British Journal of Pharmacology (2008) 154, 144–152
 0007–1188/08 $30.00
www.brjpharmacol.org

RESEARCH PAPER

A novel activity for substance P: stimulation of peroxisome proliferator-activated receptor-γ protein expression in human monocytes and macrophages

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Background and purpose: Substance P (SP) and peroxisome proliferator-activated receptor-γ (PPAR-γ) play important roles in different inflammatory conditions and are both expressed in human monocytes and macrophages. However, it is not known whether or not they interact. This study was undertaken to evaluate the effects of SP on PPAR-γ protein expression in monocytes and macrophages (MDMs: monocyte-derived macrophages) from healthy smokers and non-smokers.
Experimental approach: PPAR-γ protein was detected by western blot and quantified by calculating the ratio between PPAR-γ and β-actin protein expression. Constitutive tachykinin NK_1 receptor expression in monocytes and MDMs from healthy smokers and non-smokers was evaluated by western blot. Cytokine release was evaluated by ELISA.
Key results: In the concentration range 10^{-10}–10^{-6} M, SP stimulated PPAR-γ protein expression in monocytes and MDMs, being more effective in cells from healthy smokers. Moreover, in these cells there was a constitutively increased expression of NK_1 receptors. SP-induced expression of the PPAR-γ protein was receptor-mediated, as it was reproduced by the NK_1 selective agonist [$Sar^9Met(O_2)^{11}$]SP and reversed by the competitive NK_1 antagonist GR71251. SP-induced maximal effects were similar to those evoked by 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; an endogenous PPAR-γ agonist, and were significantly reduced by a PPAR-γ antagonist. NK_1 and PPAR-γ agonists exerted opposite effects on TNF-α release from monocytes and MDMs.
Conclusions and implications: Enhancement of PPAR-γ protein expression represents a novel activity for SP, which could contribute to a range of chronic inflammatory disorders.
British Journal of Pharmacology (2008) **154,** 144–152; doi:10.1038/bjp.2008.50; published online 18 February 2008

Keywords: substance P; PPAR-γ; monocytes; macrophages; NK_1 receptors; GR 71251; PGJ_2; TNF-α; tobacco smoke

Abbreviations: GR71251, [D-Pro^9,(spiro-γ-lactam)Leu^{10},Trp^{11}]substance P; GW9662, 2-chloro-5-nitrobenzanilide; MDM, monocyte-derived macrophage; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; PPAR-γ, peroxisome proliferator-activated receptor-γ; SP, Substance P

Introduction

Although substance P (SP) was originally described as a peptide of neuronal origin, studies in rodents and humans demonstrated its production by inflammatory cells (for example, macrophages, eosinophils, lymphocytes and dendritic cells) and suggested that this neuropeptide could be an autocrine, paracrine or endocrine regulator (Maggi, 1997; Severini *et al*., 2002; O'Connor *et al*., 2004). In monocyte/macrophages, SP stimulates the release of both arachidonic acid metabolites and proinflammatory cytokines, induces the respiratory burst and acts as a potent chemoattractant (Lotz *et al*., 1988; Brunelleschi *et al*., 1990, 1998; O'Connor *et al*., 2004; Bardelli *et al*., 2005), most of the proinflammatory effects of SP being mediated by NK_1 receptors. We previously reported that SP and selective NK_1 agonists induce superoxide anion production, tumour-necrosis factor (TNF)-α release (as well as an enhanced TNF-α mRNA expression) and triggers activation of nuclear factor-κB in human monocytes and alveolar macrophages (Brunelleschi *et al*., 1998; Bardelli *et al*., 2005). Interestingly, very relevant increases in NK_1 receptor expression (>threefold), TNF-α release (about fourfold) and nuclear factor-κB nuclear translocation (threefold) were documented in alveolar macrophages from healthy smokers as compared with non-smokers (Bardelli *et al*., 2005).

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Received 14 November 2007; accepted 28 January 2008; published online 18 February 2008

The peroxisome proliferator-activated receptor-γ (PPAR-γ), a key regulator of adipocyte differentiation, lipid storage and glucose metabolism, is expressed in a wide variety of cells, including monocytes, macrophages and foam cells (Ricote *et al*., 1998b; Tontonoz *et al*., 1998; Amoruso *et al*., 2007). Even if a recent meta-analysis raised some concerns about the serious cardiovascular effects of rosiglitazone treatment in type II diabetes patients (Nissen and Wolski, 2007), PPAR-γ agonists have been proposed as possible anti-inflammatory drugs.

We previously showed that PPAR-γ protein is constitutively present in human monocytes and that its expression is upregulated along with differentiation to monocyte-derived macrophages (MDMs); moreover, monocytes and MDMs from healthy smokers presented a significantly enhanced constitutive PPAR-γ expression, as compared with healthy non-smokers (Amoruso *et al*., 2007).

Despite the fact that both PPAR-γ and NK_1 receptors are expressed at significant levels in human monocyte/macrophages, it is not known whether or not they interact with each other.

Therefore, to evaluate such a possibility, we examined the ability of SP, as well as the selective NK_1 agonist [Sar^9 $Met(O_2)^{11}$]SP and the NK_1 antagonist GR71251, to modulate PPAR-γ protein expression in human monocytes and MDMs from healthy smokers and non-smokers. We demonstrated that, in a concentration-dependent manner, SP stimulated PPAR-γ protein expression in both cell types and that this effect was potently reduced by a PPAR-γ antagonist or an NK_1 antagonist. We also report here that SP and PPAR-γ ligands exerted divergent effects on TNF-α release, which was stimulated by SP and NK_1 agonists and inhibited by PPAR-γ agonists. However, the evidence that a PPAR-γ antagonist enhances SP-induced cytokine release further supports the possibility of cross-talk between the two receptors.

