinins enhance TNF- α mRNA expression in MO from donors and rheumatoid patients.²¹

Pulmonary diseases represent clinical conditions in which a role for tachykinins is clearly established: NKA exert potent bronchoconstrictor effects on human airways, while SP preferentially enhances plasma exudation and increases mucous secretion.^{1,2,22} NK₂ receptor expression is increased 4-fold in asthmatics, 3-fold in smokers and 2-fold in chronic obstructive pulmonary diseases

(COPD), while NK₁ receptor expression is unchanged in smokers and COPD and is increased 2-fold in asthmatics.²³ Elevated tachykinin contents have been measured in bronchoalveolar lavage from patients with pulmonary fibrosis and sarcoidosis.²⁴ Tachykinins regulate airway smooth muscle cell proliferation,²⁵ stimulate lung fibroblast proliferation and chemotaxis²⁶ and induce a respiratory burst in alveolar macrophages from sarcoid patients and healthy smokers.¹²

Pulmonary sarcoidosis is a multisystemic granulomatous disease of unknown etiology mainly affecting lung interstitium, characterized by the presence of multiple non-caseating granulomas. The preminent immunological feature is an accumulation of CD4+ T lymphocytes; alveolar macrophages, derived from circulating MO and accumulated within alveolar structures during sarcoid alveolitis, play a central role in the recruitment and activation of CD4+ lymphocytes.²⁷ Idiopathic pulmonary fibrosis is an inflammatory-fibrotic disease of unknown etiology, characterized by the accumulation of inflammatory cells in the lower respiratory tract, by alveolar epithelial injury and by progressive interstitial fibrosis²⁸ in which MO, alveolar macrophages and fibroblasts play a central role, and by releasing oxy-radicals and different cytokines.²⁹ Macrophage alveolitis in smokers is another condition in which an accumulation of activated mononuclear cells is observed: an increased number of alveolar macrophages, as well as morphophenotypical and functional pattern of macrophages similar to that observed in sarcoidosis, has been documented.^{30,31}

Therefore, we decided to evaluate the effects of SP, NKA and selective receptor agonists and antagonists on MO obtained from healthy volunteers or patients with different lung diseases (e.g. sarcoidosis, idiopathic pulmonary fibrosis, tobacco smoke-related).

MATERIALS AND METHODS

Study population

Nine patients (four male and five female), aged between 30 and 77 years, with interstitial lung diseases (ILD) were studied: four patients (1 male and 3 female) had an active sarcoidosis (SAR) and five patients (3 male and 2 female) were affected by idiopathic pulmonary fibrosis (IPF). Diagnosis was carried out on the basis of clinical, laboratory and chest X-ray data, and confirmed by histological findings on specimens from transbronchial lung biopsies during bronchoscopy. None of the patients had a medical history of asthma, and pulmonary function tests excluded actual obstruction. No patients had received steroids or other therapy at the time of the study. The other group under study was composed by four healthy smokers (HS) (all male, aged between 40 and 45 years).

Blood monocytes were also obtained from five healthy volunteers (VOL) (3 male and 2 female), aged between 28 and 58 years. This study and the research protocol were approved by a local Ethical Committee.

Isolation of peripheral blood monocytes

Peripheral blood MO were isolated from heparinized venous blood (30–40 ml) by standard techniques of dextran sedimentation (dextran T-500; Pharmacia, UK) and Ficoll-Paque (density = 1.077 g/cm³; Pharmacia) gradient centrifugation (400×g, 30 min) and recovered by thin suction at the interface. Cells were washed twice with balanced phosphate-buffered saline (PBS, pH 7.4; Sigma, UK) and resuspended at 1–2×10⁷/ml in RPMI 1640 medium, supplemented with 5% heat-inactivated fetal calf serum (Sigma), 2 mM glutamine (Sigma), 50 µg/ml streptomycin and 5 U/ml penicillin (Sigma). Cell viability, as assessed by trypan blue dye exclusion, was >98%. Purified MO populations were obtained by adhesion (see below) and assessed with the pan-macrophage monoclonal antibody anti-CD14 (Becton Dickinson, UK). Cell suspensions (100 µl) were plated in 6-well tissue culture plates (35 mm diameter, Costar, UK) and allowed to adhere for 90 min at 37°C in a humidified atmosphere containing 5% CO₂. The non adherent cells (mainly lymphocytes) were removed by three gentle washings with PBS.

