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## Low Voltage Vagal Nerve Stimulation Reduces Bronchoconstriction in Guinea Pigs Through Catecholamine Release

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### Abstract

**Objective**—Electrical stimulation of the vagus nerve at relatively high voltages (e.g., >10V) can induce bronchoconstriction. However, low voltage (2V) vagus nerve stimulation (VNS) can attenuate histamine-invoked bronchoconstriction. Here, we identify the mechanism for this inhibition.

**Methods**—In urethane-anesthetized guinea pigs, bipolar electrodes were attached to both vagus nerves and changes in pulmonary inflation pressure were recorded in response to i.v. histamine and during VNS. The attenuation of the histamine response by low-voltage VNS was then examined in the presence of pharmacologic inhibitors or nerve ligation.

**Results**—Low-voltage VNS attenuated histamine-induced bronchoconstriction ( $4.4 \pm 0.3$  vs.  $3.2 \pm 0.2$  cm H<sub>2</sub>O,  $p < 0.01$ ) and remained effective following administration of a nitric oxide synthase inhibitor, NG-nitro-L-arginine methyl ester, and after sympathetic nerve depletion with guanethidine, but not after the  $\beta$ -adrenoceptor antagonist propranolol. Nerve ligation caudal to the electrodes did not block the inhibition but cephalic nerve ligation did. Low-voltage VNS increased circulating epinephrine and norepinephrine without but not with cephalic nerve ligation.

**Conclusion**—These results indicate that low-voltage VNS attenuates histamine-induced bronchoconstriction via activation of afferent nerves, resulting in a systemic increase in catecholamines likely arising from the adrenal medulla.

### Keywords

Asthma; bronchoconstriction; catecholamine; guinea pig; vagus nerve stimulation

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Authorship Statements

All authors contributed to the design, conduction, and interpretation of this study. Each has reviewed and approved the version to be published.

## INTRODUCTION

Asthma is a complex disorder characterized by chronic inflammation, edema, and airway smooth muscle hyperresponsiveness that can be triggered by both allergic and nonallergic processes. However, despite improvements in treatments and in the understanding of its etiology, asthma continues to be a major health burden, affecting 8.5% of the population in the USA (1). Recently, vagus nerve stimulation (VNS) using a percutaneous device (Resolve RPS-1000 Stimulator and Proximity Electrode, ElectroCore LLC, Morris Plains, NJ, USA) was tested in patients with acute asthma exacerbations (ClinicalTrials.gov Identifier: NCT00762931) under an FDA Investigational Device Exemption. Although this phase 1 study was limited in size and scope, the use of percutaneous VNS was associated with respiratory improvements as measured by forced expiration volume in 1 sec and perceived dyspnea (2,3). Following the completion of this study, a new transcutaneous VNS device was developed for the treatment of bronchoconstriction that is approved for use outside the United States.

The rationale for these devices was based on our previous study that found low-voltage VNS reduced histamine-induced broncho-constrictive responses in guinea pigs and swine (4). Here, we elucidate the mechanism for this VNS-mediated reduction of histamine-induced bronchoconstriction in guinea pigs *in vivo*.

## MATERIALS AND METHODS

### Animals

Male Hartley guinea pigs (400–500 g) were acclimated to our facility for >2 weeks prior to use. They were housed in pairs at 22°C on a 12-hour light/dark cycle and provided food and water ad libitum. All animal experiments were performed under protocols approved by the Animal Care and Use Committee of Columbia University (New York, NY) and in accordance with standards established by the U.S. Animal Welfare Acts set forth in the National Institutes of Health guidelines. Animals were randomly selected to receive different treatments as defined below.

### Procedure

This study utilized a well-characterized guinea pig model of bronchoconstriction and procedures previously described (4–6). Briefly, animals were anesthetized with intraperitoneal injections of urethane (1.5 ± 0.2 g/kg) which has a long duration (>8 hours) and is without respiratory nerve interference (7). Presurgical depth of anesthesia was monitored via toe pinch reflex and respiration rate, with additional anesthesia dosed (0.2 g/kg) until the animal was no longer responsive to stimulation. Body temperature was maintained at 37°C through the use of a homeothermic heating pad. A tracheostomy was performed using a 14G angiocatheter connected to a microventilator (Model 683, Harvard Apparatus Co., South Natick, MA, USA) to provide positive pressure, constant volume ventilation (62 strokes/min, tidal volume 2.5 cc). A side tap between the ventilator and the tracheal cannula enabled airway pressure recording using a pressure transducer and data acquisition system (TSD160C with model MP100A, Biopac Systems Inc., Goleta, CA, USA). Bilateral external jugular vein catheters, using PE-50 tubing, provided independent access for continuous infusion of a paralyzing agent, succinylcholine, and for histamine, acetylcholine, or pre-treatment dosing of inhibitors. Succinylcholine (1.25 mg/kg bolus then 5 mg/kg/hour) removed any confounding effects of chest wall and diaphragm muscle contractions on airway pressure measurements. Following muscle paralysis, hemodynamic changes (hypertension and tachycardia) were monitored to assure that the depth of anesthesia was sufficient. The left carotid artery was cannulated with PE-50 tubing for

monitoring blood pressure and heart rate (HR) (Model TSD104A pressure transducer, Biopac Systems). Both cervical vagus nerves were isolated and vertically elevated above the surrounding tissue by applying slight upward traction to custom bipolar silver electrodes. This insured consistent electrical contact with the nerves while avoiding undesired electrical stimulation of surrounding structures. In some studies, both nerves were tightly ligated with 4-0 silk sutures but not transected, which ensured consistent contact pressure with the electrodes. Saline-soaked cotton was placed alongside the nerves to prevent drying. The electrodes were then connected to a Grass S88 stimulator (Grass Technologies, Quincy, MA, USA). Airway and blood pressure measurements were captured and analyzed using Acknowledge software, version 3.90 (Biopac Systems, Inc.). Following preparation, animals were allowed to stabilize for >20 min before testing commenced.

## Treatments

Intravenous bolus histamine (4–16 $\mu$ g/kg) and acetylcholine (4–10 $\mu$ g/kg) were dosed using a syringe pump (Model 11 Plus, Harvard Apparatus Co., South Natick, MA, USA) and titrated to elicit a ~2–6 cm H<sub>2</sub>O increase in pulmonary inflation pressure (Ppi). At these doses, the bronchoconstriction responses were rapid and spontaneously resolved in less than 1 min. If airway pressure failed to completely return to baseline, the expiratory port of the ventilator was occluded for 3–4 breaths to re-expand atelectatic lungs and subsequent challenges delayed to guard against deep inspiration effects (8).

