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# RANTES release by human airway smooth muscle: effects of prostaglandin $E_2$ and fenoterol

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**Abstract**

In human airway smooth muscle cells, the levels of RANTES were increased upon stimulation with interleukin-1β together with tumour necrosis factor-α (TNF-α) (10 ng $ml^{-1}$ for each). In this study, we have assessed the effects of prostaglandin $E_2$ and the $\beta_2$-adrenoceptor agonist, fenoterol on RANTES (regulated upon activation, normal T cell expressed and secreted) release by these cells. The levels of RANTES released by human airway smooth muscle cells were measured after 24 h of treatment. Prostaglandin $E_2$ and fenoterol, only in presence of a cyclo-oxygenase inhibitor indomethacin ($10^{-6}$ M), provoked a concentration-dependent reduction in RANTES release. These data suggest that, in settings where cyclo-oxygenase activity is low, both drugs may relieve the symptoms of airway diseases by reducing RANTES production. © 2001 Published by Elsevier Science B.V.

*Keywords:* RANTES; Prostaglandin $E_2$; $\beta_2$-Adrenoceptor agonist; Smooth muscle cells, human; airway

## 1. Introduction

RANTES (regulated upon activation, normal T cell expressed and secreted) is a potent chemokine for monocytes, memory T lymphocytes and eosinophils (Schall et al., 1990; Rot et al., 1992) and is produced by cells including T lymphocytes, macrophages, synovial fibroblasts and airway epithelial cells (Kameyoshi et al., 1992; Devergne et al., 1994; Marfaing-Koka et al., 1995; Rathanaswami et al., 1993; Berkman et al., 1995).

Our group and others have recently suggested that, in addition to their well characterised contractile function, airway smooth muscle is actively involved in the release of inflammatory mediators including chemokines (Johnson and Knox, 1997; Belvisi et al., 1997; Saunders et al., 1997; John et al., 1997, 1998). Indeed, airway smooth muscle releases high levels of RANTES after stimulation with inflammatory cytokines (John et al., 1997).

Furthermore, human airway smooth muscle cells, when treated with proinflammatory cytokines, have increased expression of cyclo-oxygenase-2 and consequently release elevated amounts of prostaglandins (Belvisi et al., 1997; Pang and Knox, 1997). Most recently, cyclo-oxygenase-2 expression has been demonstrated in human airway inflammation (Sousa et al., 1997), when the levels of RANTES would also be high. However, the potential influence of cyclo-oxygenase-2 activity on RANTES production in these cells has not been addressed.

Thus, the purpose of this study was to investigate the effect of cyclo-oxygenase products as well as other agents that stimulate the adenylyl cyclase pathway on RANTES release by human airway smooth muscle cells.

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## 2. Methods

### 2.1. Isolation of human airway smooth muscle cells

As described previously (Belvisi et al., 1997), tracheal rings, from either heart/heart and lung transplantation donors (2 females, 5 males, aged 27–45 years), were dissected under sterile conditions in Hanks buffer saline solution (HBSS; in mM: NaCl 136.8, KCl 5.4, $MgSO_4$ 0.8, $Na_2HPO_4 \cdot 7H_2O$ 0.4, $CaCl_2$. $2H_2O$ 1.3, $NaHCO_3$ 4.2 and glucose 5.6) supplemented with the antibiotics penicillin (100 U $ml^{-1}$), streptomysin (100 μg $ml^{-1}$) and the anti-fungal amphotericin B (2.5 μg $ml^{-1}$). The smooth muscle layer was dissected free of adherent connective tissue and cartilage; the epithelial layer was removed. The smooth muscle section was then incubated for 30 min at 37 °C in 5% $CO_2$/air in HBSS containing 10 mg $ml^{-1}$ bovine serum albumin and the enzymes collagenase (type XI, 1 mg $ml^{-1}$) and elastase (type I, 3.3 U $ml^{-1}$). After the removal of any remaining connective tissue, the smooth muscle was chopped finely and incubated for a further 150 min in the enzyme solution outlined above with the elastase content increased to 15 U $ml^{-1}$. Cells were centrifuged (100 × *g*, 5 min) at 4 °C and resuspended in Dulbecco's Modified Eagles' Medium (DMEM) containing heat-inactivated foetal calf serum (10% v/v), sodium pyruvate ($10^{-3}$ M), L-glutamate (2 mM), non-essential amino acids (1 ×) and anti-microbial agents as previously described.

