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Increased interleukin-8 release by β-adrenoceptor activation in human transformed bronchial epithelial cells

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1 The effect of β-adrenoceptor activation on release of the neutrophil chemoattractant, interleukin-8 (IL-8), was examined in human transformed bronchial epithelial cells (16HBE cells).

2 The combined β_1- and β_2-adrenoceptor agonist, isoprenaline, time- (100 nM, 2–18 h) and concentration- (1–30 nM) dependently increased IL-8 protein content in the cell culture supernatant as measured by an enzyme immunosorbent assay standardized for DNA by fluoro-colorimetry.

3 Isoprenaline (1–100 nM, 15 min) increased cyclic AMP concentration-dependently.

4 The effect of isoprenaline (100 nM) was inhibited by the β-adrenoceptor blocker propranolol (10 μM). The maximum magnitude of IL-8 increase caused by β-adrenoceptor activation was 40% of that caused by the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α: 100 ng ml^{-1}).

5 The selective β_2-adrenoceptor agonist salbutamol (1 μM), increased IL-8 protein similarily to isoprenaline and the cyclic AMP analogue, dibutyryl cyclic AMP (1 mM) produced a corresponding effect.

6 Pretreatment with isoprenaline (100 nM) followed by TNF-α (20 ng ml^{-1}) increased IL-8 additively.

7 In conclusion, β-adrenoceptor stimulation increased the release of the neutrophil chemoattractant, IL-8 in 16HBE cells, via an increase in intracellular cyclic AMP. β-adrenoceptor stimulation adds to the IL-8 increase caused by the pro-inflammatory cytokine TNF-α. If this mechanism exists *in vivo*, β-adrenoceptor activation may increase neutrophil chemotaxis into the airways.

Keywords: Cyclic AMP; isoprenaline; airway epithelial cells; neutrophil chemotaxis; IL-8; salbutamol; TNF-α

Introduction

Studies on asthmatic subjects show that regular inhalation of the β_2-adrenoceptor agonist, salbutamol, can increase both the early and the late asthmatic airway responsiveness to allergen challenge and more recent data indicate that this is associated with an increased airway inflammation involving neutrophils (Cockroft *et al.*, 1993; 1995; Gavreau *et al.*, 1995; Morley *et al.*, 1995). Until now, however, the mechanism behind this type of increased neutrophil recruitment has been unknown.

It is known that the cytokine, interleukin-8 (IL-8), causes neutrophils to migrate to the luminal side of airway epithelial cells *in vitro* (Isano *et al.*, 1995) as well as *in vivo* (Johrens *et al.*, 1992). It is also known that airway epithelial cells from humans and dogs release IL-8 in response to inflammatory stimuli *in vitro*, including tumour necrosis factor alpha (TNF-α) (Inoue *et al.*, 1994; Kwon *et al.*, 1994; Massion *et al.*, 1995; Nakamura *et al.*, 1991). Airway epithelial cells may therefore play a role in controlling neutrophil recruitment into the airway lumen. Consequently, a drug which targets airway epithelial cells and alters the release of IL-8, also possesses the potential to modulate neutrophil recruitment into the airways.

Inhaled salbutamol is likely to target airway epithelial cells and the time frame needed for salbutamol to increase the number of neutrophils in bronchoalveolar lavage (Morley *et al.*, 1995) is similar to that needed for human bronchial epithelial cells to produce the neutrophil chemoattractant IL-8 (Massion *et al.*, 1994). Hypothetically, the effect of salbutamol on neutrophil recruitment could therefore be attributed to an increased release of IL-8 from airway epithelial cells but until now, there have been no data indicating that such a mechanism exists. The present *in vitro* study was therefore designed to evaluate whether β-adrenoceptor agonists amplify neutrophil chemotaxis by increasing the release of IL-8 from human bronchial epithelial cells and, if so, whether this effect on IL-8 is mediated by β-adrenoceptors and cyclic AMP.