## VNS

To evaluate the effect of low-voltage VNS on histamine-induced increases in Ppi, VNS (25 Hz, 0.2 msec pulse duration and 0.5–2 V) was applied starting 20 sec prior to the histamine challenge and continued for 10 sec past the peak response. In each animal a voltage was chosen that minimally influenced blood pressure but reduced histamine-induced increases in Ppi. In one group of animals, the duration of low-voltage VNS effectiveness was tested where the VNS stimulation was applied for 30 sec then discontinued for 10 sec prior to the histamine challenge. The ability of low-voltage VNS to attenuate histamine-induced increases in Ppi was also tested, with the vagal nerves ligated by silk sutures either cephalic or caudal to the electrodes. In some animals, electrical stimulation of both vagal nerves with a relatively high voltage (10 V) and brief pulse trains (25 Hz, 0.2 msec pulse duration for 7 sec) was used to confirm the classic parasympathetic responses including bronchoconstriction and bradycardia.

To investigate the mechanism of action for low-voltage VNS effects on intravenous histamine-induced bronchoconstriction, atropine (1 mg/kg i.v.), *N*<sub>G</sub>-nitro-L-arginine methyl ester (L-NAME) (50 mg/kg i.v.), guanethidine (10 mg/kg i.v.), and propranolol (1 mg/kg i.v.) were administered as indicated. To examine the mechanism for the increase in blood pressure by low-voltage VNS, phentolamine (3 mg/kg i.v.) was administered as indicated.

Animals served as their own controls, with bronchoconstriction to histamine or acetylcholine compared with and without low-voltage VNS stimulation. The extent of pulmonary and hemodynamic changes following histamine challenge was determined using AcqKnowledge Software (Biopac Systems Inc.). To account for secretion-induced spike artifacts found on some tracings, all airway pressure waveforms were first filtered by replotting as maximum peak to peak waveforms followed by the application of a 1-sec smoothing algorithm. The Ppi response was then calculated as the difference between the baseline peak value over a 20-sec period preceding vs. the peak value during the histamine or acetylcholine response. To guard against adaptation, all VNS + histamine or VNS + acetylcholine challenges were preceded by and followed by control (no nerve stimulation) histamine or acetylcholine challenges. If these controls varied by >20%, the comparison was

rejected. Multiple bracketed measurements were obtained per animal and averaged to yield a single value for that animal. This averaged single value from each animal was then compared between animals.

The blood pressure data were analyzed to generate a mean arterial blood pressure (MAP) waveform and HR waveform with each, including a 5-sec smoothing algorithm. The reported values represent the percent change from a 20-sec pre-dose baseline.

### Catecholamine Levels

Blood samples were obtained from the carotid artery catheter at baseline and during low-voltage VNS, pre-histamine dosing, and immediately placed on ice. All samples were collected into 1 mM ethylenediaminetetraacetic acid, centrifuged for 15 min,  $2000 \times g$  at  $4^{\circ}\text{C}$ , and plasma was stored at  $-20^{\circ}\text{C}$  until analyzed by a dual adrenaline/noradrenaline ELISA kit (Labor Diagnostika Nord GmbH & Co. KG, Nordhorn, Germany) according to the manufacturer's instructions. In each animal, all pre-stimulation (baseline) sample results were averaged and all samples collected during stimulation were averaged. The mean value from each condition for each animal was then compared.

### Chemicals

Urethane, histamine, acetylcholine, atropine, L-NAME, guanethidine, phentolamine, and propranolol were obtained from Sigma-Aldrich (St. Louis, MO, USA) and made up in 0.9% saline. Succinylcholine (Hospira, Lake Forest, IL, USA) was diluted to 1 mg/mL with 0.9% saline and continuously infused via a Medfusion 2010 microinfuser pump (Medex, Kensington, MD, USA).

### Statistics

Results are reported as means  $\pm$  standard errors and as percent change from baseline (pre histamine challenge). For significance, pairwise comparisons were performed using a two-tailed Student's *t* distribution with 95% confidence intervals.

## RESULTS

### Model Characteristics

The application of low-voltage bilateral stimulation ( $2\text{V}$ ,  $25\text{ Hz}$ ,  $0.2\text{ msec}$  pulse width) to the vagus nerves did not increase resting pulmonary airway pressure. When the voltage was increased to 10 volts ( $25\text{ Hz}$ ,  $0.2\text{ msec}$  pulse width for 7 sec), stimulation induced the expected parasympathetic responses, including bronchoconstriction (airway pressure:  $8.8 \pm 0.2$  vs.  $15.8 \pm 1.7\text{ cm H}_2\text{O}$ ,  $p < 0.01$ ,  $N = 21$ , before VNS vs. during high-voltage VNS, respectively), hypotension (MAP:  $47.6 \pm 2.8$  vs.  $33.0 \pm 1.7\text{ mmHg}$ ,  $p < 0.01$ ), and bradycardia (HR:  $310 \pm 6$  vs.  $138 \pm 10\text{ bpm}$ ,  $p < 0.01$ ).

The low-voltage stimulation did not accentuate responses to histamine or acetylcholine. In contrast, when bronchoconstriction was induced using histamine, low-voltage VNS significantly reduced the Ppi increase ( $4.4 \pm 0.3$  vs.  $3.2 \pm 0.2\text{ cm H}_2\text{O}$ ,  $N = 26$ ,  $p < 0.01$ ) (Fig. 1). In a group of animals that were challenged with i.v. acetylcholine, low-voltage VNS also reduced the bronchoconstrictive response ( $4.8 \pm 0.9$  vs.  $3.1 \pm 0.6\text{ cm H}_2\text{O}$ ,  $N = 6$ ,  $p < 0.05$ ) (Fig. 1).

To determine the degree of contraction attributable to histamine inducing parasympathetic nerve acetylcholine release vs. histamine directly activating histamine receptors on airway smooth muscle, the muscarinic acetylcholine receptor antagonist, atropine, was administered at the conclusion of selected experiments. Atropine significantly reduced the

bronchoconstriction response to histamine ( $4.5 \pm 1.1$  vs.  $0.9 \pm 0.2$  cm H<sub>2</sub>O,  $N = 8$ ,  $p < 0.01$ ), indicating that the predominant mechanism of bronchoconstriction following i.v. histamine was via activation of parasympathetic nerves inducing the release of acetylcholine.

