Vascular Phenotypes

High throughput characterization of vascular reactivity in rats conditioned on 0.4% and 4.0 % NaCl diet.

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with
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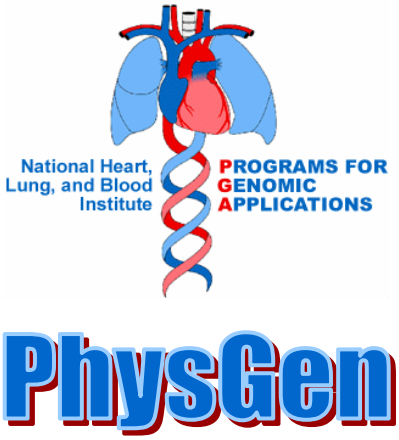
Revised 02/23/04 by Kathryn Privett



Revised 3/3/2004

I. Experimental setup for aortic ring studies (instrumentation and calibration procedures)

Instrumentation and equipment used in setup [order information listed in section VI, set up is shown in Figures 1A and 1B]:

- 8 tissue bath system with reservoirs and circulators used for relaxation/contraction protocol [Radnoti Glass Technology]
- Digi-Med tissue force analyzers [Micro Med DMSI-210]
- Grass FT-03 force transducers
- Oxygen tanks: individual tanks and regulators for delivery of 95% oxygen concentration
- Dissection station with fiber-optic light, microscope [Edmonds Scientific], surgical instruments and plexiglass dissection board with petri dishes lined with silgard for pinning of vessel.

Figure 1A: Close-up view of 8-bath isolated vessel ring set up.

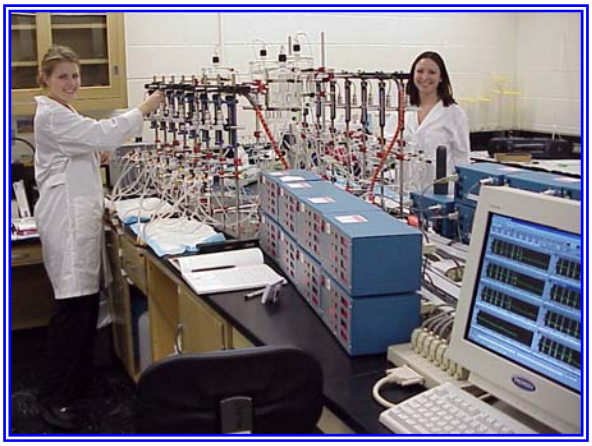


Figure 1B: View of both 8-bath isolated vessel ring set-up with tissue force analyzers and on-line recording system.

II. Experimental protocol for aortic ring studies

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

1. Turn on computer, circulating heater pump [should be 38.2^{o}C], and open oxygen tank to check gas level.
2. Turn on all Tissue Force Analyzers [TFA] using red on/off switch on front of each box. Allow 15 min. warm up before calibration procedure is initiated.
3. Fill reservoirs and vessel baths with freshly prepared PSS [Physiological salt solution; see section on Solutions for formula]. Ensure oxygen is on first before introducing PSS into the reservoir to prevent backflow into the air delivery system.

4. Reserve 150 ml of PSS for the dissection of the vessels and for the preparation of drug solutions.
5. Adjust the airflow into each bath using the valve on the left of each bath to control the airflow into the bath solution. The flow of bubbles should be the same in each bath with a constant bubbling rate.
6. Verify that each transducer is level using a carpenters' level.
7. Perform calibration of the force transducers:
   a. Adjust baseline to 0 on each TFA by selecting button #2 or "Base".
   b. Press "Base" again to zero the transducer [after this button is pressed it will light up and will remain lit until the process is complete]. Do not press any other button while this button is on or the process will be interrupted.
   c. Select button #1 to return to the menu again.
   d. Select button # 3 or "Cal". Hang a 10 g weight by a thread from the wire hook on the force transducer and allow the weight reading to stabilize. Press #3 to calibrate.
   e. Leaving weight on, select button #1 for menu and then select #1 again for the "set-up mode".
   f. A number will appear in the first window, which should read 10.00. If the reading differs by more than 0.05 from a reading of 10.00 than the calibration procedure must be repeated.
   g. To record calibration: Open the DMSI-210_8 program where an icon for each TFA will appear along the toolbar. The word "set-up" above each TFA icon indicates that the channel is in set-up mode and has not been calibrated. Select the Force Icon [has a picture of a line graph]. Tile horizontally [use Window option on menu] and view all channels individually but simultaneously. Click "run" and press start to begin recording that is indicated by the red line turning to green [green indicates the recording mode]. Hang the 10-gram weight from each transducer and allow the calibration to be recorded.

