

**COUGH AND EXPIRATION REFLEXES ELICITED BY INHALED IRRITANT GASES
ARE INTENSIFIED IN OVALBUMIN SENSITIZED MICE**

* Cheng Zhang^{1,3}, * Ruei-Lung Lin¹, Jeff Hong¹, Mehdi Khosravi², and Lu-Yuan Lee¹

Departments of ¹Physiology and ²Medicine, University of Kentucky

Lexington, Kentucky, USA

And

³Department of Respiratory Medicine, Guizhou Provincial People's Hospital

Guiyang, Guizhou, China

* Both authors contributed equally to the study.

Short Running Head: Cough hyperresponsiveness induced by allergen sensitization

Corresponding author: Lu-Yuan Lee, Ph.D.
Department of Physiology
University of Kentucky Medical Center
Lexington, Kentucky 40536-0298
USA
Telephone: 859-323-6339
Email: lylee@uky.edu

Abstract

This study was designed to determine the effect of active sensitization with ovalbumin (Ova) on cough responses to inhaled irritant gases in mice. Conscious mice moved freely in a recording chamber while the pressure change in the chamber, audio and video signals of the mouse movements were recorded simultaneously to measure the frequencies of cough reflex (CR) and expiration reflex (ER). To further verify the accuracy of cough analysis, the intra-pleural pressure was also recorded by a telemetry sensor surgically implanted in the intra-pleural space in a subgroup of mice. During the irritant gas inhalation challenge, sulfur dioxide (SO₂; 200 & 400 ppm) or ammonia (NH₃; 0.1% & 0.2%) was drawn into the chamber at a constant flowrate for 8 minutes. Ova sensitization and sham sensitization with vehicle (Veh) were performed over a 25-day period in separate groups of mice. Our results showed: 1) Both SO₂ and NH₃ inhalation challenges increased CR and ER frequencies in a concentration-dependent manner before Ova sensitization. 2) The baseline CR frequency was significantly elevated after Ova sensitization, accompanied by pronounced airway inflammation. 3) Ova sensitization also markedly augmented the responses of CR and ER to both SO₂ and NH₃ inhalation challenges; in sharp contrast, the cough responses did not change after sham sensitization in the Veh group. In conclusion, Ova sensitization caused distinct and lingering increases in baseline cough frequency, and also intensified both CR and ER responses to inhaled irritant gases, which probably resulted from an allergic inflammation-induced hypersensitivity of airway sensory nerves.

Keywords: airway, inflammation, allergen, cough, inhaled irritants

Introduction

Chronic airway inflammation induced by active sensitization with allergen significantly enhances the sensitivity of vagal bronchopulmonary C-fibers in Brown-Norway rats (22, 39, 40). These C-fibers represent a majority of the vagal afferents that innervate the entire respiratory tract, and play an important role in regulating airway functions in animals and humans under both normal and disease conditions (8, 25, 30). These sensory nerve endings can be activated abruptly by irritant gases inhaled into the respiratory tract, which triggers important airway reflex responses such as cough, bronchoconstriction and airway secretion; these reflex responses can expel and/or reduce the penetration of these harmful gases into deeper regions of the lung. On the other hand, hypersensitivity of these C-fiber afferents resulting from airway allergic inflammation (22, 34, 39) can induce cough hyperresponsiveness and excessive bronchoconstriction, which are common symptoms of allergic airway inflammation. Whether allergic inflammation alters the cough response to inhaled irritant gases in mice is yet not known.

This study was therefore designed to test the hypothesis that the cough responses to inhalation of sulfur dioxide (SO₂) and ammonia (NH₃) are elevated following induction of chronic airway inflammation by active sensitization with ovalbumin (Ova) in awake mice. SO₂ and NH₃ were chosen for this study because both are irritant gases that are present in the environment and can cause lung injury at high concentrations during occupational exposure or chemical accident (10, 11)

Both cough and expiration reflexes are airway protective functions, and their regulatory mechanisms have been studied extensively in various animal species such as guinea

pigs, dogs, cats and monkeys (29). Despite the vast and growing interest generated by the availability of various transgenic and knockout mouse models for physiological and pharmacological studies, the advances in our knowledge about the regulation of cough reflex (CR) and expiration reflex (ER) in this species have been relatively limited, in comparison with other animal species. In fact, questions have been raised as to whether the cough reflex is present in mice (1, 29). One of the reasons is probably related to the fact that cough is suppressed by anesthesia, whereas respiratory signals recorded in awake mice are inherently noisy, making it difficult to accurately analyze the CR and ER responses. Hence, another aim of this study was to characterize and differentiate the CR and ER responses to inhaled irritant gases in awake mice by recording the intra-pleural pressure directly with the telemetry technique in a subgroup of these animals.

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Methods

Thirty adult male mice (C57BL6/J) were used in this study; their average age at the beginning of the study was 12.8 wk. The procedures described below were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health, and were also approved by the University of Kentucky Institutional Animal Care & Use Committee.

Measurements of Cough Responses: During the experiment, awake mice moved freely in a plexiglass recording chamber (volume 160 ml). To prevent CO₂ accumulation and hyperthermia, room air was drawn through the chamber at a constant flow rate (200 ml/min) by a negative pressure (Fig. 1). The CO₂ concentration (CWE model CapStar-100, Ardmore PA) and temperature (PhysiTemp model TH-8, New Jersey) of the outlet air were continuously monitored, which reached steady-state peaks of ~0.7% and ~25°C, respectively. The pressure in the chamber (P_{cham}) was recorded continuously by a pressure transducer (Biopac model TSD160A, Goleta, CA); both audio and video signals of the mouse movements were also recorded simultaneously by a microphone (Zalman model Zm-Mic1; South Korea) and a video camera (Logitech model C920; Switzerland), respectively. These signals were used collectively for identifying and analyzing the cough frequency.

Breathing is inherently unstable in awake free-moving mice, which can alter the respiratory signals and affect its analysis. To further verify the accuracy of the cough analysis, the intra-pleural pressure (P_{ip}) was measured directly in 44.4% (8/18) of the animals studied for cough: the tip of a telemetry sensor (Data Sciences International

model PA-C10, St. Paul, MN; marked by the red arrow in Fig. 1) and the transmitter (green arrow) with an attached catheter were surgically implanted in the intra-pleural space and under the lower back skin, respectively, under anesthesia by isoflurane inhalation. Two to three weeks were allowed for recovery from the surgery before any measurement or experiment was performed in the animal.

Inhalation Challenges of Irritant Gases: During the inhalation challenge, a gas mixture of SO₂ or NH₃ was drawn into the chamber (replacing room air) at the same flow rate (200 ml/min) for 8 min (Fig. 1). Two concentrations of SO₂ (200 and 400 ppm) and NH₃ (0.1 and 0.2 %) balanced in air were tested in each animal; SO₂ (2500 ppm) and NH₃ (100%) contained in gas cylinders (Scott-Gross, Inc.; Lexington, KY) were diluted by air to the desired concentrations in each experiment. The mouse was placed in the recording chamber for >30 min of adaptation period before the beginning of experiment. All signals were recorded for 5 min before, 8 min during and 5 min after each inhalation challenge. At least 30 min was allowed for a full recovery between two consecutive challenges.

Ova Sensitization: Mice were randomly divided into two groups. One group (Ova group; n=18) was actively sensitized by intra-peritoneal (ip) injection of 10 µg Ova/1 mg aluminum hydroxide (Imject Alum, Thermo Scientific, Rockford, IL) in 0.3 ml of sterile saline suspension on day 1 and day 11. Beginning on day 21, the mice were treated with Ova aerosol, 30 min daily for 5 consecutive days. Aerosols were generated from Ova/saline solution (wt/vol: 1%) by an ultrasonic nebulizer (Devilbiss model 100, Port Washington, NY). During the treatment, the awake mouse was placed in a plastic restrainer and breathed aerosol through a nose cone connected directly to the aerosol

reservoir. The other group (Veh group; n=12) of mice received the ip injections and aerosol inhalations of the vehicles following the identical procedures.

