Effect of *Saiboku-to*, an Antiasthmatic Herbal Medicine, on Nitric Oxide Generation from Cultured Canine Airway Epithelial Cells

Jun Tamaoki, Mitsuko Kondo, Atsushi Chiyotani, Hisashi Takemura and Kimio Konno

First Department of Medicine, Tokyo Women's Medical College, Tokyo 162, Japan

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ABSTRACT—The effect of *Saiboku-to* (TJ-96), an antiasthmatic Kampo medicine, on the generation of nitric oxide (NO) from cultured canine tracheal epithelium was investigated using a highly specific amperometric sensor for this molecule in vitro. Immersion of the NO-selective electrode in the medium containing tracheal epithelial cells detected the baseline current of 16.8-57.0 pA, which corresponded to an NO concentration ([NO]) of 39.7±8.1 nM. Addition of TJ-96 increased [NO] in a concentration-dependent manner, the maximal increase from the baseline level and the concentration of TJ-96 required to produce a half-maximal effect (EC_{50}) being 127.5±20.1 nM ($P<0.001$) and 86±9 μg/ml, respectively. Pretreatment of cells with N^G-nitro-L-arginine methylester (L-NAME) greatly inhibited the TJ-96-induced increase in [NO], whereas N^G-nitro-D-arginine methylester (D-NAME) had no effect, and this inhibition was reversed by L-arginine but not by D-arginine. Cytochemical staining of the epithelial cells showed marked reactivity of NADPH diaphorase activity. These results suggest that NO is spontaneously released by the airway epithelium and that TJ-96 stimulates the epithelial NO generation.

Keywords: Chinese medicine, Airway epithelium, Nitric oxide, Asthma

Nitric oxide (NO) is now recognized as an important endogenous multifunctional bioregulatory molecule generated from the amino acid L-arginine by nitric oxide synthase (NOS) (1). In the respiratory system, the localization of NOS-like immunoreactivity has been found in a variety of cell types such as endothelial cells, alveolar macrophages, autonomic neurons and airway epithelial cells (2). There is increasing evidence that NO may play a role in the regulation of airway and vascular smooth muscle tone, pulmonary neurotransmission, mucociliary transport and host defense (3, 4). Recent studies have shown that NO is present in the exhaled air of various species including humans (5, 6) and that the airway epithelium may be functioning in the protection against development of airway hyperresponsiveness, a characteristic feature of asthma (7), through the synthesis of NO (8).

Saiboku-to (TJ-96) is a traditional Chinese herbal medicine that has been widely used in the treatment of asthma in Asian countries. This drug modifies allergic events by inhibiting IgE-mediated release of histamine from basophils (9) and the release of platelet-activating factor from neutrophils (10) and by preventing down-regulation of glucocorticoid and β-adrenergic receptors (11). Additionally, TJ-96 has been reported to stimulate ciliary motility (12) and Na absorption (13) in cultured airway epithelial cells. However, it is unknown whether this herbal medicine alters airway epithelial NO generation. Therefore, in the present study, we investigated the effect of TJ-96 on the generation of NO from cultured canine tracheal epithelium by a direct measurement of NO using a highly specific amperometric sensor for this molecule in vitro.

MATERIALS AND METHODS

Preparation of epithelium

Mongrel dogs of either sex, weighing 21 to 38 kg (SLC Japan, Hamamatsu), were anesthetized with intravenous pentobarbital sodium (40 mg/kg), and the trachea was rapidly removed. The tracheal mucosa was dissected from the underlying connective tissue, rinsed several times with sterile phosphate-buffered saline (PBS), and enzymatically digested with 0.1% protease type XIV (Sigma Chemical Co., St. Louis, MO, USA) at 4℃ for 24 hr. After mild agitation, the tissue sections were removed from the medium, and the cells were concentrated by centrifugation (800×*g*, 10 min). The cell pellets were washed with medium containing 5% fetal calf serum to neutralize the

