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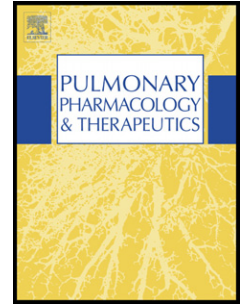
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**Salbutamol delays human eosinophil apoptosis via a cAMP-dependent mechanism**

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## Abstract

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).  $\beta_2$ -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  $\beta_2$ -agonist salbutamol.

Isolated human peripheral blood eosinophils were cultured in the absence or presence of a  $\beta_2$ -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  $\beta$ -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 ( $EC_{50}$  for the salbutamol effect was  $13.6 \pm 4.0$  and  $8.1 \pm 3.1$  nM in the absence and presence of rolipram, respectively;  $P=0.0142$ ,  $n=10$ ). In contrast, inhibition of  $Ca^{2+}$ -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 at clinically relevant concentrations decreases apoptosis in human eosinophils by activating the canonical  $\beta_2$ -receptor-adenylyl cyclase–cAMP-protein kinase A pathway.

## Key words

eosinophils, apoptosis, salbutamol, cAMP

**Abbreviations**

Apamin: Cys-Asn-Cys-Lys-Ala-Pro-Glu-Thr-Ala-Leu-Cys-Ala-Arg-Arg-Cys-Gln-Gln-His-NH<sub>2</sub> [Disulfide Bridges: 1-11, 3-15]

cAMP: cyclic adenosine-3',5'-monophosphate

Charybdotoxin: pGlu-Phe-Thr-Asn-Val-Ser-Cys-Thr-Thr-Ser-Lys-Glu-Cys-Trp-Ser-Val-Cys-Gln-Arg-Leu-His-Asn-Thr-Ser-Arg-Gly-Lys-Cys-Met-Asn-Lys-Lys-Cys-Arg-Cys-Tyr-Ser [Disulfide Bridges: 7-28, 13-33, 17-35]

COPD: chronic obstructive pulmonary disease

Iberiotoxin: pGlu-Phe-Thr-Asp-Val-Asp-Cys-Ser-Val-Ser-Lys-Glu-Cys-Trp-Ser-Val-Cys-Lys-Asp-Leu-Phe-Gly-Val-Asp-Arg-Gly-Lys-Cys-Met-Gly-Lys-Lys-Cys-Arg-Cys-Tyr-Gln [Disulfide Bridges: 7-28; 13-33; 17-35]

NS-1619: 1,3-Dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one

Paxilline: (2R,4bS,6aS,12bS,12cR,14aS)-5,6,6a,7,12,12b,12c,13,14,14a-Decahydro-4b-hydroxy-2-(1-hydroxy-1-methylethyl)-12b,12c-dimethyl-2H-pyrano[2'',3'':5',6']benz[1',2':6,7]indeno[1,2-b]indol-3(4bH)-one

PDE: phosphodiesterase

PI: propidium iodide

PI3K: phosphatidylinositol 3-kinase

PKA: Protein kinase A

Rp-8-CPT-cAMPS: 8- (4- Chlorophenylthio)adenosine- 3', 5'- cyclic monophosphorothioate, Rp- isomer

SQ-22,536: 9-(Tetrahydro-2-furanyl)-9H-purin-6-amine

## 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  $\beta_2$ -agonists. However, excessive use of  $\beta_2$ -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  $\beta_2$ -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  $\beta_2$  agonists prolong eosinophil survival remains largely unknown. For example, the roles of  $\beta$ -receptor, cAMP, protein kinase A and  $\text{Ca}^{2+}$ -activated  $\text{K}^+$ -channels are not known. Some other cAMP-increasing agents such as forskolin and prostaglandin  $\text{E}_2$  have been reported to prolong eosinophil survival [13-16] and we recently reported that a phosphodiesterase 4 inhibitor with a  $\beta_2$ -agonist also prolonged eosinophil survival [17]. This suggests that a cAMP-



