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Thoracic vagal efferent nerve stimulation evokes substance P-induced early airway bronchonstriction and late proinflammatory and oxidative injury in the rat respiratory tract

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Summary

Electrical stimulation of efferent thoracic vagus nerve (TVN) evoked neurogenic inflammation in respiratory tract of atropine-treated rats by an undefined mechanism. We explored whether efferent TVN stimulation via substance P facilitates neurogenic inflammation via action of nuclear factor-κB (NF-κB) activation and reactive oxygen species (ROS) production. Our results showed that increased frequency of TVN stimulation concomitantly increased substance P-enhanced hypotension, and bronchoconstriction (increases in smooth muscle electromyographic activity and total pulmonary resistance). The enhanced SP release evoked the appearance of endothelial gap in silver-stained leaky venules, India-ink labeled extravasation, and accumulations of inflammatory cells in the respiratory tract, contributing to trachea plasma extravasation as well as increases in blood O_2^- and H_2O_2 ROS amount. L-732138 (NK_1 receptor antagonist), SR-48968 (NK_2 receptor antagonist), dimethylthiourea (H_2O_2 scavenger) or catechins (O_2^- and H_2O_2 scavenger) pretreatment reduced efferent TVN stimulation-enhanced hypotension, bronchoconstriction, and plasma extravasation. Increased frequency of TVN stimulation significantly upregulated the expression of nuclear factor-κB (NF-κB) in nuclear protein and intercellular adhesion molecule-1 (ICAM-1) in total protein of the lower respiratory tract tissue. The upregulation of NF-κB and ICAM-1 was attenuated by NK receptor antagonist and antioxidants. In conclusion, TVN efferent stimulation increases substance P release to trigger NF-κB mediated ICAM-1 expression and O_2^- and H_2O_2 ROS production in the respiratory tract.

Although autonomic nervous system mainly innervates the airway, non-adrenergic and non-cholinergic (NANC) nervous system is important in the regulation of various airway responses via tachykinins [1–3]. Tachykinins belonging to the NANC system are released from sensory C-fiber nerve endings when stimulated and are known to induce neurogenic airway responses [1, 2, 4]. Upon stimulation, substance P (SP), neurokinin A or B

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can be released from these sensory axon terminals and cause plasma leakage at inflammation sites, acts on smooth muscle or blood vessels to regulate visceral motility and blood flow [5–7] via neurokinin type 1 (NK_1) and type 2 (NK_2) receptors. SP can induce the adhesion of neutrophils to bronchial epithelial cells [8] and endothelial cells [5]. This process may amplify proinflammatory response and oxidative stress, including the generation of intercellular adhesion molecule-1 (ICAM-1) adhesion molecules on vascular endothelium [9, 10], the release of reactive oxygen species (ROS) by macrophages, eosinophils, and neutrophils [11, 12], and the production of a wide array of cytokines involved in inflammation and the immune response. Upregulation of the oxidative stress-sensitive transcription of nuclear factor-κB (NF-κB) [13] seems to play a central role in the inflammatory response [14], and promotes ICAM-1 gene and protein expression [15–17].

Thoracic vagus nerves (TVN) harbor tachykinin-containing sensory axons, which carry sensory and preganglionic parasympathetic fibers to the trachea, bronchial trees, and lung [1, 2, 18]. Recently, an electrostimulatory therapy, using TVN afferent excitation, inhibited SP release in the spinal cord through a dynorphin-mediated neuronal interaction [19]. However, our previous result showed that selective TVN efferent stimulation can cause plasma extravasation on the ventral wall of the lower trachea and on the ipsilateral first- (main) to fourth-order bronchi [20] by an undefined mechanism. Whether TVN efferent stimulation enhances SP release to trigger proinflammatory response and oxidative stress in the respiratory tract *in vivo* has not been explored. Elucidation of the possible mechanisms by which SP modulates NF-κB translocation and ICAM-1 protein expression in response to efferent TVN stimulation of the respiratory tract is crucial to understanding the interplay between the nervous and immune systems, processes that probably play a significant role during the evolution of inflammation. In the present study, therefore, we explored the possible cellular events whether efferent TVN stimulation may increase SP releases via NK_1 or NK_2 receptor signaling to activate airway hyperactivity, to increase NF-κB translocation to nucleus and to enhance ICAM-1 expression for inflammatory cell adhesion and ROS production, and consequently led to neurogenic inflammation in the respiratory tract of atropine-pretreated rats. We also evaluated whether a NK receptor antagonist or an antioxidant supplement could effectively ameliorate the TVN efferent stimulation induced neurogenic inflammation and oxidative injury.

