Western blotting of hypothalamic proteins

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PROTOCOL STATUS

Working

We use this protocol in our group and it is working

MATERIALS TEXT

For Western blotting of hypothalamic proteins, the following solutions and reagents were used:

1X Tissue Lysis Buffer:

20 mM Tris-HCl (pH 7.5);
150 mM NaCl;
2 mM EDTA;
2 mM EGTA;
0.5% Triton X-100;
0.5% deoxycholate Na;
0.02% NaN_3;
0.25 M sucrose;
The cocktail of the protease inhibitors (Roche, Switzerland);
1 mM PMSF;
25 mM NaF;
21 mM β-glycerophosphate Na;
21 mM pyrophosphate Na;
0,1 mM orthovanadate Na.

2X SDS Sample Loading Buffer:

125 mM Tris-HCl (pH 6.8);
4% SDS;
20% Glycerol;
2% β-mercaptoethanol;
0.05% Bromophenol Blue (w/v).

SDS Running Buffer:

Dissolve 14.4 g of Glycine, 3.03 g of Tris-base and 1.0 g SDS in 800 mL of distilled water.
Add distilled water to a volume of 1 liter.

Transfer Buffer:

3.03 g Tris-base;
14.4 g Glycine;
200 ml Ethanol (96%);

Add deionized water to a volume of 1 liter.

10X TBS (Tris-buffered saline):
Dissolve 87.7 g of NaCl, 60.57 g of Tris-base in 800 mL of distilled water.
Adjust the pH to 7.5 with HCl. Add distilled water to a volume of 1 liter.
To prepare the **1X TBS-T** containing 0.1% Tween-20, 100 mL of 10X TBS should be mixed with 900 mL of distilled water and 1 mL of Tween-20.

Blocking Buffer: 1X TBS-T with 5% non-fat dry milk.

Wash Buffer: 1X TBS-T.

Primary Antibody Dilution Buffer: 1X TBS-T with 5% non-fat dry milk or 5% BSA.

Secondary Antibody Dilution Buffer: 1X TBS-T with 5% non-fat dry milk.

Blotting Membrane : Amersham Protran 0,45 μM Nitrocellulose (GE Healthcare, Life Sciences, Germany) or 0,2 μM Immun-Blot PVDF membrane (BioRad Laboratories Inc., USA).

The list and characteristics of the used antibodies for detection of AMPK, Akt-kinase and the transcriptional factor STAT3, the main components of the leptin and insulin signaling:

1. **Phospho-Akt (Ser473) (193H12) Rabbit mAb** (#4058, "Cell Signaling Technology", USA). Phospho-Akt (Ser473) (193H12) Rabbit mAb detects endogenous levels of Akt only when phosphorylated at Ser473. Species Reactivity: Human, Mouse, Rat. Monoclonal antibody is produced by immunizing rabbits with a synthetic phosphopeptide corresponding to residues around Ser473 of mouse Akt.

2. **phospho-Akt(Thr^{308}) Antibody** (#9275, "Cell Signaling Technology", USA). Phospho-Akt (Thr308) Antibody detects endogenous levels of Akt only when phosphorylated at Thr308. This antibody does not detect Akt phosphorylated at other sites or related kinases such as PKC and p70 S6 kinase. Species Reactivity: Human, Mouse, Rat. Species predicted to react based on 100% sequence homology: Chicken, Xenopus, Bovine. Polyclonal antibodies are produced by immunizing rabbits with a synthetic phosphopeptide corresponding to residues around Thr308 of mouse Akt. Antibodies are purified by protein A and peptide affinity chromatography.

3. **Phospho-AMPKα (Thr172) (40H9) Rabbit mAb** (#2535, "Cell Signaling Technology", USA). Phospho-AMPKα (Thr172) (40H9) Rabbit mAb detects endogenous AMPKα only when phosphorylated at threonine 172. The antibody detects both α1 and α2 isoforms of the catalytic subunit, but does not detect the regulatory β or γ subunits. Species Reactivity: Human, Mouse, Rat, Hamster, Monkey, D. melanogaster, S. cerevisiae. Species predicted to react based on 100% sequence homology: Chicken, Bovine, Zebrafish, Pig. Monoclonal antibody is produced by immunizing rabbits with a synthetic peptide corresponding to residues surrounding Thr172 of human AMPKα protein.

