Vascular Phenotypes

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

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National Heart, Lung, and Blood Institute
PROGRAMS FOR GENOMIC APPLICATIONS

PhysGen

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

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

- 8 tissue bath system with reservoirs and circulators used for relaxation protocol
- 8 tissue bath system with reservoirs and circulators used for 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%, 10%, 5%, and 0% oxygen concentrations
- 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.

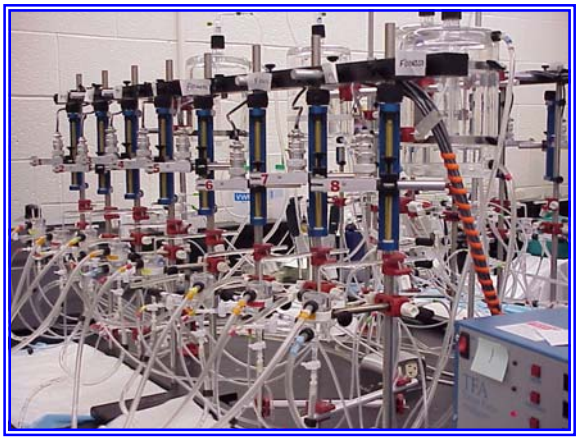


Figure 1A: Close-up view of 8-bath isolated vessel ring set up.

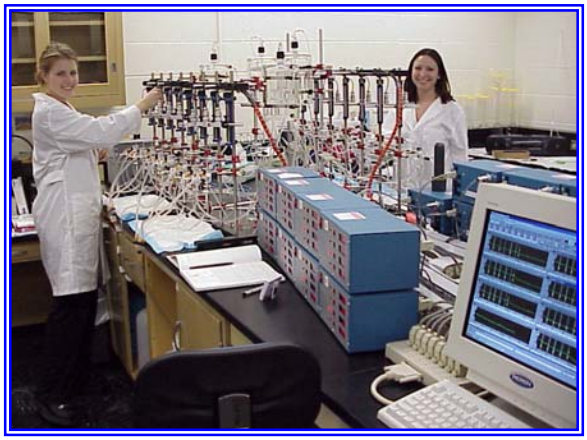


Figure 1B: View of both 8-bath isolated vessel ring set-up with tissue force analyzers and on-line recording system.

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 tanks to check gas level in each of the 4 tanks.
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 air flow into each bath using the valve on the left of each bath to control the air flow 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, pages 12-13]. Three labels are made for each rat using the described nomenclature that will be placed on the petri dish containing the vessel and on each of the two baths 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 contraction studies and the rat id label affixed to the bath, and one ring is mounted in the 8-bath setup used for the dilation studies 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 third 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 stablize for 30 minutes, washing with PSS every 10 minutes, readjusting the tension to 0 g as needed.

D. Experimental protocol:

1. **CONTRACTION PROTOCOL**-in one set of the 8 tissue baths, the contraction protocol is performed on a single aortic ring from each of 8 rats.
   a. **Phenylephrine-induced [PE] contractions**: do cumulative concentration-response curve for PE using 11 different concentrations. Follow the instructions on the "Contraction Data Sheet" [see page 13]. PE in the tissue bath will be increased by successive addition of appropriate dilutions of stock solutions to achieve bath concentrations of 10^{-9} to 10^{-4} 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 50µl of **PE Stock Solution F** to bath to achieve **300µM**

   b. **Equlibration:** Wash with PSS and allow vessels to equilibrate for 30 minutes, washing at 5-10 min. intervals.

c. **Hypoxic relaxation**: changes in the amount of force will be measured as the oxygen concentration of the tissue bath is reduced.
   - Contract the vessels with PE 10^{-6} M (5µl of 10^{-4} PE) and allow the vessels to reach a maximum response (10min.)
   - After 10 minutes at **95% O_2**, reduce oxygen concentration to **10% O_2** for 20 minutes.
   - Reduce oxygen concentration to **5% O_2** for 20 minutes.
   - Reduce oxygen concentration to **0% O_2** for 20 minutes.
   - Return oxygen concentration to **95% O_2** for 20 minutes.

2. **RELAXATION PROTOCOL**--in one set of the 8 tissue baths, the 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 Data Sheet" [see page 14]. 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} to 30 µM in the tissue bath.

