

## Full Paper

**Effect of *d*-Pseudoephedrine on Cough Reflex and Its Mode of Action in Guinea Pigs**Kiyoshi Minamizawa<sup>1</sup>, Hirozo Goto<sup>1</sup>, Yoshiaki Ohi<sup>2</sup>, Yutaka Shimada<sup>1,3,\*</sup>, Katsutoshi Terasawa<sup>3,4</sup>, and Akira Haji<sup>5</sup>*Departments of <sup>1</sup>Japanese Oriental Medicine and <sup>2</sup>Pharmacology, Faculty of Medicine, University of Toyama, Toyama 930-0194, Japan**<sup>3</sup>21st Century COE Program, University of Toyama, Toyama 930-0194, Japan**<sup>4</sup>Department of Japanese Oriental Medicine, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan**<sup>5</sup>Laboratory of Neuropharmacology, School of Pharmacy, Aichi Gakuin University, Nagoya 464-8650, Japan*

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**Abstract.** *d*-Pseudoephedrine (PSE) is one of the main ingredients of *Ephedrae herba*. Although PSE is widely applied for patients with a common cold and upper respiratory inflammation as a decongestant, the effects of PSE on cough have never been reported. In this study, we investigated the antitussive effects of intraperitoneal injection of PSE on the cough reflex induced by microinjection of citric acid into the larynx of guinea pigs. PSE decreased the number of cough reflexes dose-dependently ( $-18.3 \pm 5.0\%$  at 20 mg/kg,  $P < 0.05$ ;  $-41.1 \pm 7.2\%$  at 60 mg/kg,  $P < 0.01$ ). Furthermore, PSE (60 mg/kg) increased the threshold intensity for inducing fictive cough by electrical micro-stimulation of the nucleus tractus solitarius ( $+72.7 \pm 8.4\%$ ,  $P < 0.01$ ). On the afferent discharge of the superior laryngeal nerve, PSE suppressed the increases of amplitude and frequency when stimulated by citric acid at laryngeal mucosa. These results demonstrate that PSE possesses an antitussive effect that might be derived from both central and peripheral actions.

**Keywords:** *d*-pseudoephedrine, antitussive effect, cough reflex, nucleus tractus solitarius, superior laryngeal nerve

**Introduction**

In the previous study, we clarified the antitussive effect of *eppikahangeto*, a traditional Chinese/Japanese (*Kampo*) formula, in a laryngeal cough model of guinea pigs (1). Among the crude drugs comprising *eppikahangeto*, *Ephedrae herba* played a crucial role, as it alone exhibited an antitussive effect, while *eppikahangeto* without *Ephedrae herba* did not.

*Ephedrae herba* is one of the most important crude drugs in *Kampo* medicine. Nowadays, it is likely to be used for the purpose of weight reduction and performance enhancement in western nations (2), but it has a long history of use for the treatment of asthma, common cold, nasal congestion, and cough as a bronchodilator,

expectorant, and antipyretic (2, 3). These pharmacological actions appear to be due to a series of ephedra alkaloids such as *l*-ephedrine, *d*-pseudoephedrine (PSE), *l*-methylephedrine, and *l*-norephedrine (2–4). Among these, *l*-ephedrine and PSE account for about 90% of the total alkaloids, and they are considered to play the principle role in the pharmacological actions of *Ephedrae herba* (4).

*l*-Ephedrine is known to be a sympathomimetic agonist on both  $\alpha$ - and  $\beta$ -adrenergic receptors (2). It has also been reported that *l*-ephedrine is the most potent and the only ephedrine isomer to possess a partial agonistic activity on  $\beta_3$ -subtype adrenergic receptor, which is involved in lipolysis and non-shivering thermogenesis (5). As for PSE, its most popular application is in common cold medications to relieve nasal decongestion (6, 7). PSE is the fourth most commonly used medicine among all prescriptions and over-the-

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counter drugs in the USA (8), and its use is likely to increase further in the treatment of the common cold and respiratory problems as a substitute for phenylpropanolamine, on the basis of FDA's Public Health Advisory in 2000 (9).

