Hypersensitivity of lung vagal C fibers induced by acute intermittent hypoxia in rats: role of reactive oxygen species and TRPA1

Mei-Ya Shen¹, Ya-Ling Luo¹, Chung-Huan Yang², Ting Ruan³, and Ching Jung Lai¹

¹ Department of Physiology, Tzu Chi University, Hualien, Taiwan
² Institute of Physiology, National Yang-Ming University, Taipei, Taiwan
³ Department of Physiology, Chung-Shan Medical University, Taichung, Taiwan

RUNNING HEAD: ACUTE INTERMITTENT HYPOXIA AND AIRWAY HYPERSENSITIVITY

Address for requests and other correspondence: Dr. C. J. Lai, Department of Physiology, Tzu Chi University. No. 701, Sec. 3, Chung-Yang Rd., Hualien 97004, Taiwan. Tel. +886-3-856-5301 ext. 2133 (E-mail: cjlai@mail.tcu.edu.tw)
ABSTRACT (244 words)

Obstructive sleep apnea, manifested by intermittent hypoxia and excess production of reactive oxygen species (ROS) in airways, is associated with hyperreactive airway diseases, but the mechanism remains unclear. Sensitization of lung vagal C fibers (LVCFs) contributes to the airway hypersensitivity. We investigated the mechanisms underlying the sensitization of LVCFs with acute intermittent hypoxia (AIH), by 10 episodes of exposure to 30 s hypoxic air (0%, 5% or 10% O₂) followed by 30 s room air in anesthetized, open-chest, and artificially ventilated rats. Reflex apneic response to intravenous capsaicin (an LVCF stimulant), as measured by phrenic nerve activity, was concentration-dependently augmented by AIH. Similarly, reflex apneic response to intravenous α,β-methylene-ATP (another LVCF stimulant) was augmented by AIH (0% O₂). The reflex apnea evoked by these 2 stimulants was abolished by bilateral vagotomry, which suggests the involvement of lung vagal afferents. The AIH-augmented apneic response to these 2 stimulants was prevented by pretreatment with dimethylthiourea (a hydroxyl radical scavenger), N-acetyl-L-cysteine (an antioxidant) and HC-030031 [a transient receptor potential ankyrin 1 (TRPA1) receptor antagonist]. Consistently, electrophysiological study revealed the afferent responses of LVCFs to capsaicin or α,β-methylene-ATP were augmented by AIH and this sensitization of LVCFs was prevented by dimethylthiourea, N-acetyl-L-cysteine
and HC-030031. In contrast, AIH did not alter the afferent response of LVCFs to mechanical stimulation by lung hyperinflation. We concluded that AIH sensitizes LVCFs in rats, thus resulting in exaggerated airway reflexogenic responses to chemical stimulants, possibly by ROS action and activation of TRPA1 receptors.

**KEY WORDS:** intermittent hypoxia; lung vagal C fibers; reactive oxygen species; TRPA1 receptors
INTRODUCTION

Obstructive sleep apnea (OSA), characterized by intermittent hypoxia during sleep, is associated with several hyperreactive airway diseases including nocturnal asthma (6, 9), chronic nocturnal cough (10), and bronchial hyperreactivity (32). Patients with OSA generally show airway inflammation (47). Airway hypersensitivity is among the major mechanisms contributing to hyperreactive airways and is manifested by augmented sensory and reflexogenic responses to inhaled irritants or circulating mediators; the hypersensitivity is a prominent pathophysiological feature of airway inflammatory diseases (30, 31). The possible role of sensitization of airway afferents in the OSA-related hyperreactive airway diseases has not been explored.

Lung vagal C fibers (LVCFs) are nociceptive-like free nerve endings that are sensitive to various chemicals or inhaled irritants, which leads to activation of various airway reflexes such as cough and bronchoconstriction (13, 31). The sensitivity of LVCFs to stimulants can be markedly increased by several inflammatory mediators (11, 20, 23), thus resulting in exaggerated airway reflexes (13, 30). Thus, sensitization of LVCFs by inflammatory mediators may play an important role in the pathogenesis of airway hypersensitivity (31). Indeed, exposure to intermittent hypoxia increases breathing (16, 27). However, exposure to intermittent hypoxia can also induce pathophysiological consequences such as increased generation of reactive oxygen
species (ROS) in airways by repeated hypoxia–reoxygenation cycles (8, 44) and several forms of neuroplasticity (35, 41). Recent studies from our laboratory have shown that ROS is essential for enhanced cardiorespiratory consequences induced by intermittent hypoxia (27). ROS also participate in intermittent hypoxia-induced sensitization of carotid body sensory activity to acute hypoxia (42, 43). Several studies have reported that LVCFs can be stimulated by pulmonary ROS by activating pharmacological receptors located at nerve terminals, such as transient receptor potential ankyrin 1 (TRPA1; a nonselective cation channel) receptors (45, 46, 51). In addition, TRPA1 may have a role in the development of airway hyperreactivity (2, 7). Intermittent hypoxia may sensitize LVCFs through ROS action and activation of TRPA1 receptors but remains uninvestigated.

Acute intermittent hypoxia (AIH) has been used in the laboratory to study the mechanism of neuroplasticity in OSA (36, 56) and may elicit neuroplasticity without producing the cardiovascular abnormalities caused by chronic intermittent hypoxia (56). We hypothesized that AIH may sensitize LVCFs through ROS action and activation of TRPA1 receptors because 1) intermittent hypoxia can increase the generation of ROS in airways, 2) ROS may act on TRPA1 receptors located at LVCF terminals to sensitize these afferents, and 3) increased sensitivity of LVCFs to stimulants may lead to augmented afferent or reflex responses. We used anesthetized,
open-chest and artificially ventilated rats to investigate whether 1) AIH augments the reflex apnea evoked by chemical stimulants of LVCFs and 2) increases the excitability of LVCFs to chemical and mechanical stimulation, and whether 3) ROS and TRPA1 receptors are involved in the induction of this airway hypersensitivity by AIH. We measured reflex apnea and the excitability of LVCFs by recording phrenic nerve activity and the single-unit LVCF activity, respectively, and used 2 chemical stimulants, capsaicin and α,β-methylene-ATP, to stimulate LVCFs by activating TRP vanilloid 1 (TRPV1) receptors and P2X purinoceptors (24, 25). We mechanically stimulated LVCFs by lung hyperinflation (23, 28).

