Protocol for evaluating vascular permeability in wildtype and PRTg mice

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1) Female Tiel-PR Tg mice and wildtype control litter-mates aged 6-9 wks were ovariectimized and allowed to recover for 7-10 days.

Making estrogen and progesterone stock solutions:

For estrogen (17 beta-estradiol from Sigma), weigh out 10 mg and add to 1 ml 200-proof EtOH. This will yield a lOmg/ml solution. Mix by vortexing. Perform a 1:100 dilution by adding 5 μl of the lOmg/ml solution to 495 μl EtOH. This will leave you with the 100 μg/ml stock solution which you can keep at -20C for probably about 2 weeks.
For the progesterone stock: Weigh out 5-10 mg progesterone (Sigma) and add to the corresponding amount of EtOH to yield a 1mg/ml solution. (For example: 6.7mg progesterone would be added to 6.7ml EtOH). Mix by vortexing and store at -20C - stable
about 2 weeks.

2) The mice were treated with the following hormone regime: Inject mice subcutaneously (on their underside) for 3 days (once a day around 10-11AM) with 100 ng 17 β-estradiol (Sigma) dissolved in 0.1 ml sesame oil. [1]
- Directions: Take a 1.5ml tube and place 10 μl of the lOOug/ml stock solution into the tube.
Add 990 μl sesame oil to the same tube and vortex immediately and thoroughly (the EtOH will have a tendency to move to the top of the oil). Injecting 100 μl will deliver long estrogen. In reality, inject about 120 μl to account for some backflow leakage. Animals receiving vehicle get injected with 100 μl sesame oil alone. Make sure to go s.c. and not i.p. The skin should balloon out if you inject the 100 μl s.c. Wetting the hair with a small amount of 70% EtOH will help the injection because the skin can be more easily visualized.

3) On Days 4 and 5 the mice received no treatment.

4) On days 6-8, mice received lμg progesterone (Sigma) and 6.7 ng 17-β -estradioi in 0.1 ml sesame oil (once a day around 10-11AM).

- Directions: Take a 1.5ml tube and mix 33.5 μl of the 100 μg/ml estrogen stock solution with 466.5 μl EtOH (final volume 500μl) to make a 1:14.93 dilution and therefore a 6.7 μg/ml stock solution. Store solution at -20C for at max 2 weeks. Add 10 μl each of the 6.7 μg/ml estrogen stock and 1mg/ml progesterone stock solutions to a 1.5ml tube and then

add 980 μ l sesame oil. Vortex immediately and throughly. Injecting 100 μl will deliver 6.7ng estrogen and 1μg progesterone. In reality, inject about 110 μ1 to account for some backflow leakage.

5) 5-7 hours after receiving the last hormone treatment on day 8, the mice were injected by tail vein with 1 ml/kg 3% Evans Blue dissolved in 0.9% saline for injection.

- Directions: remove evans blue stock bottle from 4C and place on magnetic stirrer for 1-2 hours. Remove 3-4 ml and place in plastic tissue specimen container. Sonicate on setting 5 with 5-10 pulses for about 1 min. Keep tube on ice and make sure you cover the top of the container (with the probe sticking out of it) with parafilm to keep the E.B from spraying out. Filter the EB after sonication with a 0.22 μm syringe filter to remove any particles left in the solution. Weigh the mice (probably will be around 20-25g) and measure out the amount of EB (in μl) that matches their weight. Then add 0.9% saline for injection to a final volume of 100 μl (easier to inject 100μl then 20-25μl). For example for a 25g mouse, add 25 μlEB to 75 μl saline in a 1,5ml tube. Pull solution up in 0.5cc insulin syringe. Inject into tail vein using plastic restrainer and warming the tail in 45C water.
Note the time of injection - perfusion will be 20 mins following injection.

6) 15 min after injection, the mice were anesthetized with isoflurane using a 50ml conical nosecone device.[2]. Around 19 min after injection, the chest cavity was opened to expose the heart, the right atria removed, the left ventricle cut, and perfused at 120 mmHg through the ascending aorta with 1% paraformaldehyde in 0.05M citrate buffer, pH 3.5 for 2 min. The time between the chest cut and the start of perfusion was 8-15 sec and optimally occurred at the 20 min timepoint[3].

