Stimulation of Na Absorption by the Antiasthmatic Kampo Drug Saiboku-To in Cultured Airway Epithelium

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ABSTRACT—To study the effect of the Kampo drug Saiboku-to (TJ-96) on ion transport function of airway epithelial cells, we studied bioelectric properties of cultured tracheal epithelium from dogs under short-circuit conditions in vitro. Addition of TJ-96 (1 mg/ml) to the mucosal solution of the Ussing chamber increased the epithelial short-circuit current (SCC) from 6.5 ± 0.7 to 11.4 ± 1.6 μA/cm² (P < 0.001). This effect was dose-dependent, with the maximal increase from the baseline value and the concentration required to produce a half-maximal effect (EC₅₀) being 70.5 ± 12.6% (P < 0.001) and 3 μg/ml, respectively; and there were corresponding increases in transepithelial potential difference and cell conductance. Submucosal addition of TJ-96 likewise increased SCC, although the magnitude of the response was smaller as compared with the response to the mucosal addition. The TJ-96-induced increase in SCC was not affected by diphenylamine-2-carboxylate or furosemide but abolished by amiloride. Intracellular cyclic AMP levels were dose-dependently increased by TJ-96. These results indicate that TJ-96 may selectively stimulate Na absorption across the tracheal epithelium, probably through intracellular accumulation of cyclic AMP.

Saiboku-to (TJ-96) is a traditional Chinese herbal medicine that has been widely used in the treatment of bronchitis and asthma. There is increasing evidence that TJ-96 modifies allergic events through inhibition of type I (1) and IV reactions (2), reduction of the IgE-mediated release of histamine from basophils and platelet-activating factor from neutrophils (3), and prevention of the down-regulation of glucocorticoid and β-adrenergic receptors (4), thereby reducing bronchoconstriction and airway hyperreactivity.

In addition to hyperreactivity of airway smooth muscle, hypersecretion of respiratory mucus and water is another characteristic feature of asthma. Although it has been frequently recognized that treatment of asthmatic patients with TJ-96 can decrease the amount of sputum production, the mechanism of this anti-secretory action remains unknown. Because airway surface fluid consists of mucous glycoproteins released from submucosal glands and water transported across airway epithelial cells, the latter being determined by transepithelial ion transport (5), the present study was undertaken to elucidate the effect of TJ-96 on airway epithelial ion transport functions and the possible mechanism of its action. To do so, we measured bioelectric properties of canine cultured tracheal epithelium, which primarily secretes Cl and absorbs Na (6), under short-circuit conditions in vitro.
MATERIALS AND METHODS

Cell culture

Mongrel dogs of either sex weighing between 23 and 38 kg were killed with an overdose of intravenous pentobarbital sodium (60 mg/kg), and the trachea was rapidly removed (7). After dissecting submucosal tissue and blood vessels, the resected sections were placed in fresh modified Eagles’s medium containing 0.1% protease type XIV (Sigma Chemical Co., St. Louis, MO, USA) and maintained at 4°C for 24 hr. After mild agitation, the tissue sections were removed from the medium and the cells were concentrated by centrifugation (800 × g). The cell pellets were washed twice with modified Eagle’s medium containing 10% fetal calf serum to neutralize the protease. In this preparation of cells, fibroblasts and other nonepithelial cells constituted less than 1% of the total, and the viability was 90–98% as assessed by trypan blue exclusion. These cells were suspended in Hamm’s nutrient F12 medium containing 0.5% fetal calf serum, 5 µg/ml insulin, 5 µg/ml transferrin 10 ng/ml epidermal growth factor, 50 U/ml penicillin, 50 µg/ml streptomycin, and 50 µg/ml gentamicin. The cells were then plated at a density of 1.5 × 10^6/cm² using 1 ml of Hamm’s nutrient F12 medium per Linbro tissue culture multi-well plate (Flow Lab Inc., McLean, VA, USA) and then grown on nucleopore polycarbonate filters (13-mm diam., 0.45-µm pore size) at 37°C in a CO₂ incubator. Medium was changed at 24 hr and every 2 days thereafter. Because the filters are not transparent, assessment of monolayer formation was made daily by observing cell growth on the culture dish surrounding the filter by phase contrast microscopy at a magnification of ×200. On the 10th day of incubation, the cells became confluent and were used for the measurement of electrical properties. Our preliminary studies on transmission electron microscopy showed that these preparations maintained microvilli and a glycocalyx on the apical membrane and tight junctions separating the apical and submucosal membranes. It has been shown by Coleman and associates (8) that these cultured cells possess identical electrical properties as native tissues.

