Hyperglycemic Clamp

Version: 1
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Summary:

Hyperglycemic Clamps are used to assess whole-body and possibly tissue-specific insulin sensitivity during hyperglycemia in awake mice.

Reagents and Materials:

Reagent/Material	Vendor	Stock Number
CMA 402 Microdialysis Syringe Pump	Harvard Apparatus	CMA8003110
Catheter for mouse jugular vein	Instech	C20PU-MJV1458
Vascular Access Button 25ga	Instech	VABM1B/25
3-way Y connector, 25ga, sterile	Instech	SCY25
PU tubing for external use	Instech	VAHBPU-T25
Luer stub needle, 25ga	Instech	LS25
[1-14C]-2deoxy-D-glucose	Perkin Elmer	nec495001mc
D-[3H] glucose	Perkin Elmer	NET331C005MC
Dextrose 20%	Covetrus	SKU:068545
Heparin Sodium Injection, USP, 10,000 Unit/10ml	Covetrus	SKU:049130
Sodium Chloride (0.9%) Injection	Covetrus	SKU:061758
Bovine Serum Albumin	Sigma Aldrich	A8806
Luer-Lok 1ml syringe	Fisher Scientific	BD-309628
Heparinized micro-hematocrit capillary tubes	Fisher Scientific	211766

Protocol:

1. Implant catheters in carotid artery and/or jugular vein 5-7 days before clamp
2. Weigh and fast mice morning or overnight (maximum length of fast: 16 hours)
3. Infuse with HPLC-purified 3-[3H]glucose (0.05 uCi/min) during a 2-hour basal period
4. Collect blood samples (~40 ul) at the end of the basal period to estimate the rate of basal hepatic glucose production
5. Infuse 10-50% glucose (with labeled 3-[3H]glucose) at variable rates to maintain plasma glucose at basal concentrations (200~250 mg/dl). Hot-GINF protocol see below.
6. Collect blood samples (~20 ul) at 20 min intervals for the immediate measurement of plasma glucose concentration
7. Administer 1-[14C]-2-deoxy-D-glucose as a bolus (10 uCi) at 75 min after the start of clamp experiments.
8. Collect blood samples (20 ul) at 80, 90, 100, 110, and 120 min after the start of clamp experiments for the determination of plasma 3-[3H]glucose, 3H2O, and 1-[14C]-2-deoxy-D-glucose concentrations. Additional blood samples (20 ul) will be collected at the end of clamp experiments for measurement of plasma insulin concentrations.
9. Sampling Blood:

   *Blood samples in mice will be obtained via one of two methods:

   1: by initially cutting the distal 1 MM of tail and gently massaging the tail. Following the sample collection, the opened tail site and associated bleeding will be temporarily closed using a tape. For subsequent blood sampling, the tape and blood clot will be carefully removed and blood will again be collected via gentle massage of tail. This is the primary method of blood collection

   2: by blood draw via arterial catheters placed in the carotid artery. Following every blood collection, the line will be carefully infused with heparinized saline to keep the line clean and clear from clotting and coagulation
10. Euthanize the mouse and collect tissues (skeletal muscle, liver, white adipose tissue, brown adipose tissue, heart)
11. Store plasma samples at -20ºC and tissue samples at -80ºC

Whole Body Plasma Tracer Assay:

For plasma and F1 samples (dry and non-dry samples):

1. Thaw samples 10 minutes before beginning (analyze 4-6 mice at a time).
2. Quickly centrifuge samples to precipitate the drops stuck on the wall (don't spin F1/F2 with samples to avoid contamination).
3. Add 25 µl $Ba(OH)_2$ (to open tubes, ev. use the cap of a scintillation vial to avoid spills). First open samples, then F1, then F2 (avoid to touch the inside of tubes).
4. Add 25 µl $ZnSO_4$.
5. Vortex and then centrifuge 10 minutes at 12000 rpm.
6. Prepare to sets of scintillation vials (dry and non-dry).
7. For non-dry samples, add 175 µl dH_2O in scintillation vials.
8. Add 25 µl supernatant in dry and then 25 µl in non-dry samples. Change pipette tip between each sample.
9. Add 3 ml scintillation cocktail (Ultima Gold) in non-dry samples, put a cap, vortex, then run protocol #1 (don't forget blank).
10. Put dry samples in a vacuum oven overnight.
11. On the next day, add 200 µl dH_2O to dry samples, cap, vortex and let stand for 30-60'. Then, add 3 ml scintillation cocktail to dry samples, cap, vortex. Run samples in beta counter (in β-counter: first blank 3 ml scintillation cocktail), then samples with missing vial between blank and first sample as well as between mice).

