Effect of substance P on cytokine production by human astrocytic cells and blood mononuclear cells: characterization of novel tachykinin receptor antagonists

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Abstract Substance P (SP) has been reported to induce inflammatory cytokine production in human neuroglial cells and peripheral lymphoid cells as well. In order to evaluate the potency of novel non-peptide antagonists of the tachykinin receptors as inhibitors of SP-induced cytokines, we used the astrocytoma cell line U373MG and blood mononuclear cells as models of central and peripheral SP-target cells, respectively. In the first part of this study, we showed that SR 140333, an NK1 tachykinin receptor antagonist, was able to inhibit strongly the SP-induced production of interleukin (IL)-6 and IL-8 in the astrocytoma cell line. The antagonistic activity of SR 140333 toward SP-induced cytokine production was specific and could not be attributed to a general anti-cytokine effect, since cytokine release induced by another inflammatory protein such as IL-1β was not blocked by this compound. In addition, NK2 and NK3 agonist neuropeptides were at least 1000-fold less effective than SP, while SR 48968 and SR 142801 which are selective NK2 and NK3 receptor antagonists, respectively, displayed a 2.5–3 orders of magnitude lower inhibitory potency than SR 140333. All these data indicated that SR 140333 blocked SP-induced cytokine production in U373MG astrocytic cells via a specific NK1 receptor-mediated process. Since SP has also been described to trigger peripheral blood mononuclear cells (PBMNC) or monocytes to release inflammatory cytokines, we attempted, in the second part of this study, to evaluate the potential antagonistic effect of our compounds on these cells. Experiments on human PBMNC from different donors were carried out to determine first their pattern of cytokine production upon SP stimulation. Surprisingly, we noticed that SP at concentrations ranging from 0.1 to 1000 nM was unable to stimulate the release of any inflammatory cytokine tested. This raises the question of the specificity of the reported in vitro effects of SP on cytokine production by human peripheral immune cells.

Key words: Substance P; Tachykinin receptor antagonist; Cytokine; Astrocyte; Monocyte; Mononuclear cell

1 Introduction

Substance P (SP) is a neurotransmitter, member of the tachykinin family of neuropeptides. These peptides released from primary sensory nerves exert their actions through activation of G-protein coupled receptors, denoted neurokinin 1 (NK1), NK2, NK3 and having preferential affinity for SP, neurokinin A (NKA) and neurokinin B (NKB) respectively [1]. In addition to being a key mediator of pain, SP may contribute to the pathophysiology of neuronal inflammation in humans by stimulating cytokine production in the brain. Indeed, functional SP receptors have been identified in mouse, rat and human astrocytes [2–4] and these cells play important auxiliary roles in immune and inflammatory responses in the central nervous system (CNS). They act as antigen-presenting cells and produce cytokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, in response to inflammatory mediators [5]. Thus, SP-stimulated production of inflammatory cytokines by astrocytes could exacerbate inflammatory processes in the CNS and be involved in different neuropathologies. In this regard, the discovery of a high density of SP-immunoreactive astrocytes in multiple sclerosis plaques [6] is of particular importance.

Using the U373MG cell line which expresses high levels of glial acidic fibrillary protein and functional SP receptors [7], as a model of human astrocytes, we sought to evaluate whether the novel non-peptide NK1 receptor antagonist SR 140333 [8] was able to interfere with SP-triggered inflammatory cytokines in human astrocytes. For comparison, we have also studied the effects of SR 48968 and SR 142801 which are selective NK2 and NK3 receptor antagonists, respectively [9,10].

In addition to being a potent inducer of proinflammatory cytokines in the CNS, SP has also been reported to act at the periphery by stimulating the production of these mediators in human peripheral blood mononuclear cells (PBMNC) [11–13]. With regard to the importance that this peripheral influence could have in chronic inflammatory diseases and the beneficial effects that specific SP antagonists could represent, in the second part of this study we evaluated the impact of SP on the secretion of several 'acute-phase' proinflammatory cytokines from peripheral mononuclear cells of different donors.

2. Materials and methods

2.1. Peptides, cytokines and SR compounds

SP, NKA, NKB, were purchased from Bachem (Voisins-le-Bretonneux, France). Stock solutions of peptides (2 mM) were stored as frozen aliquots in pyrogen-free water. Recombinant human IL-1β was obtained from Genzyme (Paris, France). SR 140333, SR 48968 and SR 142801 [8–10] were synthesized at Sanofi Recherche (Montpellier, France) and stock solutions (2 mM in ethanol) were stored at −20°C. SR compounds were diluted to the appropriate concentrations in culture medium. The proportion of ethanol in culture media did not exceed 0.01% (v/v), which was shown to have no effect on cytokine induction.