Measurement of electrical properties

The filters on which tracheal epithelial cells were grown were mounted between two silicon-treated Ussing chambers (0.5-cm² surface area) bathed with Krebs-Henseleit solution of the following composition: 143.9 mM Na, 5.6 mM K, 1.9 mM Ca, 1.2 mM Mg, 117.6 mM Cl, 25.0 mM HCO₃, 5.6 mM acetate, 3.8 mM gluconate, 1.3 mM H₃PO₄, 1.2 mM SO₄, and 5.6 mM glucose, heated to 37°C and bubbled with 95% O₂-5% CO₂ (9). The calomel half-cells were paired to within 0.2 mV of each other. The spontaneous potential difference (PD) across the epithelium was measured with two polyethylene bridges containing 3% agar in 1 M KCl, located 1 mm from each side of the epithelial surface and connected to a calomel half-cell and a high-impedance voltmeter. Another pair of polyethylene bridges containing 3% agar in 0.9% NaCl located 10 mm from the tissue was used to pass sufficient current through the chamber and the cells to bring the transepithelial PD to zero (Fig. 1). This short-circuit current (SCC) was recorded continuously except for 3 sec every 10 min when the voltage clamp was turned off and the PD was recorded. Tissue conductance (G) in mS/cm² was calculated by dividing the measured SCC per surface area (µA/cm²) by the PD (mV).

The cells were allowed to equilibrate for 20 min to establish a baseline SCC that did not vary by more than 0.2 µA/cm² in any of the 10-min intervals thereafter, and TJ-96 (1 mg/ml) or its solvent (Eagle’s medium) alone was added to the mucosal solution. In our preliminary experiments, the response of SCC induced by TJ-96 did not show tachyphylaxis. Thus, in the studies with the dose-dependent relationship between TJ-96 and SCC of tracheal epithelium, TJ-96 at concentrations from 10 ng/ml to 1 ng/ml were cumulatively added to either the mucosal or submucosal solution. The concentration of this drug re-
required to produce a half-maximal effect (EC_{50}) was determined by linear regression analysis.

To assess whether the TJ-96-induced changes in SCC were associated with Na absorption and/or Cl secretion by the epithelium, cells were pretreated for 30 min with each of the following drugs: amiloride (10^{-4} M), a Na channel blocker (10); diphenylamine-2-carboxylate (10^{-4} M), a Cl channel blocker (11); furosemide (10^{-4} M), a Cl transport inhibitor (12), and TJ-96 (1 mg/ml) was then added to the mucosal solution. After 15 min, cells were quickly removed from the chambers, placed in ice-cold 10% trichloroacetic acid, and sonicated in a bath-type sonicator. After the extraction of trichloroacetic acid with ether, the residue was dissolved in acetate buffer. Cyclic AMP levels were determined in duplicate by 125I-radioimmunoassay, corrected for ether extraction of 87% recovery, and normalized for protein content of the cells as determined by the Lowry method (14), with bovine serum albumin as a standard.