B. Surgical removal of vessel and preparation for mounting ring in tissue bath.

1. Each rat delivered to the Vascular Phenotyping station has had the transponder read at the time the rat is brought to the lab to verify the i.d. of the rat and its corresponding group assignment and conditioning protocol [0.4% or 4.0% NaCl diet for 3 weeks prior to study]. The identification nomenclature has been described in an earlier section.
2. Data sheets for each animal are used which record the information from the animal i.d., date of study, verification of gender, body weight, and conditioning group [see Vascular Worksheets, page 11]. Two labels are made for each rat using the described nomenclature that will be placed on the petri dish containing the vessel and on the bath where the vessel ring is mounted.
3. Rat is weighed and given an intraperitoneal injection of sodium pentobarbital [60 mg/kg] to produce a deep anesthesia.

Figure 2: Close-up view of chamber for mounting aortic ring.

4. The anesthetized rat is positioned on the dissection board and the chest opened with an incision along the left and right lateral aspect of the ribcage from the midline at the floating rib to the brachial plexus. The rib cage can then be lifted up and back and clamped such to expose the thoracic cavity.
5. Remove the heart cutting the vena cava, pulmonary artery and the root of the thoracic aorta. Using gauze to absorb the blood, push the lungs to the left side of the chest cavity to expose the thoracic aorta.
6. Clamp the distal end of the aorta where the vessel passes through the diaphragm and dissect to the most proximal end yielding a length of aorta that is at least 3-5 cm.
7. Place vessel in the labeled petri dish containing room temperature PSS.
8. Gently remove remaining blood from vessel by swishing in PSS.
9. Pin the ends of the aorta to the sylgard resin in the petri dish.
10. Under the dissecting microscope, remove adhering fat and connective tissue using fine #5 Dumont forceps and small Vannas scissors and cut away the ends of the vessel.
11. Cut the aorta into three, 3 mm wide rings and insert the opened triangular wire holders through the lumen of the vessel and close the holder.
12. For each rat aorta, mount two rings [as shown in figure 2]: one ring is mounted in the 8-bath setup used for the relaxation/contraction study and the rat id label affixed to the bath. The remaining ring is left in the labeled petri dish in the event that during the setup and pre-conditioning steps, the ring is injured and must be replaced. Prior to mounting the ring in each bath, the PSS to all baths is changed.
13. Adjust tension to 1.5 g for each ring and allow the rings to equilibrate for 30 minutes.

C. *Initial pre-conditioning procedures for rings prior to initiation of either contraction or relaxation protocols.*

1. Pre-load and equilibration: set passive force to 1.5 g using tension adjustment dial for each transducer and allow rings to stabilize for 30 minutes washing with PSS every 10 minutes and readjusting tension to 1.5 g as needed during this

period. When rings are stable at 1.5 g: select button #1 for Menu; select button #2 for "base"; select button #3 to zero out the 1.5 g tension; return to menu and set-up mode by pressing button #1 twice.