### 2.2. Primary culture of human airway smooth muscle cells

Cells were placed in a tissue culture flask (75 $cm^2$) with 6 ml of supplemented DMEM and incubated at 37 °C in 5% $CO_2$/air. The cells adhere after approximately 12 h and the culture medium was replaced after 4–5 days (12 ml) and subsequently every 3–4 days. When the cells reached confluence, they were passaged into 2 × 75-$cm^2$ flasks and plated onto 96 well at seeding density of 2000 cells/well. Cells were used at passages 3–7 and at subconfluence, the cells were growth arrested by withdrawing serum for 24 h. Other supplements were still present and viability was preserved by adding bovine serum albumin (0.1%). Under these conditions, cell stained positive for smooth muscle α-actin, as we have described previously (Belvisi et al., 1997), indicative of the contractile phenotype. Then cells were treated for 24 h with test drugs containing 0% foetal calf serum, in the presence of a mixture of cytokines (interleukin-1β and tumour necrosis factor (TNF-α)) each at 10 ng $ml^{-1}$). The cyclo-oxygenase inhibitor, indomethacin ($10^{-6}$ M), was added 10 min before the addition of the cytokine mixture.

### 2.3. Measurement of RANTES by ELISA

The cytokine RANTES was measured by use of a specific sandwich ELISA.

### 2.4. Materials

Interleukin-1β, TNF-α, interferon-γ and human RANTES antibodies and protein for ELISA were purchased from R&D Systems Europe (Abingdon, Oxfordshire, UK). Amphotericin B, non-essential amino acids and sodium pyruvate were purchased from Life Technologies (Paisley, UK). All other materials were purchased from Sigma (Poole, UK).

### 2.5. Statistical analysis

Results are shown as the mean ± S.E.M. of *n* experiments; cells from at least three separate patients, replicated in triplicate, were used for each protocol. The 'normalised' data were analysed by a one-sample *t*-test. All treatments were compared to control value and $P < 0.05$ was considered to be significant.

## 3. Results

### 3.1. Release of RANTES by human airway smooth muscle cells and effect of indomethacin

Under control culture conditions, human airway smooth muscle cells released low or undetectable levels of RANTES and undetectable levels of prostaglandin $E_2$. However, when cells were treated with a combination of interleukin-1β and TNF-α (each at 10 ng $ml^{-1}$) for 24 h, they released relatively high levels of RANTES (control: 91.5 ± 48.2 pg $ml^{-1}$, $n = 18$; interleukin-1β + TNF-α: 648.9 ± 122.7 pg $ml^{-1}$, $n = 18$).