**Measurement of cyclic AMP**
We measured intracellular cyclic AMP levels, one of the important determinants for airway epithelial ion transport function (5). Cells were preincubated for 30 min with 3-isobutyl-1-methylxanthine (10^{-3} M) to inhibit cyclic AMP phosphodiesterase (13), and TJ-96 (10 ng/ml to 1 mg/ml) or its solvent alone was added to the mucosal solution. After 15 min, cells were quickly removed from the chambers, placed in ice-cold 10% trichloroacetic acid, and sonicated in a bath-type sonicator. After the extraction of trichloroacetic acid with ether, the residue was dissolved in acetate buffer. Cyclic AMP levels were determined in duplicate by 125I-radioimmunoassay, corrected for ether extraction of 87% recovery, and normalized for protein content of the cells as determined by the Lowry method (14), with bovine serum albumin as a standard.

**Drugs**
The following drugs were used: TJ-96 (Tsumura & Co., Tokyo, Japan), amiloride, furosemide (Sigma Chemicals), diphenylamine-2-carboxylate (Nacalai Tesque, Kyoto, Japan). For each day’s experiments, TJ-96 was dissolved in Eagle’s medium (100 mg/ml) and subsequently diluted by Krebs-Henseleit solution.

**Statistics**
All values are expressed as means ± S.E. Statistical analysis was performed by one-way analysis of variance or the Newman-Keuls multiple comparison test, and a P value of less than 0.05 was considered significant.

**RESULTS**

**Electrical properties**
Addition of TJ-96 (1 mg/ml) to the mucosal solution in the Ussing chamber increased SCC of canine tracheal epithelium from 6.5 ± 0.7 to 11.4 ± 1.6 μA/cm² (P < 0.001, n = 12) observed within 5 min, after which SCC remained elevated for up to 20 min, whereas the solvent of this drug alone (Eagle’s medium) had no effect (Fig. 2). There were corresponding increases in PD and G (P < 0.01, P <
0.05, respectively) in response to TJ-96 (Table 1). Submucosal application of TJ-96 at the same concentration likewise produced a rise in each of bioelectric parameters, but the magnitude of the increase was smaller as compared with the response to mucosal TJ-96 (Fig. 2, Table 1).

The stimulatory effect of TJ-96 on SCC of tracheal epithelium was dose-dependent: the maximal increases from the baseline value were 70.5 ± 12.6% (P < 0.001, n = 9) for mucosal addition and 30.3 ± 6.1% (P < 0.001, n = 8) for submucosal addition, and the EC50 values were 3 μg/ml and 20 μg/ml, respectively (Fig. 3).

The increase in tracheal SCC induced by mucosal TJ-96 (1 mg/ml) was not affected by pretreatment of cells with diphenylamine-2-

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**Table 1. Effect of Saiboku-to (TJ-96) on bioelectric properties of cultured canine tracheal epithelium**

|                | SCC (μA/cm²) | PD (mV) | G (mS/cm²) |
|----------------|--------------|---------|------------|
| Mucosal TJ-96  |              |         |            |
| before         | 6.5 ± 0.7    | 1.5 ± 0.2 | 4.3 ± 0.4 |
| after          | 11.4 ± 1.6***| 2.2 ± 0.3** | 5.2 ± 0.4* |
| Submucosal TJ-96|              |         |            |
| before         | 6.2 ± 0.8    | 1.3 ± 0.2 | 4.7 ± 0.3 |
| after          | 8.2 ± 0.6*   | 1.7 ± 0.3*| 4.8 ± 0.5 |

Definitions of abbreviations: SCC, short-circuit current; PD, potential difference; G, conductance. TJ-96 (1 mg/ml) was added to either the mucosal solution or the submucosal solution in the Ussing chamber. Values are means ± S.E. for 12 preparations. *P < 0.05, **P < 0.01, ***P < 0.001, significantly different from the value before drug administration.
carboxylate or furosemide, but it was aboli-
ished by amiloride (P < 0.001, n = 9) (Fig. 4).

**Intracellular cyclic AMP**

Addition of TJ-96 to the mucosal side in-
creased cyclic AMP levels in tracheal epithel-
ium in a dose-dependent fashion, the maximal
response (from 23.4 ± 5.2 to 78.8 ± 16.0
pmole/mg protein, P < 0.01, n = 8) was
observed at 1 mg/ml of TJ-96, whereas its sol-
vent alone was without effect (Fig. 5).

