P-Akt, p-HSP27, and p-MK2 Assays in Obese Mice

(Lale Ozcan, October 2016)

Portal Vein Insulin Infusion

Following 5 h food withdrawal, anesthetize mice with Avertin (see Avertin protocol below and never use Ketamine/Xylazine as an anesthetic). Once the mouse is anesthetized (make sure that it still can breath normally –not too deep or shallow), open the abdominal cavity and gently transfer the intestines to animal's side so that portal vein is visible (do not cut or remove any organs). Inject insulin (0.75 - 1IU kg^{-1} for DIO and 1.5 - 2 IU kg^{-1} for *ob/ob*) or PBS through the portal vein using an insulin syringe and start the timer (insulin solution should be kept on ice and the total volume should be 200 ul –filled with PBS). Keep the syringe (and do not move it) inside the portal vein for three minutes and apply 37^0C PBS over the abdominal organs during this period to prevent the tissues from drying. Three minutes after insulin or PBS injection, remove the tissues and immediately freeze them in liquid nitrogen, and keep at −80°C until processing. All the tissues (liver, epididymal adipose tissue and skeletal muscle) should be removed within 2 minutes (skeletal muscle should be devoid of surrounding fat tissue).

Protein Extraction from Tissues

For protein extraction, place the tissues in a cold lysis buffer (25 mM Tris-HCl pH 7.4, 1 mM EGTA, 1 mM EDTA, 10 mM $Na_4P_2O_7$, 10 mM NaF, 2 mM Na_3VO_4, 1% NP-40, 2 mM PMSF, 5 µg/ml leupeptin, 10 nM okadaic acid, and 5 µg/ml aprotinin). After homogenization on ice, centrifuge the tissue lysates, and use the supernatant fractions for immunoblot analysis.

Use below primary antibodies and block the membranes with 5% BSA and use 5% BSA for the primary and secondary antibodies:

For phospho Akt, use Cell signaling cat# 9271

For phospho MK2, use Cell signaling cat# 3007

For phospho hsp25, use Cell Signaling cat# 2401

Guidelines for the Use of Tribromoethanol/Avertin Anesthesia

Tribromoethanol, (manufactured as Avertin outside the U.S.), produces surgical anesthesia in most rodents, with good skeletal muscle relaxation and only a moderate degree of respiratory depression. Proper storage is essential as the decomposition products are toxic. Repeated administration to the same animal may also be unsafe.

I. Materials

2,2,2 tribromoethanol (Aldrich T4, 840.2 or equivalent)
Tert-amyl alcohol (Aldrich 15, 246-3 or equivalent)

II. Stock solution

Add 5 ml T-amyl alcohol to 5 g tribromoethanol, (tbe), in a dark bottle to make a 100% stock solution. Shake or stir gently until the solid is dissolved. Stock solution is light sensitive and evaporates rapidly. Do not leave the bottle open longer than is necessary. Label, date and refrigerate in tightly sealed, dark bottle. Yellowing of the solution indicates toxic degradation products and the stock must be replaced. (If the original solution's pH was greater than 5, a drop of Congo Red dye can be added to 5 mls anesthetic stock solution to test for decomposition products, which lower the pH. If the solution turns purple with the addition of the dye, or if crystallization or any other discoloration is noted, the anesthetic should be discarded.) Otherwise**, unused stock solution should be discarded after 6 months.**

III. Working solution

Mix 0.2 ml stock solution with approximately 7.8 ml normal saline, (or PBS), in a glass vessel, (ie. a graduated cylinder wrapped in foil or a dark bottle). Seal container, heat to improve solubility, and mix well by vortexing until dissolved. (Filter sterilization through 0.2 micron filter

is recommended). Label, date and refrigerate when not in use. (Leave out of refrigerator for approximately one hour prior to administration.) **Unused working solution should be discarded after one month.**

IV. Dosage and Anesthetic Effects

The working solution is administered intraperitoneally at 0.5-0.9ml/mouse, (approximately 0.2 ml/10 grams of body weight). (Inadvertent intravenous injection will cause death within minutes.) It will take about five minutes for the mouse to become fully anesthetized, (evidenced by lack of response to toe or tail pinch). An additional 0.05-0.1 ml can be given to effect, allowing sufficient Avertin time after administering the additional anesthetic to observe the effect. Note that the effective dosage is dependent upon the weight of the mouse. Older, fatter or lactating mice will need more anesthetic to become fully anesthetized. The mouse will remain anesthetized for approximately 30-60 minutes and recover within 1-2 hours. Anesthetized animals should be kept warm for the duration of anesthesia, including surgery.