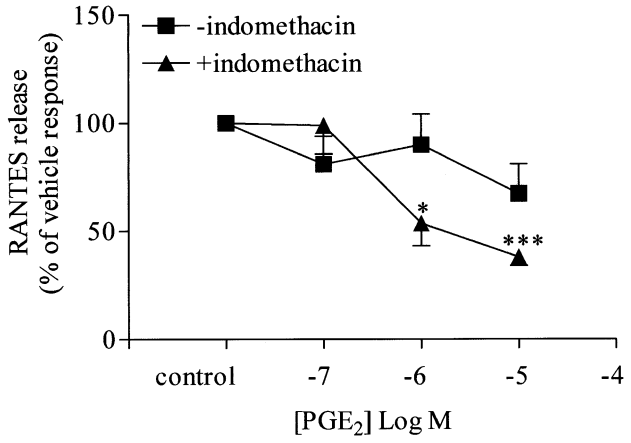


Fig. 1. Effect of exogenous prostaglandin (PG)$E_2$ ($10^{-7}$ to $10^{-5}$ M) on RANTES release by human airway smooth muscle cells stimulated with cytokines (TNF-α plus interleukin-1β, each at 10 ng $ml^{-1}$), in the absence (■) or presence (▲) of indomethacin ($10^{-6}$ M). The values are expressed as percentage of vehicle response. Results are shown as the mean ± S.E.M. of nine determinations from three patients. Each value of treatment was compared to the control one by one-sample *t*-test. * $P < 0.05$; *** $P < 0.001$.

In addition to RANTES, when cells were stimulated with interleukin-1β together with TNF-α, they released increased levels of prostaglandin $E_2$ ($23 \pm 7$ ng $ml^{-1}$) which were blocked by indomethacin ($10^{-6}$ M; data not shown), as we have previously shown (Belvisi et al., 1997, 1998). Moreover, in cells stimulated with cytokines, addition of the indomethacin ($10^{-6}$ M) induced a further increase in RANTES production (plus interleukin-1β and TNF-α: $578.5 \pm 136.4$ pg $ml^{-1}$, $n=18$; plus interleukin-1β and TNF-α plus indomethacin: $1197.6 \pm 187.7$ pg $ml^{-1}$, $n=18$). By contrast, indomethacin had no effect on the levels of RANTES released by cells not stimulated with cytokines (data not shown).

### 3.2. Effect of prostaglandin $E_2$ and fenoterol on RANTES release by human airway smooth muscle cells

Prostaglandin $E_2$ ($10^{-7}$ to $10^{-5}$ M) had no effect on RANTES release by human airway smooth muscle cells stimulated with cytokines. By contrast, when endogenous prostaglandin $E_2$ production was blocked with indomethacin ($10^{-6}$ M), exogenous prostaglandin $E_2$ inhibited RANTES released by human airway smooth muscle cells stimulated with the combination of interleukin-1β and TNF-α (Fig. 1). In separate experiments, the $\beta_2$-adrenoceptor agonist fenoterol ($10^{-8}$ to $10^{-5}$ M), like prostaglandin $E_2$, did not affect RANTES release by human airway smooth muscle cells stimulated with cytokines. However, again as was observed with prostaglandin $E_2$, fenoterol reduced RANTES release by cells stimulated with cytokines and treated with indomethacin (Fig. 2).

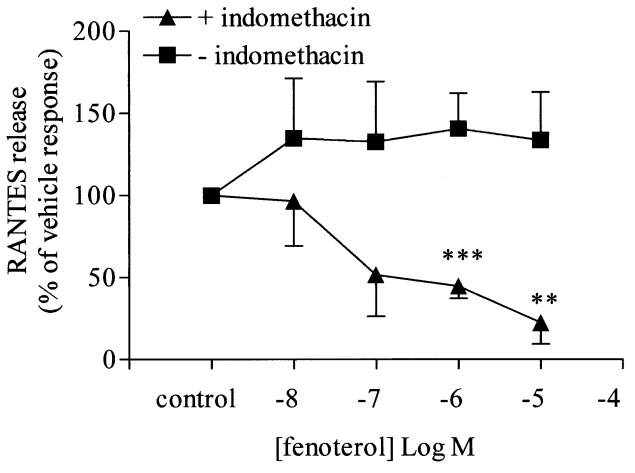


Fig. 2. Effect of fenoterol ($10^{-8}$ to $10^{-5}$M) on RANTES release by human airway smooth muscle cells stimulated with cytokines (TNF-α plus interleukin-1β, each at 10 ng $ml^{-1}$), in the absence (■) or presence (▲) of indomethacin ($10^{-6}$ M). The values are expressed as percentage of vehicle response. Results are shown as the mean ± S.E.M. of nine determinations from three patients. Each value of treatment was compared to the control one by one-sample *t*-test. ** $P<0.01$; *** $P<0.001$.