4. **Phospho-AMPKα1 (Ser485)/AMPKα2 (Ser491) Antibody** (#4185, "Cell Signaling Technology", USA). Phospho-AMPKα1 (Ser485)/AMPKα2 (Ser491) Antibody detects endogenous levels of AMPKα1/α2 only when phosphorylated at serine 485 or serine 491. This antibody does not cross-react with other related proteins. Species Reactivity: Human, Mouse, Rat, Monkey. Polyclonal antibodies are produced by immunizing rabbits with a synthetic phosphopeptide corresponding to residues surrounding Ser491 of human AMPKα2. Antibodies are purified by protein A and peptide affinity chromatography.

5. **phospho-STAT3 (Tyr^{705}) Antibody** (#9131, "Cell Signaling Technology", USA). Phospho-Stat3 (Tyr705) Antibody detects endogenous levels of Stat3 only when phosphorylated at Tyr705. The antibody does not cross-react with other Stat proteins when phosphorylated on the corresponding tyrosine residue, but has been shown to cross-react with Phospho-EGFR. Species Reactivity: Human, Mouse, Rat, Monkey. Species predicted to react based on 100% sequence homology: Chicken, Bovine, Dog. Polyclonal antibodies are produced by immunizing rabbits with a synthetic phosphopeptide corresponding to residues surrounding Tyr705 of mouse Stat3. Antibodies are purified by protein A and peptide affinity chromatography.

6. **Akt Antibody** (#9272, "Cell Signaling Technology", USA). Akt Antibody detects endogenous levels of total Akt1, Akt2 and Akt3 proteins. The antibody does not cross-react with related kinases. Species Reactivity: Human, Mouse, Rat, Hamster, Monkey, Chicken, D. melanogaster, Bovine, Dog, Pig, GuineaPig. Species predicted to react based on 100% sequence homology: Dog. Polyclonal antibodies are produced by immunizing rabbits with a synthetic peptide corresponding to the carboxy-terminal sequence of mouse Akt. Antibodies are purified by protein A and peptide affinity chromatography.

7. **AMPK-α2 Antibody** (#NB100-238, “Novus Biologicals”, USA). Species Reactivity: Human, Mouse, Rat, Bovine. Species predicted to react based on 100% sequence identity: Orangutan, Rhesus Monkey, Gorilla, Chimpanzee, White-Tufted-ear marmoset, Crab-eating macaque, African elephant, Chinese hamster, Naked mole rat and Northern white-cheeked gibbon. The immunogen recognized by this antibody maps to a region between residues 350 and 400 of human 5’-AMP-activated Protein kinase, catalytic Alpha-2 chain using the numbering givenin entry AAH69823.1 (GeneID 5563). Polyclonal antibodies are produced by immunizing rabbits.

8. **Stat3 (124H6) Mouse mAb** (#9139, “Cell Signaling Technology”, USA). Stat3 (124H6) Mouse mAb detects endogenous levels of total Stat3 protein. Species Reactivity: Human, Mouse, Rat, Monkey. Monoclonal antibody is produced by immunizing mice with a synthetic peptide corresponding to the sequence of human Stat3.

9. **GAPDH Mouse mAb**(#NB600-502, “Novus Biologicals”, USA). Species Reactivity: Human, Mouse, Rat, Porcine, Amphibian, Canine, Feline, Fish, Monkey, Primate. Hybridoma clone has been derived from hybridization of Sp2/0 myeloma cells with spleen cells of Balb/c mice immunized with human or rabbit GAPDH.

10. **Anti-mouse IgG, HRP-linked Antibody** (#7076, “Cell Signaling Technology”, USA). Affinity purified horse anti-mouse IgG (heavy and light chain) antibody is conjugated to horseradish peroxidase (HRP) for chemiluminescent detection.