Materials and methods

Drugs

One the day of experiment, non-peptide NK_1 receptor antagonist, L-732138 (*N*-acetyl-L-tryptopan-3,5-bistrifluoromethyl benzyl ester, Sigma), non-peptide NK_2 receptor antagonist, SR-48968 {(S)-*N*-methyl-*N*-[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl]benzamide} (Sanofi Recherche, France) were dissolved in 10% dimethyl sulphoxide in 0.9% saline at the concentration of 1 μmol/kg. The other groups of drug pretreatment were a H_2O_2 scavenger, dimethylthiourea (DMTU, Sigma) [21] and an O_2^- and H_2O_2 scavenger, catechins (328 mg/g of epigallocatechin gallate, 152 mg/g of epicatechin gallate, 148 mg/g of gallocatechin gallate, 132 mg/g of epicatechin, 108 mg/g of epigallocatechin, 104 mg/g of galloctechin, and 44 mg/g of catechin, Numen Biotech Co., LTD, Taipei, Taiwan) [7], which dissolved in 0.9% saline at concentration of 2.5 mg/kg.

Animals and surgery

Male Sprague–Dawley rats (age 12–14 weeks, 250–300 g) were purchased from National Laboratory Animal Center and were housed at the Experimental Animal Center, National Sun Yat-Sen University, at a constant temperature and with a consistent light cycle (light from 0700 to 1800 h). On the day of experiment, the rats were anesthetized with subcutaneous urethane (1.2 g/kg). The body temperature was kept at 36.5–37.0 °C by an infrared light and was monitored with a rectal thermometer. The study was conducted according to the guidelines of the National Science Council of the Republic of China (NSC 1997) and was approved by the Animal Care and Use Committee of the National Taiwan University.

The trachea was cannulated caudal to the larynx (PE-200) and the animal breathed spontaneously through a Pneumotrachometer (TSD

137C, Biopac Systems) connected to a flow transducer (TSD 160A, amplifier DA 100C, Biopac Systems) for monitoring airflow with a zero-flow method. Intratracheal pressure was measured by a pressure transducer (Model DP 103–24) connected to a manometer (Model CD-15-A-1-B-1, validyne). A fluid-filled PE-50 cannula was introduced into the esophagus to measure the esophageal pressure as an approximation of pleural pressure. The transpulmonary pressure (defined as the pressure difference between the intratracheal and the esophageal pressures) was measured with a manometer. Total pulmonary resistance (R_L) was calculated as previously described [22]. For electromyogram recordings, epoxy-coated stainless steel wire (50 μm; M.T. Giken Co., Ltd, Tokyo, Japan) was placed into the inner layer of airway smooth muscle of lower trachea under a dissecting microscope (Nikon, Japan). The EMG electrodes were embedded into the smooth muscle ~1–2 mm. EMG signals were connected to an EMG amplifier (EMG 100C, Biopac Systems) and recorded on the recording system [22]. All the signals collected are stored in the IBM computer (ThinkPad R40) and analyzed with software (AcqKnowledge 3.7.3 Biopac System). The rate of rise of EMG activity was calculated by dividing peak integrated EMG activity by the time to peak, and expressed the result as a percentage of the maximal activity. PE-50 catheters were placed in the left femoral artery for measurement of arterial blood pressure (ABP) and in the left femoral vein for administration of test drugs. ABP was recorded on a polygraph (DA 100C, Biopac Systems, Inc., Goleta, CA, USA) with a transducer (TCI 100, Biopac Systems).

Electrical stimulation of thoracic vagus nerve (TVN)

The surgical procedure for right TVN isolation was described previously in our laboratory [20]. Briefly, under a dissecting microscope, the right TVN localized between and under of precava vein and common carotid artery was identified and sectioned. A stainless steel bipolar electrode was placed on the peripheral end of the sectioned right TVN and fixed with Kwik-Cast (World Precision Instruments). An electric current of square-wave pulse duration of 5 ms was applied from a stimulator (model S88, Grass, Quincy, MA) through a stimulus isolation unit (model SIU5B, Grass) and a constant-current unit (model CCU1A, Grass). Our previous study showed that the increased frequency of electric stimulation dose-dependently enhanced SP release, smooth muscle contraction and hypersensitivity from the bladder pelvic nerve terminals [5]. Therefore, all rats were randomly divided into four groups, depending on different stimulus frequency 0, 1, 5, and 10 Hz, then subjected to the fixation parameters of 10 V, 5 ms. Atropine (1 mg/ml/kg) was given 20 min before TVN stimulation to inhibit the muscarinic effect.

Measurement of substance P (SP)

To explore whether efferent TVN stimulation results in elevation and release of SP in the respiratory tract, we measured plasma levels of SP [5] in the carotid arterial blood. During TVN (0–10 Hz) stimulation, 0.5 ml of blood samples were obtained from the carotid artery in the atropine-treated rats. The supernatant from plasma sample was diluted with the same volume of *buffer* A (RIK-BA-1, Peninsula Laboratory). Then each sample was passed slowly through a C18 Sep-Pak column (RIK-SEPCOL-1, Peninsula Laboratory). The column was washed with 9 ml of *buffer* A and eluted with 3 ml of *buffer* B (RIK-BB-1, Peninsula Laboratory). The eluted samples were dried by vacuum centrifugation and stored at −70 °C for later analysis. An SP enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) was used to detect the SP level. Each sample was dissolved in 1% HCl, diluted to a suitable concentration with enzyme immunoassay buffer, and assayed in duplicate. The SP, which was linked to acetylcholinesterase as a tracer, and rabbit SP antiserum were added to the sample and incubated in the assay plate at 4 °C for 18 h. Then, the wells were rinsed five times with washing buffer. Ellman's reagent was added for development the plates in each well. After development, the plates were read at 410 nm, and SP levels were calculated.