Methods

Preparation of human monocytes and monocyte-derived macrophages

This study and the research protocol were approved by the Local Ethical Committee; informed written consent was obtained by all participants. Human monocytes were isolated from fresh buffy-coat preparations of whole human blood, collected from healthy non-smokers and smokers of both sexes, as described (Amoruso *et al*., 2007). Briefly, the mononuclear cell fraction was diluted with phosphate-buffered saline (PBS, pH 7.4), layered over a Histopaque (density = 1.077 g cm^{-3}) gradient solution, centrifuged (400 *g*, 30 min, room temperature) and recovered by thin suction at the interface. The mononuclear cell layer was mixed with PBS and centrifuged for 10 min; cells were then resuspended in RPMI 1640 medium, supplemented with 5% heat-inactivated fetal bovine serum, 2 mM glutamine, 10 mM Hepes, 50 μg ml^{-1} streptomycin, 5 U ml^{-1} penicillin and 2.5 μg ml^{-1} amphotericin B. Purified monocytes were obtained by adhesion (90 min, 37 °C, 5% CO_2), non-adherent cells (mainly lymphocytes) being removed by three gentle washes with PBS; cell viability (Trypan blue dye exclusion) was usually >98% (Brunelleschi *et al*., 1998; Amoruso *et al*., 2007). Monocyte-derived macrophages (MDMs) were prepared from monocytes cultured for 8–10 days in a 5% CO_2 incubator at 37 °C in RPMI 1640 medium containing 20% fetal bovine serum, 2 mM glutamine, 10 mM HEPES and antibiotics; medium was changed every 2–3 days (Amoruso *et al*., 2007). MDMs were defined as macrophage-like cells, by evaluating surface markers CD14, MHCII, CD1a and CD68. Briefly, adherent cells were detached by gentle scraping with a plastic scraper. After three washings with sterile PBS, cells were resuspended at the final concentration of 1×10^5 cells ml^{-1} and fluorescent dye-labelled antibodies against the different surface markers (anti-CD14 from Becton Dickinson, Oxford, UK; anti-CD68 and anti-MHCII from Dako, Milan, Italy; anti-CD1a from eBioscence, San Diego, CA, USA) were added for 30 min on ice. Incubation was performed in the dark and expression of surface markers was analysed by flow cytometry.

TNF-α release in monocytes and MDMs

Cells (1×10^6) were treated in the absence or presence of the PPAR-γ agonist 15d-PGJ_2, (used at 1–10 μM) for 30 min and then challenged with SP (10^{-8}–10^{-6} M) for 24 h; supernatants were collected and stored at −20 °C. This 24-h stimulation time was chosen to ensure maximal cytokine release, as observed previously (Bardelli *et al*., 2005; Gunella *et al*., 2006). In some cases, cells were pretreated for 30 min with the NK_1 antagonist GR71251 (10^{-8}–10^{-6} M) or the PPAR-γ antagonist GW9662 (2-chloro-5-nitrobenzanilide; 10^{-6} M) and then stimulated by SP (10^{-6} M). TNF-α in the samples was estimated by ELISA (Pelikine Compact human ELISA kit) following the manufacturer's instructions (CLB/Sanquin, Amsterdam, The Netherlands). No cross-reactivity was observed with any other known cytokine; results are expressed in pg ml^{-1}.

PPAR-γ protein expression in monocytes and MDMs

Cells from healthy smokers and non-smokers were evaluated either as untreated (that is, 'basal, constitutive PPAR-γ expression') or after challenge (6 h, 37 °C, 5% CO_2) with SP (concentration range: 10^{-10}–10^{-6} M); the PPAR-γ ligand 15d-PGJ_2 (10 μM) was used for comparison. To confirm that enhancement of PPAR-γ expression is a receptor-mediated effect, cells were also challenged with the selective NK_1 agonist [$Sar^9Met(O_2)^{11}$]SP, or were pretreated for 30 min with the NK_1 antagonist GR71251 (10^{-9}–10^{-6} M) and then challenged with SP. In some experiments, cells were pretreated for 30 min with the PPAR-γ antagonist GW9662 (used at 10^{-6} M) and then stimulated by SP.

Cells (2×10^6), seeded in six-well plates, were washed twice with ice-cold PBS and scraped in lysis buffer containing 3% SDS, 0.25 M Tris and 1 mM phenylmethylsulphonyl fluoride and lysed by sonication; when necessary, cell lysates were stored at −80 °C. The determination of protein concentration was done with a Bradford-based assay. Protein samples (20 μg) were analysed by SDS-polyacrylamide gel electrophoresis (10% acrylamide) and electroblotted on nitrocellulose membrane (Protran; PerkinElmer Life Sciences,

Boston, MA, USA). Immunoblots were performed as described (Amoruso *et al*., 2007) using the following antibodies: monoclonal mouse anti-human PPAR-γ (E-8; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000 in TBS-T 5% milk) and monoclonal mouse anti-human β-actin (Sigma, St Louis, MO, USA; 1:5000 in TBS-T 3% BSA). Anti-mouse secondary antibody was coupled to horseradish peroxidase (Amersham Biosciences, Buckinghamshire, UK). Proteins were visualized with an enzyme-linked chemiluminescence detection kit according to the manufacturer's (PerkinElmer) instructions. Chemiluminescence signals were analysed under non-saturating conditions with an image densitometer (Versadoc; Bio-Rad, Hercules, CA, USA). Quantification of PPAR-γ protein was performed by calculating the ratio between PPAR-γ and β-actin protein expression; the latter was selected as reference housekeeping protein.