Methods

Cell culture media and supplements

Minimal essential Eagle's medium with Earle's balanced salt solution (MEM Earle-Eagle), phosphate buffered saline (PBS), PBS (Ca^{2+}-, Mg^{2+}-free), trypsin (STV), (0.025% STV 0.01% EDTA) penicillin-streptomycin (10 000 u ml^{-1} and 10 000 mg ml^{-1}) and foetal bovine serum (FBS) were obtained sterile from the University of California San Francisco Cell Culture Facility. Human fibronectin (FNC) was purchased from Collaborative Research (Bedford, MA, U.S.A.) and collagen (Vitrogen 100) was obtained from Celtrix Laboratories (Palo Alto, CA, U.S.A.)

Cell culture

Human transformed bronchial epithelial (i.e. 16HBE14o-, abbreviated as 16HBE) cells were chosen because of their documented ability to release IL-8 in response to inflammatory stimuli in a similar way to human primary bronchial epithelial cells (Cozens *et al.*, 1994; Gruenert *et al.*, 1995; Massion *et al.*, 1994; 1995). These cells form polarized monolayers with intact tight junctions *in vitro*. 16HBE cells were received from Dr Dieter Gruenert, Cardiovascular Research Institute, University of California, San Francisco, U.S.A. The cells were grown to confluence in MEM Earle-Eagle medium with 10% FBS and penicillin-streptomycin (P-S, 100 u ml^{-1} and 100 mg ml^{-1} respectively) on 6-well (35 mm diameter) plates coated with collagen and fibronectin (Massion *et al.*, 1994). Eighteen h prior to all experiments, cells were placed in fresh MEM Earle-Eagle medium containing 10% FBS and P-S. Immediately prior to each experiment, the cells were washed 2 times with PBS. During the up to 18 h long experiments, the FBS content was reduced to 1% to minimize the spontaneous IL-8 release without causing significant cell damage (Skerret *et al.*, 1994). Lactate dehydrogenase (LDH 320, Sigma Chemical Co., St. Louis, MO, U.S.A.) was used to assess cell damage as

described previously (Massion *et al.*, 1994). The extracellular LDH levels were the same for cells treated with isoprenaline (0.1 μM) and saline (0.9%), respectively (data not shown).

Measurement of interleukin-8

Confluent 16HBE cells were used for experiments for up to 18 h. Cell supernatant was collected and frozen at the end of each experiment. Thawed samples were diluted in commercial assay buffer (from the enzyme immunosorbent assay (EIA) kit) and measured with a commercial, highly specific EIA for human interleukin-8 (Biotrak Interleukin-8 EIA, Amersham Ltd, Buckinghamshire, England) (Massion *et al.*, 1994). For this EIA kit, the increase in IL-8 signal correlates to an increase in non-specific protein (Massion *et al.*, 1994). The current results were also confirmed with EIA kits of alternative brands. The cell fragments in the pellet remaining after centrifugation were frozen for DNA measurements.

Measurement of cyclic AMP

Confluent 16HBE cells were incubated with isoprenaline (1 nM – 1 μM) for 15 min and the metabolism was then stopped with ice cold trichloroacetic acid (6% final concentration) as previously described (Kaneko *et al.*, 1995). Cells were scraped off and frozen together with supernatant immediately after the experiment. The cell samples were then thawed, sonicated (3 × 10 s at minimum setting, Branson, model 350, Sonifier Inc., Danbury, CT, U.S.A.) and spun (5000 r.p.m., 15 min, 4°C) to obtain a clear supernatant. This supernatant was washed in water-saturated ether four times and evaporated using room air (60°C, 3 h). The supernatant samples were then rediluted in a commercial assay buffer (from the EIA kit) followed by measurement of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) with a highly specific EIA (Biotrak cAMP EIA, Amersham Ltd, Buckinghamshire, England). The cell fragments in the pellet remaining after centrifugation were frozen for DNA measurements.

Measurement of DNA

DNA measurements were used to standardize each experiment for hypothetical variations in total cell numbers (Massion *et al.*, 1994). DNA was measured by a standardized fluoro-colorimetric method as described previously (Cesarone *et al.*, 1979).