Airway Inflammatory Cell, Mediator and Cytokine Analyses: To determine the severity of airway inflammation induced by Ova-sensitization, bronchoalveolar lavage fluid (BALF) was obtained in both Ova and Veh groups of mice (n=6 in each group) at 3-5 hours after the last inhalation exposure to Ova or Veh aerosol on day 25 (no cough measurement was made in these mice). The procedures have been described in details in our previous study (26). Briefly, the BALF samples were collected and centrifuged at 1500 rpm, 4°C for 10 min. The pelleted cells were treated with 0.24-ml TRIS-buffered ammonium chloride solution (pH 7.2) to lyse red blood cells, and the remaining cells were washed with 1.2-ml PBS supplemented with Hank's buffer solution with 20% fetal bovine serum (FBS). Differential leukocyte counts were then performed on cytopspin slides (by University of Kentucky Clinical Laboratory) after Wright-Giemsa staining using standard morphologic criteria. A minimum of 500 leukocytes were counted by two different individuals, and the data were then averaged.

To compare the inflammatory mediators and cytokines released in the BALF, supernatants were transferred to other tubes for enzyme-linked immunosorbent assay (ELISA) of inflammatory mediators and cytokines including leukotrienes (LTs) C₄/D₄/E₄, thromboxane B₂ (TXB₂), interleukin (IL)-5 and tumor necrosis factor alpha (TNFα). All assays of the BALF collected from Ova and Veh groups were performed at the same time to avoid any possible experimental error and variability. Only these inflammatory mediators and cytokines were selected for measurements in this study because they are known to be upregulated during allergic inflammation (7, 16), and based upon the

previous reports, they can cause a stimulatory or sensitizing effect on airway sensory nerves (17, 21, 26, 27, 31).

Experimental Protocols and Data Analysis

Experiments were initiated after the mice had acclimatized to the chamber and experimental protocols daily for a week. Cough responses to SO₂ and NH₃ inhalation challenges during control (before Ova sensitization or Veh treatment) were studied on three separate days in each animal, and the control data were calculated as the average of the three days. Identical protocols were then carried out at the following time points: 1 (3-5 hours), 2, 3 and 10 days following the last inhalation exposure to Ova or Veh.

The signals (pressures, audio and video) recorded in each inhalation challenge were played back and analyzed; frequencies of CR and ER were calculated as the total numbers of CR and ER elicited in each minute for the entire duration of each testing condition. These numbers were independently determined by two individuals, and the data from their determinations are averaged.

For each inhalation challenge, the CR and ER frequencies were analyzed for the 5-min baseline, 8-min challenge and 5-min recovery in each animal, and the data were then pooled for all animals in that group.

Statistical Analysis

Data were analyzed with the one-way or two-way repeated-measures ANOVA. For example, in the study of how Ova sensitization affected the cough frequency response

to inhaled SO₂, one factor was the treatment effect of Ova, and the other factor was the effect of inhalation challenge. When either one-way or two-way ANOVA showed a significant positive interaction, pair-wise comparisons were made with a *post-hoc* analysis (Fisher's least significant difference). The only exception is in the BALF cell/mediator/cytokine analyses, where the paired t-test was used to evaluate the difference. A value of $P < 0.05$ was considered significant. Data are reported as means $\pm$ SEM.

Results

Breathing pattern was usually unstable in awake, free-moving mouse. The change in P_{cham} reflected the change of volume in the chamber: during inspiration in quiet breathing, the air inhaled into the lung expanded its volume due to an increase in the temperature (from room temperature to body temperature), causing the P_{cham} to increase and reach a peak at the end of each inspiration; likewise, P_{cham} continued to decrease during expiration until the lung volume reached the functional residual capacity (e.g., Fig. 2A). These changes in P_{cham} were synchronized with the changes of P_{ip} measured directly by the telemetry sensor (Fig. 2A).

Each cough was characterized by three distinct but tightly connected phases in the change of P_{cham} : it was initiated by an inspiratory effort (an increase in P_{cham}) followed by a chest compression (depicted by a sharp reverse and decrease in P_{cham}), and then a forced expiration (a sharp increase in P_{cham}) coinciding with a cough sound relatively weaker than that in larger animals (e.g., dogs, cats, etc.) and a jerking movement of the head (representative audio/video recordings are submitted with this manuscript as “Supplemental Information”). All these changes in P_{cham} were closely correlated with the synchronous changes in P_{ip} (e.g., Fig. 2A). In addition, CR, similar to that described by other investigators (6, 36, 38), were also detected in awake mice during inhalation challenge with irritant gases. A typical ER consisted of only two phases: a chest compression, with no clear or significant preceding inspiratory effort, and followed by a forced expiration synchronized with a cough sound, which could also be clearly identified by the P_{ip} signal (Fig. 2A). During both CR and ER, the peak positive P_{ip} generated by chest compression usually far exceeded 30 mmHg and the corresponding

210 ΔP_{cham} dropped more than 1 cmH₂O (e.g., Fig. 2A and 2B); during chest compression,
211 the peak P_{ip} reached 60.1 ± 4.1 mmHg during CR (n=63) and 42.1 ± 3.1 mmHg during ER
212 (n=77; $P < 0.05$), whereas the duration of chest compression was not different: 84.1 ± 5.1
213 ms during CR and 88.1 ± 6.1 ms during ER. In this study, ~20% of the responses could
214 not be easily distinguished between CR and ER (e.g., in Fig. 2B), and were categorized
215 as CR/ER in our data analysis.

216 The recorded video and audio signals generated by the mouse were also useful when
217 they were used in conjunction with the changes in P_{cham} , but they were not reliable in
218 general and frequently inaccurate when they were used alone in detecting and
219 identifying coughs in the awake mice.

220 During the control (before Ova or Veh treatment) condition, both SO₂ and NH₃ evoked
221 CRs in a concentration-dependent manner; only very mild responses to the low
222 concentrations (200 ppm of SO₂ and 0.1% of NH₃) were detected (Fig. 3 & 4). The ER
223 frequency abruptly increased in the first minute after exposure to 400 ppm of SO₂, and
224 remained elevated above the baseline during the 8 min of challenge, whereas the CR
225 frequency increased progressively during the SO₂ challenge (Fig. 3). Both CR and ER
226 frequencies gradually declined toward the baseline during the 5-min recovery (Fig. 3).
227 The peak magnitude of these responses varied between animals but was relatively
228 reproducible in the same animal; the group data showed no significant difference
229 ($P > 0.05$) in the CR, ER and CR/ER responses to SO₂ challenge between the three days
230 of control experiments. There was no difference in the control responses to SO₂ (either
231 200 or 400 ppm) between Ova and Veh groups.

232 A pronounced airway inflammation was induced by the Ova sensitization and challenge.
233 The percentages of both eosinophils and neutrophils in the BALF collected from Ova-
234 sensitized mice (n=6) at 3-5 hours after the last Ova aerosol challenge (the same time
235 that cough responses were tested in all other animals) were ~26.7 ($P<0.05$) and ~18.7
236 ($P<0.05$) folds higher than that collected from Veh-treated mice (n=6), respectively
237 (Table 1). In addition, ELISA results showed that the levels of inflammatory mediators
238 LTC₄/D₄/E₄ and TXB₂, and also the pro-inflammatory cytokines TNF α and IL-5 in the
239 BALF of the Ova-sensitized mice were significantly higher than that of the Veh mice (Fig.
240 5).

