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Award Number: W81XWH-10-2-0189

TITLE: Pulmonary Stress Induced by Hyperthermia: Role of Airway Sensory Nerves

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REPORT DATE: October 2011

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-10-2011	Final	30 Sep 2010 - 29 Sep 2011

4. TITLE AND SUBTITLE
Pulmonary Stress Induced by Hyperthermia: Role of Airway Sensory Nerves

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-2-0189

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Kentucky
Lexington, KY 40506

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Based upon the results obtained from these studies, we can draw the following conclusions: 1) Airway hyperresponsiveness developed in Ova-sensitized mice was less pronounced in TRPV1-null mice, indicating an important role of TRPV1. 2) An increase in airway temperature within the normal physiological range triggered bronchoconstriction in sensitized rats, but not in control rats. Chronic airway inflammation in sensitized animals is likely a major contributing factor in causing this response. 3) transient increase in airway resistance was generated immediately after hyperventilation with warm humid air in patients with mild asthma, but the same warm humid air challenge failed to cause any bronchoconstriction in healthy subjects. Furthermore, this bronchoconstriction is likely generated by the increase in airway temperature because hyperventilation with humidified air at room temperature did not generate any change in airway resistance in the same patients. These studies, once completed, should provide important and novel information for: 1) documenting the pulmonary stresses induced by hyperthermia in healthy individuals and in patients with sensitized airways; 2) understanding the mechanism underlying the hyperthermia-induced pulmonary dysfunction; and 3) detecting the susceptibility to heat stress in soldiers with underestimated or overlooked airway hypersensitivity such as in airway allergy or mild asthma.

15. SUBJECT TERMS
Hyperthermia, asthma, airway constriction, cough, dyspnea

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	15	19b. TELEPHONE NUMBER *(include area code)*

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Introduction

It is well documented that vagal bronchopulmonary C-fiber sensory nerves play an important role in the overall regulation of cardiopulmonary functions and protection of the airways against various environmental stresses. Stimulation of these sensory nerves is known to trigger cough, bronchoconstriction, and other cardiopulmonary reflex responses (1). Recent studies conducted in our lab have established the first evidence that the sensitivity of pulmonary C-fiber endings was markedly elevated when the intrathoracic temperature exceeded ~39.2°C (2, 3). Furthermore, this effect of hyperthermia is primarily mediated through an activation of the temperature-sensitive TRPV1 ion channels expressed on vagal bronchopulmonary C-fibers (3, 4). The working hypothesis of this TATRC project is that the expression of this TRPV1 channel is up-regulated in the airway mucosa of patients with mild asthma, allergic rhinitis and upper respiratory infection, which makes these patients more susceptible to the bronchoconstriction and other respiratory dysfunctions induced by thermal stress. There are two specific aims for the first year of this translational project: 1) To investigate the role of the TRPV1 channel in triggering the bronchoconstriction caused by airway hyperthermia, and to determine whether this acute bronchoconstrictive effect is amplified by chronic airway allergic reaction. 2) To determine if thermal stress generates various airway dysfunctions (dyspnea, airway constriction, cough, etc) in healthy volunteers, and in patients with mild asthma, allergic rhinitis and post upper respiratory infection.

Body

The USAMRC/ACURO and HRPO protocols for our proposed animal and human studies were approved on January 11th and July 20th, respectively. We initiated the proposed experiments immediately after receiving the approvals of these protocols, and have made the following major research progresses in this project:

Task 1-1:

TRPV1-null mice model: We purchased the breeding pairs from the Jackson Laboratory, and established a TRPV1-null (B6.129X1-Trpv1^{tm1Jul}/J) mice colony in our lab for carrying out the proposed study. To confirm that TRPV1 gene knock out was maintained through generations of offspring in our breeding colony, PCR experiment was performed in four TRPV1-null breeding pairs and two pairs of the offspring (one pair each from the two generations). Our results confirmed the absence of the TRPV1 gene expression in the PCR reaction product obtained from the tissue in both breeding pairs and offspring of TRPV1-null mice, whereas the tissue from the wild-type (WT; C57BL/6J) mice contained the 984 bp fragment corresponding to the TRPV1 allele.

Pulmonary nodose-jugular ganglion neurons were identified by the retrograde labeling of DiI, and a large percentage (>70%) of these neurons, mostly of small- and medium-size, express TRPV1-immunoreactivity in WT mice (e.g., Fig. 1). In contrast, TRPV1-IR was not found in any of the TRPV1-null pulmonary nodose-jugular neurons.

Capsaicin ($5x10^{-7}$ to 10^{-6} M, 3 s) evoked an inward current in pulmonary nodose-jugular ganglion neurons isolated from WT mice, but failed to evoke any response in TRPV1-null neurons. In comparison, 2-APB and acid still consistently activated the TRPV1-null neurons, although the responses were significantly smaller than that in the WT mice (Fig. 2). These data demonstrated a distinct difference in the sensitivity between pulmonary sensory neurons isolated from WT and TRPV1-null mice.

