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Stress-induced Inhibition of Protein Synthesis Initiation: Modulation of Initiation Factor 2 and Guanine Nucleotide Exchange Factor Activities following Transient Cerebral Ischemia in the Rat

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Neuronal protein synthesis is severely depressed following stress such as heat-shock, hypoxia, and hypoglycemia. Following reversible cerebral ischemia, protein synthesis is transiently inhibited in ischemia-resistant areas, but persistently depressed in vulnerable brain regions. Eukaryotic initiation factor 2 (eIF-2) activity, that is, the formation of the ternary complex eIF-2·GTP initiator ^{35}S-Met-tRNA, a rate-limiting step in the initiation of cellular protein synthesis, was studied in the rat brain during and following 15 min of transient global cerebral ischemia. At 30 min and 1 hr of reperfusion, a general decrease of eIF-2 activity by approximately 50% was seen in the postmitochondrial supernatant (PMS). In the relatively resistant neocortex and CA3 region of the hippocampus, the eIF-2 activity returns to control levels at 6 hr of reperfusion, but remains depressed in the vulnerable striatum and the CA1 region. Similarly, the activity of the guanine nucleotide exchange factor (GEF), which catalyzes the exchange of GTP for GDP bound to eIF-2, a crucial step for the continued formation of the ternary complex, is transiently reduced in neocortex but persistently depressed in striatum. The postischemic decrease in eIF-2 activity is further attenuated by agarose-bound alkaline phosphatase, and mixing experiments revealed that a vanadate-sensitive phosphatase may be responsible for the depression. Addition of partially purified GEF to PMS from postischemic neocortex restored eIF-2 activity to control levels. We conclude that ischemia alters the balance between phosphorylation and dephosphorylation reactions, leading to an inhibition of GEF and a depression of ternary complex formation. The persistent inhibition of GEF and ternary complex formation in areas vulnerable to ischemia may be due to factors causing cell death.

[Key words: ischemia, phosphatase, protein synthesis, initiation factor 2, guanine exchange factor, neuronal death, phosphorylation]

A wide range of conditions such as heat-shock, amino acid or glucose deprivation, chemical stress, and viral infection depress global cellular protein synthesis rate (Pain et al., 1980; Siekierka et al., 1985; Scorsone et al., 1987). The influence of stress, caused by transient cerebral ischemia, on protein synthesis has been intensively studied. During the reperfusion phase following a brief period of cerebral ischemia, polyribosomes disaggregate (Cooper et al., 1977; Petito and Pulsinelli, 1984a,b; Munekata et al., 1987; Deshpande et al., 1992) and protein synthesis, as measured by the incorporation of radioactive amino acids into brain proteins, is depressed (Dienel et al., 1980, 1985; Bodsch et al., 1985; Thilmann et al., 1987; Araki et al., 1990; Widmann et al., 1991). In areas vulnerable to ischemia, such as the CA1 region of the hippocampus, protein synthesis is persistently inhibited, while it is transiently depressed in the hippocampal CA3 region, dentate gyrus, and neocortex, areas relatively resistant to ischemia. The correlation between selective vulnerability to ischemia and the persistent depression of protein synthesis suggests a causal relationship between inhibition of protein synthesis and neuronal death (Bodsch et al., 1985). The mechanisms underlying the postischemic inhibition of protein synthesis are still virtually unknown. However, neuronal maturation, development, and survival are intimately associated with the presence of growth factors, which through the activation of their cell surface receptors affect among others the translation of mRNA via phosphorylation–dephosphorylation reactions (Morley and Thomas, 1991).

Cellular protein synthesis is commonly divided into an initiation, an elongation, and a termination step. It is generally recognized that the rate of polypeptide synthesis is regulated at the initiation step by the eukaryotic initiation factors (eIFs), in particular eIF-2 and eIF-4 (Nygård and Nilsson, 1990; Hershey, 1991; Morley and Thomas, 1991; Rhoads, 1991). Initiation factor 2 is believed to modulate the overall protein synthesis rate, while eIF-4 is thought to regulate the selection and translational efficiency of different mRNA species. Initiation of protein synthesis constitutes a series of intricate processes starting with the formation of a complex between GTP and eIF-2 that when binds the initiator Met-tRNA, Met-tRNAi, to form a ternary complex (eIF-2·GTP·Met-tRNAi) (Ochoa, 1983; Pain, 1986), often referred to as eIF-2 activity. The ternary complex associates with ribosomal subunits, additional eIFs, and the mRNA in a series of interactions leading to the formation of the mRNA·80S ribosomal complex. Concomitantly, GTP is hydrolyzed and eIF-2·GTP is released. The GDP bound to eIF-2 must be exchanged for GTP to regenerate eIF-2·GTP for another round of initiation. Since eIF-2 has higher affinity for GDP than for GTP, the exchange process is catalyzed by the guanine nucleotide exchange factor (GEF) (Proud, 1986). Recycling of eIF-2 is essential for a continued initiation of protein

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synthesis, and the exchange of GDP for GTP is a rate-limiting step. Two regulatory mechanisms for this process have been proposed. One involves the phosphorylation of the α-subunit of eIF-2, which in its phosphorylated state binds GEF in a stable complex with GDP (Proud, 1986; Scorsone et al., 1987; Clemens, 1989). The other mechanism involves the modulation of GEF activity by phosphorylation–dephosphorylation reactions (Dholakia and Wahba, 1988; Tuazon and Traugh, 1991).

The objective this study was to investigate whether severe stress such as cerebral ischemia alters the activity of brain eIF-2 and GEF, and whether particular protein kinases or phosphatases are involved in these processes.

Materials and Methods

The chemicals were obtained from the following sources: L-^{35}S-methionine and ^{125}I-anti-mouse IgG from Amersham (Amersham, UK); tRNA of rabbit liver type XII, aminoacyl-tRNA synthetase from *Escherichia coli,* phosphoenolpyruvate (PEP), pyruvate kinase (PK), ATP, GTP, benzoylated naphtoylated DEAE (BND)-cellulose, leupeptin, and 5[*N*-morpholino]propanesulfonic acid (MOPS) from Sigma (St. Louis, MO); nitrocellulose (NC) filter (0.2 μm, type HA) from Millipore. All other chemicals were of analytical or reagent grade. The monoclonal antibody against eIF-2α was kindly provided by Professor E. C. Henshaw (Rochester University, Rochester, NY).

