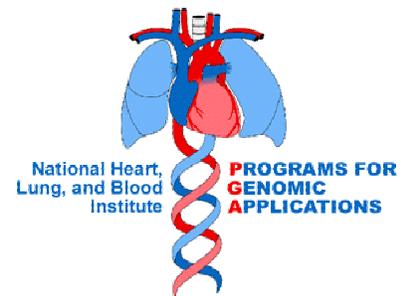


# Lung Phenotypes

**High-throughput characterization of pulmonary vascular resistance and airway responsiveness to cholinergic challenge in normoxic and chronically hypoxic rats.**

**Bernadette Cabigas and Jessica Powlas  
with  
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**PhySGen**

## I. Experimental setup for isolated lung studies (instrumentation and calibration procedures)

Instrumentation and equipment used in setup [all order information listed in section IV]:

- isolated lung perfusion set-up with reservoir and circulator
- gas tanks and regulators
- dissection station with light, surgical instruments and plexiglass dissection board on surgical lift
- whole body plethysmograph with integrator etc.

Figures 1A, 1B and 1C depict the experimental set-up as used daily.



**Figure 1A:** Close-up view of rat surgical station for animal preparation.



**Figure 1B:** Close-up view of plethysmograph used for methacholine challenge.



**Figure 1C:** Close-up view of isolated lung perfusion set-up.

## **II. Experimental protocol for isolated lung studies**

In the Lung Protocol, the daily goal of 4-6 lungs studied is achieved by performing the experiments in series rather than having 6 set-ups. The nature of the protocol and the amount of minute to minute interaction with the preparation requires this arrangement. To run these 4-6 experiments in series efficiently, the two technologists are designated “surgical tech” and “data tech” and their activities are coordinated as described below.

### ***A. Preparation of equipment and instrumentation for beginning of experimental protocol.***

1. Turn on computer, circulating heater pump [should be 37° C], and open both tanks to check gas level in the tank (tanks should be set at 2.5-3 psi for tank going to the perfusate and 10 psi for tank going to the ventilator). When the gas level falls below 500 psi, the tank should be changed.
2. The surgical tech sets up the surgical station for the day’s studies: surgical instruments are laid out next to the lift station (see Figure 1A) which is equipped with two plexiglas boards between which an absorbent surgical mat is placed. Also needed at the surgical station are:
  - 150 ml beaker of saline
  - 50 ml beaker of heparinized saline [5 ml 10,000 units/ml sodium heparin + 45 ml saline]
  - arterial cannulas made from 10 cm PE 50
  - two syringes [a 1 ml and a 3 ml] for filling catheters each with a blunted 22 g x 1” needle
  - scalpel blade [one per rat]
  - 3 ml syringe fitted with a 10cm length of catheter material for blood draw (filled with heparinized saline)
  - a capped 1 ml syringe containing 0.2 ml of heparin
  - clean gauze sponges
  - heavy fabric thread cut (roughly equivalent to 2-0 suture) lengths (approximately 30cm, length dependent on surgical tech preference)
3. During this time the Data Tech should be setting up the perfusion system and the plethysmograph.
  - a. Perfusion system setup (Figure 1B): fill the reservoir with saline and turn the circulating water bath on. The pump should be allowed to circulate saline until it is warmed by the water bath.
    - pull saline through the tubing of the perfusion system with a 10 ml syringe attached to the stopcock of the transducer. Make sure there are no bubbles. Be sure to maintain the reservoir fluid volume level to prevent the introduction of bubbles into the system if the level falls too low.
    - flush saline across the transducer filling the 2 stopcocks with saline and eliminating the air bubbles.