Ovalbumin (Ova) Sensitization: Young TRPV1-null mice and WT mice (n = 6 in each group; 20-30 g) were sensitized by i.p. injection of 10 µg Ova/1 mg aluminum hydroxide in 0.3 ml of sterile saline suspension on day 1 and day 10. Beginning on day 20, the mice were exposed to Ova aerosol, 30 min daily for 5 consecutive days. Aerosols were generated from Ova/saline solution (wt/vol 1% in sterile saline) by an ultrasonic nebulizer (Devilbiss model 100). During exposure, the unanesthetized mice were placed in a plastic restrainer and breathed aerosol through a nose cone that was connected directly to the aerosol reservoir. Control mice (n = 6 in WT group and n = 6 in TRPV1-null group) received the i.p. injections and aerosol inhalation of the vehicle following the identical procedures.

Assessment of Airway Inflammation and Hyperresponsiveness: On day 24, mice were anesthetized and mechanically ventilated via a tracheal cannula. Transpulmonary pressure, respiratory flow and integrated volume were analyzed on a breath-by-breath basis by an on-line computer for total pulmonary resistance (R_L) and dynamic lung compliance (C_{dyn}). In each animal, the dose-responses of R_L and C_{dyn} to bolus injections (20 µl) of methacholine (MCh; 10, 20, 40 and 60 µg/kg, with ~10 min recovery between injections) were determined.

To determine the presence and severity of airway inflammation induced by Ova-sensitization, this study was carried out in two groups of WT mice: control and Ova-sensitized (22-28 g; n=6 in each group). Bronchoalveolar lavage fluid (BALF) was collected at 4~6 hours after the last inhalation exposure on day 24, and the differential leukocyte count of the BALF was performed using the standard procedures and criteria.

Summary of Results: Results of our study clearly showed: 1) airway hyperresponsiveness to non-specific bronchoactive challenge (MCh dose response) was developed in Ova-sensitized WT mice (Fig. 3). Furthermore, the airway hyperresponsiveness induced by chronic exposure to Ova was less pronounced in TRPV1-null mice (Fig. 3), indicating an important role of TRPV1 in the Ova-induced airway hyperresponsiveness. 2) The percentages of both eosinophils and neutrophils in the BALF of sensitized mice were higher than those in control rats by more than 15 folds (Table 1). Eosinophils and neutrophils are primary inflammatory cells known to release a wide range of potent bronchoactive substances (e.g., cationic proteins, prostaglandins, leukotrienes, etc). These results have clearly demonstrated an inflammatory reaction in the lungs of Ova-sensitized mice.

Task 1-2:

Animal Model of Allergic Asthma: Brown Norway rats: An amended animal study protocol was submitted and approved on April 8, 2011 by the USAMRMC/ACURO to add a study using Brown Norway rats as an animal model of allergic asthma to test the originally proposed hypothesis.

Ovalbumin (Ova) Sensitization: Adult male rats were separated into 2 groups. Sensitized rats received an initial intraperitoneal injection of a suspension containing 2 mg Ova in 1 ml ImjectAlum as adjuvant. Three days later, these rats were exposed to Ova aerosol for 15 min each time, 3 times per week for 3 weeks. During exposure, the unanaesthetized rat was placed in a Plexiglas restrainer (University of Kentucky, Center for Manufacturing), and breathed spontaneously through a nose cone connected to a free stream of air/aerosol mixture under a negative-pressure exhaust hood. Ova solution (wt vol^{-1} concentration: 5% in saline) was nebulized and delivered by an ultrasonic nebulizer at a droplet size ranging from 0.5 to 5 μm. Control rats received the intraperitoneal injection and aerosol inhalation of the vehicle (isotonic saline) following the identical procedures.

Humidified Warm Air (HWA) Challenge: HWA was generated by connecting the outlet of the respirator inspiratory line to an air stone and immersing it in isotonic saline contained in a bottle that was placed in a heated water bath. HWA was then delivered directly into the lung via the tracheal tube (Fig. 4A). During either HWA or humidified room air (HRA) challenge, minute ventilation was increased to ~375% of the baseline (V_T and f at 12 ml/kg and 150 breaths/min, respectively) for 2 min. To prevent arterial hypocapnia and alkalosis, a gas mixture containing 3.5-4.0% of CO_2, 21% O_2, balance N_2 was administered via the respirator during hyperventilation.

Summary of Results: 1) In Ova sensitized rats, isocapnic hyperventilation with HWA for 2 min induced an increase in tracheal temperature (T_{tr}) from 33.4 ± 0.6°C to a peak of 40.6 ± 0.3°C (Fig. 4B) and an immediate and sustained (> 10 min) increase in R_L (from 0.12 ± 0.01 to 0.21 ± 0.02 $cmH_2O/ml/s$; n=6, P<0.01; Fig. 5). In sharp contrast, the HWA challenge produced the same increase in T_{tr}, but did not generate any increase in R_L in matching control rats (n=6, P>0.05; Fig. 5 & 6). 2) The responses in R_L were reproducible in both groups when the same HWA challenge was repeated 60-90 min later (n=5). 3) This bronchoconstrictive effect was temperature dependent; a smaller increase in peak T_{tr} (39.9 ± 0.2°C) generated a smaller, but significant increase in R_L in sensitized rats: from 0.12 ± 0.01 to 0.19 ± 0.03 $cmH_2O/ml/s$ (n=3, P<0.05; Fig. 7). In summary, an increase in airway temperature within the normal physiological range triggered bronchoconstriction in sensitized rats, but not in control rats. Chronic airway inflammation in sensitized animals is likely a major contributing factor in causing this response.