Induction of ischemia. The two-vessel occlusion model of global cerebral ischemia according to the method of Smith et al. (1984), as modified by Gustafson et al. (1989), was used. The experiments were approved by the ethical committee at Lund University. Male Wistar rats (350–400 gm) (Møllegaard A/S, Copenhagen, Denmark) were used. Before surgery the animals were fasted overnight with free access to tap water. Anesthesia was induced by placing the rat in a jar with 3% isoflurane in a mixture of oxygen/nitrous oxide (30/70%). Following intubation with a plastic tube, they were artificially ventilated by a rodent respirator (7025 Rodent Ventilator, Ugo Basile Biological Research Apparatus, Comeno, Italy). Anesthesia was maintained with 1–2% isoflurane in the oxygen/nitrous oxide (30/70%) gas mixture. An external jugular vein catheter was then inserted, and positioned in the superior caval vein. A tail artery catheter and vein catheter were also inserted to allow blood sampling, arterial blood pressure recording, and infusions of drugs. The arterial blood pressure was measured and recorded continuously until the rats could be extubated. In all animals both common carotid arteries were exposed and encircled by loose ligatures. Arterial blood samples (200 μl) were collected 15 min before ischemia and 15 min postischemia to measure blood gases and blood glucose. If blood gases could not be corrected (P_aO_2 > 90 mm Hg, P_aCO_2 35–45 mm Hg, pH 7.35–7.45) by adjustments of the rodent ventilator, or if the blood glucose was <4 mmol/liter, the rats were excluded from the study. At the end of the surgery bipolar EEG electrodes were inserted into the temporal muscles and the EEG activity was recorded (Mingograf 34, Elema-Schönander, Stockholm, Sweden) every 5–10 min before ischemia, continuously during the ischemic insult, and intermittently in the postischemic period. These electrodes were removed once postischemic EEG activity recovered. At the beginning of a 30 min steady-state period prior to induction of ischemia, the inspired isoflurane concentration was decreased to 0.5% and 150 IU/kg heparin was administered intravenously. Vecuronium (Organon Teknika, Boxtel, Holland), a muscle relaxant, was given as a bolus dose of 0.7 mg followed by an intravenous infusion of 3 mg/hr. Blood was withdrawn via the jugular catheter to a mean arterial blood pressure (MABP) of 50 mm Hg and both carotid arteries were clamped. Blood pressure was maintained at 50 mm Hg during the ischemic period by withdrawing or infusing blood through the jugular catheter. The beginning of the ischemic insult was defined as the time of onset of isoelectric EEG at an MABP of 50 mm Hg. At the end of ischemia the clamps were removed and the blood reinfused through the jugular catheter, followed by 0.5 ml of 0.6 M sodium bicarbonate. In all experiments, temperature was monitored before, during, and following ischemia (15 min of reperfusion), using thermistors placed in the rectum and subcutaneously under the scalp. Temperature control was accomplished with the aid of a heating pad and kept at approximately 37°C. Animals to be killed immediately at the end of ischemia or following 1 hr reperfusion were left on isoflurane anesthesia and the brain was frozen *in situ* with liquid nitrogen. Both vecuronium and isoflurane, administered to the animals that were allowed to recover for 6 hr postischemia, were discontinued at the end of ischemia. Following the removal of the external jugular vein catheter, all wounds were sutured. Within 45 min after the ischemic insult the animals resumed adequate spontaneous breathing and the endotracheal tube could be removed. The animals were transferred to a cage where they received supplementary oxygen. Animals killed at 6 hr postischemia were reanesthetized on isoflurane, tracheostomized, and artificially ventilated on isoflurane. The brains were then frozen *in situ.*

The brains were dissected out at −17°C. Neocortex (parietal cortex) was sampled rostral to 3.5 mm from bregma. Striatum was sampled from both hemispheres. The CA1 region and CA3 region plus dentate gyrus of the hippocampus were sampled at the dorsal aspect of the hippocampus.

Subcellular fractionation. Ten to twenty milligrams of frozen tissue were sonicated for 3 × 10 sec bursts at an output setting of 30 (Ultrasonic Homogenizer, Cole-Parmer Instrument), in a homogenization buffer containing 50 mM MOPS (pH 7.4), 0.5 mM magnesium acetate, 100 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 50 mM NaF, and 0.1 mg/ml leupeptin. In the mixing experiments or the experiments employing alkaline phosphatase treatment, NaF was omitted the homogenization buffer. Following homogenization a postmitochondrial supernatant (PMS) was obtained by centrifugation in a Sorvall SE12 rotor, 15,000 rpm for 15 min at 4°C. The fractions were stored at −70°C in aliquots or used immediately. The samples were allowed to thaw only once, since repeated freezing–thawing cycles inactivate eIF-2. The protein concentration was determined by the method of Lowry et al. (1951).

Synthesis of ^{35}S-Met-tRNAi. Initiator tRNA, ^{35}S-Met-tRNAi, was synthesized by ligating rat liver tRNA with L-^{35}S-methionine (500 mCi/mmol) in presence of *E. coli* synthetase according to the methods of Smith and Henshaw (1975). The incubation buffer (buffer A) contained 100 mM Na-cacodylate, pH 7.0, 10 mM KCl, 2 mM DTT, 10 mM magnesium acetate, and 2.5 mM ATP. The reaction was initiated by addition 140 U of *E. coli* aminoacyl-tRNA synthetase (35 U/μl) to 1 ml of buffer A containing 2 mCi of L-^{35}S-methionine and 50 U of rat liver tRNA. Following 10 min of incubation at 37°C, 15 ml of ice-cold buffer B (10 mM acetate buffer, pH 4.5, 125 mM NaCl) was added to stop the reaction and the mixture was applied on a BND-cellulose column (1 × 2 cm) preequilibrated with buffer B. The column was washed thoroughly with buffer B (1 ml/min, 30–40 ml) until A_{260} was back to baseline and was then washed with 0.5 M NaCl in buffer B with a flow rate of 0.1 ml/min. Fractions (2 ml) were collected and 2 μl was taken for measurement of specific eIF-2 activity. Fractions with highest specific activity were pooled, precipitated with 2 vol of ice-cold ethanol (99%), and centrifuged (20,000 rpm; 80,000 × *g*) for 15 min at 2°C in a Beckman 28.1 rotor. The precipitate was washed with 70% and 90% ethanol, respectively, and finally dissolved in 2 mM DTT solution to a specific radioactivity of 15,000 cpm/μl. Aliquots of 100–200 μl were stored at −80°C until further use.

