[17] Glutamate-Induced c-Src Activation in Neuronal Cells

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Introduction

Glutamate toxicity is a major contributor to pathological cell death within the nervous system and appears to be mediated by reactive oxygen species.[1] Two pathways of glutamate toxicity have been defined: receptor-initiated excitotoxicity[2] and nonreceptor-mediated ROS-dependent toxicity.[3] One model used to study oxidative stress-related neuronal death is to inhibit cystine uptake by exposing cells to high levels of glutamate.[4] The induction of oxidative stress by glutamate in this model is a primary cytotoxic mechanism in C6 glial cells,[5,6] PC-12 neuronal cells,[7,8] immature cortical neurons cells,[4] and oligodendoglia cells.[9] Murine HT hippocampal neuronal cells, lacking an intrinsic excitotoxicity pathway, have been used as a model to characterize the oxidant-dependent component of glutamate.[10–12]

In neurons and astrocytes, c-Src is present at 15–20 times higher levels than that found in fibroblasts. The specific activity of the c-Src protein from neuronal cultures is 6–12 times higher than that from astrocyte cultures, suggesting a key function of this protein in neurons.[13] Src family kinases are able to induce caspase-independent cytoplasmic events leading to cell death.[14] We have reported that activation of c-Src kinase is a key event in glutamate-induced death of HT4 neurons.[10] A subsequent study showed that Src kinase-dependent neuronal damage plays a key role in stroke

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disorder.[15] Thus, Src represents a key intermediate and novel therapeutic target in the pathophysiology of neurodegenerative disorders.

Src family proteins are regulated through reversible phosphorylation and dephosphorylation events that alter the conformation of the kinase. A tyrosine kinase termed C-terminal Src kinase (Csk), expressed ubiquitously but predominantly in lymphoid tissues and neonatal brain, has been implicated to be the upstream regulatory tyrosine kinase by virtue of its ability to inactivate several Src family kinases.[16–20] Csk phosphorylates Tyr-527 in the C-terminal tail of c-Src and thus creates a binding site for the Src homology 2 (SH2) domain, locking the molecule in an inactive state. Dephosphorylation of Src Tyr-527 increases Src kinase activity up to 10- to 20-fold. The goal of this chapter is to present a detailed description of the methods that may be used to determine glutamate-induced c-Src activation in neuronal cells.

I. Determination of Glutamate-Induced Global Protein Tyrosine Phosphorylation Profile Using L-[^{35}S]Methionine Labeling of Proteins

Reagents

Lysis buffer: phosphate-buffered saline (PBS), pH 7.4, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (v/v) sodium dodecyl sulfate, 0.25 m*M* sodium orthovanadate (Na_3VO_4), 10 m*M* phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, and 10 μg/ml pepstatin

Wash buffer: phosphate buffer saline, pH 7.4, 1 *M* sodium chloride

Antibody: monoclonal protein phosphotyrosine antibody (PY99; Santa Cruz Biotech, Santa Cruz, CA)

Agarose beads: Protein A-agarose (Santa Cruz Biotech)

Other reagents and supplies: L-[^{35}S]methionine (NEN, Boston, MA); sodium orthovanadate (Sigma, St. Louis, MO); PBS, pH 7.4; cell lifter (Costar, Corning, NY); microfuge tubes; 2× Laemmli sample buffer

Procedure

Mouse hippocampal HT4 cells, kindly provided by D. E. Koshland, Jr., University of California at Berkeley, are grown in Dulbecco's modified Eagle's medium

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supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37° in a humidified atmosphere containing 95% air and 5% CO_2.

HT4 cells (0.7×10^6) are seeded in a 100 × 20-mm plate using 10 ml growth medium.