It has been reported that *l*-ephedrine had an antitussive effect in cats and mice (10, 11) and that *l*-methylephedrine showed an antitussive effect by suppressing the cough center, not afferent signals of the peripheral nerve, in dogs and cats (12). However, the antitussive effect of PSE, a widely used ephedra alkaloid, has never been evaluated. In the present study, we investigated the effects of PSE on the cough reflex induced by citric acid microinjection into the larynx in guinea pigs. Furthermore, to address its mode of action, we investigated the effects of PSE on fictive cough evoked by electrical micro-stimulation of the nucleus tractus solitarius (NTS) as well as on the enhancement of afferent discharges of the superior laryngeal nerve (SLN) caused by citric acid application at laryngeal mucosa.

## Materials and Methods

### Animals

Male Hartley guinea pigs purchased from Japan SLC, Inc. (Hamamatsu) weighing 400–600 g were used for the experiments. The animals were placed individually into plastic cages and housed in an animal room at a temperature of  $24 \pm 2^\circ\text{C}$  and a 12-h light-dark cycle. The animals were allowed free access to standard laboratory food. All experimentation was performed in accordance with the Guide Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society and also approved by the Committee on Animal Experimentation, University of Toyama. At the end of all experiments, the guinea pigs were sacrificed with a lethal dose of pentobarbital sodium (100 mg/kg) injected intraperitoneally.

### Drugs

The following drugs were obtained from the indicated commercial sources: *d*-Pseudoephedrine hydrochloride (PSE; Alps Pharmaceutical Ind. Co., Ltd., Gifu), codeine phosphate (Takeda Pharmaceutical Co., Ltd., Osaka), citric acid (Wako Pure Chemical Ind. Ltd., Osaka), ketamine hydrochloride (Wako), xylazine hydrochloride (Sigma, St. Louis, MO, USA), pancuronium bromide (Sankyo Co., Ltd., Tokyo), and pentobarbital sodium (Dainippon Pharmaceutical Co., Ltd., Osaka) were used.

### Experiment 1: citric acid-induced laryngeal cough

The experiment was performed in accordance with the

procedure described in our previous study (1), which was based on the method of Tanaka and Maruyama (13). A guinea pig was anesthetized with an intramuscular injection of a mixed solution of ketamine (80 mg/kg) and xylazine (8 mg/kg). The animal was fixed on a bed in supine position. The trachea was exposed surgically and a polyethylene tube (inside diameter, 0.5 mm; outside diameter, 0.9 mm; length, 17.5 cm; SP-35; Natume Seisakusho Co., Ltd., Tokyo) was inserted between the seventh and eighth cartilage. The tip of the tube was advanced 15 mm toward the larynx to place it just under the larynx. Then, the tube was fixed with ligation to the accompanying sternohyoid muscle, and the other end was led out from the dorsum of the neck through a subcutaneous tunnel. All experiments were started after one-week recovery.

The cough was evaluated by whole-body plethysmography under unrestrained, unanesthetized condition. The animal was placed in a sealed chamber (5 L) into which continuous airflow (2 L/min) was fed. The implanted polyethylene tube was connected to an extension tube and led outside through a pinhole in the chamber. Firstly, non-treatment (control) trials were carried out, consisting of  $20 \mu\text{l}$  of 7.5% citric acid solution being microinjected for 5 s directly into the larynx through the tube to elicit coughing. Pressure changes derived from breath-to-breath ventilation were measured with a differential pressure transducer (TP-602T; Nihon Kohden Corp., Tokyo), and sounds were recorded with a microphone placed inside the chamber for 10 min. After 3 days, post-treatment trials were performed. PSE (20 or 60 mg/kg) was intraperitoneally injected 15 min prior to the citric acid injection. After a 4-day drug washout period, another control trial was repeated in the same manner. As a positive control, some of the animals were given codeine (60 mg/kg) intraperitoneally (1). The same animal was set up to receive different treatments in each trial. We preliminarily confirmed that intraperitoneal injection of vehicle (saline, 1.0 ml/kg) did not change the number of coughs.

As in our previous report (1), the cough reflex was determined by ventilation waveform pattern and sound according to the report of Tanaka and Maruyama (13), and the criteria for coughing employed in this study were (I) a deep initial inspiration followed by rapid large expiration, (II) expiration amplitude more than 200% of normal expiration, and (III) simultaneous sound.

