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Salbutamol delays human eosinophil apoptosis via a cAMP-dependent mechanism

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Eosinophils play a major role in asthma. One described mechanism leading to the impaired clearance of these cells from the lung is the delay in their programmed cell death (apoptosis). β2-Adrenoceptor agonists have been shown to prolong survival and delay apoptosis of eosinophils. The aim of the present study was to evaluate the mechanisms, especially the role of cAMP pathway, in the prolongation of human eosinophil survival by a selective β2-agonist salbutamol.

Isolated human peripheral blood eosinophils were cultured in the absence or presence of a β2-agonist salbutamol and the indicated antagonists/inhibitors under sterile conditions. Apoptosis was measured by using the relative DNA fragmentation assay and Annexin-V binding.

Salbutamol prolonged survival of human eosinophils and it was inhibited by a β-receptor antagonist propranolol and mimicked by cell-permeant cAMP analogues dibutyryl- and 8-bromo-cAMP. Pharmacological inhibitors of adenylyl cyclase (SQ-22,536) and protein kinase A (Rp-8-CPT-cAMPS) antagonized the effects of salbutamol. The survival-prolonging action of salbutamol was potentiated by a phosphodiesterase inhibitor rolipram (EC50 for the salbutamol effect was 13.6 ± 4.0 and 8.1 ± 3.1 nM in the absence and presence of rolipram, respectively; p = 0.0142, n = 10). In contrast, inhibition of Ca2+-activated K+-channels by apamin, charybdotoxin, iberiotoxin or paxilline did not affect the ability of salbutamol to prolong eosinophil survival.

Taken together, the present results suggest that salbutamol relevant concentrations decreases apoptosis in human eosinophils by activating the canonical β2-receptor-adenyl cyclase – cAMP-protein kinase A pathway.

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1. Introduction

Eosinophils are considered to play a major role in asthma pathogenesis and in exacerbations of chronic obstructive pulmonary disease (COPD) [1]. Eosinophils can release many toxic substances such as granule proteins and oxygen-derived free radicals, which may cause tissue injury, airway remodelling and contraction of bronchial smooth muscle [2–4]. In asthma, the number of pulmonary eosinophils is increased and their role in disease pathogenesis is suggested by the finding that their removal from inflamed areas is associated with the relief of symptoms [3]. The number of granulocytes in the lung tissue is determined by cell maturation, recruitment and death. Apoptosis (programmed cell death) is an efficient method for eosinophil and neutrophil removal from inflamed tissues without their toxic contents being released [2–4]. Finally, apoptotic cells are recognized and removed by phagocytes [5,6]. In vitro and in vivo eosinophil apoptosis is delayed by cytokines such as interleukin-5 and granulocyte-macrophage colony-stimulating factor and enhanced by glucocorticoids and death receptors i.e. Fas [2–4]. We, and others, have previously shown that eosinophil apoptosis is delayed in patients with asthma or inhalant allergy [7–9].

Bronchoconstriction in asthma or COPD is commonly relieved with β2-agonists. However, excessive use of β2-adrenoceptor agonists has been associated with increased risk of death or near death from asthma [10,11]. One possible mechanism contributing to this detrimental activity could be the prevention of eosinophil clearance by inhibition of apoptosis resulting in more severe inflammatory response and asthma symptoms. In line with this, clinically used β2-agonists, such as salbutamol, fenoterol, formoterol and salmeterol, have been reported to increase human eosinophil longevity via inhibition of apoptosis [7,12]. However, the mechanism by which β2-agonists prolong eosinophil survival remains largely unknown. For example, the roles of β-receptor, cAMP, protein kinase A and Ca2+-activated K+ channels are not known. Some other cAMP-increasing agents such as forskolin and prostaglandin E2 have been reported to prolong eosinophil survival [13–16] and we recently reported that a combination of a phosphodiesterase 4 inhibitor with a β2-agonist also prolonged eosinophil survival [17]. This suggests that a cAMP-dependent mechanism may account for how β2-agonists inhibit apoptosis of human eosinophils. In the present study we have tested this hypothesis using the short-acting β2-agonist, salbutamol.