protease. These cells were suspended in a mixture of DMEM and Ham's F-12 containing 5% fetal calf serum, 100 U/ml each of penicillin and streptomycin, 100 μg/ml gentamicin, 10 μg/ml insulin, 5 μg/ml transferrin, 25 ng/ml epidermal growth factor and 7.5 μg/ml endothelial cell growth supplement. Our preliminary studies showed that this preparation of cells was composed of 97–99% epithelial cells and 1–3% fibroblasts and other nonepithelial cells, and the viability was between 89% and 96%, as assessed by trypan blue exclusion. We plated the cells at a density of $1.5 \times 10^6/cm^2$ on a coverglass (18 × 24 mm) coated with human placental collagen (5.8 $\mu g/cm^2$, Sigma) in a Petri dish and cultured them at 37℃ in a CO_2 incubator (95% air – 5% CO_2). The medium was changed every 24 hr and on the seventh day of incubation, the tracheal epithelial cells were used for the measurement of NO generation. Our separate studies on transmission electron microscopy showed that this preparation was a monolayer culture in which non-ciliated cells containing microvilli and glycocalyx constituted more than 99% of the total. Moreover, we have previously shown that these cultured cells maintain, at least, bioelectric properties similar to those of native tissues (13).

Measurement of NO generation

The measurement of NO generation using the NO-selective electrode has been described in detail previously (14, 15). Briefly, a coverglass on which the tracheal epithelial cells were grown was mounted in a glass chamber filled with 10 ml Krebs-Henseleit (KH) 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 HCO_3, 5.6 mM acetate, 3.8 mM gluconate, 1.3 mM H_2PO_4, 1.2 mM SO_4 and 5.6 mM glucose, continuously stirred and maintained at 37℃ by a water-jacketed perfusion apparatus (model 5301; Yellow Springs Instruments Co., Ltd., Yellow Springs, OH, USA). The concentration of NO ([NO]) in the medium bathing the cells was determined by an NO meter (NO-501; Inter Medical Co., Ltd., Tokyo). The principle of the NO meter is based on the measurement of pA-order redox current between the working electrode and the counter electrode: the working electrode consisted of a 0.2-mm diam Pt/Ir alloy wire (Pt 90%, Ir 10%) coated with a three-layered membrane consisting of KCl, NO-selective resin, and normal silicon membranes, and the counter electrode was made of carbon fiber. The KCl membrane was electrochemically deposited on the Pt/Ir wire to suppress overvoltage in the discharge of NO. The NO-selective resin was coated by 0.8% nitrocellulose, and the outermost membrane was introduced to avoid a non-specific ionic effect and electrochemical reactions. The working electrode was supplied with 0.4 to 0.8 V for the electrochemical oxidation of NO. This resulting polarographic current was detected with a current-voltage counter circuit in a high input impedance preamplifier placed near the electrode pair. The current flow was proportional to the rate of diffusion of NO through the membrane, which was in turn proportional to [NO] at the outer surface of the membrane. Thus, to assess the generation of NO by the tracheal epithelium, both working and counter electrodes were immersed in the medium, and the current was continuously recorded on a pen recorder (SR-6355; Graphtec, Tokyo).

Calibration of the electrode was performed daily prior to the experiments. Using the nitrosothiol NO donor *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) as a standard (16), the relationship between the magnitude of electrical current recorded and the concentration of SNAP in KH solution was determined (Fig. 1). The current increased linearly as the SNAP concentration increased. The [NO] can be determined from the concentration of SNAP based on the facts that SNAP is decomposed thermally according to first-order kinetics (17) and that the reaction of liberated NO with O_2 occurs with third-order kinetics (18). The signal-to-noise ratio (S/N) was approximately 1 when 5 μM SNAP, which corresponded to 6.5 nM NO (14), was applied. When 0.1 mM SNAP, which corresponded to 130 μM NO, was added, the S/N was greater than 60. To test the selectivity of the electrode, we performed a preliminary study on the effects of NO_2^- and NO_3^-, which are reaction products of NO and O_2, and found that these molecules did not affect the electrode current. Because heat-decomposed SNAP exhibited only