11. **Anti-rabbit IgG, HRP-linked Antibody** (#7074, “Cell Signaling Technology”, USA). Designed for use with rabbit polyclonal and monoclonal antibodies, this affinity purified goat anti-rabbit IgG (heavy and light chain) antibody is conjugated to horseradish peroxidase (HRP) for chemiluminescent detection.

Sample preparation:

1 Homogenize a piece of hypothalamus tissue (½ of hypothalamus) in the 1X Tissue Lysis Buffer in the ratio of 1:10.

2 Keep homogenate on ice for 30 min. **00:30:00**

3 Centrifuge the homogenate at 14 000g for 10 minutes (4 °C). **00:10:00**

4 Transfer the supernatant to a new tube and discard the pellet.

5 Remove 3x3 µl of supernatant to measure the protein concentration using modified method of Lowry. Mix an aliquot of lysate containing 300 µg of protein with the Lysis Buffer to adjust to a volume of 50 µl, and then add 50 µl of 2X SDS Sample Loading Buffer.

6 Warm the samples for 5 min at 95 °C. **00:05:00**

7 Cool at the room temperature for 5 min. **00:05:00**

8 Centrifuge the samples for 5 minutes using the Microfuge 22R centrifuge (Beckman Coulter, USA). **00:05:00**

9 Load up to 10 µl of sample to each well of a 1.5 mm thick gel. Guidelines for choosing the gel percentages are based on the protein size to be detected: 8.5% gel for the analysis of 80-200 kDa proteins, and 9-14% gel for the analysis of 20-65 kDa proteins.

10 Set gel running conditions at 75 V at the beginning of electrophoresis procedure, gradually increasing it to 95 V, using SDS Running Buffer.

11 Transfer the proteins to a nitrocellulose membrane at 100 V for 1 hour, using Transfer Buffer. 01:00:00

Membrane blocking:

12 Disassemble the transfer sandwich, remove the blotted membrane, wash the membrane 3-4 times with distilled water and immediately place in the Blocking Buffer consisting of 5% non-fat dry milk/TBST.

13 Incubate the blot for 30 min-1 hour at the room temperature with the agitation.
00:30:00

Antibody Incubation:

14 The primary antibody is diluted according to the manufacturer's recommendations. In the most cases, the antibodies are diluted in the 5% BSA/TBS-T in the ratio of 1:1000.

15 Place the membrane in the primary antibody solution and incubate overnight at 4°C with the agitation.

16 Wash for 5 min with the TBS containing 0.1% Tween-20 (wash 1/3). 00:05:00

17 Wash for 5 min with the TBS containing 0.1% Tween-20 (wash 2/3). 00:05:00

18 Wash for 5 min with the TBS containing 0.1% Tween-20 (wash 3/3). 00:05:00

19 Incubate the membrane for 1 hour at the room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies diluted to 1:1000–1:5000 in 5% non-fat dry milk/TBS-T. 01:00:00

20 Wash for 5 min with the TBS containing 0.1% Tween-20 (1/3). 00:05:00

21 Wash for 5 min with the TBS containing 0.1% Tween-20 (2/3). 00:05:00

22 Wash for 5 min with the TBS containing 0.1% Tween-20 (3/3). 00:05:00

23 Wash for 5 min with the TBS without Tween-20 (1/2). 00:05:00

24 Wash for 5 min with the TBS without Tween-20 (1/2). 00:05:00

Antibody Detection:

Antibody Detection:

25 Incubate the membrane (protein side up) with 10 ml of ECL (enhanced chemiluminescence substrate) for 1 min. The final volume required is 0.125 mL/cm^2. **00:01:00**

26 Drain off the excess of detection reagent, wrap up the blots, and gently smooth out any air bubbles.

27 Place the wrapped blots, protein side up, in an X-ray film cassette and expose to X-ray film. Exposures can vary from 5 seconds to 60 minutes.