Western blotting for NK_1 receptors in monocyte and MDM membranes

Cells (3×10^6), seeded in six-well plates, were washed twice with ice-cold PBS and scraped in ice-cold PBS containing protease inhibitors (10 μg ml^{-1} aprotinin, 10 μg ml^{-1} pepstatin, 50 μg ml^{-1} leupeptin, 1 mM phenylmethylsulphonyl fluoride) and centrifuged (14 000 *g*; 30 s, 4 °C). The pellet was resuspended in 10 mM Tris-HCl, containing 1 mM EDTA and protease inhibitors, and subjected to three cycles of rapid freezing and thawing. The lysate was centrifuged (14 000 *g*; 15 min, 4 °C); the pellet (membranes) so obtained was suspended in 10 mM Tris-HCl, 10% sucrose and protease inhibitors, and used for the western blot experiments. The determination of protein concentration was done with a Bradford-based assay. Protein samples (20 μg) were analysed by SDS-polyacrylamide gel electrophoresis (10% acrylamide) and electroblotted on nitrocellulose membrane (Protran; PerkinElmer Life Sciences). Immunoblots were performed as described (Bardelli *et al*., 2005) using a rabbit polyclonal NK_1 receptor antibody (ab466; Abcam, Cambridge, UK; 1:1000 in TBS-T 5% milk) specific for human NK_1 receptors, and a monoclonal anti-Na^+/K^+ ATPase (α-subunit) antibody (clone M7-PB-E9; Sigma, St Louis, MO, USA; 1:250 in TBS-T 5% milk;). Proteins were visualized by using ECL western blotting detection reagents (PerkinElmer); Na^+/K^+ ATPase was selected as reference housekeeping membrane enzyme. Quantification of western blots was performed by densitometry using 'Quantity One, 1-D Analysis' software (Bio-Rad) and expressed as the ratio between NK_1 receptor and Na^+/K^+ ATPase protein expression.

Data and statistical analyses

Data are mean ± s.e.mean of *n* independent experiments. Concentration–effect curves for SP and for the NK_1 antagonist GR71251 were constructed; EC_{50} values (for SP) and IC_{50} values (for GR71251) were interpolated from curves of best fit. Statistical evaluation was performed by one-way or two-way ANOVA and Bonferroni post-test correction.

Drugs and analytical reagents

Fetal bovine serum was from Gibco (Paisley, UK). PBS, Histopaque, RPMI 1640, glutamine, HEPES, streptomycin, penicillin, amphotericin B, protease inhibitors, monoclonal anti-Na^+/K^+ ATPase (α-subunit) and monoclonal mouse anti-human β-actin antibodies were obtained from Sigma (Milwaukee, WI, USA). The rabbit polyclonal NK_1 receptor antibody (ab466; specific for human NK_1 receptors) was from Abcam; the monoclonal mouse anti-human PPAR-γ (E-8) antibody was from Santa Cruz. The PPAR-γ agonist 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 was from Biomol (Plymouth Meeting, PA, USA) and the PPAR-γ antagonist GW9662 was from Cayman Chemical (Ann Arbor, MI, USA). SP, [Sar^9 $Met(O_2)^{11}$]SP and GR71251 ([D-Pro^9,(spiro-γ-lactam)-Leu^{10},Trp^{11}]substance P) were from Neosystem (Strasbourg, France). Tissue-culture plates were from Nunc Ltd (Roskilde, Denmark); all cell culture reagents, with the exception of fetal bovine serum, were endotoxin free according to details provided by the manufacturer.

Results

Characterization of human monocyte/macrophage preparations

As shown in Figure 1, monocytes cultured for 8–10 days in RPMI medium supplemented with 20% fetal calf serum acquired a macrophage-like profile, which was accompanied by an increase in $CD68^+$ cells and a decrease of $CD14^+$ cells as compared with monocytes. Moreover, the absence of CD1a expression demonstrated that no differentiation towards dendritic cells occurred in our MDM preparations, as previously documented (Amoruso *et al*., 2007).

SP stimulates PPAR-γ protein expression in human monocytes and MDMs

To quantify PPAR-γ protein expression in both monocytes and MDMs from healthy smokers and non-smokers, we calculated the ratio between PPAR-γ and β-actin protein expression; in our experiments, β-actin levels were constant and stable in each cell type and were neither induced nor inhibited by the different 6-h *in vitro* treatments.

As illustrated by Figure 2a (a representative western blot of monocyte/macrophages from healthy non-smokers), constitutive PPAR-γ protein was detected in monocytes and MDMs and its expression was upregulated along with differentiation into mature macrophages. A 6-h challenge with the endogenous PPAR-γ ligand 15d-PGJ_2 (used at 10 μM) or with SP enhanced PPAR-γ expression in monocytes and MDMs from healthy non-smokers (Figure 2a). To ensure a better evaluation of SP-induced PPAR-γ expression, we performed concentration–response curves. As shown in Figure 2b, dealing with cells from five healthy non-smokers, SP, in the concentration range 10^{-10}–10^{-6} M, stimulated PPAR-γ expression in human monocytes and MDMs. Maximal effect (about twofold increase) was observed with SP 10^{-6} M and was quantitatively similar to that induced by the endogenous PPAR-γ agonist 15d-PGJ_2 (Figure 2b). The calculated EC_{50} values are similar in both cell types: 19 nM in monocytes and 17 nM in MDMs (Figure 2b). By evaluating SP-induced effects in cells obtained from four healthy smokers, we confirmed our previous observation (Amoruso *et al*., 2007) that exposure to tobacco smoke *in vivo* greatly affects PPAR-γ