Measurement of ternary complex formation. The ternary complex formation in PMS was measured as the GTP-dependent retention of ^{35}S-Met-tRNA as a ternary complex, GTP·eIF-2·^{35}S-Met-tRNAi, on NC filters according to the methods of Wong et al. (1982) with some modifications. In the absence of GTP or a GTP-regenerating pyruvate kinase system in the assay, ternary complex formation is low. The ternary complex formation increases with increasing GTP concentrations, and potassium and magnesium ions are obligatory (Dwyer and Wasterlain, 1980). For our assay conditions we chose an incubation time of 10 min, and a reaction mixture (100 μl) that contained 50 mM MOPS, pH 7.4, 1 mM DTT, 100 mM KCl, 0.75 mM magnesium acetate, 75 μM GTP, 0.25 mM PEP, 6 U/ml of PK, 150 μg/ml of BSA, 5 μl of ^{35}S-Met-tRNAi (0.4 pmol), and 20 vol% (150 μg protein) of PMS fraction. The assay was linear up to 300 μg added PMS protein. Following incubation for 10 min at 30°C, the reaction was stopped by addition of 2.5 ml ice-cold washing buffer consisting of 20 mM MOPS, pH 7.4, 0.1 mM DTT, 100 mM KCl, and 3 mM magnesium acetate, and the solution was immediately passed through an NC filter under mild suction. The filter was washed four times with 2 ml of washing buffer, dried under a heating lamp, and placed in a scintillation vial, and 10 ml of Ready-Safe cocktail (Beckman) was added. Radioactivity was counted in a Beckman LS 2800 liquid scintillation counter.

Electrophoresis and immunoblotting. Electrophoresis was carried out on a 1.5 mm, 10% SDS-polyacrylamide gel according to the method of Laemmli (1970). The samples (50 μg protein) were mixed with a solution

of 0.3 M Tris-HCl, pH 6.8, 25% mercaptoethanol, 12% SDS, 25 mM EDTA, 20% glycerol, and 0.1% bromphenol blue (5× SDS), boiled 2 min, and subjected to gel electrophoresis at a constant current of 20 mA (stacking gel) and 30 mA (separating gel). Following electrophoresis, proteins on the gel were electrotransferred onto NC (Bio-Rad Transblot, 0.2 mm) using the method of Towbin et al. (1979) with a constant current of 200 mA overnight. After transfer the NC was washed once in PBS, washed (2 × 10 min) in PBS plus 0.1% Tween 20, and then preincubated with 3% BSA in PBS for 1 hr. It was incubated with a solution containing monoclonal antibody against eIF-2α in PBS with 3% BSA for 2 hr. Finally, the NC was incubated with 0.5 mCi/ml ^{125}I-anti-mouse IgG for 1 hr. All incubations were carried at room temperature. The NC was placed in a plastic bag and exposed onto Kodak X-Omat film for about 24 hr at −80°C in a cassette with Hi-plus intensifying screen. The protein bands on the film were identified according to the molecular weight or purified eIF-2α standard run on the same gel. The autoradiograms were scanned with a laser scanner, and the relative density of the eIF-2α bands on the film was calculated and expressed as percentage of control values. The densities were linear with the amount of eIF-2 present within the range measured.

Purification of eIF-2. Initiation factor 2 was purified from calf brain according to the methods of Cales et al. (1988) and Haro and Ochoa (1979) with some added modifications. Calf brain (500 gm) was obtained from a local slaughterhouse, where cortex was dissected out and frozen in liquid nitrogen. Prior to preparation the frozen tissue was crushed into smaller pieces and homogenized in a blender for 3 × 1.5 min with ice-cold homogenization buffer (3 ml/gm tissue). The homogenization buffer consisted of 20 mM Tris-HCl, pH 7.6, 150 mM KCl, 5 mM magnesium acetate, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM EDTA, and 0.125 mM sucrose. The homogenate was centrifuged in Sorvall GSA rotor at 10,000 rpm for 15 min, and the resulting supernatant was centrifuged in a Beckman 28 rotor, at 28,000 rpm for 4 hr. The pellets (microsomal fractions) were stored at −80°C or immediately used for next purification step. The microsomal fraction was suspended with 210 ml of buffer A (20 mM Tris-HCl, pH 7.6, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, 10% glycerol) and made up up to a final KCl concentration of 0.5 M by slow addition of 4 M KCl. Following gentle stirring for 1 hr, the suspension was centrifuged at 28,000 rpm overnight. The resulting supernatant was precipitated by addition of solid ammonium sulfate to 40% saturation (22.6 gm/100 ml), stirred for 10 min, and centrifuged. The clear supernatant was precipitated again by addition of solid ammonium sulfate (11.6 gm/100 ml) to 60% saturation. The precipitated protein was collected by centrifugation, dissolved in 15 ml buffer A, and dialyzed overnight against buffer A containing 0.15 M KCl overnight. The dialyzed fraction was stored at −80°C or processed in the next chromatographic step.

Chromatography on heparin-Sepharose. Fractions (30 ml) from 1 kg of calf brain cortex were pooled and loaded onto a heparin-Sepharose column (1.6 × 12 cm) preequilibrated with buffer A and 0.15 M KCl and washed thoroughly with the same buffer (30 ml/hr). The column was eluted with a gradient (0.15–0.5 M KCl) at a flow rate of 20 ml/hr. The 4 ml fractions were collected using LKB Retriver II and monitored by LKB Uvicord S II, and 10 μl was taken to determine ternary complex formation (see above). The active fractions (0.35–0.4 M KCl) were pooled, and dialyzed against buffer A containing 0.2 M KCl and 20% sucrose for 16 hr. This fraction contained both eIF-2 and GEF, and was used as the partially purified GEF source in our experiments (Jeffrey et al., 1990).