## 4. Discussion

Airway smooth muscle was traditionally viewed as a contractile tissue responsible for maintaining airway calibre. However, it is now recognised as an important source of inflammatory mediators under certain pathophysiological conditions (Belvisi et al., 1998; Saunders et al., 1997; John et al., 1998; Chung et al., 1999). Among the other mediators, human airway smooth muscle cells release the chemoattractant RANTES as well as large quantities of prostaglandins after stimulation with cytokines such as interleukin-1 and TNF-α (John et al., 1997; Belvisi et al., 1998). In this study, we show that when human airway smooth muscle cells are stimulated with cytokines, the prostaglandins released suppress RANTES production. Furthermore, we show that when endogenous prostaglandin production is blocked, the addition of either prostaglandin $E_2$ or the $\beta_2$-adrenoceptor agonist fenoterol reduces RANTES production.

In this study, we found that when cells were stimulated with cytokines and prostaglandin production was blocked with indomethacin, RANTES production was increased. We and others have shown that the ability of human airway smooth muscle cells to release prostaglandins is regulated by two distinct isoforms of cyclo-oxygenase. Cyclo-oxygenase-1 is expressed in these cells under physiological conditions and cyclo-oxygenase-2 is induced after exposure to cytokines (Belvisi et al., 1998). Indomethacin is a potent inhibitor of both cyclo-oxygenase-1 and cyclo-oxygenase-2 (Mitchell et al., 1993; Warner et al., 1999; Belvisi et al., 1998). However, human airway smooth muscle cells treated with cytokines release greatly elevated levels of prostaglandin $E_2$ (Belvisi et al., 1997, 1998; current study) which is blocked by cyclo-oxygenase-2 selective inhibitors (Belvisi et al., 1998). Thus, under the conditions described here, as in our previous studies, it is most likely that cyclo-oxygenase-2 has the predominate role in prostaglandin $E_2$ production. Inhibition of prostaglandin production by indomethacin appears to regulate RANTES production in these cells. However, indomethacin, as with some other drugs in its class, can have effects on other pathways (Cashman, 1996). Thus, it is conceivable that indomethacin increases RANTES via a non-prostaglandin dependent pathway. However, the effects of indomethacin on RANTES released were reversed by the addition of exogenous prostaglandin $E_2$, a principal COX product in the cells (Belvisi et al., 1997).

Prostaglandin $E_2$ inhibited RANTES production in cytokine-stimulated cells, treated with indomethacin, in a concentration-dependent fashion. By contrast, in cells stimulated with cytokines but without a cyclo-oxygenase inhibitor, prostaglandin $E_2$ did not affect RANTES production. This observation suggests that endogenous prostaglandin release is sufficient to maximally inhibit RANTES production and results in saturation in the inhibitory mechanism.

Similarly to prostaglandin $E_2$, the $\beta_2$-adrenoceptor agonist fenoterol decreased RANTES production by cells treated with cytokines and indomethacin. Again, when cells were treated with cytokines but without indomethacin, fenoterol did not affect RANTES production. Fenoterol causes bronchodilation via activation of adenylate cyclase and increases in cAMP. Similarly, prostaglandin $E_2$ also activates adenylate cyclase in human airway smooth muscle cells (Narumiya, 1994). Moreover, for some functions, prostaglandin $E_2$ can induce 'cross-tolerance' or 'heterologous desentitisation' to $\beta_2$-adrenoceptor agonists at the level of adenylate cyclase activity (Pang et al., 1998). Thus, we suggest that in this setting, the endogenous production of prostaglandin $E_2$ is sufficient to maximally activate a cAMP-mediated inhibitory pathway for RANTES production.

