A ROLE OF LYSOsomAL ENZYMES IN THE MECHANISM OF MUCOLYTIC ACTION OF Bromhexine

Hiroshi Takeda, Miwa Misawa and Saizo Yanaura

Department of Pharmacology, School of Pharmacy, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142, Japan

Accepted December 9, 1982

Abstract—The effect of bromhexine on behaviors of lysosomal enzymes in the submucosal gland was investigated using canine tracheal slice preparations, with reference to the histochemical changes in acid glycoproteins (AGP) in the gland. Incubation of tracheal slices with 1% Triton X-100 or 0.0004–0.04% bromhexine for 30 min decreased the number of stained lysosomes in the glandular cells. The decrease in stained lysosomes after treatment with 1% Triton X-100 or 0.04% bromhexine was effectively prevented by addition of 5% lecithin. The number of glandular cells that were stained red (stain index R) in the combined alcian blue at pH 2.5 and periodic acid-Schiff procedure markedly increased by treatment with 1% Triton X-100 or 0.0004–0.04% bromhexine. In the bromhexine treated groups, there was a close correlation (r=0.932, P<0.01) between the increase in the number of glandular cells showing the stain index R and the decrease in the number of stained lysosomes in the cells. These findings suggest that the enzymes which are liberated from lysosomes into the cytoplasm by bromhexine may, at least in a part, be involved in the mucolytic action of the agent on AGP contained in mucus granules of the submucosal gland.

Bromhexine, N-(2-amino-3,5-dibromo benzyl)-N-cyclohexyl-methylamine, is a derivative of vasicine, an alkaloid extracted from Adhatoda vasica, an Indian plant which has been used in the treatment of cough and asthma. Bromhexine is widely used as an expectorant possessing a mucolytic property for treatment of various pulmonary diseases. In 1963, Engelhorn and Puschmann found that bromhexine had a mucolytic effect (1). Afterwards, the action was confirmed in many animal experiments and clinical trials (2–6). However, the mechanism of the mucolytic action of this drug is poorly understood (7–9). Gieseking and Caldamus (7) found that lysosome-like structures in the serous cells of human bronchial glands were increased after bromhexine treatment. It was recently shown that bromhexine altered the histochemical properties of acid glycoproteins (AGP) in canine tracheal secretory cells (8, 9).

To determine the detailed mucolytic action of bromhexine, we investigated histochemically the effect of bromhexine on the behaviors of lysosomal enzymes in canine tracheal submucosal gland, with reference to the changes in histochemical properties of AGP in the gland.

Materials and Methods

Animals

Adult male mongrel dogs weighing between 8 and 12 kg were anesthetized with sodium pentobarbital (30 mg/kg i.v.). The trachea, about 4 cm in length from the larynx, was excised after a mid-incision and cut into small pieces (about 2 mm x 2 mm) in Hanks' solution. The medium was oxygenated with a 95% O2–5% CO2 mixture and maintained...
at a temperature of 37±1°C.

**Tissue preparation and drug treatment**

The tracheal tissues were fixed with 4% cold-formaldehyde, pH 7.2, for 2 hr, and then they were washed in 0.1 M cacodylate buffer, pH 7.2, with 8% sucrose at a temperature below 4°C for 15 hr. The fixed tissues were sectioned at 10 μm thickness with a cryostat microtome at -18--20°C, and the frozen sections were mounted on a slide glass with glycerine jelly.

The frozen sections (10 μm thickness) on the slide glass were treated with 1% Triton X-100, 5% lecithin, 0.0004–0.04% bromhexine, a combination of 5% lecithin and 1% Triton X-100, or a combination of 5% lecithin and 0.04% bromhexine for 30 min at room temperature.

The drugs used were obtained from the following sources: Triton X-100 (Sigma Chem. Co.), lecithin (from eggs) (Wako Pure Chem. Co.), and bromhexine hydrochloride (Nippon Boehringer Ingelheim Co.). Drugs were dissolved in 0.05M acetate buffer, pH 5.0, with 8% sucrose.

**Staining**

After drug treatment, the tissue slices were rinsed with distilled water and stained using the following procedure:

1) lysosomal enzymes: Acid phosphatase was used as a marker enzyme for lysosomal enzymes. The localization of lysosomal enzymes in glandular cells was determined using a modified Gomori lead-salt method with cytidine-5'-monophosphoric acid as the substrate (10). Lysosome granules are stained brown with this method.