Morphometric analysis of India ink-labeled leaky blood vessels

For evaluation of the permeability-producing ability by efferent TVN stimulation, India ink (1 mg/ml/kg, over 5 s, Chroma-gesellschaft, Kongen,

Germany) was used as a tracer dye to evaluate the area density of the India ink-labeled leaky blood vessels [20]. Rats in each group (0, 1, 5, and 10 Hz) received an intravenous injection of India ink (1 ml/kg, over 5 s) followed by 10 min of TVN stimulation. Within 5 min after efferent TVN stimulation, the rat chest was opened and a cannula was inserted through a cut in the left ventricle of the heart into the aorta to perfuse the circulation with 0.05 M phosphate buffer containing 2% paraformaldehyde and 1% glutaraldehyde pH 7.4 for 2 min at a pressure of 120–140 mm Hg. The respiratory tract were removed and cut open along their ventral midline. They were processed with ethanol and toluene, and mounted with Permount (Merck). The trachea of India ink was analyzed with a microscope (Leica DMRD). The magnitude of plasma leakage was expressed by the area density of India ink-labeled blood vessels in the mucosal tissue of trachea whole mounts determined by a point counting method [20, 22].

Silver staining of endothelial cells in microvessel of airways

For measurement of endothelial gap after 10 min of TVN stimulation, four groups of rats ($n=3$ each) were perfused with 0.075 M cacodylate buffer (pH 7.4) containing 0.5% glutaraldehyde and 1% paraformaldehyde for 5 min at a pressure of 120–140 mm Hg as described previously [20]. After then, 80 ml of 0.9% NaCl, 25 ml of 5% glucose, 20 ml of 0.2% $AgNO_3$, and 50 ml of fixatives were perfused in order. The right lower trachea was mounted with Permount.

Whole blood for lucigenin- and luminol-enhanced CL determination

Our previous results showed that SP enhanced whole blood ROS production, a major source from leukocytes, after 30 min of incubation with SP [5]. For measurement of ROS in whole-blood samples, a series (30–180 min) of blood samples (0.2 ml) from the left carotid artery were obtained after 10 min of TVN stimulation. Before CL measurement, 0.1 ml of phosphate-buffered saline (pH 7.4) was added to 0.2 ml of blood sample. The CL was measured in a completely dark chamber of the Chemiluminescence Analyzing System. After 100-s background level determination, 0.5 ml of 0.1 mM lucigenin (*bis-N*-methylacridinium nitrate, Sigma, St. Louis, MO, USA) or 0.2 mM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma) in phosphate-buffered saline (pH 7.4) was injected into the sample. The CL was monitored continuously for an additional 600 s. The total amount of CL was calculated by integrating of the area under the curve and subtracting it from the background level. The assay was performed in duplicate for each sample and was expressed as CL counts/10 s for blood CL [5].

Immunoblot analysis for NF-κB and ICAM-1

We measured nuclear protein of NF-κB and total protein of ICAM-1 [7] in right lower trachea and right lung after 90 min of TVN stimulation. For protein analysis, bronchiolar samples were homogenized with a prechilled mortar and pestle in extraction buffer, which consisted of 10 mM Tris–HCl (pH 7.6), 140 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1% NP-40, 0.5% deoxycholate, 2% β-mercaptoethanol, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin. The mixtures were homogenized completely by vortexing and kept at 4 °C for 30 min. The homogenate was centrifuged at $12{,}000 \times g$ for 12 min at 4 °C, the supernatant was collected, and the protein concentrations were determined by BioRad Protein Assay (BioRad Laboratories, Hercules, CA). Primary antibody of ICAM-1 (Catalog no. AF583, R&D Systems, Minneapolis, MN) and NF-κB (Catalog no. 610868, 610869, R&D) were used. All of these antibodies cross-react with respective rat antigens. SDS-polyacrylamide gel electrophoresis (PAGE) was performed on 12.5% separation gels in the absence of urea and was stained with Coomassie brilliant blue. Proteins on the SDS-PAGE gels, each lane containing 30 μg of total protein, were transferred to nitrocellulose filters. The immunoreactive bands were detected by incubation with the antibody described above, followed by secondary antibody-alkaline phosphatase, and finally with NBT and 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (Roche Diagnostic GmbH, Mannheim, Germany) stock solution for 30 min at room temperature.