METHODS

Protocols for all surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee of Tzu Chi University.

Animal preparation. Male adult Sprague-Dawley rats were anesthetized with intraperitoneal injection of α-chloralose (100 mg/kg; Sigma-Aldrich, St. Louis, MO) and urethane (500 mg/kg; Sigma-Aldrich) dissolved in a borax solution (2%; Sigma-Aldrich). A polyethylene catheter was inserted into the jugular vein and advanced until the tip was close to the right atrium for intravenous administration of pharmacological agents. The right femoral artery was cannulated for measuring
arterial blood pressure and heart rate. During the experiments, supplemental doses of α-chloralose (20 mg/kg/hr) and urethane (100 mg/kg/hr) were administered to ensure no pain reflexes on pinching the animal's tail. The neck was opened in the midline, and a segment (~ 1 cm) of each vagus nerve was carefully isolated from the common carotid artery for later use. A short tracheal cannula was inserted just below the larynx via a tracheostomy. The rats were ventilated by use of a rodent respirator (Harvard 683; South Natick, MA) via the tracheal cannula. Tidal volume and respiratory frequency of the respirator were set at 2.0-2.5 ml and 60 breaths/min, respectively. Tracheal pressure (Ptr) was monitored by a pressure transducer (Validyne MP45-28; Northridge, CA) via a side tap of the tracheal cannula. A mid-line thoracotomy was then performed and the edges of the rib cage were retracted. The expiratory outlet of the respirator was placed under 3 to 4 cm of water to maintain a near normal functional residual capacity. Rats were paralyzed with use of pancuronium bromide (0.5 mg/kg, i.v.; Orgnon Teknika, Boxtel, The Netherlands). Periodically, the effect of pancuronium was allowed to wear off so that the extent of anesthesia could be checked. Body temperature was maintained at ~36 °C throughout the experiment by means of a servo-controlled heating blanket. Animals were killed at the end of experiments by intravenous injection of overdose of anesthetics.

*Measurement of phrenic nerve activity.* Efferent phrenic nerve activity was monitored
as an index of respiratory motor output. The phrenic nerve was isolated unilaterally, identified in the cervical region via a ventrolateral approach and cut as distally as possible. Action potentials were recorded via a bipolar electrode connected to a preamplifier (Grass P511K; Quincy, MA), filtered at 0.3–3 kHz, monitored by an audio monitor (Grass AM8), and displayed on an oscilloscope (Gould 420; Cleveland, OH).

**Recording LVCF afferent activity.** Afferent activity arising from LVCFs was recorded as described (28). Briefly, a fine afferent filament was split from the desheathed nerve trunk of the right vagus and placed on a platinum-iridium unipolar recording electrode to record afferent nerve activity. The fine nerve filament was further split until the afferent activity arising from a single unit was electrically isolated. Hyperinflation of the lung (3-4 tidal volume) by occlusion of the expiratory line of the respirator was used as the first step in searching for these fibers. Furthermore, capsaicin (1 μg/kg; Sigma) was injected as a bolus into the right atrium. Only fibers that responded to capsaicin within 2 s after the injection were studied (12). Finally, the conduction velocity of the afferent fibers was measured as described (3). Before the end of each experiment, the general locations of C fibers studied were identified within the lung structure by gently probing the tissues with use of a polyethylene rod (diameter 2 mm).

**Airway sensitization by AIH.** Hypoxic air (0%, 5% or 10% with balanced N₂) was
stored in a twenty-five liters plastic balloon connected to the inlet of the respirator through a 3-way stopcock. Ten episodes of AIH were induced by repeatedly adjusting the 3-way stopcock for 30 s of hypoxic air exposure, then 30 s of room air (RA). An air control group received the same exposure to RA. Before each AIH exposure, the animal’s lungs were hyperinflated (tracheal pressure >20 cmH₂O) to establish a constant volume history.

*Pharmacological agents.* Stock solutions of capsaicin (a selective TRPV1 activator; 250 μg/ml, Sigma-Aldrich), polygodial [a TRPA1 receptor agonist (18); 1 mg/ml, Biomol, Plymouth Meeting, PA], and allyl-isothiocyanate (AITC; another TRPA1 agonist; 30 mg/ml, Sigma-Aldrich) were prepared by dissolving the drugs in 10% Tween 80, 10% ethanol, and 80% isotonic saline. α,β-methylene-ATP (a LVCF stimulant; 2 mg/ml, Sigma-Aldrich), dimethylthiourea (a scavenger of hydroxyl radical; 500 mg/ml, Sigma), and N-acetyl-L-cysteine (a potent antioxidant; 300 mg/ml, Sigma-Aldrich) were dissolved in saline. The stock solutions of HC-030031 (a TRPA1 receptor antagonist, Tocris Cookson, Ellisville, MO) and AP18 (another TRPA1 receptor antagonist) were prepared by first dissolving in dimethyl sulfoxide (Sigma-Aldrich) at 30 mg/ml and 50 mg/ml, respectively, and further diluting with a solution containing 10% Tween 80, 10% ethanol, and 80% isotonic saline. Except for the solutions of dimethylthiourea and N-acetyl-L-cysteine, which were stored at 4°C,
others were stored at -20°C.

*Agonist injection and pharmacological pretreatment.* Solutions of capsaicin, α,β-methylene-ATP, polygodial, and AITC at desired concentrations were prepared daily by diluting the stock solution with saline on the basis of animal body weight. Capsaicin (1 μg/kg) and α,β-methylene-ATP (15 μg/kg) were injected into the vein as a bolus (0.1 ml volume). Dimethylthiourea, N-acetyl-L-cysteine, HC-030031, AP18, polygodial, and AITC (~0.4 ml) were slowly injected into the vein over 20 s at 500, 300, 3, 5, 0.1, and 3 mg/kg, respectively. Each of the injections was then flushed into the right atrium by use of an injection of 0.3 ml saline. Pretreatments with dimethylthiourea, N-acetyl-L-cysteine, vehicle of antioxidants, HC-030031, AP18, and vehicle of TRPA1 antagonists were made 15, 15, 15, 5, 5, and 5 min, respectively, before AIH exposure. The doses and treatment times with effectiveness for these drugs were adopted from previous studies (28, 33).