2) mucus glycoproteins: Mucus glycoproteins in glandular cells were stained with a combination of alcian blue (AB) at pH 2.5 and periodic acid-Schiff (PAS) technique (designated below as AB (pH 2.5)/PAS) (11–13).

**Evaluation**

To assess the drug effects, 7–9 tracheal preparations were used for each drug. Fifty slices from a trachea were treated with a drug. Ten out of the 50 slices, chosen at random, were stained and photographed at 150-fold magnification.

Both the stained lysosome number in glandular cells and the area of the cells were measured from photomicrographs of the slices, and then the stained lysosome number per unit area was calculated. Histochemical properties of mucus glycoproteins in the glandular cells were analyzed according to the method described previously (8, 9, 14–16).

**Results**

**Changes in stained lysosome number in submucosal glandular cells:** Typical photomicrographs of submucosal glands in the tracheal sections treated with 1% Triton X-100, 5% lecithin, 0.04% bromhexine, and a combination of 5% lecithin and 0.04% bromhexine are shown in Fig. 1.

The number of stained lysosomes in glandular cells was 3.97±0.41×10^3/mm^2 (mean±S.E.) in the control group. A 1% solution of Triton X-100 significantly decreased the number, while 5% lecithin had no effect. After application of bromhexine in concentrations of 0.0004, 0.004 and 0.04%, the number decreased to 2.83±0.42, 2.63±0.52 and 2.10±0.54, respectively (Fig. 2).

Changes in the number of stained lysosomes after treatment with a combination of 5% lecithin and 1% Triton X-100 or a combination of 5% lecithin and 0.04% bromhexine are shown in Table 1. The decrease in stained lysosomes after treatment with 1% Triton X-100 or 0.04% bromhexine was inhibited by the addition of 5% lecithin.

**Histochemical changes in mucus glycoproteins in submucosal glandular cells:** The changes in stain index of submucosal glandular cells with the AB (pH 2.5)/PAS procedure following applications of Triton
X-100, lecithin and bromhexine are shown in Fig. 3. The number of glandular cells, which stained blue and purple (stain index B & P), was markedly reduced by 1% Triton X-100 or 0.0004–0.04% bromhexine treatment. On the other hand, the number of glandular cells, which stained red (stain index R), markedly increased from 13.5% (control group) to 47.2, 50.8 and 52.3% following application of bromhexine at concentrations.

Fig. 1. Photomicrographs of lysosomes in canine tracheal submucosal glands stained with Novikoff's method. A: control group, B: group treated with 1% Triton X-100, C: group treated with 5% lecithin, D: group treated with 0.04% bromhexine, and E: group treated with 5% lecithin + 0.04% bromhexine. ×300.
Fig. 2. Effects of Triton X-100, lecithin and bromhexine on the number of stained lysosomes in tracheal submucosal glands. Each value represents the mean±S.E. of 7–9 experiments (Each experimental datum is the mean of results determined from 10 slices). Significant at *P<0.05 and ***P<0.001 as compared to the control value.

Table 1. Effects of lecithin on the changes in stained lysosome number induced by Triton X-100 or bromhexine in tracheal submucosal glands

| Drug                      | Number of stained lysosomes in submucosal gland (x10³/mm²) |
|---------------------------|------------------------------------------------------------|
| Control                   | (N=7)                                                      |
| 1% Triton X-100           | *** (N=9)                                                  |
| 5% Lecithin               | (N=8)                                                      |
| Bromhexine                |                                                            |
| 0.0004%                  | (N=8)                                                      |
| 0.004%                   | (N=8)                                                      |
| 0.04%                    | * (N=8)                                                    |

Each value represents the mean±S.E. of 7–9 experiments (Each experimental datum is the mean of results determined from 10 slices). Significant at ***P<0.001 as compared to the value of the 1% Triton X-100-treated group.

Fig. 3. Effects of Triton X-100, lecithin, and bromhexine on the stain indexes of glandular cells in the tracheal submucosa. Each column represents the percent of cells which stained blue (stain index B: ■ ■ ■ ■), purple (stain index P: ■ ■ ■ ■ ■), or red (stain index R: ■ ■ ■) in the combination procedure of alcian blue at pH 2.5 and periodic acid-Schiff (AB (pH 2.5)/PAS). The numbers of glandular cells evaluated were 123 (control), 141 (1% Triton X-100), 110 (5% lecithin), 152 (0.0004% bromhexine), 107 (0.004% bromhexine), and 155 (0.04% bromhexine).
of 0.0004, 0.004 and 0.04%, respectively. With a combination of 5% lecithin, the enhanced proportion of the cell number showing stain index R after 1% Triton X-100 and 0.04% bromhexine was decreased (Fig. 4).