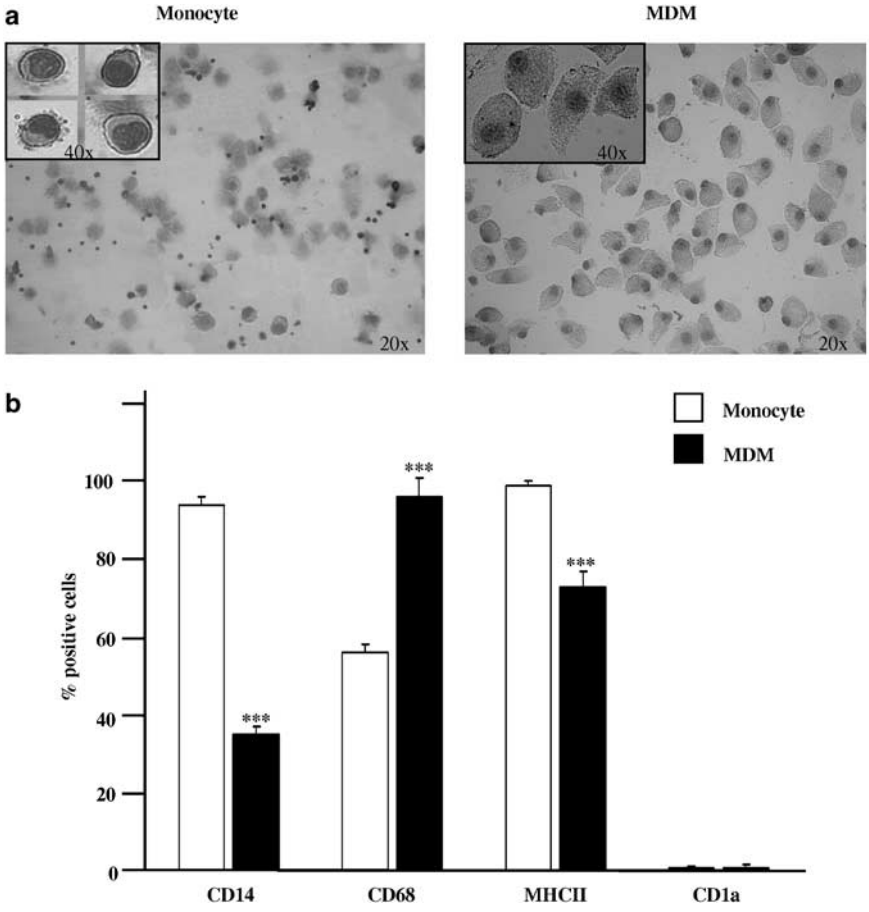

Figure 1 Morphology and phenotype of monocytes/macrophages from healthy non-smokers. In (**a**), May–Gruenewald–Giemsa stain of monocytes and monocyte-derived macrophages (MDMs) from healthy non-smokers. Magnification × 20 (magnification × 40 in the insets). In (**b**), surface marker expression in monocytes and MDMs. Data are means ± s.e.mean; $n = 4$; ***$P < 0.001$ vs monocytes.

expression. Monocytes and MDMs from healthy smokers exhibited a significantly enhanced constitutive PPAR-γ protein expression as compared with non-smokers (Figure 2c). In this case, too, SP dose dependently stimulated PPAR-γ expression, with EC_{50} values (6 nM in monocytes, 4 nM in MDMs) lower than those measured in cells from non-smokers (Figure 2c). Also in cells from healthy smokers, the maximal SP-induced effect was observed at 10^{-6} M and was quantitatively similar to that for 15d-PGJ_2 (Figure 2c).

Stimulation of PPAR-γ induced by SP was mediated by NK_1 receptors

To demonstrate that SP-induced PPAR-γ protein expression is a receptor-mediated activity, we evaluated the effects of selective NK_1 receptor agonists and antagonists. As shown in Figure 3, SP effects were reproduced, although to a minor extent, by the NK_1 selective agonist [$Sar^9Met(O_2)^{11}$]SP in both monocytes and MDMs from healthy non-smokers ($n = 5$; Figure 3a) and healthy smokers ($n = 4$; Figure 3b). At the highest concentration evaluated, 10^{-6} M, the NK_1 antagonist GR71251, which had no effect by itself, completely reversed the SP-induced effects (Figure 3). Interestingly, expression of PPAR-γ protein induced by SP was largely inhibited when cells were pretreated for 30 min with GW9662, a PPAR-γ antagonist, used at 10^{-6} M (Figure 3). GW9662, given alone, did not modify constitutive PPAR-γ protein expression (data not shown).

Over a concentration range (10^{-9}–10^{-6} M), the NK_1 receptor antagonist, GR71251, was more effective in reversing SP-induced PPAR-γ protein expression in cells from healthy smokers (Figure 4). At the highest concentration tested, this antagonist reversed almost completely this effect of SP, yielding a level of PPAR-γ protein very similar to that in untreated cells that is, basal, constitutive levels. These data were obtained by subtracting the value of basal constitutive PPAR-γ expression (in monocytes and MDMs, non-smokers and smokers) from all the determinations with SP. The calculated IC_{50} values were 84 and 38 nM in monocytes from non-smokers and smokers, respectively. In MDM, the IC_{50} values for GR71251 were 77 nM (non-smokers) and 19 nM (smokers) (Figure 4).

SP-induced cytokine release and modulation by PPAR-γ ligands

Previous reports from our and other laboratories indicated that PPAR-γ agonists inhibited the release of proinflammatory cytokines in monocyte/macrophages (Jiang *et al.*, 1998;