2. Pre-conditioning of aortic rings:
   a. Contract vessel ring with 10^{-7} M of phenylephrine (95.0 µl of 10^{-4} M stock solution to bath) and let stabilize for 5 minutes.
   b. Add 10^{-5} M acetylcholine (95 µl of 10^{-2} M ACH stock solution to bath) to the pre-contracted vessels to test for endothelial integrity (5 mins.) If the vessel relaxes, it can be used for further study. If the vessel ring fails to relax, replace the ring with the remaining ring for that aorta [the second ring], and repeat contraction and dilation steps.
   c. Wash 3 times with PSS and allow vessel to equilibrate for 30 minutes, wash at 10-minute intervals. Make sure the tension is stable and close to 0 g before starting the maximum response. Test the maximal contraction response with 80 mM K^+ (fill bath) + 10^{-5} M PE (50 µl of 10^{-3} M PE to bath).
   d. Allow rings to stabilize for 30 minutes, washing with PSS every 10 minutes, readjusting the tension to 0 g as needed.

D. Experimental protocol:

1. **RELAXATION/CONTRACTION PROTOCOL**-in one set of the 8 tissue baths, the relaxation/contraction protocol is performed on a single aortic ring from each of 8 rats.

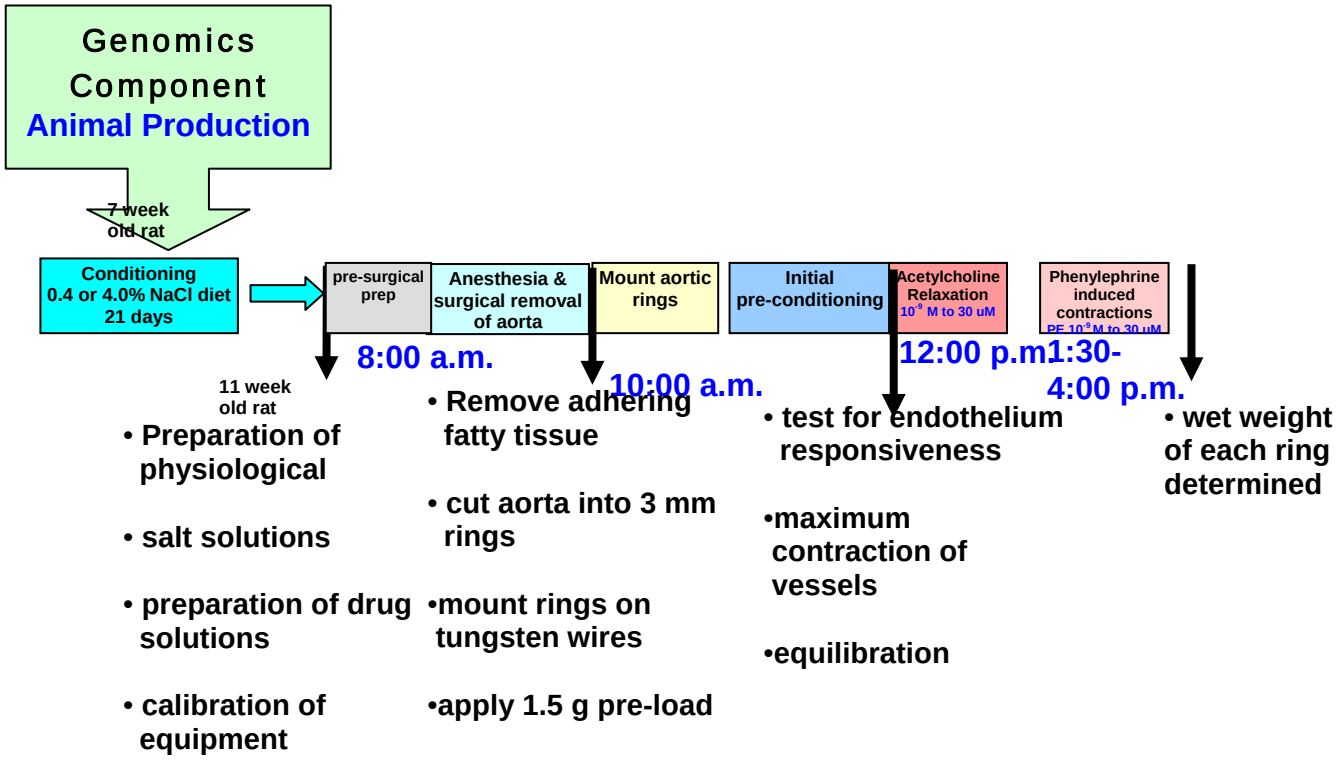
   a. **Acetylcholine relaxation**: do cumulative concentration-response curve for acetylcholine [ACH] induced relaxation at 10 different concentrations. Follow instructions on the "Relaxation/Contraction Data Sheet" [see page 11]. The amount of relaxation of the PE contracted vessel will be measured as the appropriate dilutions of stock solution are added to achieve ACH concentrations of 10^{-9} M to 30µM in the tissue bath.