2. Materials and methods

2.1. Eosinophil purification

Blood (100 ml) was obtained from individuals with normal or slightly elevated blood eosinophil counts. The donors were atopic or healthy. Patients with hypereosinophilic syndrome were excluded. Eosinophils were isolated to >98% purity under sterile conditions as previously reported [7,18,19]. The cells were resuspended at 10^6 cells ml^{-1} and cultured in Dutch modification of RPMI 1640 (1 g/L sodium bicarbonate and 20 mM HEPES), 10% fetal calf serum, antibiotics and 1-glutamine. Subjects gave informed consent to a study protocol approved by the Ethics Committee of Tampere University Hospital (Tampere, Finland).

2.2. Cell culture

Eosinophils were cultured in the absence or presence of salbutamol and the indicated inhibitors. Salbutamol was dissolved in RPMI 1640. Charybdotoxin, iberiotoxin, Rp-8-CPT-cAMPS, dibutyryl-cAMP, 8-bromo-cAMP and SQ-22,536 were dissolved in Hank’s balanced salt solution (HBSS). Apamin was dissolved in acetic acid (50 mM) and the final concentration of acetic acid in the cells was 5 mM. Acetic acid (5 mM) had a slight, but statistically significant inhibitory effect on spontaneous eosinophil apoptosis during a culture for 40 h (61.7 ± 78 vs. 58.8 ± 7.2% apoptotic cells in the absence and presence of acetic acid, n = 6, p < 0.05). Paullin, rolipram and NS-1619 were dissolved in DMSO. The final concentration of DMSO in the cells was 0.2% and did not affect apoptosis (n = 6, data not shown). A similar concentration of acetic acid (apamin experiments), HBSS or DMSO was added to the control incubations. Apamin and paullin were added 5 min before, iberiotoxin and charybdotoxin 10 min before, rolipram 20 min before and Rp-8-CPT-cAMPS and SQ-22,536 were added to the cells 30 min before salbutamol. The preincubation times were chosen based on manufacturer’s manual or literature. The cells were incubated under sterile conditions and the incubation time was 17 h for the Annexin-V binding assay and 40 h for the relative DNA fragmentation assay.

2.3. Determination of eosinophil apoptosis

Eosinophil apoptosis was determined by using the relative DNA fragmentation assay in propidium iodide (PI)-stained cells and flow cytometry (FACScan, Becton Dickinson, San Jose, CA) as previously described [7,20]. Endonuclease-catalyzed DNA fragmentation is considered a specific feature of apoptotic cell death. The cells showing decreased relative DNA content were considered to be apoptotic. The results were confirmed by showing the changes in phosphatidylserine (PS) expression on the surface of eosinophils by using the Annexin-V binding assay as previously reported [19,21]. The cells displaying positive Annexin-V FITC labelling (FITC + /PI- and FITC+ /PI+) were regarded as apoptotic [21]. Eosinophil apoptosis is expressed as percentage of apoptotic cells (number of apoptotic cells/total number of cells × 100).

2.4. Materials

Apamin, 8-bromo-cAMP, charybdotoxin, dibutyryl-cAMP, iberiotoxin, NS-1619, paullin, PI, rolipram, Rp-8-CPT-cAMPS, salbutamol, SQ-22,536 and Triton-X-100 were obtained from Sigma Chemicals Co (St Louis, MO, USA). Other reagents were purchased as follows: anti-CD16-microbeads and magnetic cell separation system (Miltenyi Biotec, Bergisch Gladbach, Germany), Ficol-Paque (Pharmacia AB, Uppsala, Sweden), antibiotics and RPMI 1640 (Dutch modification; Gibco BRL, Paisley, UK) and HBSS and RPMI 1640 (Biowhittaker, Verviers, Belgium).

2.5. Statistics

Results are expressed as the mean ± SEM. The EC_{50} was defined as the concentration of drug producing 50% of its maximal effect. Statistical significance was calculated by paired t-test or analysis of variance for repeated measures supported by the Student–Newman–Keuls or Dunnett’s test by using Instat software (GraphPad Software, San Diego, CA). Differences were considered significant if p < 0.05.