Drugs

Tumour necrosis factor alpha (TNF-α) was purchased from Genzyme Co. (Cambridge, MA, U.S.A.). The selective β_2-adrenoceptor agonist, salbutamol, the cyclic AMP analogue, dibutyryl cyclic AMP (db cyclic AMP) (Koyama *et al.*, 1991), the combined β_1- and β_2-adrenoceptor agonist (±)-isoprenaline and the combined β_1- and β_2-adrenoceptor antagonist, propranolol were purchased commercially (Sigma Chemical Co., St. Louis, MO, U.S.A.). Propranolol was initially dissolved in ethanol (10% v/v) and thereafter diluted in sterile saline (NaCl 0.9%), producing a final ethanol concentration of 0.01% in the cell culture. The corresponding ethanol concentration (0.01%) was present in experiments comparing other drugs with propranolol plus isoprenaline (data in Figure 2). In all other experiments, drugs were dissolved and diluted in sterile saline. Separate experiments indicated no difference in LDH or IL-8 production for saline compared with PBS (data not shown).

Statistics

The computer software StatView 4.01 (Abacus Concepts Inc, Berkeley, CA, U.S.A.) was used. Correlation factors were evaluated by Spearman's rank correlation (non-parametric). Single measure points were compared by Wilcoxon's signed rank test (non-parametric). Multiple measure points were compared by ANOVA followed by Fischer's PLSD test. All comparisons of drug effects on IL-8 and cyclic AMP production were conducted on samples originating from the same cell plates (paired observations). *P*-values less than 0.05 were considered statistically significant. Each experimental series was based upon at least three different cell passages. *n* equals the number of independent experiments, conducted on separate days.

Results

Effect of isoprenaline on production of IL-8 and cyclic AMP

In the range 2 – 18 h, isoprenaline (100 nM) time-dependently increased IL-8 production compared with time-matched, saline-treated control cells (Figure 1a). In the range 1 – 30 nM, isoprenaline (18 h) increased IL-8 concentration-dependently (Figure 1b). In the range 1 – 100 nM, isoprenaline increased cyclic AMP concentration-dependently (Figure 1c).

Effect of β-adrenoceptor blockade, β_2-adrenoceptor agonism and a cyclic AMP analogue

The increase in IL-8 caused by isoprenaline (100 nM), was similar to that caused by salbutamol (1 μM) and by db cyclic AMP (1 mM) (Figure 2). Pretreatment by propranolol (10 μM) significantly inhibited the isoprenaline-induced (100 nM) IL-8 increase (Figure 2). In these experiments, the isoprenaline-induced increase in IL-8 reached 39 ± 10% of the maximum IL-8 increase caused by TNF-α (100 ng ml^{-1}: 74 ± 28 pg μg^{-1} DNA) ($n = 4$).

Additive effect of β-adrenoceptor activation in IL-8 production

Pretreatment with isoprenaline (100 nM) significantly added to the IL-8 response caused by a submaximum concentration of TNF-α (Figure 3). The magnitude of this additive effect of isoprenaline pretreatment was similar to the increase caused by isoprenaline alone.

Discussion

This study demonstrates a novel mechanism by which β-adrenoceptor activation modulates the release of the neutrophil chemoattractant, IL-8, in human transformed bronchial epithelial cells (16HBE cells). β-Adrenoceptor activation increases the release of IL-8 protein and also adds to the IL-8 release caused by the pro-inflammatory cytokine, TNF-α.

The effect of the β-adrenoceptor agonist, isoprenaline alone on IL-8 release in 16HBE cells constituted 40% of the maximum increase caused by the potent cytokine, TNF-α; this effect was time- and concentration-dependent. Furthermore, the selective β_2-adrenoceptor agonist, salbutamol alone produced a similar effect to isoprenaline and the effect of isoprenaline was abolished by the β-adrenoceptor blocker, propranolol. It can therefore be concluded that in 16HBE cells, IL-8 is significantly increased by activation of β-adrenoceptors. The fact that a selective, clinically used β_2-adrenoceptor agonist also increases IL-8, urges an evaluation of the current findings in patients, although it does not exclude involvement of β_1-adrenoceptors.