Statistical analyses

All values were expressed as mean ± standard error mean (SEM). Differences in parameters

among groups were analyzed with analysis of variance. *Post hoc* analyses were performed by means of the Newman–Keuls test. For all tests, differences were considered significant if $p < 0.05$.

Results

TVN stimulation on substance P (SP) release and functional breathing patterns

In our experiments, the baseline level of ABP was 105–120 mm Hg. Atropine treatment did not significantly increase the ABP in all the animals. TVN stimulation (from 1 to 10 Hz) concomitantly evoked an immediate response in hypotension, enhanced airway EMG activity, apnea or bradypnea (decreased respiratory frequency), and calculated R_L in a frequency-dependent manner (Figure 1). During TVN stimulation, plasma SP from the carotid arterial blood increased (62 ± 9.0, 108 ± 16, 215 ± 29, 278 ± 32 ng/ml at 0, 1, 5, and 10 Hz, respectively). The cardiovascular and breathing pattern data and increased plasma SP confirmed that TVN efferent stimulation releases SP to the respiratory tract and triggered SP-mediated vasodilation and airway hypersensitivity.

Efferent TVN stimulation (0, 1, 5, and 10 Hz) significantly decreased mean ABP (0, 10.2, 27.6, and 37.8%. TVN stimulation (0, 1, 5, and 10 Hz) significantly increased the airway smooth EMG activity (2 ± 0.7, 18 ± 3, 32 ± 4, and $86 \pm 11 \mu V$) as well as calculated R_L (0.2 ± 0.01, 0.9 ± 0.1, 1.3 ± 0.5, and 2.6 ± 0.2 cm H_2O/ml/s) (Figure 2). L-732138, and SR-48968 pretreatment significantly reduced 10 Hz of efferent TVN stimulation in R_L by 30% and 69% and in smooth muscle EMG activity by 40% and 45%, respectively. Pretreatment with DMTU and catechins also significantly reduced 10 Hz of TVN stimulation in R_L by 38% and 62% and in smooth muscle EMG activity by 23% and 24%, respectively (Figure 2). These data suggest that NK_1 and NK_2 receptors and O_2^- and H_2O_2 are involved in efferent TVN stimulation induced airway hypersensitivity.

NK antagonists and antioxidants on TVN stimulation induced blood ROS generation

We used the lucigenin CL (O_2^-) and luminol CL (H_2O_2) method to examine the amounts of ROS evoked by SP in the carotid arterial blood samples. The basal lucigenin and lumino CL level detected from carotid arterial blood was approximately 85–90 counts/10 s and 75–85 counts/10 s, respectively. As shown in Table 1, after 10 min of TVN stimulation, the blood lucigenin CL (O_2^-) and luminol CL (H_2O_2) were increased significantly within 90 min in a frequency-dependent manner. The peak level of blood lucigenin CL (O_2^-) and luminol CL (H_2O_2) were found at 90 min after 10 min of TVN stimulation. The enhanced ROS activity was declined at 180 min after 10 min of TVN stimulation.

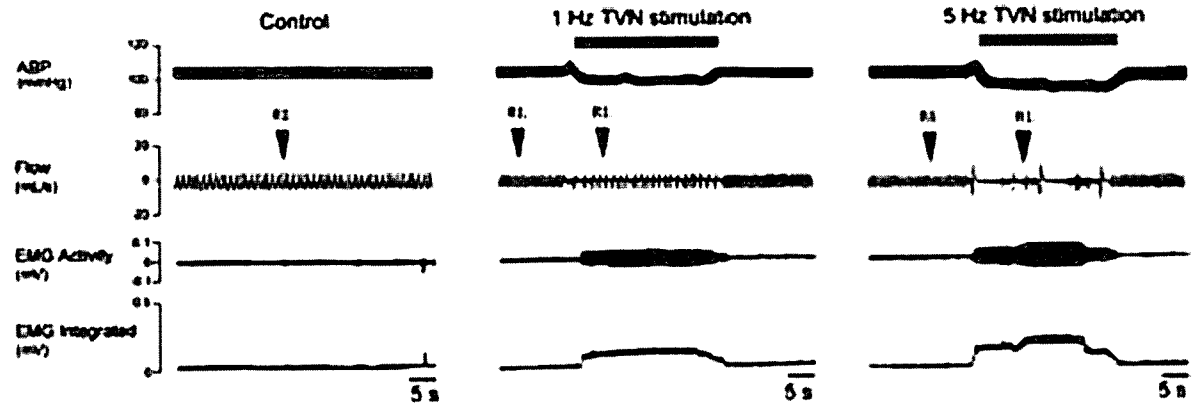


Figure 1. Experimental recordings of responses to TVN stimulation in anesthetized rats. TVN stimulation evokes hypotension (decrease in arterial blood pressure (ABP)), bradypnea (a decrease in respiratory frequency and flow) and augmented electromyographic (EMG) activity in a dose-dependent manner. Downward arrow (▼) indicates time of total pulmonary resistance (R_L) measurement.