### Pharmacologic Inhibitors

The nitric oxide synthase inhibitor, L-NAME, was administered in seven animals to deplete the inhibitory non-adrenergic non-cholinergic iNANC nerves of nitric oxide to determine their role in the VNS reduction of the histamine response. In these studies, L-NAME significantly increased baseline blood pressure ( $49 \pm 3$  vs.  $101 \pm 9$  mmHg,  $p < 0.01$ ) and the airway responsiveness to histamine ( $3.3 \pm 0.4$  vs.  $5.8 \pm 0.8$  cm H<sub>2</sub>O,  $p < 0.01$ ), as similarly reported by others (9,10). However, L-NAME did not block the ability of low-voltage VNS to attenuate bronchoconstriction ( $5.8 \pm 0.8$  vs.  $4.5 \pm 0.7$  cm H<sub>2</sub>O,  $p < 0.05$ ) (Fig. 2). To verify that the low-voltage VNS response was not through sympathetic nerves, guanethidine was used to inhibit norepinephrine release from presynaptic terminals and adequate dosing confirmed through observed sustained dramatic decreases in blood pressure. Guanethidine pretreatment did not prevent VNS from attenuating histamine-induced bronchoconstriction ( $6.1 \pm 1.2$  vs.  $4.1 \pm 0.8$  cm H<sub>2</sub>O,  $N = 6$ ,  $p < 0.05$ ) (Fig. 2). Guanethidine induced a dramatic and sustained decrease in blood pressure, indicating effective blockade of sympathetic nerves. The contribution of  $\beta$ -adrenoceptors on airway smooth muscle to the VNS attenuation of histamine-induced bronchoconstriction was examined using the nonselective  $\beta$  antagonist, propranolol. Propranolol pretreatment increased the Ppi response to histamine ( $4.2 \pm 1.3$  vs.  $10.1 \pm 2.4$  cm H<sub>2</sub>O,  $N = 6$ ,  $p < 0.05$ ) as others have also reported (11). Subsequent i.v. histamine doses were reduced to compensate for this elevated response of Ppi to histamine before testing the effect of low-voltage VNS in the presence of propranolol. After propranolol treatment, low-voltage VNS was no longer effective in attenuating the histamine bronchoconstriction ( $7.7 \pm 2.8$  vs.  $7.8 \pm 2.9$  cm H<sub>2</sub>O,  $N = 6$ , not significant [NS]) (Figs. 2 and 3).

Low-voltage VNS can increase blood pressure (3), and for most studies we titrated VNS to a level that reduced histamine bronchoconstriction while minimizing blood pressure elevation. However, to examine pressor effects more closely, in selected studies we used voltages that increased MAP and then compared the response of blood pressure with low-voltage VNS in the presence of phentolamine. With phentolamine pretreatment, the low-voltage VNS-induced increase in MAP was eliminated ( $20.8 \pm 2.1\%$  [VNS] vs.  $1.8 \pm 0.5\%$  [VNS after phentolamine pretreatment],  $N = 3$ ,  $p < 0.01$ ).

### Vagus Nerve Ligation

Bilateral vagus nerve ligation was used to identify the neural pathways governing the VNS response. When nerve ligation was caudal to the electrodes, low-voltage VNS reduced histamine-induced airway bronchoconstriction ( $3.9 \pm 0.9$  vs.  $2.1 \pm 0.6$  cm H<sub>2</sub>O,  $N = 7$ ,  $p < 0.05$ ) (Fig. 2). In contrast, when nerve ligation was cephalic to the electrode, low-voltage VNS did not reduce the histamine response ( $2.3 \pm 0.6$  vs.  $2.3 \pm 0.6$  cm H<sub>2</sub>O,  $N = 3$ , NS) (Figs. 2 and 4), indicating that an afferent nerve signal traveling toward the central nervous system was mediating the low-voltage VNS attenuation of histamine-induced bronchoconstriction.

### Low-Voltage VNS Bronchoprotection after Discontinuation of VNS

In four animals, low-voltage VNS was applied for 30 sec then discontinued for 10 sec prior to the injection of histamine and was compared with leaving the VNS on for 20 sec before and through the histamine injection. Low-voltage VNS remained effective at inhibiting bronchoconstriction when the VNS signal was interrupted 10 sec prior to i.v. histamine ( $3.6 \pm 0.7$  vs.  $2.6 \pm 0.6$  cm H<sub>2</sub>O,  $p < 0.05$ ), and was similar to applying VNS continuously during

i.v. histamine ( $3.8 \pm 0.8$  vs.  $2.3 \pm 0.6$  cm H<sub>2</sub>O,  $p < 0.05$ ) (histamine vs. histamine with VNS, respectively) (Fig. 5).

### Catecholamine Analysis

Low-voltage VNS increased plasma epinephrine levels above baseline ( $1.67 \pm 0.38$  vs.  $2.51 \pm 0.56$  ng/mL,  $N = 4$ ,  $p < 0.05$ ) and norepinephrine levels above baseline ( $2.82 \pm 0.37$  vs.  $3.22 \pm 0.33$  ng/mL,  $N = 4$ ,  $p < 0.05$ ). In three animals following cephalic nerve ligation, low-voltage VNS no longer increased catecholamine levels (epinephrine:  $0.93 \pm 0.10$  vs.  $0.79 \pm 0.17$  ng/mL, NS, and norepinephrine:  $2.73 \pm 0.55$  vs.  $2.40 \pm 0.62$  ng/mL, NS) (Fig. 6).

## DISCUSSION

The primary findings of the current study are that low-voltage nerve stimulation of guinea pig cervical vagus nerves attenuates histamine-induced increases in Ppi and that this attenuation required an intact afferent (but not efferent) vagus nerve. Secondly, the low-voltage VNS attenuation of the histamine-induced bronchoconstriction could be blocked by systemic pretreatment with a  $\beta$ -adrenoceptor antagonist. In contrast, pretreatment with a nitric oxide synthase inhibitor or depletion of sympathetic nerve terminals did not interfere with low-voltage VNS attenuation of histamine-induced increases in Ppi. Additionally, low-voltage VNS was able to attenuate acetylcholine-induced increases in Ppi. These findings confirm our previous report and identify a likely mechanism to account for this protective effect of low-voltage VNS on bronchoconstriction in our animal studies and in early human clinical trials.

Classically, stimulation of the vagus nerve with relatively high voltages (10–25 V) results in direct bronchoconstriction due to the release of acetylcholine from efferent parasympathetic nerves traveling in the cervical vagus nerve to the lung (5,6,12,13). Additionally, this high-voltage stimulation induces bradycardia (5,14). However, we have demonstrated that lower voltages attenuated histamine-induced bronchoconstriction without reaching the threshold for stimulating efferent parasympathetic lung and heart responses.

We tested whether the attenuation of histamine-induced increases in Ppi was mediated by efferent stimulation of sympathetic nerve fibers traveling within the cervical vagus nerve or by indirect stimulation of iNANC nerves in the guinea pig lung. Our results rule out both of these possibilities since ligation of the vagal nerve caudal to the electrodes did not eliminate the bronchoprotection afforded by low-voltage VNS. Moreover, pretreatment with guanethidine or L-NAME to block norepinephrine or nitric oxide release from sympathetic or iNANC nerves, respectively, did not block the bronchoprotection afforded by low-voltage VNS.

Intravenously administered histamine binds to H<sub>1</sub> receptors on the vagus nerve, inducing the release of acetylcholine, which binds to muscarinic receptors on airway smooth muscle causing constriction (15). Additionally, histamine binds directly to histamine receptors on airway smooth muscle cells, resulting in constriction. In the present study, atropine pretreatment blocked the vagal nerve contribution to the bronchoconstriction (by antagonizing the effect of acetylcholine at the airway smooth muscle), revealing that the direct airway smooth muscle response to histamine comprised ~20% of the contractile response. Direct bronchoconstriction following activation of airway smooth muscle muscarinic receptors with i.v. acetylcholine was also attenuated by low-voltage VNS. This suggests that the mechanism of bronchoprotection afforded by low-voltage VNS did not involve efferent parasympathetic fibers in the vagus nerve but was by an alternative neural pathway.