1. Following 6 hr of seeding of cells, culture medium is changed and 5 ml fresh medium is added to the plate.
2. To radiolabel the cells, 36 μCi/ml L-[^{35}S]methionine (NEN) is added to cells. Cells are incubated at 37° in a humidified incubator containing 95% air and 5% CO_2 for 12 hr.
3. After 12 hr of such incubation, cells are challenged with 10 m*M* glutamate for 30 min under standard culture conditions. To inhibit protein tyrosine phosphatase activity, cells are treated with 0.25 m*M* sodium orthovanadate (Sigma) for 15 min prior to any treatment.
4. After 30 min (or any other desired duration) incubation of cells with glutamate, cells are washed twice with 10 ml of ice-cold PBS, pH 7.4.
5. Lysis buffer (1 ml) is added to cells and cells are kept on ice for 15 min for lysis.
6. After 15 min of lysis, cells are scraped using a cell lifter and collected in 2-ml microfuge tubes.
7. Cell lysates are centrifuged at 12,000*g* for 10 min at 4° and supernatants are used for immunoprecipitation.
8. Tyrosine-phosphorylated proteins are immunoprecipitated by adding 2 μg of monoclonal protein phosphotyrosine antibody to the cell extract contained in microfuge tubes.
9. Tubes are put on a rotating shaker at 4° for 4 hr followed by another 12 hr with 40 μl [25% (v/v) stock] protein A-agarose.
10. After 12 hr of incubation with antibody and agarose beads, the samples are washed once with lysis buffer and thrice with wash buffer.
11. The immunoprecipitated material is resuspended in 40 μl of 2× sample buffer and boiled for 10 min. After centrifugation (14,000*g*, 3 min), immunoprecipitated proteins are separated on a 10% SDS–PAGE gel, and the protein tyrosine phosphorylation profile is detected by autoradiography (Fig. 1).

Notes

1. To obtain reproducible data, it is important that attention be paid to having the same cell density in repeated experiments.
2. Treatment with 0.25 m*M* sodium orthovanadate for 15 min before challenging the cells to inhibit protein tyrosine phosphatase activity is critical. It is difficult to find phosporylated proteins without inhibiting phosphatase activity.
3. After extracting the cell, the whole procedure should be performed at 4°.
4. This radioisotope-dependent method suffers from the limitation that it is a

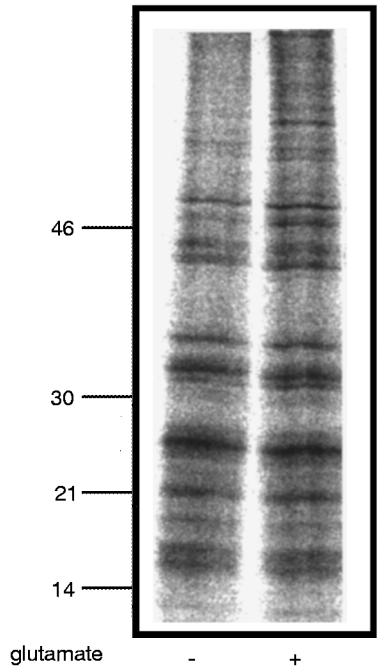

FIG. 1. Protein tyrosine phosphorylation profile in HT4 cells as determined by the method described in Section I. Glutamate treatment resulted in tyrosine phosphorylation of specific proteins.

"hot" procedure. The advantage is having sharp bands that are often difficult to get using standard Western blot detection.

II. Determination of Src Kinase Activity

Reagents

Lysis buffer: 20 m*M* HEPES–NaOH, pH 7.5; 3 m*M* $MgCl_2$, 100 m*M* NaCl, 1 m*M* dithiothreitol, 1 m*M* PMSF, 1 μg/ml leupeptin, 1 m*M* EGTA, 1 m*M* sodium orthovanadate, 10 m*M* NaF, 20 m*M* glycerophosphate, and 0.5% Nonidet P-40

Antibody: A-agarose-conjugated anti-Src family kinase antibody (Santa Cruz Biotechnology)

Reaction buffer: 40 m*M* HEPES–NaOH, pH 7.5, 10 m*M* $MgCl_2$, 3 m*M* $MnCl_2$, 0.5 m*M* dithiothreitol, 0.1 m*M* PMSF, 0.1 μg/ml leupeptin, 0.1 m*M* sodium orthovanadate, 1 m*M* NaF, and 2 m*M* glycerophosphate

Kinase activity buffer: acid-denatured enolase (Boehringer Mannheim, Germany), 10 μ*M* ATP, and 10 μCi of [γ-^{32}P]ATP (NEN)

1. Enolase processing. Weigh enolase (5 μg/sample), add ice cold 10% (500 μl) trichloroacetic acid (TCA), keep on ice for 5 minutes. Add 500 μl reaction buffer and spin at maximum speed for 5 min. Repeat this step two more times. If the assay is to be performed on the same day, keep the pellet on ice. Otherwise it may be stored at $-70°$ for weeks.