Relation between histochemical changes in mucus glycoproteins and stained lysosome number: When the number of stained lysosomes is plotted against the percent of the cell number showing stain index R, a linear correlation (r=0.932, P<0.01) is observed in the bromhexine-treated groups (Fig. 5).

Discussion

In 1965, Bürgi (2) observed that when sputum was incubated with bromhexine, fibrils in the sputum disintegrated to lower the viscosity of the mucus. He insisted that bromhexine had a direct mucolytic effect on mucus discharged from airway secretory cells into the lumen. On the other hand, Merker (17) reported in an electron microscopic study that bromhexine caused qualitative changes in mucus granules within the bronchial goblet cells of rats. Gieseking and Baldamus (7) found that consecutive administrations of bromhexine for several days markedly increased the number of lysosome-like granules in glandular serous cells of patients undergoing pneumonectomy. They suggested that the mucolytic action of bromhexine may be based on an extracellular action: bromhexine would release the lysosome-like granules out of serous cells, and the enzymes that were contained in the granules act on mucous substances to lower the viscosity. We found that bromhexine altered the histochemical properties of AGP, viscous factors (18-20) inside tracheal goblet and submucosal glandular cells, indicating that the mucolytic action of this agent would occur inside secretory cells (8, 9).

In the present study, Triton X-100 decreased the number of stained lysosomes in submucosal glands, and, moreover, lecithin prevented the decrease induced by Triton X-100. These results suggest that Triton X-100, a strong membrane labilizer (21, 22),
discharges enzymes from lysosomes in the glands, which is prevented by lecithin, a membrane stabilizer (23).

Application of bromhexine in a concentration range of 0.0004% to 0.04% decreased the number of stained lysosomes in submucosal glands, which was also prevented by an addition of lecithin. These findings suggest that bromhexine induces liberation of enzymes from lysosomes into the cytoplasm through labilizing the lysosome membrane.

After bromhexine or Triton X-100 treatment, the number of glandular cells showing stain index B & P was markedly decreased, while the number of cells showing stain index R was increased. These histochemical findings suggest that bromhexine and Triton X-100 may dissolve the high molecular weight AGP in the secretion granules of submucosal glands through a mucolytic action because it is known that AB colors blue only when one dye molecule combines with several polyanion sites of one AGP molecule (24, 25). In the present experiment, lecithin also prevented the histochemical change in glandular cells induced by bromhexine or Triton X-100 treatment, probably through the membrane-stabilizing action of lecithin.

There was observed a close correlation between the increase in glandular cells showing stain index R and the decrease in the number of stained lysosomes in the cells induced by bromhexine. This finding supports the idea that lysosomal enzymes liberated by bromhexine may be involved in the histochemical changes inside the cells.

It is concluded that the mucolytic action of bromhexine may, at least in part, be ascribed to the action of this agent to liberate lysosomal enzymes into the cytoplasm inside the secretory cells, which then dissolve AGP molecules of the mucus enzymatically. In the present study, we observed the lysosomal enzyme liberating action of bromhexine. The effect of this agent on the synthesis of these enzymes, however, still remains to be investigated.