The histamine-induced bronchoconstriction in this study had a rapid onset and recovery such that each airway and blood pressure response was completed in less than 1 min. In preliminary tests, we observed that low-voltage VNS was more effective if it preceded the histamine challenge than if administered simultaneously; therefore, we used a 20-sec pretreatment in our mechanistic investigations. In addition, we found in selected studies that low-voltage VNS remained effective even if discontinued 10 sec prior to the histamine challenge. This prolonged effectiveness is contrary to normal fast neural responses exhibited by sympathetic and parasympathetic nerves. However, it is consistent with the characteristics of iNANC nerves, which have a slow response with long duration (11,16–18).

iNANC nerves release nitric oxide and are capable of modulating both tracheal (19–21) and bronchial (17,22) smooth muscle tone. The synthesis of nitric oxide and its modulation of airway tone can be inhibited by L-NAME (9,21,22). However, in our study, VNS remained capable of attenuating bronchoconstriction in the presence of L-NAME. Since we had concerns with the ability of reagents to fully block other iNANC neurotransmitters such as vasoactive intestinal peptide, we performed vagal nerve ligation caudal to the electrodes to interrupt all efferent iNANC control of airway responses. Despite ligation, low-voltage VNS remained effective in attenuating bronchoconstriction.

In order to demonstrate iNANC nerve function, it is necessary to first isolate the response using cholinergic inhibitors, adrenergic inhibitors, and, frequently, beta antagonists prior to eliciting iNANC responses (16,23–25). These inhibitors were not necessary to demonstrate the inhibition of the histamine response by VNS in this study. Indeed, it was the failure of propranolol to attenuate VNS-induced relaxation of guinea pig tracheal pouch pressure that led Chesrown et al. (26) to conclude that their mechanism was iNANC nerves. In our study, propranolol did attenuate the low-voltage VNS reduction of bronchoconstriction. Thus, while the contribution of iNANC nerves might not have been completely eliminated, they were not the primary pathway responsible for the low-voltage VNS inhibition.

Another possible mechanism for the reduction of the histamine response during low-voltage VNS could be the release of norepinephrine from the sympathetically innervated pulmonary vasculature (27–29), with subsequent norepinephrine binding to  $\beta_2$ -adrenoreceptors on airway smooth muscle (30). This norepinephrine outflow can also bind to  $\alpha_1$  adrenergic receptors on vascular smooth muscle to increase blood pressure. When we administered the  $\alpha_1$  antagonist, phentolamine, the blood pressure response from low-voltage VNS was eliminated. However, when we administered guanethidine to deplete sympathetic nerves but not interfere with catecholamine release from the adrenal glands (31–33), low-voltage VNS remained effective at attenuating histamine-induced increases in Ppi. In addition, vagus nerve ligation between the electrode and the pulmonary vasculature would interrupt this nerve pathway and norepinephrine's release yet the VNS bronchoprotection remained. Thus, it is unlikely that this efferent sympathetic pathway was responsible for the low-voltage VNS effect.

In contrast to efferent vagus nerve ligation, ligation cephalic to the electrode blocked afferent nerve transmission and eliminated the ability of low-voltage VNS to attenuate histamine-induced increases in Ppi. Furthermore, we noticed that cephalic but not caudal ligation interfered with the ability of low-voltage VNS to increase blood pressure. The adrenal medulla is a source for both circulating norepinephrine and epinephrine and is innervated by preganglionic sympathetic splanchnic nerves. Afferent VNS has been shown to reach the hypothalamus and activate the hypothalamic-pituitary-adrenal axis (34), and direct electrical stimulation of the hypothalamus can induce the adrenal medulla to release epinephrine (35). Thus, in the present study, afferent low-voltage VNS stimulation may have

led to efferent stimulation of the adrenal medulla and the release of circulating catecholamines, which could explain the effects on airway and blood pressure responses.

The peak measured serum concentration of epinephrine in the present study was 2.35 ng/mL, equating to a concentration of approximately 18 nM. This concentration is within the reported  $K_d$  of epinephrine for the  $\beta_2$ -adrenoceptor in some studies (23.9 nM) (36) but falls well below the reported  $K_d$  (200 nM) for epinephrine binding at the  $\beta$ -adrenoceptor in other studies (37). However, significant bronchodilation has been reported in humans at a serum concentration of epinephrine similar to that reported in the present study (38). Additionally, pharmacologic receptor-effector studies have suggested the presence of “spare  $\beta$ -adrenoceptors receptors” (39) and the concept that with excess receptors present, ligand occupancy of only a small fraction of the receptor pool is necessary for full activation of the downstream cellular signaling pathway. Although spare  $\beta$ -adrenoceptors have been reported in airway smooth muscle (40,41), overexpression studies suggest the opposite (42): that the quantity of  $\beta$ -adrenoceptors present in airway smooth muscle is the rate-limiting step in activation of the  $\beta$ -adrenoceptor- $G_s$ -adenylyl cyclase signaling pathway. Additional molecular explanations for this apparent discrepancy is a concept of a rapid off rate in the kinetics of epinephrine binding such that a single molecule of ligand can rapidly and sequentially activate neighboring receptors, amplifying the signaling event, or that a single activated receptor can activate multiple molecules of the  $G_s$  protein, again leading to amplification of the signaling event (43).

Parasympathetic pulmonary nerves are normally tonically active and trigger smooth muscle contractions in response to ongoing afferent nerve activity (20,44), while circulating catecholamines have been reported to provide tonal balance (38,45–47). Using adrenalectomy techniques, several investigators (30,48,49) linked these circulating catecholamines to adrenal release and binding to  $\beta_2$ -adrenoceptors to reverse bronchoconstriction. Other studies have indicated a possible impaired sympathoadrenal response to exercise in asthmatics (50,51). Both epinephrine and norepinephrine bind to  $\beta_2$ -adrenergic receptors, and blocking these receptors with propranolol can induce bronchoconstriction in asthmatics. We found that the bronchoconstriction response to histamine was augmented by propranolol, as did others (11,30,52), while Matsu-moto (53) reported a similar finding for acetylcholine. The finding that the VNS bronchoprotection was eliminated by propranolol but not guanethidine suggests circulating catecholamines as the common modality in this and previous studies. This would also explain the temporal nature of the low-voltage VNS response that required VNS treatment to commence several seconds prior to the histamine challenge but which remained effective at reducing the histamine response even after terminating stimulation.

In an isolated lung perfusion model, it was found that during sympathetic electric field stimulation, norepinephrine levels were increased while epinephrine levels remained below detection (29). This is in agreement with the general view that the adrenal medulla is the primary source of circulating epinephrine. Since we measured an increase in plasma epinephrine levels with stimulation, it suggests that we were indeed triggering an efferent sympathetic stimulation of adrenal chromaffin cells. Furthermore, the adrenal gland secretes proportionally more epinephrine than norepinephrine. In this study, we found epinephrine made up 68.0% of the increase in catecholamines.

