

Hyperinsulinemic-euglycemic Clamp

Version: 1
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Summary:

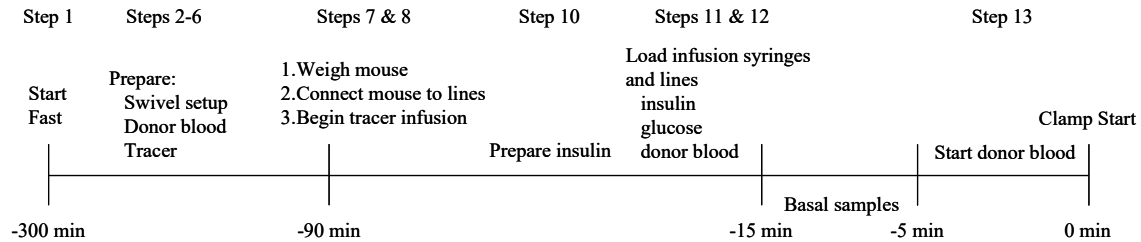
The hyperinsulinemic, euglycemic clamp or “insulin clamp” has been used in a variety of species to assess insulin action. In an insulin clamp, the rate of glucose infused to maintain euglycemia is an index of whole body insulin sensitivity. Isotopes can be used during an insulin clamp to distinguish between insulin’s effects on endogenous glucose production and glucose utilization, or to examine insulin’s effects on specific tissues and metabolic pathways. The insulin infusion rate used in a study depends on a number of factors such as whether the experiment requires a sensitive analysis of endogenous glucose production, or whether the mouse is known to be unusually insulin resistant (e.g. ob/ob). Finally the insulin clamp technique, especially when isotopes are involved, is most quantitative under steady state conditions. Therefore, clamps should be of a duration (~2 h) that is sufficient for the actions of insulin to obtain steady state. The section that follows describes, a) the technical procedures used on the day of a clamp experiment; b) preparation of insulin and tracer infusates; and c) analyses of plasma and tissue radioactivity. Familiarity with these procedures combined with laboratory demonstration provides the investigator with the necessary background to clamp the conscious mouse.

Reagents and Materials:

Reagent/Material	Vendor	Stock Number
Infusion Pumps	Harvard Apparatus	PY8 70-2208
Stand	Fisher Scientific	14-670A
Dual channel swivel	Instech Solomon	375/D/22QM
3- and 4-way stainless steel connectors	Ziggy’s Tubes and Wires	HSCY-25 or HSC4-25
Microrenathane tubing (0.033” OD)	Braintree Scientific	MRE-033
Glucose meter and strips	ACCU-CHEK aviva	
[3- ³ H]-Glucose	Perkin Elmer	NET331C
2-[1- ¹⁴ C]-deoxy-glucose	Perkin Elmer	NEC 495
Blunt needle with luer hub	Ziggy’s Tubes and Wires	LHN-E011041 25ga x 0.5”
Wire, stainless steel	Ziggy’s Tubes and Wires	W020304V-1

Clamp, extension	Fisher Scientific	05-769-7Q
Connector, hook	Fisher Scientific	14-666-18Q
0.3 N Ba(OH) ₂	Sigma Aldrich	B4059
0.3 N ZnSO ₄	Sigma Aldrich	Z2876

Protocol:



