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Procaterol inhibits IL-1β- and TNF-α-mediated epithelial cell eosinophil chemotactic activity

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ABSTRACT: Theophylline inhibits eosinophilic infiltration into the bronchial wall. It is unknown whether this is mediated by a cyclic adenosine monophosphate (c-AMP) - dependent reduction in eosinophil chemotactic activity (ECA) from bronchial epithelial cells (BEC). Therefore the effect of a β_2-agonist, procaterol and theophylline on the release of ECA from a BEC line, BEAS-2B was evaluated in response to interleukin (IL)-1β and tumour necrosis factor-α (TNF-α).

ECA was assessed using a blind-well chemotactic chamber, and the release and gene expression of cytokines were evaluated by means of enzyme-linked immunosorbent assay and reverse transcriptase polymerase chain reaction.

IL-1β and TNF-α stimulated the release of ECA from BEAS-2B cells in a dose- and time-dependent manner. Procaterol and theophylline directly inhibited eosinophil migration to IL-1β and TNF-α-conditioned medium. The pretreatment of BEAS-2B cells with the same concentrations of procaterol inhibited the release of ECA in a dose-dependent fashion. Anti-IL-8, anti-regulated on activation, normal T-cell expressed and secreted (RANTES), and anti-granulocyte-macrophage colony-stimulating factor (GM-CSF) inhibited ECA. Procaterol inhibited the release of RANTES, GM-CSF and IL-8 in a dose-dependent fashion. The effect of theophylline was less potent. Procaterol augmented cAMP levels in BEAS-2B cells in a time- and dose-dependent manner. The expression of IL-8, RANTES, and GM-CSF messenger ribonucleic acid was not inhibited by procaterol and theophylline.

These data indicate that procaterol and theophylline may directly inhibit eosinophil migration and that procaterol may further inhibit the release of eosinophil chemotactic activity from BEAS-2B cells *via* a cyclic adenosine monophosphate-dependent mechanism. This warrants further studies on the involvement of bronchial epithelial cells in the anti-inflammatory effects of procaterol and theophylline in patients with asthma.
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Eosinophil recruitment into the lung is associated with both acute and chronic pulmonary disorders [1]. During an attack of asthma, eosinophils accumulate in the bronchial wall as well as in the airway lumen. There is now strong evidence that eosinophils play an important role in the airway inflammation characteristics of asthma through their ability to release a variety of toxic and pro-inflammatory mediators. These include eicosanoids, reactive oxygen species, cytokines and basic proteins. It has been suggested that the basic proteins, major basic protein, eosinophil cationic protein and eosinophil-derived neurotoxin contribute to the damage observed in the epithelial lining of the airways of asthmatics [2]. The presence of eosinophils in bronchoalveolar lavage fluid during periods of increased airway reactivity supports a relationship between the role of eosinophils in the production of the late asthmatic response and the subsequent increase in airway reactivity [3].

Bronchial epithelial cells (BEC) release chemotactic activity for inflammatory cells, including eosinophils [4, 5]. Interleukin (IL)-8 [6, 7], granulocyte colony-stimulating factor[7], granulocyte-macrophage colony-stimulating factor (GM-CSF) [6], transforming growth factor-β (TGF-β) [8], regulated on activation, normal T-cell expressed and secreted (RANTES) [9], monocyte chemotactic protein (MCP)-1 [10], MCP-4 [11], eotaxin [12], and eicosanoids [4, 5] are thought to be involved in the release of chemotactic factors in response to a variety of stimuli. Thus, BECs actively participate in the airway pathophysiology of asthma, including the bronchial inflammation.

Conversely, BECs express β-adrenergic receptors that are downregulated by intracellular cyclic adenosine monophosphate (cAMP) and TGF-β [3]. Since type IV phosphodiesterase inhibitors inhibited microvascular leakage into airway lumina [14], BECs may possess type IV phosphodiesterase, which may be inhibited by theophylline. Thus, theophylline and procaterol have the potential to increase cAMP levels in BECs themselves. The intracellular augmentation of cAMP levels in bovine BECs by prostaglandin E_2 and dibutyryl cAMP inhibited the release of neutrophil chemotactic activity in response to *Eschericia coli* lipopolysaccharide (LPS) [15].

Because theophylline inhibits the recruitment of inflammatory cells including eosinophils into the bronchial

wall and lumen and the release of inflammatory cytokines in a variety of cells [16], the effect of theophylline and procaterol on the release of eosinophil chemotactic activity (ECA) from BECs was evaluated. In the present study, it was demonstrated that procaterol inhibited the release of ECA from BECs and directly inhibited ECA-induced chemotaxis of eosinophils. However, theophylline predominantly inhibited only the latter.

Materials and methods

Bronchial epithelial cell preparation

BEAS-2B cells (passage 27–29, the kind gift of C. Harris, National Institute of Health, Bethesda, MD, USA) were used as human BECs. They were cultured in F-12 supplemented with bovine foetal serum (Sigma, St Louis, MO, USA), penicillin (50 U·mL^{-1}, Gibco, Grand Island, NY, USA), streptomycin (50 μg·mL^{-1}, Gibco) and fungizone (2 μg·mL^{-1}, Gibco). Once the cells reached confluence, they were harvested with 0.25% trypsin and ethylenediamine tetraacetic acid (0.1%) in phosphate-buffered solution (pH 7.3) (Sigma), centrifuged at low speed ($250 \times g$, 5 min) and resuspended in fresh medium at 1×10^6 cells·mL^{-1} in 35-mm tissue culture dishes. The cells reached confluence after 5–7 days′ incubation, and were then used for experiments.