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- the left stopcock should be closed to atmosphere but open to the lung and transducer; the right one should be closed to atmosphere.
  - remove the syringe and top off the port with saline so there are no air bubbles.
  - a beaker of saline should be positioned between the perfusion pump and plethysmograph, used to flush methacholine catheter and to fill the adapter to which the PA catheter is attached.
- b. Plethysmograph setup: the pressure transducer for the plethysmograph should be filled as described above just prior to use.
- place a 10 ml syringe half filled with saline on each stopcock on either side of the transducer and force the small air bubbles off the surface of the transducer membrane by movement of the fluid-filled syringes back and forth. Turn the stopcocks such that the transducer stopcock is open to the transducer and the esophageal catheter.
  - flush the esophageal catheter and fill with saline, removing any air bubbles. Remove the syringe and top off the stopcock port with saline and replace with a 1 ml syringe filled with saline.
  - for each rat, replace the i.p. catheter tubing on the plethysmograph. The outside piece of the tubing is a 4 cm length of PE50 fitted to the metal connection on the right hand side of the box. The inside piece of tubing is a 13 cm length and passes through the center of a blunted 17 g x 1.5” needle. Mark the catheter with a black marker at a point that is 2 times the length of the needle. This will serve as an insertion stop point for the i.p. catheter and prevents i.v. administration of the methacholine.
  - Attach a saline filled 1 ml syringe marked “control” using a blunted 22 g x 1” needle adaptor to the 4 cm length of tubing. Flush the catheter with saline filling it with the volume to be used as a control volume for the specific rat ( $0.0007 \times \text{rat weight} + 0.05 \text{ ml}$ ). Clamp the catheter with Allis forceps [serrated tips covered with tygon tubing to protect catheter].
4. Preparation of solutions used for studies:
- a. **methacholine**: on Monday of each week, the methacholine doses should be made up (see Table 1). These doses are stored in 15 ml disposable, polystyrene tubes marked with the dose. Each tube is placed in a styrofoam holder next to the corresponding syringe. For each dose, a 1 ml syringe with a 22 g adaptor is prepared and marked with the dose. The methacholine should be refrigerated between experiments and the volume for each rat drawn during the time the rat is being prepared for the plethysmograph.
- b. FAPGG and MB: FAPGG stock is prepared by adding 3.25 mg of FAPGG to 13 ml of perfusate. The tube should be placed in a beaker of water placed in the heater bath to facilitate dissolution of the FAPGG. Methylene blue (MB) should be prepared monthly other Friday for use and stored in the refrigerator protected from light (*i.e.* wrapped in aluminum foil).

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**Table 1: Methacholine solutions.** Solutions for methacholine dose response to be made up at the beginning of each week and stored at 4°C.

| Solution Number | Add Together in at 15 ml Test Tube    | Dose (mg/ml) | Dose (mg/kg) |
|-----------------|---------------------------------------|--------------|--------------|
| #9              | 336mg methacholine + 12ml 0.9% saline | 28           | 22           |
| #8              | 6.75 ml #9 + 5.25 ml saline           | 15.8         | 11.15        |
| #7              | 6.75 ml #8 + 5.25 ml saline           | 8.9          | 6.9          |
| #6              | 6.75 ml #7 + 5.25 ml saline           | 5.0          | 3.65         |
| #5              | 6.75 ml #6 + 5.25 ml saline           | 2.8          | 2.2          |
| #4              | 6.75 ml #5 + 5.25 ml saline           | 1.58         | 1.16         |
| #3              | 6.75 ml #4 + 5.25 ml saline           | 0.89         | 0.69         |
| #2              | 6.75 ml #3 + 5.25 ml saline           | 0.50         | 0.41         |
| #1              | 6.75 ml #2 + 5.25 ml saline           | 0.28         | 0.20         |

**B. Methacholine Challenge:**

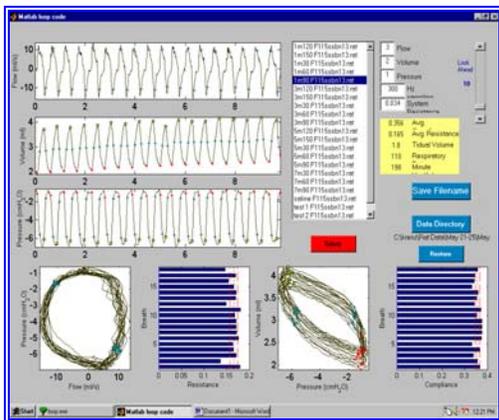
1. The Surgical Tech will anesthetize rat with i.m. [right rear leg] injection of ketamine [30 mg/kg dose; to calculate use this formula:(((weight in g)/100)\*2)/100)\*1.5; some strains require additional anesthetic (e.g. when using Sprague-Dawley rats, multiply by 2 rather than by 1.5; ketamine stock concentration 100 mg/ml] Adjust dose of anesthetic depending upon the strain of rat. All rats with SS background are more sensitive to anesthetic and the dose adjusted accordingly.
  
2. During the time of surgical preparation, the Data Tech will be preparing for data collection as follows:
  - a. open Acquisition and open the reference file named Loop Calibration which has been saved on the preceding Friday (e.g. Jan. 18 01) in the current week's folder [example: January 21-25 01].
  - b. open the Windaq Data Acquisition file creating a sub folder for the day [example: "21Jan01"]
  - c. another folder is created for the rat using the rat ID as the folder name [example "M084SD]. *Remember that the rat nomenclature has been assigned for all rats in all studies. No other designation should be used for any animal studied.*
  - d. within this folder, the data collected from the methacholine challenge is called by the rat ID followed by an underscore and m [M084SD\_m]
  - e. change the recording time to 10 hours
  
