INFLUENCE OF SELECTIVE INHIBITORS OF PHOSPHODIESTERASE 3 AND 4 ON COUGH AND AIRWAY REACTIVITY

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As the administration of many antitussive drugs is often associated with adverse effects, new alternatives are evaluated in experimental and clinical conditions. The aim of this study was to assess the influence of selective inhibitors of PDE3 (cilostazol) and PDE4 (citalopram) on cough and airway reactivity. The number of cough efforts, specific airway resistance, \textit{in vitro} airway reactivity, and differential blood cells count were measured in healthy and in ovalbumin-sensitized guinea pigs before and after administration of cilostazol or citalopram (1 mg/kg). Cilostazol significantly suppressed citric acid induced cough only in healthy guinea pigs, whereas citalopram in both healthy and ovalbumin-sensitized animals. Both PDE inhibitors decreased \textit{in vivo} and \textit{in vitro} airway reactivity to histamine and the count of monocytes and neutrophils, confirming their anti-inflammatory potential. Administration of selective PDE3 and PDE4 inhibitors may influence cough and airway reactivity in the model of ovalbumin-sensitized guinea pigs.

Key words: cough, cilostazol, citalopram, ovalbumin, phosphodiesterase inhibitors

INTRODUCTION

Several agents from a group of xanthine derivatives are historically used in the therapy of airway diseases associated with cough and inflammation (bronchial asthma, chronic obstructive pulmonary disease). They are generally considered as non-selective inhibitors of phosphodiesterase (PDE); however, in therapeutically relevant plasma concentrations several other mechanisms are involved in their effects (1, 2). In previous studies the antitussive effects of xanthine derivatives was confirmed, with no particular discussion about the underlying mechanism (3, 4).

Furthermore, low specificity of xanthine derivatives, interactions with other drugs, and a narrow therapeutic range can often lead to an occurrence of adverse effects, which can limit their use as antitussives (5, 6). Thus, the use of selective
(PDE3, PDE4) or dual (PDE3/4, PDE4/7) PDE inhibitors in the therapy of cough-related conditions could be beneficial. Selective inhibitors of PDE have attracted increasing attention in the therapy of respiratory diseases (7). PDE isoenzymes play an important role in the regulation of airways diameter and smooth muscle functions. The presence of major PDE isoforms, PDE3 and PDE4, in the airways was confirmed; both hydrolyzing cAMP. However, airway smooth muscle contains more PDE isoenzymes, e.g., PDE1, 3, 4, 5, and 7.

PDE represent 11 superfamilies of metalophosphohydrolases, hydrolyzing cAMP and cGMP to their inactive metabolites (8, 9). Inhibition of PDE3 seems to be the most suitable target in affecting airway reactivity and cough. PDE3 is expressed in airway smooth muscle, myocardium, vessels, and gastrointestinal tract. However, inhibitors of PDE4 are considered as the most important therapeutic tools. The first generation inhibitor of PDE4 is rolipram, which was not introduced into clinical practice due to its adverse effects (nausea, vomiting). A second generation of PDE4 inhibitors (roflumilast, cilomilast) has a better perspective, as they maintained anti-inflammatory and immunomodulating effects with lower incidence of adverse effects (10). Nowadays, there are no relevant data about their antitussive effects.

In the present study, to elucidate the participation of PDE3 and PDE4 isoenzymes in cough and bronchoconstriction, effects of selective inhibitors of PDE3 (cilostazol) and PDE4 (citalopram) on cough and airway reactivity were assessed.

**MATERIAL AND METHODS**

The study protocol was approved by a local Ethics Committee at the Jessenius Faculty of Medicine, Comenius University in Martin, Slovakia. Forty eight healthy, male guinea pigs (250-350 g) were used for the study. They were kept in an animal house and had food and water ad libitum. In three groups of animals (n=8 each), airway hyperresponsiveness was induced with ovalbumin antigen (n=8) and the other three groups was used as a control (n=8). From the three groups, the first one was left without treatment, the second was treated with PDE3 inhibitor cilostazol (Sigma Aldrich, Germany), and the third with PDE4 inhibitor citalopram (Sigma Aldrich, Germany), both at a dose of 1 mg/kg.