Effects of procaterol and theophylline pretreatment of bronchial epithelial cells on their response to IL-1β and TNF-α

BEAS-2B cells were exposed to recombinant human IL-1β (500, 50 and 5 pg·mL^{-1}, Genzyme, Cambridge, MA, USA) and tumour necrosis factor-α (TNF-α·1,000, 100 and 10 U·mL^{-1}, Genzyme) for 12, 24 and 48 h. In some experiments, BEAS-2B cells were pretreated with clinical therapeutic concentrations of procaterol (1,000, 100 and 10 ng·mL^{-1} (3,100, 310 and 31 nM); Otsuka Pharmaceutical Co., Tokushima, Japan) and theophylline (20, 10 and 5 μg·mL^{-1} (111, 56 and 28 μM), Otsuka Pharmaceutical Co.) for 30 min, and then exposed to IL-1β and TNF-α for 24 or 48 h. IL-1β and TNF-α were tested for LPS contamination, and LPS was <1 ng·mL^{-1}. These cytokines did not cause BEAS-2B cell injury (no deformity of cell shape, no detachment from the culture dish and >98% of cells were viable by trypan blue exclusion) after a 48-h incubation. The culture supernatant fluid was harvested and frozen at -80°C until assayed. At least six separate BEAS-2B cell supernatant fluids were harvested from cultures for each experimental condition.

Measurement of eosinophil chemotactic activity

Eosinophils were isolated by a modification of the method of Hansel *et al.* [17] utilizing a magnetic cell separation system (MACS). Briefly, venous blood, anticoagulated with 13 mM trisodium citrate, was obtained from normal human volunteers and diluted 1:1 with phosphate-buffered saline (pH 7.3). Diluted blood was overlayered on an isotonic Percoll solution (density 1.082 g·mL^{-1}) (Sigma), and then centrifuged at $1,000 \times g$ for 30 min at 4°C. The supernatant and mononuclear cells at the interface were carefully removed, and erythrocytes in the sediment were lysed using two cycles of hypotonic water lysis. Isolated granulocytes were washed twice with piperazine-N,N′-bis-(2-ethansulphonic acid) PIPES buffer (25 mM PIPES, 50 mM NaCl, 5 mM KCl, 25 mM NaOH, 5.4 mM glucose, pH 7.4) containing 1% defined calf serum (DCS; Hyclone Laboratories, Logan, UT, USA), and an approximately equal volume of anti-CD16 conjugated with magnetic particles (Miltenyi Biotec, Bergisch Gladbach, Germany) was added to the cell suspension. After a 60 min incubation on ice, 5 mL of PIPES buffer containing 1% DCS was added to the cell/antibody mixture. Resuspended cells were loaded on to the separation column positioned in the strong MACS magnetic field. Cells were subjected to three separation cycles with 5 mL PIPES buffer containing 1% DCS. The purity of the eosinophils, assessed using Randolph′s stain was >94%, viability was >98%. Purified eosinophils were washed twice in PIPES buffer containing 1% DCS. The eosinophils were resuspended in Gey′s solution at 2.0×10^6 cells·mL^{-1} and used for the chemotaxis assay.

Chemotaxis was assayed in 48-well microchemotaxis chambers (Neuro Probe, Cabin John, MD, USA), as previously described [18]. The bottom wells of the chamber were filled with 25 μL of fluid containing the chemotactic stimulus or media in duplicate. A 10-μm-thick polyvinylpyrrolidone-free polycarbonate filter, with a pore size of 5 μm for eosinophil chemotaxis, was placed over the bottom wells. The silicone gasket and upper piece of the chamber were applied, and 50 μL of the cell suspension was placed into the upper wells above the filter. The chambers were incubated in humidified air 5% CO_2 for 180 min at 37°C to measure ECA. Cells which had not migrated were wiped away from the filter. The filter was then immersed in methanol for 5 min, stained with Diff-Quik (American Scientific Products, McGraw Park, IL, USA) and mounted on a glass slide. Cells that had completely migrated through the filter were counted in 10 random high-power fields (HPF; × 1,000) per well using light microscopy.

In order to determine whether the migration was due to movement along a concentration gradient (chemotaxis) or stimulation of random migration (chemokinesis), a checkerboard analysis was performed using BEAS-2B cell supernatant fluid harvested at 24 h in response to 50 pg·mL^{-1} IL-β and 100 U·mL^{-1} TNF-α. To do this, various dilutions of BEAS-2B cell supernatant fluids (1:1, 1:4, 1:16, 1:64 and 1:256) were placed below the membrane and above the membrane with target cells.

Direct effects of procaterol and theophylline on eosinophil migratory response

Theophylline and procaterol have the potential to attenuate the migratory response of eosinophils to chemotactic factors. The direct inhibitory effects of theophylline and procaterol on BEAS-2B cell conditioned medium harvested at 24 h in response to IL-1β and TNF-α was assessed. The concentrations of theophylline and procaterol added to the supernatant fluids were adjusted to the same concentrations as those used for BEAS-2B cell pretreatment. BEAS-2B cell conditioned medium plus theophylline and procaterol was tested for eosinophil chemotaxis and compared with BEAS-2B cell supernatant fluid, pretreated with theophylline and procaterol, in response to IL-1β and TNF-α.

Effects of anti-IL-8, anti-GM-CSF, and anti-RANTES on eosinophil chemotactic activity release in response to IL-1β and TNF-α

The neutralizing antibodies to human IL-8, GM-CSF and RANTES were purchased from Genzyme (Cambridge, MA, USA). These antibodies were obtained from mouse and added to BEAS-2B cell supernatant fluids, which were harvested after 24 h incubation with IL-1β and TNF-α at the suggested concentrations, to inhibit the migratory potential of these cytokines. These antibodies inhibited eosinophil migration in response to recombinant human IL-8, GM-CSF and RANTES (data not shown). The supernatant fluids and antibodies were coincubated for 30 min at 37°C and then used for chemotactic assay. These antibodies did not influence the chemotactic response to LPS-activated serum (data not shown). To assess the effect of immunoglobulin G (IgG), nonimmune IgG (Genzyme) was added to the supernatant and used as negative control for the antibodies.