There are several limitations to our study. The increases in catecholamine concentrations we reported were modest, but this was in keeping with the study design. The guinea pig model of bronchoconstriction used here utilized urethane anesthesia, which is known to accentuate blood pressure elevations from stimulation (54). In addition, increases in VNS voltage could negatively affect the HR and blood pressure. We therefore adjusted



stimulation to minimize these effects. Although the caudal ligation technique would minimize the neuronal chronotropic and inotropic cardiac contribution, it would not interfere with the afferent mediated central nervous system responses leading to adrenal medulla stimulation. It is possible that higher voltages could increase the secretion of circulating catecholamines and have greater utility for treating bronchoconstriction without cardiovascular implications.

In a study of conscious individuals with an implanted vagus nerve stimulator for treating epilepsy, a test with increasing levels of stimulation was not associated with increases in blood pressure and HR due to the natural ability of the body to compensate (55). In two studies using low-dose infusions of epinephrine (38,45), the infusions produced dose-related bronchodilation in asthmatic patients prior to any changes in blood pressure or HR. However, norepinephrine was not effective (45). Therefore, the increase in epinephrine levels from low-voltage VNS that we found may have utility for treating asthma without concurrent changes in blood pressure or HR.

## CONCLUSION

This study has confirmed that low-voltage vagal nerve stimulation can attenuate histamine-induced bronchoconstriction in guinea pigs. The primary mechanism for this effect is not through blockade of efferent vagally induced bronchoconstriction, nor iNANC nerves, nor norepinephrine outflow from sympathetic innervated pulmonary vasculature. Rather, this study suggests that the response is through an afferent nerve pathway leading to efferent stimulation of chromaffin cells in the adrenal medulla and the release of catecholamines into the systemic circulation. These catecholamines, especially epinephrine, bind to  $\beta_2$ -adrenergic receptors to relax airway smooth muscle. The induced release of low levels of epinephrine by VNS would be in a manner that is tolerable and without local complications associated with epinephrine infusions. This mechanism may explain the improvements in FEV<sub>1</sub> and the perception of dyspnea that were associated with percutaneous VNS in asthma patients that had been reported (3), but this must be confirmed in future studies.