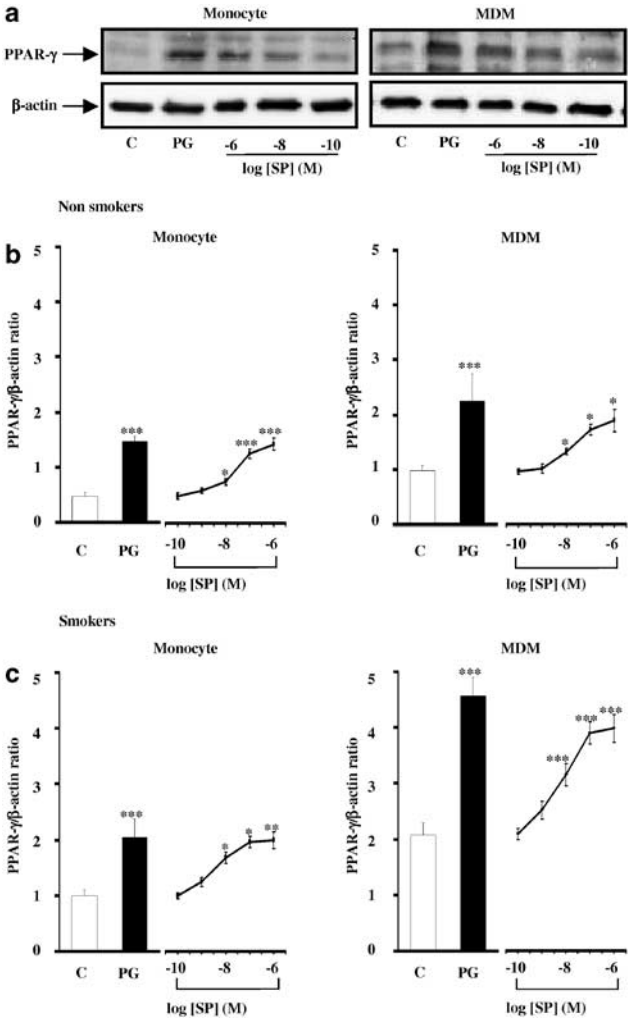

Figure 2 Concentration-dependent effects of substance P (SP) on peroxisome proliferator-activated receptor-γ (PPAR-γ) protein expression in human monocytes and monocyte-derived macrophages (MDMs) from healthy non-smokers. A representative western blot of PPAR-γ and β-actin in monocytes and MDMs from a non-smoker male volunteer (in **a**); SP-induced PPAR-γ expression in human monocytes and MDMs from five healthy non-smokers (in **b**) and four healthy smokers (in **c**). Cells were challenged for 6 h in the absence (C, control) or presence of 15d-PGJ_2 (15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; PG, 10 μM; shown for comparison) and SP (10^{-10}–10^{-6} M). Results are expressed as PPAR-γ/β-actin ratio. Means ± s.e.mean; $n = 4$–5. ***$P < 0.0001$, **$P < 0.001$, *$P < 0.05$ vs control.

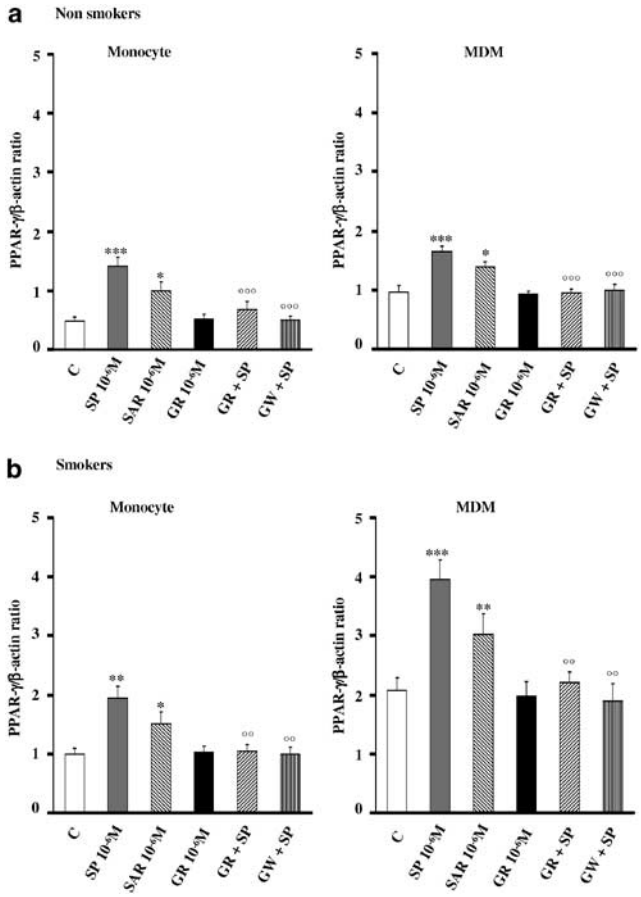

Figure 3 Effects of selective NK_1 receptor agonists and antagonists on peroxisome proliferator-activated receptor-γ (PPAR-γ) protein expression. Monocytes and monocyte-derived macrophages (MDMs) from non-smokers (**a**) and smokers (**b**) were challenged for 6 h with substance P (SP, 10^{-6} M), the NK_1 selective agonist [$Sar^9Met(O_2)^{11}$]SP (SAR, 10^{-6} M), the NK_1 antagonist GR71251 ([D-Pro^9,(spiro-γ-lactam)Leu^{10},Trp^{11}]substance P; GR, 10^{-6} M), a combination of SP + GR71251, or SP + PPAR-γ antagonist GW9662 (2-chloro-5-nitrobenzanilide; GW, 10^{-6} M). Results are expressed as PPAR-γ/β-actin ratio. Means ± s.e.mean; $n = 4$–5. ***$P < 0.0001$, **$P < 0.001$, *$P < 0.05$ vs control; °°°$P < 0.0001$, °°$P < 0.001$ vs SP.

Ricote *et al*., 1998b; Amoruso *et al*., 2007), whereas SP and NK_1 agonists induced the release of TNF-α, IL-1β and IL-6 (Lotz *et al*., 1988; Brunelleschi *et al*., 1998; Bardelli *et al*., 2005). Besides confirming these data, we now provide evidence of the interaction between SP and PPAR-γ.

