

Bilateral Vagotomy as a Tool for Determining Autonomic Involvement in Airway Responses in Mouse Models of Asthma

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Abstract

This chapter describes the use of bilateral vagotomy as a tool for determining autonomic regulation of airway responses to the exogenous bronchoconstrictor thromboxane mimetic U46619 in an acute model of asthma in the mouse. Mice receive a sensitization of ovalbumin (OVA) and adjuvant followed by 3 days of OVA aerosol to induce allergic airway disease characterized by bronchoalveolar lavage (BAL) eosinophilia, increased mucus production, and elevated IgE and IL-13. Using a small animal ventilator (Flexi-vent) and the forced oscillatory technique fit to the constant phase model of the lung, a variety of features associated with human asthma can be evaluated in mouse models. For example, this protocol describes the methods to evaluate central and peripheral airway mechanics, airway resistance (R_{aw}) and tissue damping (G), and tissue elastance (H) in response to U46619. The contribution of autonomic nerves in this response is determined by severing both the left and right vagus nerves prior to aerosol challenge.

Key words Vagus nerve, Autonomic nervous system, Lung mechanics, Asthma

1 Introduction

Asthma is one of the most common respiratory diseases, affecting approximately 300 million adults and children worldwide [1]. Phenotypically, asthma is a heterogeneous disease manifesting in many subtypes and is affected by interactions of both environmental and genetic factors. This disease is typically characterized by three principal characteristics: chronic airway inflammation; airway hyperresponsiveness (AHR); and reversible airflow obstruction [2]. Airway smooth muscle (ASM) plays a central role in regulating bronchomotor tone; however, ASM can constrict in response to many agents, either directly via receptors on the muscle, or indirectly through the activation of immune cells or nerves.

Sensory and parasympathetic nerves extending into the lungs may contribute significantly to airway obstruction and hyperreactivity

in the diseased lung through the release of neurotransmitters that can elicit effects on further neurotransmission, mucus secretion, ASM, epithelial cells, and inflammatory cells [3–7]. Furthermore, dysregulation of these neurotransmitters, airway receptors, or the response of nerves during a disease state may lead to an alteration of normal airway tone or an increase/decrease in nerve excitability and signal transduction. One hypothesis is that asthma represents an imbalance or dysregulation of the autonomic nervous system.

The vagus nerve contains the majority of airway sensory afferents (reviewed in [8]), approximately 75 % of which are unmyelinated C-fibers, which span the entire respiratory tract from trachea to the central airways to the parenchyma [9]. While typically quiescent during tidal breathing, C-fiber activation provides excitatory input to neuronal pathways driving autonomic output to the airways, resulting in bronchoconstriction, mucus secretion, and vasodilation [10, 11]. Additionally, the vagus nerve also contains parasympathetic (or cholinergic) nerves, which are the primary controllers of human and animal airways. Specifically, the vagus nerve carries efferent cholinergic fibers that synapse in small ganglia within the airway wall, from which short postganglionic fibers innervate airway smooth muscle and submucosal glands [12]. Therefore, the vagus nerve contains the vast majority of nerves that are responsible for both sampling and controlling the airways.

Due to the complex nature of the disease and the limitations of mechanistic studies in humans, animal models are crucial for the study of neural involvement in the asthmatic response. By conducting a bilateral vagotomy prior to measuring airway responses to an inhaled or intravenous stimulus, one can assess the contribution of airway nerves/neurotransmitters to that response. The mouse is an ideal model for this assessment, as models of asthma, methods of *in vivo* airway mechanistic monitoring, and genetic manipulation are all well established. Here, we describe the use of bilateral vagotomy as a tool for determining autonomic regulation of airway responses to an exogenous bronchoconstrictor in an acute model of asthma in the mouse.

2 Materials

2.1 Equipment and Supplies

1. Computer/monitor.
2. Flexi-vent system (Scireq respiratory equipment) including the following pieces of equipment: Base Unit; EC controller unit; XC accessories controller; Module M1; Scireq Aeroneb plug-in; Scireq EKG plug-in (*see Note 1*); Manometer; and PEEP trap.
3. Scireq FV-AN-A1 aerosol base.
4. Aeroneb pro aerosol cup.