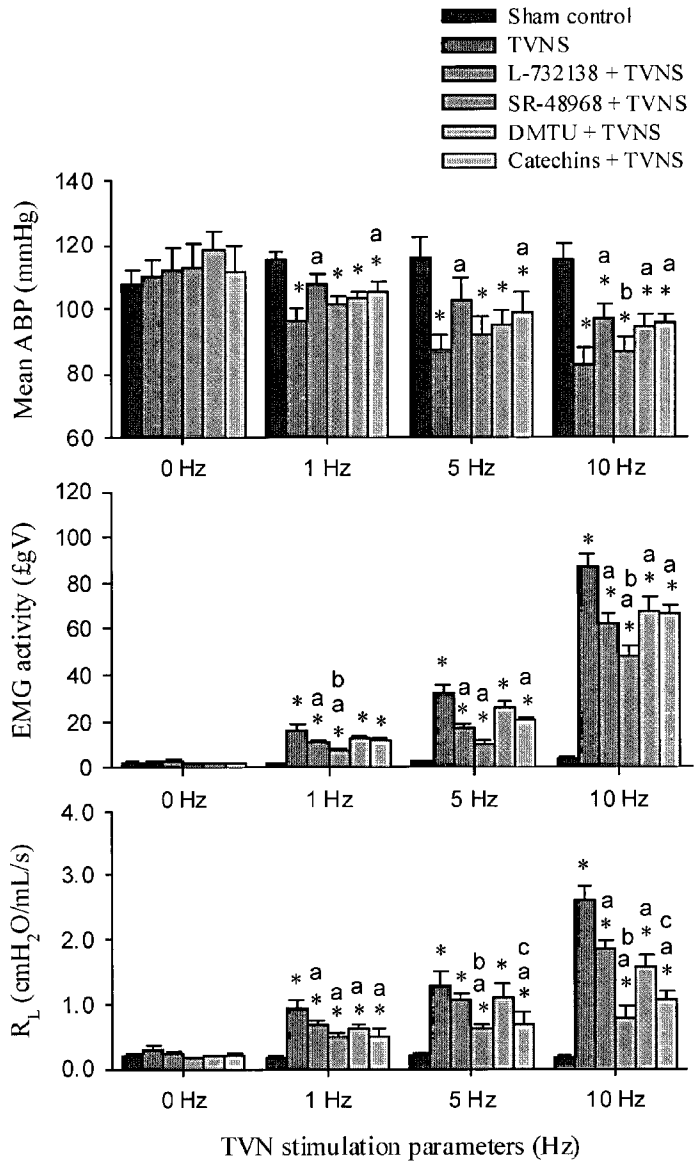


Figure 2. Effects of tachykinin antagonists, DMTU and catechins on TVN stimulation enhanced hypotension in mean arterial blood pressure (ABP), airway smooth muscle EMG activity, and total pulmonary resistance (R_L). TVN stimulation evokes hypotension and increases in EMG activity and R_L in a frequency-dependent manner. Pretreatment of L-732138 (NK_1 receptor antagonist), SR-48968 (NK_2 receptor antagonist), DMTU (H_2O_2 scavenger), or catechins (O_2^- and H_2O_2 scavenger) attenuated the response of hypotension and increases in EMG activity and R_L by TVN stimulation. $^*p < 0.05$ vs. respective baseline control without TVN stimulation. $^ap < 0.05$ vs. TVN stimulation alone. $^bp < 0.05$ SR-48968 vs. L-732138 treatment. $^cp < 0.05$ catechins vs. DMTU treatment. For all tests, significant difference (*,a–c) between the groups was performed by analysis of variance and post hoe contracts by means of the Newman–Keults test.

NK antagonists and antioxidants on TVN stimulation induced plasma extravasation

We explored the possible mechanism how efferent TVN stimulation evoked plasma extravasation in the trachea by silver staining [20] and India ink staining techniques [22, 23]. As shown in Fig. 3B, silver-stained endothelial gaps (indicated by ↑) were significantly observed in the leaky vessels of TVN stimulated trachea when compared to those with sham control (Figure 3a). We also found that the appearance of accumulations of inflammatory

Table 1. ROS activity indicated by lucigenin or luminol chemiluminescence in carotid arterial blood after sham control and different frequency of right TVN stimulation (TVNS).