Tasks 2-1 & 2-2:

We have made substantial progresses in our human study, despite that we are near nine months behind our projected time table due to the preparation and review process of our HRPO protocol.

Hypothesis & Specific Aims: To test our hypothesis that hyperthermia can activate the TRPV1 expressed in airway sensory nerves and trigger reflex bronchoconstriction in patients with mild asthma, this study was carried out to compare the airway responses to an increase in airway temperature induced by hyperventilation with humidified warm air between healthy volunteers and mild asthmatics.

Methods: Spirometry & body plethysmography measurements were performed in four young healthy volunteers (age range: 24 to 46 years) and four young patients with mild and stable asthma (age: 22 to 40 years); the patients were off asthma therapy for at least 2 weeks, and tests were performed on different days in each subject. Each subject was challenged with isocapnic hyperventilation (40% MVV) of filtered room air in two different temperatures and relative humidity (RH): room air, 22°C (room temperature) and RH: 60-70%; and warm air, 49°C (hyperthermia) and RH: 65-75%.

Measurements and Main Results: Hyperventilation with warm humidified air triggered an immediate increase in airway resistance (Raw) in mild asthmatics (e.g., Fig 8A); baseline Raw = 2.14 ± 0.16 cmH_2O/L/sec (mean ± SEM) and after warm humid air peak Raw = 4.41 ± 0.65 cmH_2O/L/sec (Fig. 9A; $p<0.05$), whereas Raw did not change significantly in the same group of patients after hyperventilation with humidified air at room temperature (Fig. 8A & 9A). In a distinct contrast, hyperventilation with warm humidified air did not trigger any detectable immediate increase in Raw in healthy volunteers (n=4); baseline Raw = 1.42 ± 0.18 cmH_2O/L/sec and after warm humid air peak Raw = 1.67 ± 0.18 cmH_2O/L/sec ($P>0.05$). Furthermore, increasing airway temperature also consistently elicited bouts of cough in asthmatic patients, but not in healthy individuals.

Summary of Results and Tentative Conclusions: The results of this study have clearly shown that a transient increase in airway resistance was generated immediately after hyperventilation with warm humid air in patients with mild asthma, but the same warm humid air failed to cause any bronchoconstriction in healthy subjects. Hyperventilation with warm humid air also triggered coughs in these patients, suggesting an involvement of the airway sensory nerves. Furthermore, this bronchoconstriction is likely generated by the increase in airway temperature because hyperventilation with humidified air at room temperature did not generate any change in airway resistance in the same patients. Continuing studies are required to further analyze the responses statistically in a larger number of subjects, and to uncover the underlying mechanisms.

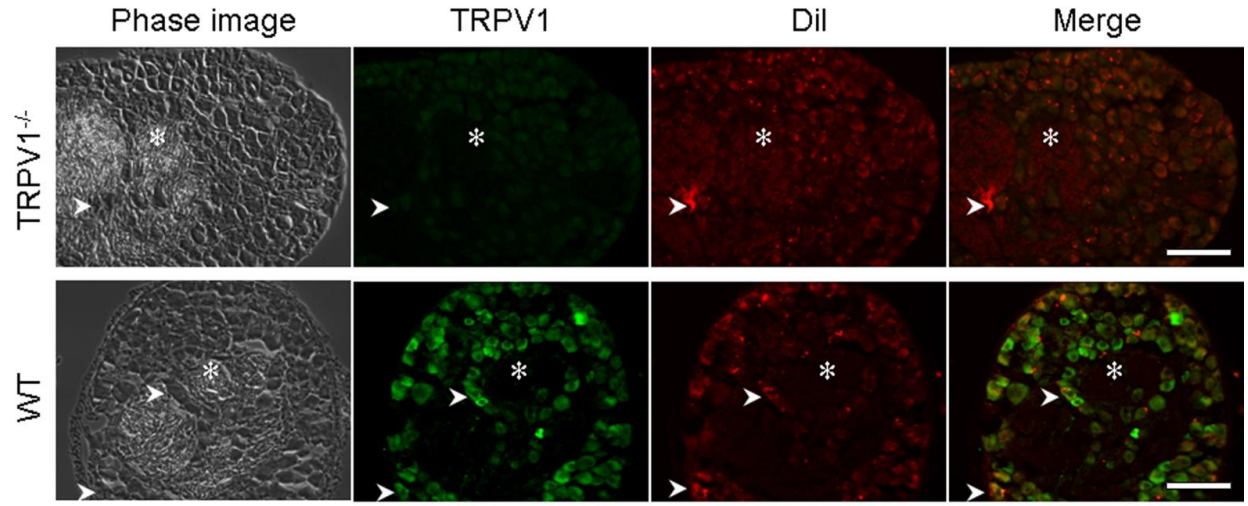

Fig. 1. Representative photographs illustrating TRPV1 immunohistochemistry and DiI-labeling in nodose/jugular ganglia of WT and TRPV1-null (-/-) mice. Arrowheads are added to depict pulmonary (DiI-labeled) neurons. Note that there is no TRPV1-immunoreactivity in any of the neurons in the TRPV1-/-l ganglion. Asterisks depict cross sections of axon bundles. Scale bar, 100 μm.