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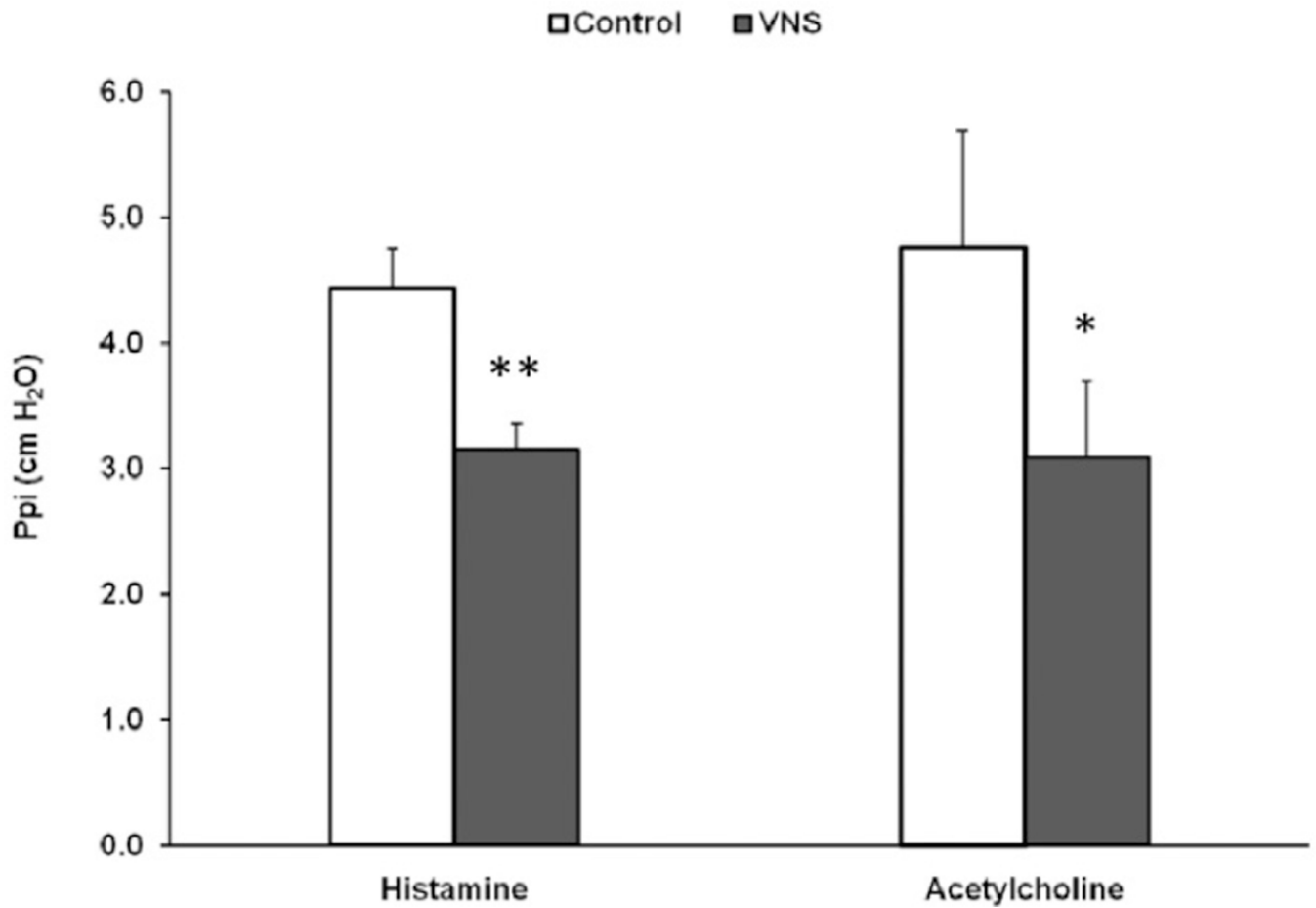
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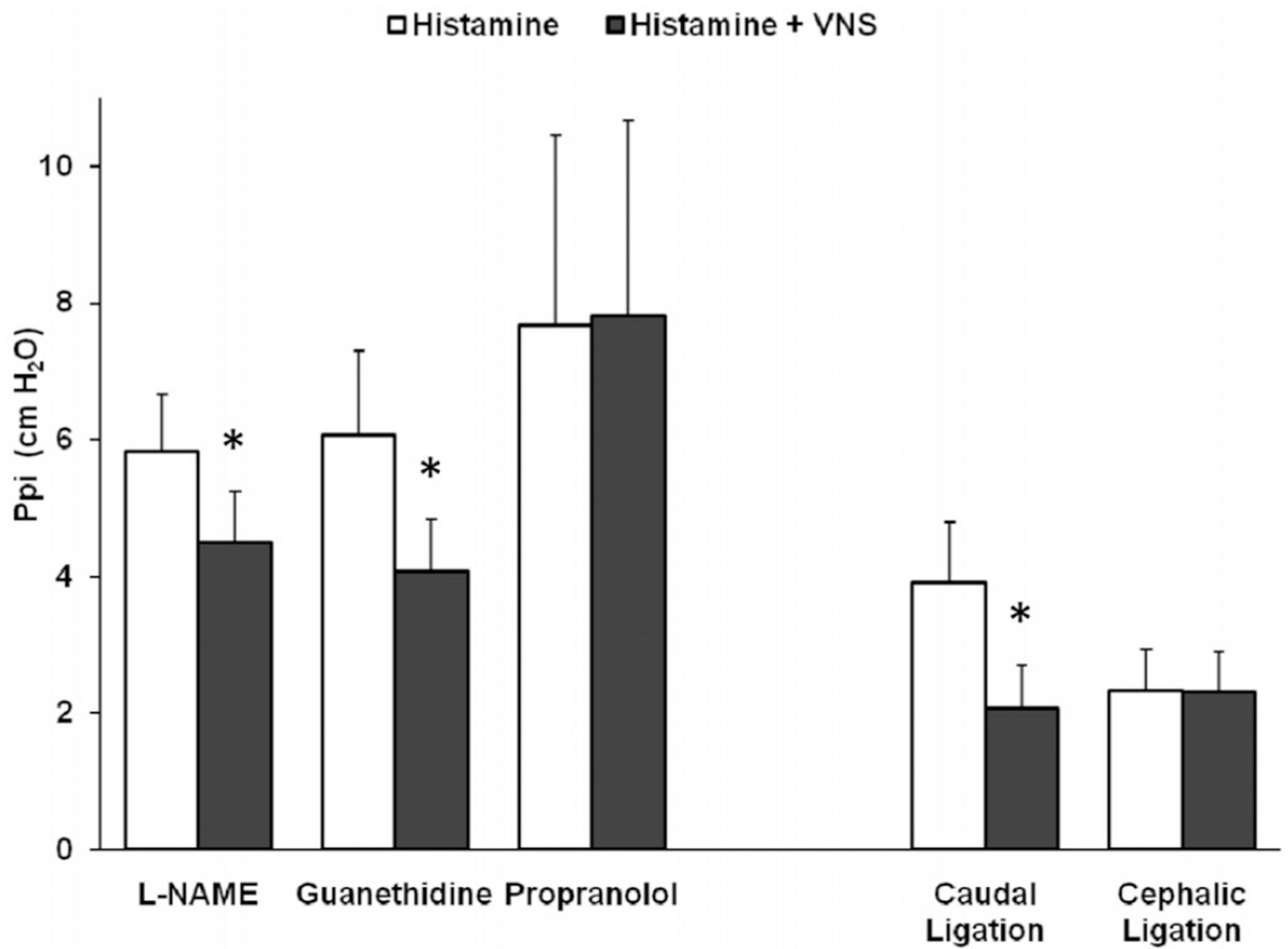
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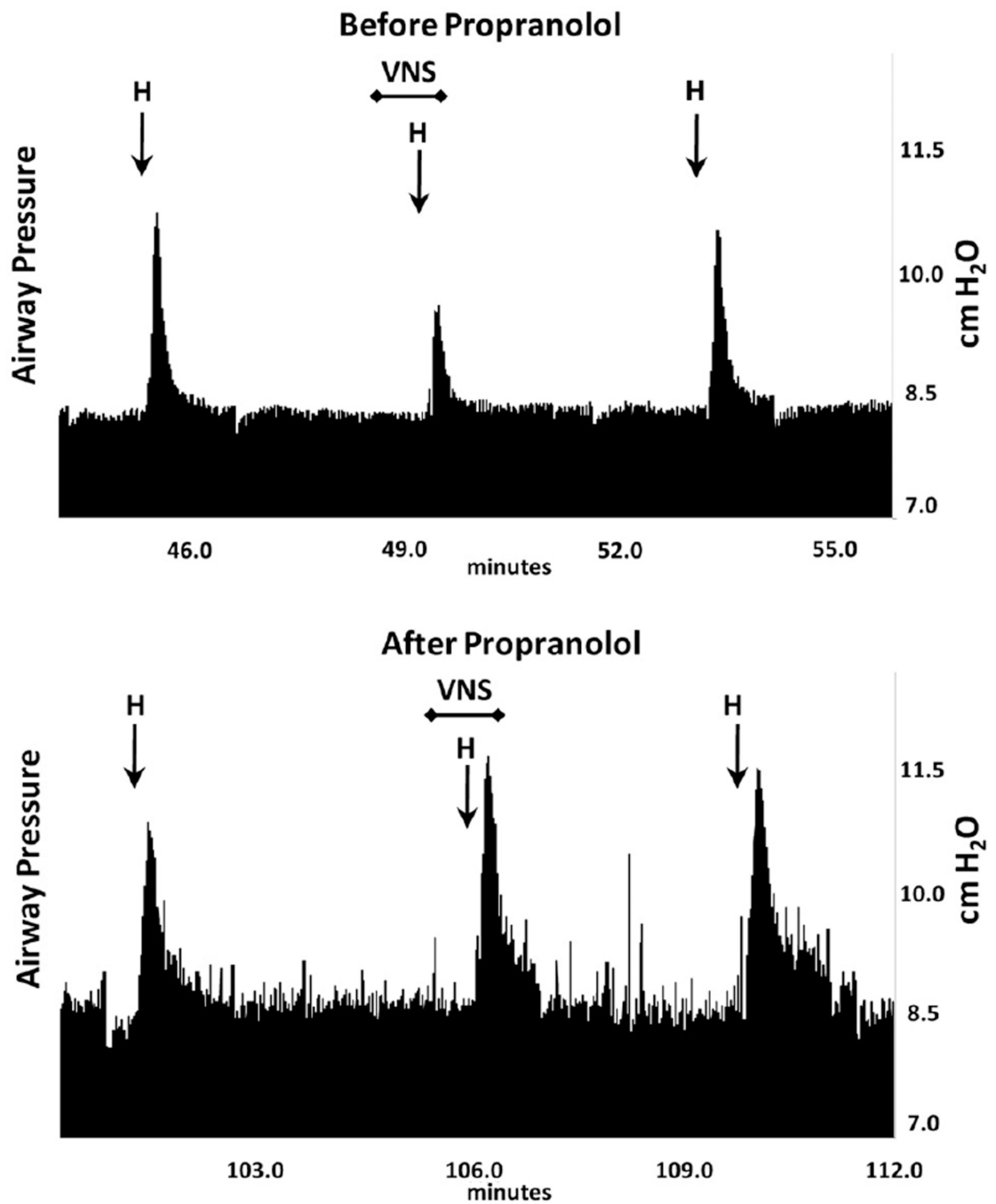
**Figure 1.**

Pulmonary inflation pressure (Ppi) was monitored during histamine ( $N=26$ ) or acetylcholine ( $N=6$ ) administration (control) and compared with the response when low-voltage vagus nerve stimulation (VNS) treatment was applied 20 sec before and during the administration of histamine or acetylcholine (VNS). \* $p < 0.05$  and \*\* $p < 0.01$  compared with respective controls.