In 16HBE cells, isoprenaline concentration-dependently increased the intracellular 2nd messenger cyclic AMP at similar concentrations to those at which IL-8 was increased. The cyclic AMP analogue, db cyclic AMP also increased IL-8 with a similar magnitude, thereby indicating the involvement of intracellular cyclic AMP as a 2nd messenger. In addition, the effect of cyclic AMP on IL-8 release was supported by ex-

periments showing that the cyclic AMP-increasing pituitary adenylate cyclase-activating peptide (PACAP 1-27) also increases IL-8 release in 16HBE cells (data not shown).

The current study is the first to show that β-adrenoceptor agonists increase the release of the neutrophil chemoattractant IL-8, by increasing cyclic AMP, although the exact intracellular mechanism remains to be characterized. However, the idea that cyclic AMP may increase the release of pro-inflammatory cytokine is not entirely new. A recent study shows that the release of the pro-inflammatory cytokine, IL-1β, is increased via a cyclic AMP elevating pathway in human transformed monocytes (Chandra *et al.*, 1995). In the case of IL-1β, cyclic AMP induces the IL-1β promoter construct and this induction requires an intact cyclic AMP response element. It remains to be determined whether the mechanism is similar for IL-8.

Separate experiments (data not shown) demonstrated that isoprenaline also increased IL-8 release in primary dog tra-