Chromatography on CM-Sephadex C-50. A CM-Sephadex C-50 column (1.2 × 5 cm) was equilibrated with buffer B (20 mM MOPS, pH 7.6, 1 mM DTT, 0.1 mM EDTA, 10% glycerol) containing 0.2 M KCl. The pooled eIF-2 fractions were applied and the column washed with equilibration buffer and eluted with a 0.2 M–0.5 M KCl gradient in buffer B. Two-milliliter fractions were collected, and ternary complex formation was determined in 5 μl. The active fractions were pooled, and if necessary, the sample was chromatographed on an additional CM-Sephadex C-50 column. Finally, the pooled fractions were concentrated on an Amicon Ultrafiltration cell with YM30 filter, and dialyzed against buffer B overnight. This fraction contained 80% pure eIF-2, and was used to form the eIF-2·^{3}H-GDP complex for GEF activity measurements.

Treatment with agarose-bound alkaline phosphatase. When alkaline phosphatase (AlP) was used to treat samples, the tissue was first homogenized in a buffer identical to that described above but with NaF omitted. Agarose-bound AlP (agAlP) was washed three times with homogenization buffer (adjusted to pH 8.1 with MOPS) and finally suspended in homogenization buffer containing zinc chloride (375 mM) or vandate (100 μM). The PMS was mixed and incubated with AlP for 10 min at 25°C and the agAlPase was spun down. The eIF-2 activity was determined in the supernatant. In the control experiments the agAlP was boiled for 5 min prior to mixing with PMS.

Measurement of GEF activity. The activity of GEF in PMS was measured by the dissociation of eIF-2·^{3}H-GDP in the presence of GDP and Mg^{2+} (Matts et al., 1988). The binary complex was prepared by incubating eIF-2 with ^{3}H-GDP in absence of Mg^{2+}. The reaction mixture contained 50 mM MOPS, pH 7.6, 1 mM DTT, 100 mM KCl, 1 mg/ml creatine phosphokinase (CPK; E.C.2.7.3.2., type 1 from rabbit muscle; Sigma), 80% pure eIF-2 (4 μg), and ninefold molar excess of ^{3}H-GDP. Following 20 min of incubation at 30°C, the mixture was cooled on ice and adjusted to 2 mM magnesium acetate with 0.5 M magnesium acetate to stabilize the complex. The eIF-2·^{3}H-GDP complex was stored on ice until use.

Samples (10 μg protein) were incubated with 5 pmol of eIF-2·^{3}H-GDP complex (in 10 μl) for 10 min at 30°C in 150 μl of assay buffer containing 50 mM MOPS, pH 7.6, 1 mM DTT, 1 mg/ml CPK, 1.0 mM magenisum acetate, 100 mM KCl, and 40 μM GDP. The reaction was terminated by addition of 2.5 ml of ice-cold wash buffer (50 mM MOPS, pH 7.6, 0.5 mM DTT, 3 mM magnesium acetate; 100 mM KCl) and immediately filtered through NC filter (Millipore, type HA, 0.45 μm). After washing with 3 × 2.5 ml wash buffer, the filters were dried under a heating lamp and the radioactivity determined by liquid scintillation counting.

Phosphorylation of eIF-2. Incorporation of ^{32}P into partially purified eIF-2 was conducted in a reaction mixture (35 μl) containing 50 mM MOPS, pH 7.4, 1 mM DTT, 25 mM NaF, 0.5 mg/ml creatine phosphokinase (CPK), 0.25 mM creatine phosphate (CP), 0.4 mM ATP containing 250 μCi/ml ^{32}P-ATP (3000 Ci/mmol; Amersham), 2 mM magnesium acetate, 0.1 mg/ml protein kinase inhibitor (from bovine heart; Sigma), 1.5 μg of eIF-2, and 10 μg of PMS protein (Pollard et al., 1989). The KCl concentration was 150 mM or 80 mM, and in some experiments 0.25 mM $CaCl_2$ or 0.25 mM EGTA was added to the reaction mixture. The reaction was performed at 30°C for 20 min and stopped by addition of 5× SDS buffer. After boiling in a water bath for 2 min, the sample was applied onto a 6–16% SDS-polyacrylamide gradient gel or 10% SDS-polyacrylamide gel and the electrophoresis carried out at 15 mA (stacking gel) and 25 mA (separating gel). Following electrophoresis, the gels were fixed in 10% acetic acid and 15% 2-propanol, stained with Coomassie bright blue, and dried in the LKB gel dryer. The dried gel was apposed to a Kodak-X-Omat film for about 24 hr in a cassette with Hi-plus intensifying screen. The eIF-2α bands were identified by the molecular weight and the distance of migration of partially purified eIF-2α.

Mixing experiments. Cortex PMS (150 μg) from control rats was mixed with cortex PMS obtained from rats killed 30 min postischemia (150 μg). The samples were preincubated for 10 min at 25°C in either the presence or absence of protein kinase or phosphatase inhibitors. Initiation factor 2 activity was then measured as described above. The ternary complex formation in the mixtures thus represents the sum of eIF-2 activity in the PMS from sham-control group and the 30 min reperfusion group.

Results

Ternary complex formation during and following ischemia

Ternary complex formation in hippocampus CA1 and CA3 plus dentate gyrus (DG), striatum, and neocortex is shown in Figure 1. At the end of 15 min of ischemia eIF-2 activity in CA1 and neocortex significantly increases by 20–30% compared to sham-operated animals. During reperfusion following 15 min ischemia, eIF-2 activity transiently decreases at 30 min and 1 hr postischemia. This decrease is seen in all brain regions studied and amounts to approximately 50% of the sham-control group. At 1 hr following 10 and 20 min of ischemia, a depression of eIF-2 activity of a similar magnitude is also seen in neocortex (data not shown). In neocortex and the CA3 region of the hippocampus, eIF-2 activity is not different from sham-controls at 6 hr of reperfusion but is still depressed by 20% in the striatum and by 30% in the CA1 region of the hippocampus.