dependent mechanism may account for how  $\beta_2$ -agonists inhibit apoptosis of human eosinophils. In the present study we have tested this hypothesis using the short-acting  $\beta_2$ -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 >99% purity under sterile conditions as previously reported [7,18,19]. The cells were resuspended at  $10^6$  cells  $\text{ml}^{-1}$  and cultured in Dutch modification of RPMI 1640 (1g/L sodium bicarbonate and 20 mM HEPES), 10% fetal calf serum, antibiotics and L-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 ( $61.7 \pm 7.8$  vs.  $58.8 \pm 7.2$  % apoptotic cells in the absence and presence of acetic acid,  $n=6$ ,  $p<0.05$ ). Paxilline, 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 paxilline 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 x100).

### *2.3 Materials*

Apamin, 8-bromo-cAMP, charybdotoxin, dibutyryl-cAMP, iberiotoxin, NS-1619, paxilline, 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, Bergish Gladbach, Germany), Ficoll-Paque (Pharmacia AB, Uppsala, Sweden), antibiotics and RPMI-1640 (Dutch modification; Gibco BRL, Paisley, UK) and HBSS and RPMI-1640 (Biowhittaker, Verviers, Belgium).

### *2.4 Statistics*

Results are expressed as the mean  $\pm$  SEM. The EC<sub>50</sub> 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,  $49 \pm 5$  % 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,  $41 \pm 6$  % of the cells were Annexin-V<sup>+</sup>, whereas the corresponding figure in the presence of salbutamol (100 nM) was  $26 \pm 4$  % (n=5, p<0.01).

Human eosinophils express exclusively  $\beta_2$ -receptor [3]. To evaluate, whether the effect of salbutamol is mediated via  $\beta_2$ -receptors, the ability of propranolol, a non-selective  $\beta$ -adrenoceptor antagonist to block the effect of salbutamol was evaluated. In the presence of propranolol, salbutamol (100 nM) failed to significantly inhibit human eosinophil apoptosis ( $8.9 \pm 0.7$  % inhibition of apoptosis by salbutamol in the absence of propranolol and  $3.3 \pm 1.0$  % inhibition of apoptosis in the presence of propranolol (10  $\mu$ M), mean  $\pm$  SEM, n=5, p<0.05 for the inhibitory effect in the absence and presence of propranolol). This suggests that the mechanism of action of salbutamol involves  $\beta_2$ -receptor activation.

#### *3.1 Effect of analogues of cAMP on spontaneous eosinophil apoptosis*

Many of the effects of  $\beta_2$ -agonists are mediated via the adenylyl cyclase – cAMP and protein kinase A pathway.  $\beta_2$ -agonists 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 (Figure 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,  $41 \pm 6$  % of the cells were Annexin-V<sup>+ve</sup>, whereas the corresponding figures in the presence of cAMP analogues were:  $9 \pm 1$  % (in the presence of 1000  $\mu$ M dibutyryl-cAMP) and  $12 \pm 1$  % (in the presence of 1000  $\mu$ M 8-bromo-cAMP) (n=5, mean  $\pm$  SEM, both  $p < 0.001$  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 Effects of adenylyl cyclase and phosphodiesterase inhibitors on eosinophil apoptosis in the presence of salbutamol*

It is well-established that the  $\beta_2$ -adrenoceptor can couple via the heterotrimeric stimulatory G-protein (Gs) to adenylyl cyclase (AC) [22]. To evaluate, whether adenylyl cyclase is involved in the action of salbutamol, we employed a pharmacological inhibitor of adenylyl cyclase, SQ-22,536. In the presence of SQ-22,536 (100  $\mu$ M), salbutamol did not reduce the number of apoptotic cells as it did in the absence of the adenylyl cyclase inhibitor (Figure 3A).