Quantification of IL-8, GM-CSF and RANTES proteins in BEAS-2B cell supernatant fluids

Because IL-8, RANTES and GM-CSF antibodies inhibited ECA in the supernatant fluids, the concentration of IL-8, RANTES and GM-CSF in BEAS-2B cell supernatant fluids in response to IL-1β (50 pg·mL^{-1}) and TNF-α (100 U·mL^{-1}) treatment, in the presence or absence of procaterol and theophylline, for 24 h were measured by means of enzyme-linked immunosorbent assay according to the manufacturers' directions. IL-8, RANTES and GM-CSF kits were purchased from Amersham (Amersham, UK). The minimum concentration detected by these methods was 10.0 pg·mL^{-1} for, IL-8, 15.6 pg·mL^{-1} for RANTES and 2.0 pg·mL^{-1} for GM-CSF.

Evaluation of IL-8, RANTES and GM-CSF messenger ribonucleic acid expressions

The secretion of IL-8, RANTES and GM-CSF was inhibited by procaterol and theophylline. Reverse transcriptase polymerase chain reaction (PCR) was used to evaluate the messenger ribonucleic acid (mRNA) expression of IL-8, RANTES and GM-CSF in BEAS-2B cells after 6 h stimulation with 50 pg·mL^{-1} of IL-1β and 100 U·mL^{-1} TNF-α. Total ribonucleic acid (RNA) was extracted from BEAS-2B cells as previously described [19]. One microgram of total RNA was reverse transcribed into complementary deoxyribonucleic acid (cDNA) using a cDNA synthesis kit (Boeringer Mannheim, Mannheim, Germany) and then amplified for ≥27 cycles in a Perkin Elmer Gene Amp PCR System 9600 (Norwich, CT, USA) (denaturation for 30 s at 94°C, primer annealing for 30 s at 55°C, and primer extension for 30 s at 72°C). The IL-8, RANTES and GM-CSF sense, antisense, and probe sequences used are shown in table 1.

Preliminary studies indicated that >27 cycles were subsaturating for the mRNAs tested, and thus was appropriate for comparison of the relative levels of mRNA between groups. PCR products were separated by means of electrophoresis on a 3% agarose gel and were visualized by means of exposure to ^{32}P-labelled probes followed by autoradiography. PCR band densities were determined using the National Institutes of Health (NIH) image analytical program (NIH, Bethesda, MD, USA) on unaltered computer-scanned images. β-Actin mRNA, which has been shown not to change on stimulation, was measured in both normal and stimulated RNA samples at each point, using the same cDNA as analysed for cytokines. The integrated optical density measurements of 10 separate β-actin samples did not vary by >33% from the mean indicating the expected variation in results from this experimental technique.

Measurement of cyclic adenosine monophosphate levels in BEAS-2B cells in response to procaterol

Intracellular cAMP levels were measured in BEAS-2B cells in response to various concentrations (1.0, 10, 100 and 1,000 ng·mL^{-1}) of procaterol for a variety of time periods (2.5, 5, 7.5, 10, 15 and 20 min), as previously reported [20]. The cAMP radioimmunoassay kit was purchased from Amersham (Arlington Heights, IL, USA).

Statistics

In experiments in which multiple measurements were made, differences between groups were tested for significance using one-way variance analysis, applying Duncan's multiple range test to data at specific time and dose points. In experiments in which a single measurement was made, the differences between groups were tested for

Table 1. – Interleukin (IL)-8, regulated on activation, normal T-cell expressed and secreted (RANTES) and granulocyte-macrophage colony-stimulating factor (GM-CSF) sense, antisense and probe nucleotide sequences

	Strand	Nucleotide sequence
RANTES	Sense	5′-ATA TTC CTC GGA CAC CAC AC-3′
	Antisense	5′-CAC GTC CAG CCT GGG GAA GG-3′
	Probe	5′-ACA CCA GTG GCA AGT GCT CCA ACC CAG CAG-3′
GM-CSF	Sense	5′-TGA ACC TGA GTA GAG ACA CTG C-3′
	Antisense	5′-TGA CAA GCA GAA AGT CCT TCA G-3′
	Probe	5′-ATG TTT GAC CTC CAG GAG CCG ACC TGC CTA-3′
IL-8	Sense	5′-AAC ATG ACT TCC AAG CTG GC-3′
	Antisense	5′-ACT GGC ATC TTC ACT GAT TC-3′
	Probe	5′-TTG AGA GTG GAC CAC ACT GCG CCA ACA CAG-3′
β-Actin	Sense	5′-TGA CCC AGA TCA TGT TTG AG-3′
	Antisense	5′-TCA TGA GGT AGT CAG TCA GG-3′
	Probe	Human cDNA

A: adenine; T: thymine; C: cytosine; G: guanine; cDNA: complementary deoxyribonucleic acid.

significance using Student's paired t-test. In all cases, a p-value <0.05 was considered significant. Data in figures are expressed as mean±SEM.

Results

Release of eosinophil chemotactic activity from BEAS-2B cells in response to IL-1β and TNF-α

IL-1β and TNF-α stimulated the release of ECA from BEAS-2B cells in a time- and dose-dependent fashion (fig. 1). ECA release was observed in response to 50 pg·mL^{-1} IL-1β and 100 U·mL^{-1} TNF-α after 12 h. IL-1β and TNF-α induced the release of significant amounts of ECA at 5 pg·mL^{-1} and 10 U·mL^{-1}, respectively.

Checkerboard analysis revealed that the supernatant fluids of BEAS-2B cells stimulated by IL-1β and TNF-α induced eosinophil migration in the presence of a gradient across the membrane in a concentration-dependent manner. However, a smaller increase in eosinophil migration was observed in the absence of a gradient (data not shown). Thus, the migration of eosinophils was predominantly consistent with a chemotactic rather than a chemokinetic mechanism.