To assess whether the changes in eIF-2 activity were due to

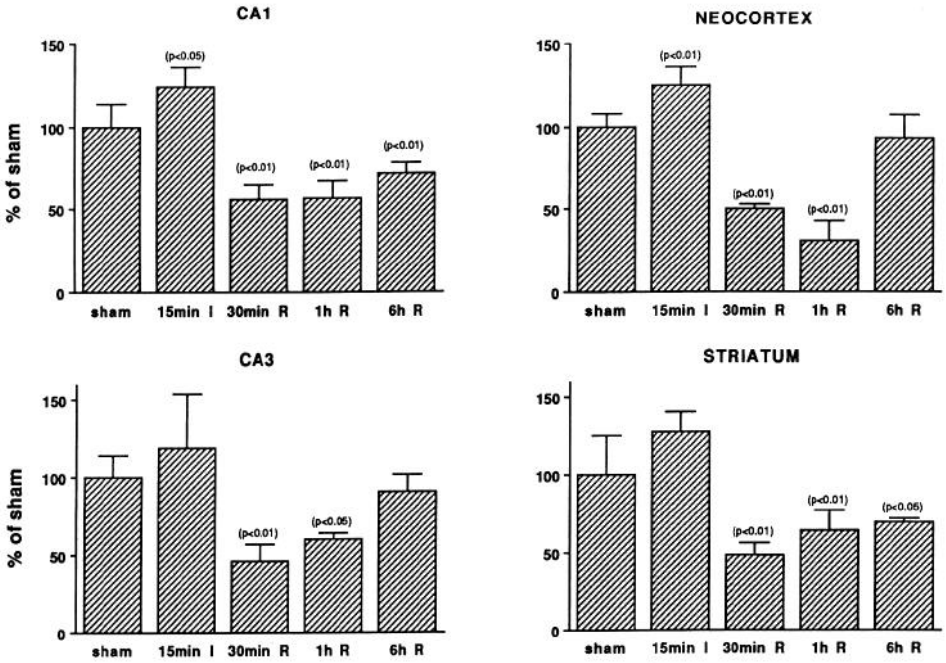

Figure 1. Initiation factor 2 activity measured as ternary complex formation in the PMS of CA1 and CA3+DG (*CA3*), neocortex, and striatum of sham-operated rats and rats exposed to 15 min ischemia, and 15 min ischemia followed by 30 min, 1 hr, and 6 hr of reperfusion (*R*). Data are expressed as percentage change from the mean value of the sham-operated group. Data are means ± SD with $n = 4$ in each group. The p values represent significant differences compared to the sham-operated group using Dunnett's test.

variations in the levels of eIF-2 protein, the eIF-2α levels were determined on Western blots (Fig. 2). There are no alterations in the immunoreactivity of the eIF-2α in neocortex or striatum during reperfusion.

Phosphorylation of eIF-2

To elucidate whether there were any changes in the activity of the enzyme(s) responsible for eIF-2 phosphorylation during reperfusion, the ^{32}P incorporation into partially purified eIF-2 was measured under conditions favoring eIF-2 kinase activity. Figure 3 shows the ^{32}P incorporation into the α- and β-subunits of eIF-2 following incubation of PMS from brains exposed to ischemia and different reperfusion times with ^{32}P-ATP. There is no difference in ^{32}P incorporation into the eIF-2α band between the sham-control group and the reperfusion groups. A significant (41%) increase in the phosphorylation of a 50 kDa band, most probably corresponding to the β-subunit of eIF-2 (Tuazon and Traugh, 1991), is seen during reperfusion (Fig. 4). The phosphorylation of eIF-2 is higher at 80 mM potassium concentration than at 150 mM, but is not influenced by calcium ions (0.25 mM) or EDTA (0.25 mM).

Treatment with alkaline phosphatase

agAlP dephosphorylates the phosphorylated form of eIF-2, eIF-2α(P) (Thomas et al., 1984). To demonstrate the ability of agAlP to dephosphorylate eIF-2α(P) in our assay, partially purified eIF-2 and agAlP, or boiled agAlP, were mixed and incubated with ^{32}P-ATP. The presence of agAlP decreases the ^{32}P incorporation into eIF-2α by 17 ± 7% ($n = 4$, $p < 0.05$, Student's t test) compared to the incorporation in the presence of boiled agAlP. Figure 5 shows the eIF-2 activity in the PMS from neocortex of the 30 min reperfusion group following agAlP treatment. A small but significant *decrease* in eIF-2 activity is seen, which is abolished when vanadate, a phosphatase inhibitor, is added to the incubation mixture.

Mixing experiments

When PMS from brains exposed to 15 min of ischemia followed by 30 min of reperfusion are mixed with PMS from sham operated rats, the eIF-2 activity in the mixture (MIX) is approximately 80% of the sham-control group (Fig. 6). The expected value should be the sum of the eIF-2 activity in the sham-control

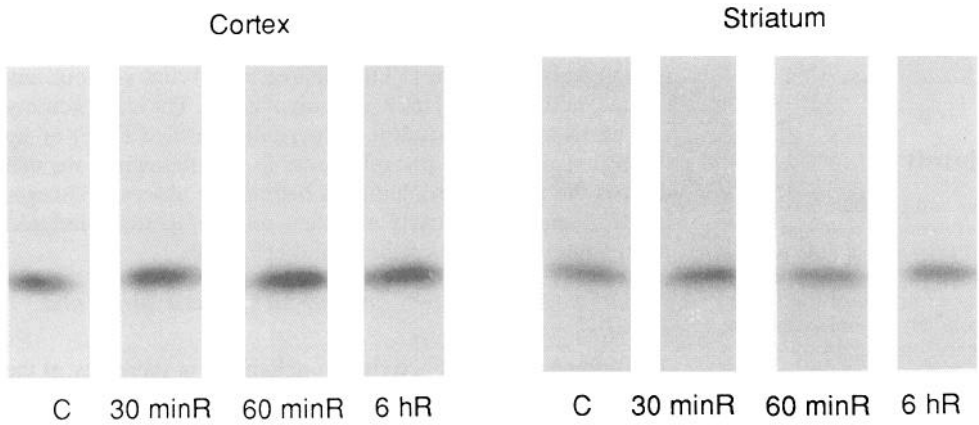

Figure 2. Changes in the levels of initiation factor 2α in neocortex and striatum of sham-operated rats (*C*) and rats exposed to 15 min of ischemia, and 15 min of ischemia followed by 30 min, 1 hr, and 6 hr of reperfusion (*R*). The levels are visualized as immunoreactivity on Western blots of PMS obtained from rat neocortex and striatum. The PMS (50 μg protein) was separated on a 10% SDS-polyacrylamide gel. Essentially similar results were obtained in four different series of samples.