Superoxide anion production

Adherent monocytes (0.6–1×10⁶/dish) were washed twice with PBS and then challenged with increasing concentrations of SP, NKA or selective tachykinin receptor agonists ([Sar⁹Met(O₂)¹¹]SP: NK₁ agonist; [β-Ala⁸]-NKA(4–10): NK₂ agonist) for 30 min. These effects were compared with those evoked by standard stimuli, e.g. the bacterial peptide N-formylmethionyl-leucyl-phenylalanine (FMLP) and phorbol 12-myristate 13-acetate (PMA), a direct activator of protein kinase C. In the experiments with tachykinin receptor antagonists (CP 96 345: NK₁ antagonist; MEN 10 627: NK₂ antagonist), MO were preincubated for 15 min with these drugs and then challenged with tachykinins.

Superoxide anion production was evaluated by superoxide dismutase (SOD)-inhibitable cytochrome C reduction. The absorbance changes were recorded at 550 nm in a Beckman spectrophotometer and expressed as nmol cytochrome C reduced/10⁶ monocytes/30 min.²¹

Experiments were performed in duplicate and control values (e.g. basal O₂⁻ production) were subtracted from all determinations. All results are expressed as mean ± SEM. Statistical analysis was performed by Student's *t*-test.

The apparent affinity (pK_b) of a given antagonist was calculated as follows: pK_b = log [DR–1] – log [B], where

DR (dose-ratio) was calculated as the concentration of the agonist required in the presence of a given concentration of antagonist to produce the same response as the concentration of the agonist in the absence of the antagonist and [B] was the molar concentration of the antagonist.

Chemicals

The compounds used and their sources were as follows: SP, NKA, [β-Ala⁸]-NKA(4–10), [Sar⁹Met(O₂)¹¹]SP and CP 96 345 (Peninsula, UK); SOD, PMA, FMLP, PBS, RPMI 1640 and cytochrome C type VI (Sigma, UK). MEN 10 627 was donated from Dr C.A. Maggi, Menarini Laboratories, Firenze, Italy.

RESULTS

Superoxide anion production evoked by standard stimuli

Blood MO were collected from four patients with active sarcoidosis (SAR), five patients with IPF, four HS and five VOL. Basal values (O₂⁻ production from unstimulated MO) were significantly higher in SAR and HS (6.91 ± 1 and 5.97 ± 0.13 nmol cytochrome C reduced/10⁶ MO/30 min respectively; *n* = 4; *P* < 0.05) than in VOL (1.32 ± 0.4 nmol cytochrome C reduced/10⁶ MO/30 min; *n* = 5) or PF (3.19 ± 1.3 nmol cytochrome C reduced/10⁶ MO/30 min; *n* = 5). These values were subtracted from those observed after challenge with tachykinins or standard stimuli to obtain the net O₂⁻ production. No significant variations among experimental groups were observed after challenge with PMA 10⁻⁷ M (a near maximal concentration): it produced 22.83 ± 0.6, 23.9 ± 1.9, 21.86 ± 2 and 22.5 ± 3 nmol cytochrome C reduced/10⁶ MO/30 min (*n* = 4–5) in HS, SAR, IPF and VOL, respectively. Similar results were also obtained with the bacterial peptide: FMLP 10⁻⁷ M produced 13.2 ± 0.6, 11.4 ± 0.9, 13.3 ± 0.4 and 8.4 ± 2 nmol cytochrome C reduced/10⁶ MO/30 min in HS, SAR, IPF and VOL respectively.

Superoxide anion production evoked by tachykinins

In the concentration range 10⁻¹²–10⁻⁶ M, the mammalian neuropeptides SP and NKA dose-dependently evoked O₂⁻ production in MO of all the four groups; as expected, maximal activation was achieved at micromolar concentration and was less than those observed by standard stimuli, reaching 3–4 nmol cytochrome C reduced/10⁶ MO/30 min.

At 10⁻⁶ M, NKA produced 2.97 ± 0.26 nmol cytochrome C reduced/10⁶ MO/30 min (*n* = 4) in cells obtained from HS; 3.56 ± 0.25 nmol cytochrome C reduced/