2. Preparation of cell extracts. To determine Src kinase activity, 1.6×10^6 HT4 cells are seeded in 140 $\times$ 20-mm plates containing 20 ml growth media. Following 12 hr of seeding, cells are activated with glutamate for 30 min as described in Section I. Cells are harvested and lysed in 1 ml of lysis buffer. Cell lysates are centrifuged at 12,000*g* for 10 min at 4°. The protein content of the sample is determined using the Pierce BCA protein assay kit (Rockford, IL).

Other reagents and supplies: cell lifter (Costar, Corning, NY) and microfuge tubes.

Kinase Assay

1. The cell extract (750 μg protein) is placed in a 1.5-ml microfuge tube.
2. The total volume of the cell extract is made to 1 ml using lysis buffer.
3. Src kinase is immunoprecipitated from the extract by adding 2 μg of protein A-agarose-conjugated anti-Src family kinase antibody.
4. The microfuge tubes are placed on a rotating shaker at 4° for 4 hr.
5. The immune complex is separated by centrifugation at 6000 rpm for 5 min in a refrigerated centrifuge.
6. The beads are washed twice with the lysis buffer (500 μl/wash) followed twice with reaction buffer (500 μl/wash).
7. Substrate mix is prepared by adding 20 μl of reaction buffer to the enolase pellet. Mix by vortexing and make sure that the pellet is dissolved.
8. Add 1 μl of 10 m*M* ATP (final 10 μM) and 10 μCi of [γ-^{32}P]ATP to each sample.
9. The kinase assay is performed for 10 min at 22° with 5 μg of acid-denatured enolase as substrate in 30 μl of the reaction buffer containing 10 μM ATP and 10 μCi of [γ-^{32}P]ATP per sample.
10. The kinase reaction is stopped by adding 10 μl of 4$\times$ Laemmli sample buffer. The mixture is boiled for 5 min and subjected to 10% SDS–PAGE.
11. The gel is dried and the radioactivity incorporated into enolase is determined using a phosphoimager (Molecular Dynamics, Sunnyvale, CA) as shown in Fig. 2.

Notes

1. Denatured enolase serves as a good substrate for this kinase assay. If the phosphorylation of any other specific physiological substrate is to be studied, enolase may be replaced.

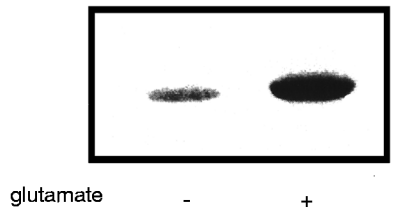

FIG. 2. Activity of Src kinase immunoprecipitated from HT4 cells. Glutamate treatment increased Src kinase activity.

III. Immunolocalization of Src and Phospho-Src

Reagents

Fixing solution: 3.7% formaldehyde in PBS pH 7.4, PBS-T: PBS containing 0.2% Triton X-100

Blocking buffer: PBS containing 0.2% (v/v) Triton X-100 and 1% (w/v) bovine serum albumin

Mounting media: GEL/MOUNT biomeda (Fisher Scientific)

Primary antibodies: anti-Src (c-Src B-12; Santa Cruz Biotech), anti-phospho-Src (Upstate Biotechnology, Lake Placid, NY)

Secondary antibodies: FITC-conjugated donkey anti-mouse IgG antibody and RhodamineRed-conjugated donkey anti-rabbit IgG antibody (Jackson Immunoresearch, West Grove, PA)

Microscope: Nikon E800, MetaMorph version 4.5 software (Universal Imaging Corp., West Chester, PA)

Other reagents and supplies: poly-L-lysine (Sigma); coverslips (Fisher Scientific); glass slides (Fisher Scientific); parafilm; and aluminum foil

Cell Culture

HT4 cells (0.1×10^6) are seeded on 22 × 22-mm (0.13 to 0.16 mm thick) autoclaved poly-L-lysine (0.01%)-coated coverslips at 37° in a humidified atmosphere containing 95% air and 5% CO_2 for 12 hr before challenging with glutamate.