      - Contract the vessels with 10^{-6}M Phenylephrine (5.0 **µl** of stock solution 10^{-3} M)
      - Add 50µl of **ACH Stock Solution A** to bath to achieve **10^{-9} M**
      - Add 150µl of **ACH Stock Solution A** to bath to achieve **3nM**
      - Add 50µl of **ACH Stock Solution B** to bath to achieve **10^{-8} M**
      - Add 150µl of **ACH Stock Solution B** to bath to achieve **30nM**
      - Add 50µl of **ACH Stock Solution C** to bath to achieve **10^{-7} M**
      - Add 150µl of **ACH Stock Solution C** to bath to achieve **300nM**
      - Add 50µl of **ACH Stock Solution D** to bath to achieve **10^{-6} M**
      - Add 150µl of **ACH Stock Solution D** to bath to achieve **3µM**
      - Add 50µl of **ACH Stock Solution E** to bath to achieve **10^{-5} M**
      - Add 150µl of **ACH Stock Solution E** to bath to achieve **30µM**

b. **Equilibration:** Wash with PSS and allow vessels to equilibrate for 30 minutes, washing at 5-10 min. intervals.
c. **Phenylephrine-induced [PE] contractions**: do cumulative concentration-response curve for PE using 12 different concentrations. Follow the instructions on the "Relaxation/Contraction Data Sheet" [see page 11]. PE in the tissue bath will be increased by successive addition of appropriate dilutions of stock solutions to achieve bath concentrations of 10^{-9} M to 300µM as the following dilution instructions would indicate:

- Add 50µl of **PE Stock Solution A** to bath to achieve **10^{-9} M**
- Add 150µl of **PE Stock Solution A** to bath to achieve **3nM**
- Add 50µl of **PE Stock Solution B** to bath to achieve **10^{-8} M**
- Add 150µl of **PE Stock Solution B** to bath to achieve **30nM**
- Add 50µl of **PE Stock Solution C** to bath to achieve **10^{-7} M**
- Add 150µl of **PE Stock Solution C** to bath to achieve **300nM**
- Add 50µl of **PE Stock Solution D** to bath to achieve **10^{-6} M**
- Add 150µl of **PE Stock Solution D** to bath to achieve **3µM**
- Add 50µl of **PE Stock Solution E** to bath to achieve **10^{-5} M**
- Add 150µl of **PE Stock Solution E** to bath to achieve **30µM**
- Add 50µl of **PE Stock Solution F** to bath to achieve **10^{-4} M**
- Add 150µl of **PE Stock Solution F** to bath to achieve **300µM**

Vascular Relaxation/Contraction Protocol



III. Solutions

A. Salt solutions:

PHYSIOLOGICAL SALT SOLUTION [PSS]

	MW	mM	20X Salt Stock (2 L)	20X Buffer (2 L)
NaCl	58.4	119.0	278.0g	
KCl	74.6	4.7	14.0g	
$MgSO_4$ $7H_2O$	246.5	1.17	11.52g	
$CaCl_2$ $2H_2O$	147.02	1.6	9.4g	
NaH_2PO_4	120.0	1.18	3.1g	
$NaHCO_3$	84.0	24.0		80.8g
EDTA	372.24	0.03		0.4g
Dextrose	180.16	5.5		
HEPES	260.3	5.0		52.06g

Directions for mixing to make 2 liters of PSS:

