EFFECT OF PILOCARPINE ON BEHAVIOR OF MUCUS GLYCOPROTEINS OF CANINE TRACHEAL SECRETORY CELLS

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Abstract—Behavior of mucus glycoproteins in tracheal secretory cells after treatment with pilocarpine was investigated histologically and histochemically using the isolated canine trachea. Following pilocarpine treatment, the number of total and neutral glycoprotein-containing goblet cells was reduced concentration-dependently. The number of acid glycoprotein (AGP)-containing goblet cells was not altered with $10^{-7}$ and $10^{-6}$ M pilocarpine, but significantly decreased with $10^{-5}$ and $10^{-4}$ M pilocarpine. The thickness of the acini of submucosal glands significantly decreased, and the ratio of acinar inner diameter to tracheal wall thickness increased in $10^{-5}$ and $10^{-4}$ M pilocarpine. AGP content in glandular cells increased in $10^{-7}$ and $10^{-6}$ M pilocarpine, but markedly decreased at concentrations of $10^{-5}$ and $10^{-4}$ M. Pilocarpine treatment caused an increase in N-acetylhexosamine concentration in the incubation fluid. Total saccharide concentration in the incubation fluid decreased in $10^{-7}$ and $10^{-6}$ M pilocarpine, but was not apparently altered at concentrations of $10^{-5}$ and $10^{-4}$ M. These findings suggest that lower concentrations of pilocarpine stimulate synthesis of AGP in goblet and glandular cells much more preferentially than it stimulates discharge of AGP from the cells, while at higher concentrations, the AGP-discharge effect overcomes the stimulation in synthesis.

Excessive mucus secretion in the respiratory tract is an important feature in chronic obstructive pulmonary diseases such as chronic bronchitis, cystic fibrosis and bronchial asthma. The mucin component of airway secretions consists of high molecular glycoproteins which are synthesized and secreted by both goblet cells in the airway epithelium and submucosal glandular cells in the lamina propria mucosae (1). The viscosity of mucus is definitely dependent on its content of acid glycoprotein (AGP) (2–4).

Pilocarpine has been found to increase the respiratory tract fluid volume in vivo (5–7). However, only a few histological and histochemical studies on the effect of pilocarpine on airway secretory tissues have been reported (8, 9). To understand the detailed mechanism of the secretagogue effect of pilocarpine on airway tissues, it is necessary to study the behavior of glycoproteins in the secretory cells following application of this agent.

In the present study, using histochemical and histological techniques, we attempted to estimate both the synthesis and discharge of glycoproteins in goblet cells and sub-
mucosal glands after pilocarpine treatment.

MATERIALS AND METHODS

Animals: Healthy male mongrel dogs weighing between 10 and 14 kg were anesthetized with sodium pentobarbital (30 mg/kg i.v.). The trachea, about 10 cm in length, was excised from the larynx after a midincision.

Drug treatment: Pieces of the trachea (about 2 cm long) were preincubated for 30 min in a 40 ml-organ bath containing Hanks solution. The bathing medium was oxygenated with a 95% O2-5% CO2 mixture and maintained at a temperature of 37±1°C. After preincubation, the tracheal segment was treated for 30 min with pilocarpine hydrochloride (Torii) at the concentrations being tested (10⁻⁷ to 10⁻⁴ M). The segment was then fixed as described below for histological and histochemical studies, and the incubation fluid was stored at -20°C until the total saccharide, N-acetylhexosamine and protein in the fluid was analyzed.

Tissue preparation: The tracheal tissues were prefixed using a two-step procedure: First, with a 1:1 ratio of 2.5% glutaraldehyde in 0.2 M Millonig's phosphate buffer (pH 7.4) and Hanks solution at room temperature for 30 min and secondly, with 2.5% glutaraldehyde in the buffer below 4°C for 1 hr. The prefixed tissues were cut into small pieces (about 5 mm×5 mm), and they were then postfixed with a mixture of 2% paraformaldehyde and 2.3% glutaraldehyde in 0.2 M Millonig's phosphate buffer (pH 7.4) below 4°C overnight.