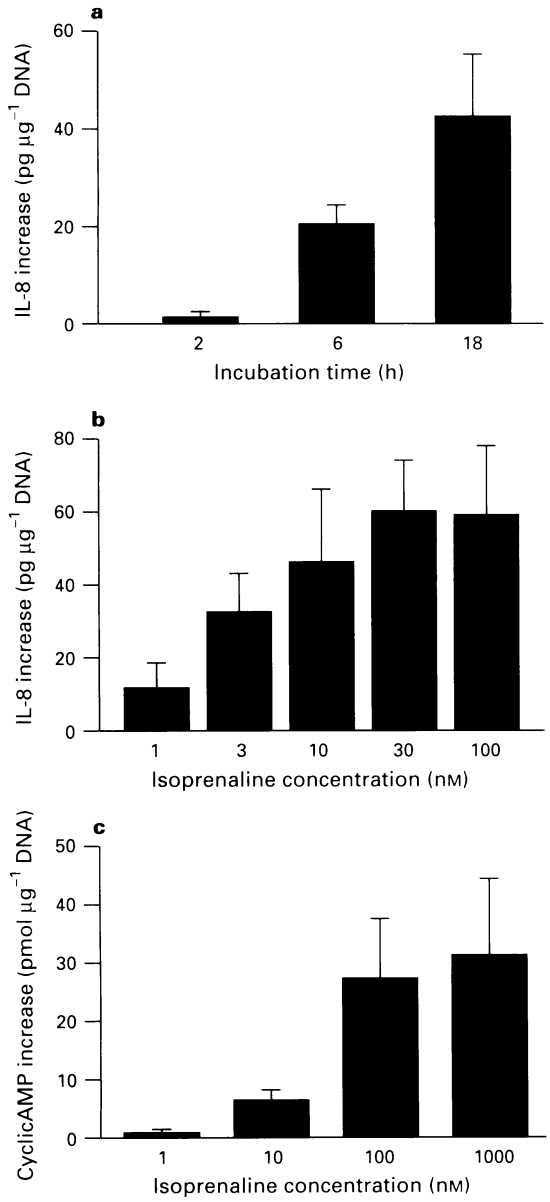

Figure 1 Increasing effect of isoprenaline on interleukin-8 (IL-8) and intracellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) in human transformed bronchial epithelial (16HBE) cells as measured by enzyme immunosorbent assay (EIA). (a) In the range 2–18 h, the effect of isoprenaline (100 nM) was time-dependent (Spearman's rank correlation: $r=0.9$, $P=0.001$, $n=15$). Basal (inherent) IL-8 release in saline-treated cells was 21 ± 4, 44 ± 13 and 102 ± 22 pg μg^{-1} DNA, at 2, 6 and 18 h respectively and was thus also time-dependent (Spearman's rank correlation: $r=0.8$, $P=0.004$, $n=15$). (b) In the range 1–30 nM, the effect of isoprenaline (18 h) was concentration-dependent (Spearman's rank correlation: $r=0.6$, $P<0.05$, $n=20$). In these experiments, basal IL-8 release in saline-treated cells was 217 ± 50 pg μg^{-1} DNA ($n=5$). (c) From 1–100 nM, isoprenaline (15 min) increased cyclic AMP concentration-dependently (Spearman's rank correlation: $r=0.8$, $P<0.01$, $n=12$). Basal cyclic AMP production in saline-treated cells was 0.928 ± 0.363 pmol μg^{-1} DNA ($n=4$). All data in the figures refer to increase in IL-8 above basal release in saline-treated cells and all data are standardized for DNA using fluoro-colorimetry. Mean values with s.e.mean.

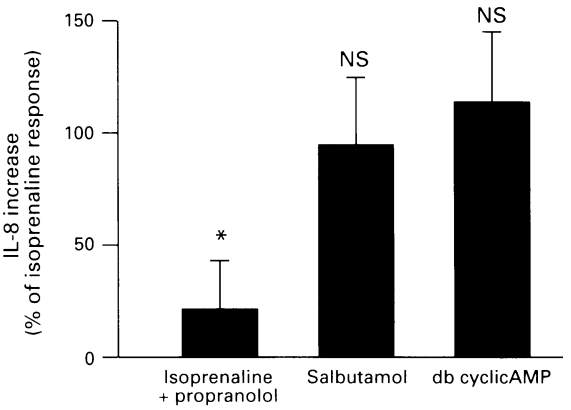

Figure 2 Increasing effect of isoprenaline (100 nM, 18 h) with and without pretreatment with propranolol (10 μM, 15 min preincubation, 18 h coincubation) on IL-8 release measured by EIA standardized for DNA in 16HBE cells compared with salbutamol (1 μM) and dibutyryl cyclic AMP (1 mM) (18 h for all drugs). Data are presented as a percentage of the isoprenaline reference response (% of response to 100 nM isoprenaline at 18 h) which constituted 20 ± 4 pg μg^{-1} DNA. *$P<0.05$ and NS) $P>0.05$ (ANOVA, Fischer's PLSD) compared (paired design) with the isoprenaline reference response. All data refer to increase in IL-8 above basal release in saline-treated cells. Basal IL-8 production in saline-treated cells was 108 ± 20 pg μg^{-1} DNA ($n=4-5$). Mean values with s.e.mean ($n=4-5$).

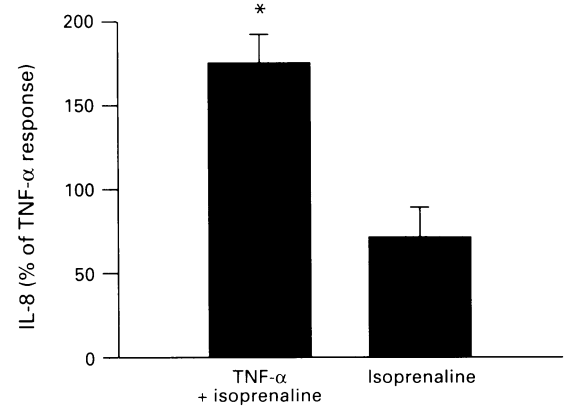

Figure 3 Increasing effect of isoprenaline (100 nM, 15 min preincubation, 18 h coincubation) on release of IL-8 induced by tumour necrosis factor alpha (TNF-α, 20 ng ml^{-1}, 18 h) as measured by EIA and standardized for DNA in 16HBE cells (see also Figure 1). Data are presented as the percentage of the reference response (28 ± 4 pg μg^{-1} DNA) to TNF-α (% of TNF-α response). *$P<0.05$ (Wilcoxon's signed rank) compared (paired design) with the reference response. The increasing effect of isoprenaline on the TNF response was plus 74 ± 19% and the increase caused by isoprenaline alone was 71 ± 20% of the TNF reference response ($P>0.05$). All data refer to increase above basal IL-8 release in saline-treated cells. Basal IL-8 release in saline-treated cells was 66 ± 11 pg μg^{-1} DNA ($n=5$). Mean values with s.e.mean ($n=5$).

cheal epithelial cells and in Calu-3 cells, a human epithelial adenocarcinoma cell line (Shen *et al.*, 1994). The effect of β-adrenoceptor activation on IL-8 production may thus be a common feature in various airway epithelial cells, at least *in vitro*.