References

1) Engelhorn, R. and Puschmann, S.: Pharmakologische Untersuchungen über eine Substanz mit sekretolytischer Wirkung. Arzneimittelforsch. 13, 474–490 (1963)
2) Bürgi, H.: In-vitro-Untersuchungen mit dem Sekretolytikum Bisolvon. Praxis 54, 1327–1330 (1965)
3) Bruce, R.A. and Kumar, V.: The effect of a derivative of vasicine on bronchial mucus. Br. J. Clin. Pract. 22, 289–292 (1968)
4) Hamilton, W.F.D., Palmer, K.N.V. and Gent, M.: Expectorant action of bromhexine in chronic obstructive bronchitis. Br. Med. J. 3, 260–261 (1970)
5) Cobbin, D.M., Elliott, F.M. and Reubuck, A.S.: The mucolytic agent bromhexine (Bisolvon) in chronic lung disease. Aust. N.Z. J. Med. 1, 137–140 (1971)
6) Aylward, M.: A between patient double-blind comparison of s-carboxymethyl cysteine and bromhexine in chronic obstructive bronchitis. Curr. Med. Res. Opin. 1, 219–227 (1973)
7) Gieseking, R. and Baldamus, U.: Electron-microscopic findings in human bronchial mucosa after treatment with Bisolvon. Batr. Klin. Tuberk. 137, 1–18 (1968)
8) Yanaura, S., Takeda, H., Nishimura, T. and Misawa, M.: Histological and histochemical changes of tracheal secretory cells following bromhexine treatment. Folia Pharmacol. Japon. 77, 559–568 (1981) (Abs. in English)
9) Yanaura, S., Takeda, H., Nishimura, T. and Misawa, M.: Effect of bromhexine on the tracheal secretory cells with enhanced mucus synthesis by pilocarpine treatment. Folia Pharmacol. Japon. 78, 17–25 (1981) (Abs. in English)
10) Novikoff, A.B.: Lysosomes in the physiology and pathology of cells: contributions of staining methods. In Ciba Foundation Symposium on Lysosomes, Edited by Reuck, A.V.C. and Cameron, M.P., p. 36–77, Little Brown, Boston (1963)
11) Lamb, D. and Reid, L.: Histochemical types of acidic glycoprotein produced by mucous cells of the tracheobronchial glands in man. J. Pathol. 98, 213–229 (1969)
12) Jones, R. and Reid, L.: The effect of pH on alcian blue staining of epithelia acid glycoprotein.
II. Human bronchial submucosal gland.

Histochem. J. 5, 19–27 (1973)

13) Jones, R., Baskerville, A. and Reid, L.: Histochemical identification of glycoproteins in pig bronchial epithelium: (a) normal and (b) hypertrophied from enzootic pneumonia. J. Pathol. 116, 1–11 (1975)

14) Yanaura, S., Takeda, H. and Misawa, M.: Secretagogue action of glyceryl guaiacolate in tracheal submucosal glands. Folia Pharmacol. Japon. 79, 57–64 (1982) (Abs. in English)

15) Yanaura, S., Takeda, H., Nishimura, T. and Misawa, M.: Effect of pilocarpine on behavior of mucus glycoproteins of canine tracheal secretory cells. Japan. J. Pharmacol. 32, 29–35 (1982)

16) Yanaura, S., Takeda, H. and Misawa, M.: Behavior of mucus glycoproteins of tracheal secretory cells following L-cysteine methyl ester treatment. J. Pharmacobiodyn. 5, 603–610 (1982)

17) Merker, H.J.: Elektronenmikroskopische Untersuchungen über die Wirkung von N-Cyclohexyl-N-methyl-(2-amino-3,5-dibrombenzyl)-ammonium-chlorid auf das Bronchialepithel der Ratte. Arzneimittelorsch. 16, 509–516 (1966)

18) Sturgess, J., Palfrey, A.J. and Reid, L.: Rheological properties of sputum. Rheol. Acta 10, 36–43 (1971)

19) Charman, J., Lopez-Vidriero, M.T., Keal, E. and Reid, L.: The physical and chemical properties of bronchial secretions. Br. J. Dis. Chest 68, 215–227 (1974)

20) Iravani, J. and Melville, G.N.: Mucoctilai function in the respiratory tract as influenced by physicochemical factors. Pharmacol. Ther. 2, 471–498 (1978)

21) Novikoff, A.B.: Lysosomes and related particles. In The Cell, Biochemistry. Physiology, Morphology. Edited by Bracet, J. and Mirsky, A.E., Vol. II, p. 423–488, Academic Press, New York (1961)

22) Koenig, H.: Lysosomes in the nervous system. In Lysosomes in Biology and Pathology, Edited by Dingle, J.T. and Fell, H.B., Vol. II, p. 111–162, North-Holland, Amsterdam (1975)

23) Coldman, M.F., Gent, M. and Good, W.: Relationships between osmotic fragility and other species-specific variables of mammalian erythrocytes. Comp. Biochem. Physiol. 34, 759–772 (1970)

24) Scott, J.E., Quintarelli, G. and Dellovo, M.C.: The chemical and histochemical properties of alcian blue. I. The mechanism of alcian blue staining. Histochemie 4, 73–85 (1964)

25) Quintarelli, G., Scott, J.E. and Dellovo, M.C.: The chemical and histochemical properties of alcian blue. II. Dye binding of tissue polyanions. Histochemie 4, 86–98 (1964)