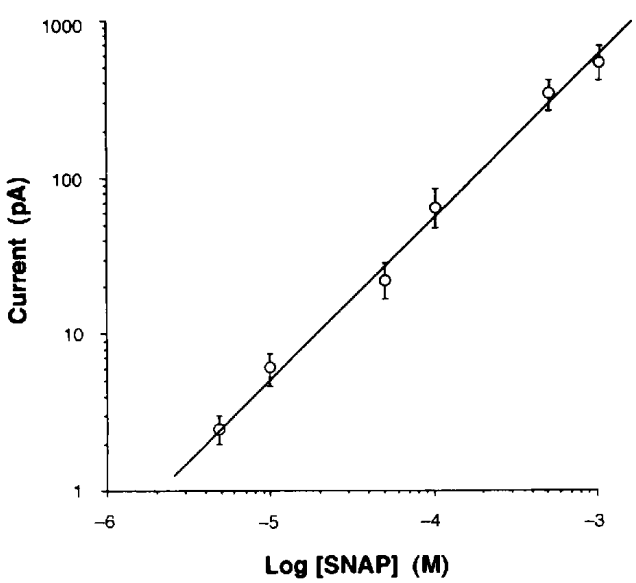

Fig. 1. Relationship between the concentration of *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) in Krebs-Henseleit solution at 37℃ and the electrical current detected by a nitric oxide-selective electrode. Data are means ± S.E.M., n = 18–26 for each point.

2% of the original current and because the NO scavengers oxyhemoglobin (4×10^{-6} M) and 2-phenyl-4,4,5,5-tetramethylimidazole-3-oxide-1-oxyl (PTIO, 3×10^{-5} M) suppressed the SNAP (5×10^{-5} M)-induced current by 94±3% (n=6) and 89±7% (n=5), respectively, there seemed no significant direct effect of SNAP itself on the electrode current.

Effect of TJ-96 on NO release

The electrodes were transferred from KH solution without cells in which the electrical current was zero into the solution bathing the cells in the chamber, and the current was measured. When the baseline current became stable, TJ-96 (100 μg/ml) or its vehicle (PBS) alone was added to the chamber and the response was monitored at least for 10 min. To confirm whether the response of the current was actually due to the released NO, the cells were incubated for 15 min with either N^G-nitro-D-arginine methylester (D-NAME, 10^{-3} M) or N^G-nitro-L-arginine methylester (L-NAME, 10^{-3} M), an inhibitor of NOS (19), and TJ-96 (100 μg/ml) was subsequently added to the chamber. Then, after the response to TJ-96 reached a plateau, D-arginine and L-arginine at 10^{-3} M were consecutively added.

We also examined the concentration-dependent effect of TJ-96 on NO generation. TJ-96 was added to the chamber in the absence and presence of 10^{-3} M D-NAME or L-NAME in a cumulative manner from 1 to 1000 μg/ml, and the highest recorded value of the current in response to each concentration was determined.

Cytochemistry of NADPH diaphorase

Since all the NOSs characterized to date are active in histochemical assay for NADPH diaphorase (20), we examined the existence of NOS in cultured canine tracheal epithelial cells by cytochemical staining of NADPH diaphorase activity. The cells grown on a coverglass were fixed in fresh 2% buffered paraformaldehyde for 10 min and rinsed with PBS. The coverglass was then immersed in a reaction mixture consisting of 0.25 mg/ml nitroblue tetrazoium, 1 mg/ml NADPH and 0.5% Triton X-100 in 0.1 M Tris buffer at pH 7.6. After 30 min, the cells were washed with PBS, and NADPH diaphorase staining was assessed by a microscope at a magnification of ×200. In the control experiment, the substrate was omitted in the reaction medium and *p*-nitrophenylphosphate (1.5×10^{-3} M) was included to inhibit endogenous phosphatases that may convert NADPH to NADH and cause false-positive staining by NADH dehydrogenases (21).

Drugs

The following drugs were used: TJ-96 (extract granules; Tsumura Co., Tokyo), SNAP, PTIO (Inter Medical Co., Ltd.), oxyhemoglobin, L-NAME, D-NAME, L-arginine, D-arginine, nitroblue tetrazoium, NADPH, *p*-nitrophenylphosphate (Sigma). TJ-96 was dissolved in PBS at 1 mg/ml, vortexed for 30 min, and centrifuged (200×*g*, 10 min). Then the supernatant was taken and passed