As shown in Table 1, SP-induced TNF-α release from monocytes isolated from healthy smokers and non-smokers was inhibited, in a concentration-dependent manner, by both the PPAR-γ agonist 15d-PGJ_2 and the NK_1 receptor antagonist GR71251. When cells were pretreated for 30 min with the PPAR-γ antagonist GW9662, used at 10^{-6} M, and then challenged by SP, an enhanced cytokine release ($P < 0.05$; $n = 4$) was observed. In keeping with our previous data (Bardelli *et al*., 2005; Gunella *et al*., 2006; Amoruso *et al*., 2007), cells from smokers released higher amounts of TNF-α, compared with non-smokers (Table 1). Similar results were also obtained in MDMs (data not shown).

NK_1 receptor expression in monocytes and MDMs from healthy smokers and non-smokers

Our previous observations demonstrated the presence of authentic NK_1 receptor in human alveolar macrophages, a threefold enhanced expression being observed in healthy smokers (Bardelli *et al*., 2005). We now confirmed these observations in peripheral monocytes and MDMs, too. The western blot experiments performed in cells from four healthy smokers and five healthy non-smokers (Figure 5) clearly indicated that MDMs have a higher membrane expression of NK_1 receptors than monocytes, and that cells from smokers (Figure 5b) have higher NK_1 receptor content

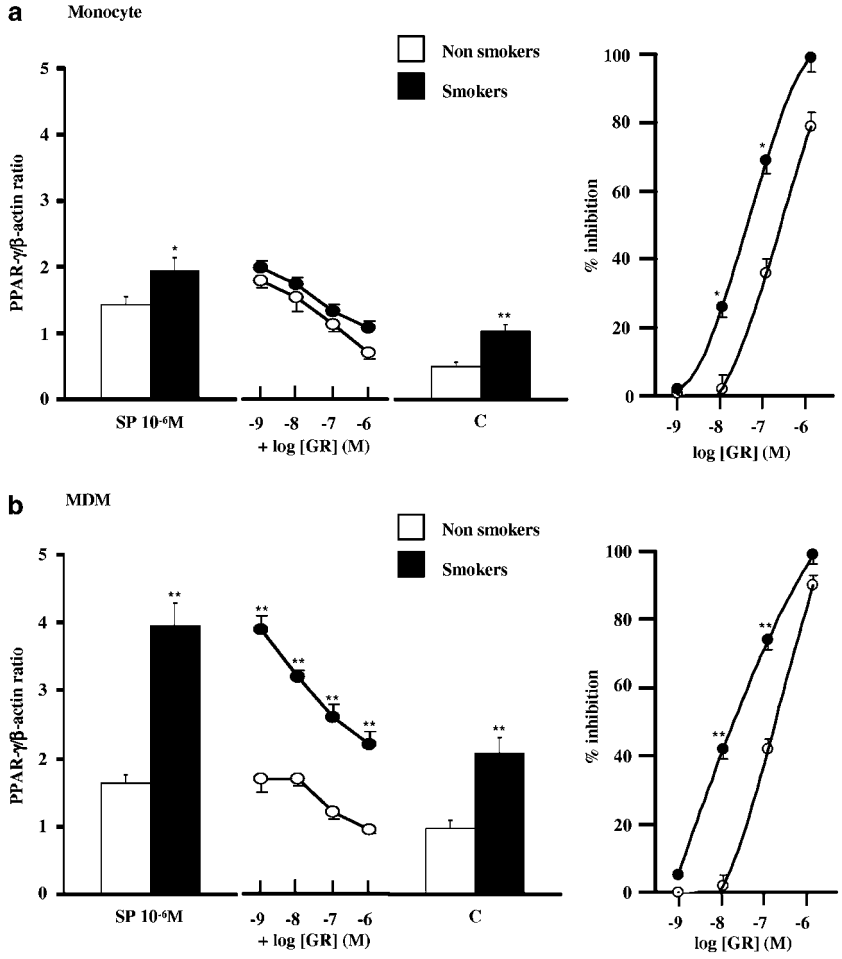

Figure 4 Substance P (SP)-induced peroxisome proliferator-activated receptor-γ (PPAR-γ) expression was mediated by NK_1 receptors: reversal by the NK_1 antagonist GR71251 ([D-Pro^9,(spiro-γ-lactam)Leu^{10},Trp^{11}]substance P). Monocytes (in **a**) and monocyte-derived macrophages (MDMs) (in **b**) from non-smokers and smokers were challenged for 30 min with increasing concentrations of GR71251 (10^{-9}–10^{-6} M) and then exposed to SP 10^{-6} M. Control, unstimulated (C) cells and SP-stimulated cells are shown for comparison. Results are expressed as PPAR-γ/β-actin ratio (on the left) and as % inhibition of SP-induced effect (on the right). Means ± s.e.mean; $n = 4$. $^{**}P < 0.001$, $^{*}P < 0.05$ vs non-smokers. See text for further details.

Table 1 TNF-α release (pg ml^{-1}) in monocytes isolated from healthy smokers and non-smokers

	Non-smokers (n = 4)	*Smokers* (n = 4)
Control	110 ± 10	280 ± 15
Control + 15d-PGJ_2 (10^{-6} M)	90 ± 10	268 ± 10
Control + 15d-PGJ_2 (10^{-5} M)	63 ± 8**	170 ± 15**
SP (10^{-8} M)	200 ± 20*	420 ± 12*
SP (10^{-6} M)	270 ± 15**	600 ± 15**
GR (10^{-8} M) + SP (10^{-6} M)	200 ± 10	520 ± 10
GR (10^{-6} M) + SP (10^{-6} M)	125 ± 15°	290 ± 12°°
GW (10^{-6} M) + SP (10^{-6} M)	340 ± 10°°	740 ± 15°°
15d-PGJ_2 (10^{-6} M) + SP (10^{-6} M)	238 ± 12	560 ± 8
15d-PGJ_2 (10^{-5} M) + SP (10^{-6} M)	140 ± 10°°	310 ± 10°°

GR, GR71251 ([D-Pro^9,(spiro-γ-lactam)Leu^{10},Trp^{11}]substance P), NK_1 antagonist; GW, GW9662 (2-chloro-5-nitrobenzanilide), PPAR-γ antagonist; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; PPAR-γ, peroxisome proliferator-activated receptor-γ; SP, substance P; TNF-α, tumour-necrosis factor-α.
Data are means ± s.e.mean. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control, unstimulated monocytes; $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$ vs SP (10^{-6} M).

than monocytes/macrophages from non-smokers (Figure 5a). The calculated NK_1 receptor: Na^+/K^+ ATPase protein ratios in Figure 5c summarize the results from the western blots.

