INTRODUCTION

PDE4 inhibitors increase cyclic adenosine monophosphate (cAMP) level in cells and inhibit various stages of the inflammatory process. cAMP is a classical second messenger synthesized from adenosine triphosphate (ATP) with the participation of a group of enzymes named adenylyl cyclases (1). It’s involved in the regulation of levels of numerous hormones, cytokines and cell-mediated response to signals from neurotransmitters (2). cAMP is the main indicator for protein kinase A (PKA) - cAMP begins the cascade of reactions leading to changes in the cell function by modulating the activity of PKA (3). cAMP is decomposed to 5’nucleotidemonophosphoran by a specific phosphodiesterase (PDEs). The various isoforms of PDE4 are present in all mobile immune and structural cells of the respiratory tract (4-7). The clinical application of PDE4 inhibitors are limited by side effects of gastrointestinal nature (nausea, vomiting, diarrhea). Several multicenter clinical trials have been conducted on the treatment of non-infected airway inflammatory diseases using PDE4 inhibitors showing highly inconsistent results (8-11). Recently it has been shown that roflumilast can decrease the exacerbations of chronic obstructive pulmonary disease (COPD). Orally given roflumilast used alone improves FEV1 (forced expiratory volume in one second) by 48 mL (12), but it gives better results in combination with salmeterol - 49 mL, and tiotropium - 80 mL (13).

Eotaxins (eotaxin-1/CCL11, eotaxin-2/CCL24, eotaxin-3/CCL26) are the strongest chemotactic agents for eosinophils. Inhibitors of phosphodiesterase 4 (PDE4), the enzyme decomposing cAMP, are anti-inflammatory agents which act through cAMP elevation and inhibit numerous steps of allergic inflammation. The effect of PDE4 inhibitors on eotaxin expression is not known in details. The aim of our study was to evaluate the influence of PDE4 inhibitors: rolipram and RO-20-1724 on expression of eotaxins in bronchial epithelial cell line BEAS-2B. Cells were preincubated with PDE4 inhibitors or dexamethasone for 1 hour and then stimulated with IL-4 or IL-13 alone or in combination with TNF-α. After 48 hours eotaxin protein level was measured by ELISA and mRNA level by real time PCR. Results: PDE4 inhibitors decreased CCL11 and CCL26 expression only in cultures co-stimulated with TNF-α. In cultures stimulated with IL-4 and TNF-α rolipram and RO-20-1724 diminished CCL11 mRNA expression by 34 and 37%, respectively, and CCL26 by 43 and 47%. In cultures stimulated with IL-13 and TNF-α rolipram and RO-20-1724 decreased expression of both eotaxins by about 50%. These results were confirmed at the protein level. The effect of PDE4 inhibitors on eotaxin expression in BEAS-2B cells, in our experimental conditions, depends on TNF-α contribution.

Key words: bronchial epithelial cells, eotaxins, phosphodiesterase 4 inhibitors, tumor necrosis factor-α, interleukin 4, interleukin 13
The effect of PDE4 inhibitors on key cells and their mediators which participate in the pathogenesis of asthma or COPD has been shown by numerous reports. PDE4 inhibitors block the production of TNF-α, IL-2, IL-5, IL-4, interferon γ (INF-γ), prostaglandin 2 (PGE₂), many neutrophil enzymes and proliferation of T lymphocytes (27, 28). It was shown that PDE4 inhibitors are the most effective in the reduction of inflammatory cell influx into lungs and IL-4, IL-13, TNF-α levels in the bronchoalveolar fluid (BALF) compared with other PDE3 inhibitors (PDE1-isobuty-1-methylxanthine, PDE3-cilostazol, PDE5-sildenafil, zaprinast, theophiline-nonselective) (29, 30). PDE3 inhibitors are suppose to attenuate the effect of PDE4 inhibitors (used in combination). PDE3 inhibitors can be helpful in cough treatment (31) and as vasodilator in the vascular smooth muscle environment with prolonged exposure to nitric oxide (NO) (32). Rolipram inhibits eotaxin-1 secretion in smooth muscle cells stimulated with TNF-α (33). Sabatini et al. showed that rolflumilast N-oxide decrease eotaxin release in TNF-α-treated human lung fibroblasts (34). Santamaria et al. showed that rolipram inhibits eotaxin-mediated activation and migration of eosinophils (35). Rolipram also affects cell adhesion molecules that appear on eosinophils after eotaxin, but not IL-5 action (36). PDE4 inhibitors selectively diminish the influx of eosinophils into airways during experimental asthmaic reaction in an animal model (37). Silva et al. have shown that rolipram reduces the number of eosinophils and concentration of eotaxin in the BALF of sensitized guinea pigs after inhalation challenge with specific allergen (38).

