**Effect of inhaled steroids on cholinergic transmission in human isolated bronchi**

J. Říčný*, K.-D. Höhle*, K. Racké**, I. Wessler*

**ABSTRACT:** Steroids have been found to facilitate cholinergic transmission in skeletal muscle, but possible effects in airways smooth muscles have not been studied. Therefore, choline acetyltransferase (CAT) activity, tissue content of stored acetylcholine and release of newly-synthesized [3H]acetylcholine were measured in freshly-dissected human bronchi.

All lung tissue was obtained from patients with lung cancer at thoracotomy. Group I bronchi were obtained from patients who also suffered from chronic obstructive bronchitis and had been treated for at least 6 weeks before surgery with daily doses of four puffs of flusinolid. Group II bronchi were obtained from patients who did not suffer from chronic obstructive airways disease and had not been treated with steroids.

Neither CAT activity (3.1 nmol·h⁻¹·mg protein⁻¹) nor acetylcholine tissue content (260 pmol·100 mg⁻¹) or electrically evoked [3H]acetylcholine release (about 2,000 dpm·100 mg⁻¹) differed in the two groups.

This cross sectional study indicates that inhaled steroids do not change cholinergic transmission beyond the level observed in the airways obtained from patients with lung cancer who do not suffer from chronic obstructive airways disease and have not been treated with steroids. This suggests that inhaled steroids can be given chronically without the induction of a facilitatory side-effect on cholinergic transmission within the airways.

**Efferent activity in the pulmonary vagal nerve plays an important role in controlling airway diameter, ion transport and secretion [1–3]. Experimental evidence has been presented that cholinergic transmission may be enhanced in acute or chronic airways diseases. For example, cholinergic transmission in the airway wall is thought to be facilitated by various inflammatory intermediates (tachykinins, thromboxane, serotonin) [4]. In addition, the activity of choline acetyltransferase (CAT), the key enzyme for acetylcholine synthesis, was found to be significantly enhanced in the nasal mucosa obtained from actively sensitized guinea-pigs [5]. Steroids are basically applied to suppress airway inflammation. However, possible facilitatory effects of these compounds on cholinergic transmission have to be considered. For example, in skeletal muscle (rat diaphragm) it has been reported that steroids increase the uptake of the precursor choline and its incorporation into acetylcholine [6]. It is not known whether steroids mediate a similar effect within the smooth muscle of the airways. Therefore, in the present study, CAT activity, tissue content of acetylcholine and release of newly-synthesized [3H]acetylcholine were determined in freshly dissected human bronchi.**

**Material and methods**

**Patients**

The protocol for this study was approved by the local review board for human studies (Ethik Kommission Landesärztekammer Rheinland-Pfalz). All bronchi (segmental) used were obtained from patients with lung cancer, immediately after thoracotomy and resection of a lobe or right/left lung. Lung tissue was separated into two groups. Group I bronchi were obtained from patients who suffered, in addition to lung cancer, from chronic obstructive bronchitis and had been continuously treated with inhaled steroids for at least 6 weeks before surgery (flusinolid 0.25 mg·puff⁻¹; 4 puffs·day⁻¹). Group II bronchi were obtained from patients who did not suffer from obstructive airways disease and had not been treated with inhaled steroids. Sex and age were comparable in both groups (table 1). It has to be considered that a possible effect of chronic obstructive airways disease (COAD) itself on cholinergic transmission could not be studied because freshly dissected tissue of appropriate patients (lung cancer, COAD without inhalation of steroids) was not available.
Table 1. – Possible effects of inhaled steroids on cholinergic transmission (CAT activity, acetylcholine tissue content, release of newly-synthesized [3H]acetylcholine) in human isolated bronchi

<table>
<thead>
<tr>
<th>Group</th>
<th>Pt</th>
<th>Age yrs</th>
<th>Sex M/F</th>
<th>CAT activity nmol·h⁻¹·mg protein⁻¹</th>
<th>Acetylcholine tissue content pmol·100 mg⁻¹</th>
<th>[3H]acetylcholine release dpm·100 mg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>3.1±0.5</td>
<td>6</td>
<td>56±14</td>
<td>5/1</td>
<td>260±100</td>
<td>8</td>
</tr>
<tr>
<td>Group II</td>
<td>3.1±0.4</td>
<td>8</td>
<td>65±13</td>
<td>6/2</td>
<td>250±30</td>
<td>8</td>
</tr>
</tbody>
</table>

Segmental bronchi were isolated and analytical measurements were performed as described in Methods. Group I contained patients treated with inhaled flusinolid (4 puffs·day⁻¹ for a period of at least 6 weeks before surgery). Group II patients had not been treated with steroids. Data presented as means±SEM of n experiments. CAT: choline acetyltransferase; M: male; F: female; Pt: patient.

Tissue preparation

Immediately after resection, macroscopically normal bronchi (segmental bronchi) were dissected and placed in oxygenated physiological salt solution of the following composition (in mM): NaCl 125, KCl 2.7, CaCl₂ 1.8, MgCl₂ 1.0, NaH₂PO₄ 0.4, NaHCO₃ 24, D-glucose 5.4, choline 0.001. The medium was aerated with 5% CO₂ (v/v) in O₂. The pH of the aerated solution was 7.30. After transport to the laboratory, bronchi were carefully cleaned from adhering connective tissue and from cartilage. Tissue was weighed and homogenized mechanically in high performance liquid chromatography (HPLC)-buffer and then injected in to the HPLC system (20 µl).