3. Results

3.1. Effect of salbutamol on spontaneous eosinophil apoptosis

Isolated and cultured human eosinophils undergo spontaneous apoptosis in the absence of life-supporting cytokines. After a culture for 40 h, 49 ± 5% of eosinophils were apoptotic (n = 10). Incubation of the cells with salbutamol decreased the number of...
cells showing decreased relative DNA content in a concentration-dependent manner (Fig. 1A–C) suggesting that salbutamol inhibited eosinophil apoptosis. The maximal inhibition of apoptosis produced by 100 nM salbutamol was 21%. To confirm that salbutamol inhibits eosinophil apoptosis, Annexin-V binding assay was employed. Salbutamol reduced the number of eosinophils expressing PS on the outer leaflet of the cell, a typical hallmark of apoptosis (Fig. 1D–E). In the absence of salbutamol, 41 ± 6% of the cells were Annexin-V <sup>+/ve</sup>, whereas the corresponding figure in the presence of salbutamol (100 nM) was 26 ± 4% (<i>n</i> = 5, <i>p</i> < 0.01).

Human eosinophils express exclusively <b>2-receptor</b> [3]. To evaluate, whether the effect of salbutamol is mediated via <b>2-receptor</b>, the ability of propranolol, a non-selective <b>-adrenoceptor</b> antagonist to block the effect of salbutamol was evaluated. In the presence of propranolol, salbutamol (100 nM) failed to significantly inhibit human eosinophil apoptosis (8.9 ± 0.7% inhibition of apoptosis by salbutamol in the absence and presence of propranolol), meaning that salbutamol could be related to cAMP pathway in general, we used cell-permeant analogues of cAMP. Dibutyryl-cAMP and 8-bromo-cAMP inhibited reduced relative DNA content in human eosinophils in a concentration-dependent manner (Fig. 2A,B). Furthermore, dibutyryl-cAMP and 8-bromo-cAMP reduced the number of eosinophils expressing PS on the outer leaflet of the cell. In the absence of cAMP analogues, 41 ± 6% of the cells were Annexin-V <sup>+/ve</sup>, whereas the corresponding figures in the presence of cAMP analogues were: 9 ± 1% (in the presence of 1000 μM dibutyryl-cAMP) and 12 ± 1% (in the presence of 1000 μM 8-bromo-cAMP) (<i>n</i> = 5, mean ± SEM, both <i>p</i> < 0.001 vs. control). As the effect of salbutamol was mimicked by cell-permeant analogues of cAMP, the mechanism how salbutamol inhibits apoptosis in human eosinophils might involve cAMP pathway.

3.2. Effect of analogues of cAMP on spontaneous eosinophil apoptosis

Many of the effects of <b>-agonists</b> are mediated via the adenylyl cyclase–cAMP and protein kinase A pathway. <b>-Agonists</b> are known to activate adenylyl cyclase in eosinophils and to increase the levels of cAMP [3]. To investigate whether the effect of salbutamol could be related to cAMP pathway in general, we used cell-permeant analogues of cAMP. Dibutyryl-cAMP and 8-bromo-cAMP inhibited reduced relative DNA content in human eosinophils in a concentration-dependent manner (Fig. 2A,B). Furthermore, dibutyryl-cAMP and 8-bromo-cAMP reduced the number of eosinophils expressing PS on the outer leaflet of the cell. In the absence of cAMP analogues, 41 ± 6% of the cells were Annexin-V <sup>+/ve</sup>, whereas the corresponding figures in the presence of cAMP analogues were: 9 ± 1% (in the presence of 1000 μM dibutyryl-cAMP) and 12 ± 1% (in the presence of 1000 μM 8-bromo-cAMP) (<i>n</i> = 5, mean ± SEM, both <i>p</i> < 0.001 vs. control). As the effect of salbutamol was mimicked by cell-permeant analogues of cAMP, the mechanism how salbutamol inhibits apoptosis in human eosinophils might involve cAMP pathway.

