EXPERIMENTAL PROTOCOLS

This primer contains general information regarding a standard reactivity experimental protocol. A typical experimental protocol for vascular reactivity experiments involves equilibration of the mounted vessel segment, a wake-up protocol, a functional test of the mounted vessel segment, and the actual experiment, which may involve a concentration-response curve to one or several vasopressors and/or vasodilators. First, each component of an entire experimental protocol is discussed, and then step-by-step instructions are listed on how to perform a full vascular reactivity protocol.

EQUILIBRATION

Once a vessel has been mounted (regardless of whether it is a mouse aortic ring on pins or rat mesenteric artery on wires), the vessel needs to equilibrate in the Myograph Chamber before reliable, reproducible results can be obtained during the experiments. The equilibration period allows the vascular ring preparation to heat up to experimental temperature slowly while giving the preparation time to reset ion gradients that may have been disturbed during dissection and cleaning. The equilibration period also allows the preparation time to achieve a stable level of passive tension, regardless of whether the normalization for small vessels or a pre-determined passive tension was used. Typically, equilibration takes no more than 1 hour.

THE WAKE-UP PROTOCOL

The purpose of performing a wake-up protocol is to:

1. Re-activate the mechanical, functional, and signaling properties of the vessel segment.
2. Check that responses to different types of stimuli are normal and verify that the functionality of the vascular ring preparation was not damaged during the dissection or mounting procedures.
3. Check whether the vessel segment meets inclusion/exclusion criteria to be used for experimentation. For example, if the DMT normalization module from ADInstruments is used to determine the passive tension for the vessel segment, a vessel segment would have to develop a stimulated tension equivalent to an effective active pressure above 13.3 kPa or 100mm Hg. This is only an example, and it is up to the end-user to determine appropriate inclusion/exclusion criteria for the vessels being used for their particular experiments.

The wake-up protocol is performed after the vessel segment has been heated, equilibrated and stretched with the appropriate passive tension. The present procedure is to be used on rat mesenteric arteries. Another procedure may be needed for other animal species and tissue or vessel types.

The wake-up protocol consists of a series of stimuli and washout periods. The stimuli chosen will depend on the mounted vessel. For example, noradrenaline (also known as norepinephrine) is a good agonist to use to stimulate contractions in mesenteric resistance arteries. Methoxamine and cirazoline (α_1 receptor agonists) are also good agonists to use in the mesenteric resistance arteries. If using mouse or rat aorta for reactivity experiments, the α_1 receptor agonist, phenylephrine, would be a good choice. If other resistance arteries are used, these agonists may not stimulate for optimal contractions.

KPSS (high potassium physiological salt solution) should always be a part of the wake-up protocol. The number of stimuli, the order, and length of time for each stimulus may need to be optimized, depending on the vessel chosen for observation. The step-by-step protocol at the end of this primer lists a wake-up protocol that is ideal for rat mesenteric resistance arteries and may prove to be a good starting point for other vessels of interest.

FINAL VASCULAR FUNCTION ASSESSMENT

One of the last components of the wake-up protocol is an agonist-mediated contraction and test for endothelial function. The contraction should be a contraction elicited by a submaximal concentration of a vasopressor of choice. For example, in mouse aorta, 10^{-6} mol/L phenylephrine in an endothelium-intact vessel segment would be an appropriate stimulus. In mouse mesenteric artery, 10^{-6} mol/L or 10^{-5} mol/L norepinephrine (noradrenaline) in an endothelium-intact vessel segment would be an appropriate stimulus. This contraction can be used as a final stimulus in the wake-up protocol. In addition, this stimulus can be used in two ways:

1. To assess whether the vascular smooth muscle is contracting properly.
2. It can be used as the contraction needed to test an endothelium-dependent relaxation, if intact endothelial experiments are to be performed.

It is recommended that endothelial function (or lack of) is evaluated. Stimulating a vessel segment with acetylcholine or carbachol causes the release of nitric oxide (NO) from the endothelial cells. If the endothelium is undamaged after dissection and mounting, a substantial relaxation will occur. If the endothelium is removed or damaged during dissection and mounting, a partial relaxation or no relaxation to acetylcholine or carbachol will be observed.

The purpose of checking endothelial function, therefore, is:

1. To check whether the endothelium is intact. The procedure is performed to verify that the endothelium was not damaged during dissection or mounting.
2. To verify that endothelium was sufficiently removed (denuded) if an experiment requires removal of the endothelium.