The expression of eotaxins in stimulated human normal lung epithelial cells or established lung epithelial cell lines is well characterized (39-42). Also a decrease of eosinophils in airways during experimental asthmatic reaction in animals treated with PDE4 inhibitors is well documented. However direct effects of PDE4 inhibitors on eotaxin production by bronchial epithelial cells are not definitely established.

The aim of the presented study was to evaluate the effect of PDE4 inhibitors, rolipram and RO-20-1724 on eotaxin expression in human bronchial epithelial cells.

MATERIALS AND METHODS

Cell culture

Human bronchial epithelial cells BEAS-2B (American Type Culture Collection, Rockville, MD, USA) were cultured in Airway Epithelial Cell Growth Medium (PromoCell, USA, no. cat. C-21160) with 100 U/mL penicillin, 100 µg/mL streptomycin (PAA, Austria) at 37°C in 5% CO₂. The experiments were performed in Dulbecco’s Modified Eagle’s Medium (DMEM) without serum (Sigma Aldrich, Germany).

Stimulation of the cells

When the cells reached 80% confluence they were preincubated with 10 µM rolipram (Sigma Aldrich, Germany), RO-20-1724 (Calbiochem, Germany) or dexamethasone (Sigma Aldrich, Germany). Compounds were dissolved in 96% ethanol. After 1 hour the cells were stimulated with IL-4, IL-13 (25 ng/mL) or TNF-α (10 ng/mL) purchased from R&D Systems (Minneapolis, USA) for 48 hours. Cytokines were dissolved in water and minimal amount of bovine serum albumin (BSA).

ELISA

Culture supernatants were used to detect eotaxin-1, eotaxin-2 and eotaxin-3 using an ELISA from R&D Systems (USA) according to the manufacturer’s instructions (cat. no. DTX00, DCC240B, DCC260B). The sensitivity of tests was: 5 pg/mL, 2.5 pg/mL, and 2.33 pg/mL for CCL11, CCL24 and CCL26 respectively. The optical density was read at 450 nm with correction at 570 nm on StarFax 2000 microplate reader (USA).

RNA extraction and determination of eotaxin gene expression level

Total RNA was isolated from cells using Trizol (Invitrogen, USA). The purity and concentration of isolated RNA was measured using 260/280 nm absorbance ratio at spectrophotometer (Beckman, USA) type DU650. One microgram of RNA was used for reverse transcription using quantitative SuperScript III First-Strand Synthesis SuperMix (Invitrogen, USA). Real-time quantitative PCR evaluation was performed with an ABI-Prism 7500 Sequence Detector System (Applied Biosystems, USA). For real-time PCR, 1 µl of cDNA was amplified in a 25 µl PCR volume containing Power SYBR Green PCR master mix (Applied Biosystems, USA) with 150 nmoles of specific primers. Each sample was measured in duplicate. The sequences of primers were as follows: Eotaxin-1: forward, 5’CTCGCCTGGGCAAGCTTCTGTC3’; reverse, 5’GGCTTTGGAGATTTGTTG3’ (227 bp), eotaxin-2: forward, 5’CACATCACCTCCACCGGCT3’; reverse, 5’GGGTGCCAGATTCCTGAGACGG3’ (288 bp). Eotaxin-3: forward, 5’GGAACTCAGGACACGTCAGG3’; reverse, 5’CTCTGAGGAGAACACCTCTC3’ (354 bp) GAPDH: forward, 5’GAAGTTGAAAGTTCGAGT3’; reverse, 5’GAAGATGGTTTGGGATTT3’ (226 bp).