Discussion

The results presented in this paper show, for the first time, that SP, by activation of NK_1 receptors, enhanced PPAR-γ protein expression in human monocytes/macrophages, suggesting the possibility of a physiologically relevant cross-talk between the two receptors.

PPAR-γ is expressed in a wide variety of cells, including human monocytes and macrophages, its expression being stimulated by endogenous (for example, 15d-PGJ_2, oxidized low-density lipoproteins, advanced glycation end products) or exogenous ligands, mainly thiazolidinedione antidiabetic drugs (Nagy *et al*., 1998; Ricote *et al*., 1998a, 1999; Tontonoz *et al*., 1998; Scher and Pillinger, 2005; Amoruso *et al*., 2007). Despite a number of diverging reports (Nagy *et al*., 1998; Chinetti *et al*., 2000; Desmet *et al*., 2005), most experimental data indicated that the anti-inflammatory potential of PPAR-γ mainly resides in the ability of PPAR-γ agonists to inhibit monocyte/macrophage activation and expression of inflammatory molecules, that is, TNF-α, IL-6, IL-1β, inducible nitric oxide synthase, gelatinase B and COX-2 (Chinetti *et al*., 1998; Jiang *et al*., 1998; Ricote *et al*.,

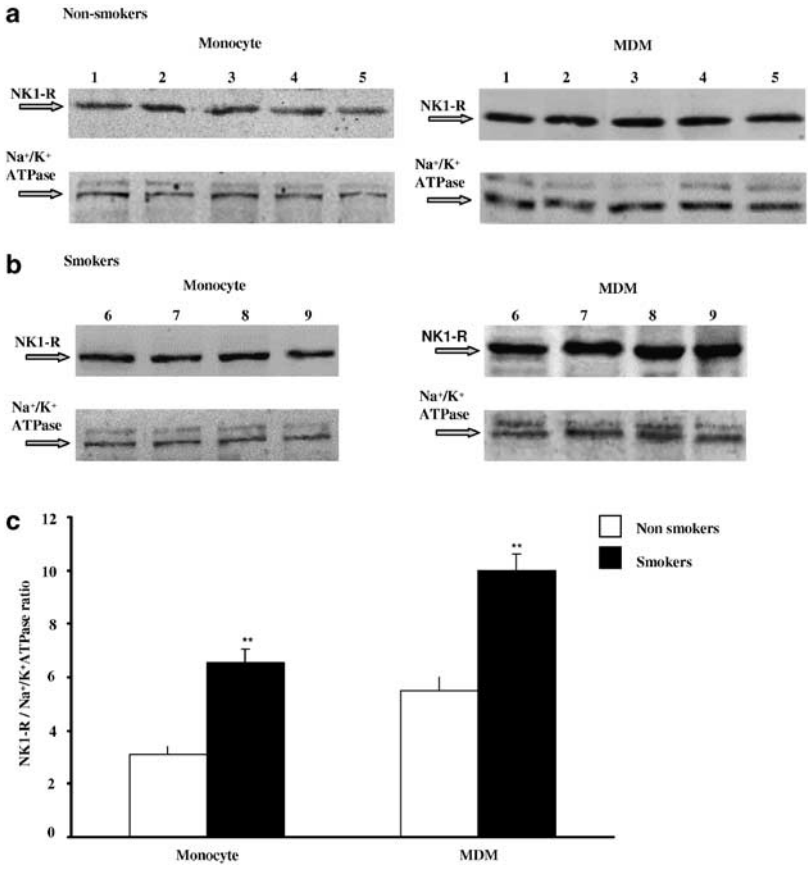

Figure 5 Western blots of NK_1 receptors (NK_1-R) and Na^+/K^+ ATPase. In (**a**), monocytes and monocyte-derived macrophages (MDMs) from non-smokers (lanes 1–5 = different healthy non-smokers). In (**b**), monocytes and MDMs from smokers (lanes 6–9 = different healthy smokers). In (**c**), NK_1 receptor and Na^+/K^+ ATPase ratio in monocytes and MDMs from non-smokers ($n=5$) and smokers ($n=4$). Means ± s.e.mean; $^{**}P<0.001$ vs non-smokers. See text for further details.

1998a, b, 1999; Subbaramaiah *et al*., 2001; Amoruso *et al*., 2007).

Interestingly, the anti-inflammatory effects of PPAR-γ have been demonstrated in animal models of arthritis, ischaemia reperfusion, inflammatory bowel diseases and chronic airway inflammation (see Daynes and Jones, 2002; Scher and Pillinger, 2005 and Belvisi *et al*., 2006), that is, pathological conditions in which a role for SP has long been established (Mantyh *et al*., 1995; Maggi, 1997; Keeble and Brain, 2004; O'Connor *et al*., 2004; Keeble *et al*., 2005; Reed *et al*., 2005). Moreover, previous studies indicate that NK_1 receptors are upregulated at inflamed sites in many tissues, including joints and intestine (Mantyh *et al*., 1995; Keeble and Brain, 2004; Keeble *et al*., 2005; Reed *et al*., 2005). By using a rabbit polyclonal anti-human NK_1 receptor antibody, we first demonstrated that NK_1 receptor expression was potently upregulated in alveolar macrophages from healthy smokers as compared with non-smokers (Bardelli *et al*., 2005). At variance from those experiments, in which we used total cell extracts (Bardelli *et al*., 2005), we have now employed membrane extracts to evaluate NK_1 receptor expression in monocytes and MDMs. The use of membrane extracts allows us to evaluate only those NK_1 receptors that are present and functionally active (see also below, cytokine experiments) on cell membranes. Consistent with our previous observations (Bardelli *et al*., 2005), we report here that cells from healthy smokers have more than double the NK_1 receptor expression of cells from non-smokers and that MDMs have significantly higher NK_1 receptor content than monocytes.