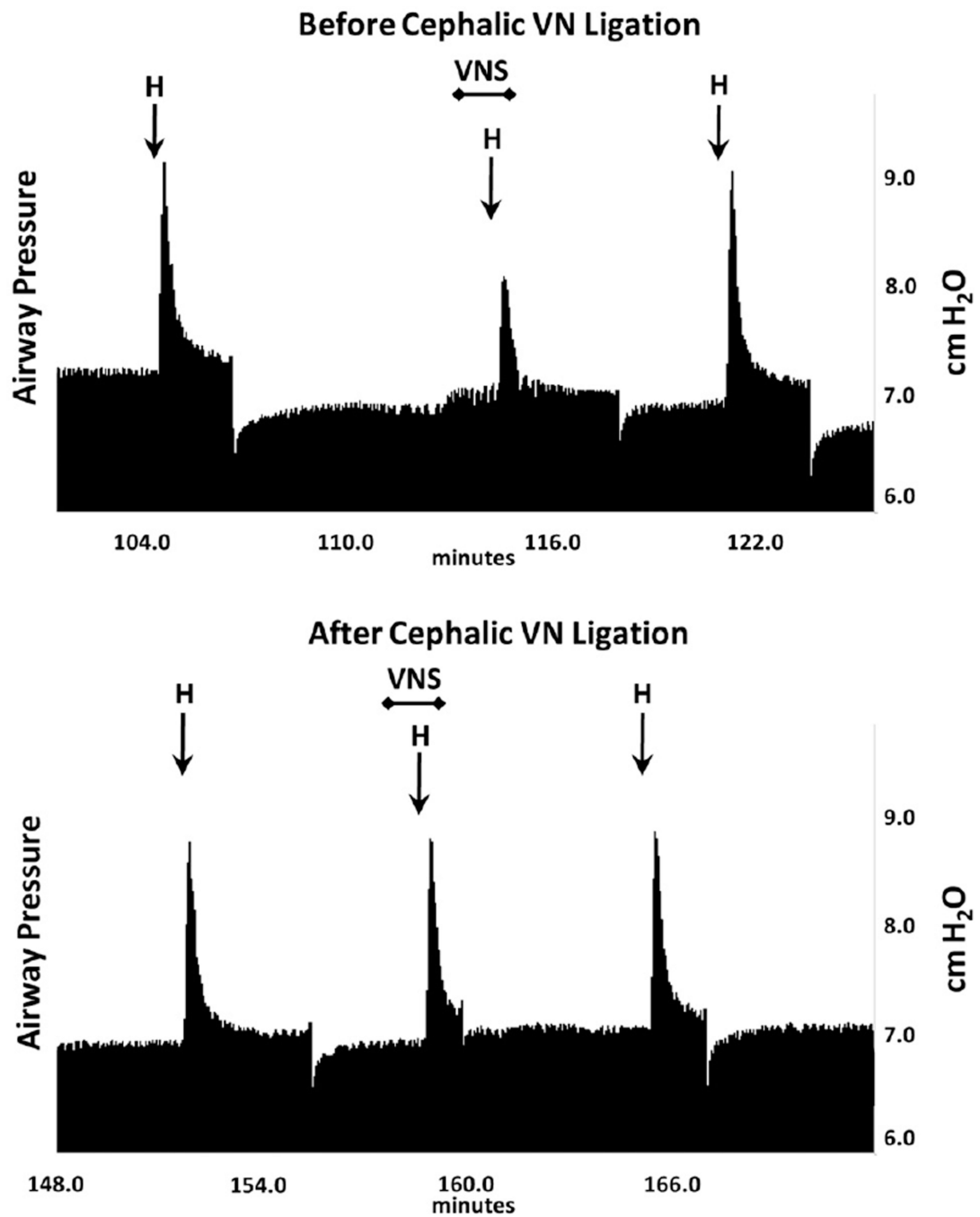


**Figure 2.**

Following treatment with chemical inhibitors or ligation of the vagus nerve, the pulmonary inflation pressure (Ppi) response to histamine was compared without and during low-voltage vagus nerve stimulation (VNS). *N*<sub>G</sub>-nitro-L-arginine methyl ester (L-NAME), *N* = 7, Guanethidine, *N* = 6, Propranolol, *N* = 6, Caudal Ligation, *N* = 7, Cephalic Ligation, *N* = 3. \**p* < 0.05.

