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Action of *N*-acylated ambroxol derivatives on secretion of chloride ions in human airway epithelia

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ABSTRACT

We report the effects of new *N*-acylated ambroxol derivatives (TEI-588a, TEI-588b, TEI-589a, TEI-589b, TEI-602a and TEI-602b: a, aromatic amine-acylated derivative; b, aliphatic amine-acylated derivative) induced from ambroxol (a mucolytic agent to treat human lung diseases) on Cl^- secretion in human submucosal serous Calu-3 cells under a $Na^+/K^+/2Cl^-$ cotransporter-1 (NKCC1)-mediated hyper-secreting condition. TEI-589a, TEI-589b and TEI-602a diminished hyper-secretion of Cl^- by diminishing the activity of NKCC1 without blockade of apical Cl^- channel (TEI-589a > TEI-602a > TEI-589b), while any other tested compounds including ambroxol had no effects on Cl^- secretion. These indicate that the inhibitory action of an aromatic amine-acylated derivative on Cl^- secretion is stronger that that of an aliphatic amine-acylated derivative, and that 3-(2,5-dimethyl)furoyl group has a strong action in inhibition of Cl^- secretion than cyclopropanoyl group. We here indicate that TEI-589a, TEI-589b and TEI-602a reduce hyper-secretion to an appropriate level in the airway, providing a possibility that the compound can be an effective drug in airway obstructive diseases including COPD by reducing the airway resistance under a hyper-secreting condition.

Introduction

The surface fluid covering the airway plays an important role in protection of the lung from bacterial/viral infection by forming low viscosity leading to clearance of mucus [1]. Airway epithelia provide the fluid containing salts, water and immune proteins by secreting Cl^- mediated via $Na^+/K^+/2Cl^-$ cotransporter (NKCC) [2]; especially, submucosal glands respond to local mediators or stimulation of various pathways, resulting in production of the fluid. The serous cells of the glands in the human lung express a lot of amounts of the cystic fibrosis transmembrane regulator (CFTR) channel [3] secreting Cl^- when the cells are stimulated by adrenergic and/or cholinergic stimulations which increase intracellular cyclic AMP (cAMP) and/or Ca^{2+} concentrations [4,5].

Ambroxol, a synthetic mucolytic drug, is known as a drug with mucokinetic and stimulatory action on patients with hyper-secretion, is reported to stimulate surfactant synthesis in the lung, and is effective in patients with mucus hyper-secretion including chronic bronchitis, emphysema and obstructive airways diseases such as chronic obstructive pulmonary disease (COPD) [6,7]. However, the mechanism of the ambroxol action on cell function is unknown.

In this study, we examined the action of ambroxol-derivatives on hyper-secretion of chloride ions driven by $Na^+/K^+/2Cl^-$ cotransporter, which is the most major ion transporter participating in Cl^- secretion. We here report that one of ambroxol-derivatives showed an inhibitory action on Cl^- secretion under a hyper-secreting condition to keep a moderate level of Cl^- secretion.

Materials and methods

Solutions. The solution used in this study contained (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 5 glucose gassed with 21% O_2/79% N_2 (pH 7.4).

Chemicals. We produced six kinds of derivatives of ambroxol as shown in Fig. 1: TEI-588a, TEI-588b, TEI-589a, TEI-589b, TEI-602a and TEI-602b; a, aromatic amine-acylated derivative; b, aliphatic amine-acylated derivative. Ambroxol hydrochloride (2-amino-3,5-dibromo-*N*-(trans-4-hydroxycyclohexyl)benzylamine-hydrochloride) was obtained from Sigma–Aldrich (St. Louis, MO, U.S.A.).

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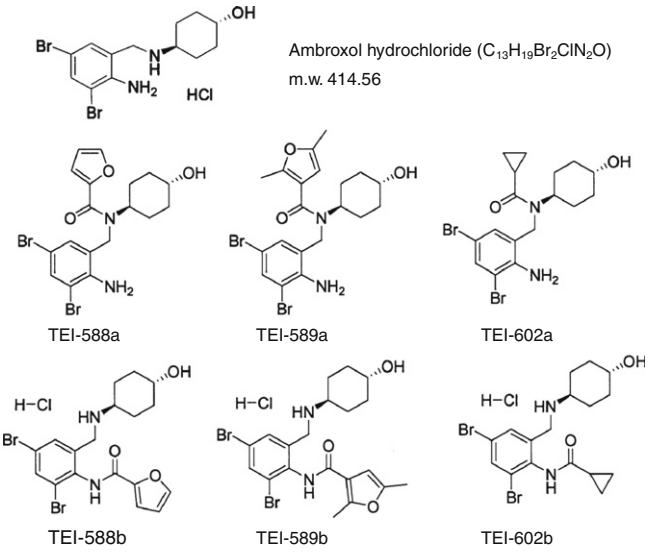

Fig. 1. Structures of ambroxol and its *N*-acylated ambroxol derivatives. We tested 6 derivatives and ambroxol.

Terbutaline, benzamil and 5-nitro 2-(3-phenylpropylamino)-benzoate (NPPB) were also obtained from Sigma–Aldrich; bovine serum from GIBCO (Grand Island, NY, U.S.A.); Dulbecco's Modified Eagle's Medium and Ham's F-12 from Invitrogen (Carlsbad, CA, U.S.A.). All other chemicals not listed above were obtained from Sigma–Aldrich.

Solvents of chemicals. Ambroxol, its derivatives, benzamil and NPPB were dissolved in dimethyl sulfoxide (DMSO), the final concentration of which was 0.1%. DMSO of 0.1% had no significant effects on the short-circuit current (Isc) or the transepithelial conductance (Gt). Terbutaline was dissolved in water.