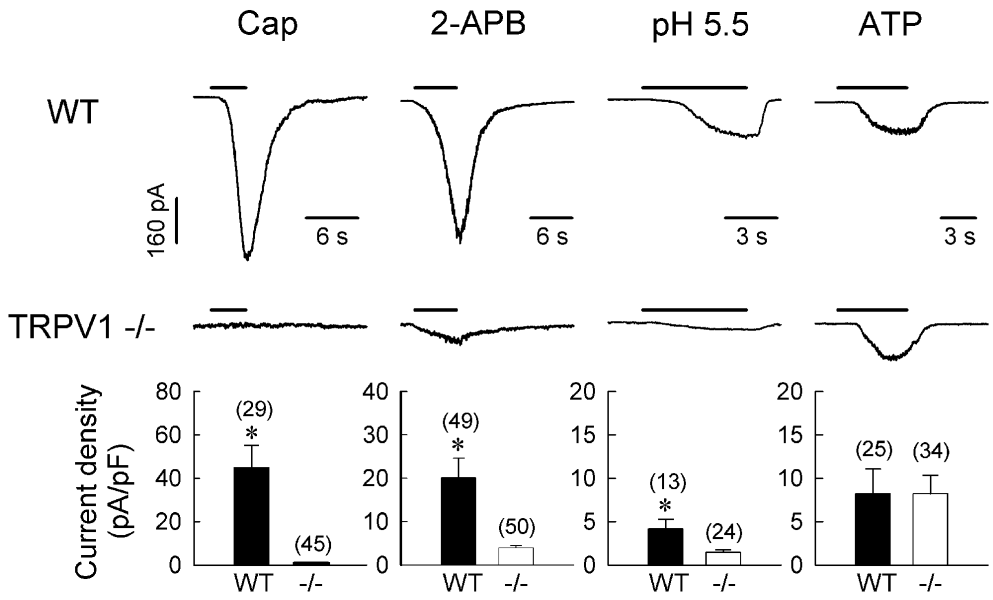

Fig. 2. A comparison of the responses to chemical stimulations in nodose-jugular neurons between TRPV1-null (-/-) and wild-type (WT) mice. *Upper panels:* representative experimental records of current responses (whole-cell, perforated patch clamp, voltage-clamp mode) to capsaicin (Cap; 1 μM, 3 s; WT, 25.1 pF; TRPV1-/-, 20.6 pF), 2-APB (0.3 mM, 6 s; WT, 18.5 pF; TRPV1-/-, 20.1 pF), acid (pH 5.5, 6 s; WT, 22.5 pF; TRPV1-/-, 18.3 pF), and ATP (1 μM, 6 s; WT, 21.4 pF; TRPV1-/-, 22.8 pF). *Lower panels:* group data in means ± SEM; *, $P < 0.05$, significant difference between WT and TRPV1-/- neurons.