241 In 3-5 hours after the last Ova aerosol inhalation, a distinct increase in the baseline
242 (before the SO₂ challenge) CR frequency was found in the Ova-sensitized mice (Fig. 6A,
243 top panel); in comparison, there was no significant change in the baseline ER and
244 CR/ER frequencies (Fig. 6A, middle panels). The total cough frequency
245 (CR+ER+CR/ER) at baseline remained elevated for >24 hours, and then gradually
246 declined toward control. In contrast, no change in the baseline CR, ER or CR/ER
247 frequency was detected in the Veh group (Fig. 6A). The responses of CR, ER and
248 CR/ER to SO₂ challenge at low concentration (200 ppm) did not increase significantly
249 after Ova sensitization (Fig. 7A). However, the total cough response (CR+ER+CR/ER)
250 to SO₂ challenge at high concentration (400 ppm) was elevated to >200% of the control
251 (pre-Ova) responses in the Ova group (Fig. 6B); and both CR and ER increased in a
252 similar pattern. The enhanced cough sensitivity persisted for >48 hours after the last
253 exposure to Ova aerosol (Fig. 6B). In sharp contrast, there was no difference in CR, ER

254 or CR/ER responses to SO₂ (400 ppm) between before and after the Veh treatment in
255 the Veh group (Fig. 6B).

256 A potentiating effect of Ova sensitization similar to that on the SO₂ response was also
257 found in the total cough response (CR+ER+CR/ER) to NH₃ challenges at both low and
258 high concentrations (Fig. 6C & 7B) in a separate group of Ova-sensitized mice. The
259 cough responses to the NH₃ challenges were not tested in Veh-treated mice.

260

Discussion

In this study, we have characterized the cough reflex and expiration reflex elicited by inhaled irritant gases in awake mice. Furthermore, we have demonstrated that active sensitization with Ova increased the baseline cough frequency and markedly intensified the responses of cough and expiration reflexes to SO₂ and NH₃ inhalation challenges (Fig. 6 and 7). In sharp contrast, the sham sensitization with vehicle did not cause any change in the cough frequency and sensitivity to these inhaled irritant gases (Fig. 6).

Both SO₂ and NH₃ are colorless gases with a pungent odor and common irritant gases to human respiratory tract. SO₂ in the environmental air is derived primarily from the burning of fossil fuels (coal, oil), and can reach a high concentration in work places such as at power plants or from copper smelting (11). NH₃ is generated by nature as part of the nitrogen cycle in the environment, and is produced in large quantity for industrial use (e.g., agriculture fertilizers or household cleaning products) (10). Both SO₂ and NH₃ can cause lung injury and other harmful health effects to humans at high concentrations or during sustained exposure in the case of chemical accident or unregulated occupational conditions (10, 11). Sensory receptors that detect these irritant gases in the respiratory tract play an important role in performing the first-line pulmonary defense function by eliciting cough and expiration reflexes, which expel and limit the further inhalation of these harmful gases, and protect the rest of the body.

In this study, both SO₂ and NH₃ inhalation challenges increased the frequencies of CR and ER in a concentration-dependent manner (Fig. 3 and 4). The lower concentrations of SO₂ (200 ppm) and NH₃ (0.1%) used in this study were only slightly above the threshold for triggering CR and ER in mice. The cough reflex elicited by inhaled SO₂ or

284 NH_3 is probably elicited by activation of C-fibers and/or rapidly adapting receptors
285 (RARs) located in the airways and lung (4, 17, 25) and/or tracheal cough receptors (3).
286 Indeed, using a single-fiber recording technique, we have shown that SO_2 or NH_3
287 inhaled at the concentrations similar to that used in this study stimulated both these
288 afferents in anesthetized mice (unpublished data; submitted in this manuscript as
289 “Supplemental Information”).

290 The expiration reflex is known to be elicited by stimulation of sensory endings located in
291 the upper airways, particularly in the larynx and upper trachea (33, 36, 38). In this study,
292 both P_{cham} and the P_{ip} signal recorded by telemetry showed that the ER started with a
293 forced expiration without a preceding inspiratory effort (e.g., Fig. 2), which is consistent
294 with the observations reported by previous investigators in other species (33, 36, 38) .
295 During the inhalation challenge with SO_2 or NH_3 in awake mice, CR and ER occurred
296 frequently in combination and rapid succession; two of these examples are shown in Fig.
297 2B. In these cough epochs, the second “cough” is usually an ER, identified based on
298 our criteria: forced expiration without a clear preceding inspiration. Whether the absence
299 of inspiratory effort was due to the rapid and brisk onset of the succeeding “cough” in
300 the epoch cannot be determined in this study. In any case, this may have accounted for
301 the relatively higher ER frequency than the CR frequency during the SO_2 or NH_3
302 inhalation challenge at control (before the Ova sensitization; Fig. 3). Because both CR
303 and ER are airway defense responses against inhaled irritants, they were pooled for
304 data analysis in the evaluation of the overall cough responses to irritant gas inhalations
305 in this study (Fig. 4 & 7).

306 We cannot determine the relative contributions of the different types of vagal afferents in
307 the Ova sensitization-induced cough hyperresponsiveness in this study, but a possible
308 involvement of certain key factors should be considered. SO₂ can form sulfuric acid and
309 activate transient receptor potential vanilloid type 1 (TRPV1) and acid-sensing ion
310 channels (ASICs) (5, 14); both these channels are abundantly expressed in the
311 bronchopulmonary C-fibers (25). NH₃ has been shown to stimulate both TRPV1 and
312 transient receptor potential ankyrine type 1 (TRPA1) receptors (9). Both TRPV1 and
313 TRPA1 receptors are ligand-gated non-selective cation channels (32), and known to
314 play important roles in the inflammation-induced hypersensitivity of vagal
315 bronchopulmonary afferents (25, 30). Recent studies have demonstrated a striking
316 synergistic effect in pulmonary sensory nerves when TRPA1 and TRPV1 were activated
317 simultaneously, which may play an important role in the regulation of airway reflex
318 functions such as cough during airway inflammatory reaction (18, 24, 28).

319 A number of the pathophysiological features of human allergic asthma have been
320 previously described in mice actively sensitized with Ova using the same protocol as in
321 the present study (7). For example, acute inhalation challenge of Ova aerosol triggered
322 both early- and late-phases of bronchoconstriction in Ova-sensitized mice after 30
323 minutes and 3-6 hours, respectively, demonstrating the airway hyperreactivity to the
324 antigen challenge (7). The early-phase bronchoconstriction is mediated primarily by
325 mast-cell derived mediators, and the more sustained late-phase response is
326 characterized by airway obstruction and inflammatory reaction resulting from the action
327 of various endogenous inflammatory mediators and cytokines released from the
328 resident inflammatory cells (e.g. mast cells, macrophages, etc.) as well as the recruited

329 inflammatory cells (neutrophils and eosinophils) (2). Furthermore, the bronchomotor
330 responses to methacholine challenges were also markedly elevated in Ova-sensitized
331 mice, indicating the airway hyperresponsiveness to non-specific bronchoactive
332 challenge (7). Hence, these airway responses in Ova-sensitized mice closely resemble
333 the clinical features and pattern of airway hyperresponsiveness triggered by inhaled
334 antigens in patients with allergic asthma (13, 35, 37). In this study, the elevated baseline
335 cough frequency and enhanced cough responses to inhaled irritant gases were
336 observed at 3-5 h after the last Ova aerosol challenge in sensitized mice (Fig. 6A),
337 coinciding with the time of late-phase airway hyperresponsiveness (7).