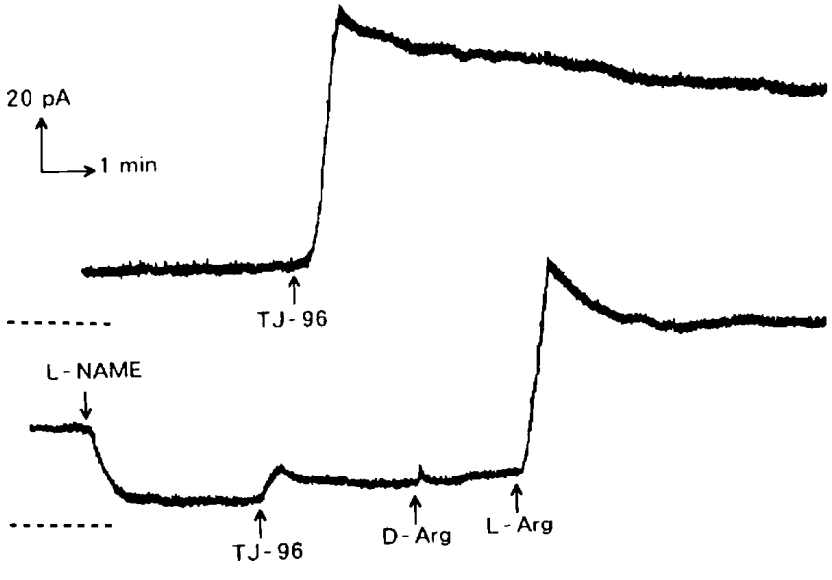

Fig. 2. Representative tracing of electrical current in the Krebs-Henseleit solution bathing the cultured canine tracheal epithelial cells. After equilibration, TJ-96 (100 μg/ml) was added to the chamber in the absence (upper panel) and presence of N^G-nitro-L-arginine methylester (L-NAME, 10^{-3} M, lower panel). When the response reached a plateau, D-arginine (D-Arg, 10^{-3} M) and L-arginine (L-Arg, 10^{-3} M) were consecutively added. (·····: zero current). Typical of 10 and 8 experiments for upper panel and lower panel, respectively.

through a millipore filter (pore size, 0.45 μm); this filtered supernatant was used for the experiments on NO generation.

Statistics

All values are expressed as means±S.E.M. Statistical analysis was performed by ANOVA, and a P value of less than 0.05 was considered statistically significant.

RESULTS

Generation of NO

The polarographic current detected by the NO-selective electrode in the KH solution bathing the cultured canine tracheal epithelium is shown in Fig. 2. Immersion of both the working and counter electrodes in the incubation medium produced a baseline current with a variation of 16.8–57.0 pA, which corresponded to [NO] at 39.7±8.1 nM (n=14). Addition of TJ-96 (100 μg/ml) elicited an increase in the current that peaked within 1 min (42.2±7.0 to 133.6±18.5 pA, $P<0.001$, n=10) and gradually decreased during the 10-min observation period. Pretreatment of cells with L-NAME (10^{-3} M) per se reduced the baseline current from 44.7±6.8 to 8.1±3.3 pA ($P<0.001$, n=8) and inhibited the response to TJ-96 (100 μg/ml) by approximately 86% ($P<0.001$), whereas D-NAME (10^{-3} M) had no such effects. This inhibition of the current by L-NAME was restored by the subsequent addition of L-arginine (10^{-3} M), but not by D-arginine (10^{-3} M). As shown in Fig. 3, TJ-96 increased [NO] in a concentration-dependent manner, the maximal increase from the baseline level and the concentration of TJ-96 required to produce a half-maximal effect (EC_{50}) being 127.5±20.1 nM ($P<0.001$) and 86±9 μg/ml, respectively (n=12). This effect of TJ-96 on NO generation was greatly attenuated by L-NAME but not by D-NAME.

NADPH diaphorase activity

Cultured canine tracheal epithelial cells showed marked reactivity to NADPH diaphorase staining, where the labeling was confined to the cytoplasmic region, leaving the nucleus unstained. In contrast, no staining was observed in the control experiment in which the substrate was omitted from the reaction medium (Fig. 4).

DISCUSSION

Our in vitro studies demonstrate that cultured canine tracheal epithelial cells are spontaneously generating NO under baseline conditions and that this generation is stimulated by *Saiboku-to*, TJ-96, a Chinese antiasthmatic herbal medicine.