3.3. Effects of adenylyl cyclase and phosphodiesterase inhibitors on eosinophil apoptosis in the presence of salbutamol

It is well-established that the <b>-adrenoceptor</b> can couple via the heterotrimeric stimulatory G-protein (G<sub>s</sub>) to adenylyl cyclase.
in the absence and presence of rolipram (1 μM). Pharmacologically, these data suggest that inhibition of cAMP breakdown by PDE4 should move the concentration–response curve of salbutamol to the left. To evaluate this, we determined the concentration–response curves of salbutamol in the absence and presence of rolipram (1 μM). In the presence of rolipram, the concentration curve (Fig. 3B) of salbutamol was shifted to the left. This is indicated in the reduction of the EC50 value for salbutamol effect by rolipram (EC50 for the salbutamol effects 13.6 ± 4.0 and 8.1 ± 3.1 nM in the absence and presence of 1 μM rolipram, respectively; mean ± SEM, p = 0.0142, n = 10).

3.4. Effect of a protein kinase A inhibitor on eosinophil apoptosis in the presence of salbutamol

To evaluate whether the effects of salbutamol are mediated via protein kinase A (PKA), we employed a pharmacological inhibitor of type I and II of PKA, namely, Rp-8-CPT-cAMPS. Rp-8-CPT-cAMPS occupies cAMP binding sites at the regulatory subunit of PKA and prevents the holoenzyme from dissociation and activation [23,24]. To evaluate that this inhibitor is effective, its effect (at 100 μM) was tested on the survival-prolonging effect of cell-permeant cAMP analogues. Rp-8-CPT-cAMPS (100 μM) reduced the effects of dibutyryl-cAMP (10 μM) and 8-bromo-cAMP (100 μM) on eosinophil apoptosis (Table 1). Similarly, the inhibitory effect of salbutamol on eosinophil apoptosis was reversed by Rp-8-CPT-cAMP (Fig. 4).

3.5. Role of Ca2+-activated K+-channels in the effect of salbutamol on eosinophil apoptosis

Targets of PKA include potassium channels (e.g. large-conductance calcium activated potassium channel and ATP-sensitive potassium channel) that open upon phosphorylation, resulting in the efflux of K+ from the cell down its concentration gradient leading to membrane rectification (repolarisation). Gating of K+-channels may also be effected by the direct interaction of Gαs with the channel independently of cAMP and PKA [22]. Pharmacological blockade of large-conductance channel (BKCa) with charybdotoxin, and the more selective ibotenate, prevents hyperpolarisation and β2-adrenoceptor-mediated relaxation in smooth muscle cells [22]. To evaluate, whether BKCa channels have a role in salbutamol-induced survival of human eosinophils, the effect of salbutamol was tested in the presence and absence of charybdotoxin and ibotenate. Neither toxin significantly affected salbutamol-induced inhibition of eosinophil apoptosis (Fig. 5A,B). To further confirm this, we used a chemically distinct BKCa blocker, paxilline. Similarly, paxilline did not affect salbutamol-induced eosinophil survival (data not shown, n = 6, p = 0.43). To exclude small-conductance/ATP-type Ca2+-activated K+-channels, we used a pharmacological antagonist apamin. However, apamin did not affect the effect of salbutamol on eosinophil apoptosis (data not shown, n = 6, p = 0.97). Whether the mechanism of salbutamol involves activation of BKCa channels, then a direct activation of BKCa channels should have a similar survival-prolonging effect on eosinophils. However, a direct activator of BKCa, NS-1619 did not inhibit but enhanced apoptosis of human eosinophils (Fig. 5C). Taken together, the above data suggest that the BKCa does not play a role in the survival-prolonging effect of salbutamol in human eosinophils.

4. Discussion

In the present study we have shown that a β2-agonist salbutamol prolongs human eosinophil survival by inhibiting apoptosis. The effects of salbutamol were abolished by a β-receptor blocker and mimicked by cell-permeant CAMP agonists. Similarly, the effect of salbutamol was reversed by pharmacological inhibitors of adenylyl cyclase and PKA and is enhanced by an inhibitor of PDE4. In contrast, the blockade of Ca2+-activated K+-channels did not reverse the effects of salbutamol and an opener of BKCa did not mimic the effects of salbutamol.