The additive effect of β-adrenoceptor activation with other IL-8 producing mechanisms was also demonstrated in the current study. Cyclic AMP was increased by pretreatment with isoprenaline for 15 min (Kaneko *et al.*, 1995) followed by co-incubation during 18 h of isoprenaline and TNF-α, a cytokine implicated as a mediator in airway inflammation (Thomas *et al.*, 1995; Maestrelli *et al.*, 1995). Because the isoprenaline response and the TNF-α response were additive, the current data support the idea that β-adrenoceptor activation can add to a pro-inflammatory chemotactic signal, for example from airway epithelial cells to neutrophils.

This study used foetal calf serum (1%) during relatively long experiments. It could therefore be argued that calf serum would be required as a co-factor for β-agonist-induced IL-8 release (Skerret *et al.*, 1994; Aitken *et al.*, 1996). However, separate experiments showed that isoprenaline caused a moderate and concentration-dependent increase in IL-8 after 6 h drug incubation without foetal calf serum (data not shown). Thus, the current demonstration of IL-8 release caused by β-agonists cannot be attributed to co-factors in foetal calf serum.

In contrast to the case of IL-8 released by β-agonists, the use of foetal calf serum may have contributed to the time-dependent increase in basal (inherent) release of IL-8 (Skerret *et al.*, 1994; Aitken *et al.*, 1996). However, the basal release of IL-8 may also be due to the disruption and isolation of epithelial cells (Shibata *et al.*, 1996), which is supported by the fact that virtually no IL-8 mRNA is detected in human airway epithelium *in situ* without inflammatory stimulation (Inoue *et al.*, 1994). It is therefore unlikely that the basal IL-8 production *in vitro* is physiologically relevant.

To recruite neutrophils, the IL-8 release from bronchial epithelial cells has to be significant because of the diffusion gradient to the microvascular system, provided that epithelial cells are the exclusive effector cells. However, in addition to bronchial epithelial cells, vascular endothelial cells, eosinophils, monocytes, macrophages and neutrophils can also release IL-8 under certain experimental conditions (Schröder, 1989; Inoue *et al.*, 1994; Karakurum *et al.*, 1994; Massion *et al.*, 1994; Kita *et al.*, 1995; Zhong *et al.*, 1995). It is therefore possible that cell types other than bronchial epithelial cells are also involved as effector cells increasing the release of IL-8 protein in response to β-adrenoceptor activation.

β-Adrenoceptor stimulation using isoprenaline and salbutamol as well as direct stimulation using db cyclic AMP caused a significant and reproducible increase in IL-8 after 18 h incubation in transformed human bronchial epithelial (16HBE) cells. This time course was thus similar to the time course of increased neutrophil recruitment into the airways caused by β-adrenoceptor activation in human subjects (Gavreau *et al.*, 1995) and in guinea-pigs *in vivo* (Morley *et al.*, 1995). The similarities in time course do not *per se* prove that the currently demonstrated mechanism is the same as in the *in vivo* studies, but it underlines this possibility. The fact that at least 6 h of incubation time was required for the present demonstration of a significant increase in IL-8 release is compatible with previous data showing that IL-8 release is controlled mainly at the transcriptional level (Nakamura *et al.*, 1991; Massion *et al.*, 1994).

In conclusion, this *in vitro* study demonstrates a novel mechanism by which β-adrenoceptor activation can increase release of the neutrophil chemoattractant protein IL-8 in airway epithelial cells, via an increase in intracellular cyclic AMP. Because airway epithelial cells may control neutrophil influx into the airway and also constitute primary targets for inhaled β-adrenoceptor agonists, the current findings may have important pharmacotherapeutical implications in asthma. However, clinical significance cannot be judged *in vitro* and therefore the functional significance of the current findings needs to be evaluated *in vivo*.

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