For cDNA amplification, a 10-minute incubation at 95°C was performed to activate AmpliTaqGold DNA Polymerase followed by 45 cycles, each of 15 s and at 95°C and 1 min at 60°C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control in order to normalize eotaxin-1, eotaxin-2 and eotaxin-3 expression levels. The results were expressed as relative quantification units (fold change).

Relative quantification values were calculated by the 2-∆ΔCT method. The cycle thresholds (CT) for the target amplicon and for the endogenous control (GAPDH) were determined for each sample. Differences were calculated between these two CTs and called ∆CT, in order to account for the differences in the amount of total nucleic acid taken for each reaction. The value of ∆CT for unstimulated cells (calibrator) was subtracted from the ∆CT of each sample and termed ∆ΔCT. The value of the target normalized to the endogenous control for experimental samples relative to that in the calibrator, was then calculated by the formula 2-∆∆CT.

Statistical analysis

Data were analyzed using the Mann-Whitney U-test. P-value <0.05 was considered statistically significant.

RESULTS

Preliminary results

Our preliminary experiments were made to evaluate the optimal culture condition for CCL26 production; because this chemokine respond to almost all cytokines used, and has the highest expression in BEAS-2B cells. The results of the preliminary trials are shown in Fig. 1-3. IL-4 and IL-13 stimulated the CCL26 production in dose dependent manner (Fig. 1). CCL26 concentration peaked at 48 hour after cytokine stimulation and the addition of TNF-α intensified this effect (Fig. 2) also in dose dependent manner on protein level (Fig. 3).
All data concern eotaxin-1 and eotaxin-3 only, since eotaxin-2 could not be detected as protein or mRNA in any experimental variant.

**Effect of tumor necrosis factor-α on eotaxin expression**

The effects of TNF-α on CCL11 production by BEAS-2B stimulated with Th2 cytokines and the effects of the PDE4 inhibitors on CCL11 production in the presence/absence of TNF-α are shown on Fig. 5. Fig. 6 shows analogous data concerning CCL26. TNF-α significantly increased the production of the both CCL11 and CCL26 in cultures stimulated with IL-4 or IL-13. In cell cultures stimulated with IL-4 and TNF-α the mean concentration of CCL11 was increased from undetectable level (Fig. 5A) to 213 pg/mL (Fig. 5C) and from 10 (Fig. 5B) to 109 pg/mL in culture stimulated with IL-13 (Fig. 5D). TNF-α increased production from 363 (Fig. 6A) to 1237 pg/mL (Fig. 6C) and from 431 to 1096 pg/mL in cultures stimulated with IL-4 or IL-13, respectively. Notably in unstimulated cultures treated with TNF-α, the level of eotaxins did not exceed a few pg/mL (Fig. 4). Moreover, we observed inhibition of CCL26 release in cultures stimulated TNF-α alone (Fig. 3).

**Effect of PDE4 inhibitors on eotaxin expression**

As is shown on Fig. 4, the levels of CCL11 in non-stimulated culture were below their detection limits; the CCL26...
concentration reached only 20 pg/mL. Fig. 4 demonstrate that neither of the two PDE4 inhibitors, nor dexamethasone influenced the release of CCL11 and CCL26 in unstimulated cultures of BEAS-2B cells. In cultures stimulated with Th2 cytokines, the concentration of CCL26 was significantly higher than CCL11 and reached 750 pg/mL and 670 pg/mL versus undetectable level and 10 pg/mL after stimulation with IL-4 or IL-13, respectively. It is noteworthy that in our experimental conditions the concentration of CCL11 in cultures stimulated by IL-4 was below limit of the detection (Fig. 5A).

We observed significant differences in the effect of PDE4 inhibitors on eotaxin expression measured both at the protein and mRNA level in the cell cultures stimulated with the Th2 cytokines which depends on the presence of TNF-α.