**Antigen-induced airway hyperresponsiveness**

Sensitization of animals by the antigen ovalbumin, which causes airway reactivity changes on immunological basis, was performed during 14 days (11, 12). The allergen (1% ovalbumin) was administered on the 1st day of sensitization intraperitoneally (0.5 ml) and subcutaneously (0.5 ml), on the 3rd day intraperitoneally (1 ml), and on the 14th day only by inhalation (3 min). The airway reactivity to mediators of bronchospasm was followed in vivo immediately after the inhalation and in vitro after sacrificing the animal. In treated groups, cilostazol or citalopram were administered 30 min before the nebulization.
**Cough reflex assessment**

The method of chemically-induced cough was used for assessing the cough reflex (11, 13). We used citric acid aerosol in concentration of 0.6 M in saline for cough provocation. The inhalation of citric acid in double chamber plethysmograph lasted 2 min. During this time and during the following 2 min, a well trained observer evaluated visually the number of cough efforts. To distinguish cough from sneezing or movement artifacts, subsequent evaluation of the computer records of air flow in nasal chamber was performed.

**Evaluation of in vivo airway reactivity**

*In vivo* airway reactivity was evaluated using a double chamber whole body plethysmograph immediately after administration of bronchoconstrictors (11). Specific airway resistance and its changes after a short-term inhalation (2 min) of bronchoconstricting agents (citric acid and histamine in a concentration of $10^{-6}$ mol/l in saline) were considered as an indicator of the *in vivo* reactivity changes. For comparison, reactivity after nebulization of saline was used. Between two exposures there was an interval of minimum 5 min. During intervals, fresh air was insufflated into the nasal chamber.

**Evaluation of in vitro airway reactivity**

After sacrificing the animals, trachea and lungs were immediately excised. Tracheal strips (approximately 15 mm) were cut on the opposite side of a smooth muscle. Lung tissue strips (2 x 2 x 15 mm) were cut from the margin of upper lobes of both lungs. The strips were mounted between two hooks and placed into the 30 ml organ chambers containing Krebs-Henseleit buffer (NaCl 110.00 mmol/l, KCl 4.80 mmol/l, CaCl$_2$ 2.35 mmol/l, MgSO$_4$ 1.20 mmol/l, KHPO$_4$ 1.20 mmol/l, NaHCO$_3$ 25.00 mmol/l, and glucose 10 mmol/l in glass-distilled water). The chambers were maintained at 36.5 ±0.5°C and aerated continuously with a mixture of 95% O$_2$ and 5% CO$_2$ to maintain pH 7.5 ±0.1. One of the hooks was connected to a force transducer (TENSIL10, RES Martin, Slovakia) and an amplifier (TEMES 1052, RES Martin, Slovakia), and tension changes were recorded online using special computer software (TEMES 1, RES Martin, Slovakia). The tissue strips were initially set to 4 grams of tension for 30 min (loading phase). Then, in each strip the tension was readjusted to a baseline value of 2 grams for another 30 min (adaptation phase). During both periods, the tissue strips were washed at 10 min intervals. Cumulative doses of histamine ($10^{-8}$ to $10^{-3}$ mol/l, Sigma-Aldrich, Germany) were added after the adaptation phase had been finished and a continuous recording of contractions was made (14, 15). Data of the tracheal and lung tissue reactivity are showed in grams (g) of the smooth muscle tension.

**Hematological assay**

Samples of blood were taken immediately after sacrificing the animals and total white blood cells (WBC) count was determined in Bürker’s chamber after staining by Türk. Differential leukocytes count in blood was evaluated microscopically after panchromatic staining by Pappenheim and a relative count of neutrophils, monocytes, basophils, eosinophils, and lymphocytes was determined (in %) (16).

**Statistical analysis**

Data are shown as means ±SE. For illustration of cough efforts changes, box plots were used depicting mean, median, 25$^{th}$ and 75$^{th}$ percentile, minimum, and maximum. For statistical analysis, one-way ANOVA was used. A P<0.05 was considered statistically significant.
RESULTS

Intraperitoneal administration of PDE3 inhibitor cilostazol at a dose of 1 mg/kg 30 min before the experiment significantly decreased the number of cough efforts in healthy guinea pigs. In ovalbumin-sensitized animals cilostazol did not influence cough significantly, suggesting its weaker effect in inflammation (Fig. 1A). PDE4 inhibition by citalopram at the same dose led to a significant suppression of cough in both healthy and ovalbumin-sensitized guinea pigs (Fig. 1B).