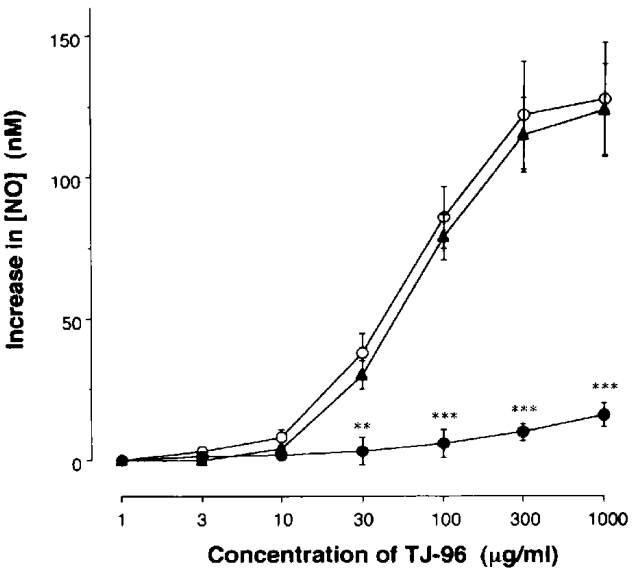

Fig. 3. Concentration-dependent effects of TJ-96 on release of nitric oxide (NO). TJ-96 was cumulatively added to Krebs-Henseleit solution bathing the cultured canine tracheal epithelium in the absence (control, open circles) and presence of N^G-nitro-D-arginine methylester (D-NAME, 10^{-3} M; closed triangles) or N^G-nitro-L-arginine methylester (L-NAME, 10^{-3} M; closed circles). Responses are expressed as the increase in the concentration of NO ([NO]) in the solution. Data are means±S.E.M., n=12 for each point. **$P<0.01$, ***$P<0.001$, significantly different from control values.

The multipurpose messenger molecule NO is generated from the amino acid L-arginine by NOS and has been identified as part of the transduction mechanism of the soluble guanylyl cyclase and also as an immunologically derived effector molecule. In the respiratory tract, endothelial cells (22), vascular smooth muscle cells (23), mast cells (24), alveolar macrophages (25), polymorphonuclear leukocytes (26) and epithelial cells (2) are potential sources of NO. The epithelial layer in asthmatic patients is often damaged, and the degree of epithelial damage is associated with the degree of airway hyperresponsiveness (27). A diminished production of epithelium-derived relaxing factors caused by the epithelial damage may contribute to the increased airway response in these patients. Nijkamp and coworkers (8) have recently shown that NO secreted by the airway epithelium may protect against the development of airway hyperresponsiveness. Therefore, stimulation of epithelial NO generation by TJ-96 seems desirable in the treatment of asthma.

Because NO is destroyed in less than a second by oxidation in the biological environment, the measurement of this molecule has been difficult. In most studies, the notion that NO is generated has been derived from the enantiomer specific effects of methylester derivatives of arginine analogues including L-NAME, but this conclu-