3. After the rat has become sedated with the administration of ketamine, Inactin is given i.p. [dose 0.0375g/kg; to calculate, use this formula: (((weight in g)/100)\*2.5)/50)\*1.5; 1g/20ml stock frozen in 1ml aliquots] The animal is placed on its back when fully anesthetized and each leg taped to the dissection board. The Surgical Tech will then prepare the rat as follows:
  - a. cut vertically a 2 cm incision down the neck region just under the mandible; expose the trachea placing sutures under the trachea and loosely tie

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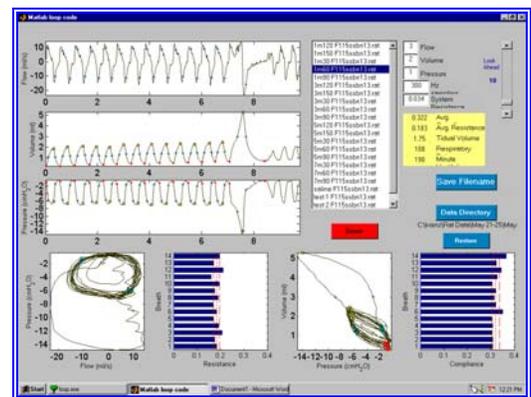
- b. using the scapula, cut between the cartilage rings cutting high up on the trachea so there is plenty of trachea distal to the cut for later procedures.
  - c. insert the tracheal cannula until the metal tubing is completely inside the trachea. Tie the cannula in place with both sets of ties being careful to preserve as much length of trachea as possible.
  - d. isolate the external carotid artery on either the left or right side of the neck. Take care not to disturb the vagus nerve that runs alongside the artery by carefully dissecting the artery free. Place 2 sutures (fabric thread) around the carotid pulling one anterior and tying off and the other posterior and applying some tension on the suture to stop blood flow temporarily during insertion of the catheter. Keep the vessel moist with saline.
  - e. cut the carotid with a small cut using the Vannas scissors and advance the prepared catheter into the artery up to the level of the posterior tie; release the tie and advance the catheter further about 20 mm and tie into place.
  - f. remove the syringe and allow the blood to flow out of the catheter until the line is clear of heparinized saline, reattach the syringe and withdraw 1 ml of blood from the rat into the attached syringe quickly and then remove syringe and carefully deliver blood into tubes provided by the PGA Biochemistry Core Laboratory for collection of samples for analysis by Marshfield Laboratory. 400  $\mu$ l is delivered into the purple EDTA tube and 600  $\mu$ l into the yellow serum separator tube. The EDTA tube should be inverted immediately and repeatedly to prevent clotting. [*Note: when collecting samples from rats conditioned in the hypoxia chamber, be sure that the full volume is withdrawn and delivered to the tubes. The hematocrit is significantly higher in hypoxic animals, which then delivers a lower volume of serum or plasma for biochemical measurements.*] Samples are immediately turned over to the Core Lab staff for processing. The measurements made and the list of biochemical phenotypes are given in a separate section under Biochemical Phenotypes (page 11).
  - g. after removing the blood collection syringe, connect the syringe containing heparin and flush the catheter with 0.05 ml of heparin. Tie the catheter into place with both ties.
4. Remove the rat from the surgical table and put into the plethysmograph.
  5. The saline filled esophageal catheter is inserted into the anesthetized rat through the mouth and attached to the esophageal port (attached to the pressure transducer) on the plethysmograph. The tracheal cannula is attached to the tracheal port on the plethysmograph (open to the atmosphere).
  6. Insert an intraperitoneal catheter into the rat abdomen [below the diaphragm] using a 16/17g x 1" needle.
  7. Adjust the esophageal catheter position such that it is in the pleural cavity and not inserted past the diaphragm. The waveforms displayed on channel one of the computer screen will confirm the location. Make sure the pressure waveform plateaus are as flat as possible without excessive notching. A small waveform may indicate a bubble in the catheter, which can be cleared by flushing a small amount of saline through the catheter.

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8. Once the waveforms indicate the proper position of the esophageal catheter, close the plethysmograph. Watch the volume channel for drift and adjust, if necessary, by using the balance knob for the volume channel on the Grass recorder. When satisfied, record 5-9 seconds of data.
9. Data Tech will open Windaq Playback and review the breaths. Making sure that the compression is set at 1 (F7), hit F4 twice to zero the recorder and scroll a section that does not show any abnormal breaths. Cut this section (5-9 seconds) by using Ctrl V and save it as a test file in the rat's folder [example "test1\_M084.rat]. Close Playback and open Loop.exe. This program cannot operate if Playback is still open. Check the settings in Loop to be sure that Pressure=channel 1, Volume = channel 2, Flow = channel 3 and that the cannula resistance is between 0.034 and 0.044. Open the test file with the control breaths and the Loop program will now calculate: compliance, resistance, tidal volume, respiratory rate, and minute ventilation. The resulting graph of volume vs. pressure should have open loops (Figure 2A). Individual breaths can be eliminated if the remaining breaths look normal (Figure 2B). If they are stacked, there is a drift in volume (Figure 2C).