- To make the PFA: dissolve lOg PFA in about 200-300ml dH20 at 50-60C. Once in solution add 14.7g sodium citrate. Bring the total volume up to about 800-900ml with dH20. pH the solution to 3.5 (you will need to add a lot of acid). Top the PFA off with dH20 to the final volume of 1L. Filter solution with #50 Whatman paper.

7) After perfusion, the first 4 cm of intestine located immediately distal to the stomach was resected and the lumen flushed with fixative. The uterine horns were also dissected and each tissue was blotted between 2 pieces of Whatman filter paper for 10 sec (place a full pipette blue tip container on top of paper and count to 10) and weighed (wet weight). The tissues were placed in 1 ml of fomiamide (in labeled glass 7ml solvent-saver type tubes) o/n at 56°C to extract the Evans Blue. The following day, the tissues were removed from the formamide and the A_{620} of the samples and standard curve was measured on a
spectophotometer and the results were expressed as ng Evans Blue/tissue and ng Evans Blue/mg tissue[4].

Directions for reading samples:

The amount of EB in the experimental samples will be calculated by interpolating to a standard curve. To make the standard curve, perform a 1:1000 dilution of the 3% stock solution (30mg/ml) by adding 1 μl stock: 1000 μl formamide. This will make a 30 μg/ml solution. Label 8 1.5ml tubes 1-8 for each of the points in the standard curve. The first point on the standard curve will be 12.9 μg/ml. Make this by adding 430 μl of the 30 μml solution to 570 μl formamide.
Then perform a 2x dilution series by adding 500 μl of the 12.9 μg/ml solution to 500 μl formamide and repeat each time adding 500 μl of the more concentrated EB solution to 500 μl formamide. At the end, the standard curve points will be 12.9, 6.45, 3.225, 1.6125, 0.806, 0.403, 0.2015, and 0.1μg /ml. By the time you get to the last 2 dilutions, the solution will look pretty much clear by eye.

Load the Evans Blue protocol in the SoftMax program. In the template window, label the wells for the 8 standards and all the experimental samples (use some system such as M1 duo and M1ut to denote which mouse and tissue is in each well). Add 200 μI/well formamide and blank the plate. Make sure to remove all of the formamide from the crevices of the well by suction and add 200 μI/well of each of the standards and experimental samples to a microtiter plate. Be careful to add the correct solution to the correct well and to not cross-contaminate. Read the plate and print out the curve and the unknowns grid. The numbers in the MeanResult column are the amount of EB (in μg/ml). Since the samples were extracted in 1ml, this will give you the amount (in μg, or you can convert to ng) of EB/tissue. To get the amount of EB in ng/mg tissue, multiply the values in the MeanResult column by 1000 (to convert them to ng) and divide them by the wet weight of the tissue.

[1]. It is difficult to handle perfusing more than 6-8 mice in one day. Therefore if a greater number of mice are to receive treatment, it is best to stagger the hormone injections so that no more than 6-8 are to receive Evans Blue on the final day.

[2] Using an inhaled anesthetic is preferred as indictable such as avertin seem to stimulate vascular leakage in the intestine. However, the mouse's breathing must be carefully monitored and the amount of isoflurane inhalation adjusted accordingly to ensure that it does not die before the 20 injection time has elapsed.

[3] If many mice (5-8 mice) are going to be perfused, it may be easiest if you time the injections and perfusions to be overlapping (i.e. inject one mouse and then after waiting 10-15 min, inject the next mouse so that after you are done perfusing the first, the next mouse will be ready in a few minutes). Place the intact mice on ice until you are done with the perfusions and then dissect the tissue.

[4] After reading the samples, the 200 μl aliquot in the microtiter plate can be discarded. Store the remaining 800 μl in the solvent saver tubes indefinitely as they can be re-read on the spectrophotometer in the future should the need arise.