The ability to affect the airway reactivity was evaluated with respect to both selective PDE inhibitors. Specific airway resistance measured in the whole body double chamber plethysmograph was used as a marker of \textit{in vivo} airway reactivity. Administration of citalopram caused a significant decrease of specific

\begin{figure}
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\includegraphics[width=\textwidth]{image}
\caption{Fig. 1. A - Number of cough efforts during (2 min) and after (2 min) inhalation of citric acid aerosol in healthy, ovalbumin-sensitized and cilostazol (Panel A) or citalopram (Panel B) pre-treated guinea pigs. Diamond represents the mean, thick middle line the median, lower and upper sides of the box plot the 25th and 75th percentile, respectively, and error bars the minimum and maximum. *P<0.05 vs. control.}
\end{figure}
airway resistance after citric acid as well as histamine nebulization. The pre-
treatment with cilostazol did not change significantly in vivo airway reactivity in
healthy animals (Fig. 2A). In the ovalbumin-sensitized groups, administration of
both cilostazol and citalopram decreased in specific airway resistance after
inhalation of ovalbumin. Moreover, cilostazol pretreatment caused a significant
decrease in specific airway resistance after inhalation of histamine (Fig. 2B).

In vitro testing of airway smooth muscle tissue strips contractility to
cumulative doses of histamine demonstrated a significant suppression of strips
reactivity, with the predominance in lungs tissue samples (Fig. 3).

Hematological examination was focused on changes in total WBC count and
in their differential count. We found that sensitization with ovalbumin led to a
significant increase in circulating WBC with increasing representation of
neutrophils and eosinophils at the expense of lymphocytes. Administration of

![Fig. 2. Specific airway resistance after inhalation of saline, citric acid and histamine in healthy, non-
sensitized (Panel A) and ovalbumin-sensitized (Panel B) guinea pigs before (control) and after
administration of selective PDE inhibitors. *P<0.05 vs. control.]
cilostazol and citalopram to healthy guinea pigs caused a significant increase of WBC count. However, increased neutrophil representation was not accompanied by increased monocytes and eosinophils. In ovalbumin-sensitized guinea pigs, decrease in relative count of neutrophils, monocytes, and eosinophils compared to non-treated animals was observed; these changes were statistically significant only in the citalopram group (Table I).

**DISCUSSION**

Inhibition of PDE, particularly PDE3 and PDE4, has been previously confirmed as a suitable target for influencing the airway inflammation as well as contractility of airway smooth muscle (7).

In our experiments, cilostazol, a selective inhibitor of PDE3, showed antitussive effects only in healthy guinea pigs; however, *in vivo* airway reactivity was decreased in ovalbumin-sensitized animals. Similar results were observed by Matsuda et al (17), who considered inhibition of PDE3 as the most suitable manner for influencing cough and airway reactivity. Our results are in accordance with other studies, where cilostazol in clinical conditions decreased the cough reflex sensitivity to capsaicin in patients with bronchial asthma (18). In a recent study by Fujimura and Liu (19), the antitussive effect of olprinon (PDE3

*Fig. 3. Airway reactivity to cumulative doses of histamine in *in vitro* lung strips of healthy and ovalbumin-sensitized guinea pigs without or after pretreatment with cilostazol (Panel A) and citalopram (Panel B). §P<0.05 vs. control, *P<0.05 vs. ovalbumin.*
inhibitor) to capsaicin was confirmed in normal and sensitized guinea pigs with negligible effects on eosinophilia in bronchoalveolar lavage fluid. However, this effect was not observed after PDE4 inhibition with SB207499.

The inhibition of PDE4 seems to be a highly perspective choice in the therapy of chronic inflammatory diseases associated with airway hyperresponsiveness. Inhibitors of PDE4 (rolipram, citalopram, cilomilast, roflumilast, piclamilast) have predominantly anti-inflammatory and immunomodulating effects with markedly reduced adverse effects comparing to non-selective PDE inhibitors (10).

Our results support these findings, demonstrating the suppression of cough and airway reactivity by the PDE3 inhibitor cilostazol and the PDE4 inhibitor citalopram in both healthy and ovalbumin-sensitized guinea pigs. Previously published data describe the antitussive effects of PDE3 inhibitors (17, 20, 21). In our experiments, PDE3 inhibitor cilostazol suppressed cough only in healthy guinea pigs, whereas PDE4 inhibitor citalopram in both healthy and ovalbumin-sensitized animals.

This discrepancy could be explained by the participation of PDE4 in inflammation and more significant anti-inflammatory effect of PDE4 inhibitors. Paradoxically, the significant antitussive effect and decrease in in vitro airway reactivity after administration of citalopram in ovalbumin-sensitized guinea pigs was not accompanied by in vivo airway reactivity (specific airway resistance) changes, suggesting an involvement of other mechanisms in this process.