1. Surgical catheterization of the carotid artery and jugular vein in mice at least 5 days prior to the day of the study (refer to protocol for Surgical Catheterization of the Carotid Artery and Jugular Vein).
2. Weigh mouse and start fast (suggested starting time between 7:00 and 8:00 AM) by placing mouse in a plastic container with fresh bedding.
3. Set up microrenathane tubing to swivel and connectors as needed (see Figure 1).
4. Collect donor blood ([Reagent 1](#)).
5. Prepare tracers, if needed ([Reagent 3](#), [Reagent 4](#)).
6. If using [3-³H]glucose, draw tracer infusate into a 1 ml syringe and place it securely into the pump slot.
7. Fill the arterial line with heparinized saline. Leave the sampling syringe connected to the top of the swivel. Fill the venous line with non-heparinized saline (or tracer, if using [3-³H]glucose). Plug the free end of the 3 cm microrenathane tubing going into the venous port of the swivel (**A** in Figure 1) or the free end of the 3 cm tubing going into the 3-way connector if using [3-³H]glucose (**B** in Figure 1) with a stainless steel plug. Filling these lines prevents infusion of air into the mouse.
8. If using [3-³H]glucose, weigh the mouse and hook it up to the microrenathane tubing ~100 minutes prior to clamp start as shown in Figure 1.
9. Begin baseline primed-continuous tracer infusion at least 90 minutes prior to clamp start. We typically use a 1.2 μCi prime followed by a 0.04 μCi/min continuous infusion.
10. Prepare Protocol sheets using mouse weight, insulin dose, sample times and volumes.
11. Prepare insulin ([Reagent 2](#)).
12. Once donor blood and insulin are prepared, draw glucose infusate (50% dextrose mixed with or without [3-³H]glucose), insulin infusate, and donor blood into separate 1 ml syringes and place them securely into the pump slots.
13. Connect 10 cm microrenathane tubing to each infusate syringe, and connect tubing to a 4-way connector (see Figure 1). Fill each line with glucose, insulin, and donor blood infusates up to the 4-way connector.

14. Once the last basal sample is taken, stop the baseline tracer infusion pump if using $[3\text{-}^3\text{H}]\text{glucose}$ and connect the 4-way connector to the 3 cm microrenathane tubing connected to the infusion port of the swivel, and begin infusion of donor blood.
15. Once donor blood reaches the mouse, start study by beginning insulin and glucose infusion.
16. To take blood samples for analysis of plasma metabolites:
 - Insert a blunt syringe into the free end of the arterial line prior to releasing the clamp.
 - Release the clamp and draw the desired volume of blood into the syringe.
 - Clamp the arterial line and remove the syringe.
 - Dispense the blood from the syringe into a 0.5 ml EDTA tube and mix by “flicking” the tube.
 - Centrifuge for 1 min at 16,000 g. Transfer the plasma to an appropriately labeled 1.5 ml tube. Plasma can be temporarily stored on ice but should be permanently stored at -20°C .
 - While the blood is being centrifuged, insert the sampling syringe back into the arterial line. Pull back slightly on the plunger to draw any air bubbles into the sampling syringe. Very slowly, give back the $\sim 20 - 40 \mu\text{l}$ of blood originally taken such that the arterial line becomes lightly red to clear.