*Experimental design and protocols.* In this study, 349 rats (weight 310-460 g) were divided into 45 study groups to perform 8 series of experiments. In each rat, only one exposure of air or AIH was performed. In 198 rats used for LVCF responses, only one afferent fiber was studied from one rat. In series 1, the potentiating effect of AIH (10 episodes of either 0, 5, 10, or 21% O₂, each treatment: n = 8) on the apneic responses to capsaicin was investigated in 4 groups of rats. Also, the potentiating effect of AIH
(0 or 21% O₂, each treatment: n = 8) on the apneic responses to α,β-methylene-ATP was investigated in another 2 groups of rats. In these groups with 0% O₂ AIH, vagotomy was additionally performed at 32 min after termination of AIH. Five minutes later, apneic responses to capsaicin or α,β-methylene-ATP injection was measured. Apneic responses to these chemical stimulants were analyzed from phrenic nerve activity. In series 2, suppressive effects of pretreatment with dimethylthiourea, N-acetyl-L-cysteine, 1.5 mg/kg HC-030031, 3.0 mg/kg HC-030031, the vehicle of N-acetyl-L-cysteine or dimethylthiourea (vehicle 1) or the vehicle of HC-030031 (vehicle 2) (each treatment: n = 8) on 0% O₂ AIH-induced potentiation of the apneic responses to injection of capsaicin were investigated in 6 groups of rats. Also, the suppressive effects of pretreatment with dimethylthiourea, N-acetyl-L-cysteine, 3.0 mg/kg HC-030031, vehicle 1 or vehicle 2 (each treatment: n = 8) on this potentiating response of AIH to α,β-methylene-ATP was examined in another 5 groups of rats. The apnea responses to these chemical stimulants were measured as described in series 1.

In series 3, the potentiating effects of AIH on the LVCF responses to injection of capsaicin and α,β-methylene-ATP were investigated in 4 and 2 groups of rats (each group: n = 8), respectively, with the same protocols described in series 1. To study whether AIH could also potentiate the LVCF responses to mechanical stimulation, 2 additional groups of rats (each group: n = 8) were subjected to either 0 or 21% O₂ AIH
exposure. The LVCF responses to lung hyperinflation (Ptr = 30 cmH\textsubscript{2}O for 10 s) were measured just before, 10 min, and 30 min after the end of AIH. In series 4, suppressive effects of pretreatment with dimethylthiourea, N-acetyl-L-cysteine, 1.5 mg/kg HC-030031, 3.0 mg/kg HC-030031, AP18, vehicle 1 or vehicle 2 (the vehicle of HC-030031 or AP18) (each treatment: n = 8) on 0% O\textsubscript{2} AIH-induced potentiation of the LVCF responses to injection of capsaicin were investigated in 7 groups of rats. Additionally, the suppressive effects of pretreatment with dimethylthiourea, N-acetyl-L-cysteine, 3.0 mg/kg HC-030031, vehicle 1 or vehicle 2 (each treatment: n = 8) on this potentiating response of AIH to α,β-methylene-ATP was also evaluated in another 5 groups of rats. The LVCF responses to these chemical stimulants were measured at time points as described in series 1. In series 5, the effectiveness of the receptor blockade by the TRPA1 antagonist was checked in one group of rats (n = 6). The LVCF responses to injection of polygodial were measured before and 45 min after treatment with HC-030031. In series 6, the possible deleterious effects of pharmacological pretreatments on LVCFs were studied in 4 groups of rats. The LVCF responses to injection of capsaicin were measured before and after pretreatment with dimethylthiourea, N-acetyl-L-cysteine, HC-030031 or AP18 (each treatment, n = 6). In series 7, the sensitizing effect of the TRPA1 activation by its agonist AITC on LVCFs was studied in one group of rats (n = 8). The responses of LVCFs to injection of
capsaicin were measured before, 10 min, and 30 min after treatment with AITC. In series 8, the effects of 0 and 21% O₂ AIH on PaO₂ at baseline, the last hypoxic period of AIH, the last RA period of AIH, and 30 min after termination of AIH were measured in 2 groups of rats.

Data analysis and statistics. For the studies of apneic reflex, expiratory duration (Tₑ) was analyzed as the interval between 2 successive phrenic bursts (29). The baseline Tₑ was calculated on a breath-by-breath basis as the average value over the 10-breath period immediately before injection of chemical stimulants. To compare the apneic responses evoked by different experimental conditions, the apneic ratio was calculated as the longest Tₑ occurring during the first 5 breaths after injection of chemical stimulants divided by the baseline Tₑ. For studies of C-fiber afferent activity, averaged baseline fiber activity was calculated over 10-s intervals; peak responses were defined as the maximal value averaged 2-s and 10-s intervals for injection of 2 chemical stimulants and lung hyperinflation, respectively. In all studies, mean arterial blood pressure (MABP) and heart rate (HR) were continuously analyzed at 1-s intervals. Baseline MABP and HR were calculated as the mean value over the 10-s period immediately preceding chemical stimuli; the peak response was defined as the minimum 2-s average during 20 s after administration of stimuli. All physiological signals were analyzed with use of a computer equipped with an analog-to-digital
converter (Gould DASA 4600) and software (BioCybernatics 1.0, Taipei, Taiwan).

Data for 2 groups were compared by Student’s $t$ test. Data for 3 or more groups were compared by one-way ANOVA or two-way mixed factorial ANOVA, then Neuman-Keuls test as appropriate. A $P < 0.05$ was considered statistically significant. All data are presented as mean ± SE.