Indirect Immunofluorescence Staining

1. Wash cells three times with PBS. Make sure that there is no cell culture medium left and check under microscope that cells are still attached to coverslips.
2. To fix cells, add 500 μl of fixing solution and keep cells at room temperature for 10 min.
3. Remove fixing solution and wash cells three time with PBS.

4. Add 1 ml of PBS-T for 20 min at room temperature to permeabilize cells.
5. Wash cells twice with 1 ml PBS-T and add 1 ml blocking buffer for 45 min at room temperature to block nonspecific antibody binding.
6. Wash cells with 1 ml PBS-T three times.
7. Take a 100 × 20-mm plate, place a piece of parafilm, and add 50 μl (1.5 μg/sample or 1 : 50 dilution) primary antibody for anti-src and anti-phospho-src.
8. Place the coverslip (cells face down) on the antibody solution placed on the parafilm.
9. Place some wet tissue paper by the side of the 100 × 20-mm plates to have some humidity.
10. Incubate the coverslip under humid conditions overnight at 4°.
11. The next day, place the plates containing the coverslips at room temperature for 45 min.
12. Gently flip the coverslip (cells face up) and wash three times with 1 ml PBS-T.
13. Incubate the coverslip with secondary antibody (FITC-conjugated donkey anti-mouse IgG antibody, 1 : 100 dilution; and RhodamineRed-conjugated donkey anti-rabbit IgG antibody, 1 : 100 dilution) for 45 min at room temperature in the dark.
14. Wash cells with 2 ml PBS three times.
15. Add one drop of mounting medium containing antifade reagent on the glass slide. Put coverslips (cells face down) carefully on mounting media.

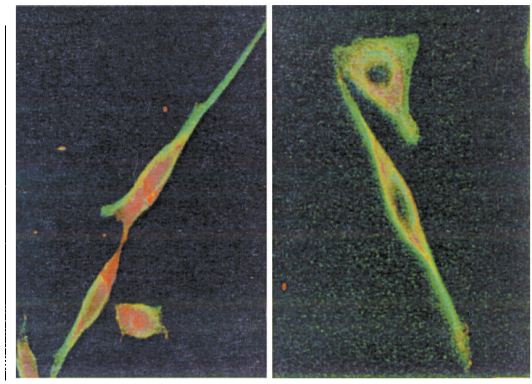

FIG. 3. HT4 cells immunostained with anti-Src or antiphospho-Src. Images are taken with 60× magnification (CFI Plan Apochoromat 60× oil, N.A. 1.40). Fluorescence analysis is performed using Metamorph Imaging software. FITC (representing Src) and rhodamine (representing phospho-Src) images are digitally overlayed. Less phospho-Src (Rhodamine; red) signal is visible in glutamate-treated cells (B versus A), suggesting glutamate-induced dephosphorylation (activation) of c-Src.

16. Keep for at least 30 min to dry mounting media before observing under a microscope.

Note

Avoid bubbles during antibody incubation and mounting of coverslips.

Microscopy

We recommend performing fluorescence microscopy using a Nikon E800 microscope with a 0.5 to 100× objective. Its differential interference contrast (DIC, Nomarski) optics allows excellent three-dimensional imaging of cells. Imaging is performed using a Photometrics Sen Sys CCD digital camera and MetaMorph 4.5 software. Simultaneous images of FITC/rhodamine fluorescence via epifluorescence and cell morphology via DIC are obtained.

Notes

1. A mercury lamp allows high magnification imaging with DIC and Planapochoromatic objectives provide the best correction for all aberrations.
2. Sensitivity and resolution with a Photometrics Syn CCD digital camera are exceptional, especially for fluorescence imaging.
3. The MetaMorph software allows image processing, contrast enhancement, color overlays, and intensity measurement. Dual color and overlay images are shown in Fig. 3.

[18] Measurement of Inflammatory Properties of Fatty Acids in Human Endothelial Cells

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Introduction

Fatty acids can modulate inflammatory responses in numerous tissues, including the vascular endothelium. At least two different independent pathways can be responsible for these effects. These pathways are linked to either (1) eicosanoid production or (2) redox-regulated gene expression. Traditionally, lipid-mediated cellular inflammatory reactions have been linked to the release of arachidonic acid from the cellular membranes, activation of cyclooxygenases, and lipoxygenases

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