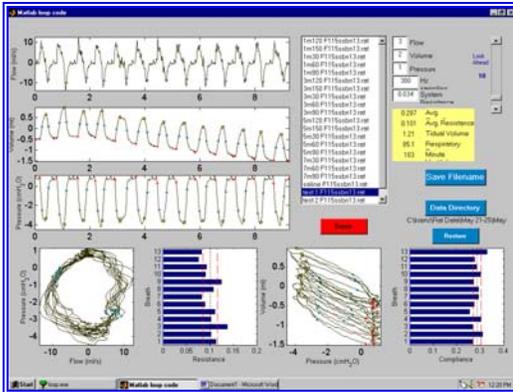


**Figure 2A:** Sample loop data file with no drift or bad breaths. Both loops (pressure v. flow and volume v. pressure) are 'open'.



**Figure 2B:** Sample loop data file containing at least one 'bad' breath. Eliminating this breath would open the loops.

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**Figure 2C:** Sample loop data file demonstrating drift in volume channel. The loops are stacked, slowly drifting downward.

10. The methacholine challenge protocol can be performed when the control data is qualitatively determined to be appropriate. Using the methacholine solutions prepared above, fill 1 ml syringes with 0.0007 ml/gram body weight of solution 1, 3, 5, 7, 9. Use a separate syringe for each dose. The following protocol is performed to deliver the dose-response methacholine challenge:
  - a. Data Tech starts recording (F4), starts the timer set at 2.5 min. and the Surgical Tech will introduce saline into the i.p. catheter (same volume as the methacholine doses).
  - b. methacholine is injected at 2.5 minute intervals with doses 1, 3, 5, 7, and 9 delivered successively.
  - c. breath-by-breath pulmonary resistance and compliance are measured over the data collection period until the rat gives a suitable reaction by increasing resistance and decreasing compliance as indicated by decreasing volume and flow waveforms and increasing pressure waveforms with decreasing breath frequency.
  - d. the Data Tech should be marking the record [both electronically (ctrl space) and on the Grass recorder (signal marker)] at the beginning of each 2.5 minute interval to indicate the delivery of the next dose of methacholine. If the volume balance is stable, it is possible to electronically mark every 30 seconds, which is helpful when cutting the data for analysis later. Double mark (electronically) the 2.5 minute time point, and single mark the 30 second time points.
  - e. the rat is finished when respiratory arrest occurs, or all doses have been used.
11. Following the methacholine challenge, the animal is removed from the plethysmograph and returned to the surgical station for removal of the lung for perfusion. During this period, the Data Tech performs the following data manipulations:
  - During the surgical time the Data Tech can be cutting data from Playback. It is essential to have the playback compression at 1 (F7). The data should be cut in the order as follows:

**Table 2: Data Cutting and Naming Formats**

| Time of data cutting  | Naming format                            |
|---|--|
| 20 second previous to dose 1  | Saline rat name (i.e. saline M084SD.rat) |
| 0-30 seconds post Methacholine dose 1   | 1m30 M084SD.rat                          |
| 30-60 seconds post Methacholine dose 1  | 1m60 M084SD.rat                          |
| 60-90 seconds post Methacholine dose 1  | 1m90 M084 SD.rat                         |
| 90-120 seconds post Methacholine dose 1   | 1m120 M084 SD.rat                        |
| 120-150 seconds post Methacholine dose 1  | 1m150 M084 SD.rat                        |
| 0-30 seconds post Methacholine dose 3   | 3m30 M084 SD.rat                         |
| 30-60 seconds Methacholine does 3   | 3m60 M084 SD.rat                         |
| Continue in this manner until all the data is cut. The Data Tech should also note on the rat's data sheet what doses are given. |  |

- The saline file should have approximately 20 sec of data. The file name for the saline data must begin with an 's' (saline M084SD.rat). The files from the methacholine doses should contain at least 9 sec of data and begin with the dose number (1, 3, 5, 7, 9).