Interestingly, we found that fenoterol was somewhat more efficacious as an inhibitor of RANTES release than prostaglandin $E_2$. It is unlikely that $\beta_2$-adrenoceptors were up-regulated in our cells (Shore et al., 1997; Koto et al., 1996). However, it is possible that the inhibitory effect of prostaglandin $E_2$ was limited by itself via an auto-functional antagonism. The inhibitory effects of either fenoterol or prostaglandin $E_2$ on RANTES production was partial. This suggests that there are cAMP independent as well as dependent pathways modulating RANTES release in these cells.

In conclusion, when human airway smooth muscle cells are activated with cytokines, they release RANTES which is limited by endogenous prostaglandin (possibly prostaglandin $E_2$) production. Furthermore, in the presence of a cyclo-oxygenase inhibitor, exogenous prostaglandin $E_2$ as well as the $\beta_2$-adrenoceptor agonist fenoterol reduces RANTES production by these cells. Thus, in these cells, endogenous cyclo-oxygenase activity has a protective role in the limitation of chemokine production. These observations add weight to the hypothesis that nonsteroidal anti-inflammatory drugs, including the new cyclo-oxygenase-2 selective drugs, may exacerbate airway inflammation associated with diseases such as asthma or chronic obstructive pulmonary disease.

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## References

Belvisi, M.G., Saunders, M.A., Haddad, E.-B., Hirst, S.J., Yacoub, M.H., Barnes, P.J., Mitchell, J.A., 1997. Induction of cyclo-oxygenase-2 by cytokines in human cultured airway smooth muscle cells. Br. J. Pharmacol. 120, 910–916.

Belvisi, M.G., Saunders, M., Yacoub, M., Mitchell, J.A., 1998. Expression of cyclo-oxygenase-2 in human airway smooth muscle is associated with profound reductions in cell growth. Br. J. Pharmacol. 125, 1102–1108.

Berkman, N., Robichaud, A., Krishnan, V.L., Roesems, G., Robbins, R.A., Jose, P.J., Barnes, P.J., Chung, K.F., 1995. Expression of RANTES in human airway epithelial cells: effect of corticosteroids and interleukin-4, -10 and -13. Immunology 87, 599.

Cashman, J.N., 1996. The mechanisms of action of NSAIDs in analgesia. Drugs 52 (Suppl. 5), 13–23.

Chung, K.F., Patel, H.J., Fadlon, E.J., Rousell, J., Haddad, E.B., Jose, P.J., Mitchell, J.A., Belvisi, M.G., 1999. Induction of eotaxin expression and release from human airway smooth muscle cells by IL-1beta and TNFalpha: effects of IL-10 and corticosteroids. Br. J. Pharmacol. 127, 1145–1150.

Devergne, O., Marfaing-Koka, A., Schall, T.J., Leger-Ravet, M.B., Sadick, M., Crevon, M.C., Kim, K.J., Schall, T.T., Galanaud, P., Emilie, D., 1994. Production of the RANTES chemokine in delayed-type hypersensitivity reactions: involvement of macrophages and endothelial cells. J. Exp. Med. 179, 1689.

John, M., Hirst, S.J., Jose, P., Robichaud, A., Witt, C., Twort, C., Berkman, N., Barnes, P.J., Chung, K.F., 1997. Human airway smooth muscle cells express and release rantes in response to th-1 cytokines: regulation by Th-2 cytokines. J. Immunol. 158, 1841–1847.

John, M., Au, B.T., Jose, P.J., Lim, S., Saunders, M., Barnes, P.J., Mitchell, J.A., Belvisi, M.G., Chung, K.F., 1998. Expression and release of interleukin-8 by human airway smooth muscle cells: inhibition by Th-2 cytokines and corticosteroids. Am. J. Respir. Cell Mol. Biol. 18, 84–90.

Johnson, S.R., Knox, A.J., 1997. Synthetic functions of airway smooth muscle in asthma. Trends Pharmacol. Sci. 18, 288–292.

Kameyoshi, Y., Dörschner, A., Mallet, A.I., Christophers, E., Schröder, J., 1992. Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils. J. Exp. Med. 176, 587.