5. Tygon tubing.
6. Luer connector kit (Harvard apparatus).
7. Flexi-vent 5.1 (or newer) software.
8. Heating pad (*see Note 2*).
9. Ultrasonic nebulizer (DeVillbiss Health Care).
10. Forceps: Two sets small curved forceps (45–90°) and one set fine, curved Dumont #7 forceps.
11. Scissors: Straight and angled (45–90°).
12. 1 cm³ syringe for injecting/anesthetizing animals.
13. 26 G 3/8" needles for injecting animals.
14. 4-0 Silk Suture thread (Ethicon), 2 pieces/mouse.
15. 1.0 mm × 20 mm endo-tracheal tube (Harvard apparatus) with attached luer adaptor.
16. p200 and p1000 pipettes.
17. Reditip general-purpose pipette tips (200 and 1,000 µl).
18. Kim wipes.
19. Lab tape.
20. 15 ml conical tubes.
21. 1.5 ml Eppendorf tubes.
22. 500 ml glass beaker.
23. 100 ml graduated cylinder.
24. 100 ml glass solution jar.
25. 100 ml crimp-top glass injection vials.
26. Aluminum foil.

2.2 Chemical Reagents and Supplies

1. 100 % EtOH.
2. 70 % EtOH.
3. 0.9 % Saline (in non-sterile bottle and 20 ml sterile injection vials).
4. Urethane (Sigma).
5. Pancuronium bromide (Sigma).
6. Grade V Ovalbumin (≥98 %) (Sigma).
7. Alhydrogel (Brenntag).
8. U46619 (Cayman Chemical, 10 mg/ml).

2.3 Reagent Preparation

1. 1 % Ovalbumin (OVA): Mix 1 g of OVA in 100 ml of 0.9 % saline. Filter the solution.
2. Urethane—anesthetic (500 mg/ml stock solution): Weigh out 50 g of urethane crystals into a large graduated beaker (*see Note 3*). Add pre-warmed (not boiling) distilled water and bring the solution up to 100 ml. Continue to heat on low

heat and stir until the crystals are completely in solution. Cool the solution to room temperature. Dilute 1:4 in distilled water for 125 mg/ml working solution. Place into glass injection vials (with crimp tops) and wrap in aluminum foil to protect from light.

3. Pancuronium bromide—paralytic agent (8 mg/ml stock solution): Add 50 mg of pancuronium bromide powder to 6.25 ml of 0.9 % saline in a 15 ml conical tube. Cover with aluminum foil to protect from light and keep the stock refrigerated. To prepare working stock, dilute stock 1:100 in 0.9 % saline to make a 0.08 mg/ml working solution by injecting 200 μ l of stock directly into a 20 ml, 0.9 % saline injection vial. Keep the working solution on ice.
4. U46619 (thromboxane analog, 10^{-2} M stock) (*see Note 4*): Add 100 μ l of U46619/methyl acetate solution into an Eppendorf tube and slowly evaporate off the methyl acetate using nitrogen. Add 200 μ l of 100 % EtOH and resuspend the oil residue to make a 10^{-2} M stock. Dilute the stock 1:10 in 0.9 % PBS to make a 10^{-3} M dose. Dilute the 10^{-3} M dose 1:10 in 0.9 % PBS to make a 10^{-4} M dose. Dilute the 10^{-4} M dose 1:10 in 0.9 % PBS to make a 10^{-5} M dose.

2.4 Mice

1. Adult mice, 6–12 weeks old (*see Note 5*).

3 Methods

3.1 Mouse Model of Allergic Airway Disease

1. Add 200 μ l of 1 % OVA solution to 10 ml of alhydrogel and mix well.
2. Sensitize mice on day 1 by i.p. injection with 200 μ l of OVA + alhydrogel.
3. On days 14–16 challenge mice with aerosolized 1 % OVA for 1 h/day using an exposure chamber equipped with an ultrasonic nebulizer.
4. On day 17 proceed with airway evaluations.