2.2. Cell preparations and cultures

The human astrocytoma cells U373MG were obtained from the American Type Culture Collection (Rockville, MD, USA). They were grown in Dulbecco's modified Eagle's culture medium supple-

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mented with 10% fetal bovine serum (FBS), penicillin (50 U/ml) and streptomycin (50 μg/ml). PBMNC were isolated from heparinized blood, obtained from healthy donors, by centrifugation over Ficoll Paque (Pharmacia Biotech, Uppsala, Sweden). For cytokine induction, U373MG cells were plated in sextuplicate at $0.5–1 \times 10^5$/well in 96-well microplates and incubated overnight in medium with 1% FBS. Cells were then washed twice with serum-free medium and treated in a total volume of 200 μl with the various stimuli in 1% FBS culture medium containing 15 μg/ml of the endotoxin inhibitor polymyxin B sulfate (Sigma, Saint Quentin Fallavier, France) for an additional 18–24 h. PBMNC were identically treated except for the overnight preincubation and for the presence of polymyxin when LPS (*E. coli* 055:B5, Sigma) was used as stimulus. Cell-free supernatant samples were stored at −20°C prior to cytokine assays.

2.3. Cytokine assays

IL-1β, IL-6, IL-8, and TNFα, were measured using ELISA kits (R&D Systems, Abingdon, UK) according to the manufacturer's specifications. Data were expressed as mean ± S.D. of triplicate assays performed with supernatants collected and pooled from six wells. Unless otherwise stated, each experiment was performed at least twice.

3. Results

3.1. SR 140333 blocks SP-induced inflammatory cytokines in human astrocytes

In accordance with previous data [7], SP induces in U373 MG astrocytoma cells a concentration-dependent release of IL-6 with an EC_{50} of 3 nM (Fig. 1A). In addition, we showed for the first time that SP was also able to stimulate IL-8 secretion by these cells within the same range of concentrations (Fig. 1B). In the presence of SR 140333, production of both IL-6 and IL-8 induced by 10 nM SP was maximally inhibited for concentrations of antagonist ranging from 10^{-7} to 10^{-6} M and IC_{50} was evaluated in both cases at about 2 nM (Fig. 1C,D). SR 140333 alone did not affect cytokine release (not shown). The inflammatory cytokines IL-1 and TNFα were not detected in the supernatants of SP-stimulated U373MG cells and it is noteworthy that SP-induced cytokine production by these cells is not mediated by undetectable amount of IL-1 [14].

Astrocytes are, however, sensitive to stimulation by IL-1β, a cytokine which could also play a role in some neurologic diseases as an inducer of inflammatory cytokines [5]. We therefore evaluated the impact of a cotreatment with both SP and IL-1β. As shown in Fig. 2, IL-1β alone increased IL-6 secretion in a concentration-dependent manner. What we showed in this study is that the presence of 100 nM SP potentiated the effect of IL-1β at all concentrations tested. The addition of 1 μM SR 140333 to the cells cotreated with IL-1β and SP allowed suppression of the enhancing effect of SP without affecting the stimulating effect of IL-1β. Indeed, SR140333 reduced IL-6 levels in cultures costimulated with both stimulating agents to those observed with IL-1β alone. This clearly indicated that SR 140333 specifically blocked cytokine production induced by SP without interfering with another stimulus. The SP-receptor specificity of cytokine inhibition by SR 140333 was further strengthened by comparative studies with specific NK2 and NK3 agonists and antagonists.