### Experiment 2: fictive cough induced by NTS stimulation

The experiment was performed according to the procedure reported by Ohi et al. (14, 15). A guinea pig was anesthetized with inhalation of 1.5%–2.0%

halothane in oxygen-enriched air. The animal was intubated and catheterized into the femoral vein for drug administration. Artificial ventilation was started after the intravenous injection of pancuronium bromide (2.0 mg/kg initially, followed by 0.2 mg/kg per hour). Tracheal pressure was maintained between 1- and 6-cm H<sub>2</sub>O. End-tidal CO<sub>2</sub> was maintained at 4.5%–5.5%. The head of the animal was fixed on a stereotaxic frame in prone position. Decerebration was performed by aspirating the brain rostral to the midcollicular transection. To record cough reflex discharges, the right phrenic nerve (PN) and left iliohypogastric nerve (IHN) were isolated from surrounding tissue, cut distally, and mounted on bipolar electrodes in a mineral oil pool. Halothane anesthesia was discontinued, and then, to avoid any influence of anesthesia, the recording of nerve discharges was not begun until more than 3 h had elapsed after the surgery. The central inspiratory and expiratory outputs were monitored by recording the efferent discharges of PN and IHN, respectively. A coaxial microelectrode (outer diameter, 0.35 mm) was inserted into the rostral NTS, extending 0–1.5 mm rostral to the obex, 0.5–1.5 mm lateral to the midline, and 0.5–1.5 mm deep from the dorsal. A series of repetitive pulses (0.1-ms pulse width, 100 pulses at 10 Hz) were used to stimulate the NTS. An increased PN discharge that was immediately followed by a large burst of IHN discharge was defined as a fictive cough response (15). To find the most effective area to evoke the cough, the electrode was systematically positioned, and the area where the cough was induced by a minimum intensity was explored. The minimum threshold intensity to induce cough response was evaluated before and 15 min after intraperitoneal injection of PSE (20 or 60 mg/kg). We preliminarily confirmed that intraperitoneal injection of vehicle (saline, 1.0 ml/kg) did not change the minimum threshold intensity to induce the cough response.

#### *Experiment 3: afferent discharge of SLN induced by laryngeal stimulation*

A guinea pig was anesthetized with intramuscular injection of a mixed solution of ketamine (80 mg/kg) and xylazine (8 mg/kg). The animal was fixed in supine position. The trachea was gently exposed surgically and cut between the fourteenth and fifteenth cartilage for intubation to the caudal segment. Following this, the seventh cartilage of the rostral segment of the trachea was ligated. A silicon tube for the use of citric acid injection was cannulated from between the fifth and sixth cartilage toward the larynx. The right SLN was isolated carefully from the surrounding tissues and cut distal to the ganglion. This distal cut end was attached

to bipolar electrodes in the mineral oil pool.

After the afferent signals of SLN were being stably recorded, laryngeal mucosa was stimulated by an injection of citric acid solution (7.5%, 100  $\mu$ l) for 15 s through the tube, and changes in the SLN afferent signals were recorded in the control group. In the post-treatment group, PSE (20 or 60 mg/kg) was injected intraperitoneally 15 min before the citric acid application. In the control group, only vehicle (saline, 1.0 ml/kg) was similarly injected.

#### *Data acquisition and analysis*

Signals were amplified and filtered (30–3000 Hz band-pass) (AR-601G in experiment 1, MEG-1200 in experiments 2 and 3; Nihon Kohden Corp.). All data were digitized (4000 Hz sampling rate) with PowerLab/4s (ADInstruments, Castle Hill, Australia), displayed on a screen, and stored on hard disk using signal-processing software (Chart 5, ADInstruments).