Effects of BEAS-2B cell pretreatment on eosinophil chemotactic activity and direct inhibition with procaterol and theophylline

Procaterol and theophylline inhibited the release of ECA in response to 50 pg·mL^{-1} IL-1β (fig. 2a and b) and 100 U·mL^{-1} TNF-α (fig. 2c and d) in a dose-dependent manner after 24 h. Similar effects were observed after 48 h (data not shown). Although procaterol and theophylline significantly inhibited ECA release in response to 5 pg·mL^{-1} IL-1β and 10 U·mL^{-1} TNF-α in a dose-dependent fashion at both 24 and 48 h, they did not inhibit the release of ECA in response to 500 pg·mL^{-1} of IL-1β and 1,000 U·mL^{-1} of TNF-α at both 24 and 48 h (data not shown).

Direct addition of procaterol and theophylline to IL-1β and TNF-α conditioned medium significantly attenuated the eosinophil migratory response in a dose-dependent fashion (fig. 2). The effect of pretreatment of BEAS-2B cell monolayers with the same concentrations of procaterol was, though significant, small. Although pretreatment of BEAS-2B cells with theophylline at a concentration of 20 μg·mL^{-1} significantly, but slightly, inhibited the release of ECA ($p<0.05$), the effects of theophylline on eosinophil migration was primarily a direct effect on eosinophils.

Inhibition of eosinophil chemotactic activity by anti-IL-8, anti-RANTES and anti-GM-CSF

IL-8-, RANTES-, and GM-CSF-neutralizing antibodies inhibited ECA release from BEAS-2B cell monolayers in response to IL-1β and TNF-α (table 2), respectively. The addition of the three antibodies together inhibited 60–70% of ECA in the supernatant fluids in response to

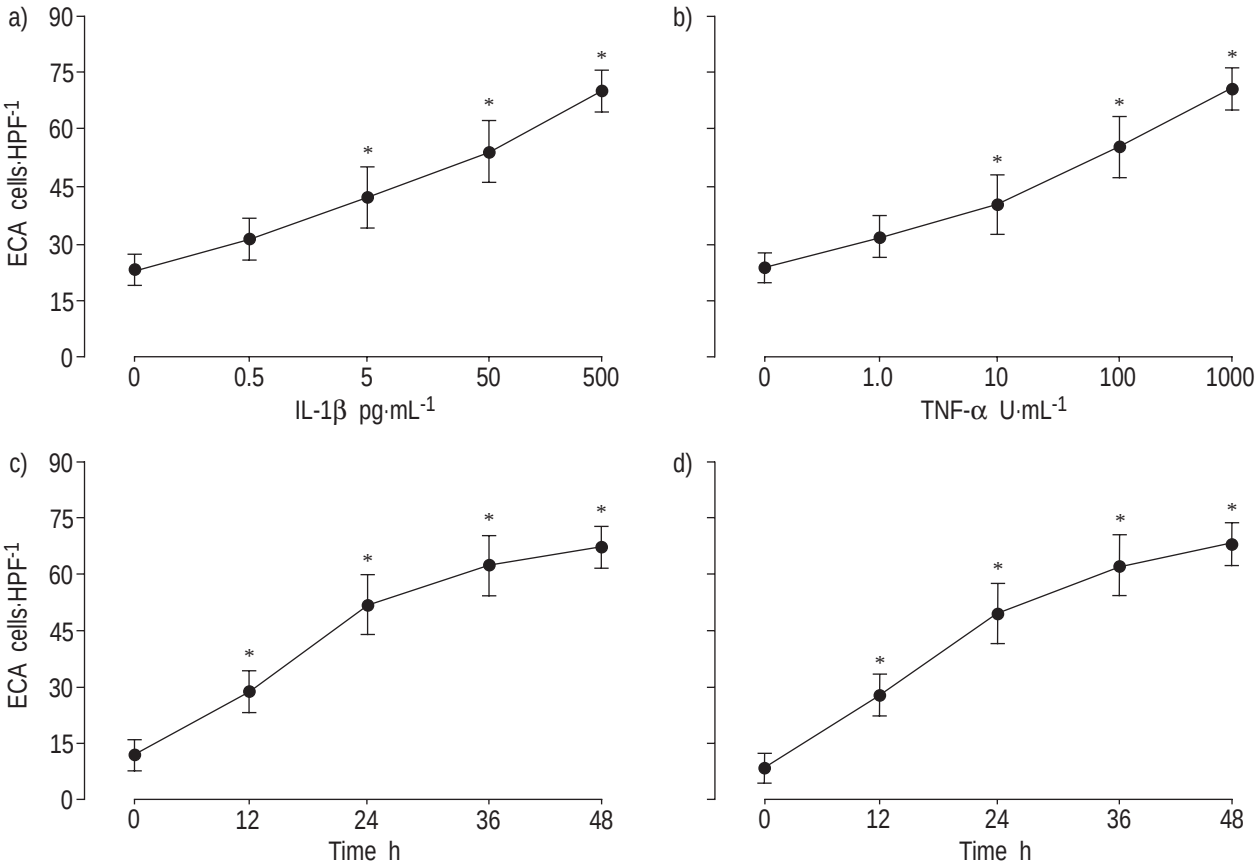

Fig. 1. – Release of eosinophil chemotactic activity (ECA) in response to: a, c) interleukin (IL)-1β; and b, d) tumour necrosis factor-α (TNF-α) from BEAS-2B cell monolayers (n=6). Data are expressed as mean±SEM. HPF: high-power field. *: $p<0.01$ *versus* unstimulated supernatant fluid.