Sample	Group				
	Sham control	30 min	60 min	90 min	180 min
Lucigenin CL					
1 Hz ($n=5$)	91 ± 7.0	558 ± 27*	809 ± 26*	1136 ± 43*	673 ± 18*
5 Hz ($n=6$)	96 ± 5.0	672 ± 16*	1073 ± 61*a	1989 ± 106*a	796 ± 33*
10 Hz ($n=5$)	85 ± 8.0	965 ± 23*b,c	1557 ± 89*b,c	2847 ± 189*b,c	879 ± 26*
Luminol CL					
1 Hz ($n=5$)	79 ± 7.0	192 ± 23*	359 ± 24*	778 ± 68*	553 ± 17*
5 Hz ($n=6$)	82 ± 11	258 ± 14*	568 ± 20*a	1057 ± 109*a	578 ± 16*
10 Hz ($n=5$)	81 ± 3.0	634 ± 65*b,c	1211 ± 83*b,c	1684 ± 112*b,c	785 ± 21*

Data are displayed as counts/10 s and expressed as mean ± SEM.
*$p \geq 0.05$ vs. sham control value.
[a]$p < 0.05$ between 1 and 5 Hz groups.
[b]$p < 0.05$ between 1 and 10 Hz groups.
[c]$p < 0.05$ between 5 and 10 Hz groups.

cells is indicated in the TVN stimulated trachea (Figure 3b).

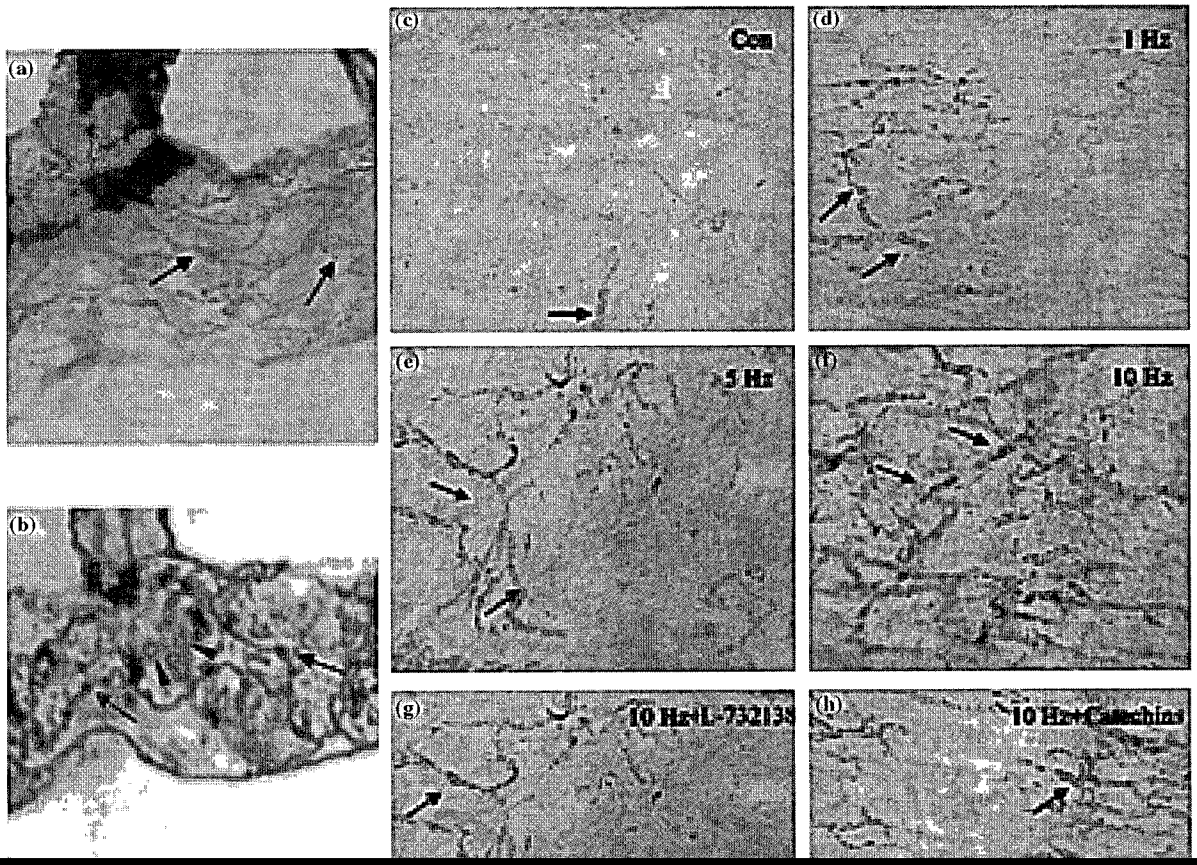
India ink stain can be retained by basement membrane while leakage and can easily be identified as a severity index of neurogenic inflammation [20, 22]. Efferent TVN stimulation increased India ink labeled plasma extravasation in a frequency-dependent manner (Table 2 and Figure 3) when compared to sham control (Figure 3c). This data suggests increased SP may contribute to significant plasma extravasation. As shown in Table 2 and Figure 3, pretreatment of L-732138 significantly ($p < 0.05$) reduced the India-ink intensity of plasma extravasation by 62% (1 Hz), 26% (5 Hz), and 39% (10 Hz), whereas SR-48968 had no significant inhibition on TVN stimulation induced plasma extravasation. Pretreatment of catechins significantly ($p < 0.05$) reduced the India-ink intensity of plasma extravasation by 55% (1 Hz), 36% (5 Hz), and 33% (10 Hz), whereas DMTU did not significantly reduced TVN stimulation induced plasma extravasation.