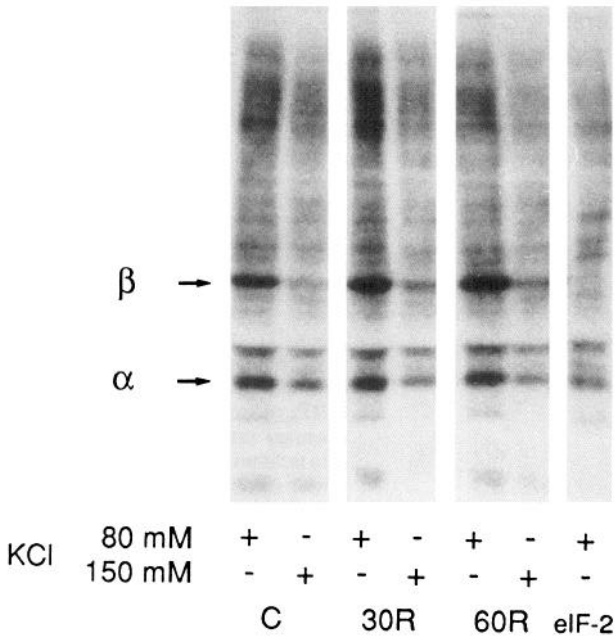

Figure 3. An autoradiogram of PMS from control rats (*C*) and rats exposed to 15 min of ischemia and followed by 30 min (*30R*) and 1 hr (*60R*) of reperfusion, incubated in the presence of ^{32}P-ATP and under conditions favoring eIF-2α kinase activity. The incubation mixture (10 μg of PMS protein) was separated on a gradient SDS-polyacrylamide gel (6.5–16%). In one slot (*eIF-2*) a mixture was loaded containing purified eIF-2 but no PMS. Incubations were performed at 80 mM and 150 mM KCl. The α- and β-subunit bands of eIF-2 are indicated.

group and the 30 min reperfusion group, that is, 155% of sham-controls. Vanadate (100 μM), a phosphatase inhibitor, added to the samples prior to mixing, increases eIF-2 activity by 40% compared to the sham-control group, a value close to the sum

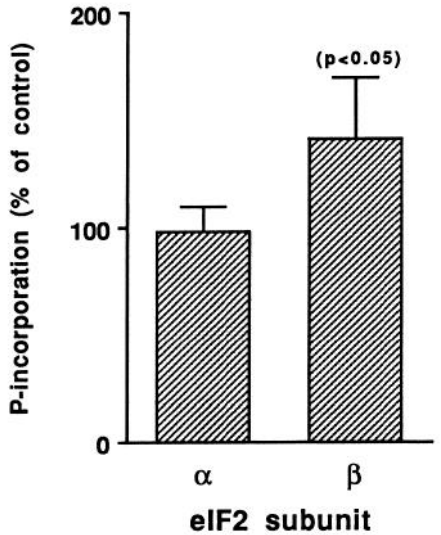

Figure 4. The ^{32}P incorporation into the α- and β-subunits of eIF-2 in brain PMS from rats exposed to 15 min of ischemia followed by 30 min of reperfusion, and separated on an SDS-polyacrylamide gel (see Fig. 3). The protein bands were excised and the radioactivity counted. Phosphate incorporation is expressed as the percentage of the ^{32}P incorporation into PMS of control (sham-operated) animals. Data are given as means ± SD with $n = 4$ in each experimental group. The p value represents significant differences compared to the sham-operated group using Student's t test.

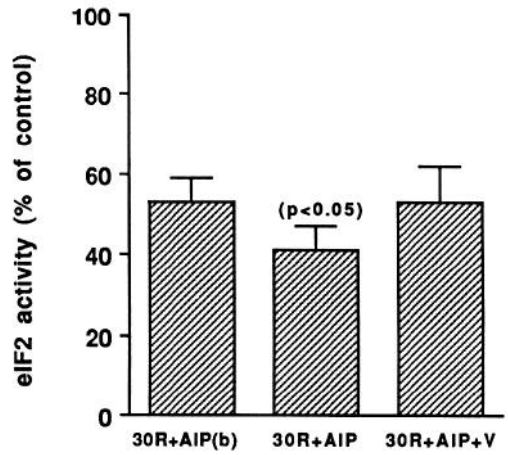

Figure 5. The effect of added AIP, active or boiled (*b*), in the absence and presence of vanadate (*V*), on the ternary complex formation in PMS from neocortex of rats exposed to 15 min of ischemia followed by 30 min of reperfusion (*R*). The data are expressed as percentage of the values from the corresponding sham-operated group. Zinc chloride (325 mM) was added as an activator of AIP. Data are means ± SD with $n = 4$ in each experimental group. The p value represents significant differences compared to the AIP(b) group using Dunnett's test.

of the activity in the sham-control group and the 30 min reperfusion group. Other phosphatase inhibitors such as okaidate (0.5 μM), zinc chloride (100 μM; Fig. 6), or molybdate (100 μM) and sodium fluoride (25 mM), or the protein kinase inhibitors staurosporine (62 nM) and H-7 (5 μM) do not affect the eIF-2 activity in the mixture (data not shown). Also, the different inhibitors do not influence the eIF-2 activity in the PMS form the sham-control group or the 30 min reperfusion group.

Changes in GEF activity following ischemia

Figure 7 shows the changes in GEF activity in neocortex and striatum PMS, measured as the exchange of ^{3}H-GDP bound to eIF-2 for free GDP. In striatum the dissociation rate is persistently decreased by 30% during reperfusion, while in neocortex there is a transient 50% decrease at 30 min and 1 hr of reperfusion that recovers by 6 hr postischemia. When GEF is added to cortical PMS (sham-control or 30 min reperfusion), the ternary complex formation increases (Fig. 8). At the highest GEF concentrations, the ternary complex formation is not significantly different between the sham-control and the 30 min reperfusion groups.

Discussion

This investigation shows that following transient cerebral ischemia, the eIF-2 and GEF activities are reversibly inhibited in brain regions resistant to ischemia, and persistently depressed in vulnerable structures. This depression occurs without any changes in the levels of the α-subunit of eIF-2. The eIF-2 activity can be restored by addition of partially purified GEF, or by inhibition of protein phosphatases. In the following, we will discuss the possible mechanisms behind the observed changes in eIF-2 and GEF activity and their relation to stress-induced depression of protein synthesis and cell death.