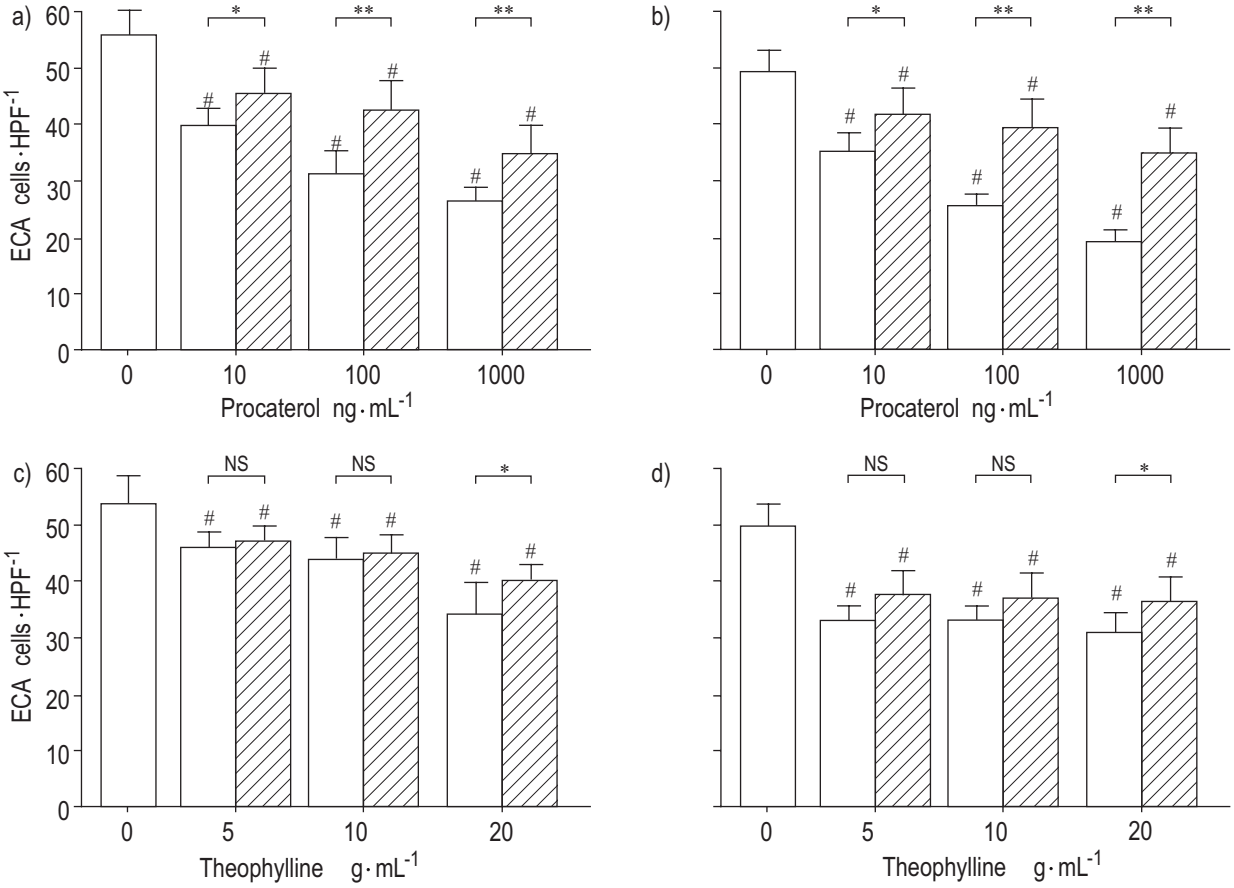

Fig. 2. – Effects of: a, b) procaterol; and c, d) theophylline on eosinophil migratory response to: a, c) interleukin (IL)-1β; and b, d) tumour necrosis factor-α (TNF-α). □: effects of procaterol or theophylline pretreatment on the release of eosinophil chemotactic activity (ECA) from BEAS-2B cell monolayers in response to IL-1β or TNF-α stimulation; ▨: direct effects of procaterol or theophylline on eosinophil migration in response to IL-1β- or TNF-α-stimulated BEAS-2B cell conditioned medium. Data are expressed as mean±SEM. HPF: high-power field; NS: nonsignificant. *: $p<0.05$; **: $p<0.01$; #: $p<0.01$ *versus* IL-1β or TNF-α.

IL-1β and TNF-α. Nonimmune IgG did not have any effect on ECA released from BEAS-2B cell monolayers in response to IL-1β and TNF-α.

Procaterol and theophylline inhibition of IL-8, RANTES and GM-CSF secretion from BEAS-2B cell monolayers in response to IL-1β and TNF-α

The release of GM-CSF elicited by TNF-α was lesser than that brought about by IL-1β. Procaterol inhibited the release of GM-CSF in response to TNF-α in a dose-dependent fashion. However, the inhibition of release in response to IL-1β was limited, but significant (fig. 3a and b). The release of GM-CSF in response to TNF-α, but not to IL-1β was also inhibited significantly by theophylline at a concentration of 20 mg·mL^{-1}.

IL-1β and TNF-α stimulated the release of IL-8 similarly. Procaterol slightly, but significantly, inhibited the release of IL-8 in response to IL-1β and TNF-α from BEAS-2B cell monolayers at each dose examined (fig. 3c and d). The release of IL-8 in response to both IL-1β and TNF-α was also inhibited significantly by theophylline at a concentration of 20 μg·mL^{-1}.