338 At approximately the same time that cough responses were measured as described
339 above, the percentages of both eosinophils and neutrophils in the BALF of another
340 group of Ova-sensitized mice were elevated to >15 folds higher than those in the Veh
341 mice in this study (Table 1), revealing an infiltration of inflammatory cells in the airways.
342 Eosinophils and neutrophils are primary inflammatory cells known to release a wide
343 range of potent bronchoactive substances (e.g., cationic proteins, lipoxygenase and
344 cyclooxygenase metabolites of arachidonic acid, etc). Indeed, inflammatory mediators
345 ($\text{LTC}_4/\text{D}_4/\text{E}_4$ and TXB_2) and pro-inflammatory cytokines (IL-5 and $\text{TNF}\alpha$) were also
346 significantly elevated in the BALF of Ova-sensitized mice in this study (Fig. 5). Many of
347 these mediators and cytokines are known to activate or sensitize bronchopulmonary C-
348 fibers; for example, TXA_2 , the precursor of TXB_2 , is a potent stimulator of
349 bronchopulmonary C-fibers (21, 27). LTC_4 can also stimulate the C-fiber afferents and
350 trigger the release of tachykinins in isolated trachea/bronchus of guinea pigs (31).
351 Although $\text{TNF}\alpha$ does not activate these airway sensory receptors directly, it can

markedly enhance their sensitivity (19, 26). Moreover, eosinophil granule-derived cation proteins such as major basic proteins can exert potent stimulatory and sensitizing effects on bronchopulmonary C-fibers (15, 23). All these evidences seem to suggest that an increase in the C-fiber sensitivity is probably involved in the increased cough responses to inhaled irritant gases in Ova-sensitized mice. This notion is in agreement with the finding that the cough sensitivity to inhalation of aerosolized capsaicin, a selective and potent chemical activator of the TRPV1 receptors expressed in C-fiber sensory neurons, is enhanced in asthmatic patients (12). In addition, in Ova-sensitized rats, capsaicin sensitivity was detected in a large percentage of the vagal myelinated afferents including RARs, which normally do not respond to capsaicin (39). Furthermore, immunohistochemical evidence shows that sensitization and challenge with Ova significantly upregulated the expression of TRPV1 in bronchopulmonary neurons in rat nodose ganglia, mainly in neurofilament-positive (myelinated) neurons (39).

Previous investigators have questioned whether the cough reflex is present in mouse (1, 29). This uncertainty is probably related to the technical difficulties in making direct and accurate measurements of the physiological variables characterizing the cough action (tracheal pressure, expiratory flow rate, muscle EMG, etc.) in awake mice (6, 20, 29), due to their small body size and high breathing rate at rest (>250 breaths per minute). In this study, we have used the telemetry technique to measure directly the change in intra-pleural pressure in a subgroup of mice. Our results have yielded definitive evidence illustrating the actions of both cough and expiration reflexes in awake mice that are consistent with those described in other species (3, 36, 38). Certain limitations of the experimental approach used in this study should be noted; for example, the

375 telemetry method requires surgical implantation and care in each animal as well as the
376 cost for purchase of the equipment, which may be prohibitive for a large-scale study.
377 Nonetheless, our results have shown that collective measurements of the change in the
378 chamber pressure together with the audio and video signals can also accurately and
379 reliably determine the cough frequency in awake mice, as verified by the simultaneous
380 recording of the telemetry signal in this study.

381 In conclusion, this study has demonstrated that Ova sensitization caused distinct
382 increases in both baseline cough frequency and cough responses to inhaled irritant
383 gases in awake mice. The cough hyperresponsiveness probably resulted from an
384 allergic inflammation-induced hypersensitivity of airway sensory nerves. Further
385 investigations are required to uncover the signaling pathways and mechanisms involved.

386

387 **Perspectives and Significance:**

388 This study demonstrated that airway allergic inflammation increased the baseline cough
389 frequency and intensified the cough responses to inhaled irritant gases such as SO₂
390 and NH₃ in awake mice. The direct recording of intra-pleural pressure using the
391 telemetry technology in a subgroup of mice has yielded the definitive evidence and
392 further characterized the actions of cough and expiration reflex in this species. This
393 murine model may serve as a useful tool for the future studies of the underlying
394 mechanisms of cough hyperresponsiveness associated with airway inflammation and
395 for testing potential therapeutic interventions.

396

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List of References:

1. **Belvisi MG, and Bolser DC.** Summary: animal models for cough. *Pulmonary pharmacology & therapeutics* 15: 249-250, 2002.
2. **Busse WW, and Lemanske RF, Jr.** Asthma. *The New England journal of medicine* 344: 350-362, 2001.
3. **Canning BJ, Mazzone SB, Meeker SN, Mori N, Reynolds SM, and Undem BJ.** Identification of the tracheal and laryngeal afferent neurones mediating cough in anaesthetized guinea-pigs. *The Journal of physiology* 557: 543-558, 2004.
4. **Canning BJ, Mori N, and Mazzone SB.** Vagal afferent nerves regulating the cough reflex. *Respiratory physiology & neurobiology* 152: 223-242, 2006.
5. **Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, and Julius D.** The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389: 816-824, 1997.
6. **Chen L, Lai K, Lomask JM, Jiang B, and Zhong N.** Detection of mouse cough based on sound monitoring and respiratory airflow waveforms. *PloS one* 8: e59263, 2013.
7. **Cieslewicz G, Tomkinson A, Adler A, Duez C, Schwarze J, Takeda K, Larson KA, Lee JJ, Irvin CG, and Gelfand EW.** The late, but not early, asthmatic response is dependent on IL-5 and correlates with eosinophil infiltration. *The Journal of clinical investigation* 104: 301-308, 1999.
8. **Coleridge JC, and Coleridge HM.** Afferent vagal C fibre innervation of the lungs and airways and its functional significance. *Reviews of physiology, biochemistry and pharmacology* 99: 1-110, 1984.
9. **Dhaka A, Uzzell V, Dubin AE, Mathur J, Petrus M, Bandell M, and Patapoutian A.** TRPV1 is activated by both acidic and basic pH. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 29: 153-158, 2009.
10. **Division-of-Toxicology.** Public Health Statement: Ammonia (CAS#: 7664-41-7). edited by Department of Health And Human Services PHS. Atlanta, GA: Agency For Toxic Substances And Disease Registry, 2004.
11. **Division-of-Toxicology.** Public Health Statement: Sulfur Dioxide (CAS#: 7446-09-5). edited by Department of Health And Human Services PHS. Atlanta, GA: Agency For Toxic Substances And Disease Registry, 1998.
12. **Doherty MJ, Mister R, Pearson MG, and Calverley PM.** Capsaicin responsiveness and cough in asthma and chronic obstructive pulmonary disease. *Thorax* 55: 643-649, 2000.
13. **Gauvreau GM, El-Gammal AI, and O'Byrne PM.** Allergen-induced airway responses. *The European respiratory journal* 46: 819-831, 2015.
14. **Gu Q, and Lee LY.** Characterization of acid signaling in rat vagal pulmonary sensory neurons. *American journal of physiology Lung cellular and molecular physiology* 291: L58-65, 2006.
15. **Gu Q, Wiggers ME, Gleich GJ, and Lee LY.** Sensitization of isolated rat vagal pulmonary sensory neurons by eosinophil-derived cationic proteins. *American journal of physiology Lung cellular and molecular physiology* 294: L544-552, 2008.
16. **Henderson WR, Jr., Tang LO, Chu SJ, Tsao SM, Chiang GK, Jones F, Jonas M, Pae C, Wang H, and Chi EY.** A role for cysteinyl leukotrienes in airway remodeling in a mouse asthma model. *American journal of respiratory and critical care medicine* 165: 108-116, 2002.
17. **Ho CY, Gu Q, Lin YS, and Lee LY.** Sensitivity of vagal afferent endings to chemical irritants in the rat lung. *Respiration physiology* 127: 113-124, 2001.
18. **Hsu CC, and Lee LY.** Role of calcium ions in the positive interaction between TRPA1 and TRPV1 channels in bronchopulmonary sensory neurons. *Journal of applied physiology (Bethesda, Md : 1985)* 118: 1533-1543, 2015.