RESULTS

*Enhancement of the reflex apnea response to chemical stimuli by AIH.* At baseline, rat groups did not differ in respiratory frequency, TI, TE, heart rate, or MABP (one-way ANOVA, $P > 0.05$). Right-atrial injection of capsaicin evoked an inhibitory response of phrenic nerve activity which resulted in reflex apnea appearing as a prolonged TE, as previously described (29, 34). This reflex apnea resulted in an increased apneic ratio, reflecting the magnitude of the apneic response. At 10 (apneic duration: $5.63 \pm 0.71$ s) and 30 min (apneic duration: $5.66 \pm 0.65$ s) after 0% O$_2$ AIH, the apneic ratio induced by capsaicin was higher than RA challenge (Figs. 1 and 2A). At 30 min after 5% O$_2$ AIH but not 10% O$_2$ AIH, the reflex apneic ratio induced by capsaicin was higher than RA challenge (Fig. 2A). In addition, the enhanced reflex apnea was nearly abolished by bilateral cervical vagotomy (Fig. 2A). After vagotomy, the baseline TE and capsaicin-induced apnea were $2.12 \pm 0.18$ and $2.33 \pm 0.16$ s at 30 min after 0% O$_2$.
AIH. RA challenge did not augment the capsaicin-induced reflex apnea (Figs. 1 and 2A). The baseline TE with RA and 10%, 5%, and 0% O2 AIH was 1.39 ± 0.14, 1.43 ± 0.17, 1.35 ± 0.22, and 1.44 ± 0.15 s (one-way ANOVA, $P > 0.05$; Table 2). The baseline TE at 10 and 30 min after 10%, 5% or 0% O2 AIH did not differ from their corresponding values before challenge (one-way ANOVA, $P > 0.05$). Thus, the baseline TE had returned to the control level before we measured the apneic response.

The reflex apnea response to $\alpha,\beta$-methylene-ATP was also enhanced with 0% O2 AIH (Fig. 3). The apneic ratio induced by $\alpha,\beta$-methylene-ATP remained higher at 30 min after AIH than with RA alone (4.45 ± 0.42 vs 2.89 ± 0.28). Furthermore, the AIH-enhanced reflex apnea response to $\alpha,\beta$-methylene-ATP was abolished by bilateral cervical vagotomy (Fig. 3A).

**Involvement of ROS and TRPA1 receptors in AIH enhanced-reflex apnea.** We assessed the roles of ROS and TRPA1 receptors in 0% O2 AIH-enhanced reflex apnea response to capsaicin in 6 groups of rats. The 0% O2 AIH-enhanced apneic ratio to capsaicin was totally prevented by pretreatment with dimethylthiourea, N-acetyl-L-cysteine or 3.0 mg/kg HC-030031 but not their vehicles or 1.5 mg/kg HC-030031 (Fig. 2B and C). Similarly, the 0% O2 AIH-increased reflex apneic response to $\alpha,\beta$-methylene-ATP was completely abolished by pretreatment with dimethylthiourea, N-acetyl-L-cysteine or 3.0 mg/kg HC-030031 but not their vehicles (Fig. 3B and C).
Hypersensitivity of LVCFs by AIH. We studied 32 LVCFs in the 4 groups, and all were localized within the lung structure. The mean conduction velocity of 22 LVCFs was $1.13 \pm 0.07$ m/s (range 0.79–1.84 m/s); the conduction velocity of the remaining 10 fibers was not measured. The baseline activity of the LVCFs studied was irregular and sparse ($0.04 \pm 0.01$ impulses/s; $n=32$), with a distinct sensitivity to capsaicin injection (1 μg/kg) and weak or no response to lung hyperinflation. Bolus injection of capsaicin (1 μg/kg) in the right atria of rats immediately evoked an intense and short burst of discharge before RA or AIH exposure, with no significant difference among the 4 study groups (RA: mean 8.04 ± 0.56 impulses/s; 10% O₂ AIH: 8.71 ± 0.57 impulses/s; 5% O₂ AIH: 8.83 ± 0.70 impulses/s; 0% O₂ AIH: 8.29 ± 0.39 impulses/s; $P > 0.05$). However, the peak activity to capsaicin injection was markedly enhanced at 10 and 30 min after 0% O₂ AIH as compared with the control (Figs. 4D, and 5A). AIH potentiated the LVCF response in a concentration-dependant manner; 5% O₂ or 0% O₂ but not 10% O₂ AIH enhanced the LVCF response to capsaicin (Figs. 4B, C, D, and 5A). In contrast to the effect of AIH, RA did not change the afferent response of LVCFs to capsaicin (Figs. 4A and 5A). In addition, the baseline activity of LVCFs did not significantly differ among the 4 groups.

Enhancement of the afferent response with α,β-methylene-ATP was similar to that with capsaicin. Augmented response to α,β-methylene-ATP was also found at 30
min after 0% O₂ AIH (Fig. 6A). At 30 min after RA or 0% O₂ AIH, the mean peak response induced by α,β-methylene-ATP was 9.94 ± 0.54 impulses/s with RA and 15.21 ± 1.73 impulses/s with AIH exposures.

All LVCFs showed weak response to lung hyperinflation (Fig. 7), which is similar to what we found previously (28). In sharp contrast to chemical stimuli, the afferent response to constant-pressure lung inflation (Ptr = 30 cmH₂O; 10 s) was not augmented with 0% O₂ AIH as compared with RA. At 30 min after RA or 0% O₂ AIH, the mean peak response induced by lung hyperinflation was 0.44 ± 0.05 impulses/s with RA and 0.53 ± 0.06 impulses/s with AIH exposures.

Involvement of ROS and TRPA1 receptors in AIH-induced hypersensitivity of LVCFs.