Therefore, we are now suggesting that the increased expression of NK_1 receptor in the cell surface of monocyte/macrophages from healthy smokers is not only associated with, but could also be responsible for, the higher PPAR-γ expression induced by SP in smokers. This suggestion mainly comes from the following experimental results we obtained.

First, SP, at physiological concentrations and in a concentration-dependent manner, stimulated PPAR-γ protein expression in human monocytes and MDMs, with a maximal effect similar to the endogenous PPAR-γ agonist 15d-PGJ_2 and a greater efficiency in cells from healthy smokers (as demonstrated by the lower EC_{50} values). The EC_{50} values we calculated for SP-induced PPAR-γ expression in monocytes and MDMs from non-smokers (19 and 17 nM, respectively) are identical to the K_D value (19 nM) reported by Hartung *et al*. (1986) for SP-binding studies in guinea-pig macrophages, whereas lower EC_{50} values (4 and 6 nM, in MDMs and monocytes, respectively) were documented in cells from smokers.

Then, we have also demonstrated that SP-induced PPAR-γ protein expression was a receptor-mediated effect, as it was

reproduced by the NK_1 selective agonist [$Sar^9Met(O_2)^{11}$]SP and reversed by the competitive NK_1 antagonist GR71251. Interestingly, GR71251 is more potent in cells from healthy smokers than non-smokers; IC_{50} values were 84 and 77 nM (monocytes and MDMs, respectively) in non-smokers and 38 and 19 nM (monocytes and MDMs, respectively) in smokers. In isolated spinal cord preparations of neonatal rats, GR71251 was demonstrated to cause a rightward shift of the concentration–response curve for SP with a pA2 value of 6.14 (Guo *et al*., 1993). The IC_{50} values we measured are far below the previously reported pA2; however, it must be noted that, apart from the different experimental models and the possible variations in affinity due to the different species (human and rat), in human monocytes/macrophages, SP acts at concentrations lower than those used by Guo *et al*. (1993).

Finally, there are two major experimental results that, in our opinion, indicate the relevance of cross-talk between SP and PPAR-γ: the ability of a PPAR-γ antagonist to potently reduce SP-induced PPAR-γ expression, as well as the ability of PPAR-γ ligands to affect SP-induced TNF-α release. As is well-known, SP stimulates proinflammatory cytokine release (Lotz *et al*., 1988; Lee *et al*., 1994; Delgado *et al*., 2003; Bardelli *et al*., 2005), whereas PPAR-γ agonists reduce it (Chinetti *et al*., 1998; Jiang *et al*., 1998; Ricote *et al*., 1998a, b, 1999; Subbaramaiah *et al*., 2001; Amoruso *et al*., 2007). In our experiments, SP-induced TNF-α release was inhibited, in a concentration-dependent manner, by the endogenous PPAR-γ ligand 15d-PGJ_2 and increased in the presence of GW9662, a PPAR-γ antagonist.

Cytokine release is a complex phenomenon, which involves several signal pathways and it is tightly regulated, often in a stimulus- and cell-specific manner (Bondeson *et al*., 1999; Andreakos *et al*., 2004). We can postulate a scheme involving different signal transduction pathways but, at this stage, such evaluation is beyond the scope of the paper. It is nevertheless tempting to speculate that the ability of 15d-PGJ_2 to inhibit SP-induced TNF-α release *in vitro* could similarly affect cytokine release *in vivo*. Interestingly, human monocytes and macrophage cell lines have been shown to express higher levels of NK_1 receptors in response to TNF-α and other cytokines (Ho *et al*., 1997; Marriott and Bost, 2000; Simeonidis *et al*., 2003; Arsenescu *et al*., 2005), and SP has been shown to participate in positive feedback loops, in which it enhances the production of cytokines that, on their own, increase SP secretion and/or NK_1 receptor stimulation (Reinke and Fabry, 2006). So, the documented level of expression of a given receptor in a given condition is the resultant of the interplay between various factors. We have disclosed the existence of such interplay by demonstrating that SP, a well-known proinflammatory mediator, is able to enhance the expression of PPAR-γ, a suggested anti-inflammatory receptor. Although the clinical relevance of these results remains to be elucidated, it is worth reminding that tobacco smoke potently affects both PPAR-γ expression and SP/NK_1 receptor function. A number of experimental observations have described the acceleration, by tobacco smoke, of the progression of atherosclerosis through different mechanisms, and epidemiological and clinical findings indicate that smokers have an increased risk to develop atherosclerosis (Taylor *et al*., 1998).

The results of this study demonstrate that monocytes and MDMs from healthy smokers present an enhanced NK_1 receptor expression and that, in both cell types, SP stimulates PPAR-γ expression with a greater efficiency, compared with monocytes/macrophages from non-smokers. This represents a novel activity for SP, which could play a role in chronic inflammatory conditions, such as atherosclerosis, rheumatoid arthritis and inflammatory bowel diseases.

Acknowledgements

We thank Dr Marcella Tarditi from the Transfusion Service, Borgomanero (Novara, Italy) for kindly providing buffy-coat preparations from healthy smokers and non-smokers. This work was supported by Regione Piemonte and PRIN 2004 (prot. 2004065227) grants.

Conflict of interest

The authors state no conflict of interest.

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