3.2 Flexi-Vent Calibration (See Note 6)

1. Fill the PEEP trap with distilled water, and set the interior tube 2–3 cm below the surface.
2. Open the Flexi-vent 5.1 software.
3. Select “Start new experiment from template” from the pop-up menu.
4. Choose the appropriate template from the pop-up menu (*see Note 7*).
5. When prompted, name your experiment.
6. When prompted, record the operator name.

7. Enter first subject information in order to proceed with calibration (identifier, strain, group, weight, gender).
8. Follow directions given by the calibration wizard to prime the nebulizer.
9. In the “Channel Selection” pop-up window select both Cylinder Pressure and Airway Pressure. To calibrate the transducers measuring cylinder and airway pressures, connect the transducers to a manometer so that a known pressure can be applied to both transducers. The pressure calibration wizard will guide you through the steps required to properly calibrate the pressure channels and will warn you if they were not calibrated correctly (click the Help button on the wizard for more detailed instructions).
10. Next select “EKG” and proceed with “known full-scale values” set as -3 to $+1$ mV.
11. Dynamic tube calibration: This allows you to perform all dynamic calibration measurements required to characterize the ventilator compartment in a single step. This must be completed before ventilating the subject. The Tube Calibration wizard will automatically display at the beginning of a new measurement after autocalibration and pressure calibration have been completed. Before starting the dynamic tube calibration make sure that the endotracheal tube (ETT) is attached to the Flexi-vent. Follow the instructions supplied by the wizard.
12. Start default ventilation and prepare to connect the mouse.

3.3 Intubation and Vagotomy Preparation

1. Turn on the 37°C water bath heating pad.
2. Weigh each mouse.
3. Anesthetize a single mouse by IP injection of 1 g/kg of urethane (weight in grams $\times 16 = \mu\text{l}$ of working solution).
4. Once the mouse is fully anesthetized (as assessed by toe pinch), secure it to the heating pad using lab tape.
5. Spread 70% EtOH on the throat of the mouse to wet the fur. Lift the skin to make a vertical cut on the throat to expose salivary glands. Separate the salivary glands with forceps to visualize the muscle surrounding the trachea. Lift the muscle and cut with straight scissors to expose the trachea.
6. Isolate the right and left vagus nerve and tease away from carotid artery using small, curved forceps.
7. Pass fine, curved Dumont forceps underneath the left vagus nerve and slowly open forceps to separate the nerve fiber from the surrounding tissue. Repeat for the right vagus nerve.
8. Pass suture string underneath both the left and right vagus nerve (*see Fig. 1*).

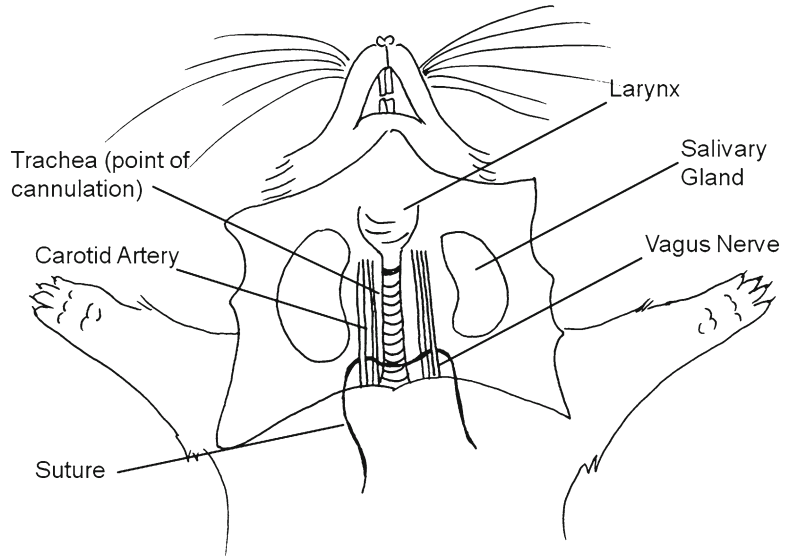


Fig. 1 Drawing depicting the preparation of the suture for bilateral vagotomy. Control sham operations are simulated by lifting and releasing of the suture. Bilateral vagotomy is conducted by lifting the nerves with the suture and then severing both the left and right vagus nerves