We investigated the role of ROS and TRPA1 receptors in 0% O₂ AIH–induced LVCF hypersensitivity to chemical stimuli. 0% O₂ AIH–induced LVCF hypersensitivity to capsaicin was completely prevented by pretreatment with dimethylthiourea, N-acetyl-L-cysteine, or 3.0 mg/kg HC-030031 (Figs. 5B and C). Pretreatment with AP18, another TRPA1 antagonist also suppressed the AIH-induced potentiation of LVCF responses to capsaicin and its suppressive effect was similar to that of HC-030031 (Fig. 5C). However, pretreatment with 1.5 mg/kg HC-030031 only slightly attenuated the AIH-induced LVCF hypersensitivity to capsaicin (Fig. 5C). Similarly, the potentiating effect of 0% O₂ AIH–induced LVCF hypersensitivity to
α,β-methylene-ATP was totally abolished by pretreatment with dimethylthiourea, N-acetyl-L-cysteine or 3.0 mg/kg HC-030031 (Figs. 6B and C). In contrast, pretreatment with their vehicles did not change the AIH-induced LVCF hypersensitivity to chemical stimuli (Figs. 5 and 6). In addition, the response of LVCFs to injection of polygodial was totally blocked after pretreatment with HC-030031; the mean peak response to polygodial before and after pretreatment with 3.0 mg/kg HC-030031 was 6.23 ± 0.95 and 0.28 ± 0.13 impulses/s, respectively. To further assess the role of TRPA1 receptors, AITC (a TRPA1 agonist) was administered to activate TRPA1 receptors. Without the exposure to AIH, LVCF response to capsaicin injection was significantly augmented at 10 min and 30 min after the treatment of AITC; the mean peak response to capsaicin before, 10 min after, and 30 min after AITC administration was 7.23 ± 0.27, 8.75 ± 0.43, and 8.35 ± 0.48 impulses/s, respectively.

Effect of various pharmacological pretreatments on the LVCF responses to capsaicin injection. We investigated the possible deleterious effects of pretreatment with antioxidants or TRPA1 antagonists on LVCF response to capsaicin. The response of LVCFs to capsaicin injection was not significantly affected after pretreatment with dimethylthiourea, N-acetyl-L-cysteine, HC-030031 or AP-18 (Table 3).

Cardiovascular responses to capsaicin injection after AIH exposure. Capsaicin
injection produced reflex apnea and LVCF activation but also induced hypotension and bradycardia. At 30 min after 0% O₂ AIH exposure, capsaicin injection lowered MABP more than RA exposure in the reflex apneic study (Table 1). In addition, the capsaicin-induced MABP suppression in 0%O₂ AIH groups pretreated with either dimethylthiourea, N-acetyl-L-cysteine or 3.0 mg/kg HC-030031 did not significantly differ from that in RA group (one-way ANOVA, $P > 0.05$). In contrast, bradycardia caused by capsaicin injection was not significantly altered between RA and 0% O₂ AIH groups. Bilateral cervical vagotomy attenuated but did not totally eliminate the capsaicin-induced hypotension and bradycardia. At 10 or 30 min after 0% O₂ AIH challenge, the baseline MABP (10 min: 103.3 ± 6.6 mmHg; 30 min: 101.6 ± 6.7 mmHg) and HR (10 min: 324.2 ± 17.8 beats/min; 30 min: 323.4 ± 17.9 beats/min) did not significantly differ from their corresponding values before challenge (MABP: 101.9 ± 5.5 mmHg; HR: 328.4 ± 17.6 beats/min). In the LVCF fiber study, capsaicin-induced hypotension and bradycardia did not significantly differ among these groups.

Changes in PaO₂ after AIH challenge. With 0% O₂ AIH, the mean PaO₂ at baseline, the last hypoxic period of AIH, the last RA period of AIH, and 30 min after AIH was 99.3 ± 1.7, 29.4 ± 2.1, 82.8 ± 3.2, and 100.2 ± 2.2 mmHg, respectively. In fact, the change in PaO₂ (≅ 80 mmHg) at the end of 10 episodes of 0%O₂ AIH was not great
because the last 30 s AIH challenge was RA. Thus at the time we took blood samples for PaO₂ measurement, the animals actually had been ventilated with RA for 30 s. Additionally, the PaO₂ was depressed by AIH but returned to the baseline level at 30 min after the challenge. With RA, the mean PaO₂ at baseline, end of 10 episode RA challenge, and 30 min after RA challenge was 100.3 ± 3.4, 101.3 ± 5.7 and 99.5 ± 2.0 mmHg, respectively

**DISCUSSION**

The results of the present study show for the first time that an exposure of the lung to acute and short term of AIH (10 episodes; 0%, 5%, 10% O₂) produced a concentration-dependant augmented reflex apnea to intravenous injection of capsaicin, compared with those of RA rats. In addition, 0% O₂ AIH significantly potentiated the reflex apnea to α,β-methylene-ATP. Bilateral vagotomy abolished the apneic response to chemical stimulants after AIH, which suggests the involvement of lung vagal afferents. Importantly, our results suggest that the action of ROS and functioning of TRPA1 receptors play critical roles in the sensitizing effects of AIH because these effects were greatly attenuated by pretreatment with dimethylthiourea (a hydroxyl radical scavenger), N-acetyl-L-cysteine (an antioxidant) or HC-030031 (a TRPA1 receptor antagonist). Electrophysiological recording of LVCF afferent activity further
confirmed the significance of ROS and TRPA1 receptors in the sensitizing effects of 0% O\textsubscript{2} AIH on LVCFs to chemical stimulants. In contrast, AIH did not alter the afferent response of LVCFs to lung hyperinflation. Taken together, these results suggest that AIH may sensitize LVCFs to chemical stimulants by activating the TRPA1 receptors through ROS.