### **C. Lung Extraction and Perfusion Experiment**

1. The Surgical Tech should make up the FAPGG and Methylene Blue reagents for this portion of the experiment as indicated below in section III [Solutions].
2. Administer the remaining 0.15 ml of heparin through the carotid catheter and remove the rat from the plethysmograph to the surgical station.
3. Clamp the trachea and open the chest from the xyphoid process to the neck with a midline incision. Cut the descending aorta, esophagus, and inferior vena cava to exsanguinate the animal. Remove the blood from the chest cavity with gauze sponges.
4. Pull the thymus gland away from the pulmonary artery and lungs. Grasp the lower portion of the heart and turn it so as to visualize the conus arteriosus and pulmonary artery.
5. Insert a suture under the pulmonary artery and loosely tie. Make an incision high on the conus arteriosus and insert a cannula (PE 240) into the pulmonary artery and secure with the suture.
6. Dissect the heart free and place in a saline soaked gauze sponge for later determination of right ventricular free wall weight and left ventricle and septum weight.
7. Remove the lungs from the chest and tie a tracheal tube into place. Pull the esophagus away from the removed lungs.
8. During this procedure the Data Tech is preparing for data acquisition during this next part of the experimental protocol:
  - open WinDaq Acquisition and open the reference file named Lung Calibration which has been saved on the preceding Friday in the current week's folder.
  - open a rat data file (M084SD\_p)

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- change the recording time to 10 hours
9. The Data Tech will also see that the spectrophotometer is set up for measurements as follows:
    - the spectrophotometer should be on
    - choose 'test'
    - choose 'multiwavelength'
    - choose 'stored tests': choices are MB and FAPGG
    - MB will be read first, although FAPGG will be collected first
    - 'run samples'
    - 'measure blank'
  10. The tracheal tube and pulmonary arterial cannula are connected to the ventilation and perfusion system, respectively. Before connecting the pulmonary artery, inflate the lungs by pinching off the expired air tubing. Make sure the flow rate on the perfusion pump is set at 2 ml/min before connecting the PE240 tubing from the lung (pulmonary artery) to the perfusion system.
  11. Clamp the pulmonary arterial (PE240) cannula, cut the crimped end of the cannula, fill the cannula with saline, connect the cannula to the metal nipple, release the clamp and allow the perfusate to flow at 2 ml/min. The roller pump provides the pulmonary arterial inflow of the perfusate, and the venous effluent exits via the vestige of the severed left atrium at atmospheric pressure. Allow the bloody perfusate to drain from the lungs while keeping the perfusate reservoir full. Once the perfusate looks clear (no blood), begin recirculating the perfusate.
  12. Recirculate the perfusate by collecting it in the reservoir with a funnel under the lung. Begin recording on the computer (F4). Recheck the zero pressures (airway and perfusion pressures) with the lung present and measure the pulmonary arterial pressure at the following flow rates: 40, 30, 20, 10, 5, and 0 ml/min. These steps will generate the Pressure-Flow curves. The Data Tech records the rates at each flow plateau on the data sheet (while the computer is recording) while the Surgical Tech decreases the flow to the next level.
  13. Following the final flow step, the Surgical Tech allows the bloody perfusate to drain from the lung, making sure the reservoir never runs out of perfusate. Set the perfusate pump at 10 ml/min, fill the reservoir with fresh perfusate, and allow the volume to come to a predetermined level (12-13 ml perfusate in reservoir).
  14. The timer is set so that perfusate flows through the lung for 64 sec during which a "blank" is collected prior to administration of the FAPGG. At the end of the 64 sec, the reservoir is nearly empty (1 ml of perfusate remaining).
  15. FAPGG solution is added to the reservoir and the timer started which activates the pump. From 59-64 seconds, the Data Tech collects the effluent from the lung into a tube marked "venous sample". The Data Tech should then collect a 1 ml sample from the reservoir in a tube marked "arterial sample".
  16. Spin the three FAPGG tubes (blank, venous, and arterial sample) for 1 min. @ 5000 x g to precipitate any sediment. The perfusate volume in the reservoir is allowed to fall to near zero and 12 ml of pre-equilibrated perfusate containing methylene blue is then added to the stirred reservoir. A timer connected to the

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perfusion pump is started simultaneous to the addition of the methylene blue containing perfusate.

17. When the timer reaches 59 seconds, a venous effluent perfusate sample is collected in a microcuvette until 65 seconds. Read the sample immediately at 665 and 750 nm (print values). The sample is left in the spectrophotometer to be read again at 3 minutes (print values again after 3 min and label "venous + 3").
18. After the sample is collected from the lung, the Data Tech collects a 1 ml sample from the reservoir. This is the arterial sample and is to be read after the "venous + 3 min" sample (print values). After the samples have been read (venous, venous + 3, arterial), decant them into tubes marked "MB V" and MB A". The samples and the blank should be centrifuged for 1 minute @5000 x g.
19. During this time that the MB samples are spinning, the Data Tech pipettes the samples for analysis from the FAPGG samples and reads them in the spectrophotometer (venous and arterial) and prints values.
20. Re-run the MB samples after being centrifuged. The venous sample only needs to be read one time after spinning (no venous +3). Print values after each sample.