These 2 functional parameters can be used as inclusion/exclusion criteria. If the vessel does not contract or relax according to the end-user's acceptable criteria, new vessels can be mounted if desired. Again, depending on the vessel and the conditions, these inclusion/exclusion criteria will need to be decided upon and assessed by the end-user.

THE ACTUAL EXPERIMENT: CONCENTRATION-DEPENDENT RESPONSES, PRESSURE CURVES OR FLOW-MEDIATED DILATION

After the wake-up protocol is completed, the actual experiment can be conducted. A typical experiment involving concentration- dependent responses would consist of concentration-response curves to one or several agonists in the presence or absence of antagonists. Depending on the agonists, multiple concentration-response curve protocols can be performed in the same vessel segment. If the agonists used are water-soluble and their effects are easy to wash out, then animals can be preserved and data collection can be maximized by performing several concentration-responses to different agonists in the same vessel preparation.

A typical pressure curve, especially if myogenic responses are being evaluated, would involve incremental pressure steps at regular intervals to establish the range in which the myogenic response occurs and whether changes in the myogenic response has developed. The range of pressures tested and the intervals between pressure steps, as well as the magnitude of each pressure step, may vary, depending on the vessel tested.

A typical flow-mediated dilation experiment in a DMT pressure myograph would involve varying the inlet pressure (P1) and outlet pressure (P2) in order to create a pressure gradient across the

vessel, thereby inducing flow through the mounted vessel. The flow through the vessel would then induce shear-stress, which in turn will cause the intact, healthy endothelium to mediate a relaxation or dilation. Several factors MUST be taken into account when performing flow-mediated dilation experiments:

1. The vessel must be mounted on the cannulas in the proper orientation. Essentially, the vessel needs to be mounted in such a way so that flow in the vessel mounted in the pressure myograph chamber is in the same direction as what the vessel experiences *in vivo*. Otherwise, the vessel will give little to no flow-mediated responses.

2. The cannula tips must be matched in terms of resistance. It is not enough to measure the tips and assume that the same tip diameters will result in the same resistance to flow. Resistance must be measured and cannulas matched according to the measured resistance. The resistance can be measured electrically or physically measured in terms of resistance to flow.

3. The cannula tip diameters must also be similar in internal diameter of the mounted vessel. The internal diameter of the mounted vessel, however, is the internal diameter of the vessel at the working pressure (mean pressure *in vivo* or working pressure of the isolated and mounted vessel in the pressure myograph). If the pressurized internal diameter of the mounted vessel is not similar to the external diameter of the glass cannula, laminar flow cannot be achieved, and flow-mediated responses as well as flow measurements will be inaccurate or nonexistent.

EXAMPLE OF STEP-BY-STEP EXPERIMENTAL PROCEDURE

Dissection and mounting

1. Isolate organ, vascular bed, or isolated artery (arteries; i.e. aorta or carotid arteries) of choice from species of interest.
2. Place the organ, vascular bed, or isolated artery into a beaker containing cold or room temperature PSS.
3. Transfer the organ, vascular bed, or isolated artery in a dissection dish for cleaning. Cleaning is best done under a dissection microscope in a petri dish coated with some kind of material such as Sylgard so as to be able to pin the organ or vessel down without causing major damage to the vessels in general. The petri dish should contain room temperature PSS.
4. Cut the vessel to an appropriate length (~2mm). Use a cut up plastic metric ruler under the dissecting microscope as a reference for length.
5. Fill the chambers of the 620M with 5 ml of room temperature PSS in each.
6. Transfer a single ring into a chamber of the 620M.
7. Mount the artery on the wire (25 µm tungsten or 40 µm stainless steel) or the pins, depending on the internal diameter of the vessel being studied. The mounting procedure on wires can be found 5.2. In addition, watch the accompanying video sent with your system for step-by-step visual instructions.
8. Place the chambers with the mounted arteries on the interface and plug the transducer cables into the back of the interface.

Equilibration

1. Turn on the heat, which should be preset to 37 °C.
2. Turn on your gas to bubble your chambers.
3. Wait 20 minutes without touching the vessels or system. This allows the vessels to slowly heat up.
4. Perform your normalization using the DMT normalization module on LabChart for small vessels to

determine the optimal passive tension. If using larger arteries such as mouse aortic rings, a length-tension relationship will need to be performed to determine optimal passive tension to conduct the reactivity at.