When eosinophils were cultured in the presence of a phosphodiesterase 4 (PDE4) inhibitor rolipram (1  $\mu$ M) and salbutamol (100 nM), 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  $\mu$ M). In the presence of rolipram, the concentration curve

(Figure 3B) of salbutamol was shifted to the left. This is indicated in the reduction of the  $EC_{50}$  value for salbutamol effect by rolipram ( $EC_{50}$  for the salbutamol effects  $13.6 \pm 4.0$  and  $8.1 \pm 3.1$  nM in the absence and presence and of 1  $\mu$ M rolipram, respectively; mean  $\pm$  SEM,  $P=0.0142$ ,  $n=10$ ).

### *3.3 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-8CPT-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  $\mu$ M) was tested on the survival-prolonging effect of cell-permeant cAMP analogues. Rp-8-CPT-cAMPS (100  $\mu$ M) reduced the effects of dibutyryl cAMP (10  $\mu$ M) and 8-bromo-cAMP (100  $\mu$ M) on eosinophil apoptosis (Table 1). Similarly, the inhibitory effect of salbutamol on eosinophil apoptosis was reversed by Rp-8-CPT-cAMP (Figure 4).

### *3.4 Role of $Ca^{2+}$ -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  $G_{s\alpha}$  with the channel independently of cAMP and PKA [22]. Pharmacological blockade of large-conductance channel ( $BK_{Ca}$ ) with charybdotoxin, and the more selective iberiotoxin, prevents hyperpolarisation and  $\beta_2$ -adrenoceptor-mediated relaxation in smooth muscle cells [22]. To evaluate, whether  $BK_{Ca}$  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 iberiotoxin. Neither toxin significantly affected salbutamol-

induced inhibition of eosinophil apoptosis (Figure 5A,B). To further confirm this, we used a chemically distinct BK<sub>Ca</sub> 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 Ca<sup>2+</sup>-activated K<sup>+</sup> 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 BK<sub>Ca</sub> channels, then a direct activation of BK<sub>Ca</sub> channels should have a similar survival-prolonging effect on eosinophils. However, a direct activator of BK<sub>Ca</sub>, NS-1619 did not inhibit but enhanced apoptosis of human eosinophils (Figure 5C). Taken together, the above data suggest that the BK<sub>Ca</sub> 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  $\beta_2$ -agonist salbutamol prolongs human eosinophil survival by inhibiting apoptosis. The effects of salbutamol were abolished by a  $\beta$ -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 Ca<sup>2+</sup>-activated K<sup>+</sup>-channels did not reverse the effects of salbutamol and an opener of BK<sub>Ca</sub> did not mimic the effects of salbutamol.

Human eosinophils express  $\beta_2$ -receptors and agonists such as isoprenaline and salbutamol elevate the cAMP content and activate PKA in eosinophils [3]. The effects and mechanisms of  $\beta_2$ -agonists on eosinophil functions such as NADPH oxidase activation, degranulation, chemotaxis, adhesion and lipid mediator production are well characterized [3]. However, studies investigating the mechanism of the inhibitory action of  $\beta_2$ -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 prostaglandin E<sub>2</sub> as well as cAMP analogue dibutyryl-cAMP have been shown to prolong eosinophil survival [12-16].

Recently, Machida and coworkers [12] reported that isoprenaline, a non-selective  $\beta$ -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  $\beta_2$ -agonists and cAMP-elevating agents, it is tempting to speculate, that  $\beta_2$ -agonist mediated eosinophil survival could follow  $\beta$ -receptor-adenylyl 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 EC<sub>50</sub> 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 fact, a recent report shows that the mean concentration of salbutamol in bronchoalveolar lavage fluid in children after salbutamol inhalation (200  $\mu$ g 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- $\alpha$ , interferon  $\alpha/\gamma$  or TLR9 activation [21,31,32]. One may argue that the effect of salbutamol is small and thus clinically irrelevant. Salbutamol is classified as a short-acting  $\beta_2$ -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 the remaining time [7]. The results indicated that even a short exposure (2 hours) 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 to 6 times daily (meaning administration of a new dose of salbutamol every 4 to 6 hours). 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.