The PDE4 inhibitors had slightly effects on eotaxin expression in cultures stimulated by Th2 cytokines alone, both at the protein (Fig. 5A, 5B, Fig. 6A, 6B) and the mRNA levels (Fig. 7A, 7B; Fig. 8A, 8B). Statistically significant effects were observed only in the two cases: CCL11 protein concentration in the culture stimulated with IL-13 was lowered by pretreatment with rolipram (Fig. 5B) and CCL26 gene expression surprisingly was increased in cells pretreated with rolipram before stimulation with IL-4 (Fig. 8A).

The situation was different when TNF-α was used in co-stimulation with the Th2 cytokines. In the most cases, pretreatment of cells with PDE4 inhibitors decreased the concentrations of CCL11 and CCL26 in culture media regardless of the cytokine used, although the effect was statistically significant in two cases only. They were: the effect of rolipram on the production of CCL11 (Fig. 5C) and the effect of RO-20-1724 on the concentration of CCL26 (Fig. 6C), both in culture stimulated with IL-4 and TNF-α. Only in one case the pretreatment with a PDE4 inhibitor increased the mean level of the eotaxin protein but not in statistically significant way. It was the CCL26 protein in the culture stimulated with IL-13 after pretreatment with RO-20-1724 (Fig. 6D).

The most pronounced effects of PDE4 inhibitors on inhibition of eotaxin production in cells stimulated with the cytokines in the presence of TNF-α was observed at the mRNA level (Fig. 7CD, Fig. 8CD). In cells stimulated with IL-4 and

Fig. 4. Effect of cytokines, TNF-α, PDE4 inhibitors, and dexamethasone on CCL11 and CCL26 release by BEAS-2B cells. Eotaxin accumulation in the medium was measured by ELISA at 48 h after stimulation. The mean values and S.D. of 6-10 determinations from 3-5 independent experiments are shown. Rol-Rolipram (10 µM); RO-Ro-20-1724 (10 µM); Dx-dexamethasone (10 µM); IL-4 (25 ng/mL); IL-13 (25 ng/mL). A - CCL11, B - CCL26.

Fig. 5. Effect of PDE4 inhibitors and dexamethasone on CCL11 release by BEAS-2B cells stimulated by IL-4 (A) or IL-13 (B); and co-stimulated with TNF-α (C,D). Eotaxin accumulation in the medium was measured by ELISA at 48 h after stimulation. The mean values and S.D. of 10 determinations from 5 independent experiments are shown. Rol-Rolipram (10 µM); RO-Ro-20-1724 (10 µM); Dx-dexamethasone (10 µM); IL-4 (25 ng/mL); IL-13 (25 ng/mL). *p<0.05 compared with cells stimulated with IL-13 (B); *p<0.05 compared with cells stimulated with appropriate cytokine+TNF-α (C,D). (A,B) - CCL11 after stimulation with IL-4 or IL13; (C,D) - after co-stimulation with TNF-α.
TNF-α the both inhibitors decreased the mRNA level of both chemokines: for CCL11 the decrease was by 34 and 37% for rolipram and RO-20-1724, and for CCL26-43 and 47%, respectively. For cells stimulated with IL-13 and TNF-α, only rolipram had a statistically significant effect (expression of both eotaxins was decreased by ca. 50%). The effect of RO-20-1724, although also inhibitory was not statistically significant.

**Effect of dexamethasone on eotaxin expression**

Dexamethasone, used in our experiments as a reference inhibitor of allergic reactions, was the most effective in inhibition of the synthesis of eotaxins regardless of the stimulation agent used.