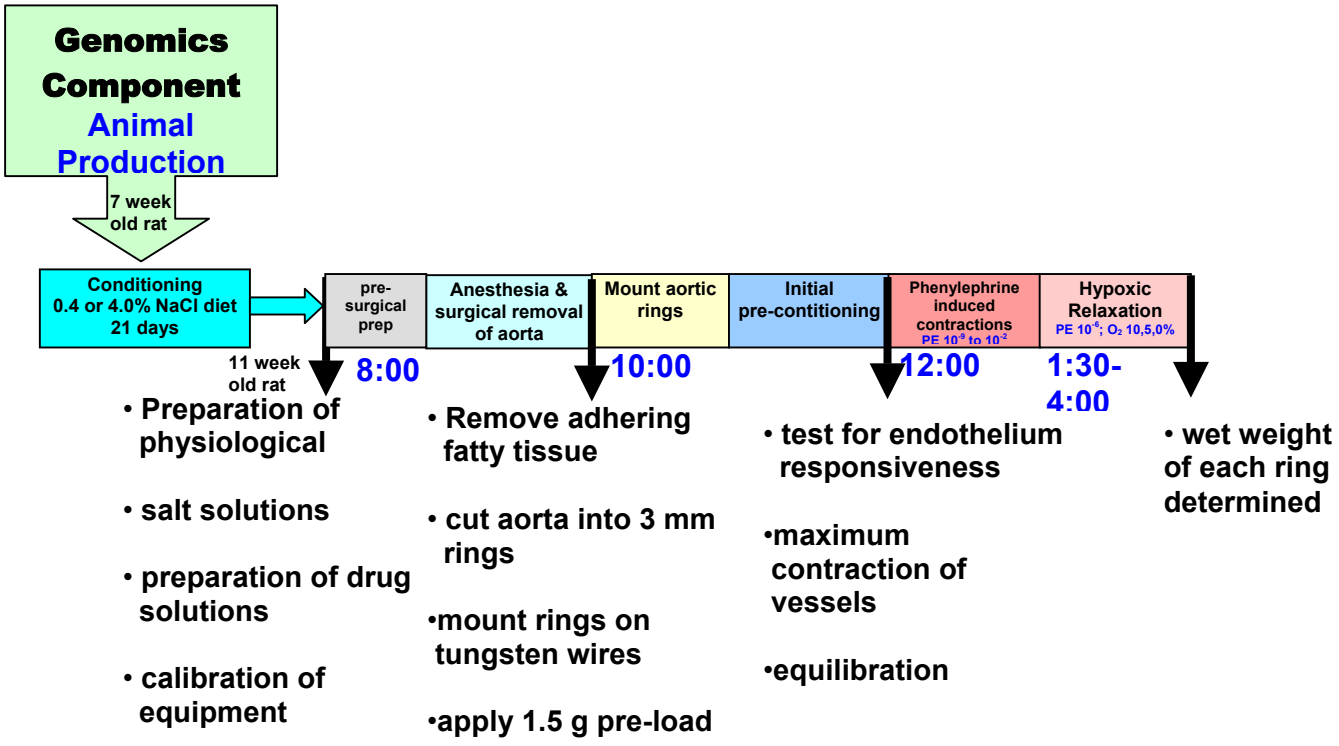
      - Contract the vessels with 10^{-6}M Phenylephrine (5.0 µl of stock solution 10^{-4} M)
      - Add 50µl of **ACH Stock Solution A** to bath to achieve **10^{-9} M**
      - Add 150µl of **ACH Stock Solution A** to bath to achieve **3nM**
      - Add 50µl of **ACH Stock Solution B** to bath to achieve **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. **Equlibration:** Wash with PSS and allow vessels to equilibrate for 30 minutes, washing at 5-10 min. intervals.