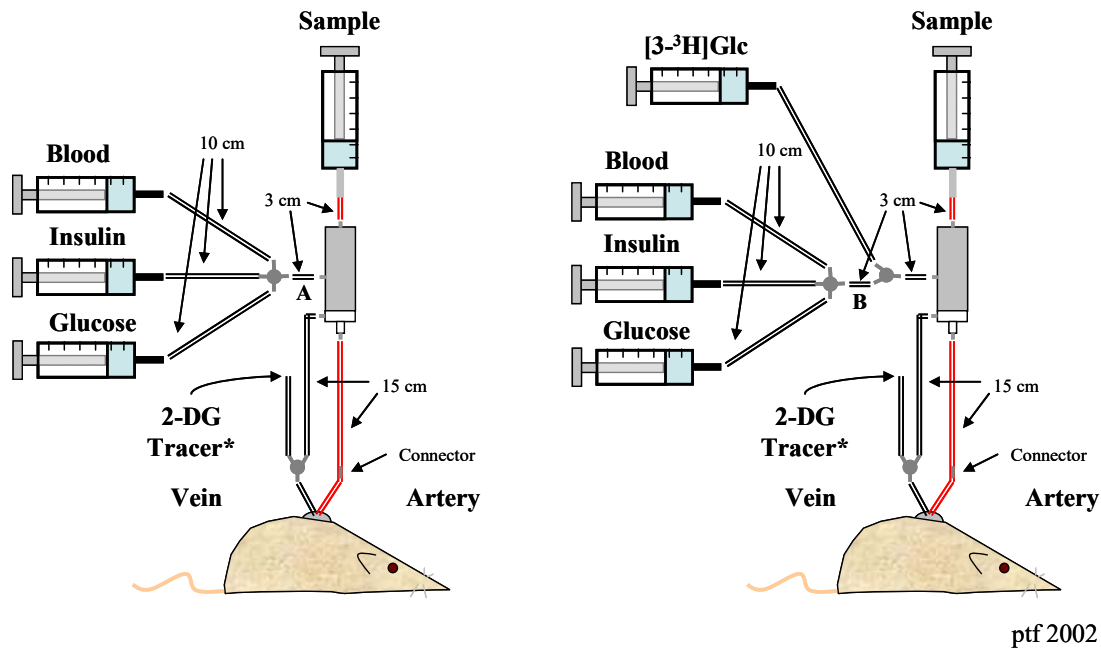


Figure 1. Scheme illustrating the connection of catheters, dual channel swivel, and infusates in relation to the mouse when clamping without (left panel) or with (right panel) $[3\text{-}^3\text{H}]\text{glucose}$. Diagram is not to scale. *This port is generally for 2-deoxyglucose (2DG) tracer bolus administration, but can be used for other infusates as well. A and B indicate the 3 cm microrenathane tubing filled with non-heparinized saline and capped with a stainless steel plug (see step 6).

17. Analysis of radioactivity (when applicable)

Plasma Glucose and 2-Deoxyglucose Radioactivities

Reagents and supplies:

Saline
 Saturated benzoic acid (3.4 g/l)
 0.3 N $\text{Ba}(\text{OH})_2$ diluted 1:3 with ddH_2O . Prepared fresh.

0.3 N ZnSO₄ diluted 1:3 ml with ddH₂O. Prepared fresh.
 1.5 ml tubes
 20 ml borosilicate scintillation vials

Processing ¹⁴C- or ³H-2-deoxyglucose standards:

1. Add 196 µl ddH₂O to 4 µl of standard (saved from bolus of 2-deoxyglucose) and vortex.
2. Pipette 50 µl diluted standard into a 1.5 ml tube and add 950 µl ddH₂O.
3. Pipette 100 µl into scintillation vial.
4. Add 900 µl ddH₂O and 10 ml scintillation fluid.
5. Measure radioactivity in a liquid scintillation counter.

Processing [3-³H]glucose standards:

1. Dilute tracer standards (both for baseline and the clamp period) 1:200 in saturated benzoic acid (5 µl in 995 µl).
2. Add 10 µl of this diluted standard directly to each of three scintillation vials labeled CS. Add 990 µl ddH₂O and cap.
3. Add 10 µl of the diluted standard directly to each of three separate scintillation vials labeled CSE and set aside for drying later (see *Plasma processing* below).
4. Add 10 µl of the diluted standard to each of three 1.5 ml tubes. To each tube add 10 µl saline + 100 µl diluted Ba(OH)₂ (prior to ZnSO₄) + 100 µl diluted ZnSO₄.
5. Vortex and spin down for 5 minutes at 16,000 g.
6. Transfer 100 µl of the supernatant to each of three scintillation vials labeled CRS and set aside to dry later (see *Plasma processing* below).

Plasma processing:

1. Add 10 µl of plasma sample and 10 µl saline to a 1.5 ml tube.
2. Add 100 µl diluted Ba(OH)₂ solution and 100 µl diluted ZnSO₄ (add Ba(OH)₂ prior to ZnSO₄).
3. Vortex and centrifuge (5 min at 16,000 g).
4. Pipette 100 µl of supernatant into scintillation vial.
5. Dry in oven with a trap for ³H₂O. Include standards (CSE and CRS) for drying at this time.^{a,b}
6. Add 1 ml ddH₂O and 10 ml scintillation fluid.
7. Measure radioactivity in a liquid scintillation counter.
 - a. To determine ³H₂O accumulation, a measure of glycolytic rate, pipette 50 µl of the supernatant into a second set of vials and do not dry. ³H₂O will be the difference in radioactivity between these non-dried samples and the dried samples (correcting for the difference in the volume of supernatant used).
 - b. Analysis of samples containing only radioactive 2-deoxyglucose does not require that they be dried. Simply add 900 µl ddH₂O and 10 ml scintillation fluid to the 100 µl of supernatant in the scintillation vial.