9. Make a horizontal incision between the second and third tracheal rings using angled scissors and insert the ETT.
10. Pass suture thread underneath the trachea using the ETT for support. Tie the suture thread tightly to secure the ETT in place.

3.4 Measuring Airway Responses in Mice

1. Attach the mouse to the Flexi-vent via the ETT (with default ventilation equal to 150–200 breaths/min).
2. Attach EKG leads to the mouse in the Lead II configuration.
3. Inject 0.8 mg/kg of pancuronium bromide (weight in $g \times 10 = \mu\text{l}$ of working solution to inject).
4. Wait 5 min for the paralytic drug to take effect before starting the experiment.
5. Double click on the desired script (*see Note 8*) on the lower right-hand side of the screen to start the experiment. The Experiment wizard will automatically display and guide you through the experiment.
6. Record baseline parameters.
7. Simultaneously sever both the left and right vagus nerves by lifting up on the suture string and cutting the nerves with straight scissors. Alternatively, a surgical sham for control animals is conducted by simply lifting the nerves via the suture string and releasing them intact.
8. Repeat baseline measurements (*see Note 9*).

9. When prompted, add 100 μl of 10^{-5} M U46619 to the nebulizer cup, record the concentration on the wizard window, and continue with the experiment (*see Note 10*).
10. After aerosolization is complete, remove excess liquid from the nebulizer cup by gently dabbing the membrane with an absorbent wipe.
11. Repeat **steps 9** and **10** for the 10^{-4} and 10^{-3} M doses of U46619.
12. When the script is complete, remove the mouse from the ventilator, stop ventilation, and select “switch subjects” from the toolbar.
13. Enter the next animal’s information into the pop-up window and repeat the protocol.

3.5 Calculations

1. Scireq Flexi-vent 5.1 software automatically fits the data to the constant phase model and calculates airway mechanics parameters (R_{aw} , G , and H) from the recorded raw data when the data is exported.
2. To export data from the Flexi-vent software, select “File” from the top left of the screen and then select “export data” from the drop-down menu. An export wizard will direct the saving of the exported data in a folder or a disk of your choice.
3. The final data is typically expressed as percent change from baseline. To calculate this, the raw data is transferred to an excel spreadsheet and the dose–response of each animal is divided by its average baseline and then multiplied by 100 for each parameter of interest. Excluded data points (automatically detected by the system and denoted with a minus sign when exported) are manually removed before calculations.

4 Notes

1. The EKG plug-in is not required, but it is recommended for monitoring the heart rate in order to evaluate the depth of anesthesia and to ensure that the mouse is still alive during measurement of airway responses.
2. A water bath heating pad is recommended for precise body temperature regulation. If a commercial heating pad is used, monitoring of the animal’s body temperature is recommended. Body temperature should be kept at or below 37 °C.
3. Urethane is a toxicant and should only be opened and weighed out in a chemical fume hood.
4. Make U46619 stock and dilutions fresh daily: do not store. It is recommended to purchase U46619 in methyl acetate and

to store it as 100 μ l aliquots. One aliquot is sufficient for up to 18 mice.