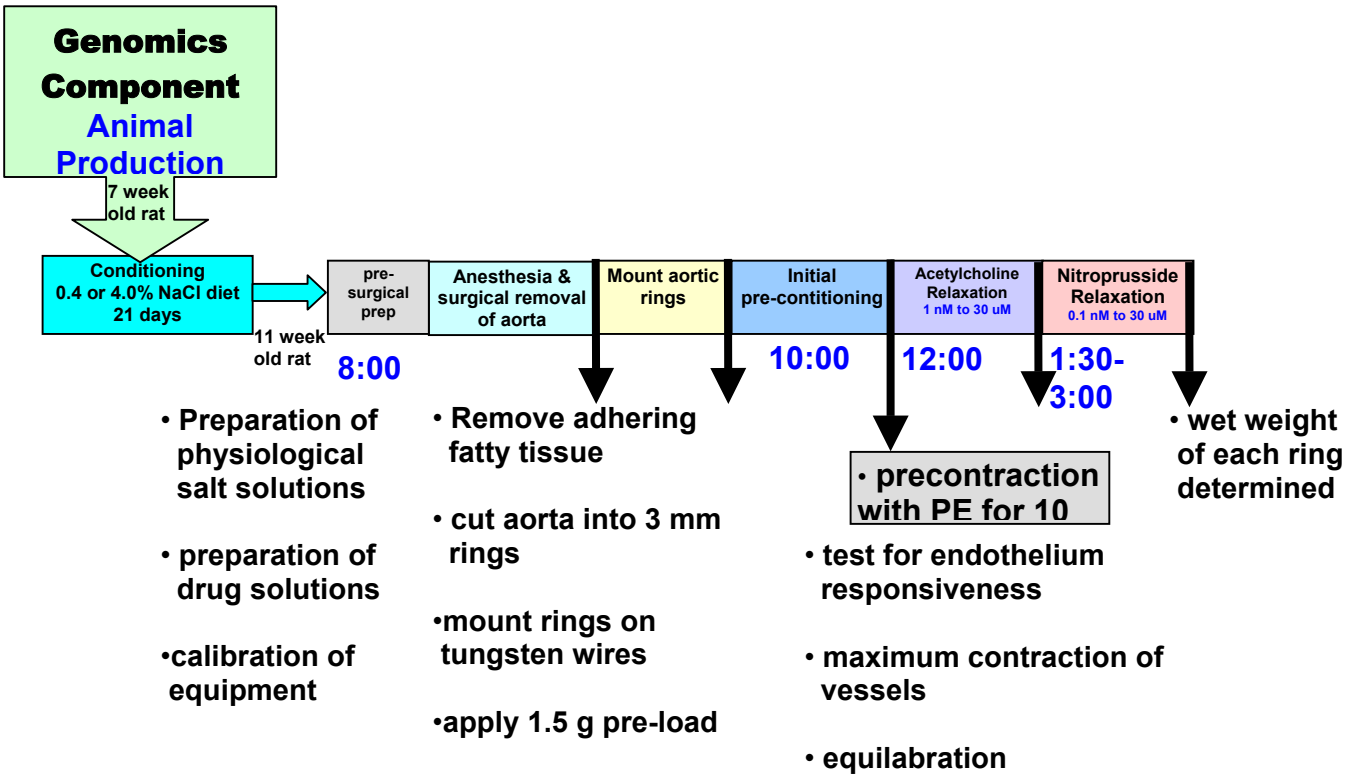
   c. **Sodium nitroprusside relaxation:** a cumulative concentration-response curve for sodium nitroprusside (SNP)-induced relaxation is determined at 10 different concentrations at 50 µl and 150 µl amounts. Follow the directions on the "Relaxation Data Sheet". The amount of relaxation of the PE contracted vessel will be measured as the appropriate dilutions of stock solution are added to achieve SNP bath concentrations of 10^{-10} M to 3 µM.

- Contract the vessels with 10^{-6}M Phenylephrine (5 µl of stock solution 10^{-4} M)
- Add 50µl of **SNP Stock Solution A** to bath to achieve **10^{-10} M**
- Add 150µl of **SNP Stock Solution A** to bath to achieve **.3nM**
- Add 50µl of **SNP Stock Solution B** to bath to achieve **10^{-9} M**
- Add 150µl of **SNP Stock Solution B** to bath to achieve **3nM**
- Add 50µl of **SNP Stock Solution C** to bath to achieve **10^{-8} M**
- Add 150µl of **SNP Stock Solution C** to bath to achieve **30nM**
- Add 50µl of **SNP Stock Solution D** to bath to achieve **10^{-7} M**
- Add 150µl of **SNP Stock Solution D** to bath to achieve **300nM**
- Add 50µl of **SNP Stock Solution E** to bath to achieve **10^{-6} M**
- Add 150µl of **SNP Stock Solution E** to bath to achieve **3µM**

Vascular Contraction Protocol



Vascular Relaxation 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 70 tubes of 200 microliters aliquots to be used for making working stock. Label tubes (date, and concentration) and store them in the freezer.

Working stock: The *day of the experiment*, take 181.7 microliters of stock 1 (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 with for 5 stock solutions. Make two tubes for each stock solution.

100µl of 10^{-3} M stock solution for **900µl** PSS for **stock solution D** (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)

3. **Sodium Nitroprusside (SNP) stock solutions:** MW: 298; a vasodilator, nitric oxide donor that elicits an endothelium-independent dilation. Only to be prepared for the relaxation experiments.

   NOTE: Prepare stock solution and dilutions prior to use. This substance is very light sensitive. Keep solutions in dark glass or plastic bottles and use immediately.

   Stock solution: To make a 10^{-3} M stock solution: dissolve 15 mg in 50 ml of PSS.