The release of RANTES elicited by TNF-α was greater than that brought about by IL-1β. Procaterol inhibited the release of RANTES in response to IL-1β and TNF-α from BEAS-2B cell monolayers dose-dependently (fig. 3e and

Table 2. – Inhibition of eosinophil chemotactic activity (ECA) released from BEAS-2B cell monolayers in response to IL-1β and tumour necrosis factor-α (TNF-α) by anti-interleukin (IL)-8, anti-granulocyte-macrophage colony-stimulating factor (GM-CSF) and anti-regulated on activation, normal T-cell expressed and secreted (RANTES)

	ECA cells·HPF^{-1}						
	No antibody	Anti-RANTES	Anti-GM-CSF	Anti-IL-8	Three Abs^{+}	Nonimmune IgG$^{\#}$	F-12§
IL-1β	54±4	33±3*	39±4*	37±27*	17±6*	55±6	11±3
TNF-α	48±6	30±5*	38±6*	39±6*	16±4*	51±6	11±3

Data are presented as mean±SEM (n=6). $^{+}$: the three antibodies (Abs) used together; $^{\#}$: representing the effect of immunoglobulin G (IgG) itself on ECA. §: unstimulated cells. F-12: Ham's F-12 medium; HPF: high-power field. *: $p<0.01$ *versus* IL-1β or TNF-α.

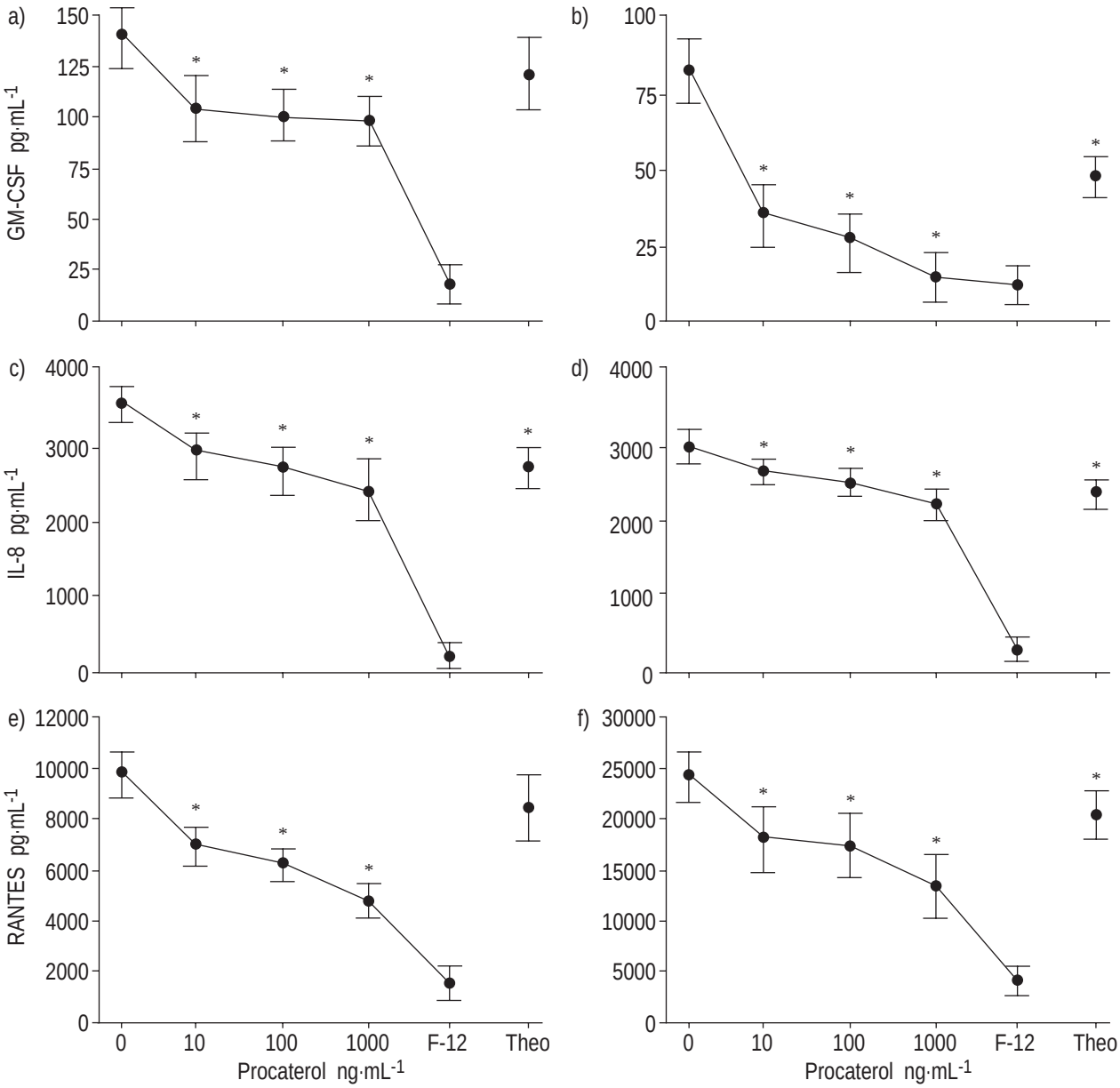

Fig. 3. – Procaterol and theophylline inhibition of the release of: a, b) granulocyte-macrophage colony-stimulating factor (GM-CSF); c, d) interleukin (IL)-8; and e, f) regulated on activation, normal T-cell expressed and secreted (RANTES) from BEAS-2B cell monolayers in response to: a, c, e) IL-1β; and b, d, f) tumour necrosis factor-α (TNF-α). Data are presented as mean±SEM. F-12 represents release from unstimulated BEAS-2B cell monolayers (control). Theo: theophylline (20 μg·mL^{-1}). *: p<0.01 *versus* IL-1β or TNF-α.

f). The release of RANTES in response to TNF-α, but not to IL-1β was inhibited significantly by theophylline at a concentration of 20 mg·mL^{-1}.

Theophylline and procaterol additively, inhibited the release of IL-8, RANTES and GM-CSF in response to IL-1β at the maximum concentration tested (table 3).