**DISCUSSION**

In this study we show that the inhibitory effects of PDE4 inhibitors on eotaxin expression may be dependent on TNF-α contribution. To our knowledge this is the first study concerning direct effects of rolipram and RO-20-1724 on eotaxin expression in an established line of human bronchial epithelial cells. There are some earlier publications about the effects of PDE4 inhibitors on eotaxin expression (33-35, 38) but those studies were performed using experimental systems different from ours (animal models, other type of cultured cells, different eotaxin stimulators and their concentrations, different duration of culture), therefore their results cannot be directly compared with the present ones.
Most authors of similar studies chose earlier time point for measure eotaxin release, because it is known that bronchial epithelial cells begin eotaxin production ca. 2 hours after stimulation (20, 39). It was also shown that although concentration of CCL11 in pulmonary epithelial cell lysates was stimulation (20, 39). It was also shown that although epithelial cells begin eotaxin production ca. 2 hours after cytokine stimulation. Our experiments partly confirm those their experiments CCL26 was the only one detected as protein expression was induced by stimulation with IL-13 or IL-4. In with the same cell line, CCL11, CCL26, but not CCL24 mRNA expression was induced by stimulation with IL-13 or IL-4. In their experiments CCL26 was the only one detected as protein after cytokine stimulation. Our experiments partly confirm those observations. We also did not find the CCL24 protein or mRNA but we detected the CCL11 protein, although its concentration was extremely low. Banwell et al., studying another bronchial epithelial cell line, also did not detect CCL24 gene expression after similar cytokine stimulation (20). On the other hand Komiya et al. (40), in experiments performed with BEAS-2B cells stimulated with IL-13 or IL-4 with/without TNF-α found all three eotaxin proteins. In their studies the concentrations of CCL11, CCL24 and CCL26 after stimulation with IL-4 were: 3766, 19, and 36305 pg/mL, respectively, thus incomparably higher than in our experiments. It seems that the use of the different medium (LHC-8; DMEM/HAM’s F12K+5% FCS), ELISA kit (made by authors using newly established antibody against CCL11, CCL24, CCL26), and concentrations of cytokines could be the reason for the apparent absence of the CCL24 protein in our experiment. Nevertheless, despite the different eotaxin protein concentrations reported, we also observed the highest production of CCL26 after IL-4 or IL-13 stimulation, and the same proportions of produced eotaxin similarly to Komiya et al.

It is known that the key players in eotaxin expression are IL-4, IL-13 and TNF-α. Numerous authors have shown that either Th2 cytokine or TNF-α alone are able to stimulate bronchial epithelial cells to produce eotaxins but this effect is much more stronger when they are added together. Fujisawa et al. showed that CCL11 expression in BEAS-2B cells depends on TNF-α addition to IL-4 (10 ng/mL) in dose dependent manner (42). It was shown that TNF-α alone can effectively induce CCL11 but not CCL26 production (20, 39, 43), probably because of differences in the structure of their promoters. TNF-α act through NFkB pathway and Th2 cytokines with STAT6. CCL11 promoter region contains binding sites for the NFkB and STAT6 factors which lay near and even overlap (44) since such dependency was not shown in CCL26 promoter. This explains the impact of TNF-α on CCL11 expression.

In our experiments TNF-α affected expression of CCL11 more than the expression of CCL26. Simultaneous stimulation with a Th2 cytokine and TNF-α enhanced about 100-fold (IL-13) and about 200-fold (IL-4) compared to Th2 cytokine alone. In the case of CCL26 this increase was only about 15-fold. Such stronger effect of TNF-α towards CCL11 was observed by other authors as well (20, 41, 43).

In our experiments the PDE4 inhibitors decreased production of eotaxins only in BEAS-2B cells co-stimulated with TNF-α with one exception - CCL11 reduction after rolipram preincubation and IL-13 stimulation. This effect was not confirm with RO-20-1724, probably the dose of RO-20-1724 was too low to inhibit protein release - the decrease on molecular level was shown but without statistical significance. It may be discussed on a biochemical as well as a molecular level. Firstly, in cells treated with both, TNF-α and the cytokine, the concentration of intracellular cAMP will be dependent on the summarized effects of the stimulators. Intercellular concentration of cAMP is regulated by a balance between its synthesis and decomposition (45). A few authors proved that eotaxin expression is connected with the process of cAMP evaluation in bronchial epithelial cells. cAMP probably got indirect effect on eotaxin expression. The activity of the main kinases which take part in eotaxin expression pathway are dependent on PKA - a main cAMP effector. After stimulation with IL-4 both forskolin - the direct activator of adenylyl cyclase, and 8Br-cAMP - the cAMP analog, decrease CCL11 production after IL-4+TNF-α or IL-13+TNF-α respectively (C,D). (A,B) - CCL 26 after stimulation with IL-4 or IL-13; (C,D) - after co-stimulation with TNF-α.
and IL-13 stimulation. But the increase of the drug dose cause the increase of the toxicity and side effects.