As there are no relevant data about antitussive effects of PDE4 inhibitors, our results indicate their efficiency in influencing cough. Furthermore, application of

<p>| Table 1. Number of white blood cells (WBC) and their differential count. |
|-----------------------------|------------------|------------------|------------------|------------------|------------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ovalbumin</th>
<th>Cilostazol</th>
<th>Ovalbumin +cilostazol</th>
<th>Citalopram</th>
<th>Ovalbumin +citalopram</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (x10^9/l blood)</td>
<td>0.90 (0.12)</td>
<td>2.54 (0.38)*</td>
<td>1.72 (0.14)*</td>
<td>3.80 (0.56)†</td>
<td>2.28 (0.29)*</td>
<td>2.65 (0.23)</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>83.3 (5.1)</td>
<td>40.6 (5.0)*</td>
<td>47.5 (3.8)*</td>
<td>56.4 (6.6)</td>
<td>66.2 (11.1)</td>
<td>61.5 (6.2)</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>12.1 (4.7)</td>
<td>52.1 (4.6)*</td>
<td>47.1 (3.4)*</td>
<td>37.9 (5.9)</td>
<td>30.3 (10.9)*</td>
<td>34.4 (5.8)†</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>3.7 (0.7)</td>
<td>5.0 (0.9)</td>
<td>4.0 (0.7)</td>
<td>3.8 (0.6)</td>
<td>2.6 (0.2)</td>
<td>2.4 (0.4)†</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>0.49 (0.3)</td>
<td>2.1 (0.8)*</td>
<td>1.1 (0.2)</td>
<td>1.9 (0.8)</td>
<td>0.9 (0.4)</td>
<td>1.8 (0.6)</td>
</tr>
<tr>
<td>Basophils (%)</td>
<td>0.4 (0.2)</td>
<td>0.4 (0.1)</td>
<td>0.1 (0.0)*</td>
<td>0 (0)†</td>
<td>0 (0)*</td>
<td>0 (0)†</td>
</tr>
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Values are means ±(SE). *P<0.05 vs. control, †P<0.05 vs. ovalbumin.
dual inhibitors (PDE3/4) should be considered, as previous findings demonstrate a more significant airway relaxing effect of siguazodan (PDE3 inhibitor) compared with rolipram (PDE4 inhibitor). On the other side, their simultaneous administration leads to additive relaxation, suggesting an interaction or even synergism between the inhibition of PDE3 and PDE4 (22). Similarly, inhalant administration of zardaverine (dual inhibitor of PDE3/4) leads to significant bronchodilation (7).

Evaluating changes in total and differential WBC count, a significant increase in WBC, with relative increases in neutrophils and eosinophils, was detected. A similar trend showed administration of cilostazol and citalopram to healthy guinea pigs. On the other side, it is necessary to emphasize that administration of cilostazol and citalopram to ovalbumin-sensitized guinea pigs increased WBC count, confirming their known influence on T-lymphocytes. Along with this, in both cases, a significant decrease in neutrophils, and in case of citalopram also monocytes, was observed, confirming stronger anti-inflammatory effect of PDE4 inhibitors. Similarly to the results obtained by Fujimura and Liu (19), no significant changes in relative eosinophil count were observed, although a tendency to its decrease was indicated.

Selective inhibitors of PDE, particularly PDE3 and PDE4, have been extensively studied for their anti-inflammatory action. Several clinical studies testing a second generation of PDE4 inhibitors (piclamilast, cilomilast, roflumilast) confirm their bronchodilating, anti-inflammatory, and antitussive effects previously demonstrated in experimental conditions. That warrants further testing of other PDE isoforms (23-25).

In conclusion, administration of both PDE3 (cilostazol) and PDE4 (citalopram) inhibitors leads to antitussive and bronchodilating effects in healthy and ovalbumin-sensitized guinea pigs, suggesting their possible use in therapy of cough associated with chronic inflammatory conditions of airways.

Acknowledgements: Authors thank to M. Repcakova, M. Duchonova and P. Kuzma for technical assistance. The study was supported by Grants VEGA No. 1/0072/08, No. 1/3375/06, Grant of Ministry of Health No. 2005/13-MFN-05, and Grant ESF SOP LZ 2005/NP1-027 - 11230100433.

Conflicts of interest: no conflicts of interest were declared in relation to this article.

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Received: May 21, 2008.
Accepted: September 2, 2008.

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