- Mix 100 ml of **20X Salt Stock** + 1800 ml distilled water + 100 ml of **20X Buffer Stock**
- Add 0.28 g of NaH_2PO_4
- Add 1.98 g glucose

HIGH POTASSIUM PSS [HPPSS]

	MW	mM	Quantity (g/L)
NaCl	58.4	43.7	2.554g
KCl	74.6	80.0	5.964g
$MgSO_4$ anhydrous	120.4	1.17	0.1409g
$CaCl_2$ $2H_2O$	147.02	1.6	0.4704g
NaH_2PO_4	120.0	1.18	0.1416g
$NaHCO_3$	84.0	18.0	1.512g
EDTA [0.5 M solution]	372.24	0.03	60.0 µl
Dextrose	180.16	5.5	1.802g
HEPES	260.3	5.0	1.3015g

Directions for mixing: To make 1 liter of HPPSS:

- Add all salts to distilled water and q.s. to one liter adding $CaCl_2$ last
- Adjust pH to 7.4 with 1 N HCl

0.01 M SODIUM BISULFITE BUFFER

Concentrated HCl	830.0µl
$Na_2S_2O_3$	5.01 g
NaCl	9.00 g
Distilled H_2O	1000.0 ml

Store at 4^0C. Stable for 4 months.

B. Drug stock solutions:

1. **Phenylephrine stock solutions:**
MW: 203.7; a sympathomimetic agent that stimulates the alpha adrenergic receptors. Prepare this stock solution every week and keep it in dark by wrapping the tube in foil, as it is light sensitive.

Stock Solutions: 10mM stock solution: To 10 ml Sodium Bisulfite Buffer, add 20mg of Phenylephrine, to make 10 ml of 10^{-2} M stock solution.

1mM stock solution: Dilute 1 ml of the 10^{-2} M stock solution in 9ml of Sodium Bisulfite Buffer to get a 10^{-3} M solution.

Working Stock: the day of the experiment made dilutions in amber vials labeled A,B,C,D,E and F.

2000µl of 10^{-2} M stock solution for **stock solution F** (10^{-2} M)
2000µl of 10^{-3} M stock solution for **stock solution E** (10^{-3} M)
200µl of 10^{-3} M stock solution into **1800µl** PSS for **stock solution D** (10^{-4} M)
20µl of 10^{-3} M stock solution into **1980µl** PSS for **stock solution C** (10^{-5} M)
20µl of 10^{-4} M stock solution into **1980µl** PSS for **stock solution B** (10^{-6} M)
20µl of 10^{-5} M stock solution into **1980µl** PSS for **stock solution A** (10^{-7} M)

2. **Acetylcholine (ACH) (1 mM and 0.1 mM) stock solutions:**
MW: 181.7; an endothelium-dependent vasodilator that is used to assess the integrity and function of the endothelium of the prepared rings.

NOTE: Prepare stock solutions to aliquot and freeze so that the working stock can be prepared fresh each day. Ach is very unstable and care must be used to ensure the potency of this compound.

Stock solution: Take one 150 mg vial and add 15 ml saline = 10 mg/ml. From this, make 49 tubes of 125 microliters aliquots to be used for making working stock ACH1. Make another 49 tubes of 181.7 microliters aliquots to be used for making working stock ACH2. Label tubes (date, and concentration) and store them in the freezer.

Working stock: The *day of the experiment*, take one ACH2 tube containing181.7 microliters (10 mg/ml) and bring up to 1 ml (add 818.3 microliters of PSS = 1.817 mg in 1 ml of solution = 1.817 mg/ml = 10^{-2} M). In relaxation experiments, make a 1:10 dilution of the working stock solution to get a 10^{-3} M solution or "solution E". This solution will be subsequently diluted.

Label two 1.5ml micro-centrifuge tubes for each stock solution (A,B,C,D and E). You will have 10 tubes for 5 stock solutions. Make two tubes for each stock solution.