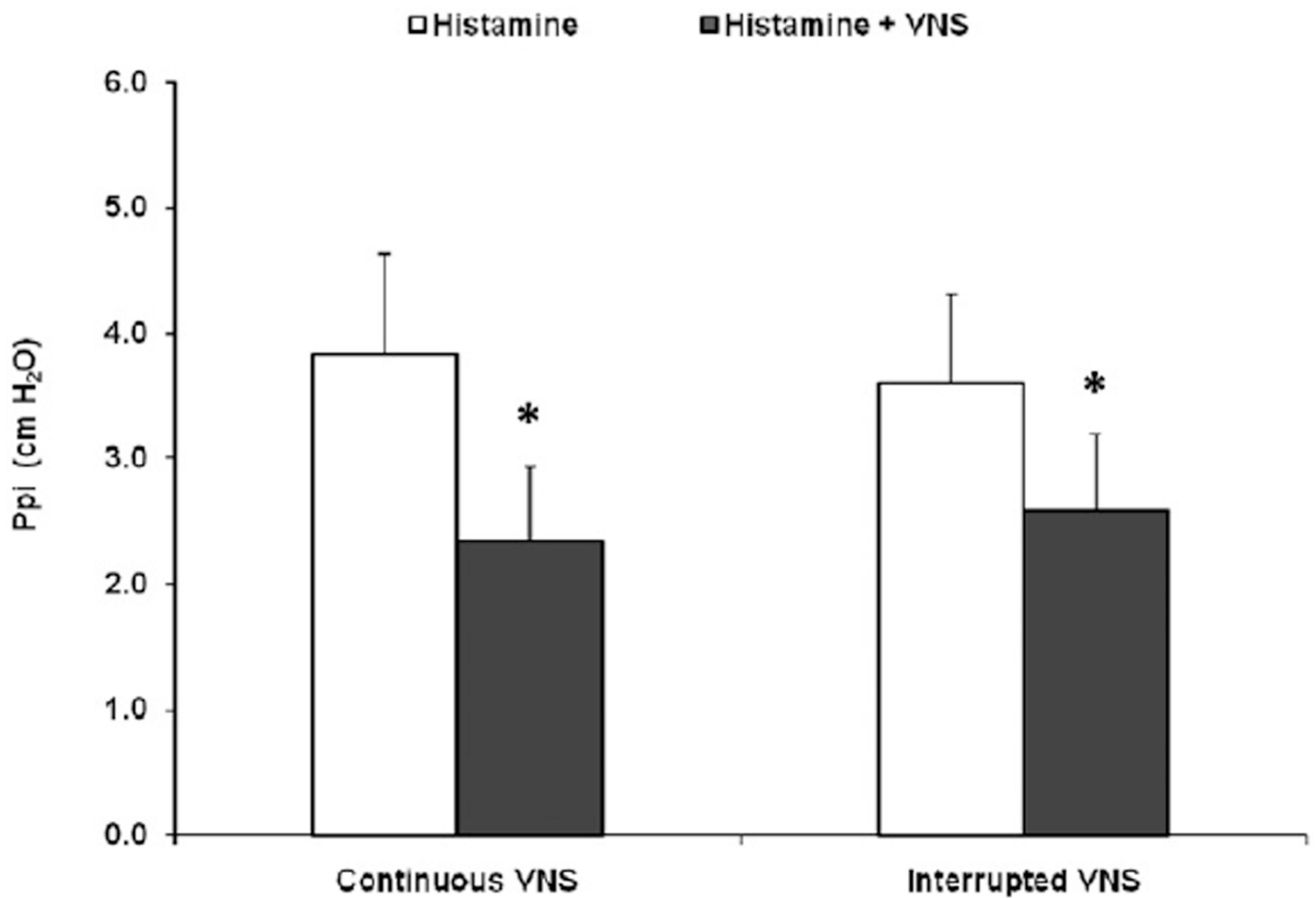


**Figure 3.** Representative traces of the airway pressure responses to i.v. histamine alone (H) and during low-voltage vagus nerve stimulation (VNS). Upper trace demonstrates responses before propranolol was administered. Bottom trace shows the responses in the presence of propranolol.



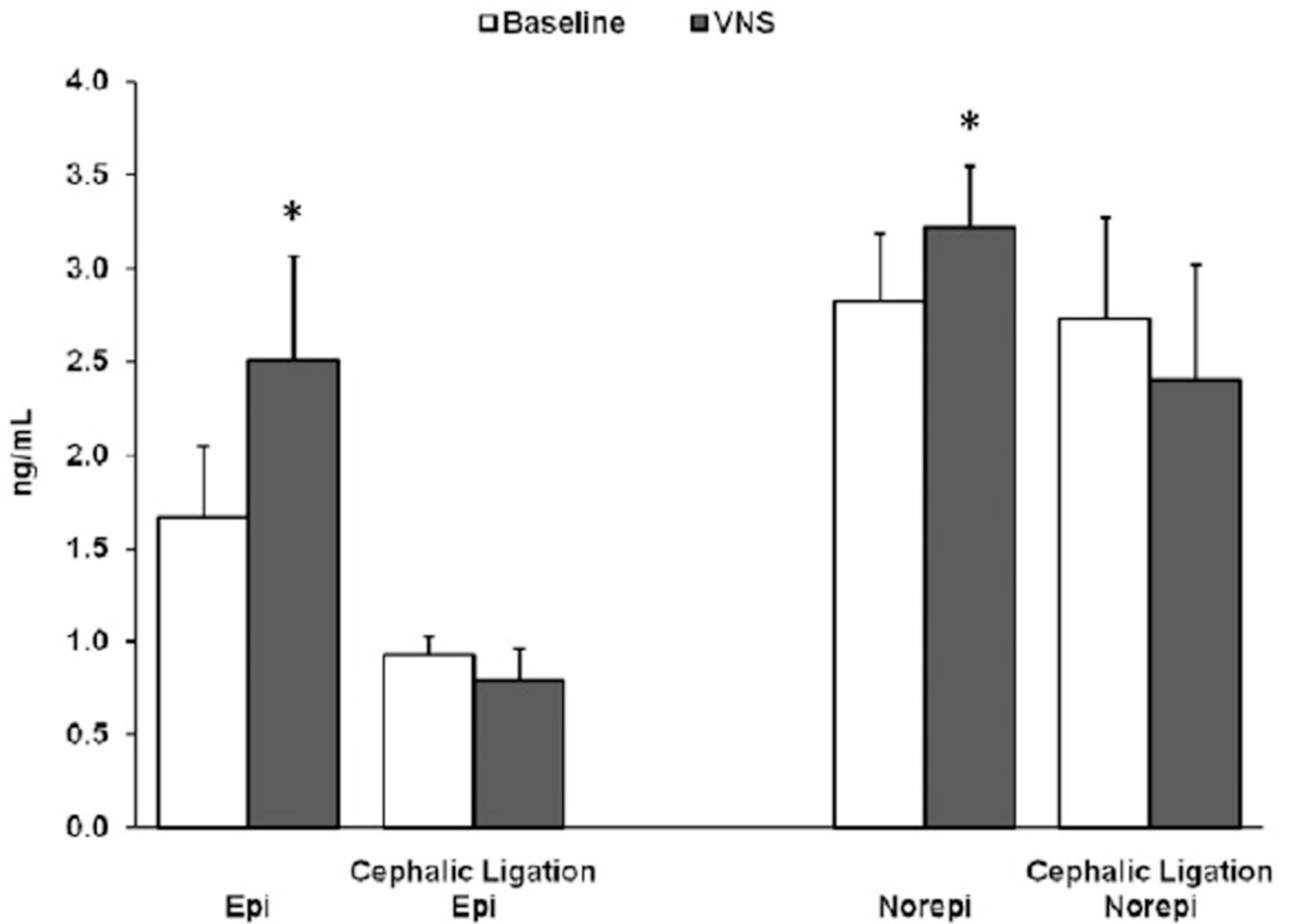
**Figure 4.** Representative traces of the airway pressure responses to i.v. histamine (H) alone and during vagus nerve stimulation (VNS). Upper trace shows responses prior to placement of a ligature around the vagus nerve. Bottom trace shows i.v. histamine responses without and during low voltage VNS after the vagus nerve was ligated cephalic to the electrodes.





**Figure 5.**

Pulmonary inflation pressure (Ppi) was monitored in five animals and the responses to i.v. histamine and to i.v. histamine plus a low-voltage vagus nerve stimulation (VNS) treatment compared. In the comparisons on the left, VNS was applied for 20 sec before and continued through the administration of i.v. histamine (Continuous VNS). In the comparisons on the right, the low-voltage VNS treatment was applied for 30 sec and then discontinued for 10 sec prior to administration of i.v. histamine (Interrupted VNS). \* $p < 0.05$ .



**Figure 6.**

Arterial epinephrine (Epi) and norepinephrine (Noepi) levels were measured at baseline and during vagus nerve stimulation (VNS),  $N = 4$ . Three of these animals underwent ligation of the vagus nerve cephalic to the electrodes. Following ligation, epinephrine (Cephalic Ligation Epi) and norepinephrine (Cephalic Ligation Noepi) levels were remeasured at baseline and during VNS. \* $p < 0.05$ .