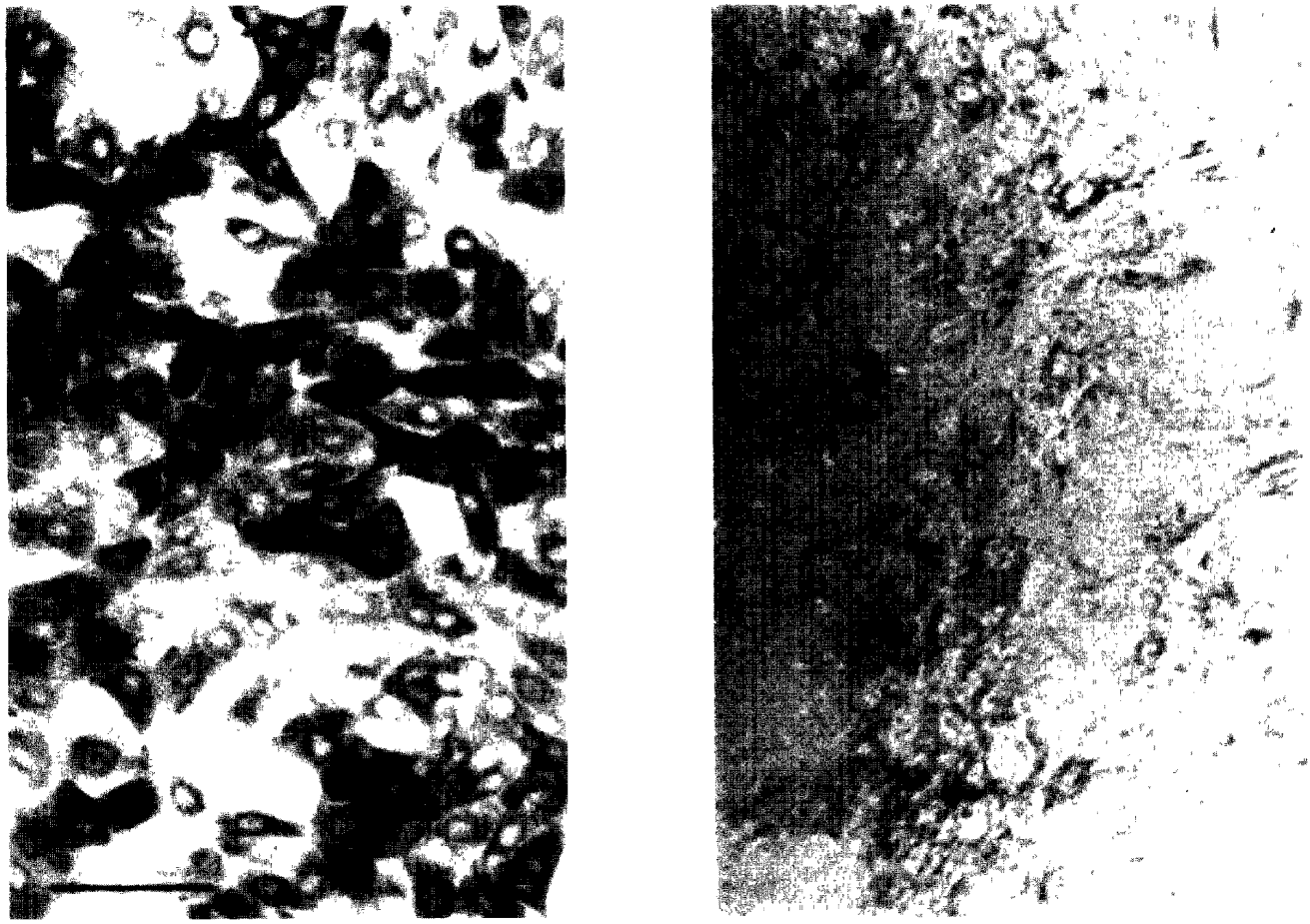

Fig. 4. Histochemical staining with NADPH diaphorase activity showing a strong blue reaction product in cultured canine tracheal epithelium (left panel). In the control experiment, NADPH was omitted and *p*-nitrophenylphosphate was included in the reaction medium (right panel). Calibration bar = 15 μm. Typical of 14 experiments for each.

sion is based on the premise that these drugs are specific inhibitors of NOS. Moreover, although the concentration of NO in exhaled air has recently been measured by a chemiluminescence analyzer (6, 28), this method, which requires purging the sample solution with inert gas to remove NO from its environment, is tedious and time-consuming and, most importantly, does not permit quantification of NO in its physiological environment in the presence of other constituents. We thus adopted a recently-developed specific amperometric sensor for NO (14). In the present study, immersion of the NO-selective electrode in the solution bathing the canine tracheal epithelial cells detected the current, indicating that NO is spontaneously released by these cells. This notion is also supported by the finding that the cells showed a potent NADPH diaphorase activity, which reflects the existence of NOS (20). Addition of TJ-96 increased NO generation from the tracheal epithelial cells in a concentration-dependent manner. Pretreatment of the cells with L-NAME greatly inhibited this increase, whereas D-NAME had no effect, and the L-NAME-induced inhibition was reversed by L-arginine but not by D-arginine.

TJ-96 is a blended medicine made from ten crude herbal drugs, and it is uncertain which crude drugs are responsible for epithelial NO generation. Bredt and colleagues (29) reported that NOS can be stoichiometrically phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II and cyclic AMP-dependent kinase, with each kinase phosphorylating a different site on this enzyme, and we have recently shown that intracellular accumulation of cyclic AMP but not elevation of Ca^{2+} concentration plays an important role in the generation of NO in canine airway epithelium (30). Although we did not assess the effect of TJ-96 on the cyclic AMP-dependent pathway in the present study, *Zizyphi fructus*, one of the constituents of TJ-96, exhibits cyclic AMP-like bioactivities (31), and TJ-96 is capable of increasing airway epithelial cyclic AMP contents (12). Therefore, we speculate that enhancement of cyclic AMP synthesis might be involved in the TJ-96-induced NO generation by airway epithelial cells.

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