5. Make a note of what the passive tension is.
6. Wash the vessels with bubbled, warmed (37 °C) PSS by pressing the “All Channels” button on the front of the interface to evacuate the chambers of buffer.
7. Immediately replace the buffer (5mL) with fresh, bubbled, warmed (37 °C) PSS.
8. Allow the artery to continue equilibrating for another 20 minutes.
9. During the next period of equilibration, if the tension slips or changes, continue to adjust the passive tension so that it remains at the noted passive tension in step 5.
10. After 20 minutes from the first wash (now 40 minutes since the start of equilibration), do another single wash with warmed, bubbled PSS.
11. Once a total of 60 minutes has passed, the Wake-up Protocol can be initiated.

THE WAKE-UP PROTOCOL

1. Reset the baseline values to “zero” by using the “zero” function menu of the interface.
2. Use a combination of PSS containing 60mM K+ (60mM KPSS) and a contractile agonist (either norepinephrine for mesenterics or some other agonist for other types of resistance arteries) for the wake-up protocol.
3. Start the wake-up protocol by stimulating the vessel with KPSS. Stimulate for 3 minutes.
4. Wash out 4 times with regular PSS over 5 minutes and wait 5 minutes.
5. Repeat steps 3 and 4.
6. While the vessel is in regular PSS, stimulate the vessel with 10μM noradrenaline* and wait 3 minutes.
7. Wash out 5 or 6 times with regular PSS over 20 minutes.
8. Stimulate the vessel with KPSS. Wait until the contraction is stable at a plateau. This will take more than 5 minutes, and can take up to 10 or 15 minutes, maybe longer depending on the vessel examined.
9. Wash out 4 times with regular PSS over 5 minutes or until the vessel has returned to baseline. Wait 5 minutes.
10. Stimulate the vessel with KPSS + 10μM noradrenaline*. Stimulate for 3 minutes.
11. Wash out 5 or 6 times over 20 minutes with regular PSS. Continue washes if the vessel hasn’t reached baseline.

**Note: use the appropriate agonist for your vessel that causes a robust contraction.*

FINAL VASCULAR FUNCTION ASSESSMENT

1. Add contractile agonist* used during the wake-up protocol so that the final concentration in the bath is 1μM (5uL of 10^{-3} M stock to the 5mL bath). Allow contraction to occur for 3 to 5 minutes.
2. DO NOT WASH THE VESSEL!
3. Add acetylcholine (Ach) or carbachol at a final concentration of 10μM in the bath (5uL of 1 x 10^{-2} M stock Ach or carbachol to 5mL bath). Allow to relax for 2 minutes.
4. Wash the contractile agonist and Ach or carbachol from the vessel preparation. Wash at least 5 to 6 times over 15 to 20 minutes to completely eliminate the effects of the vasopressor and Ach or carbachol.
5. Preparation is now ready for experiments.

Concentration responses

Example of agonist concentration response experiment:

5.0 uL of 10^{-6} M to get 10^{-9} M in the 5 mL bath
1.0 uL of 10^{-5} M to get $3x10^{-9}$ M in the 5 mL bath
3.5 uL of 10^{-5} M to get 10^{-8} M in the 5 mL bath
1.0 uL of 10^{-4} M to get $3x10^{-8}$ M in the 5 mL bath
3.5 uL of 10^{-4} M to get 10^{-7} M in the 5 mL bath
1.0 uL of 10^{-3} M to get $3x10^{-7}$ M in the 5 mL bath
3.5 uL of 10^{-3} M to get 10^{-6} M in the 5 mL bath
1.0 uL of 10^{-2} M to get $3x10^{-6}$ M in the 5 mL bath
3.5 uL of 10^{-2} M to get 10^{-5} M in the 5 mL bath

Each concentration should be added cumulatively without washing the previous addition out. Once the concentration response is complete, the vessels can be washed completely (5 to 6 washes over 20 minutes), and another concentration response can be performed with a different agonist.

For example, the first concentration response can be norepinephrine (NE). This can be washed out, the EC80 can be determined from the concentration response. The NE EC80 can then be used to contract the vessel again. An acetylcholine (Ach) or carbachol concentration response can be performed to assess endothelial function once a stable contraction (a plateau) is reached after addition of the NE EC80. A 3rd concentration response can be performed similar to the 2nd, only using sodium nitroprusside or nitroglycerin for endothelium-independent relaxation.

These final concentrations will need to be modified depending on the agonist(s) used, the range of efficacy for that agonist, and the volume of buffer used in the chambers.