Koto, H., Mak, J.C., Haddad, E.B., Xu, W.B., Salmon, M., Barnes, P.J., Chung, K.F., 1996. Mechanims of impaired beta-adrenoceptor-induced airway relaxation by interleukin-1beta in vivo in the rat. J. Clin. Invest. 98, 1780–1787.

Marfaing-Koka, A., Devergne, O., Gorgone, G., Portier, A., Schall, T.J., Galanaud, P., Emilie, D., 1995. Regulation of the production of the RANTES chemokine by endothelial cells: synergistic induction by IFN plus TNF$\alpha$ and inhibition by IL-4 and IL-13. J. Immunol. 154, 1870.

Mitchell, J.A., Akarasereenont, P., Thiemermann, C., Flower, R.J., Vane, J.R., 1993. Selectivity of nonsteroid anti-inflammatory drugs as inhibitors of constitutive and inducible cyclo-oxygenase. Proc. Natl. Acad. Sci. U. S. A. 90, 11693–11697.

Narumiya, S., 1994. Prostanoid receptors: structure, function and distribution. Ann. N. Y. Acad. Sci. U. S. A. 744, 126–138.

Pang, L., Knox, A.J., 1997. Effect of interleukin-1$\beta$, tumour necrosis factor-$\alpha$ and interferon-$\gamma$ on the induction of cylo-oxygenase-2 in cultured human airway smooth muscle cells. Br. J. Pharmacol. 121, 579–587.

Pang, L., Holland, E., Knox, A.J., 1998. Role of cyclo-oxygenase-2 induction in interleukin-1beta induced attenuation of cultured human airway smooth muscle cell cyclic AMP generation in response to isoprenaline. Br. J. Pharmacol. 125, 1320–1328.

Rathanaswami, P., Hachicha, M., Sadick, M., Schall, T.J., McColl, S.R., 1993. Expression of the cytokine RANTES in human rheumatoid synovial fibroblasts: differential regulation of RANTES and interleukin-8 genes by inflammatory cytokines. J. Biol. Chem. 268, 5834.

Rot, A., Krieger, M., Brunner, T., Bischoff, S.C., Schall, T.J., Dahinden, C.A., 1992. RANTES and macrophage inflammatory protein 1$\alpha$ induce the migration and activation of normal human eosinophil granulocytes. J. Exp. Med. 176, 1489.

Saunders, M.A., Mitchell, J.A., Seldon, P.M., Barnes, P.J., Giembycz, M.A., Belvisi, M.G., 1997. Release of granulocyte macrophage-colony stimulating factor by human cultured airway smooth muscle cells: suppression by dexamethasone. Br. J. Pharmacol. 120, 545–546.

Schall, T.J., Bacon, K., Toy, K.J., Goeddel, D.V., 1990. Selective attraction of monocytes and T lymphocytes of the memory phenotype of cytokine RANTES. Nature 347, 669.

Shore, S.A., Laporte, J., Hall, I.P., Hardy, E., Panettieri, R.A., 1997. Effect of IL-1 beta on responses of cultured human airway smooth muscle cells to bronchodilator agonists. Am. J. Respir. Cell Mol. Biol. 16, 702–711.

Sousa, A.R., Pfister, R., Christie, P.E., Lane, S.J., Nasser, S., Schmitz-Schumann, M., Lee, T.H., 1997. Enhanced expression of cyclooxygenase isoenzyme 2 (COX-2) in asthmatic airways and its cellular distribution in aspirin-sensitive asthma. Thorax 52, 940–945.

Warner, T.D., Giuliano, F., Vojnovic, I., Bukasa, A., Mitchell, J.A., Vane, J.R., 1999. Nonsteroid drug selectivities for cyclo-oxygenase-1 versus cyclo-oxygenase-2 correlate with human gastrointestinal toxicity: a full *in vitro* analysis. Proc. Natl. Acad. Sci. U. S. A. 96, 7563–7568.