After fixation, the tissues were dehydrated with alcohol, infiltrated with paraffin, and sectioned in 4 μm slices. The tissue slices were stained with a combination of alcian blue (AB) at pH 2.5 or pH 1.0 and periodic acid-Schiff (PAS) (designated as AB (pH 2.5)/PAS or AB (pH 1.0)/PAS).

Histochemical and histological parameters:

The following histological parameters were evaluated: goblet cell number within a range of 500 μm of each section, acinar outer (A0) and inner diameters (A₁), thickness of acinus (A₀–A₁), Reid index (GWR) (10), and the ratio of acinar inner diameter to the tracheal wall thickness (A₁WR) of a submucosal gland (Fig. 1).

Changes in glycoproteins within goblet and glandular cells were assessed histochemically in terms of the following parameters (11–16): with the AB (pH 2.5)/PAS procedure, all AGP stain blue (stain index of B) or purple (stain index of P) and neutral glycoproteins (NGP) stain red (stain index of R); with the AB (pH 1.0)/PAS procedure, only sulphated glycoproteins (SGP) of the AGP stain blue (stain index of B) or purple (stain index of P).

To assess the effect of pilocarpine, five tracheas were used for each concentration. Fifty slices were made from a trachea treated with the agent. Ten out of the 50 slices, chosen at random, were stained and photographed at 150 fold magnification.

Analysis of macromolecular components secreted into incubation fluid: The protein
concentration of the incubation fluid was determined by the Folin-phenol method (17). The total saccharide concentration was determined by the Anthrone method (18). The N-acetylhexosamine concentration was estimated according to the method of Reissig et al. (19).

RESULTS

Typical photomicrographs of goblet cells and submucosal glands in a tracheal section treated with pilocarpine 10^-4 M are shown in Fig. 2.

Effects of pilocarpine on goblet cells: Following pilocarpine treatment, the number of goblet cells that were stained positively with AB (pH 2.5)/PAS greatly decreased, and additionally, the color of the positive cells became lighter. The number of positive goblet cells containing glycoprotein was 36.9±1.4 (S.E.) (N=5) in the control group. Pilocarpine significantly decreased the number to 29.0±0.6 at 10^-7 M, 26.3±1.7 at 10^-6 M, 16.1±1.3 at 10^-5 M and 12.0±0.6 at 10^-4 M (Fig. 3). The number of AGP-containing goblet cells which showed a stain index of B+P was 21.5±0.6 in the control group. The numbers after pilocarpine treatment were 22.7±1.4 at 10^-7 M, 23.1±1.5 at 10^-6 M, 13.6±1.1 at 10^-5 M and 11.2±0.6 at 10^-4 M. Pilocarpine caused a significant and concentration-dependent decrease in the number of NGP-containing goblet cells which showed a stain index of R (Fig. 3). The number of SGP-containing goblet cells which showed a stain index of B+P with AB (pH 1.0)/PAS decreased at pilocarpine concentrations above 10^-5 M (Fig. 4).

Effects of pilocarpine on submucosal glandular cells: The acinar outer diameter

Fig. 2. Photomicrographs of canine tracheal secretory cells stained with AB (pH 2.5)/PAS at 300X magnification. Goblet cells (GC) and submucosal glands (SG) are indicated by arrows. (A) and (B): the control group, and (C) and (D): the 10^-4 M pilocarpine treated group.
(A°) of the submucosal gland was 34.4±1.4 μm (N=5) in the control group. After application of pilocarpine at concentrations of 10⁻⁷ and 10⁻⁶ M, the diameter increased to 36.4±3.4 and 40.6±7.2 μm, respectively. It showed a slight decrease to 33.2±3.0 (10⁻⁵ M) and 29.2±2.8 μm (10⁻⁴ M) at higher concentrations. The acinar inner diameter (A') of the gland increased from 13.6±1.2 (control) to 19.4±3.0 (10⁻⁵ M) and 17.8±2.4 μm (10⁻⁴ M).