$\beta_2$ -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  $\beta_2$ -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  $\beta_2$ -agonists on eosinophils may be also affected by the findings that, excessive use of  $\beta_2$ -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  $\beta_2$ -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  $\beta_2$ -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 EC<sub>50</sub> value of salbutamol from 13.6 to 8.1 nM. This suggests that in the presence of a PDE4 inhibitor even a smaller dose of a  $\beta_2$ -agonist can lead to prolonged eosinophil survival. This suggests that the effect of this combination on pulmonary eosinophilia should be studied before the large-scale use of a combination of these drugs in daily clinical practice.

Taken together, we have shown that a  $\beta_2$ -agonist salbutamol prolongs human eosinophil survival by inhibiting apoptosis at clinically relevant concentrations and the mechanism involves the classical  $\beta$ -receptor – adenylyl cyclase – cAMP – PKA pathway. These results suggest that caution should be exercised when  $\beta_2$ -agonists are used regularly on a daily basis in the absence of glucocorticoids in the treatment of eosinophilic diseases such as asthma.

## 5. Acknowledgements

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**Table 1.**

The effect of an inhibitor of protein kinase A, Rp-8-CPT-cAMPS, on eosinophil apoptosis in the absence and presence of cAMP analogues

	Medium (percentage inhibition)	Rp-8-CPT- cAMPS (100 $\mu$ M) (percentage inhibition)	P-value for the difference in inhibition of apoptosis (%) by cAMP analogue in the absence and presence of Rp-8-CPT-cAMPS.
solvent control	61.7 $\pm$ 5.0	60.4 $\pm$ 4.4	
	49.7 $\pm$ 4.8 <sup>***</sup>	53.4 $\pm$ 4.4 <sup>**</sup>	
Dibutyryl-cAMP (10 $\mu$ M)	(19.7 $\pm$ 2.7 %)	(11.9 $\pm$ 1.6 %)	p=0.0008
	55.0 $\pm$ 5.6 <sup>**</sup>	59.1 $\pm$ 6.1	
8-bromo-cAMP (100 $\mu$ M)	(12.0 $\pm$ 2.4%)	(2.9 $\pm$ 2.7 %)	p=0.001

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  $\pm$  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 is shown in the absence and presence of Rp-8-CPT-cAMPS, respectively. P-values were obtained by t-test.

## Figure Captions

**Figure 1. A.** The effect of salbutamol on spontaneous eosinophil apoptosis during a culture for 40 h. Apoptosis was determined by DNA fragmentation assay. \*\* indicates  $p < 0.01$  and \*\*\*  $p < 0.001$  as compared with the solvent control as calculated by analysis of variance for repeated measures supported by the Dunnett's test. Mean  $\pm$  SEM,  $n=10$ . Representative graphs from relative DNA fragmentation assay of PI-stained eosinophils (B,C) and Annexin V-FITC (FL1) and uptake of PI (PI; FL2) (D,E) are shown. Solvent control is shown in (B,D) and the effect of 100 nM salbutamol in (C,E). In (B,C) M1 represents cells with decreased relative DNA content and the figures in top right hand corner represent total percentage of cells with decreased relative DNA content. In (D,E) figures in top right hand corner represent total percentage of annexin V-FITC<sup>+ve</sup> and PI<sup>-ve</sup> and annexin V-FITC<sup>+ve</sup> and PI<sup>+ve</sup> eosinophils. Representative graphs of experiments with similar results using eosinophils from 5-10 different donors are shown.