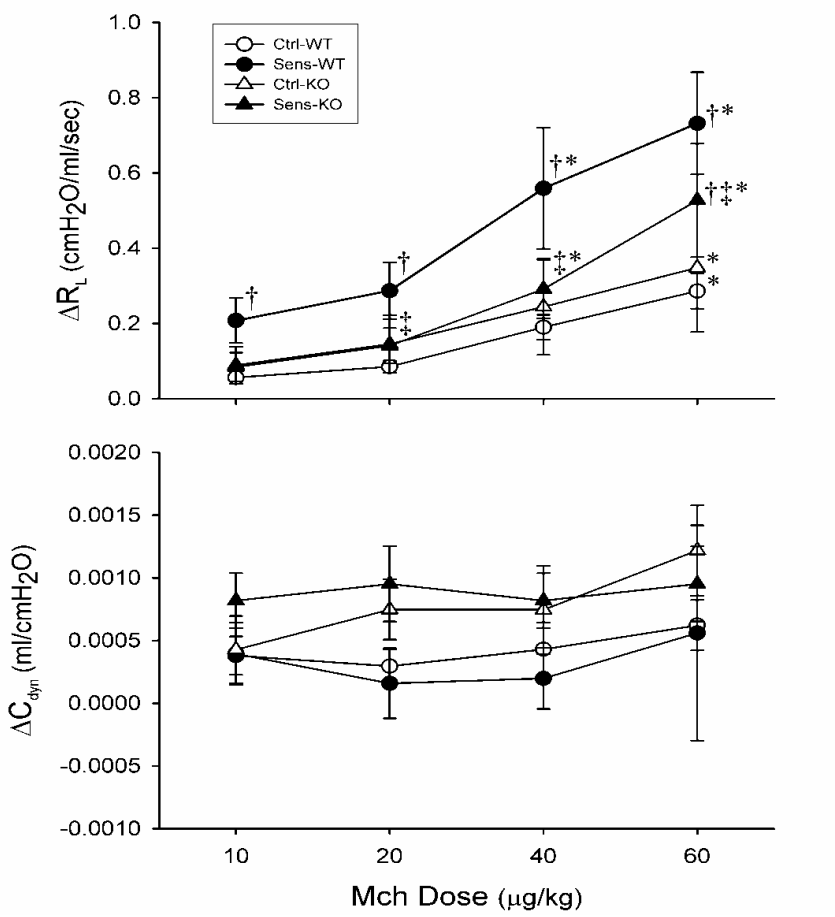

Fig. 3. Responses of R_L (total pulmonary resistance) and C_{dyn} (dynamic lung compliance) to intravenous injections of methacholine (MCh; n=6 in each group) in wild type (WT; circles) and TRPV1-null (KO; triangles) mice; each group was further divided into 2 subgroups: control (open symbols) and Ova-sensitized mice (closed symbols). The peak responses were averaged over 20 consecutive breaths after each MCh injection in each animal. Data represent means ± SEM. * : $P < 0.05$, significantly different from the response to MCh 10 ug/kg. † : significant difference comparing corresponding data between control and Ova-sensitized groups. ‡ : significant difference comparing corresponding data between WT and TRPV1-null groups.

Table 1. Differential leukocyte counts in BALF collected at 4-6 hours after the acute Ova challenge. N = 6 in each group; data are presented as means ± SEM. * ($P < 0.01$), significant difference between control and Ova-sensitized groups.

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

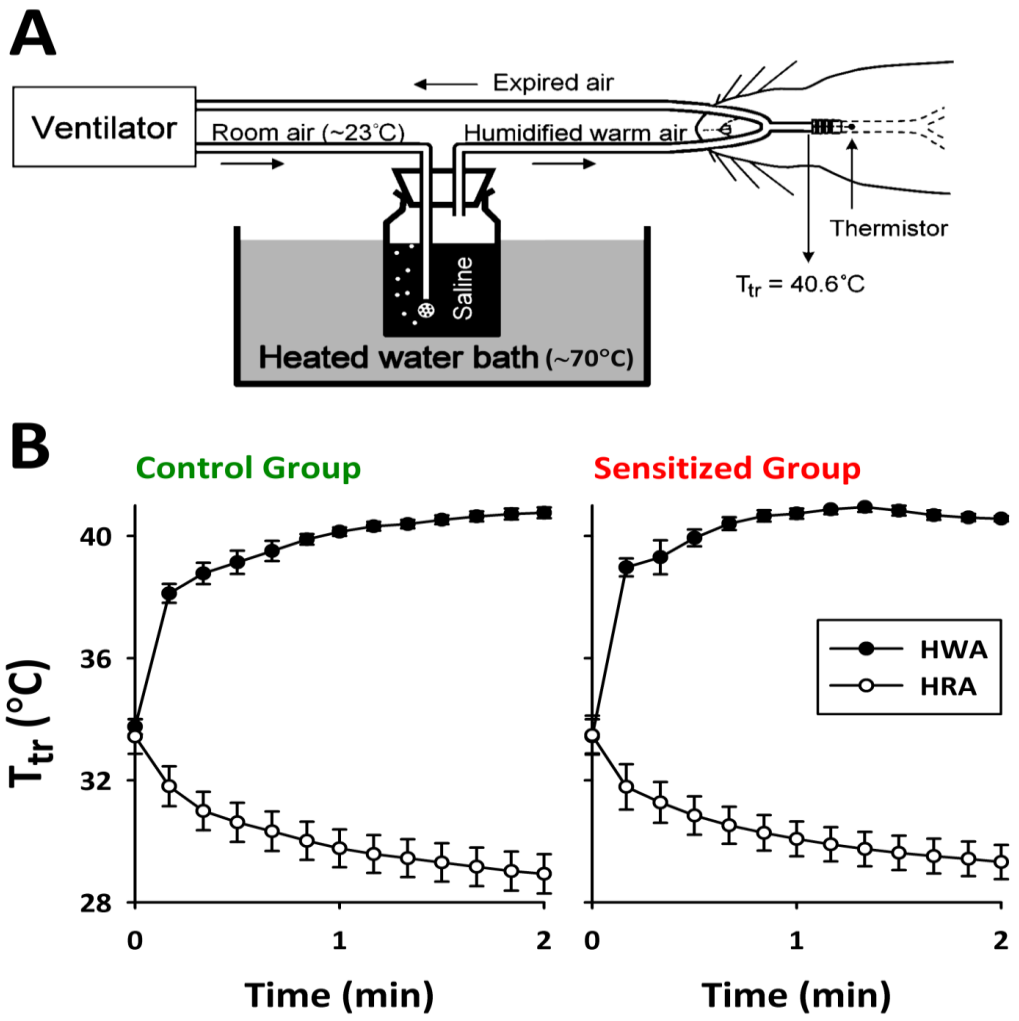

Fig. 4. *A*: schematic drawing of the experimental setup for delivery of humidified warm air (HWA) into the trachea of anesthetized Brown-Norway rats. T_{tr}, tracheal temperature. *B*: change in T_{tr} during the 2-min hyperventilation with HWA and humidified room air [HRA; water bath kept at room temperature (~23°C)] in control (left panel) and sensitized animals (right panel). Data were means ± SE of 6 animals.