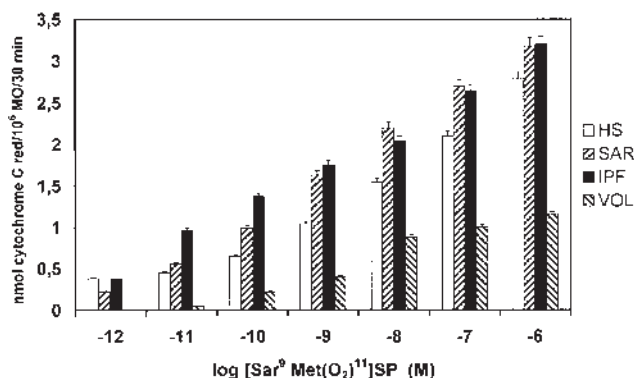


Fig. 1 Effects of the NK₁ selective agonist [Sar⁹Met(O₂)¹¹]SP on superoxide anion production in monocytes from different patients: healthy volunteers (▨), healthy smokers (□), patients with active sarcoidosis (▨) and patients with idiopathic pulmonary fibrosis (■). Mean ± SEM; *n* = 4–5. * *P* < 0.05.

10⁶ MO/30 min (*n* = 4) in SAR; 3.87 ± 0.19 and 3.36 ± 0.5 nmol cytochrome C reduced/10⁶ MO/30 min (*n* = 5) in IPF and VOL respectively. Similar effects, from a quantitative point of view, were determined by SP, which, at 10⁻⁶ M, produced 2.95 ± 0.19, 3.6 ± 0.13, 3.67 ± 0.24 and 3.02 ± 0.2 nmol cytochrome C reduced/10⁶ MO/30 min (*n* = 4–5) in HS, SAR, IPF and VOL respectively.

When selective tachykinin receptor agonists were used, the NK₂ selective agonist [β-Ala⁸]-NKA(4–10) evoked a dose-dependent (10⁻¹²–10⁻⁶ M) respiratory burst and maximal activation in the four groups (2.58 ± 0.19, 3.51 ± 0.24, 3.52 ± 0.27 and 2.89 ± 0.3 nmol cytochrome C reduced/10⁶ MO/30 min in HS, SAR, IPF and VOL respectively; *n* = 4–5) was similar to that of NKA. On the contrary, as depicted in Figure 1, the NK₁ selective agonist [Sar⁹Met(O₂)¹¹]SP showed a reduced activity in MO from VOL as compared to other groups: O₂⁻ production was significantly reduced at all the concentrations evaluated and reached only 1.15 ± 0.12 nmol cytochrome C reduced/10⁶ MO/30 min (*n* = 5) at 10⁻⁶ M (Fig. 1).

The amount of maximal respiratory burst was not significantly varied among groups and agonists (with the only exception of the NK₁ selective agonist), while the potency of each agonist was susceptible to important variations. As reported in Table 1, dealing with ED₅₀ values for mammalian tachykinins and the two selective agonists in all the four groups, significant differences were observed. Monocytes isolated from HS demonstrated a reduced sensitivity to NK₂ receptor stimulation as compared to the three other groups: ED₅₀ values were 2.54 nM for NKA and 8.43 nM for [β-Ala⁸]-NKA(4–10) in HS to be compared with 0.014 nM and 0.04 nM respectively in VOL (Table 1). Although less pronounced, differences were also recorded among groups for NK₁ receptor stimulation (Table 1).

Table 1 Effect of substance P, neurokinin A and selective NK₁ and NK₂ agonists on superoxide anion production in human monocytes

Agonists	ED ₅₀ (nM)			
	HS	SAR	IPF	VOL
NKA	2.54	0.25	0.46	0.014
[β-Ala ⁸]-NKA(4–10)	8.43	3.90	7.25	0.04
SP	1.43	1.01	1.23	0.05
[Sar ⁹ Met(O ₂) ¹¹] SP	2.62	5.53	1.8	2.15

HS: healthy smokers; SAR: sarcoidosis; IPF: idiopathic pulmonary fibrosis; VOL: healthy volunteers.

Effects of tachykinin selective antagonists on superoxide anion production

The NK₁ selective antagonist CP 96 345 and the NK₂ selective antagonist MEN 10 627, which were preincubated 15 min in our experiments, had no effect on the respiratory burst by themselves. At 1 nM, both antagonists shifted to the right (1–2 orders of magnitude, depending on the agonist and the clinical condition), the dose-response curves for the relative agonists: pK_B values for MEN 10 627 vs NKA were 10.04, 10.63, 10.41 and 10.81 in HS, SAR, IPF and VOL. When MO were stimulated by [β-Ala⁸]-NKA(4–10), the pK_B values for MEN 10 627 were 9.47, 10.55, 10.6 and 10.8 in HS, SAR, IPF and VOL.