***D. DNA sample collection and processing of Marshfield samples.***

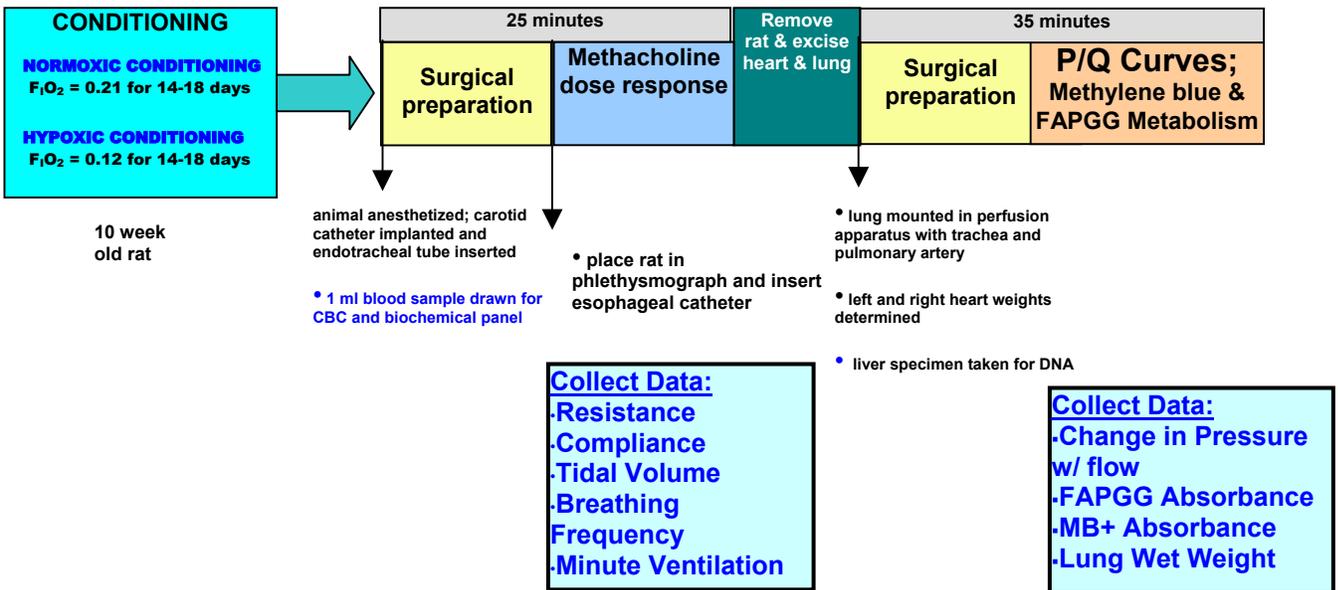
1. A small sample of liver tissue is removed from the rat and placed in the labeled tube and placed in the -80°C freezer.
2. The samples collected initially in the serum separator tubes should be put in the refrigerator for transfer by the PGA Biochemical Core Lab to Marshfield Laboratories for analysis.

***E. Preparation for next rat***

1. While the Data Tech is processing the samples for spectrophotometry, the Surgical Tech should anesthetize the next animal for study.
2. The Surgical Tech can then cut the lungs down from the perfusion setup. The lungs are cut away as well as the trachea and extraneous tissue. Do not remove the cannula or the pressure flow curve data will be negated. The lung tissue should be weighed after removal. The lung tissue is then placed in the oven (temp 65-75°C) to dry for at least 48 hours before obtaining the dry weight.
3. The right ventricle should be dissected free from the left ventricle and the ventricular weights recorded.
4. The Data Tech begins recording data, rechecks the zero values for the airway and perfusion pressures, and performs a pressure flow curve without the lungs. This pressure flow curve is subtracted from the curve obtained with the lungs.
5. The Surgical Tech should pour saline into the reservoir after the perfusate is allowed to run out. This rinses the perfusion pump prior to the next experiment.
6. At the end of the day, the pump should be washed with distilled water.