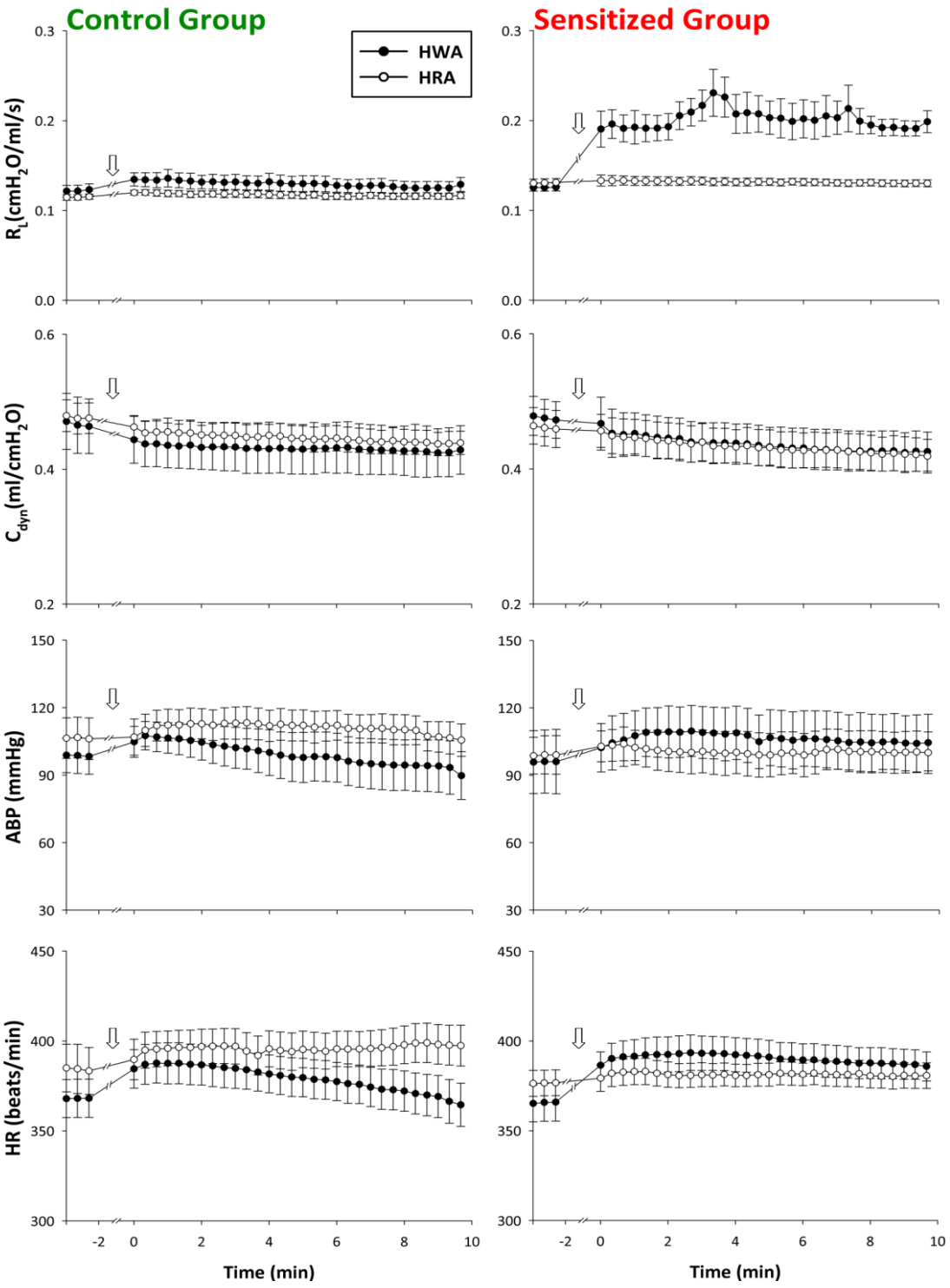

Fig. 5. Effect of hyperventilation with HWA (humidified warm air) and HRA (humidified room air) on R_L, C_{dyn}, ABP and HR in control (left panel) and sensitized animals (right panel). Responses were not recorded during hyperventilation (arrows), which was administered between -2 and 0 min. Data before *time -2* represent baseline values. Each data point was averaged over 20 consecutive breaths. Data were means ± SE of 6 animals.