Initiation factor 2 activity and protein synthesis during and following ischemia

The finding that eIF-2 activity is unchanged, or increases, at the end of an ischemic period demonstrates that the capacity of the

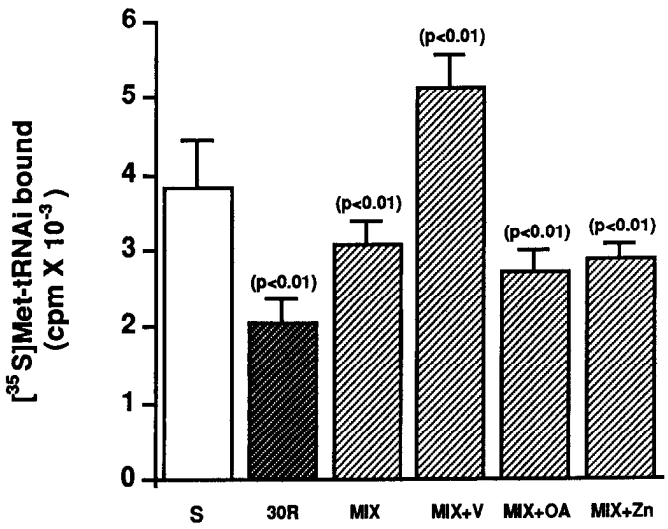

Figure 6. The ternary complex formation in cortical PMS obtained from sham-operated animals and animals exposed to 15 min of ischemia followed by 30 min of reperfusion (*30R*) and 1:1 mixtures (*MIX*) thereof. Homogenization buffer (*MIX*), 100 μM vanadate (*V*), 500 nM okaidate (*OA*), or 100 μM zinc chloride (*Zn*), respectively, was added to the mixtures, which were preincubated for 10 min at 25°C prior to the determination of ternary complex formation. Data are means ± SD with $n = 4$ in each experimental group. The p values represent significant differences compared to the sham-operated group using Dunnett's test.

tissue to form the ternary complex is not affected by ischemia per se. This is in agreement with the electron microscopic observations that the characteristic rosettes formed by polyribosomes are relatively intact during ischemia (Petito and Pulsinelli, 1984a,b; Munekata et al., 1987). Also, the polysome profile from ultracentrifugation studies, and *in vitro* ^{14}C-phenylalanine incorporation rate into proteins of PMS from ischemic brains are similar to that found in the control groups (Cooper et al., 1971). At reperfusion a rapid dissolution of polysomes into monosomes occurs, which is characteristic of an inhibited initiation of protein synthesis (Cooper et al., 1971; Petito and Pulsinelli, 1984a,b; Munekata et al., 1987; Widmann et al., 1991). Our observations, that eIF-2 activity decreases during reperfusion following ischemia, strongly suggest that the depression of protein synthesis early during reperfusion is due to the inhibition of eIF-2 activity. This also implies that the early postischemic inhibition of protein synthesis is a stress reaction similar to that found in tissue and cell cultures exposed to other stress condition, such as amino acid deprivation (Flaim et al., 1982; Everson et al., 1989), serum deprivation (Duncan and Hershey, 1985; Montine and Henshaw, 1989), heat-shock (Duncan and Hershey, 1984), and viral infection (Siekierka et al., 1985; Duncan, 1990). Later during reperfusion, at 6 hr postischemia, regional differences in the eIF-2 activity are evident. (We did not consider longer periods of reperfusion since the proliferation of glia cells may obscure changes occurring in neurons.) In neocortex and CA3+DG, where 15 min ischemia is not sufficient to cause neuronal damage, eIF-2 activity recovers to control levels, while in the vulnerable striatum and CA1 region, eIF-2 activity is still depressed. This suggests that factors inhibiting protein synthesis initiation at the eIF-2 step remain activated late into the reperfusion phase in cells particularly sensitive to ischemia, and may be part of the mechanism causing ischemic neuronal damage.

Regulation of eIF-2 activity during reperfusion

Evidently, processes that inhibit eIF-2 are activated early during the reperfusion phase. Alternatively, such processes are activated already during ischemia but require restoration of cellular functions such as ATP formation for inhibition. The inhibition could be due to a degradation of eIF-2 or a modulation of its activity through a regulatory protein or through covalent modifications of eIF-2.

The immunoblotting experiments demonstrate that the levels of eIF-2α in the postischemic brain do not change up to 6 hr postischemia. The decrease in eIF-2 activity immediately after ischemia is thus not due to degradation of eIF-2. Phosphorylation of the eIF-2 α-subunit is another possible cause of inhibition, since in reticulocytes and HeLa cells, eIF-2α is phosphorylated by eIF-2 kinases activated by deprivation of heme or by the presence of double-stranded RNA, respectively (Panniers and Henshaw, 1983; Rowlands et al., 1988; Sarre et al., 1989). The phosphorylated eIF-2α binds GEF with a 1000-fold higher affinity than the unphosphorylated form, and since eIF-2 is in excess of GEF, phosphorylation of eIF-2α by 30% is sufficient to trap essentially all cellular GEF (Panniers and Henshaw, 1983; Proud, 1986; Matts et al., 1988; Rowlands et al., 1988), which will inhibit initiation. An activation of an eIF-2α kinase immediately after ischemia could be envisioned. However, our data do not favor eIF-2α phosphorylation as a main cause of postischemic inhibition of ternary complex formation. First, treatment of postischemic PMS with agarose-bound alkaline phosphatase, which dephosphorylates eIF-2α(P), did not restore eIF-2 activity toward control levels, but in fact, slightly attenuated the activity. Second, there are no indications that protein kinases phosphorylating the α-subunit are activated

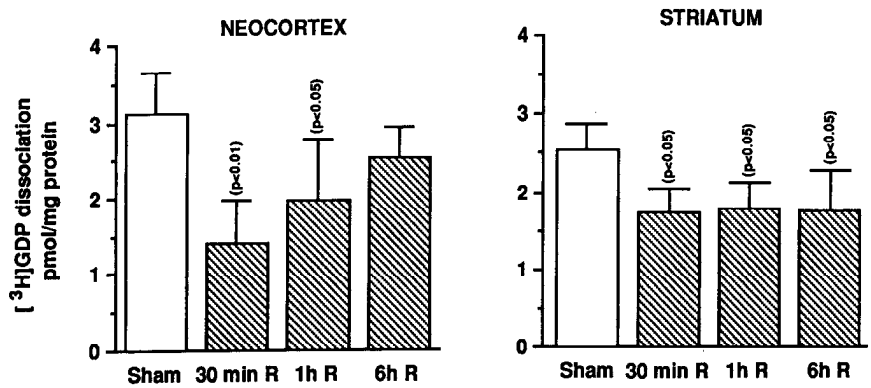

Figure 7. The GEF activity measured as the dissociation rate of preformed ^{3}H-GDP·eIF-2 in the presence of excess GDP, in PMS from neocortex and striatum of sham-operated rats and rats exposed to 15 min of ischemia followed by 30 min, 1 hr, and 6 hr of reperfusion (*R*). Data are means ± SD with $n = 4$ in each experimental group. The p values represent significant differences compared to the sham-operated group using Dunnett's test.