AIH induces long-term facilitation of phrenic and hypoglossal motor output in control rats (35). In the present study, we first demonstrated that 10 episodes of intermittent hypoxia markedly augmented the sensitivity of LVCFs to intravenous injection of capsaicin and α,β-methylene-ATP, thereby triggering the reflex apnea. Despite the elevated afferent sensitivities to these LVCF stimulants, AIH did not increase baseline LVCF activity and the sensitivity of LVCFs to lung hyperinflation, which seems to suggest a synergistic rather than additive effect. The importance of LVCFs in detecting the onset of pathophysiological conditions and triggering various airway reflexes has been well documented (13, 31). For example, activation of these C fibers immediately elicits pulmonary chemoreflexes (e.g., apnea, bradycardia and hypotension), rapid shallow breathing, cough, airway irritation, and dyspneic sensation (13, 30, 31, 40). Our results suggest that LVCF hypersensitivity may be responsible for altering various respiratory responses in intermittent hypoxia-exposed animals and OSA patients. Our finding that only 0% and 5% O\textsubscript{2} but not 10% O\textsubscript{2} AIH
potentiated the apneic response and LVCF sensitivity to capsaicin suggest that this sensitizing effect depends on the severity of AIH. These findings agree with observations that airway inflammation such as oxidative stress in patients with OSA is positively related to disease severity (14). In our preliminary study, we found that exposure to 10% O₂ AIH for a longer duration (20 episodes) may induce LVCF hypersensitivity. Thus, this sensitizing effect may also depend on duration of AIH. Indeed, the sensitizing effect of AIH we observed could last for > 30 min. It is possible that the impact of the AIH effect may be enhanced by chronic intermittent hypoxia such as in OSA.

The exact mechanisms that cause the cardiorespiratory consequences of intermittent hypoxia are not entirely understood, but increasing evidence suggests that ROS are important for these responses associated with intermittent hypoxia (53). This suggestion is not surprising because ROS generation during the re-oxygenation phase is mediated by a series of cellular responses to intermittent hypoxia (44). Although the source of ROS is not well understood, the lungs are known to be a rich source of ROS generation during intermittent hypoxia exposure (39). Furthermore, circulating neutrophils and monocytes may be sources of ROS production in OSA (17, 49). Several lines of evidence suggest that excess ROS production contributes to various cardiorespiratory consequences in intermittent hypoxia-exposed animals (26, 27, 39).
and OSA patients (15, 50). Furthermore, ROS mediates intermittent hypoxia-induced sensitization of carotid body sensory activity to the hypoxic response (42, 43). ROS not only sensitize the carotid sinus nerves but also the capsaicin-sensitive afferent fibers. Recent studies demonstrated that laryngeal acid-pepsin insult can evoke laryngeal airway hyperreactivity through sensitization of the capsaicin-sensitive laryngeal afferent fibers by ROS (54, 55). This concept is supported by the present finding that both AIH-enhanced reflex apnea and LVCF sensitivity to capsaicin and $\alpha,\beta$-methylene-ATP were significantly prevented by dimethylthiourea (a hydroxyl radical scavenger) or N-acetyl-L-cysteine (an antioxidant), so ROS may be a causative factor in the development of afferent hypersensitivity. We did not identify the types and sources of ROS responsible for eliciting LVCF hypersensitivity after AIH exposure. Some reports indicated that activation of NADPH oxidase produces superoxide anion during intermittent hypoxia (22, 57). Indeed, NADPH oxidase-derived ROS was found essential for evoking sensory plasticity of the carotid body by chronic intermittent hypoxia (41).

How ROS mediate the AIH-induced sensitization of LVCFs remains unclear. TRPA1, a member of the TRP family of ion channels, is expressed in LVCF sensory terminals (4, 37, 38) and may be a major oxidant sensor in the lungs (4, 52). TRPA1 can be activated by various chemical stimulants, including ROS (5, 48). However,
production of some arachidonic acid metabolites under oxidative stress may also activate TRPA1 receptors and then cause stimulation of LVCFs (33). Our results obtained from measurements of reflex apneic response and single-unit LVCF recording have demonstrated that administration of TRPA1 antagonists (HC-030031 or AP18) prevented the AIH-enhanced reflex apnea and LVCF sensitivity to capsaicin and \( \alpha,\beta \)-methylene-ATP. The specific blocking effect of HC-030031 on TRPA1 receptors was confirmed by the complete blocking of the afferent response to its corresponding receptor agonist, polygodial. In addition, pretreatment with antioxidants or TRPA1 antagonists nearly suppressed the AIH-enhanced reflex apnea and LVCF sensitivity to chemical stimulants, but pretreatment with their vehicles did not alter the responses. The suppressive effects of these pharmacological pretreatments were unlikely due to possible deleterious effects of these drugs because the LVCF responses to capsaicin were unaffected by these pretreatments. Therefore, activation of TRPA1 receptors may be required for the ROS-mediated hypersensitivity of LVCFs by AIH and subsequent reflex apnea. This notion is supported by our finding that pre-activation of TRPA1 receptors by AITC alone could mimic the sensitizing effect of AIH, although the effect was milder than that induced by AIH. A recent study (38) reported that TRPA1 is co-expressed with TRPV1 in a subset of mouse pulmonary C-fiber sensory neurons. Chemical mediators such as ROS and
arachidonic acid metabolites can also activate TRPV1 located at airway sensory nerve terminals (1, 46). Therefore, the interaction between TRPV1 and TRPA1 may participate in the AIH-induced sensitization of LVCFs observed in the present study.

In this study, capsaicin injection induced bradycardia and hypotension, both of which are also reflex consequences resulting from stimulation of LVCFs (31). However, exposure to 0% O₂ AIH only potentiated the capsaicin-induced decrease in MABP, but not HR in the reflex study, and failed to augment responses of MABP and HR in the electrophysiological study. The lack of potentiation effects of AIH on these cardiovascular responses may be due to the differential impact of anesthesia on respiratory and cardiovascular controls in the reflex study and due to the fact that the right vagus nerve was cut in the electrophysiological study.

**Perspectives and Significance**

The present study reveals that AIH may produce LVCF-mediated airway hypersensitivity in rats that depends on both the involvement of ROS and activation of TRPA1 receptors. In the setting of OSA, intermittent hypoxia occurs during sleep and may increase oxidative stress in the airways (8). TRPA1 receptors have been proposed as a major sensor for ROS in the airways (5). It is reasonable to speculate that excess ROS in airways can act on TRPA1 located at terminals of LVCFs to induce airway hypersensitivity. Additionally, increased thoracic pressure swings can produce
inflammation (19, 21), indicating the possible effects sensitization from thoracic pressure swings with hypoxic stimulated breathing. Thus, our findings provide a possible mechanism to understand the pathogenesis of hyperreactive airway diseases in patients with OSA. Thus, modulations of the ROS level and TRPA1 receptors in the airways are possible target choices for potential therapeutic regimes to OSA-induced hyperreactive airway diseases.