Human eosinophils express β2-receptors and agonists such as isoprenaline and salbutamol elevate the CAMP content and activate PKA in eosinophils [3]. The effects and mechanisms of β2-agonists on eosinophil functions such as NADPH oxidase activation, degranulation, chemotaxis, adhesion and lipid mediator production are well-characterised [3]. However, studies investigating the mechanism of the inhibitory action of β2-agonists on eosinophil apoptosis are lacking. Previously, we have shown that inhibition of CAMP hydrolysing phosphodiesterases 3 or 4 by pharmacological inhibitors such as cilostazol and rolipram is able to enhance eosinophil survival in the absence and presence of salbutamol [17]. Furthermore, some CAMP-elevating agents, such as forskolin and AR-A67810, the number of cells showing reduced relative DNA content was further decreased indicating reduced apoptosis (n = 12, p < 0.05). This suggests that the effect of salbutamol is mediated via cAMP. Pharmacologically, these data suggest that inhibition of cAMP breakdown by PDE4 should move the concentration–response curve of salbutamol to the left. To evaluate this, we determined the concentration–response curves of salbutamol in the absence and presence of rolipram (1 μM). In the presence of rolipram, the concentration curve (Fig. 3B) of salbutamol was shifted to the left. This is indicated in the reduction of the EC50 value for salbutamol effect by rolipram (EC50 for the salbutamol effects 13.6 ± 4.0 and 8.1 ± 3.1 nM in the absence and presence of 1 μM rolipram, respectively; mean ± SEM, p = 0.0142, n = 10).

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prostaglandin E₂ as well as cAMP analogue dibutyryl-cAMP have been shown to prolong eosinophil survival [12–16].

Recently, Machida and co-workers [12] reported that isoprenaline, a non-selective β-agonist inhibits apoptosis of murine lung eosinophils by activating a phosphatidylinositol 3-kinase (PI3K)/Akt pathway. Interestingly however, they reported that a PI3K inhibitor wortmannin also inhibited cAMP analogue enhanced eosinophil survival. Even though the PI3K-Akt pathway is not classically considered as a signalling pathway of β₂-agonists and cAMP-elevating agents, it is tempting to speculate, that β₂-agonist mediated eosinophil survival could follow β-receptor-adenyl cyclase–cAMP–PKA–PI3K–Akt pathway as recent reports suggest [25–27].

In the present study, salbutamol was shown to inhibit eosinophil apoptosis over a concentration range of 10–100 nM. The mean maximum concentration of salbutamol in plasma after a single 4 mg tablet is 30–60 nM [28,29]. The EC₅₀ for the survival-prolonging effect of salbutamol in this study was 13.6 nM and in the presence of a PDE4 inhibitor rolipram it was 8.1 nM. After inhalation of salbutamol the peak plasma concentrations have been reported to be 7.5–23 nM [30]. However, the concentration of salbutamol in the lungs is inevitably much higher as compared with the concentration in the blood. In a recent report shows that the mean concentration of salbutamol in bronchoalveolar lavage fluid in children after salbutamol inhalation (200 μg via MDI) was 668 nM [31]. When considering the clinical importance, of the present finding, it should be kept in mind that the maximal bronchodilating effect of salbutamol in guinea-pig trachea or isolated human bronchi is obtained at concentrations of 100–1000 nM [32].

Thus, the concentrations of salbutamol used and found to inhibit eosinophil apoptosis in the present study are clinically relevant. As compared with the effect of survival-prolonging cytokines interleukin-5 or granulocyte-macrophage colony-stimulating factor (maximally 70–90% inhibition of eosinophil apoptosis) [2], the effect of salbutamol (maximal inhibition of apoptosis 20–25%) is relatively small. However, it is of similar magnitude than the well-characterised effects of TNF-α, interferon γ or TLR9 activation [21,33,34]. One may argue that the effect of salbutamol is small and thus clinically irrelevant. Salbutamol is classified as a short-acting β₂-agonist. Keeping this in mind, we have earlier performed time-response experiment, where the cells were exposed to salbutamol for a shorter time and then salbutamol was replaced by drug-free medium for a remaining time [7]. The results indicated that even a short exposure (2 h) to salbutamol followed by wash-out and replacement of salbutamol by drug-free medium for a remaining time is enough to reduce apoptosis in human eosinophils during a prolonged culture similar to that used in the present study. In the treatment of acute asthma, according to the current guidelines, salbutamol, is administered as an inhalation 4–6 times daily (meaning administration of a new dose of salbutamol every 4 h). Typically, this treatment lasts some days. Thus, we consider the setting of the present experimentation (i.e. incubation of cells for 40 h) with salbutamol as highly clinically relevant.