19. **Hu Y, Gu Q, Lin RL, Kryscio R, and Lee LY.** Calcium transient evoked by TRPV1 activators is enhanced by tumor necrosis factor- α in rat pulmonary sensory neurons. *American journal of physiology Lung cellular and molecular physiology* 299: L483-492, 2010.
20. **Iwata T, Ito I, Niimi A, Ikegami K, Marumo S, Tanabe N, Nakaji H, Kanemitsu Y, Matsumoto H, Kamei J, Setou M, and Mishima M.** Mechanical Stimulation by Postnasal Drip Evokes Cough. *PLoS one* 10: e0141823, 2015.
21. **Karla W, Shams H, Orr JA, and Scheid P.** Effects of the thromboxane A2 mimetic, U46,619, on pulmonary vagal afferents in the cat. *Respiration physiology* 87: 383-396, 1992.
22. **Kuo YL, and Lai CJ.** Ovalbumin sensitizes vagal pulmonary C-fiber afferents in Brown Norway rats. *Journal of applied physiology (Bethesda, Md : 1985)* 105: 611-620, 2008.
23. **Lee LY, Gu Q, and Gleich GJ.** Effects of human eosinophil granule-derived cationic proteins on C-fiber afferents in the rat lung. *Journal of applied physiology (Bethesda, Md : 1985)* 91: 1318-1326, 2001.
24. **Lee LY, Hsu CC, Lin YJ, Lin RL, and Khosravi M.** Interaction between TRPA1 and TRPV1: Synergy on pulmonary sensory nerves. *Pulmonary pharmacology & therapeutics* 35: 87-93, 2015.
25. **Lee LY, and Yu J.** Sensory nerves in lung and airways. *Comprehensive Physiology* 4: 287-324, 2014.
26. **Lin RL, Lin YJ, Geer MJ, Kryscio R, and Lee LY.** Pulmonary chemoreflex responses are potentiated by tumor necrosis factor- α in mice. *Journal of applied physiology (Bethesda, Md : 1985)* 114: 1536-1543, 2013.
27. **Lin S, Li H, Xu L, Moldoveanu B, Guardiola J, and Yu J.** Arachidonic acid products in airway nociceptor activation during acute lung injury. *Experimental physiology* 96: 966-976, 2011.
28. **Lin YJ, Lin RL, Ruan T, Khosravi M, and Lee LY.** A synergistic effect of simultaneous TRPA1 and TRPV1 activations on vagal pulmonary C-fiber afferents. *Journal of applied physiology (Bethesda, Md : 1985)* 118: 273-281, 2015.
29. **Mackenzie AJ, Spina D, and Page CP.** Models used in the development of antitussive drugs. *Drug Discov Today Dis Models* 1: 297-302, 2004.
30. **Mazzone SB, and Undem BJ.** Vagal Afferent Innervation of the Airways in Health and Disease. *Physiological reviews* 96: 975-1024, 2016.
31. **McAlexander MA, Myers AC, and Undem BJ.** Inhibition of 5-lipoxygenase diminishes neurally evoked tachykinergic contraction of guinea pig isolated airway. *The Journal of pharmacology and experimental therapeutics* 285: 602-607, 1998.
32. **Nilius B, Owsianik G, Voets T, and Peters JA.** Transient receptor potential cation channels in disease. *Physiological reviews* 87: 165-217, 2007.
33. **Poljacek I, Rose MJ, Corrie LW, Wang C, Jakus J, Barani H, Stransky A, Polacek H, Halasova E, and Bolser DC.** Short reflex expirations (expiration reflexes) induced by mechanical stimulation of the trachea in anesthetized cats. *Cough (London, England)* 4: 1, 2008.
34. **Riccio MM, Myers AC, and Undem BJ.** Immunomodulation of afferent neurons in guinea-pig isolated airway. *The Journal of physiology* 491 (Pt 2): 499-509, 1996.
35. **Spahn JD.** Asthma biomarkers in sputum. *Immunology and allergy clinics of North America* 27: 607-622; vi, 2007.
36. **Tatar M, Hanacek J, and Widdicombe J.** The expiration reflex from the trachea and bronchi. *The European respiratory journal* 31: 385-390, 2008.
37. **Waserman S, Olivenstein R, Renzi P, Xu LJ, and Martin JG.** The relationship between late asthmatic responses and antigen-specific immunoglobulin. *The Journal of allergy and clinical immunology* 90: 661-669, 1992.
38. **Widdicombe J, and Fontana G.** Cough: what's in a name? *The European respiratory journal* 28: 10-15, 2006.

- 496 39. **Zhang G, Lin RL, Wiggers M, Snow DM, and Lee LY.** Altered expression of TRPV1 and sensitivity to
497 capsaicin in pulmonary myelinated afferents following chronic airway inflammation in the rat. *The*
498 *Journal of physiology* 586: 5771-5786, 2008.
- 499 40. **Zhang G, Lin RL, Wiggers ME, and Lee LY.** Sensitizing effects of chronic exposure and acute
500 inhalation of ovalbumin aerosol on pulmonary C fibers in rats. *Journal of applied physiology*
501 *(Bethesda, Md : 1985)* 105: 128-138, 2008.

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Figure Legends

Fig. 1. *Left panel:* schematic diagram of the experimental set-up. Mic: microphone for recording audio signal; Temp: air temperature in the chamber measured by a thermometer. *Right panel:* X-ray pictures illustrating the positions of the telemetry sensor (red arrow) and transmitter (green arrow) implanted in the intra-pleural space and under the lower back skin of a mouse, respectively. The telemetry catheter was painted with a radio opaque ink (Creative Materials Inc.) for identifying its position.

Fig. 2. A. Representative experimental records illustrating the characteristic features of pressure changes and the audio signal generated by a single cough (CR; *Left*) and a single expiration reflex (ER; *Right*). B. Additional experimental records (from left to right): a CR (closed arrowhead); an ER (open arrowhead); a cough epoch consisting of a combination of CR and ER; and a cough epoch of CR/ER and ER. CR/ER (asterisk) depicts the cough response that could not be distinguished between CR and ER. P_{cham} : pressure inside the chamber; P_{ip} : intra-pleural pressure recorded via the telemetry sensor in awake mice.

Fig. 3. Changes in frequencies of cough reflex (CR) and expiration reflex (ER) in response to inhalation challenges of SO_2 (A) and NH_3 (B) during control (before ovalbumin sensitization or vehicle treatment). CR/ER represented the response that could not be distinguished between CR and ER. Responses were recorded for 5 min

before (baseline), 8 min during (shaded areas) and 5 min after the inhalation challenge (recovery). Responses to both low and high concentrations of each of the irritant gases (SO₂: 200 ppm and 400 ppm; NH₃: 0.1% and 0.2%) were tested, and at least 30 min elapsed between two consecutive challenges. Each test was repeated on three consecutive days in each mouse, and the data were averaged. Each data point represents mean $\pm$ SEM of all the animals tested.