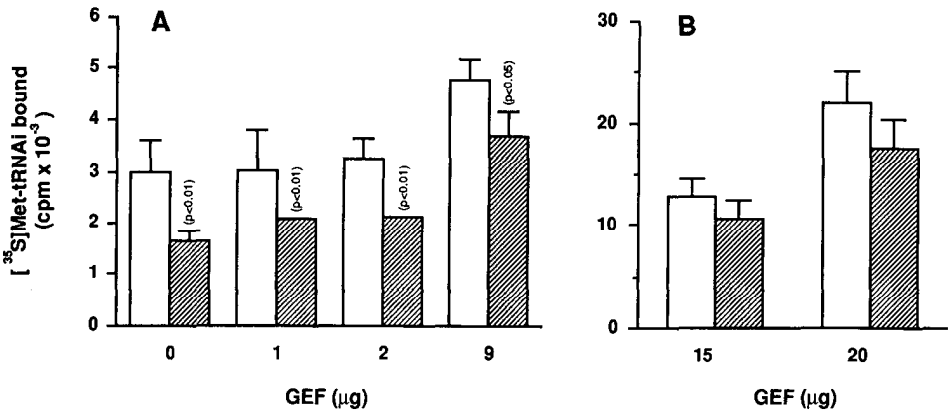

Figure 8. Effect of increasing amounts of partially purified GEF (μg protein) on ternary complex formation in cortical PMS from control rats (*open bars*) and rats exposed to 15 min of ischemia and 30 min of reperfusion (*hatched bars*). Data are means ± SD with n = 4 in each experimental group, with statistical comparison with corresponding control group using Student's t test.

postischemia (Fig. 3). Third, the mixing experiments showed that an inhibitor of eIF-2 is present in the postischemic PMS, able to depress eIF-2 activity in control PMS. The action of the inhibitor is blocked by vanadate, a phosphatase inhibitor, but not by protein kinase inhibitors, suggesting that a phosphatase is involved in the postischemic inhibition of eIF-2. This phosphatase is not inhibited by okaidate, fluoride, molybdate, or zinc ions, suggesting that it may be a protein tyrosine phosphatase (Lau et al., 1989; Shenolikar and Nairn, 1991).

Several investigations have also demonstrated that eIF-2 activity can be inhibited without any correlative changes in eIF-2α phosphorylation, indicating that other mechanisms, such as changes in the GEF activity, may regulate ternary complex formation (Jacobsen et al., 1983; Gross and Rubino, 1989; De Stefano et al., 1990; Huang and Schneider, 1990; Jefferey et al., 1990). The activity of GEF in the postischemic brain decreases in parallel with the decline in eIF-2 activity, indicating that GEF regulates the levels of eIF-2·GTP available for ternary complex formation. Also, the decrease in postischemic ternary complex formation could be reversed when partially purified GEF fraction is added to postischemic PMS. Therefore, our data suggest that modulation of the GEF activity may be responsible for the inhibition of postischemic ternary complex formation.

The mechanisms by which GEF activity is regulated are not fully understood. Purified GEF is phosphorylated by casein kinase II (CKII) (Tuazon and Traugh, 1991), which increases the GEF activity fivefold (Dholakia and Wahba, 1988). CKII is activated following stimulation of growth factor receptors (Sommercorn et al., 1987; Klarlund and Czech, 1988), which have an inherent tyrosine kinase activity, and may regulate protein synthesis through a kinase–phosphatase cascade (Morley and Thomas, 1991). The potential involvement of a tyrosine phosphatase in the postischemic inhibition of ternary complex formation implies that a tyrosine kinase may directly or indirectly regulate GEF. Consequently, the depressed postischemic GEF activity could be due to a defect in the activation of growth factor receptors or changes in the intracellular signaling process associated with growth factor receptors (Heidenreich and Toledo, 1989; Montine and Henshaw, 1989).

Phosphorylation of the β-subunit of eIF-2

The enhanced *in vitro* phosphorylation of the β-subunit of eIF-2 seen in the postsichemic PMS (Figs. 3, 4) needs to be commented upon. The change in the ^{32}P incorporation into eIF-2 is the net effect of the activities of the protein kinases and phosphatases. Several protein kinases phosphorylate the β-subunit: CKII, protein kinase C, and the ribosomal protein S6 kinase (Tuazon et al., 1989). Protein kinase C and CKII are less likely candidates since the assay conditions do not favor measurements of CKII or protein kinase C activity. The ribosomal protein S6 kinase may be responsible for the enhanced β-subunit phosphorylation. An interesting feature is that potassium ions inhibit the eIF-2β phosphorylation, which suggests that the kinase is activated (or a phosphatase is inhibited) by low potassium concentrations. So far, there is no experimental evidence indicating that the β-subunit affects eIF-2 activity, and the relevance of an increased phosphorylation of the β-subunit for the postischemic depression of protein synthesis is at present unclear. However, the phosphorylation could influence the binding of the 43S initiation complex to the AUG codon of the mRNA, thereby influencing the selection of mRNA to be transcribed (Donahue et al., 1988).

In conclusion, we propose that ischemia induces an imbalance in the postischemic phosphorylation–dephosphorylation reactions, resulting in a depression of GEF activity. This leads to a decreased exchange rate of GTP for GDP bound to eIF-2, inhibiting ternary complex formation and protein synthesis. The imbalance in protein phosphorylation may be caused by the activation of a vanadate-sensitive factor, presumably a phosphatase (tyrosine phosphatase). The inhibition of ternary complex formation is persistent in the ischemia-sensitive brain regions, and the processes causing the inhibition may also be involved in ischemic cell death.

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