# Timeline:

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### F. Data processing

1. Transfer the data daily to PGAData on Thor to Process/Lung/Rat Data Files and into the week/day/rat file. Also transfer the data to Raw/Lung/month of collection/week/day/rat.
2. Open Loop.exe and make an AVG file for each rat. Name the file ratID.ave (i.e. M084SD.avg). View each file (saline through each methacholine dose) and eliminate bad breaths, save after viewing each file.
3. Open Lung5.xlt, enable macros. On entry page, fill in cells with information (name, date, stressor, weight, anesthetic, etc.). Do not enter values in yellow cells. These cells contain calculated values using entered data.
  - a. Methacholine data: push 'Get AVG data'; find AVG file in folder from rat (created using Loop.exe)
  - b. Push 'Computer Meth'; look to see that calculated curve seems to fit through experimental values. If not, make a note to recheck values.
  - c. Pressure flow: enter values for Q lung and Q cannula; push 'Pressure Flow'; look at curve to see that calculated values fit through experimental values. If not make a note to recheck values.
  - d. Enter absorbance values from the spectrophotometer for FAPGG and MB.
  - e. Save file as rat name (i.e. M084SD.xls)
  - f. Column B on 'output' page eventually is copied into the FINAL spreadsheet for the rat strain.

### III. Solutions

**Kreb's Stock solutions:**

|                                      |        |                        |
|--------------------------------------|--------|------------------------|
| KCl                                  | 7.0 g  | 100 ml deionized water |
| CaCl <sub>2</sub> •2H <sub>2</sub> O | 7.37 g | 100 ml deionized water |
| MgSO <sub>4</sub> •7H <sub>2</sub> O | 5.8 g  | 100 ml deionized water |
| KH <sub>2</sub> PO <sub>4</sub>      | 3.2 g  | 100 ml deionized water |

Make stock solutions monthly.

**Perfusate Solution:**

|                    | 300 ml  | 400 ml | 500 ml | 800 ml | 1000 ml | 1500 ml | 2000 ml |
|--------------------|---------|--------|--------|--------|---------|---------|---------|
| 4 Stock Solutions: | 1.5 ml  | 2.0 ml | 2.5 ml | 4 ml   | 5 ml    | 7.5 ml  | 10.0 ml |
| NaCl               | 2.07 g  | 2.76 g | 3.45 g | 5.52 g | 6.9 g   | 10.35 g | 3.8 g   |
| Glucose            | 0.30 g  | 0.40 g | 0.50 g | 0.80 g | 1.0 g   | 1.5 g   | 2.0 g   |
| NaHCO <sub>3</sub> | 0.63 g  | 0.90 g | 1.05 g | 1.80 g | 2.1 g   | 3.15 g  | 4.2 g   |
| BSA (4%)           | 12.00 g | 16.0 g | 20.0 g | 32.0 g | 40 g    | 60 g    | 80 g    |

1. Fill a glass beaker with deionized water.
2. Place the beaker on a magnetic stirrer/hot plate. Heat the water to 37°C. The stirrer should be set at a low speed.
3. Add the specified amount of each of the four stock solutions (see above table). Add NaCl, glucose, NaHCO<sub>3</sub>, BSA (4%) (see above table for amount).
4. After the BSA has dissolved, add additional deionized water to bring the perfusate volume up to the required amount.

### IV. Diet

Animals are fed Teklad low salt (0.4% NaCl) chow, order # 3075S, from weaning through the end of the studies. Please see <http://www.teklad.com/index.htm> for more information.

### V. Worksheets

Following is the worksheet for the Lung protocol.