**DISCUSSION**

Our in vitro experiments demonstrate that
TJ-96, an antiasthmatic Kampo drug, stimu-
lates the absorption of Na without affecting
the secretion of Cl by canine tracheal epitheli-
um. This notion is based on the findings that TJ-96 dose-dependently increased
SCC, a bioelectric parameter that reflects net
ion movement across the epithelium (15), and
that this increase was abolished by pretreat-
ment of cells with amiloride that inhibits Na
conductance at the apical membrane (10) but
not by the Cl transport blocker diphenyl-
amine-2-carboxylate (11) or furosemide, an in-
hibitor of Cl transport (12). Simultaneous in-
creases in PD and G are compatible with our
notion because these parameters reflect the
electromotive force produced by ion transport
and the membrane permeability for such elec-
trolytes, respectively (16).

The response of SCC induced by the muco-
sal addition of TJ-96 was greater than that in-
duced by the submucosal addition. The reason
for this difference is uncertain, but one pos-
sible explanation would be that because the
amiloride-sensitive Na channel is localized to
the apical membrane of tracheal epithelium
(5), the concentration of TJ-96 at the site of
drug action could be decreased during its pas-
sage through the cell from the submucosal
membrane to the apical membrane.

It has been known that airway epithelial
cells absorb Na and secrete Cl (6) and that
these processes are correlated to the move-
ment of fluid toward and from the lumen, re-
spectively (17). Although intracellular regula-
tion of Cl secretion has been extensively
studied (18), the mechanism of epithelial Na
absorption is poorly understood. Cullen and
Welsh (19) showed that Na absorption in
canine airway epithelium is stimulated by
endogenous cyclic AMP. In agreement with this study, our finding that TJ-96 but not its solvent increased the accumulation of intracellular cyclic AMP suggests a possible contribution of this cyclic nucleotide to the absorption of Na across the airway epithelium. In addition, TJ-96 is a blended herbal medicine made from ten crude drugs, in which Zizyphi fructus has been reported to possess cyclic AMP-like bioactivities (20). Thus, the observed action of TJ-96 in our study might be attributed at least in part to Zizyphi fructus. To confirm this, further studies on the effect of this crude drug alone would be valuable.

The change of Na absorption from the airway lumen into epithelial cells and the subsequent alteration of transepithelial electrochemical gradient can promote water movement across the airway mucosa, thereby presumably affecting the volume and rheological properties of airway surface fluid and the depth of the periciliary layer that interacts with ciliary beating. Thus, stimulation of Na absorption by TJ-96 probably reduces the amount of water in the airway lumen and influences mucociliary transport function in the respiratory tract.

TJ-96 is considered to be one of the antiasthmatic drugs because of its inhibitory action on the release of chemical mediators including histamine and platelet-activating factor (3) and its stimulatory action on airway β-adrenergic receptors and glucocorticoid receptors (4). Since asthma and bronchitis are associated with airway inflammation accompanied by a significant accumulation of mucus and water in the airway, it seems reasonable to speculate that among a variety of pharmacological actions, the stimulatory effect of TJ-96 on the Na absorption across the airway epithelium could be beneficial in treating patients with airway hypersecretion and that this action might participate in the effectiveness of this drug on airway diseases.

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REFERENCES

1. Koda, A., Nishiyori, T., Nagai, H., Matsuura, N. and Tsuchiya, H.: Anti-allergic actions of crude drugs and blended Chinese traditional medicines: effect on type I and IV allergic reactions. Folia Pharmacol. Japon. 80, 31–41 (1982) (Abs. in English)

2. Nishiyori, T., Nakatomi, I., Matsuura, N., Nagai, H. and Koda, A.: Effect of Chinese blended medicine, Saiboku-to, on type IV allergic reaction. Japan. J. Allergol. 32, 317–323 (1983)