Tissue 2-Deoxyglucose Phosphate Radioactivity

Reagents and supplies:

0.3N Ba(OH)₂
 0.3N ZnSO₄
 0.5% perchloric acid
 Liquid nitrogen
 Ice
 20 ml borosilicate scintillation vials
 5 ml and 13 ml large polypropylene tubes w/ caps

1.5 ml tubes
5N KOH and 10 N HCl
Forceps
Homogenizer
pH meter

1. Keep tissues and 5 ml tubes in liquid nitrogen and make sure that the end of the forceps that will come in contact with tissue is cooled in liquid nitrogen.
2. Weigh each tissue sample (~60 mg), transfer to 5 ml tube in liquid nitrogen and record weight.
Note: you will not obtain 60 mg in all tissues (e.g. soleus muscle). In those cases, simply weigh out the entire amount of tissue obtained.
3. For each sample, place on ice the 5 ml tube containing the tissue and homogenize in 1.5 ml 0.5% perchloric acid. Keep the homogenized sample on ice while the remaining tissues are homogenized.
4. Centrifuge tubes for 20 min at 2000 g at 4°C.
5. Place tubes on ice and transfer 1.25 ml of the supernatant to 13 ml tubes. If you are unable to extract 1.25 ml of supernatant, note the volume of supernatant you are able to extract.
6. Neutralize each sample (pH ~7.5) using KOH and HCl. Record the total volume of KOH and HCl used to neutralize each sample.
7. Centrifuge tubes for 10 min at 2000 g at 4°C.
8. Label two scintillation vials (A and B) for each tissue, and number consecutively

For the A vials (measurement of 2-deoxyglucose phosphate and 2-deoxyglucose)
Add 125 µl of neutralized supernatant and 875 µl ddH₂O

For the B vials (measurement of 2-deoxyglucose only)
Pipette 250 µl of neutralized supernatant into 1.5 ml tubes
Add 125 µl of stock 0.3N BaOH
Add 125 µl of stock 0.3N ZnSO₄
Centrifuge at 16,000 g for 5 minutes
Add 250 µl of supernatant and 750 µl ddH₂O to vials

9. Add 10 ml of scintillation fluid to each vial, shake and measure radioactivity in liquid scintillation counter.
10. Deoxyglucose phosphate is the difference between radioactivity in A and B.

Reagent Preparation:

[Reagent 1](#)

[Reagent 2](#)

[Reagent 3](#)

[Reagent 4](#)

Reagent 1: Donor Blood

1. Collect ~ 1 ml of blood from donor mouse in 0.5 ml EDTA tubes.
2. Centrifuge blood (1 min at 16,000 g) and save plasma for preparation of insulin (see below).
3. Resuspend red blood cells (RBC) with heparinized saline (10U/mL).
4. Centrifuge (1 min at 16,000 g), discard supernatant, and resuspend RBC with an equal volume of heparinized saline. Transfer resuspended RBC (donor blood) to a 1.5 ml tube

Reagent 2: Insulin solution

1. Using the plasma obtained from donor mice, make a 3% plasma solution in saline by placing 6 ml of saline in a test tube, removing 180 μ l and adding 180 μ l plasma.
2. Remove 180 μ l of this 3% saline-plasma solution, place in a tube and add 20 μ l U-100 insulin (making a tenfold dilution to create U-10 solution).
3. Transfer 5 ml of 3% saline-plasma solution to a new test tube.
4. Calculate and add U-10 insulin to achieve desired insulin infusate concentration based on mouse weight, pump infusion rate, and desired insulin infusion rate.

Reagent 3: [3-³H]Glucose Infusate

1. For baseline infusion, dry down 16 μ Ci HPLC-purified [3-³H]glucose per mouse. Reconstitute in 400 μ l of saline. Save 20 μ l for standards.
2. For clamp period, dry down 40 μ Ci HPLC-purified [3-³H]glucose per mouse. Reconstitute in 650 μ l of desired concentration of dextrose. Save 20 μ l for standards.

Reagent 4: 2-Deoxyglucose (¹⁴C or ³H) Bolus

1. Dry down 13 μ Ci of 2-deoxyglucose per mouse.
2. Reconstitute in 52 μ l of saline. Save 4 μ l for standard. The remaining 48 μ l is given as the 2-deoxyglucose bolus.