100µl of 10^{-2} M stock solution into **900µl** PSS for **stock solution E** (10^{-3} M)
100µl of 10^{-3} M stock solution into **900µl** PSS for **stock solution D** (10^{-4} M)
10µl of 10^{-3} M stock solution into **990µl** PSS for **stock solution C** (10^{-5} M)
10µl of 10^{-4} M stock solution into **990µl** PSS for **stock solution B** (10^{-6} M)
10µl of 10^{-5} M stock solution into **990µl** PSS for **stock solution A** (10^{-7} M)

IV. Diet

Low Salt protocol animals are fed Teklad low salt (0.4% NaCl) chow, order # 3075S, from weaning through the end of the studies. High Salt protocol animals are fed Teklad low salt (0.4% NaCl) chow, order # 3075S, from weaning through 7 weeks. At 8 weeks they are put on Teklad high salt (4.0% NaCl) chow, order # TD01454. Please see http://www.teklad.com/index.htm for more information.

V. Worksheets

Included is a worksheet for the relaxation/contraction protocol.

Relaxation/Contraction Protocol

Procedure for Vascular Relaxation/Contraction Protocol	
DATE:	
SPECIES:	
ID #:	Cowley - PGA
PROJ #:	092-00-2
DIET:	

Bath:	#1	#2	#3	#4	#5	#6	#7	#8
Rat ID #:								
SEX:								
BIRTH:								
RAT WT (g):								
AORTA WT (mg):								
Surgeon:								

Initial Pre-Conditioning Procedures

Relax/Contr	Drug	Time	µl Amt.	#1	#2	#3	#4	#5	#6	#7	#8
R	PSS	30 min	n/a								
C	PE	5 min	5								
R	ACH1	5 min	5								

Maximum Contraction

Relax/Contr	Drug	Time	µl Amt.	#1	#2	#3	#4	#5	#6	#7	#8
R	PSS	10 min	n/a								
C	KCl + PE	10 min	50								

Relaxation Experiment

Relax/Contr	Drug	Time	µl Amt.	#1	#2	#3	#4	#5	#6	#7	#8
R	PSS	30 min	n/a								
C	PE -6	10 min	5								
R	ACH **A**	6 min	50								
R	ACH **A**	6 min	150								
R	ACH **B**	6 min	50								
R	ACH **B**	6 min	150								
R	ACH **C**	6 min	50								
R	ACH **C**	6 min	150								
R	ACH **D**	6 min	50								
R	ACH **D**	6 min	150								
R	ACH **E**	6 min	50								
R	ACH **E**	6 min	150								

Contraction Experiment

Relax/Contr	Drug	Time	µl Amt.	#1	#2	#3	#4	#5	#6	#7	#8
R	PSS	30 min	n/a								
C	PE **A**	6 min	50								
C	PE **A**	6 min	150								
C	PE **B**	6 min	50								
C	PE **B**	6 min	150								
C	PE **C**	6 min	50								
C	PE **C**	6 min	150								
C	PE **D**	6 min	50								
C	PE **D**	6 min	150								
C	PE **E**	6 min	50								
C	PE **E**	6 min	150								
C	PE **F**	6 min	50								
C	PE **F**	6 min	150								

VI. Order information

A. Micro Med
8088 Vine Crest Ave #3
Louisville, KY 40222-4683
1-800-326-8096 Diane/Customer Service
1-502-515-4292
Fax 1-502-515-1259

Tissue Force Analysis System Part # SYS210/8
Digi Med System Integrator-Part # DMSI 210/8
REG # D200/895127 & #D200/895126
S/N's 00189-00196 00181-00188
Transducer Adaptor Cables Part # TXD-320

B. Radnoti Glass Technology
227 W. Maple Ave
Monrovia, CA 91016
1-800-428-1416
Fax 1-626-303-2998

2-8 Unit Tissue Bath System Less XDR Part# 159928-X1
1 - Digital Premier Bath - 14 LTR Part # HA-723466
Part # 160171 5-10ML Glass Hooks (Package of 6)
Part # 120143-2 Oxygen Bubbler for Reservoir