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Original Contribution

Molecular control of the amount, subcellular location, and activity state of translation elongation factor 2 in neurons experiencing stress

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ABSTRACT

Eukaryotic elongation factor 2 (eEF-2) is an important regulator of the protein translation machinery whereby it controls the movement of the ribosome along the mRNA. The activity of eEF-2 is regulated by changes in cellular energy status and nutrient availability and by posttranslational modifications such as phosphorylation and mono-ADP-ribosylation. However, the mechanisms regulating protein translation under conditions of cellular stress in neurons are unknown. Here we show that when rat hippocampal neurons experience oxidative stress (lipid peroxidation induced by exposure to cumene hydroperoxide; CH), eEF-2 is hyperphosphorylated and ribosylated, resulting in reduced translational activity. The degradation of eEF-2 requires calpain proteolytic activity and is accompanied by accumulation of eEF-2 in the nuclear compartment. The subcellular localization of both native and phosphorylated forms of eEF-2 is influenced by CRM1 and 14.3.3, respectively. In hippocampal neurons p53 interacts with nonphosphorylated (active) eEF-2, but not with its phosphorylated form. The p53–eEF-2 complexes are present in cytoplasm and nucleus, and their abundance increases when neurons experience oxidative stress. The nuclear localization of active eEF-2 depends upon its interaction with p53, as cells lacking p53 contain less active eEF-2 in the nuclear compartment. Overexpression of eEF-2 in hippocampal neurons results in increased nuclear levels of eEF-2 and decreased cell death after exposure to CH. Our results reveal novel molecular mechanisms controlling the differential subcellular localization and activity state of eEF-2 that may influence the survival status of neurons during periods of elevated oxidative stress.
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Proteins determine the structural and functional phenotypes of cells by regulating intrinsic metabolic and homeostatic processes and the responses of cells to environmental signals. Rates of protein synthesis are influenced by a variety of factors, including nutrient availability, energy metabolism, growth factors, aging, and disease states [1–3]. When the organism and its cells are under energetic and oxidative stress, protein translation is limited to proteins critical for the survival and specific functions of the cells, including an array of adaptive stress response proteins [4]. Neurons are particularly vulnerable to oxidative stress and associated membrane lipid peroxidation, which can destabilize cellular calcium homeostasis and trigger apoptosis [5–7], a form of programmed cell death mediated, in part, by p53 [8]. Unmitigated lipid peroxidation contributes to the dysfunction and degeneration of neurons in both acute CNS injuries and neurodegenerative disorders including Alzheimer and Parkinson diseases [9].

Protein synthesis is a complex process that determines both qualitative and quantitative features of the proteome [1,10]. If a particular protein is no longer required, inhibition of the initiation step of translation occurs; however, specific control of the elongation phase to rapidly alter production of particular proteins occurs under conditions such as heat shock and stimulation by hormones and growth factors [11–16]. In addition, some diseases are caused by abnormalities in elongation factors [17].

Elongation factor-2 (eEF-2) is a fundamental regulatory protein of the translational elongation step [12] that catalyzes the movement of the ribosome along the mRNA. eEF-2 is regulated by several mechanisms including phosphorylation [12], mono-ADP-ribosylation [18,19], and protein–protein interactions [20,21]. A role for eEF-2 in cellular stress responses is highlighted by the fact that eEF-2 is sensitive to oxidative stress [22,23] and that it must be active, at least transiently, to drive the synthesis of proteins that help cells mitigate the adverse effects of oxidative

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stress or activate apoptosis if the extent of damage overwhelms the repair capacity.

Here we elucidate roles for eEF-2 in the cellular responses of neurons elicited by oxidative stress. We found that when exposed to low doses of cumene hydroperoxide (CH), a compound that induces membrane lipid peroxidation, eEF-2 undergoes calpain-mediated degradation, phosphorylation and ADP-ribosylation, and interaction with p53. The subcellular localization of eEF-2 is regulated by at least three proteins, 14-3-3, chromosome region maintenance 1 (CRM1), and p53. Interaction of eEF-2 with p53 in the nucleus may facilitate neuronal recovery from subapoptotic levels of oxidative stress.

Materials and methods

Cell cultures

Cultures of hippocampal neurons were prepared from embryonic day 18 rat brains, as described previously [24]. Dissociated neurons were plated at a density of 5×10^5 cells/cm^2 on dishes coated with polyethyleneimine. Neurons were grown in Neurobasal medium supplemented with B27 (Invitrogen, Carlsbad, CA, USA). All experimental treatments were performed on 7-day-old cultures. HCT116 human colon carcinoma cells, and p53-null derivatives thereof, were supplied by Dr. B. Vogelstein [25] and were grown in McCoy's medium with 10% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere. Hippocampal neurons were pretreated with or without 50 μM MDL28170 (Calbiochem, San Diego, CA, USA) for 45 min or 50 μM E-64d (Calbiochem) for 3 h, followed by exposure to 0, 10, or 15 μM CH (Sigma–Aldrich, St. Louis, MO, USA) for 3 h.