ACKNOWLEDGEMENTS

The authors thank Dr. Yu Ru Kou (National Yang-Ming University, Taipei, Taiwan) for his valuable advises regarding revision of this manuscript and Laura Smales for help with language editing.

GRANTS

This study was supported by grants from Tzu Chi University, Taiwan (TCIRP95004-02 and TCIRP98002-03), and the National Science Council, Taiwan (NSC97-2320-B-320-004 and NSC98-2628-B-320-003-MY3).
REFERENCES


7. **Caceres AI, Brackmann M, Elia MD, Bessac BF, del Camino D, D'Amours M, Witek JS, Fanger CM, Chong JA, Hayward NJ, Homer RJ, Cohn L, ...


20. **Gu Q, Ruan T, Hong JL, Burki N, Lee LY.** Hypersensitivity of pulmonary C fibers induced by adenosine in anesthetized rats. *J Appl Physiol* 95: 1315-1324;


33. **Lin YS, Hsu CC, Bien MY, Hsu HC, Weng HT, Kou YR.** Activations of TRPA1 and P2X receptors are important in ROS-mediated stimulation of capsaicin-sensitive lung vagal afferents by cigarette smoke in rats. *J Appl*


46. **Ruan T, Lin YS, Lin KS, Kou YR.** Sensory transduction of pulmonary reactive


FIGURE LEGENDS

Fig. 1. Reflex apnea induced by intravenous injection of capsaicin (1 μg/kg) in four anesthetized, open-chest, and artificially ventilated rats under 4 treatments: 10 episodes of room air (RA; A), 10%O₂ (B), 5%O₂ (C), and 0%O₂ (D) acute intermittent hypoxia (AIH). Capsaicin solution (0.1 ml) was first slowly injected into the catheter (dead space 0.2 ml), then flushed into the right atrium (at the arrow) as a bolus with saline. Apneic duration to capsaicin induction was the longest expiratory time (duration between phrenic inspirations) within the first 5 breaths after capsaicin injection before and 10 and 30 min after RA or AIH exposure; dashed lines indicate the apneic duration to capsaicin. AP, phrenic action potential; Ptr, tracheal pressure; ABP, arterial blood pressure.

Fig. 2. Effect of AIH on reflex apnea response to capsaicin. A: before and 10 and 30 min after RA or AIH exposure. After rats underwent vagotomy, the apneic response to capsaicin injection at 30 min after 0% O₂ AIH exposure was measured [VAG+AIH (0%O₂)]. Magnitude of the reflex apneic response was reflected by the apneic ratio, calculated as the longest expiratory duration (duration between phrenic inspirations; Tₑ) during the first 5 breaths after capsaicin injection divided by baseline Tₑ. B: response to capsaicin in 0%O₂ AIH rats after various pretreatments: vehicle-1: vehicle
of N-acetyl-L-cysteine (NAC) or dimethylthiourea (DMTU); DMTU; NAC. C: response with 0% O₂ AIH after pretreatment with vehicle-2 (vehicle of HC-030031), 1.5 mg/kg HC-030031 [HC(1.5)] or 3.0 mg/kg HC-030031 [HC(3.0)]. *, P < 0.05 compared with RA; †, P < 0.05 compared with 5% O₂ AIH; #, P < 0.05 compared with 0% O₂ AIH or Vehicle+AIH. Data are mean ± SE of 8 rats.

Fig. 3. Effect of 0% O₂ AIH on reflex apneic response to α,β-methylene-ATP (α,β-meATP; 15 µg/kg). A: response to α,β-meATP injection before and 10 and 30 min after RA or 0% O₂ AIH. B-C: response to α,β-meATP in 0% O₂ AIH rats after pretreatment with vehicle-1, NAC, DMTU, vehicle-2, or 3.0 mg/kg HC-030031 [HC(3.0)]. *, P < 0.05 compared with RA; #, P < 0.05 compared with 0% O₂ AIH or Vehicle+AIH. Data are mean ± SE of 8 rats. See legend of Fig. 2 for detail.

Fig. 4. Effect of AIH on response of lung vagal C fibers (LVCFs) to capsaicin (1 µg/kg) in four anesthetized, open-chest, and artificially ventilated rats. LVCF recordings in response to capsaicin injection (arrow) before and 10 and 30 min after exposure to 10 episodes of RA (A), 10% (B), 5% (C), and 0% (D) O₂ AIH. AP, LVCF action potential; Ptr, tracheal pressure; ABP, arterial blood pressure. See legend of Fig. 1 for detail.
Fig. 5. Effect of AIH on response of LVCFs to capsaicin injection after experimental interventions. Fiber activity (FA) represents peak FA (average over 2-s intervals) and baseline FA (average over 10-s intervals). A: before and 10 and 30 min after RA or AIH. B-C: responses to capsaicin injection in 0%O₂ AIH rats after pretreatment with vehicle-1, NAC, DMTU, vehicle-2, 1.5 mg/kg HC-030031 [HC(1.5)], 3.0 mg/kg HC-030031 [HC(3.0)] or AP18. *, P < 0.05 compared with response to RA; †, P < 0.05 compared with 5% O₂ AIH; #, P < 0.05 compared with 0% O₂ AIH or Vehicle+AIH. Data are mean ± SE of 8 rats. See legend of Fig. 2 for detail.

Fig. 6. Effect of 0% O₂ AIH on response of LVCFs to α,β-meATP. A: before and 10, 30 min after RA or 0% O₂ AIH. B-C: responses to α,β-meATP after pretreatment with vehicle-1, NAC, DMTU, vehicle-2, or 3.0 mg/kg HC-030031 [HC(3.0)]. *, P < 0.05 compared with RA; #, P < 0.05 compared with Vehicle+AIH group. Data are mean ± SE of 8 rats. See legend of Fig. 2 for detail.

Fig. 7. Effect of 0% O₂ AIH on the response of LVCFs to lung hyperinflation (Ptr = 30 cmH₂O). In the upper panels, periods between the 2 vertical dashed lines indicate the duration of lung hyperinflation; data were averaged over 1 s to give mean values
to plot responses over time. In the lower panels, fiber activity (FA) represents the peak
FA (average over 10-s intervals) and the baseline FA (average over 10-s intervals).