Effects of procaterol and theophylline on IL-8, RANTES and GM-CSF messenger ribonucleic acid expression in response to IL-1β and TNF-α

IL-1β and TNF-α augmented the expression of IL-8 mRNA. Procaterol and theophylline did not attenuate the increased expression of IL-8 mRNA in response to IL-1β and TNF-α (table 4).

Although BEAS-2B cells did not release detectable amount of RANTES into the supernatant fluids in the steady state, BEAS-2B cells constitutively expressed RANTES mRNA. IL-1β and TNF-α, further augmented the expression of RANTES mRNA. Procaterol and theophylline did not attenuate the expression of RANTES mRNA.

IL-1β and TNF-α did not augment the expression of GM-CSF mRNA even when assessed after a 40-cycle amplification (table 4). Procaterol slightly augmented the expression of GM-CSF mRNA in response to IL-1β.

Cyclic adenosine monophosphate concentrations in BEAS-2B cells in response to procaterol

Procaterol induced increases in intracellular cAMP levels in a time- and dose-dependent manner (fig. 4).

Table 3. – Additive effects of theophylline and procaterol on release of interleukin (IL)-8, regulated on activation, normal T-cell expressed and secreted (RANTES) and granulocyte-macrophage colony-stimulating factor (GM-CSF) from BEAS-2B cells in response to IL-1β

	No inhibitor	Theophylline	Procaterol	Theophylline + procaterol
IL-8 pg·mL^{-1}	3556±345	2659±234*	2245±359*	2011±298*
RANTES pg·mL^{-1}	9860±1290	5129±872*	8390±975	5010±790*
GM-CSF pg·mL^{-1}	140±19	97±21*	115±14	83±19*

Data are presented as mean±SEM (n=5). *: p<0.01 *versus* IL-1β alone.

Discussion

In the present study, the effect of therapeutic concentrations of procaterol and theophylline on the release of ECA from BEC line, BEAS-2B, cells in response to IL-1β and TNF-α were evaluated. Procaterol inhibited both the release of ECA and ECA-induced chemotaxis of eosinophils, but theophylline inhibited the latter. Antibodies directed against RANTES, GM-CSF and IL-8 inhibited ECA in BEAS-2B cell conditioned medium in response to IL-1β and TNF-α. Although procaterol inhibited the release of RANTES, GM-CSF and IL-8, the effect of theophylline was less potent. Procaterol induced the increase in cAMP concentration in BEAS 2B cells in a time- and dose- dependent fashion. The expression of IL-8, RANTES, and GM-CSF mRNA induced by IL-1β and TNF-α was not inhibited by procaterol and theophylline. These data suggest that procaterol may inhibit the release of ECA from BECs.

The mechanisms accounting for the recruitment of inflammatory cells to the airway are still uncertain. BECs have the potential to release ECA in response to acetylcholine and substance P [21]. Although inflammatory cells, including alveolar macrophages, lymphocytes, neutrophils and eosinophils, have the potential to release ECA, significant amounts of ECA were released from BECs in response to IL-1β and TNF-α in the present study. The release of ECA from BECs may play an important role in eosinophil recruitment in the airways.

Identification of the ECA released from BECs in response to IL-1β and TNF-α was not fully performed. However, the chemotactic factors released in response to IL-1β and TNF-α included, IL-8, GM-CSF and RANTES. The concentrations of IL-8, RANTES and GM-CSF of the ECA in the present study reached the concentration of ECA previously reported. Although BECs have the potential to release MCP-4, eotaxin and eicosanoids as ECA [4, 5, 11, 12], IL-8, GM-CSF and RANTES together accounted for 60–70% of the total chemotactic activity in the present study.

Table 4. – Ratios of cytokine messenger ribonucleic acid: β-actin*

	RANTES	IL-8	GM-CSF
Control	0.77±0.08	0.00	0.00
TNF-α 100 U·mL^{-1}			
No inhibitor	1.04±0.12	0.65±0.06	0.11±0.03
Proc 1000 ng·mL^{-1}	1.20±0.21	0.81±0.11	0.00
Proc 100 ng·mL^{-1}	0.88±0.09	0.64±0.08	0.10±0.04
Proc 10 ng·mL^{-1}	0.89±0.10	0.73±0.08	0.02±0.01
Theo 20 µg·mL^{-1}	1.22±0.21	0.95±0.11	0.08±0.02
IL-1β 50 pg·mL^{-1}			
No inhibitor	0.90±0.08	0.85±0.09	0.05±0.01
Proc 1000 ng·mL^{-1}	0.87±0.08	1.03±0.09	0.09±0.03
Proc 100 ng·mL^{-1}	0.90±0.08	0.95±0.11	0.08±0.03
Proc 10 ng·mL^{-1}	1.08±0.09	0.83±0.09	0.07±0.01
Theo 20 µg·mL^{-1}	1.26±0.21	0.95±0.08	0.08±0.01

Data are presented as mean±SEM (three experiments). *: assessed using National Institutes of Health image analyser. RANTES: regulated on activation, normal T-cell expressed and secreted; IL: interleukin; GM-CSF: granulocyte-macrophage colony-stimulating factor; TNF-α: tumour necrosis factor-α; Proc: procaterol; Theo: theophylline.

Although IL-8 has been discounted as a mediator of eosinophil diapedesis and function, there is growing evidence that IL-8 is a potent eosinophil chemoattractant. SEHMI *et al*. [22] showed that the eosinophil chemotactic responsiveness to IL-8, as measured using Boyden chambers, was associated with blood eosinophilia, related to both atopic and nonatopic diseases. SHULE *et al*. [23] reported that the disease state (allergen challenge) is important for IL-8 responsiveness. The maximum response was observed at a concentration of IL-8 of 10^{-8} M (80 ng·mL^{-1}) as required for neutrophils, and IL-8 was two orders of magnitude more potent than platelet-activating factor (PAF) as an eosinophil chemoattractant in this assay [22]. It is now clear that *in vitro* cytokine priming of eosinophils from normal [22] and atopic donors [24] produced a migrational response to IL-8 at a concentration of 10^{-10} M (800 pg·mL^{-1}). In the present study, BEAS-2B cell supernatant fluid contained GM-CSF, which has the potential to prime eosinophils and to induce an increase in nondirectional chemokinetic activity in response to IL-8 [24]. The concentration of IL-8 in the supernatant fluids was 1,000–4,000 pg·mL^{-1}. Thus the eosinophil migratory response to IL-8 in the present study may be due to both a direct response to IL-8 and a priming effect of GM-CSF in the supernatant fluids.