3.2. SR 140333 inhibits cytokine production via a specific NK1 receptor-mediated process

Extensive binding studies in different models [8,15,16] have demonstrated that SR 140333 is a highly selective NK1 receptor antagonist. On a functional basis, the experiments depicted in Fig. 3 confirmed that profile in the model of cytokine induction in astrocytoma cells. First, the NK1 agonist SP was compared to NKA and NKB which are selective agonist neuropeptides of NK2 and NK3 receptors, respectively. Fig. 3A shows that SP was 3–3.5 orders of magnitude more potent than either NKA or NKB as an IL-6 inducer. Second, SR 140333 was compared to SR 48968 and SR 142801 which are selective and potent antagonists of NK2 and NK3 respectively [9,10]. As shown in Fig. 3B, IL-6 inhibition by SR 140333 was 2.5–3 orders of magnitude more potent than that obtained

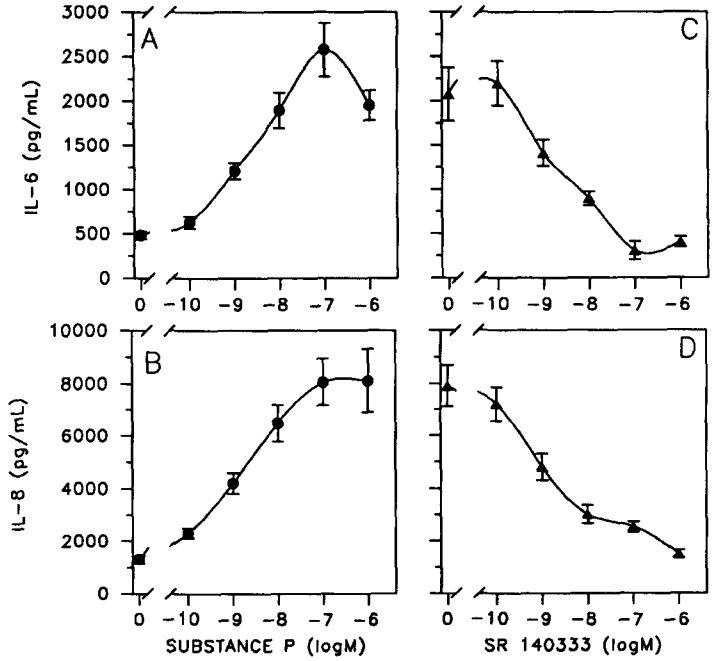

Fig. 1. Effects of SP and SR 140333 on IL-6 and IL-8 production by U373MG cells. Cells were incubated as mentioned in Section 2 with the indicated concentrations of SP (A,B) or simultaneously treated with 10 nM SP and the indicated concentrations of SR 140333 (C,D).

with either NK2 or NK3 receptor antagonists. These results strongly argued for a selective involvement of NK1 receptors in cytokine induction from SP-stimulated U373 MG astrocytoma cells.

3.3 Effect of SP on cytokine release by human PBMNC

SP has been reported to induce at the periphery, on human monocytes or mononuclear cells in general, an effect similar to that described above on astrocytes [11–13]. In order to evaluate our different neurokinin antagonists on these peripheral target cells, we first evaluated the impact of SP on different inflammatory cytokines produced by human PBMNC. A typical experiment is depicted in Fig. 4 where IL-1β, IL-6 and TNFα were measured. Whereas LPS (100 ng/ml), a well-known inflammatory cytokine inducer, triggered PBMNC to release the three cytokines, no significant effect of SP, at concentrations ranging from 10^{-10} to 10^{-6} M, was in any case observed above basal level. Furthermore, the peripheral blood cells of three other donors identically treated demonstrated the same high sensitivity to LPS and the same absence of response to SP (not shown).

Since direct treatment of PBMNC with SP had no effect, we next studied its potential effect on cells costimulated with concentrations of LPS ranging from 0.1 to 100 ng/ml. As shown in Fig. 5, LPS alone, as expected, induced a concentration-dependent increase in IL-6 release by human PBMNC. Cotreatment with 1 μM SP was devoid of any effect even at the lowest suboptimal concentrations of LPS.

