Assay of Hepatic p-CaMKII in Fasting Mice

Ozcan *et al.*, *Cell Metabolism* 2012

One group of mice is fasted overnight and the other group kept ad libitum. The fasting period should not be longer than 14 hours. After humane euthanasia following IRB guidelines, the liver is immediately (within seconds) frozen in liquid nitrogen and kept at -80C until processing.

For protein extraction, tissues are placed in a cold lysis buffer, which is 25 mM Tris-HCl pH 7.4, 1 mM EGTA, 1 mM EDTA, 10 mM $Na_4P_2O_7$ (sodium pyrophosphate), 10 mM NaF, 2 mM Na_3VO_4, 1% NP-40, 2 mM PMSF, 5 mg/ml leupeptin, 10 nM okadaic acid, and 5 mg/ml aprotinin. After homogenization on ice, the tissue lysates are centrifuged, and the supernatant fractions are used for immunoblot blot analysis. At this step, it is crucial that all the procedures (lysis and centrifugation) are performed on ice. Note that the protease and phosphatase inhibitors—PMSF, leupeptin, okadaic acid. and aprotinin—should be added just before starting the lysis. Do not use liver that has been frozen and thawed. Rather, cut a non-thawed piece from the frozen samples.

Tissue extracts are electrophoresed on 4-20% gradient SDS-PAGE gels (Invitrogen) and transferred to 0.22 µM nitrocellulose membranes. The membranes are blocked for 1 h at room temperature in Tris-buffered saline, 0.1% Tween 20 (TBST), containing 5% (w/v) nonfat milk. The membranes are then incubated with primary antibody in TBST containing 5% nonfat milk or BSA at 4°C overnight, followed by incubation with the appropriate secondary antibody coupled to horseradish peroxidase. Proteins are detected by ECL chemiluminescence (Pierce).

For primary anti-p-CaMKII and antibody, we use Novus # NB300-184 or NBP1-64741. These antibodies are advertised as p-CaMKII alpha and p-CaMKII delta, however, we tested these antibodies in cells and tissues where only the gamma isoform is expressed and got positive results with all the conditions. Ozcan *et al.*, *Cell Metabolism* 2012, shows a representative immunoblot. In our hands, p-CaMKII antibody from Cell Signaling only recognizes the “total CaMKII”, not p-CaMKII. Moreover, we complement our p-CaMKII immunoblot data with a direct CaMKII assay using a kit from Promega.