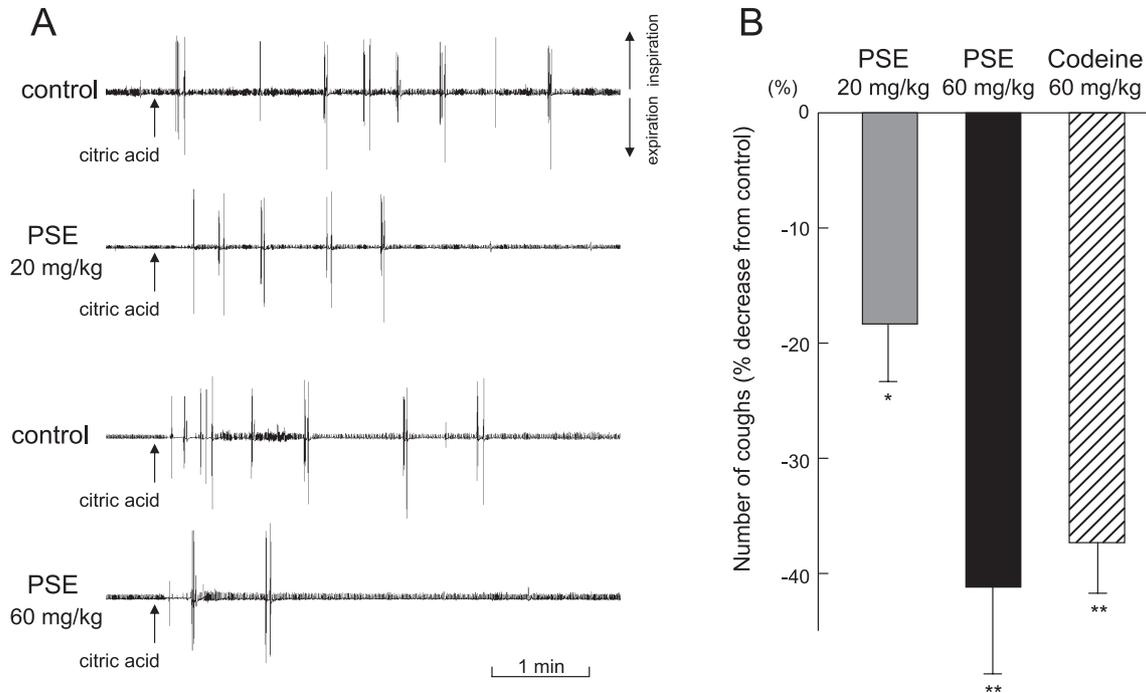
In experiment 1, the number of coughs in a 10-min period after laryngeal stimulation was counted, and the percent decrease in the count of the post-treatment trial from that of the control trial was calculated. In experiment 2, the percent increase in threshold intensity of the post-treatment trial from that in the control trial was calculated. In experiment 3, the one-second averages of amplitude and frequency of afferent discharges were measured. The percent increases of these peak values after laryngeal stimulation from those just before laryngeal stimulation (baseline) were calculated.

Data were presented as the mean  $\pm$  S.E.M. Statistical analysis was performed by Wilcoxon signed-ranks test in experiments 1 and 2 and by Bartlett's test for equality of variances and one-way analysis of variance followed by Scheffe's test for post-hoc comparison in experiment 3.  $P < 0.05$  was considered to be statistically significant.

## **Results**

#### *Effect of PSE on citric acid-induced laryngeal cough*

Figure 1A shows typical examples of ventilation pressure waveforms in guinea pigs before (control) and after PSE-treatment (post-treatment). The waveform represented constant and stable inspiration (upward) and expiration (downward) at baseline. The injection of citric acid induced a large deflection in the waveform. Cough responses were identified in accordance with our previous report (1). One injection produced  $21.3 \pm 1.5$  coughs in 10 min ( $n = 23$ ). Both a low dose (20 mg/kg) and a high dose (60 mg/kg) of PSE decreased the number of coughs, but there was no effect on the magnitude. Furthermore, PSE did not influence basal



**Fig. 1.** Effects of *d*-pseudoephedrine (PSE) on citric acid-induced laryngeal cough in guinea pigs. A: Plethysmographic waveforms of ventilation in control and post-treatment trials. Large flow deflections in waveforms represent cough reflexes. Citric acid solution (7.5%, 20  $\mu$ l) was injected into the larynx. PSE and codeine were administered intraperitoneally to animals 15 min prior to application of citric acid in post-treatment trials. B: Number of coughs after PSE- and codeine-treatments (percent decrease from control). Each bar represents the mean  $\pm$  S.E.M. (20 mg/kg of PSE,  $n = 6$ ; 60 mg/kg of PSE,  $n = 10$ ; 60 mg/kg codeine,  $n = 7$ ). \* $P < 0.05$ , \*\* $P < 0.01$  (compared to the control).

ventilation. Figure 1B summarizes the percent decrease in the number of coughs post-treatment from that in the control, showing that this effect of PSE is dose-dependent. The antitussive potency of the high dose of PSE was similar to that of codeine (60 mg/kg).