4. Discussion

In this study we clearly showed that SR 140333 potently and selectively inhibited the inflammatory cytokines IL-6 and IL-8 induced by SP-stimulation of the NK1 receptor in the human astrocytoma cell line U373MG. In the CNS, the induction of inflammatory cytokines by SP-stimulated astrocytes may be involved in the pathophysiology of inflammatory neurodegenerative disorders. SP-immunoreactive astrocytes have been localized to multiple sclerosis plaques and may exacerbate immune-mediated oligodendrocytes destruction through the local release of cytokines [6]. IL-6 has been localized to senile plaques in the brain of Alzheimer's disease (AD) patients and accumulating evidence suggests that this cytokine may mediate neurodegeneration by potentiating a local acute-phase response [17–19] including the production of amyloid precursor protein (APP) and protease inhibitors such as α_1-antichymotrypsin and α_2-macroglobulin. These two potent inhibitors prevent normal APP processing and favor the pathological degradation of APP into β-amyloid plaques [17,19]. IL-6 has also been shown to induce nerve growth factor (NGF) which potentiates the neurotoxicity of β-amyloid peptides and contribute to neuritic plaques [20,21]. IL-1β, which has also been found in AD brains, is another cytokine which could play a major role in neurodegeneration by inducing astrocytosis and stimulating IL-6 release from astrocytes [22]. In this respect, the observation made in this study that SP induced additional IL-6 release in IL-1β-stimulated astrocytes is of particular importance. Thus, SP as intrinsic inducer or enhancing agent of inflammatory cytokines involved in neurodegenerative diseases may participate in the pathological process of such disorders. With regard to the understanding of the pathophysiological responses specifically induced or enhanced by SP in the brain, the availability of potent SP antagonists such as SR 140333 can be of major interest.

In addition to its well-documented effect on inflammatory cytokine release by cells of the CNS, SP has also been reported to stimulate at the periphery the production of these mediators by human blood monocytes or mononuclear cells [11–13]. We therefore sought to evaluate the potency of our compound SR 140333 on these SP-stimulated peripheral target cells. Surprisingly, in our hands, no effect of SP, alone or associated with suboptimal concentrations of LPS, was observed on IL-1, IL-6 and TNFα production from human per-

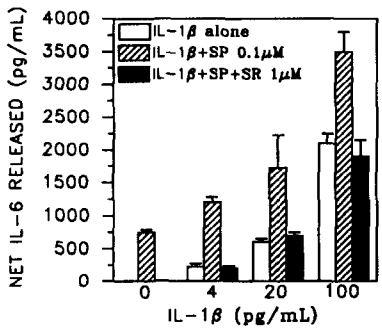

Fig. 2. Potentiation by SP of IL-1β-induced IL-6 secretion in U373MG cells. Cells were treated with increasing amounts of IL-1β or costimulated with the indicated concentrations of IL-1β and 0.1μM SP in the presence or absence of 1 μM SR 140333. Results are expressed as net cytokine release after substraction of basal level (540 ± 62 pg/ml).

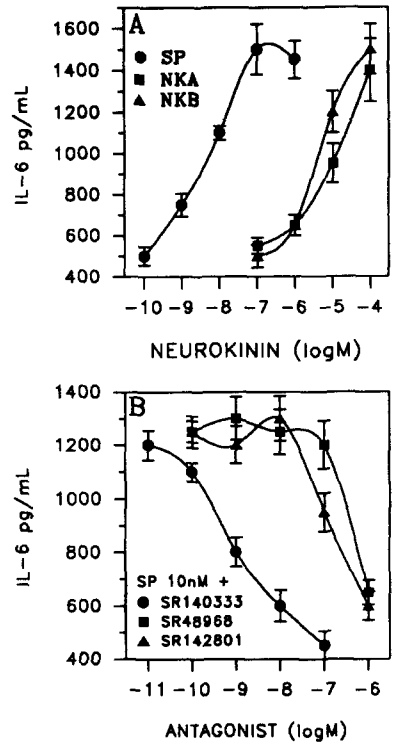

Fig. 3. Effects of different selective neurokinin-receptor agonists or antagonists on IL-6 production. U373MG cells were treated with various concentrations of the following agonists (A): SP (NK1), NKA (NK2) and NKB (NK3), or cotreated with 10 nM SP and the following antagonists (B): SR140333 (NK1), SR 48968 (NK2) and SR 142801 (NK3) (basal level = 430 ± 45 pg/ml).

ipheral blood cells of different donors. Under similar conditions, using purified monocytes instead of total mononuclear cells, and measuring IL-1β secretion as a marker of inflammatory response upon SP stimulation, we noted the same absence of effect of the neuropeptide (not shown). These results are in contrast to the reports mentioned above indicating that SP, at nanomolar concentrations, can directly stimulate cytokines such as IL-1, TNFα and IL-6 in peripheral blood monocytes [11,12] or mononuclear cells [13]. Our data are rather in agreement with the fact that no NK1 receptor has been detected on human peripheral monocytes or B-cells [23,24]. Recently, Jeurissen et al. [24], using membrane preparations from human monocytes, have found a 'non-classical' SP receptor which binds SP with an apparent affinity of 224 nM which is almost 800-fold lower than the binding affinity of SP reported on the U373MG astrocytes (K_d = 0.29 nM) [16]. The same group showed at a functional level that the stimulation by SP of this non-classical receptor on human monocytes leads to the secretion of IL-6 only at 30 and 100 μM SP [25]. These high micromolar concentrations do not correlate the apparent affinity of SP for this 'atypical' receptor. Such high concentrations of SP are more in accordance with the K_d for non-specific binding of SP to lipid bilayers of the membranes (2.7–220 μM) [26]. This non-specific and receptor-independent effect of SP has been shown to activate