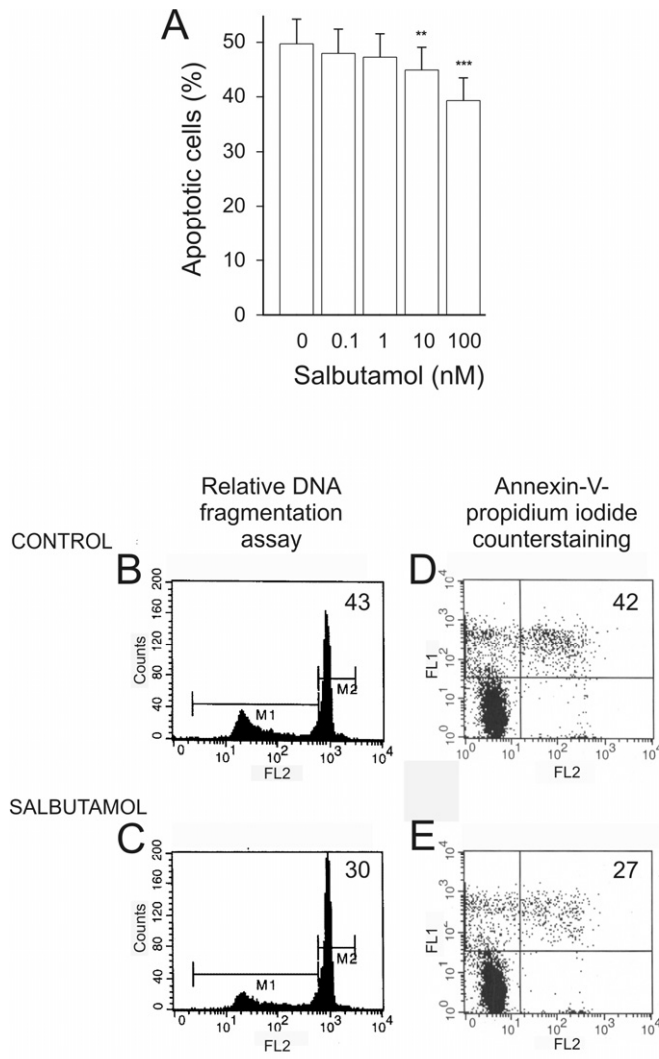
**Figure 2.** The effects of cAMP analogues dibutyryl-cAMP (A) and 8-bromo-cAMP (B) on spontaneous eosinophil apoptosis during a culture for 40 h. Apoptosis was determined by DNA fragmentation assay. \*\* indicates  $p < 0.01$  and \*\*\*  $p < 0.001$  as compared with the solvent control as calculated by analysis of variance for repeated measures supported by the Dunnett's test. Mean  $\pm$  SEM,  $n=5$ .

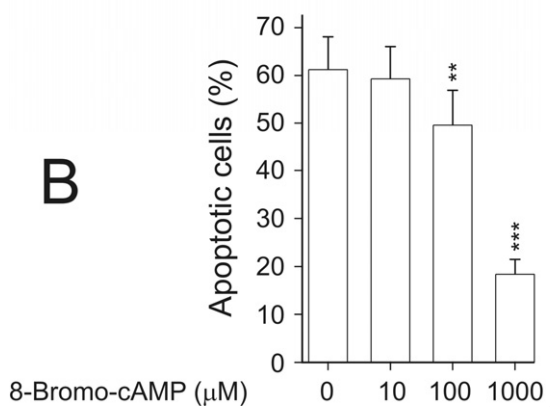
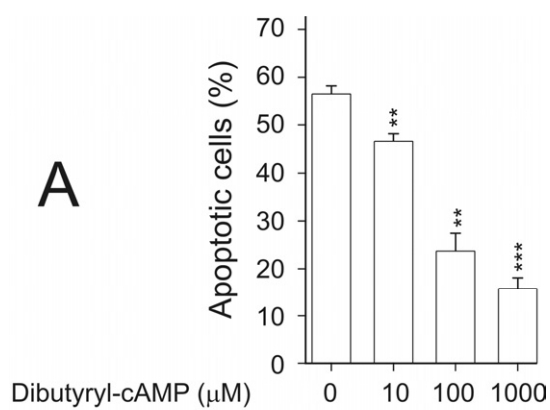
**Figure 3.** The effect of adenylyl cyclase inhibitor SQ-22,536 (A) and a PDE4 inhibitor rolipram (B) on eosinophil apoptosis in the presence and absence of salbutamol during a culture for 40 h. In (B) is shown the concentration-response curves of salbutamol in the presence and absence of rolipram (1  $\mu$ M). Apoptosis was determined by DNA fragmentation assay. \*\* indicates  $p < 0.01$ , and \*\*\*  $p < 0.001$  as calculated by analysis of variance for repeated measures supported by the Student-Newman-Keuls test. ns, not significant. The inhibitory effect of SQ-22536 on spontaneous apoptosis was statistically significant ( $P < 0.01$ ). Mean  $\pm$  SEM,  $n=6$  (A) and  $n=10$  (B).