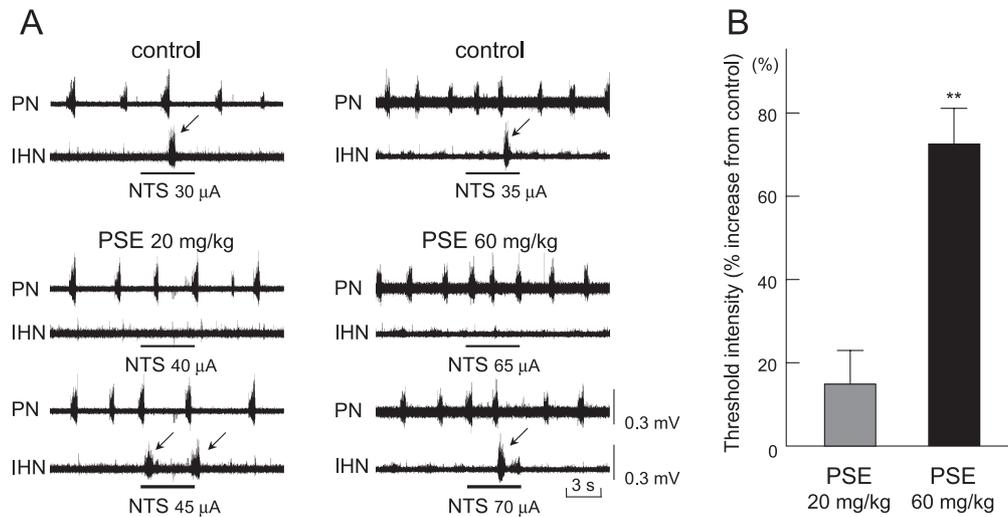
#### *Effect of PSE on fictive cough induced by NTS stimulation*

Figure 2A illustrates representative neuronal discharges of PN and IHN. In normal respiration, PN showed an augmenting discharge during inspiration and no discharge during expiration. IHN displayed no discharge during inspiration and a small augmenting or no discharge during expiration. The electrical stimulation of NTS induced one or two fictive cough responses during or just after the stimulation, which were identified by an abrupt large discharge in the IHN that was preceded by a large PN discharge. The average threshold intensity of micro-stimulation was  $48.8 \pm 4.9 \mu$ A ( $n = 12$ ). The area at which a fictive cough was induced by a minimum intensity was calculated at  $480 \pm 48 \mu$ m rostral and  $950 \pm 28 \mu$ m lateral to the obex and  $708 \pm 68 \mu$ m deep from the dorsal surface of the brainstem ( $n = 12$ ). These values were consistent with those of the previous report (15), suggesting that the

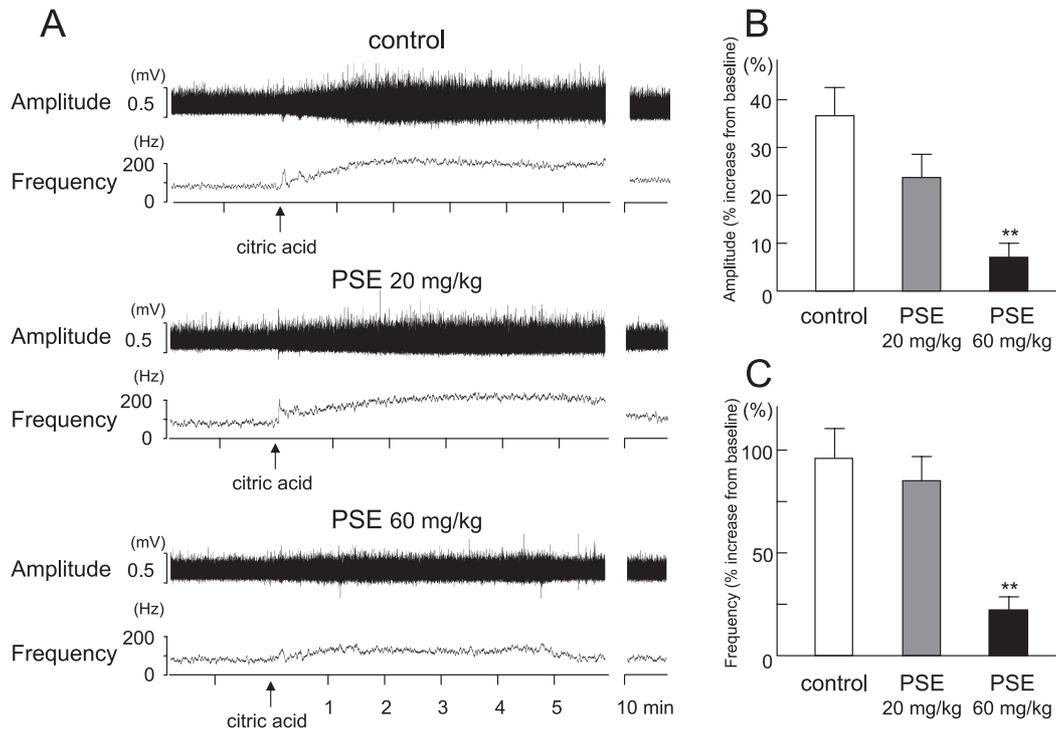
area was localized in the NTS. Figure 2B shows the percent increase of threshold intensity for evoking fictive cough in post-treatment trials from that in control trials. A high dose (60 mg/kg) of PSE significantly increased the threshold intensity, whereas a low dose (20 mg/kg) of PSE did not.