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|   |  |                   |  |                            |  |                                |  |       |  |
|---|--|-------------------|--|----------------------------|--|--------------------------------|--|-------|--|
| <b>Rat Number:</b>                                  |  |                   |  | <b>Rat Status:</b>         |  |                                |  |       |  |
| <b>Date:</b>  |  | <b>Strain:</b>    |  | <b>Sex:</b>                |  |                                |  |       |  |
|   |  |                   |  |                            |  | Lung Prep<br>C:\Rat Data Sheet |  |       |  |
| <b>Body Weight (g) :</b>                            |  |                   |  |                            |  |                                |  |       |  |
| <b>Drug Dosages</b>                                 |  | Ketamine (mL)     |  | Inactin (mL)               |  | Methacholine (mL per dose)     |  |       |  |
| <b>Stock Solutions:</b>                             |  | 100 mg/mL         |  | 0.05 mg/mL                 |  |                                |  |       |  |
| <b>Initial Dose</b>                                 |  |                   |  |                            |  |                                |  |       |  |
| <b>Additional Dosages:</b>                          |  |                   |  |                            |  |                                |  |       |  |
| <b>Cardio/Pulmonary Weights</b>                     |  |                   |  |                            |  | <b>Rat Observations</b>        |  |       |  |
| <b>Heart:</b>                                       |  | <b>Lungs:</b>     |  |                            |  |                                |  |       |  |
| RV (g)-   |  |                   |  |                            |  |                                |  |       |  |
| LV (g)-   |  | Dry (g) -         |  |                            |  |                                |  |       |  |
| <b>Hematocrit Measurements</b>                      |  | RBC/Total         |  |                            |  |                                |  | %     |  |
| Tube 1  |  |                   |  |                            |  |                                |  |       |  |
| Tube 2  |  |                   |  |                            |  |                                |  |       |  |
| <b>AVERAGE:</b>                                     |  |                   |  |                            |  |                                |  |       |  |
| FAPGG   |  | 340nm             |  |                            |  |                                |  | 400nm |  |
| <b>Venous (Centrifuged)</b>                         |  |                   |  |                            |  |                                |  |       |  |
| <b>Arterial (Centrifuged)</b>                       |  |                   |  |                            |  |                                |  |       |  |
| MB+   |  | 665nm             |  | 750nm                      |  |                                |  |       |  |
| <b>Venous</b>                                       |  |                   |  |                            |  |                                |  |       |  |
| <b>Venous + 3 min.</b>                              |  |                   |  |                            |  |                                |  |       |  |
| <b>Arterial</b>                                     |  |                   |  |                            |  |                                |  |       |  |
| MB+ (centrifuged)                                   |  |                   |  | <b>Pressure Flow Curve</b> |  |                                |  |       |  |
| <b>Venous</b>                                       |  |                   |  | Flow Rate                  |  | P lung                         |  |       |  |
| <b>Arterial</b>                                     |  |                   |  | P cannula                  |  | P difference                   |  |       |  |
| Airway pressure at beginning of Pressure Flow Curve |  |                   |  | 40                         |  |                                |  |       |  |
|   |  |                   |  | 30                         |  |                                |  |       |  |
|   |  |                   |  | 20                         |  |                                |  |       |  |
|   |  |                   |  | 10                         |  |                                |  |       |  |
| <b>Control</b>                                      |  | <b>Compliance</b> |  | <b>Resistance</b>          |  |                                |  |       |  |
| sal150 (20 sec)                                     |  |                   |  | 5                          |  |                                |  |       |  |
|   |  |                   |  | 0                          |  |                                |  |       |  |
| <b>Methacholine Dose</b>                            |  | <b>Compliance</b> |  | <b>Resistance</b>          |  | <b>Methacholine Dose</b>       |  |       |  |
| <b>Soln. #1 - 0.28 mg/ml</b>                        |  |                   |  |                            |  | <b>Soln. #5 - 2.8 mg/ml</b>    |  |       |  |
| 1m30  |  |                   |  |                            |  | 5m30                           |  |       |  |
| 1m60  |  |                   |  |                            |  | 5m60                           |  |       |  |
| 1m90  |  |                   |  |                            |  | 5m90                           |  |       |  |
| 1m120   |  |                   |  |                            |  | 5m120                          |  |       |  |
| 1m150   |  |                   |  |                            |  | 5m150                          |  |       |  |
| <b>Soln. #2 - 0.50 mg/ml</b>                        |  |                   |  |                            |  | <b>Soln. #6 - 5.0 mg/ml</b>    |  |       |  |
| 2m30  |  |                   |  |                            |  | 6m30                           |  |       |  |
| 2m60  |  |                   |  |                            |  | 6m60                           |  |       |  |
| 2m90  |  |                   |  |                            |  | 6m90                           |  |       |  |
| 2m120   |  |                   |  |                            |  | 6m120                          |  |       |  |
| 2m150   |  |                   |  |                            |  | 6m150                          |  |       |  |
| <b>Soln. #3 - 0.89 mg/ml</b>                        |  |                   |  |                            |  | <b>Soln. #7 - 8.9 mg/ml</b>    |  |       |  |
| 3m30  |  |                   |  |                            |  | 7m30                           |  |       |  |
| 3m60  |  |                   |  |                            |  | 7m60                           |  |       |  |
| 3m90  |  |                   |  |                            |  | 7m90                           |  |       |  |
| 3m120   |  |                   |  |                            |  | 7m120                          |  |       |  |
| 3m150   |  |                   |  |                            |  | 7m150                          |  |       |  |
| <b>Soln. #4 - 1.58 mg/ml</b>                        |  |                   |  |                            |  | <b>Soln. #8 - 15.8 mg/ml</b>   |  |       |  |
| 4m30  |  |                   |  |                            |  | 8m30                           |  |       |  |
| 4m60  |  |                   |  |                            |  | 8m60                           |  |       |  |
| 4m90  |  |                   |  |                            |  | 8m90                           |  |       |  |
| 4m120   |  |                   |  |                            |  | 8m120                          |  |       |  |
| 4m150   |  |                   |  |                            |  | 8m150                          |  |       |  |