Changes in acinar thickness (A°−A') and the ratio of acinar inner diameter to wall thickness (A'WR) are shown in Table 1. Pilocarpine at 10⁻⁷ and 10⁻⁶ M increased the thickness from 21.2±1.4 (control) to 24.8±3.4 and 29.0±6.4 μm, respectively, whereas at higher concentrations, it significantly decreased the thickness to 13.4±1.4 (10⁻⁵ M) and 11.6±1.2 μm (10⁻⁴ M). An application of higher concentrations resulted in a significant increase in A'WR.

Pilocarpine treatment increased the Reid index (GWR) by 17.8 (10⁻⁷ M), 26.0 (10⁻⁶ M), 28.8 (10⁻⁵ M) and 31.1% (10⁻⁴ M).

Changes in the stain index of submucosal glandular cells are shown in Fig. 5. Stain index of B for AB (pH 2.5)/PAS increased

Fig. 3. Number of goblet cells stained by AB (pH 2.5)/PAS per 500 μm length of the tracheal epithelium. Each curve represents the number of goblet cells containing total (○), acid (□—□), acid and neutral glycoprotein (△—△), or neutral glycoprotein (●—●) and stain index of B (×—×). Each value represents the mean±S.E. of 5 experiments (value for each experiment is the average determined from 5 slices). Significant at *P<0.01 and **P<0.001 as compared to the control.

Table 1. Effects of pilocarpine on the thickness of acinus and A'WR of tracheal submucosal glands

<table>
<thead>
<tr>
<th>Condition</th>
<th>Thickness of acinus (μm)</th>
<th>A'WR</th>
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<tbody>
<tr>
<td>Control</td>
<td>21.2±1.4</td>
<td>0.054±0.005</td>
</tr>
<tr>
<td>Pilocarpine 10⁻⁷ M</td>
<td>24.8±3.4</td>
<td>0.053±0.007</td>
</tr>
<tr>
<td>10⁻⁶ M</td>
<td>29.0±6.4</td>
<td>0.055±0.005</td>
</tr>
<tr>
<td>10⁻⁵ M</td>
<td>13.4±1.4**</td>
<td>0.103±0.014*</td>
</tr>
<tr>
<td>10⁻⁴ M</td>
<td>11.6±1.2***</td>
<td>0.104±0.011**</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.E. of 5 experiments (Each experiment at value is the average determined from 10 slices). Significant at *P<0.05, **P<0.01 and ***P<0.001 as compared to the control.

Fig. 4. Number of goblet cells stained by AB (pH 1.0)/PAS per 500 μm length of the tracheal epithelium. This curve represents the number of goblet cells containing sulphated glycoprotein (stain index of B+P). Other explanations are as in Fig. 3.
Fig. 5. Effects of pilocarpine on the stain index of tracheal submucosal glandular cells. (A): Each column represents the percent of the cells with a stain index of B ( ), P ( ) or R ( ) in the AB (pH 2.5)/PAS procedure. The cells with stain index of B and P contain acid glycoprotein and those with stain index of R contain neutral glycoprotein. The number of glandular cells determined for type of staining in this experiment are 97 (control), 101 (10^-7 M pilocarpine), 94 (10^-6 M), 108 (10^-5 M) and 102 (10^-4 M). (B): Each column represents the percent of the cells with stain index of B ( ) and P ( ) in the AB (pH 1.0)/APS procedure. The cells with stain index of B and P contain sulphated glycoprotein. The number of glandular cells determined for type of staining are 115 (control), 124 (10^-7 M pilocarpine), 104 (10^-6 M), 111 (10^-5 M) and 103 (10^-4 M).

from 52.0% (control) to 76.1% (10^-7 M) and 80.2% (10^-6 M), whereas it decreased to 15.0% (10^-5 M) and 7.2% (10^-4 M). Stain index of B for AB (pH 1.0)/PAS also showed similar changes.