3. Miyamoto, T., Takaishi, T., Morii, H., Kuriyama, M. and Nakamura, T.: The actions of Saiboku-to (TJ-96) on histamine release and the production of platelet-activating factor in human leukocytes. In Medicines of Plant Origin in Modern Therapy: A Symposium Report, p. 12–13, Oxford Clinical Communications, Oxford (1990)

4. Nakajima, S., Doi, Y., Yamasaki, K., Tohda, Y. and Sunaga, S.: Effect of Saiboku-to (TJ-96) on beta receptors and steroid receptors. In Medicines of Plant Origin in Modern Therapy: A Symposium Report, p. 16–17, Oxford Clinical Communications, Oxford (1990)

5. Welsh, M.J.: Electrolyte transport by airway epithelia. Physiol. Rev. 67, 1143–1184 (1987)

6. Boucher, R.C. and Larsen, E.H.: Comparison of ion transport by cultured secretory and absorptive airway epithelia. Am. J. Physiol. 254, C535–C547 (1988)

7. Tamaoki, J., Sakai, N., Isono, K., Kanemura, T. and Takizawa, T.: Effects of platelet-activating factor on bioelectric properties of cultured tracheal and bronchial epithelia. J. Allergy Clin. Immunol. 87, 1042–1049 (1991)

8. Coleman, D.L., Tuet, I.K. and Widdicombe, J.H.: Electrical properties of dog tracheal epithelial cells grown in monolayer culture. Am. J. Physiol. 246, C355–C359 (1984)

9. Tamaoki, J., Ueki, I.F., Widdicombe, J. and Nadel, J.A.: Stimulation of Cl secretion by neurokinin A and neurokinin B in canine tracheal epithelium. Am. Rev. Respir. Dis. 137, 899–902 (1988)

10. Al-Bazzaz, F.J. and Zevin, R.: Ion transport and metabolic effects of amiloride in canine tracheal mucosa. Lung 162, 357–367 (1984)

11. DiStefano, A., Wittmer, M., Schlatter, E., Lang,
H.J., Englert, H. and Greger, R.: Diphenylamine-2-carboxylate, a blocker of the Cl⁻ conductive pathway in Cl⁻-transporting epithelia. Pflugers Arch. 405, S95–S100 (1985)

12 Widdicombe, J.H., Nathanson, I.T. and Highland, E.: Effect of “loop” diuretics on ion transport by dog tracheal epithelium. Am. J. Physiol. 245, C388–C396 (1983)

13 Beavo, J.A., Rogers, N.L., Crofford, O.B., Hardman, J.G., Sutherland, E.W. and Newman, E.V.: Effects of xanthine derivatives on lipolysis and on adenosine 3',5'-monophosphate phosphodiesterase activity. Mol. Pharmacol. 6, 597–603 (1970)

14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275 (1951)

15 Macknight, A.D.C., DiBona, D.R. and Leaf, A.: Sodium transport across toad urinary bladder: a model “Tight” epithelium. Physiol. Rev. 60, 615–715 (1980)

16 Al-Bazzaz, F.J.: Regulation of salt and water transport across airway mucosa. Clin. Chest Med. 7, 259–272 (1986)

17 Welsh, M.J., Widdicombe, J.H. and Nadel, J.A.: Fluid transport across the canine tracheal epithelium. J. Appl. Physiol. 49, 905–909 (1980)

18 Nadel, J.A., Widdicombe, J.H. and Peatfield, A.C.: Regulation of airway secretions, ion transport and water movement. In Handbook of Physiology. The Respiratory System, Edited by Fishman, A.P., Fisher, A.B. and Geiger, S.R., Vol. 1, p. 419–445, American Physiological Society, Bethesda (1985)

19 Cullen, J.J. and Welsh, M.J.: Regulation of sodium absorption by canine tracheal epithelium. J. Clin. Invest. 79, 73–79 (1987)

20 Cyong, J.-C., Hanabusa, K. and Otsuka, Y.: Studies on the cyclic AMP-like substance found in Zizyphi Fructus. (Proc. Symp.) Wakan-Yaku 12, 1–7 (1979)