Cell culture. In this study, we used Calu-3 cells (American Type Culture Collection, Manassas, VA, U.S.A.) that are functionally and morphologically analogous to human airway serous cells, and cultured on polyester porous membranes using the same method as previously described [8,9]. Experiments measuring Isc and Gt were performed after culture for 12–15 days.

Measurements of Isc and Gt. We rinsed the Calu-3 cell monolayers grown on polyester porous membranes with the same solution as the experimental solution, and transferred Calu-3 cells to a modified Ussing chamber (Jim's Instrument, Iowa City, IA, U.S.A.) designed for holding the cup with polyester porous membrane [10–12]. The bathing solution was stirred with 21% O_2/79% N_2 or 5% CO_2/95% O_2. We measured Isc and Gt with an amplifier VCC-600 (Physiologic Instrument, San Diego, CA) as previously reported [13–16]. We used the NPPB-sensitive Gt as an indicator of the CFTR channel conductance (macroscopic channel activity). A positive current represents a net flow of anion from the basolateral to the apical solutions; i.e., the Cl^- secretion is represented as a positive current (Isc) in this study. The NPPB-sensitive Isc was used as an indicator of Cl^- secretion.

Application of terbutaline, ambroxol-derivatives, benzamil, and NPPB. We added benzamil of 10 μM to the apical solution to abolish the ENaC-mediated Na^+ transport (absorption) 10 min before application of terbutaline (10 μM, a β_2-agonist) to the basolateral solution in the Ussing chamber [17]. Therefore, the remaining Isc after benzamil application did not contain Na^+ absorption, but was composed of Cl^- secretion. Sixty minutes after application of terbutaline, we applied ambroxol-derivatives, the structures of which are shown in Fig. 1, to the apical and basolateral solutions in the Ussing chamber. To measure Cl^- secretion and conductance, we applied NPPB 60 min after addition of ambroxol-derivatives.

Data presentation and statistics. Results are expressed as mean ± standard error (SE). The difference between groups was evaluated by a two-way ANOVA. If ANOVA indicated a significant difference, the Dunnett's multiple comparison test was used to identify statistically significant difference vs. control group. When the SE bar is not shown, the amplitude of SE is less than the symbol size.

Results and discussion

Calu-3 cells highly express functional β_2-adrenergic receptor/adenylate cyclase system [4,18,19]. Terbutaline is well recognized as a specific β_2-adrenergic agonist that activates adenylate cyclase, leading to an increase of the cytosolic cAMP content (the second

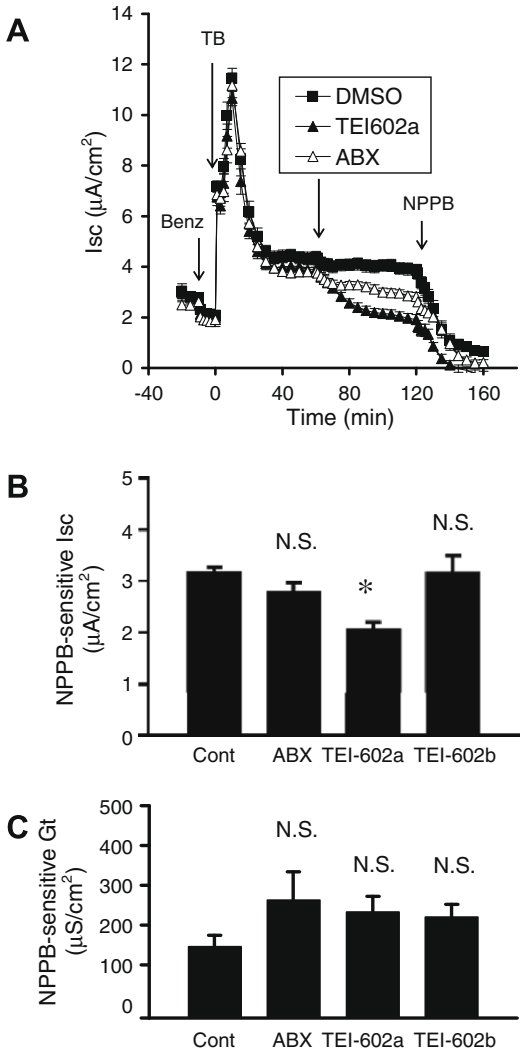

Fig. 2. (A) Time courses of Isc. Benzamil (Benz; 10 μM) added to the apical solution 10 min before application of terbutaline (TB, a specific β_2-adrenergic agonist). Benzamil diminished the Isc. Benzamil at the concentration of 10 μM abolishes the ENaC-mediated Na^+ transport. Terbutaline applied to the basolateral solution induced a biphasic increase; a transient one followed by a steady one. TEI-602a (100 μM), ambroxol (100 μM) or DMSO (solvent control for TEI-602a and ambroxol) was applied bilaterally 60 min after addition of terbutaline. NPPB of 200 μM applied to the apical solution. (B) The NPPB-sensitive Isc. Ambroxol of 100 μM, TEI-602a of 100 μM and TEI-602b of 100 μM were applied. TEI-602a significantly diminished the NPPB-sensitive Isc, while ambroxol or TEI-602b had no significant effects on the NPPB-sensitive Isc. (C) The NPPB-sensitive Gt. Ambroxol of 100 μM, TEI-602a of 100 μM and TEI-602b of 100 μM were applied. Ambroxol, TEI-602a or TEI-602b had no significant effects on the NPPB-sensitive Gt. $*p < 0.001$, $n = 10$ for DMSO, $n = 5$ for ambroxol, $n = 9$ for TEI-602a, and $n = 5$ for TEI-602b.