ROS activates NF-κB and ICAM-1 in trachea and lung by TVN stimulation

SP and ROS triggered early cellular signal transduction pathways responsible for the activation of NF-κB, resulting in up-regulation of the ICAM-1 gene in the insulted tissue [7, 24]. The proinflammatory response of NF-κB translocation and the ICAM-1 protein expression in the right lower trachea and lung obtained at the 90 min period after 10 min of TVN stimulation is displayed in Figure 4. The expression of NF-κB in nuclear protein and ICAM-1 in total protein of the right side of trachea and lung after TVN stimulation was assessed by immunoblotting with antibodies against NF-κB and ICAM-1. Expression of NF-κB (50 and 65 kDa) and ICAM-1 (85 kDa) was detected in control tissues. Efferent TVN stimulation potentiated NF-κB and ICAM-1 expression in a frequency-dependent manner. Pretreatment of L-732138 (NK_1 receptor antagonist), SR-48968 (NK_2 receptor antagonist), DMTU (H_2O_2 scavenger) or catechins (O_2^- and H_2O_2 scavenger) reduced TVN stimulation (10 Hz) induced NF-κB and ICAM-1 upregulation. These results suggest that TVN stimulation evoked proinflammatory response of NF-κB and ICAM-1 expression is triggered by SP evoked NK_1 and NK_2 receptor activation and O_2^- and H_2O_2 signaling.

Discussion

The capsaicin-sensitive, tachykinin-containing afferent fibers innervated the mammalian airway can be activated by electrical stimulation of TVN [20]. The activation of sensory nerve endings enhanced SP release, which subsequently triggered an efferent neural reflex and inflammatory response in the airway [12, 22]. Recently, TVN afferent excitation inhibited SP release in the spinal


(a)
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(d)
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(g)
10 Hz+L-732138
(h)
10 Hz+Catechins

Table 2. Effects of neurokinin receptor antagonists and antioxidants on vascular permeability of rats undergoing sham and different frequency of electrical stimulation of right thoracic vagus nerve (TVNS).

Pretreatment	Sham ($n=3$)	1 Hz ($n=5$)	5 Hz ($n=5$)	10 Hz ($n=5$)
TVNS	1.5±0.2%	8.5±3.4%*	13.4±2.9%*	18.7±4.6%*
L-732138+TVNS	1.9±0.6%	3.2±0.2%*[a]	9.8±1.6%*[a]	11.5±2.7%*[a]
SR-48968+TVNS	1.2±0.7%	6.9±21.%*	12.3±3.1%*	16.9±3.4%*
DMTU+TVNS	1.1±0.1%	6.4±0.9%*	10.7±4.5%*	15.3±2.9%*
Catechins+TVNS	1.6±0.3%	3.8±1.5%*[a]	8.6±1.9%*[a]	12.6±3.2%*[a]

The vascular permeability was quantified by the area density of India ink-labeled blood vessels, which were presented as mean ± SEM in each category.
The p-value was calculated based on the comparison between the same operation group of 1, 5, and 10 Hz.
*$p<0.05$ vs. respective sham control level.
[a]$p<0.05$ vs. TVNS group.

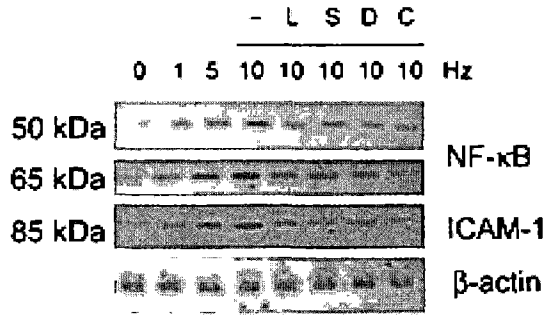

Figure 4. Western blot analysis of NF-κB (65 and 50 kDa) in nuclear protein and ICAM-1 (85 kDa) in total protein in rat bronchus subjected to TVN stimulation. The expression in NF-κB and ICAM-1 was increased by TVN stimulation in a frequency-dependent manner. At 10 Hz TVN stimulation, the enhanced NF-κB and ICAM-1 expression was downregulated by L-732138 (L), SR-48968 (S), DMTU (D), or catechins (C) pretreatment.

tachykinins may activate NK_1 receptor mediated vascular permeability and plasma protein extravasation in the airway [25] and activate NK_2 receptor provoked bronchoconstriction [25, 26]. Our previous data showed that electrical stimulation of TVN from 1 to 10 Hz increased the release of SP, bronchoconstriction (increases in EMG activity and R_L), and India ink-labeled plasma extravasation in the rat trachea in a frequency-dependent manner. Pretreatment of NK_1 (L-732138) or NK_2 receptor antagonists (SR-48968) significantly reduced TVN stimulation enhanced EMG activity and R_L. However, NK_2 receptor antagonist (SR-48968) is more potent than NK_1 receptor antagonist (L-732138) in reduction of bronchoconstriction.