   General dilution of stock solutions for delivery of variable concentrations to bath for dose response determinations for phenylephrine, acetylcholine and sodium nitroprusside:

   Prepare **Master Stock Solution** – **(10^{-3} M)** (label as **"Stock Solution F 10^{-3} M"**)
   200µl of 10^{-3} M stock solution for **1800µl** PSS for **stock solution E** (10^{-4} M)
   20µl of 10^{-3} M stock solution into **1980µl** PSS for **stock solution D** (10^{-5} M)
   20µl of 10^{-4} M stock solution into **1980µl** PSS for **stock solution C** (10^{-6} M)
   20µl of 10^{-5} M stock solution into **1980µl** PSS for **stock solution B** (10^{-7} M)
   20µl of 10^{-6} M stock solution into **1980µl** PSS for **stock solution A** (10^{-8} M)

Label with brightly colored tape, arrange in ice bucket in order.

Add 50µl of **Stock Solution A** to bath to achieve **10^{-10} M**
Add 150µl of **Stock Solution A** to bath to achieve **0.3nM**
Add 50µl of **Stock Solution B** to bath to achieve **10^{-9} M**
Add 150µl of **Stock Solution B** to bath to achieve **3nM**
Add 50µl of **Stock Solution C** to bath to achieve **10^{-8} M**
Add 150µl of **Stock Solution C** to bath to achieve **30nM**
Add 50µl of **Stock Solution D** to bath to achieve **10^{-7} M**
Add 150µl of **Stock Solution D** to bath to achieve **3nM**
Add 50µl of **Stock Solution E** to bath to achieve **10^{-6} M**
Add 150µl of **Stock Solution E** to bath to achieve **3µM**

IV. Worksheets

Included is a worksheet for the contraction protocol and one for the relaxation protocol.

Procedure for Vascular Ring Studies - Contraction

DATE:	
SPECIES:	
ID #:	
PROJ #:	
DIET:	

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

Initial Pre-Conditioning Procedures

(Start)		(Response)					(Max. Response)					
Time	**Drug**	**Time**	**µl Amt.**	**[Bath]**	**#1**	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**
	PSS	30 min	n/a									
	PE	5 min	5									
	ACH	5 min	5	-5								
	PSS	10 min	n/a									
	KCl +PE	10 min	50									
	PSS	30 min										

Contraction Experiment

(Start)		(Response)					(Max. Response)					
Time	**Drug**	**Time**	**µl Amt.**	**[Bath]**	**#1**	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**
	PE **A**	6 min	50	-9								
	PE **A**	6 min	150									
	PE **B**	6 min	50	-8								
	PE **B**	6 min	150									
	PE **C**	6 min	50	-7								
	PE **C**	6 min	150									
	PE **D**	6 min	50	-6								
	PE **D**	6 min	150									
	PE **E**	6 min	50	-5								
	PE **E**	6 min	150									
	PE **F**	6 min	50	-4								
	PE **F**	6 min	150									
	PSS	30 min	n/a									
	PE	20 min	n/a									
	10% O2	20 min	n/a									
	5% O2	20 min	n/a									
	0% O2	20 min	n/a									
	95% O2	20 min	n/a									

Procedure for Vascular Ring Studies - Relaxation

DATE:	
ID #:	
PROJ #:	
SPECIES	
DIET:	

	1	2	3	4	5	6	7	8
SEX:								
BIRTH:								
ID CARD								
BLOOD PRESSURE								
RAT WT (g):								
AORTA WT (g):								

Initial Pre-Conditioning Procedures

(Start)		(Response)					(Max. Response)					
Time	**Drug**	**Time**	**µl Amt.**	**[Bath]**	**#1**	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**
	PSS	30 min	n/a	-								
	PE	5 min	5									
	ACH	5 min	5									
	PSS	10 min	n/a	-								
	KCl + PE	10 min	50	-								

Relaxation Experiment

(Start)		(Response)					(Max. Response)					
Time	**Drug**	**Time**	**µl Amt.**	**[Bath]**	**#1**	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**
	PSS	30 min	n/a	-								
	PE -6	10 min	5									
	ACH **A**	6 min	50									
	ACH **A**	6 min	150									
	ACH **B**	6 min	50									
	ACH **B**	6 min	150									
	ACH **C**	6 min	50									
	ACH **C**	6 min	150									
	ACH **D**	6 min	50									
	ACH **D**	6 min	150									
	ACH **E**	6 min	50									
	ACH **E**	6 min	150									
	PSS	30 min	n/a	-								
	PE -6	10 min	5									
	SNP **A**	6 min	50									
	SNP **A**	6 min	150									
	SNP **B**	6 min	50									
	SNP **B**	6 min	150									
	SNP **C**	6 min	50									
	SNP **C**	6 min	150									
	SNP **D**	6 min	50									
	SNP **D**	6 min	150									
	SNP **E**	6 min	50									
	SNP **E**	6 min	150									

V. 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