Fig. 4. Group data of total cough responses (CR+ER+CR/ER) to inhalation challenges of SO₂ (A) and NH₃ (B) during control (before ovalbumin sensitization or vehicle treatment). Data (means $\pm$ SEM) represent the group responses averaged over the 5-, 8- and 5-min durations of baseline, during challenge and recovery, respectively; for details, see legend of Fig. 3. *, significantly ($P < 0.05$) different from the baseline data. #, significantly ($P < 0.05$) different from the responses to the low-concentration challenge.

Fig. 5. Effect of ovalbumin (Ova)-sensitization on inflammatory mediators and cytokines in the bronchoalveolar lavage fluid (BALF) collected from the vehicle (Veh)-treated group (open bars) and Ova-sensitized group (closed bars). BALF was collected at 3-5 hours after the last exposure to Ova or Veh aerosol. TNF α : tumor necrosis factor alpha; IL-5: interleukins 5; LTC₄/D₄/E₄: leukotrienes C₄/D₄/E₄; TXB₂: thromboxane B₂. Each data point represents mean $\pm$ SEM (n = the number of mice studied). Data were

analyzed by the t-test. *, significantly different from the Veh group ($P < 0.05$).

Concentrations of TNF α and IL-5 were not detectable in the Veh group.

Fig. 6. Effects of Ova sensitization and Veh treatment on the frequencies of cough reflex (CR) and expiration reflex (ER) in response to inhalation challenges of SO₂ and NH₃ at different time points after the treatment. CR/ER represented the response that could not be distinguished between CR and ER. A, baseline cough frequency averaged over the 5-min duration immediately before the irritant gas inhalation challenge; B and C, cough responses to SO₂ (400 ppm) and NH₃ (0.2%) inhalation challenges, respectively, averaged over the 8-min duration. In A, n=12 in the Ova-sensitized group and n=6 in the Veh-treated group; this difference is due to that the response to NH₃ was not tested in the Veh group (C). Each data point represents mean $\pm$ SEM. *, significantly ($P < 0.05$) different from the data obtained before Ova sensitization or Veh treatment. #, significantly ($P < 0.05$) different from the corresponding data point in the Veh group.

Fig. 7. Potentiating effects of Ova-sensitization on the total cough responses (CR+ER+CR/ER) to inhalation challenges of both low and high concentrations of SO₂ (A) and NH₃ (B) in awake mice. Data (means $\pm$ SEM) represent the group responses averaged over the 5-, 8- and 5-min durations of baseline, during challenge and recovery, respectively. *, significantly ($P < 0.05$) different from the baseline data (open

567 bars). #, significantly ($P < 0.05$) different from the corresponding data obtained before
568 Ova sensitization.