In early studies performed on endothelial cells by Koga et al. TNF-α was shown to reduce intercellular cAMP content at least partially increasing cyclic nucleotide phosphodiesterase activity (48). Data presented by Emalia et al. showed that TNF-α reduced also adenylyl cyclase activity in airway smooth muscle cells (49). Those observations may explain the effectiveness of PDE4 inhibitors in limiting eotaxin expression only in cultures co-stimulated with TNF-α. May be, in our experiments the effectiveness of PDE4 inhibitors were conditioned by a sufficiently low cAMP concentration. On the other hand there are only our assumptions because we optimized experimental conditions mainly for CCL26 and not assessed the levels of cAMP. Relationship of cAMP level and the effectiveness of PDE4 inhibitors in various culture conditions needs further experimental findings. We can suppose that in our experimental conditions the whole cAMP concentration after pretreatment with PDE4 inhibitors and stimulations with IL-4, IL-13, TNF-α was big enough to cause the decrease of eotaxin expression only in the cultures of cells treated simultaneously with Th2 cytokines and TNF-α.

Secondly, the effects of PDE4 inhibitors may also be explained at the level of transcription factors and protein kinases. Thus: induction of CCL11 and CCL26 production in response to TNF-α plus IL-4 stimulation of human lung fibroblast involved activation of NFκB and STAT6 with participation of p38 mitogen-activated protein kinase (MAPK) for both chemokines and, additionally by MEK (MAPK/ERK) for CCL26. It is known that rolipram precludes NFκB binding to gene promoters by stopping the phosphorylation and degradation of IkBα subunit, which blocks the breakdown of the IkBα-NFκB complex and activation of the NFκB subunit (50). This effect is probably indirect - authors conclude that rolipram partly inhibited p65 subunit translocation to the nucleus. Some authors have suggested that the increase of PKA activity after cAMP elevation can inhibit MAPK, ERK and c*NK and in this way affect the IL-4 signaling pathway (51, 52). There are no reports regarding the effect of increased cAMP level on STAT6 pathway. Full elucidation of the mechanisms of PDE4 inhibitors' action on eotaxin expression needs further research, and the effect of PDE4 inhibitors on STAT6 should be determined.

In conclusion, we have demonstrated that the effect of PDE4 inhibitors on eotaxin expression in cultured bronchial epithelial cells, stimulated with IL-4 or IL-13 may be depended on TNF-α. Increased levels of the both IL-4 and IL-13 as well as TNF-α are characteristic for the airway of asthmatic patients, thus their lung epithelium is under continuous stimulation for eotaxin production. The fact that PDE4 enzymes are widely present in the respiratory tract predisposes their inhibitors to local application. Therefore it seems that PDE4 inhibitors can be especially helpful in local - inhaled. Much lower dose of inhaled form of PDE4 inhibitor is needed to stop the late phase airflow obstruction and inflammatory call influx into lungs than orally given form. Moreover only inhaled administration of PDE4 inhibitor is effective in the improvement of lung function in allergen challenged animals. These results are supportive of the concept that inhaled PDE4 inhibition will have beneficial effects in the lungs that may not be seen with oral PDE4 inhibitors (53, 54).

Conflict of interests: None declared.

REFERENCES

35. Santamaria LF, Palacios JM, Beleta J. Inhibition of eotaxin-mediated human eosinophil activation and migration by the selective cyclic nucleotide phosphodiesterase type 4 inhibitor roflumipraz. Br J Pharmacol 1997; 121: 1150-1154.


Received: December 28, 2010
Accepted: June 10, 2011

Author's address: Dr. Magdalena Paplińska, Medical University of Warsaw Department of Internal Diseases, Pneumonology and Allergology, 1A Banacha Str., 02-097 Warsaw, Poland; E-mail: mpaplinska@wum.edu.pl