Pilocarpine-induced changes in macromolecular components in incubation fluid: N-Acetylhexosamine concentration in the incubation fluid was 1.92±0.32 mg/40 ml (N=5) in the control group. After treatment with pilocarpine, a slight increase to 2.24±0.39 mg/40 ml at 10^-7 M was observed, and significant increases were observed at higher pilocarpine as follows: 3.52±0.60 at 10^-6 M, 4.16±0.39 at 10^-5 M and 5.12±1.18 mg/40 ml at 10^-4 M (Fig. 6).

Total saccharide concentration in the incubation fluid significantly decreased after pilocarpine was added at 10^-7 and 10^-6 M, while it was not apparently altered at 10^-5 and 10^-4 M. Total protein concentration tended to decrease in 10^-7 and 10^-6 M pilocarpine, and to increase in at 10^-5 and 10^-4 M.

DISCUSSION

Mucus glycoproteins in airway secretions are synthesized in and secreted from goblet and submucosal glandular cells (1). The AGP content of mucus glycoproteins is a principal factor in determining the viscosity of the secretions (2-4).

Only few histochemical and histological studies have been reported on the secretagogue effect of pilocarpine in airway tissues. In 1975, Horstmann et al. (8) found that an intraperitoneal injection of pilocarpine to rats increased transparent granules and vacuoles at the proximal part of tracheal goblet cells. Sturgess and Reid (9) reported that total goblet cell number and PAS- or AB-
stained cell number increased after repeated and large doses of pilocarpine in rats. Detailed effects of pilocarpine on the behavior of glycoproteins in airway secretory cells, however, have not been documented yet.

In the present study, the number of total glycoprotein- and NGP-containing goblet cells was decreased by application of pilocarpine into the incubation medium. On the other hand, the number of AGP- and SGP-containing goblet cells was not altered at concentrations of up to $10^{-5}$ M pilocarpine. These results suggest that the synthesis of AGP within the cells may keep up with its discharge or that discharge of AGP, unlike NGP, may not occur at lower concentrations of the agent. It was found histochemically using AB (pH 2.5)/PAS that pilocarpine at low concentrations increased the AGP content in submucosal glandular cells, whereas at high concentrations the content markedly decreased. These findings suggest that low concentrations of pilocarpine may stimulate synthesis of AGP in submucosal glandular cells much more strongly than it stimulates discharge of AGP from these cells, while at high concentrations discharge of AGP may overcome its synthesis. That is, the threshold concentration of pilocarpine needed to stimulate synthesis of AGP should be lower than that required to stimulate discharge of AGP. The experiment using AB (pH 1.0)/PAS suggests that the AGP synthesized in glandular cells due to a stimulant action of pilocarpine, may be mostly SGP, and not siaIylated glycoprotein.

The conclusion on the relationship between the effects of pilocarpine on synthesis and discharge of glycoproteins is supported by the finding that a decrease in thickness and an increase in A'WR of submucosal glands were produced only at high concentrations of the agent. Incorporation of saccharide in Hanks medium into the incubated trachea was markedly enhanced during treatment with low concentrations of pilocarpine. This also indicates a vigorous synthesis of glycoproteins in the secretory cells. Chakrin et al. (20) showed that incorporation of $^3$H-glucosamine into a tracheal segment parallels the SGP content in the secretory tissue.

GWR, an index which was devised by Reid (10), increased in a concentration-dependent manner with pilocarpine treatment, suggesting that pilocarpine induces hypertrophy of the glands.

The present results indicate that pilocarpine produces different effects on the behavior of mucus glycoproteins in the tracheal secretory cells and that the type of effect depends on its concentration.

REFERENCES

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prenaline and pilocarpine on (a) bronchial mucus-secreting tissue and (b) pancreas, salivary glands, heart, thymus, liver and spleen. Brit. J. exp. Path. 54, 388–403 (1973)