For F2 samples (only dry samples):

1. Add 75 µl $Ba(OH)_2$.
2. Add 75 µl $ZnSO_4$ and vortex.
3. Centrifuge 10 minutes at 12000 rpm.
4. Add 100 µl supernatant.
5. Put samples in vacuum oven overnight.
6. On next day, proceed as with dry samples (see point 11 above).

Hot-GINF method for mouse clamp:

Background. The Yale MMPC typically performs hyperglycemic clamps in the conscious mouse with a constant infusion of 3-[^{3}H] glucose (0.1 µCi/min) to trace glucose turnover. In this method, the glucose tracer is dissolved in the insulin infusate and delivered at 4.2 µL/min for the full duration of the clamp. For incompletely understood reasons, measurements of glucose specific activity using this delivery method sometimes yield physiologically impossible negative values for endogenous glucose production. To avoid this problem, some groups utilize a hot-GINF method in which the glucose tracer is dissolved in the glucose infusate and therefore delivered at a variable rate. A key limitation of the hot-GINF approach is the requirement that the investigator estimate the approximate steady-state glucose infusion rate.

Method.

- The F1 solution is prepared as usual
- The F2 solution can be prepared in stocks. If you expect different groups of mice to require different glucose infusion rates, prepare different stocks.

$$\frac{0.1\ \frac{\mu Ci}{min} * 1000 * V}{F} = \mu Ci\ glucose\ to\ dry\ down = \mu L\ glucose\ stock\ to\ dry\ down$$

F = estimated glucose infusion rate (µL/min). *V* = desired stock volume in mL.

- Resuspend dried glucose in 20% dextrose (D20) stock. Measure glucose concentration of D20 – typically ~16.7%
- Store stock at 4°
- Estimate total volume needed for each mouse (400-800 µL)
- At end of clamp, aliquot hot-GINF infusate as usual for F2 solution

Calculations.

- Normally, the R_d calculation in the clamp spreadsheet uses a constant tracer infusion rate (dpm/min). To account for the variations with hot-GINF, insert a new column called Tracer Infusion in your sheet. Use the following formula:

$$\text{Tracer inf.}\left(\frac{dpm}{min}\right) = \frac{\text{F2}\left(\frac{dpm}{\mu L}\right) * \text{F2 dil.factor (80)} * \text{GINF for previous 10 min}\left(\frac{\mu L}{min}\right)}{1000}$$

- The R_d calculation is the same as before, but now uses a new tracer infusion rate for each time point:

$$R_d\left(\frac{mg}{kg * min}\right) = \frac{\text{Tracer infusion rate}\left(\frac{dpm}{min}\right) * 1000}{\text{Specific activity}\left(\frac{dpm}{mg}\right) * \text{body weight (g)}}$$

Reagent Preparation:

Reagent: Artificial Plasma

	Molecular Weight	500mL
115mM NaCl	58.44	3.36 g
5.9mM KCl	74.55	0.22 g
1.2mM $MgCl_2$, $6H_2O$	203.3	0.122 g
1.2mM NaH_2PO_4, H_2O	137.99	0.083 g
1.2mM $NaSO_4$	142.04	0.0856 g
2.5mM CaCl, $2H_20$	147.02	0.184 g
25mM $NaHCO_3$	84.01	1.052 g
4% BSA		20 g

1. Weigh out above compounds
2. pH to 7.45
3. Filter with 0.22um and store at -20ºC

Reagent: 3-[3H] Glucose Infusate

1. Basal Infusion: dry down 9 µCi HPLC-purified [3-^{3}H] glucose per mouse. Reconstitute in 670 µl of saline.
2. Clamp Infusion: please see Hot GINF Preparation

Reagent: 1-[14C]-2-deoxy-D-glucose Bolus Infusion

1. Dry down 15 µCi of 2-deoxyglucose per mouse.
2. Reconstitute in 15 µl of saline.