Fig. 1

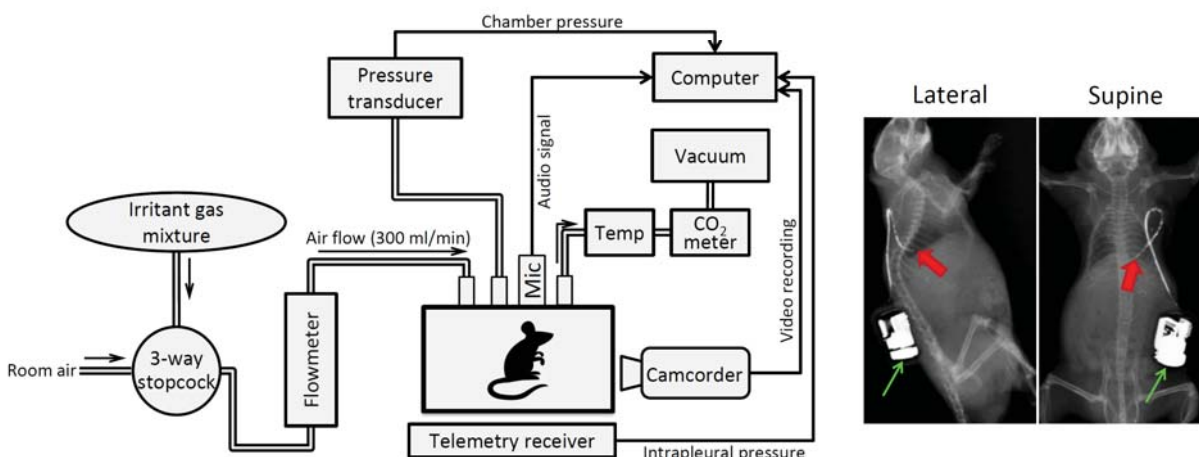


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Fig. 2

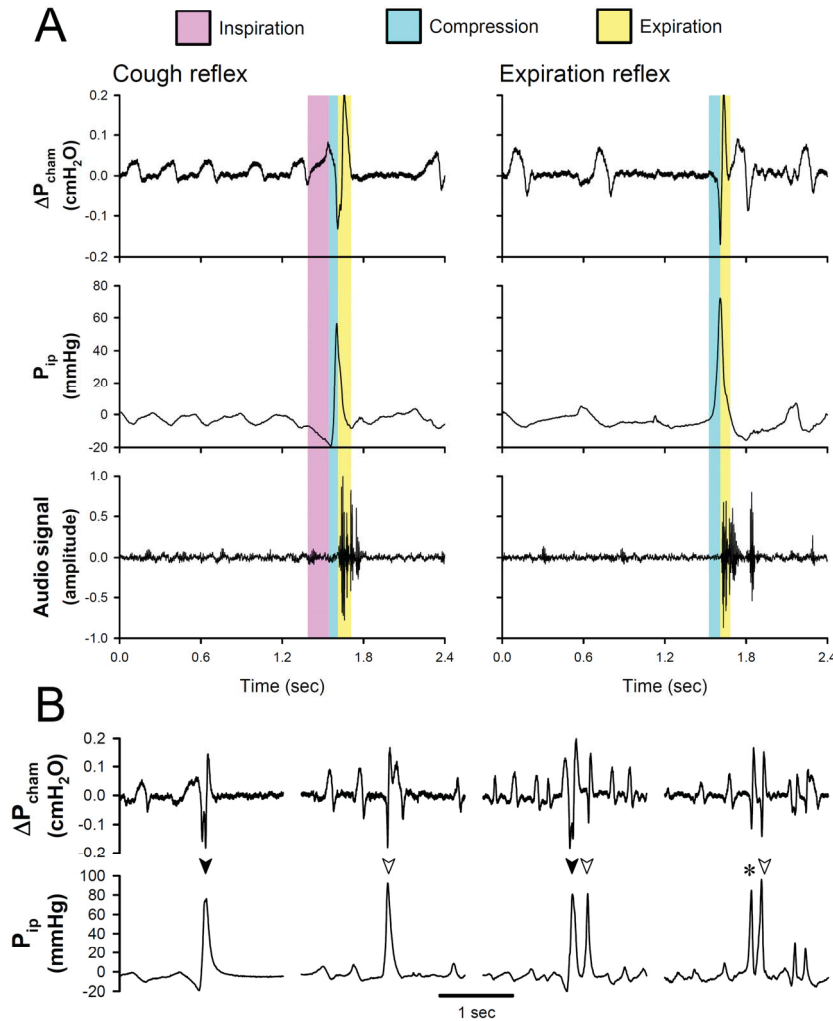


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Fig. 3

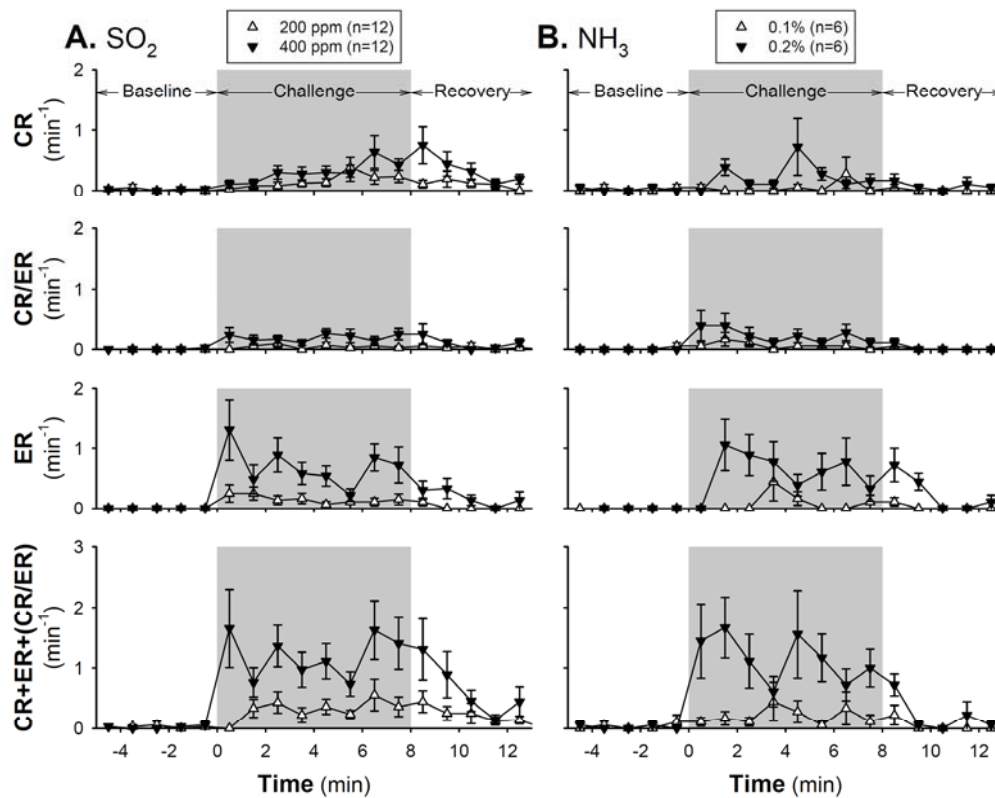


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Fig. 4

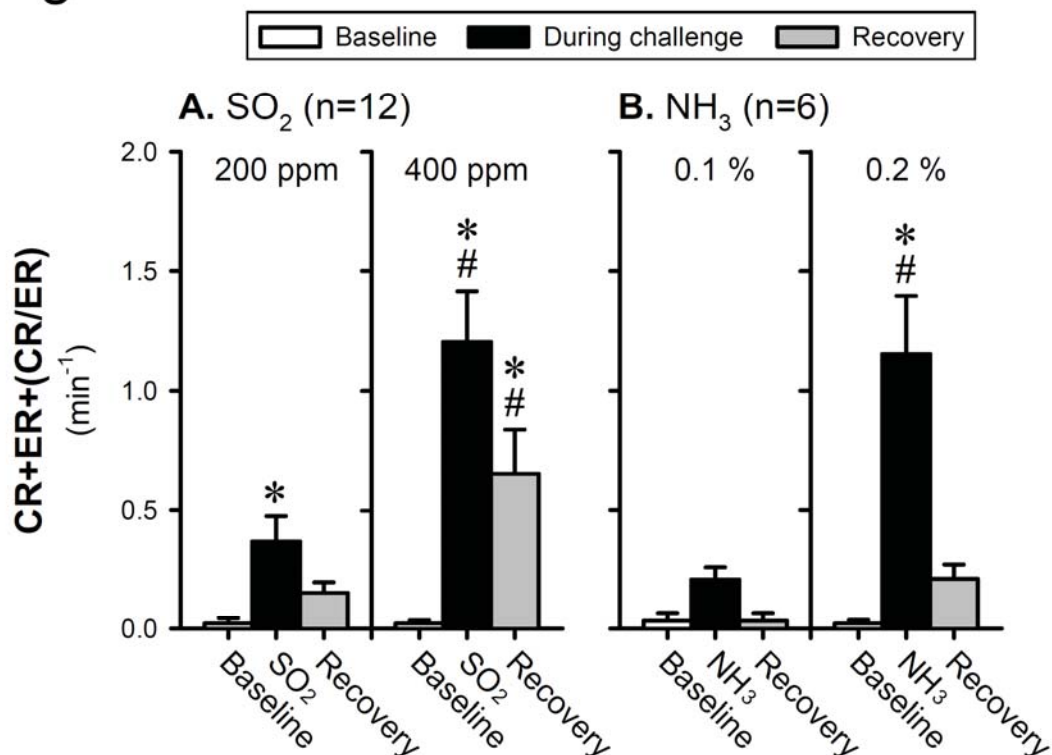


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Table 1. Effect of ovalbumin sensitization on differential cell counts of leukocytes in bronchoalveolar lavage fluid (BALF) of mice.

	Eosinophils (%)	Neutrophils (%)	Basophils (%)	Lymphocytes (%)	Monocytes (%)
Control (n=6)	0.22 ± 0.16	0.48 ± 0.22	0.03 ± 0.03	10.55 ± 0.76	88.71 ± 0.52
Sensitized (n=6)	5.95 ± 0.59*	8.89 ± 2.19*	0.12 ± 0.07	18.62 ± 1.41*	66.46 ± 2.32*

Data were analyzed by the t-test. *, significantly different from control group (P<0.01)