In the present study, theophylline and procaterol directly attenuated eosinophil migration in response to BEAS-2B cell conditioned medium stimulated by IL-1β and TNF-α. Although eosinophils express β-adrenoreceptors and contain phosphodiesterase [25, 26], the effects of theophylline and procaterol might occur *via* increased intracellular cAMP concentrations. However, the cAMP concentration in eosinophils in response to theophylline did not correlate with the inhibition of eosinophil migratory response (data not shown). Although the mechanisms underlying eosinophil migratory inhibition by theophylline are uncertain, the anti-inflammatory effects of theophylline and procaterol may involve the direct inhibition of ECA-induced chemotaxis in eosinophils.

Since procaterol induced an increase in intracellular cAMP levels in BEAS-2B cells in a time- and dose-dependent manner, the mechanism of procaterol inhibition of release of these cytokines from BEAS-2B cells seems to involve intracellular augmentation of cAMP levels. In support of this, it has been reported that BEC activation by

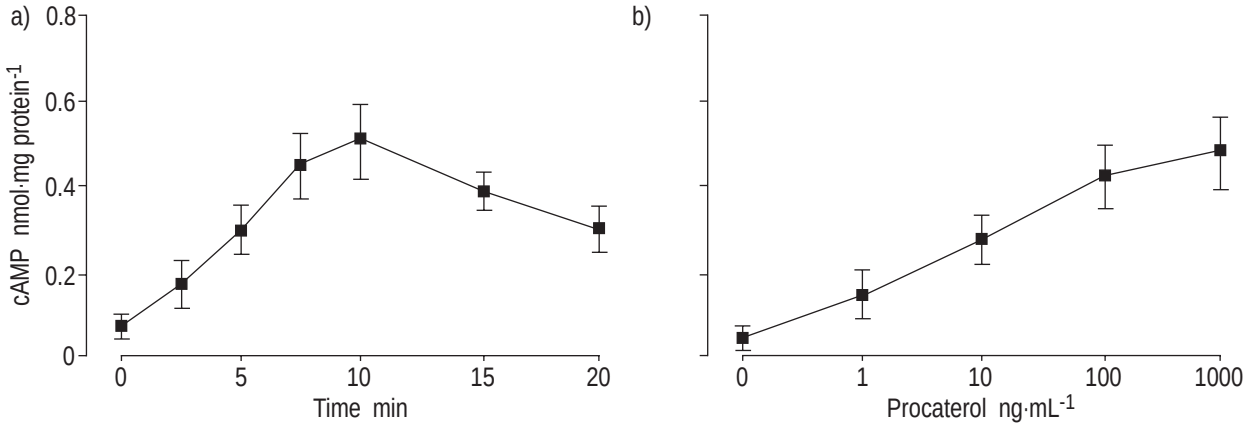

Fig. 4. – Time (a; 1,000 ng·mL^{-1} procaterol)- and dose (b; 10-min incubation)-dependent increases in cyclic adenosine monophosphate (cAMP) concentration in BEAS-2B cells in response to procaterol (n=3).

E. coli LPS is inhibited by dibutynyl cAMP and prostaglandin E_2 [15].

The reduction in eosinophil recruitment caused by β-adrenergic agonists is controversial. TEIXEIRA *et al.* [27] reported that both short- and long-acting β_2-adrenoreceptor agonists inhibited the eosinophil accumulation induced by PAF and in a passive cutaneous anaphylactic reaction. In contrast, KRAFT *et al.* [28] showed that salmeterol did not inhibit inflammatory changes assessed by means of bronchoalveolar lavage in patients with nocturnal asthma, and PIZZICHINI *et al.* [29] showed that salmeterol did not have any significant effects on sputum and blood inflammatory changes 7–48 h after allergen challenge. MANOLITSAS *et al.* [30] reported that the number of total and activated eosinophils, expressed as cells·mm^2 bronchial biopsy^{-1}, increased after treatment with albuterol. The present results suggest that procaterol might reduce eosinophil recruitment by direct inhibition of the migratory response and release of ECA.

It has been reported that a β_2-agonist, sulbutamol, does not inhibit release of IL-8 from BECs in response to 2 ng·mL^{-1} TNF-α [6]. Inhibition of ECA release by procaterol and theophylline occurred at a concentration of 100 U·mL^{-1} TNF-α and 50 pg·mL^{-1} IL-1β in the present study. Procaterol and theophylline did not inhibit the release of ECA at concentrations >1,000 U·mL^{-1} TNF-α or 500 pg·mL^{-1} IL-1β. Thus, differences in the stimulating potential may explain the discrepancy regarding the effects of β_2-agonist on release of IL-8.

In conclusion, the effect of procaterol on eosinophil migration in response to BEAS-2B cell-derived eosinophil chemotactic activity was primarily a direct effect on eosinophils. The additional effect of preincubation of procaterol with BEAS-2B cells during culture was, though significant, small. The effect of theophylline occurred *via* a direct effect on eosinophils. However, the release of eosinophil chemotactic cytokines, *i.e.* interleukin-8, regulated on activation, normal T-cell expressed and secreted, and granulocyte-macrophage colony-stimulating factor was inhibited by procaterol.

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