#### *Effect of PSE on afferent discharge of SLN induced by laryngeal stimulation*

Figure 3A shows the typical afferent discharge of SLN together with the frequency. SLN displayed tonic discharges and constant frequencies. Application of citric acid onto the laryngeal mucosa immediately provoked increases in both the amplitude and frequency of afferent discharges. This response reached a peak in 2–4 min, and gradually returned to the baseline level within 10 min. Figures 3B and 3C summarize the percent increases in amplitude and frequency, respectively, in control and PSE-treated groups. A low dose of PSE (20 mg/kg) tended to inhibit the afferent discharges, but the effect was not statistically significant. A high dose of PSE (60 mg/kg) significantly suppressed the increase of afferent discharges of the citric acid-stimulated SLN.



**Fig. 2.** Effects of *d*-pseudoephedrine (PSE) on fictive cough induced by electrical stimulation (0.1-ms pulse width, 100 pulses at 10 Hz) to the nucleus tractus solitarius (NTS). A: A set of recordings of the phrenic nerve (PN) and iliohypogastric nerve (IHN) discharges before (control) and 15 min after intraperitoneal administration of PSE. Oblique arrows indicate fictive cough reflexes. B: Threshold intensity after PSE-treatments (percent increase from control). Each bar represents the mean  $\pm$  S.E.M. (20 mg/kg of PSE,  $n = 5$ ; 60 mg/kg of PSE,  $n = 7$ ), \*\* $P < 0.01$  (compared to the control).



**Fig. 3.** Effects of *d*-pseudoephedrine (PSE) on afferent discharges of the superior laryngeal nerve (SLN) induced by laryngeal stimulation with citric acid. A: A set of recordings of the raw afferent discharges (Amplitude) and their frequencies (Frequency). Citric acid solution (7.5%, 100  $\mu$ l) was injected into the larynx. PSE was administered intraperitoneally to animals 15 min prior to application of citric acid. B: Percent increase of amplitude in control and PSE-treated groups. C: Percent increase of frequency in the control and PSE-treated groups. Each bar represents the mean  $\pm$  S.E.M. ( $n = 6$ ), \*\* $P < 0.01$  (compared to the control).

## Discussion

The present study demonstrated, using a laryngeal

cough model of guinea pig, that PSE decreased the number of coughs, demonstrating the antitussive effect of PSE. Furthermore, PSE increased the threshold

intensity for the fictive cough induced by micro-electrical stimulation of NTS, and it suppressed an increase in the afferent discharges of SLN stimulated by application of citric acid onto the laryngeal mucosa. These results suggest that the antitussive effect of PSE may be due not only to its central but also its peripheral action.

Cough reflexes usually start from stimuli to the airway, which affect the airway sensory receptors such as “irritant” receptors (rapidly adapting stretch receptors, RARs) and C-fiber receptors to evoke afferent signals (16, 17). The signals are conducted via the vagal nerve and its branches. Signals from the larynx, known as one of the most sensitive sites to tussigenic stimuli (18), are conducted mainly through SLN and terminate at NTS (19). It was demonstrated that the main route of afferent signals from the supraglottic area was SLN and that from the subglottic area was the recurrent laryngeal nerve (20). Appropriate signals evoke cough responses in the central nervous system (CNS), and the cough pattern generator makes the efferent motoneuronal signals that produce the three phases of cough — inspiration, compression, and expulsion (17). Antitussive agents are considered to suppress the responsiveness of one or more elements of this reflex pathway. Drugs that act outside the CNS are classified as peripheral antitussive drugs, and those acting inside the CNS are called central antitussive drugs (21). These agents may work on the cough-pattern generator or suppress the signals that activate the generator (22). Codeine, one of the most popular antitussive drugs, is generally considered to be a selective centrally acting agent (15, 23).