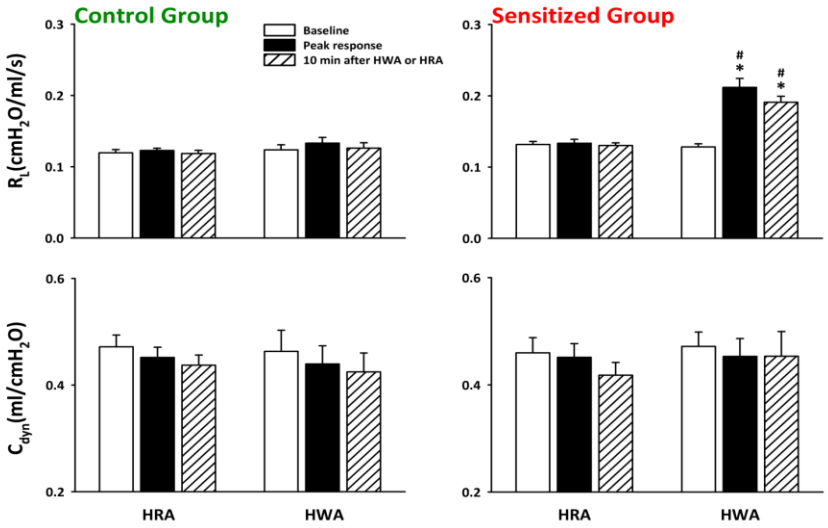

Fig. 6. Comparison of the responses of R_L and C_{dyn} to hyperventilation with HWA (humidified warm air) and HRA (humidified room air) in control (left panel) and sensitized animals (right panel). Open bars represent the baseline data averaged over 1 min before, closed bars represent the peak responses averaged over 1 min after, and hatched bars represent the responses 10 min after the HWA or HRA challenge. Data were means ± SE of 6 animals. *Significantly different from baseline ($P < 0.05$); #significant difference when corresponding data between HWA and HRA were compared ($P < 0.05$).

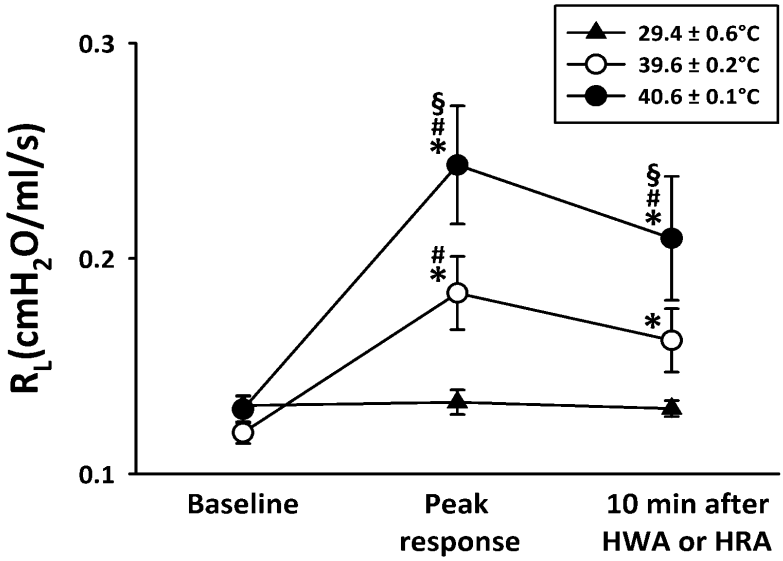

Fig. 7. Temperature-dependent effect of the HWA challenge on R_L in the Ova-sensitized animals. Animals were treated with three different tracheal temperatures: high (40.6°C), intermediate (39.6°C), and room air (30.6°C) temperature. 60-90 minutes were allowed to elapse between two challenges. Data were means ± SE of 6 animals, only 2 of 6 animals were treated with room air temperature. *Significantly different from baseline ($P < 0.05$); #significant difference from room air temperature ($P < 0.05$); §significant difference from intermediate temperature ($P < 0.05$).

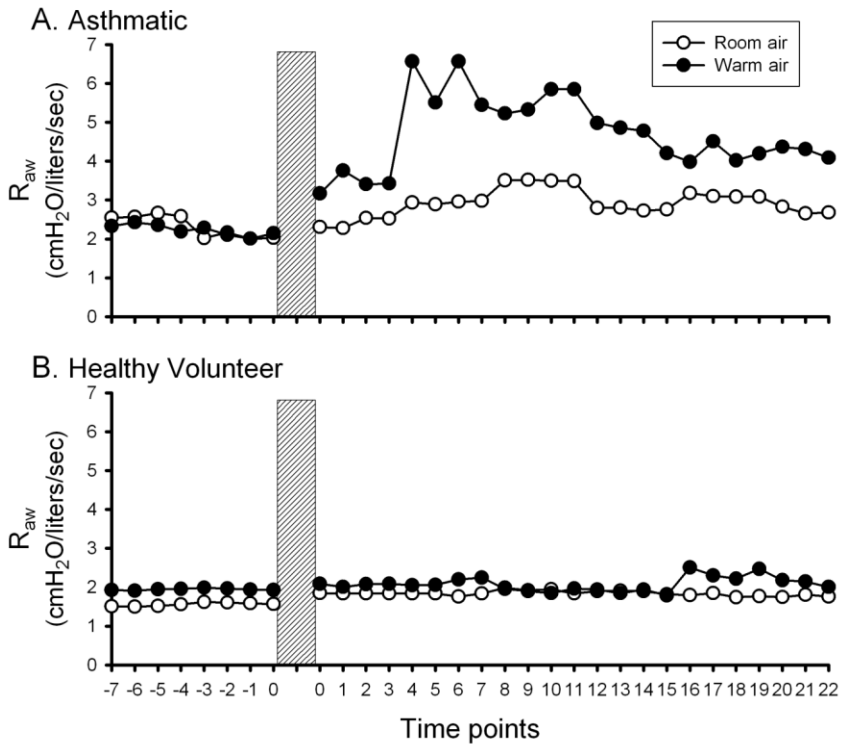

Fig. 8. Effects of isocapnic hyperventilation with humid air at room temperature and warm temperature for 4 min (shaded area) on airway resistance (Raw) in a mild asthmatic subject (Panel A) and in a healthy individual (Panel B).