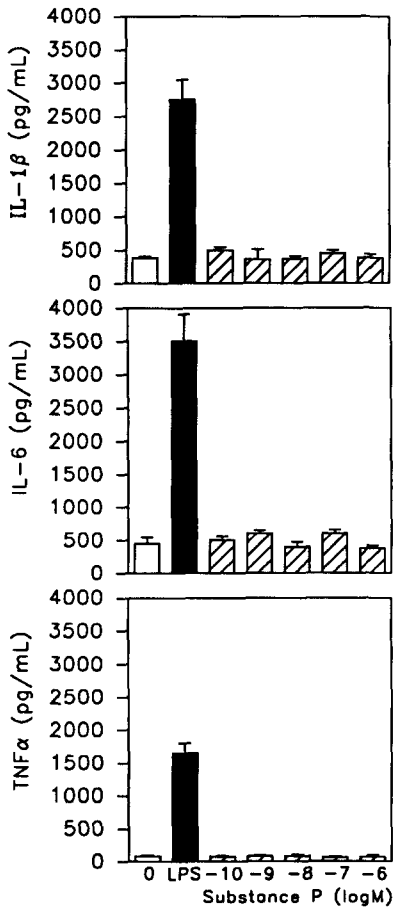

Fig. 4. Absence of effect of SP on the secretion of IL-1β, IL-6 and TNFα by human peripheral blood mononuclear cells. Human PBMNC were cultured with the indicated concentrations of SP. LPS as positive control was used at 100 ng/ml.

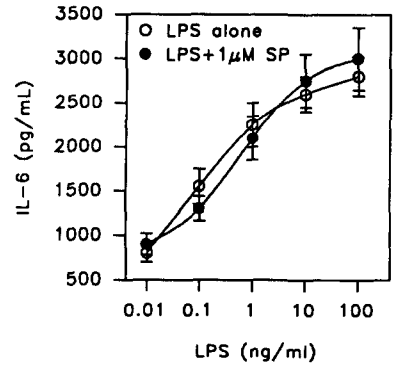

Fig. 5. Effect of the association SP and LPS on IL-6 release by human PBMNC. Cells were treated with the indicated concentrations of LPS alone or associated with 1 μM SP (basal level = 525 ± 55 pg/ml).

directly G-proteins in the cytosolic compartment [27] and has been proposed to be the basis for the SP-induced histamine release in mast cells [28] and the intracellular rise of Ca^{2+} in T-lymphocytes [29]. All these reports demonstrate that several levels of discrepancies exist about both the type of SP binding structure on monocytes and the range of SP concentrations required to stimulate them. Although our data do not negate or support the existence for a classical or an atypical SP-receptor, we clearly and repetitively demonstrated that SP at physiologically relevant concentrations was unable to stimulate the secretion of inflammatory cytokines by peripheral lymphoid cells.

In contrast to its absence of effect on these peripheral targets, SP has been confirmed in this study, in a cellular model representative of astrocytes of the central nervous system, as a very effective inducing or potentiating agent of this type of mediators. Functional SP receptors have been identified in mouse and rat primary astrocytes [30,31] and enhanced secretion of inflammatory cytokines has been demonstrated upon SP stimulation of these cells [32,33]. This indicates that the pro-inflammatory effect of SP observed on astrocytoma cell lines can be extended to normal primary astrocytes. With regard to the possible deleterious effect of SP-stimulated inflammatory mediators in certain brain diseases, drugs such as SR 140333 that interfere with SP-driven neurological responses may represent a new therapeutic approach to cytokine-mediated neuropathologies. This may have particular relevance to Alzheimer's disease as amyloid plaques are found in the highest density in the olfactory system [34], an area of the brain also richly innervated by SP-containing neurons [35,36] and where local overproduction of inflammatory cytokines by glial cells could mediate acute phase protein production and amyloidogenesis [19].

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