As a consequence of SP-immune and inflammatory cell interaction, a variety of substance, such as histamine, cytokines, and ROS are released [5, 12, 27, 28]. Our previous reports showed that in whole blood and leukocytes incubated with 30 min of different concentration of SP, ROS activity, especially O_2^-, was increased with SP concentration in a dose-dependent manner [5, 29]. ROS are known to be involved in changes in muscle tone, vascular smooth muscle strip contraction [30] and increased neural activity/conduction velocity in vitro by mechanisms such as alterations in membrane conductance, calcium homeostasis, calcium-dependent processes, and eicosanoid and nitric oxide metabolism [31]. In our study, we first demonstrated that efferent TVN stimulation enhanced SP-induced O_2^- and H_2O_2 ROS activity by the adhered leukocytes in the circulation of respiratory tract *in vivo*. The application of dimethylthiourea (H_2O_2 scavenger) or catechins (O_2^- and H_2O_2 scavenger) significantly reduced TVN stimulation evoked bronchoconstriction, however, catechins seem to be more potent than dimethylthiourea in reduction of bronchoconstriction. On the other hand, efferent TVN stimulation displayed significant endothelial gaps, accumulations of inflammatory cells, and enhanced plasma extravasation in the trachea. The appearance of inflammatory cells in the trachea may be the origin of increased ROS activity. Pretreatment of catechins, but not of dimethylthiourea, significantly inhibited TVN stimulation induced plasma extravasation. These results suggest that SP release by TVN stimulation evoked O_2^- and H_2O_2 mediated bronchoconstriction and O_2^--mediated plasma extravasation in the rat trachea.

In organs subjected to oxidative stress, the overproduced ROS triggered early cellular signal

transduction pathways responsible for the activation of NF-κB, resulting in up-regulation of the ICAM-1 gene in the vascular endothelium and subsequent tissue accumulation of activated neutrophil accumulation [7, 32, 33]. In our study, TVN stimulation increased NF-κB expression in nuclear protein, resulting in up-regulation of the ICAM-1 protein in the respiratory tract (trachea and lung). Antidromic TVN stimulation can enhance SP release from the sensory nerve ending of lower trachea and lung epithelial cells. SP induces the synthesis of proinflammatory cytokines, such as interleukin-6, tumor necrosis factor-α, or interleukin-12 in monocyte/macrophages [4, 23, 27], induces histamine release from mast cells [5], and the expression of cell adhesion molecules in endothelial cells [24, 34]. Three different cell type-specific pathways leading to NF-κB activation by cytokines. Interleukin-1 stimulation of lymphoid cells generates ROS, which are required for IκB-α degradation and NF-κB activation [27]. In monocytes, ROS are produced in response to interleukin-1 and are necessary for NF-κB activation. Finally, epithelial cells do not generate ROS after interleukin-1 stimulation, but do rapidly activate NF-κB through an acidic sphingomyelinase/ceramide-dependent transudation pathway [35]. It is thought that the release p50/p65 NF-κB dimmer can translocate to the nucleus and activate target genes by binding with high affinity to NF-κB elements in their promoters [36]. It has been reported that ROS such as O_2^- and H_2O_2 accelerate leukocyte adherence to endothelial cells, enhance transendothelial migration of leukocytes, and increase the expression of such adhesion molecules as ICAM-1 on endothelial cells [5, 37]. ROS not only increase microvascular leakage [22] but also heightens inflammatory cell-endothelial cell interaction [5, 7]. This enhanced expression in NF-κB and ICAM-1 can be partly abrogated by NK_1 receptor antagonist, NK_2 receptor antagonist, dimethylthiourea, and catechins, which exert antioxidant (scavenging ROS activity) and anti-inflammatory activity on TVN stimulation enhanced oxidative stress. However, NK_1 receptor antagonist and catechins are more efficient in reduction of plasma extravasation, indicating a regulatory role of NK_1 receptor activation and O_2^- production during TVN stimulation.

In summary, this study suggests that efferent TVN stimulation enhances SP release to trigger NK_1 and NK_2 receptors mediated immediate responses in vasodilation, airway smooth muscle contraction, increases in total pulmonary resistance, appearance of endothelial gaps, plasma extravasation, and accumulations of inflammatory cells in the respiratory tract. The increased oxidative stress evoked by increased SP and accumulated inflammatory cells could enhance the expression in proinflammatory NF-κB and ICAM-1, and production of O_2^- and H_2O_2 activity in the respiratory tract of atropine-treated rats. Selective NK receptor antagonists and antioxidants pretreatment can attenuate TVN stimulation evoked airway hyperactivity, proinflammatory response, and oxidative stress.

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