Cell viability

Cell viability was determined using an MTS assay (Promega, Madison, WI, USA) and a lactate dehydrogenase (LDH) activity assay (Roche cytotoxicity detection kit; Mannheim, Germany) according to the manufacturer's instructions.

Determination of hydroperoxides using the FOX reagent

The protocol for lipid peroxidation measurements [26] was adapted for a microplate reader. Forty micrograms of proteins was incubated with 90 μl of H_2SO_4 for 30 min. After addition of 100 μl of FOX reagent (0.5 mM ferrous ammonium sulfate, 0.2 mM xylenol orange, and 200 mM sorbitol in 25 mM H_2SO_4) the mixture was incubated at room temperature for 45 min, protected from light. The formation of ferric ions was detected by measuring the resulting colored complex with xylenol orange at 540 nm.

Immunoblot and immunoprecipitation analysis

Hippocampal neurons and cell lines were lysed in RIPA buffer (20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, and 1 mM sodium orthovanadate) containing protease inhibitors. The homogenized cells were centrifuged at 12,000*g* for 20 min at 4 °C. Protein content of the samples was estimated with a Pierce BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Protein samples were separated by SDS–PAGE (10% acrylamide) and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) at 120 V for 1 h. The membranes were incubated with blocking buffer (5% dry milk in 20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20) for 1 h at room temperature. Membranes were then incubated overnight at 4 °C in blocking solution containing the following antibodies: eEF-2 (1:5000), phospho-eEF-2 (1:1000) (Cell Signaling, Danvers, MA, USA); β-actin (1:5000; Sigma); p53 (1:500), pan-14.3.3 (1:1000), hnRNP (1:2000), and α-tubulin (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation, the membranes were washed in 20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20 and incubated with peroxidase-conjugated anti-immunoglobulin secondary antibodies. The proteins were visualized using a chemiluminescence kit from Pierce. The bands were analyzed by densitometry using the ImageJ analysis software (NIH).

Immunoprecipitation of proteins was performed on a rotator overnight at 4 °C using 800 μg of cell lysate and 4.0 μg of either anti-p53 or anti-eEF-2, or appropriate IgG as control, and the Catch and Release Reversible Immunoprecipitation system (Upstate Biotechnology, Billerica, MA, USA) according to the manufacturer's protocol. The proteins were eluted in 70 μl of elution buffer, and 20 μl was subjected to SDS–PAGE and immunoblot analysis.

ADP-ribosylation assay

The assay was performed as described previously [27]. Briefly, 50 μg of cell lysates was incubated in ADP-ribosylation buffer (20 mM Tris–HCl, 1 mM EDTA, 50 mM dithiothreitol (DTT); pH 7.4) with 500 ng of FP59 and 5 μM 6-biotin-17-NAD (Trevigen, Gaithersburg, MD, USA) for 30 min at 37 °C. Samples were separated by SDS–PAGE followed by immunoblotting. The biotin–ADP-ribose–eEF-2 complexes were detected using streptavidin–IR conjugated antibody (Rockland Immunochemicals, Gilbertsville, PA, USA) and a Typhoon 9400 scanner (GE Healthcare, Pittsburgh, PA, USA).

Subcellular fractionation

Hippocampal neurons were subfractionated as described previously [28]. Briefly, neurons were centrifuged at 20,000*g* for 20 s at 4 °C and resuspended in buffer A (10 mM Hepes, 2 mM $MgCl_2$, 15 mM KCl, 0.1 mM EDTA, 0.1% NP-40, 1 mM DTT; pH 7.6) containing protease inhibitors and incubated 7 min on ice. HCT116 human colon carcinoma cells were subfractionated as described previously [29]. Cells were incubated on ice for 10 min in 800 μl of lysis buffer containing 20 mM Tris (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 0.3% Igepal CA-630, and protease inhibitors. Cells were centrifuged at 1000*g* for 10 min to separate the cytoplasmic fraction of the cell extract. The nuclei were lysed by incubation on ice for 45 min with RIPA buffer containing protease inhibitors. The lysate was then centrifuged at 20,000*g* for 20 min. The resulting supernatant was used as the nuclear fraction of the cell extract. The subcellular fractions were separated by SDS–PAGE and analyzed by immunoblot using antibodies against cytoplasmic and nuclear marker proteins.

Nascent protein synthesis assay

HCT116 cells were plated on six-well plates, pretreated with CH, washed with warm phosphate-buffered saline (PBS), and supplemented with methionine-free Dulbecco's modified Eagle's medium (Invitrogen) for 35 min to deplete methionine reserves, after which 50 μM L-azidohomoalanine (AHA; Invitrogen) was added for 30 min. The cells were lysed and proteins were extracted by ultrasonication in RIPA buffer containing protease inhibitors. AHA-incorporating proteins were labeled with tetramethylrhodamine (TAMRA) using the Click-iT Protein Reaction Buffer Kit (Invitrogen). The TAMRA-labeled proteins in the gel were assayed using a Typhoon 9400 scanner (GE Healthcare).