In the experiment of the fictive cough induced by NTS micro-stimulation, PSE showed a central antitussive effect. Since NTS is the first relay nucleus of tussigenic afferent signals as described above and since codeine (3 mg/kg, i.v.) decreased the number of coughs at each stimulus to NTS from  $1.50 \pm 0.55$  to  $0.17 \pm 0.41$  ( $P < 0.01$ ) in the same experimental model (15), the present result reveals that PSE may have a central effect by acting on the afferent terminals/fibers and/or the NTS relay neurons related to the cough response. It is well established that serotonin (5-HT) receptors, in particular 5-HT<sub>1A</sub> receptors, play an important role in the cough-depressant activities of centrally acting antitussive drugs. Kamei et al. (24) reported that intraperitoneal injection of 8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT), a 5-HT<sub>1A</sub> agonist, decreased the number of coughs dose-dependently on capsaicin-induced cough reflex in rats. This antitussive effect of 8-OH-DPAT was blocked by the prior administration of methysergide and spiperone, whereas ketanserin

had no effect. The antitussive effects of codeine and dextromethorphan, narcotic and non-narcotic antitussive agents, respectively, were also antagonized by methysergide and spiperone, but these cough-depressant effects were not reduced by ketanserin. Kamei et al. (25) also reported that codeine and dextromethorphan, both centrally acting antitussive drugs, increased the release of 5-HT from slices of the rat NTS. These results suggested that these centrally acting antitussives interact with 5-HT<sub>1A</sub> receptors in the NTS. The mechanism of the central antitussive effect of PSE would become clearer with the examination of the effect of 5-HT-receptor antagonists against the effect of this agent.

A central antitussive effect is the common mechanism of other ephedra alkaloids (10, 11). Furthermore, PSE showed a suppressive effect on the response of SLN to chemical stimulation of laryngeal mucosa. It is unlikely that PSE directly inhibits the potential generation of action such as local anesthetics do. Rather, this drug may influence the sensitivity of peripheral receptors responding to tussigenic stimulation. This result, therefore, leads us to postulate that PSE may suppress the signals of A $\delta$  and C fibers, which are suggested to relate to “cough receptors” (16, 26, 27). Furthermore, the suppression of afferent discharges of SLN by PSE may play some important roles in its antitussive effect on the laryngeal cough. As for the peripheral effect of ephedra alkaloids, little has been investigated. To the best of our knowledge, there is only one study, which reported that *l*-methylephedrine, a precursor of phenylpropanolamine, did not influence the stretch receptor impulses on the vagal nerve during artificial ventilation in guinea pigs (12).

Cough is one of the most common symptoms, and the common cold is the most usual cause of acute cough. Chronic cough, which can profoundly disturb the quality of life of the afflicted, is sometimes caused by gastroesophageal reflux disease or post-nasal drip syndrome (28). The former causes laryngitis (29), and the latter stimulates the larynx (30). There is a report that the level of substance P in nasal lavage fluid, which affects the larynx and increases cough sensitivity, is elevated in patients with chronic nonproductive cough (31). As mentioned, the larynx is related to coughs caused by various clinical conditions. The present study indicates that PSE might be useful for patients suffering from unnecessary cough. In fact, it is reported that the use of PSE as decongestant improves chronic cough, while the newer antihistamines are ineffective (32). This may be due to the antitussive effect rather than any decongestive effect of PSE.

In the present study, PSE showed a level of efficacy on the citric acid-induced laryngeal cough equal to that

of codeine. Codeine is one of the most effective and popular antitussive drugs, but it has adverse effects such as constipation, nausea, risk for addiction, and suppression of normal breathing. Codeine is not recommended for asthmatic patients, and its use is strictly restricted in most countries and areas. Additionally, PSE has less cardiac effect than ephedrine (3) because it has less potency on  $\beta$ -adrenergic receptors (5). This is also an advantage for the use of PSE over ephedrine.

In conclusion, the present study revealed that PSE not only has a central antitussive effect but also a potential peripheral antitussive effect against laryngeal cough. We consider the use of PSE for the treatment of laryngeal cough to be entirely reasonable.

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