Fig. 5

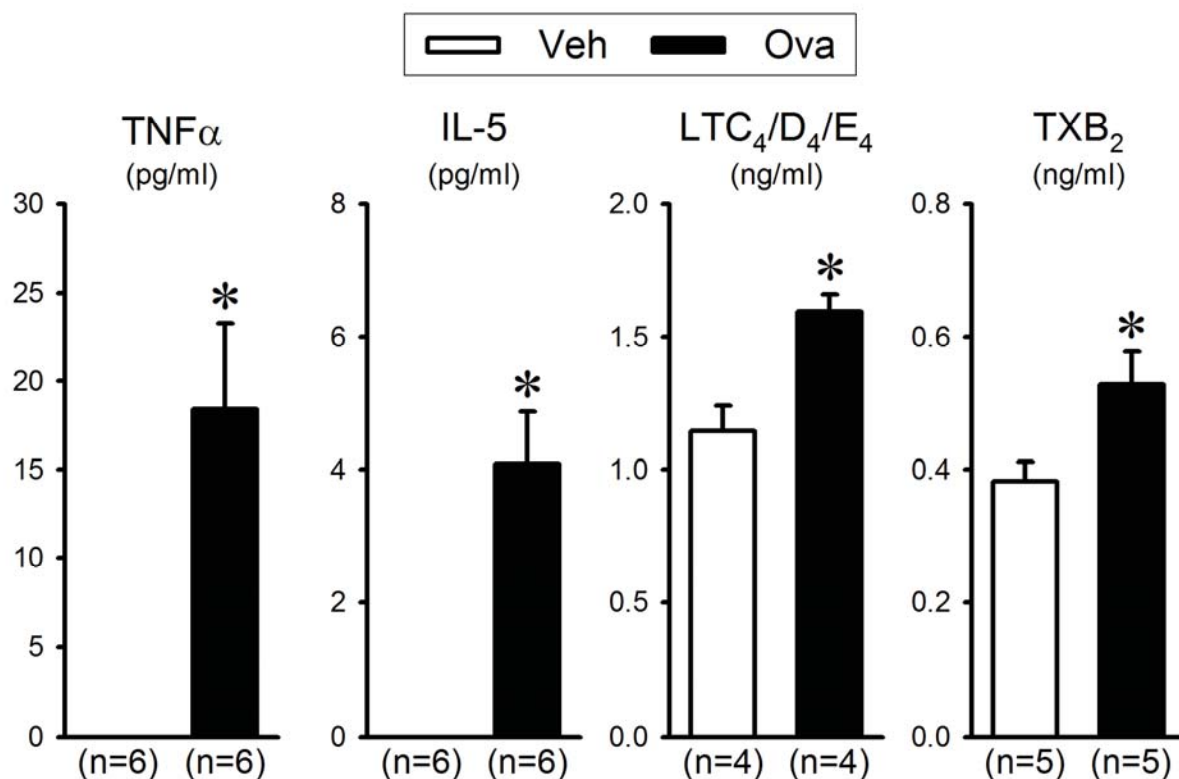


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Fig. 6

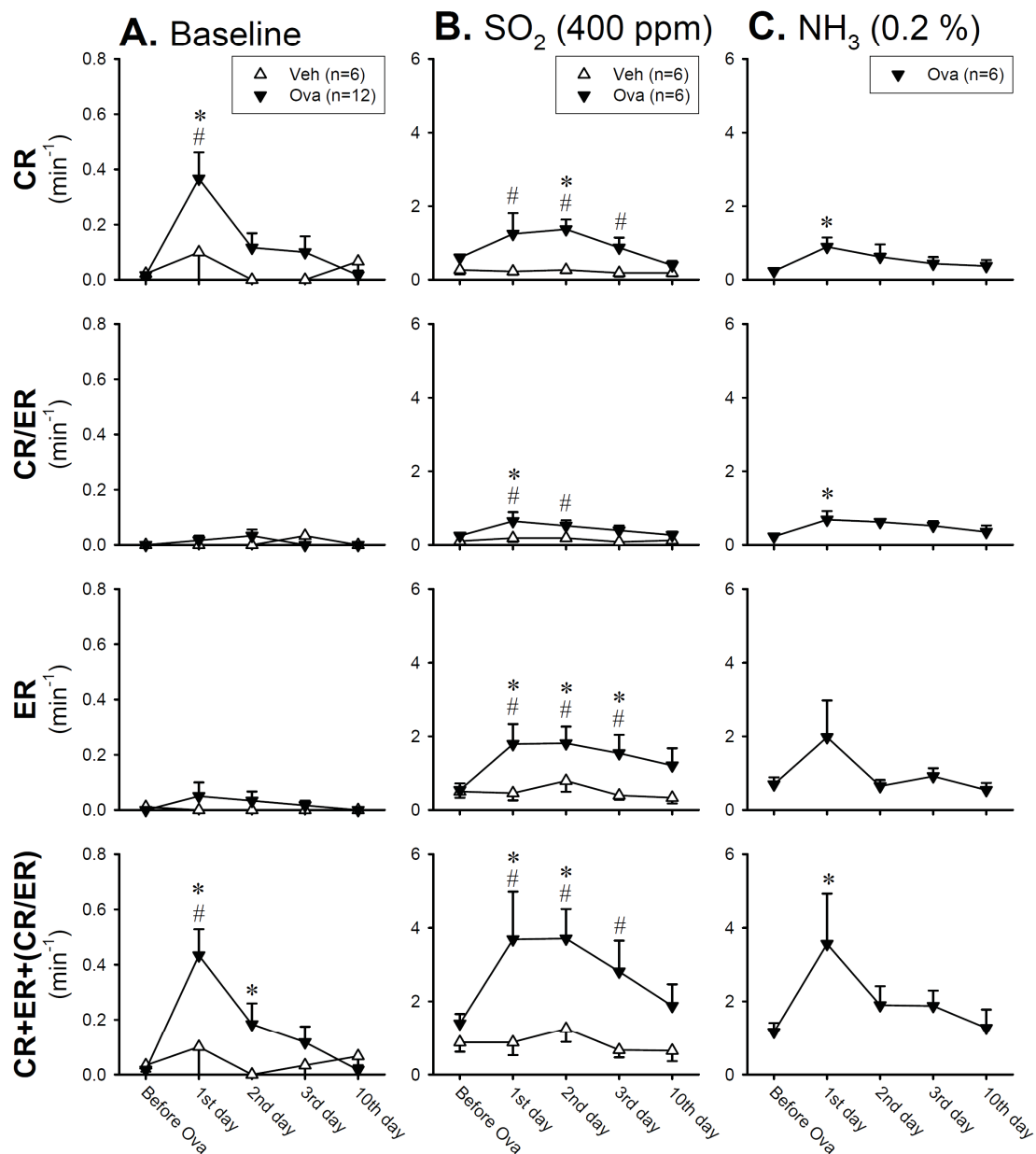
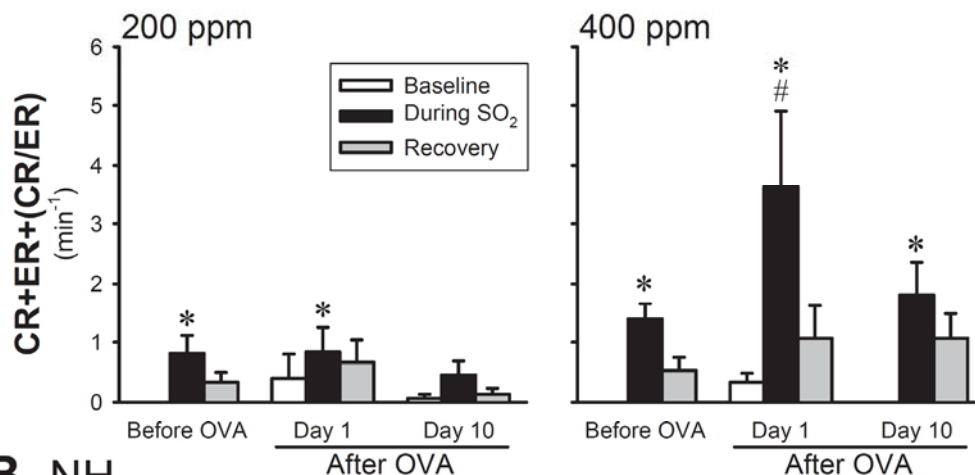


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Fig. 7

A. SO₂



B. NH₃

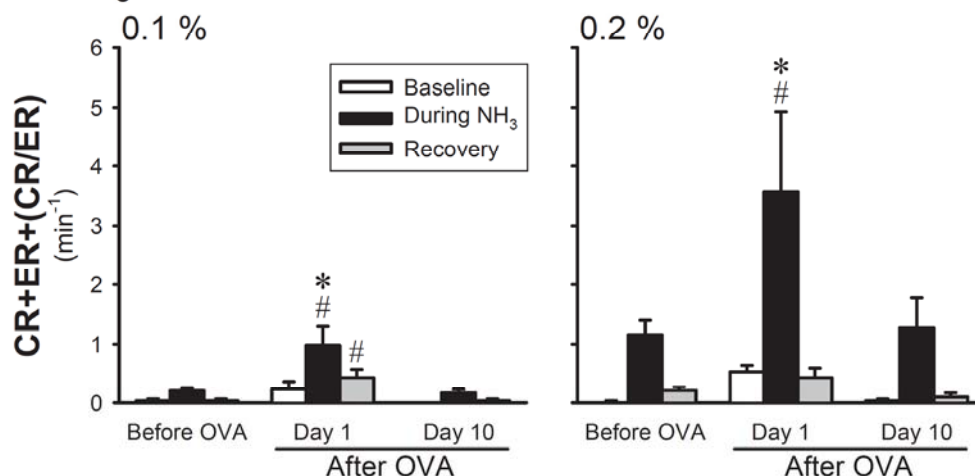


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