β₂-Agonists have other anti-inflammatory effects on eosinophils [3] that may partly counteract their effects on eosinophil apoptosis. However, compelling evidence has been presented that the number or proportion of eosinophils in induced sputum during airway challenge is increased when patients are previously treated with regular salbutamol as compared with placebo [35–37]. In addition, there are several studies reporting no significant change in eosinophil numbers after treatment with salbutamol or other β₂-agonists [3]. However, these studies include only ten or tens of patients per study and are thus significantly underpowered to detect a ~20% increase in eosinophil counts. The survival-prolonging action of β₂-agonists on eosinophils may also be affected by the findings that, excessive use of β₂-adrenoceptor agonists is associated with increased risk of death or near death from asthma [10]. Furthermore, regular treatment of chronic asthma with a long-acting β₂-agonist salmeterol in patients most of whom were not on inhaled glucocorticoids, resulted in increased morbidity and mortality due to asthma [11].

Currently, inhibitors of PDE4 are under development for asthma and, in particular, COPD, which also has an eosinophilic component [38]. In the present study, a combination of a PDE4 inhibitor with a β₂-agonist produced a further delay in eosinophil apoptosis. Furthermore, inclusion of the PDE4 inhibitor rolipram in the assay led to a shift in the concentration–response curve of salbutamol to the left as illustrated in the significant decrease in the EC₅₀ Value of

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**Table 1**

<table>
<thead>
<tr>
<th>Solvent control</th>
<th>Medium (percentage inhibition)</th>
<th>Rp-8-CPT-cAMPS (100 μM) (percentage inhibition)</th>
<th>p-value for the difference in inhibition of apoptosis (%) by cAMP analogue in the absence and presence of Rp-8-CPT-cAMPs.</th>
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</thead>
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<td>Medium</td>
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<td>60.4 ± 4.4</td>
<td><strong>0.0008</strong></td>
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<tr>
<td>Dibutyryl-cAMP</td>
<td>49.7 ± 4.8** (19.7 ± 2.7%)</td>
<td>53.4 ± 4.4** (11.9 ± 1.6%)</td>
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</tr>
<tr>
<td>8-Bromo-cAMP</td>
<td>55.0 ± 5.6** (12.0 ± 2.4%)</td>
<td>59.1 ± 6.1 (2.9 ± 2.7%)</td>
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Eosinophils were cultured for 40 h in the absence or presence of cAMP analogues and/or Rp-8-CPT-cAMPS. Apoptosis was determined by DNA fragmentation assay. The cells showing decreased relative DNA content were considered to be apoptotic. Results represent the percentage of apoptotic eosinophils and are expressed as the mean ± SEM, n = 9. "**" indicates p < 0.01 and "***"p < 0.001 as compared with the corresponding control in the absence of cAMP analogues as calculated by analysis of variance for repeated measures supported by the Student–Newman–Keuls test. In parenthesis is shown the percentage inhibition of apoptosis by cAMP analogue as compared with the corresponding solvent control. In the fourth column the p-value for the difference in percentage inhibition of apoptosis by cAMP analogues in shown in the absence and presence of Rp-8-CPT-cAMPs, respectively. p-values were obtained by t-test.
The results suggest that caution should be exercised when assessing the effect of a BKCa opener NS-1619 on spontaneous eosinophil apoptosis during a culture for 40 h. In (C) is shown the effect of a PDE4 inhibitor even a smaller dose of a 2-agonist salbutamol from 13.6 to 8.1 nM. This suggests that in the presence of a PDE4 inhibitor even a smaller dose of a 2-agonist salbutamol prolongs human eosinophil survival by inhibiting apoptosis at clinically relevant concentrations and the mechanism involves the classical β-receptor—adenylyl cyclase—cAMP—PKA pathway. These results suggest that caution should be exercised when β2-agonists are used regularly on a daily basis in the absence of glucocorticoids in the treatment of eosinophilic diseases such as asthma.

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