Data are mean ± SE of 8 rats. *, $P < 0.05$ compared with corresponding baseline.
Table 1. Average mean arterial blood pressure and heart rate to capsaicin injection (1 μg/kg) at 30 min after experimental challenges.

<table>
<thead>
<tr>
<th>Study</th>
<th>Challenge</th>
<th>Capsaicin injection</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>- ΔMABP, mmHg</td>
<td>- ΔHR, Beats/min</td>
<td></td>
</tr>
<tr>
<td>Reflex apneic study</td>
<td>RA</td>
<td>13.6 ± 1.1</td>
<td>51.2 ± 4.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AIH (10%O₂)</td>
<td>13.3 ± 1.5</td>
<td>59.8 ± 9.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AIH (5%O₂)</td>
<td>17.6 ± 2.8</td>
<td>64.0 ± 6.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AIH (0%O₂)</td>
<td>18.4 ± 2.2*</td>
<td>53.2 ± 6.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DMTU+AIH (0%O₂)</td>
<td>10.8 ± 1.2</td>
<td>48.2 ± 3.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NAC+AIH (0%O₂)</td>
<td>13.7 ± 1.4</td>
<td>49.3 ± 3.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vehicle-1+AIH (0%O₂)</td>
<td>19.0 ± 2.1*</td>
<td>57.5 ± 5.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HC(1.5)+AIH (0%O₂)</td>
<td>19.2 ± 1.4*</td>
<td>58.6 ± 7.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HC(3.0)+AIH (0%O₂)</td>
<td>14.7 ± 3.0</td>
<td>52.0 ± 5.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vehicle-2+AIH (0%O₂)</td>
<td>18.5 ± 2.3*</td>
<td>55.8 ± 6.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AIH (0%O₂)+VAG</td>
<td>7.8 ± 1.3*</td>
<td>21.4 ± 2.6*</td>
<td></td>
</tr>
<tr>
<td>C-fiber study</td>
<td>RA</td>
<td>10.8 ± 2.3</td>
<td>42.5 ± 6.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AIH (10%O₂)</td>
<td>11.8 ± 2.4</td>
<td>48.8 ± 8.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AIH (5%O₂)</td>
<td>14.5 ± 2.1</td>
<td>46.6 ± 7.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AIH (0%O₂)</td>
<td>15.0 ± 2.5</td>
<td>41.9 ± 9.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DMTU+AIH (0%O₂)</td>
<td>11.0 ± 3.0</td>
<td>35.5 ± 6.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NAC+AIH (0%O₂)</td>
<td>10.2 ± 2.9</td>
<td>34.0 ± 7.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vehicle-1+AIH (0%O₂)</td>
<td>13.9 ± 1.8</td>
<td>43.8 ± 9.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HC(1.5)+AIH (0%O₂)</td>
<td>10.3 ± 1.9</td>
<td>41.1 ± 5.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HC(3.0)+AIH (0%O₂)</td>
<td>11.8 ± 2.2</td>
<td>37.6 ± 9.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vehicle-2+AIH (0%O₂)</td>
<td>14.3 ± 2.3</td>
<td>39.9 ± 8.8</td>
<td></td>
</tr>
</tbody>
</table>

Data in each group are mean ± SE of 8 rats. MABP, mean arterial blood pressure; HR, heart rate; -ΔMABP and -ΔHR = peak response – baseline. *, P < 0.05 compared with RA. See legend of Fig. 2 for detail.
Table 2. Effect of experimental challenges on baseline expiratory time.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Before challenge</th>
<th>10 min after challenge</th>
<th>30 min after challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>1.39 ± 0.14</td>
<td>1.37 ± 0.13</td>
<td>1.40 ± 0.14</td>
</tr>
<tr>
<td>AIH (10%O₂)</td>
<td>1.43 ± 0.17</td>
<td>1.45 ± 0.16</td>
<td>1.42 ± 0.17</td>
</tr>
<tr>
<td>AIH (5%O₂)</td>
<td>1.35 ± 0.22</td>
<td>1.33 ± 0.17</td>
<td>1.30 ± 0.18</td>
</tr>
<tr>
<td>AIH (0%O₂)</td>
<td>1.44 ± 0.15</td>
<td>1.41 ± 0.14</td>
<td>1.43 ± 0.15</td>
</tr>
</tbody>
</table>

Data in each group are mean ± SE of 8 rats. Baseline data were calculated as the mean over the 10-breath period immediately preceding the capsaicin injection in the reflex study. See legend of Fig. 2 for detail. Data before and after challenge did not significantly differ (P > 0.05).
Table 3. Average peak responses of lung vagal C fibers to capsaicin injection in RA rats before and after pretreatment with antioxidants or TRPA1 antagonists.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>FA (impulses/s)</th>
<th>Before RA</th>
<th>10 min after RA</th>
<th>30 min after RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMTU</td>
<td>8.10 ± 0.70</td>
<td>8.21 ± 0.73</td>
<td>8.30 ± 0.81</td>
<td></td>
</tr>
<tr>
<td>NAC</td>
<td>7.86 ± 0.41</td>
<td>8.10 ± 0.25</td>
<td>8.42 ± 0.92</td>
<td></td>
</tr>
<tr>
<td>HC (3.0)</td>
<td>7.79 ± 0.66</td>
<td>7.90 ± 0.80</td>
<td>7.95 ± 0.73</td>
<td></td>
</tr>
<tr>
<td>AP18</td>
<td>8.12 ± 0.78</td>
<td>8.48 ± 0.72</td>
<td>8.01 ± 0.83</td>
<td></td>
</tr>
</tbody>
</table>

Data in each group are mean ± SE of 6 rats. See Figure 2 for full names of drugs.

Fiber activity (FA) represents peak FA (average over 2-s intervals) to capsaicin injection (1 μg/kg).
Figure 4

A. RA

B. AIH (10% O2)

C. AIH (5% O2)

D. AIH (0% O2)