Estimation of CAT activity, acetylcholine content and release of newly-synthesized [3H]acetylcholine

Enzymatic assay was performed according to FONNUM [7]. In brief, pulverized tissue was incubated in extraction buffer (10 mM NaPO₄, 100 mM NaCl, 2 mM ethylene diamine tetra-acetic acid (EDTA), 0.5% (v/v) Triton X-100; incubation volume was three times wet weight; pH 7.4). After standing on ice for 15 min, samples were centrifuged (5 min, 4,000 rpm at 0°C; 20 µl of supernatant was added to the assay mixture (50 mM NaPO₄, 300 mM NaCl, 8 mM choline chloride, 0.1 mM physostigmine sulphate, 10 mM EDTA, 0.15% Triton X-100 (v/v), 0.2 mM [3H]AcCoA (specific activity 12 dpm·pmol⁻¹)). Test tubes were incubated (30 min) in a water bath (37°C). Enzymatic reaction was stopped after 30 min by transferring the assay mixture to a scintillation vial containing a mixture of 5 ml 10 mM NaPO₄ and 2 ml acetonitrile/tetraphenylboron (5 mg·ml⁻¹) to extract synthesized radioactive acetylcholine in the organic phase. Thereafter, the organic phase was transferred into a new vial, scintillation cocktail added and tritium content measured by liquid scintillation spectrometry. Protein assay was performed according to MARKWELL et al. [8].

Acetylcholine was measured in the supernatant (20 µl) of homogenized bronchi by a cationic exchange HPLC-EC detection method as described previously [9, 10]. The BAS 481 microbore system was used (West Lafayette, USA). Release of newly synthesized [3H]acetylcholine was measured as described previously [10–12]. After labelling of segmental bronchi by incubation with [3H]choline (specific radioactivity 0.8 MBq·nM⁻¹) and a 60 min subsequent wash-out, tritium outflow was measured in 3 min intervals. [3H]acetylcholine release was evoked 12 min after the end of the wash-out by transmural stimulation (four 20 s trains at 15 Hz, with 5 s rest between each train) and calculated from the difference between stimulated and basal tritium outflow [10–12]. The results were analysed by independent t-tests. Values of p less than 0.05 were considered to be statistically significant.

Results

In homogenized bronchi obtained from Group I, CAT activity amounted to 3.1±0.5 nmol·h⁻¹·mg protein⁻¹, and an identical enzyme activity was found in group II (3.1±0.4 nmol·h⁻¹·mg protein⁻¹) (table 1). Likewise, the tissue content of acetylcholine did not differ between the two groups. In group I 260±100 pmol acetylcholine was found in 100 mg of homogenized bronchi, and in Group II tissue content of stored acetylcholine was 250±30 nmol·100 mg (table 1).

Bronchi were incubated with [3H]choline to allow synthesis of [3H]acetylcholine and to measure the stimulated release of newly-synthesized [3H]acetylcholine. In human bronchi obtained from group I and labelled with [3H]choline, a subsequent transmural electrical stimulation caused the release of 1,500±400 dpm·100 mg⁻¹ [3H]acetylcholine. In group II bronchi, stimulated release of [3H]acetylcholine was higher (2,300±400 dpm·100 mg⁻¹) but the difference was not statistically significant (table 1).
The CAT activity detected in the present study was comparable to other tissues containing cholinergic neurons. For example, a similar enzyme activity has been estimated in human cortical neurons, whereas in subcortical regions (striatum and thalamus) a 10 fold higher activity was found [13]. The substantial enzyme activity detected in the present study is indicative of an intensive cholinergic innervation of human airways; a similar conclusion has been drawn from histological studies [14].

The present results clearly demonstrate that the parameters studied, i.e. CAT activity, tissue content of stored acetylcholine and release of newly-synthesized $[^3H]$acetylcholine did not differ between patients with lung cancer, and patients with lung cancer associated with COAD and treated with inhaled steroids. Bearing in mind that CAT activity was identical in both groups, it can also be concluded that uptake of choline did not differ between the two groups. For obvious reasons, freshly dissected tissue from the appropriate control group (patients with obstructive airways disease not taking steroids) was not available. Chronic inflammation, however, is thought to enhance but not to reduce cholinergic transmission. For example, tachykinins are thought to increase in the biophase during inflammation and may facilitate cholinergic transmission [4]. Moreover, in the nasal mucosa of rats it was found that active sensitization caused an increase in the CAT activity [5]. Thus, following the concept of an enhanced cholinergic transmission in inflammatory airways, the present results may suggest an inhibitory effect of steroids on cholinergic transmission within human airways smooth muscles, whereas a facilitatory effect can be excluded. In contrast, in skeletal muscles a facilitatory effect of steroids has been found. Dexamethasone and related compounds were found to enhance choline uptake, acetylcholine synthesis and the tissue store of acetylcholine in the terminals of the rat phrenic nerve [6, 15, 16]. Therefore, the authors proposed that steroids enhance cholinergic transmission in skeletal muscles under in vivo conditions. In fact, in in vitro studies indirect evidence was presented that dexamethasone increased both the resting and stimulated release of acetylcholine from motor end-plates [15].

In conclusion, the present experiments have shown that long-term application of inhaled steroids did not change cholinergic transmission in the airways beyond the level which has been measured in lung tissue obtained from patients who did not suffer from obstructive airways disease and had not been treated with inhaled steroids. The chronic use of inhaled steroids did not enhance CAT activity and acetylcholine release in the present in vitro experiments. It is concluded that, in patients also, the long-term inhalation of steroids does not exert a facilitatory side-effect on cholinergic transmission within the airways, a condition which may contribute to the wide acceptance of this important drug.

References