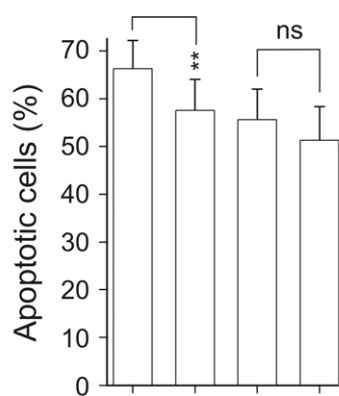
**Figure 4.** The effect of protein kinase A inhibitor Rp-8-CPT-cAMPS on eosinophil apoptosis in the presence and absence of salbutamol during a culture for 40 h. Apoptosis was determined by DNA fragmentation assay. \*\*\* indicates  $p < 0.001$  as calculated by analysis of variance for repeated measures supported by the Student-Newman-Keuls test. ns, not significant. Mean  $\pm$  SEM,  $n=10$ .

**Figure 5.** The effect of BK<sub>Ca</sub> blockers charybdotoxin (A) and iberiotoxin (B) on eosinophil apoptosis in the presence and absence of salbutamol during a culture for 40 h. In (C) is shown the effect of a BK<sub>Ca</sub> opener NS-1619 on spontaneous eosinophil apoptosis during a culture for 40 h. Apoptosis was determined by DNA fragmentation assay. \* indicates  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  as calculated by analysis of variance for repeated measures supported by the Student-Newman-Keuls test (A,B) or Dunnett's test (C). Mean  $\pm$  SEM,  $n=7$  (A,B) and  $n=5$  (C).

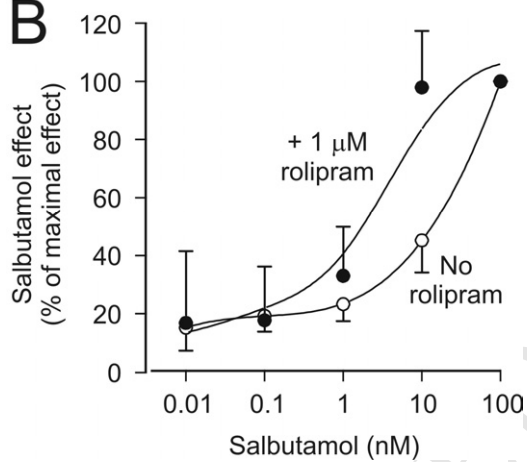


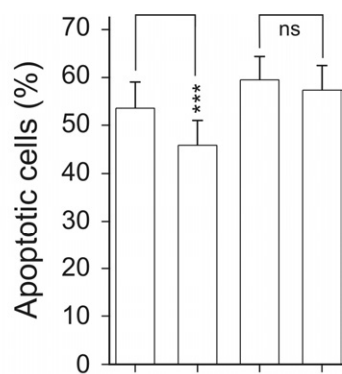




**A**

Salbutamol (100 nM)	SQ-22,536 (100 μM)
-	-
+	-
-	+
+	+

**B**



Salbutamol (100 nM)            -    +    -    +  
Rp-8-CPT-cAMPS (100  $\mu$ M)    -    -    +    +

ACCEPTED MANUSCRIPT