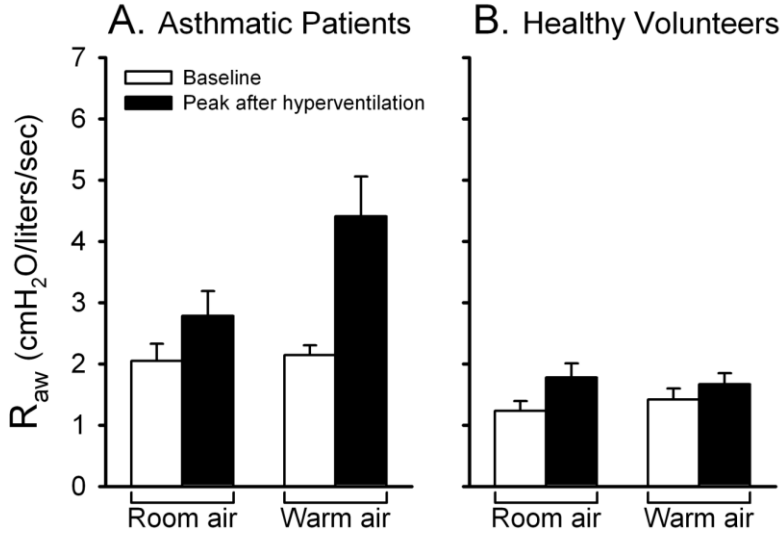

Fig. 9. Effect of hyperventilation with warm humid air on peak airway resistance (Raw) in mild asthmatic patients (Panel A; n=4) and in healthy volunteers (Panel B; n=4). Peak and baseline Raw were averaged over 4 and 8 consecutive breaths before and after hyperventilation, respectively, in each subject. Data are mean ± SEM.

Key Research Accomplishments

Despite the fact that we did not initiate the animal and human studies until three and nine months, respectively, after the starting date of this TATRC contract due to the required approval of the study protocols, we have made major research progresses in this project. More importantly, our results have clearly demonstrated the feasibility and the potential significance of these studies.

These studies, once completed, should provide important and novel information for: 1) documenting the pulmonary stresses induced by hyperthermia in healthy individuals and in patients with sensitized airways; 2) understanding the mechanism underlying the hyperthermia-induced pulmonary dysfunction; and 3) detecting the susceptibility to heat stress in soldiers with underestimated or overlooked airway hypersensitivity such as in airway allergy or mild asthma.

Reportable Outcomes

Publications Anticipated: The preliminary data obtained from these studies will be submitted as abstracts and presented in the 2012 Experimental Biology meeting. We also expect to report these new findings in a full manuscript and submit them for publications when we finish the proposed studies in Task 1-2 and Tasks 2-1 & 2-2 in the next 4-6 months.

Employment Generated by this TATRC Contract: Salaries of the employees listed below are paid in part or in full with the funds provided by this research contract:

Lu-Yuan Lee, Ph.D., Principal Investigator (30% effort)
Don Hayes, M.D., Co-investigator (20% effort), terminated on June 30, 2011 (Dr. Hayes took a faculty position at the Ohio State University)
Mahdi Khosravi, M.D., Co-investigator (20% effort), started on July 1, 2011
Paul B. Collins, B.S., RRT, Supervisor of Pulmonary Function Laboratory (10% effort).
Richard Kryscio, Ph.D., Co-investigator (3% effort), Consultant for Biostatistics (3% effort)
Marcus Geer, B.S., Lab Technician (100% effort; newly hired to work on this project)
Emy Lin, Ph.D., Postdoctoral Scholar (100% effort; newly hired to work on this project)
Chayse Martin, Part-time Lab Assistant (35% effort; newly hired to work on this project) terminated on May 1, 2011 (Mr. Martin entered medical school in 2011)
Robert Morton, Part-time Senior Research Analyst (20% effort; hired to work on this project)

Conclusions

Based upon these results, we can draw the following conclusions:

1) Airway hyperresponsiveness developed in Ova-sensitized mice was less pronounced in TRPV1-null mice, indicating an important role of TRPV1.
2) An increase in airway temperature within the normal physiological range triggered bronchoconstriction in sensitized rats, but not in control rats. Chronic airway inflammation in sensitized animals is likely a major contributing factor in causing this response.

3) A transient increase in airway resistance was generated immediately after hyperventilation with warm humid air in patients with mild asthma, but the same warm humid air challenge failed to cause any bronchoconstriction in healthy subjects. Furthermore, this bronchoconstriction is likely generated by the increase in airway temperature because hyperventilation with humidified air at room temperature did not generate any change in airway resistance in the same patients.
4) Continuing studies will be required to determine whether the effect of hyperthermia is primarily mediated through an activation of the temperature-sensitive TRPV1 channel expressed on vagal bronchopulmonary C-fibers, and if TRPV1 expression is up-regulated in the airway mucosa of patients with chronic inflammation.

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Appendices

None.