5. The weight of the mice should be approximately 20 g, although the M1 module is capable of measuring airway parameters in subjects from 15 to 40 g. Female mice tend to be smaller than male mice, so it is recommended to either normalize mixed-sex experiments by weight (requiring a larger range of ages) or conduct single-sex experiments normalized by age.
6. In order to obtain good data, it is crucial to perform pressure, flow, and auxiliary channel calibrations before you start an experiment. Scireq recommends performing a calibration at least once a day before experiments.
7. The template should be created using the Template wizard with the following parameters: Mode: Quasi-sinusoidal; Tidal Volume: 10 ml/kg; Pressure Limit: 30 cm H₂O; Frequency: 150–200 breaths/min for adult mice. Typical perturbations used for FOT measurements are total lung capacity (TLC) and Quick Prime-3. Scireq technical support can also provide a Guide to Flexi-vent Template Creation.
8. Several default scripts are included with the software; however, we suggest modifying those scripts (in Microsoft Notepad™) so that the mouse is subjected to 1–2 TLC maneuvers prior to baseline measurements to ensure normalized lung resistance. At least 3 Quick Prime-3 measurements, separated by 10 s, should be conducted for baseline parameters (both before and after vagotomy/sham). Following baseline measurements, we recommend aerosolizing for 20 s at 50 %, then recording Quick Prime-3 every 10 s for 3 min, and then prompting for a loop back to aerosolize the next dose. This can be repeated for as many doses as desired.
9. A slight drop in baseline parameters can be seen following vagotomy due to the loss of intrinsic airway tone that is generally maintained by constitutive, low-level release of acetylcholine from parasympathetic nerves [13].
10. Alternatively, other bronchoconstricting agents or antigens can be used (i.e., OVA, histamine, serotonin). Additionally, intravenous challenge can replace the aerosol challenge when appropriate.

References

1. Masoli M, Fabian D, Holt S, Beasley R (2004) The global burden of asthma: executive summary of the GINA Dissemination Committee report. *Allergy* 59:469–478
2. Busse WW, Lemanske RF Jr (2001) Asthma. *N Engl J Med* 344:350–362
3. Myers AC, Udem BJ (1993) Electrophysiological effects of tachykinins and capsaicin on guinea-pig bronchial parasympathetic ganglion neurones. *J Physiol* 470:665–679
4. Watson N, Maclagan J, Barnes PJ (1993) Endogenous tachykinins facilitate transmission

- through parasympathetic ganglia in guinea-pig trachea. *Br J Pharmacol* 109:751–759
5. Dakhama A, Kanehiro A, Makela MJ, Loader JE, Larsen GL, Gelfand EW (2002) Regulation of airway hyperresponsiveness by calcitonin gene-related peptide in allergen sensitized and challenged mice. *Am J Respir Crit Care Med* 165:1137–1144
 6. Veres TZ, Rochlitzer S, Shevchenko M, Fuchs B, Prenzler F, Nassenstein C, Fischer A, Welker L, Holz O, Muller M et al (2007) Spatial interactions between dendritic cells and sensory nerves in allergic airway inflammation. *Am J Respir Cell Mol Biol* 37:553–561
 7. Barnes PJ (1992) Modulation of neurotransmission in airways. *Physiol Rev* 72:699–729
 8. Groneberg DA, Quarcoo D, Frossard N, Fischer A (2004) Neurogenic mechanisms in bronchial inflammatory diseases. *Allergy* 59:1139–1152
 9. Agostoni E, Chinnock JE, De Daly MB, Murray JG (1957) Functional and histological studies of the vagus nerve and its branches to the heart, lungs and abdominal viscera in the cat. *J Physiol* 135:182–205
 10. Canning BJ, Fischer A (2001) Neural regulation of airway smooth muscle tone. *Respir Physiol* 125:113–127
 11. Mazzone SB, Canning BJ (2002) Evidence for differential reflex regulation of cholinergic and noncholinergic parasympathetic nerves innervating the airways. *Am J Respir Crit Care Med* 165:1076–1083
 12. Barnes PJ (1986) Neural control of human airways in health and disease. *Am Rev Respir Dis* 134:1289–1314
 13. Allen IC, Hartney JM, Coffman TM, Penn RB, Wess J, Koller BH (2006) Thromboxane A2 induces airway constriction through an M3 muscarinic acetylcholine receptor-dependent mechanism. *Am J Physiol Lung Cell Mol Physiol* 290:L526–L533