Pressure Curves

An example of a pressure curve experiment may involve something similar to the following but will vary in starting and ending pressures depending on the vessel in which compliance or myogenic responses are being measured in:

1. Depressurize the vessel to 10mmHg. The pressure should be set so that there is no flow through the vessel.
2. Allow the vessel to equilibrate. This will be evident when the diameters (outer, inner or both) are stable and not changing. This may be about 5 minutes.
3. Increase the pressure to 20mmHg. The pressure should be increased in a way so that no flow is occurring through the vessel. For example, if both P1 and P2 are used to control the internal pressure, then both P1 and P2 should be raised to 20mmHg.
4. Allow the vessel to equilibrate to the new pressure for 5 minutes.
5. Repeat steps 3 and 4, increasing the pressure by increments of 10mmHg until a peak pressure is reached. Depending on the vessel, this could be has high as 180mmHg.
6. If desired, the same curve can be performed in the absence of calcium. If done, the PSS buffer should contain EGTA to chelate any residual calcium.

*IF COMPLIANCE IS BEING EVALUATED THROUGH A PRESSURE CURVE, THE PRESSURE CURVE SHOULD BE PERFORMED ENTIRELY IN THE ABSENCE OF CALCIUM.

Flow-Mediated Dilation

An example of a flow-mediated dilation experiment will be conducted similarly to what is described here but may vary in terms of actual flows measured and pressure settings used. Important points to remember when performing a flow-mediated dilation experiment are as follows:

- The vessel must be mounted in the correct orientation, meaning that a response to flow will only occur if the vessel is mounted in the chamber in the correct orientation, similar to what the vessel experienced *in vivo*. The flow in the DMT pressure myograph will always be from P1 to P2.
- The cannula tip outer diameter must be equivalent to the inner diameter of the mounted vessel at the working pressure that the flow experiment is going to be performed at.
- The cannulas need to be matched for resistance and not tip diameters, as similar tip diameters are not the only factor that will determine resistance in the mounting cannulas.

Once these criteria are met, then the flow-induced responses will be more consistent and reproducible from preparation to preparation. An example of a flow-mediated dilation protocol may look like the following:

1. After the vessel has been equilibrated and a wake-up protocol has been performed, the vessel should be started with no flow, but the system should be set up so that the flow program is turned on.
2. To view changes (dilation) induced by flow, the vessel can be pre-constricted with a low concentration of vasopressor (i.e. EC50), or the vessel can be set at a pressure that will cause significant myogenic tone.
3. To set no flow in the system while the flow program is on, P1 and P2 should be equal. As an example in this protocol, if the starting or working pressure for the vessel is 60mmHg, then P1 and P2 should be set to 60mmHg.
4. Flow in the DMT pressure myograph system will begin when a pressure gradient between P1 and P2 is established. In the DMT pressure myograph system, P1 will always be higher than P2 in order to establish flow. If a P2 value higher than P1 is entered, the system and/or software will not allow P2 pressure to be higher than the P1 pressure and will not allow you to enter the higher P2 value as well as give you an error message in the MyoVIEW software. Flow will be measured by the inline DMT flow- meter (model 161FM or 162FM).
5. When beginning flow by modifying P1 and P2, the changes that occur to P1 and P2 must be equal. In this example, the working, starting pressure was designated as 60mmHg, meaning P1 and P2 should be set at 60mmHg. To initiate flow, P1 should be raised while P2 is lowered by the same amount. For example, to start a small amount of flow, a small pressure gradient needs to be established by setting P1 to 61mmHg and P2 lowered to 59mmHg (2mmHg difference). The mean pressure of P1 and P2 is, therefore, maintained at 60mmHg.
6. Allow flow to mediate the response. The flow-meter will monitor flow continuously.
7. Once the flow-mediated response and flow is stable, new P1 and P2 pressures can be entered while maintaining the mean working pressure. In this example, the next pressures entered could be P1 = 63mmHg and P2 = 57mmHg, again the mean pressure between P1 and P2 being maintained at 60mmHg (6mmHg difference).
8. Continue to change P1 and P2 to increase the pressure gradient, thereby increasing the luminal flow.
9. At some point, the amount of flow monitored by the flow-meter will no longer cause further response in the tissue. When this occurs, the experiment is complete, and if the vessel is to be returned to a no-flow state, P1 and P2 should be reset to the original working pressure. In this example, P1 and P2 would be set to 60mmHg.