CP 96 345 antagonized SP-evoked respiratory burst with pK_B values of 10.56, 10.27, 10.4 and 10.6 in HS, SAR, IPF and VOL. Similar values were measured when [Sar⁹Met(O₂)¹¹]SP was used to induce O₂⁻ production in MO from the four groups.

DISCUSSION

This study demonstrates that mammalian tachykinins SP and NKA, as well as the selective NK₁ agonist [Sar⁹Met(O₂)¹¹]SP and the NK₂ selective agonist [β-Ala⁸]-NKA(4–10), induce O₂⁻ production in MO obtained from human volunteers, healthy smokers or patients with ILD. All agonists act dose-dependently: maximal effects are observed at micromolar concentrations, but the respiratory burst is just detectable at concentrations as low as 10⁻¹²–10⁻¹¹ M. Selective NK₁ and NK₂ antagonists competitively antagonize tachykinin-evoked O₂⁻ production from MO, further confirming the involvement of NK₁ and NK₂ receptors.

However, according to the clinical situation of the donor (healthy volunteer, healthy smoker, patient with active sarcoidosis or patient with idiopathic lung fibrosis), some variations are observed. Since circulating MO are the precursors of alveolar macrophages, the most abundant inflammatory cell type in the lung, an altered

responsiveness of these cells might exert a profound influence at pulmonary level.

As a general rule, and with only the exception of [Sar⁹Met(O₂)¹¹]SP, the efficacy (evaluated by measuring maximal O₂⁻ production) of the agonists used is similar, while the potency (that is, the ED₅₀ of the different drugs) varies considerably, according to the clinical condition. Alveolar macrophages obtained from patients with sarcoidosis or IPF are known to have a higher respiratory burst than MO from the same individuals; but it has also been reported that MO from patients usually produce per se (that is, in the absence of added stimuli) more elevated oxy-radicals.³²

Tobacco smoke greatly affects MO responsiveness: smokers have a greater O₂⁻ release³³ and a depressed capacity to release cytokines, including TNF- α , than non-smokers.³⁴ Cigarette smoke induces the surface expression of cell adhesion molecules such as ICAM-1, ELAM-1 and VCAM-1 and favours transendothelial migration of MO.³⁵ In rodents, tobacco smoke enhances airway responsiveness to SP, mainly by inactivating neutral endopeptidase,³⁶ stimulating primary afferent sensory nerves and releasing tachykinins in the lung.^{37,38}

However, according to our results, MO from healthy smokers present a reduced sensitivity to NK₁ and NK₂ receptor stimulation (as compared to MO from volunteers or IPF patients), as evidenced by the higher ED₅₀ values: we have no definite explanation for this fact, but some attempts can be afforded. Endogenous SP (about 20–50 pg/3 \times 10⁶ cells) is released by freshly isolated human blood MO and 7 to 10 day cultured macrophages:¹⁷ cigarette smoke could potentiate this SP release (as it occurs in guinea-pig lung³⁷), probably leading to desensitization and/or down-regulation.

Mutual interactions between TNF- α and tachykinins have been described in different models³⁹ and TNF- α was shown to induce SP expression by sympathetic ganglia:⁴⁰ since TNF- α is reduced in MO from smokers,³⁴ this cytokine could be implicated. Moreover, a recent paper by Emms and Rogers⁴¹ demonstrates that, in the guinea-pig, cigarette smoke exposure reduced the magnitude of subsequent bronchoconstriction induced by NKA by 40%, in keeping with the results reported here.

Conversely, if MO are capable to release SP (as well as to be activated by neurokinins) and this tachykinin, together with NKA, induces airway smooth muscle and fibroblast proliferation, important effects on IPF could be envisaged. As a matter of fact, MO obtained from patients with sarcoidosis or IPF present (these data) a good sensitivity to both NK₁ and NK₂ receptor stimulation.

It is also worth noting that the apparent affinities (pK_B) of the two selective antagonists measured in our experiments (around 10) are higher than the corresponding affinities measured in other models:¹ however, this is not

surprising, because the affinities of natural agonists, too, are elevated. Furthermore, such a situation has been previously documented in guinea-pig alveolar macrophages, which are largely derived from circulating MO.⁴² In conclusion, these data demonstrate that NK₁ and NK₂ receptor stimulation trigger superoxide anion production in MO from healthy donors, healthy smokers and patients with IPF.

The small number of patients evaluated for each clinical condition does not allow a definitive appraisal: more experiments are required to do so. However, these data suggest that sensitivity to a given tachykinin could vary according to different clinical conditions.

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