Possible mechanisms involved in the down-regulation of translation during transient global ischaemia in the rat brain

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The striking correlation between neuronal vulnerability and down-regulation of translation suggests that this cellular process plays a critical part in the cascade of pathogenetic events leading to ischaemic cell death. There is compelling evidence supporting the idea that inhibition of translation is exerted at the polypeptide chain initiation step, and the present study explores the possible mechanism/s implicated. Incomplete forebrain ischaemia (30 min) was induced in rats by using the four-vessel occlusion model. Eukaryotic initiation factor (eIF)2, eIF4E and eIF4E-binding protein (4E-BP1) phosphorylation levels, eIF4F complex formation, as well as eIF2B and ribosomal protein S6 kinase ($p70^{S6K}$) activities, were determined in different subcellular fractions from the cortex and the hippocampus [the CA1-subfield and the remaining hippocampus (RH)], at several post-ischaemic times. Increased phosphorylation of the α subunit of eIF2 (eIF2α) and eIF2B inhibition paralleled the inhibition of translation in the hippocampus, but they normalized to control values, including the CA1-subfield, after 4–6 h of reperfusion. eIF4E and 4E-BP1 were significantly dephosphorylated during ischaemia and total eIF4E levels decreased during reperfusion both in the cortex and hippocampus, with values normalizing after 4 h of reperfusion only in the cortex. Conversely, $p70^{S6K}$ activity, which was inhibited in both regions during ischaemia, recovered to control values earlier in the hippocampus than in the cortex. eIF4F complex formation diminished both in the cortex and the hippocampus during ischaemia and reperfusion, and it was lower in the CA1-subfield than in the RH, roughly paralleling the observed decrease in eIF4E and eIF4G levels. Our findings are consistent with a potential role for eIF4E, 4E-BP1 and eIF4G in the down-regulation of translation during ischaemia. eIF2α, eIF2B, eIF4G and $p70^{S6K}$ are positively implicated in the translational inhibition induced at early reperfusion, whereas eIF4F complex formation is likely to contribute to the persistent inhibition of translation observed at longer reperfusion times.

Key words: cerebral ischaemia, initiation factors, ribosomal protein S6 kinase, protein phosphorylation, protein synthesis.

INTRODUCTION

The striking correlation between neuronal vulnerability and disregulation of protein synthesis suggests that this cellular process plays a critical role in the cascade of pathogenetic events leading to ischaemic cell death [1–3]. Protein synthesis is completely inhibited during ischaemia *in vivo* and the cerebral blood flow threshold for protein synthesis is even higher than the perfusion rate required to maintain ATP production, suggesting that factors other than energy supply are involved in the down-regulation of translation [4]. More recently, findings have shown ischaemia-induced alterations in the translational machinery components, which might, in turn, negatively influence translational recovery during reperfusion [5,6].

Upon reoxygenation after an incomplete forebrain ischaemia, inhibition of protein synthesis persists for many hours, recovery being dependent on the duration of the ischaemic period. Post-ischaemic recovery of translation is much slower than that of energy metabolism, and persistent inhibition of protein synthesis in ischaemia-vulnerable neuronal cells preceding neuronal death is observed [1–4,7,8]. Whether or not this impaired machinery is the cause or the consequence of any forms of ischaemic neuronal death remains unknown; however, there is no doubt that knowledge of the mechanism/s involved in the control of translation during incomplete forebrain ischaemia would provide clues to interventions aimed at preventing cell death.

Inhibition of protein synthesis during reperfusion is associated with polysome disaggregation, indicating translational control mainly at the rate-limiting initiation step [9,10]. The control of initiation is most commonly exerted on two processes. The first corresponds to ternary complex formation between eukaryotic initiation factor (eIF)2, GTP and Met-$tRNA_i$, which is prior to 43 S initiation complex formation. eIF2B is also required at this stage for guanine-nucleotide exchange on eIF2, in order to regenerate active eIF2–GTP; the phosphorylated α subunit of eIF2 [eIF2α(P)] acting as a competitive inhibitor of eIF2B activity [11]. Most situations that lead to eIF2α phosphorylation represent cellular stress [12–14]. Ischaemia is a severe form of stress and the proven increase in eIF2α phosphorylation during reperfusion, which triggers eIF2B inactivation and suppression of global protein synthesis, represents a well documented example [15–18].

Abbreviations used: DTT, dithiothreitol; eIF, eukaryotic initiation factor; eIF2α, α subunit of eIF2; eIF2α(P), phosphorylated α subunit of eIF2; 4E-BP1, eIF4E-binding protein; eIF4E(P), phosphorylated eIF4E; IEF, isoelectric focusing; m^7GTP, 7-methylguanosine triphosphate; PMS, post-mitochondrial supernatant; $p70^{S6K}$, ribosomal protein S6 kinase; RH, remaining hippocampus; SK, salt-washed supernatant; 5′-TOP, 5′-terminal oligopyrimidine tract.

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The second initiation process subjected to fine control is the recruitment of mRNA to a ribosome, a complex process, which facilities its transfer to the 43 S initiation complex, resulting in the 48 S initiation complex. This step requires the participation of eIF4-family initiation factors, eIF4F and eIF4B. eIF4F executes the main function of bridging the mRNA and the ribosome, and it is a complex of different polypeptides: eIF4E, a 24 kDa cap-binding protein controlled by physiological regulators, the 4E binding protein family; eIF4A, an RNA-dependent ATPase and RNA helicase; and eIF4G, a large polypeptide containing binding sites for eIF4E, eIF4A, eIF3 and polyadenylated tail-binding protein [11,19]. An additional control, at least for mRNAs containing a 5′-terminal oligopyrimidine tract (5′-TOP), which involves ribosomal protein S6 phosphorylation, has been characterized [20]. eIF4E dephosphorylation, as well as eIF4G cleavage, during ischaemia have been reported [5,6]. Additional possible regulatory mechanism/s that might contribute to post-ischaemic impairment of the initiation step have not been explored.

Together, these reported findings do not prove that phosphorylation of eIF2α is the only mechanism responsible for the down-regulation of translation during reperfusion. The aim of the present study was to explore the potential mechanisms of translational regulation during transient global cerebral ischaemia in the rat. In the present paper we extend previously carried out studies related to eIF2α, eIF2B and eIF4E that were achieved during ischaemia and/or at shorter reperfusion times exclusively in the cortex [5,15,16]. In addition, we report the first findings with ribosomal protein S6 kinase ($p70^{S6K}$), eIF4E-binding protein (4E-BP1) and eIF4F formation.

MATERIALS AND METHODS

Materials

Pure eIF2, for the eIF2B assay, was obtained from calf cortex as described previously [21]. The late Dr E. C. Henshaw and Dr A. Nieto kindly provided us with antibodies raised against eIF2α and eIF4GI respectively. A commercial monoclonal antibody raised against eIF4E was purchased from Affiniti Research Products Ltd (Mamhead, Exeter, Devon, U.K.). Polyclonal antibodies against 4E-BP1 and $p70^{s6}$ kinase, S6 peptide and Protein A/G PLUS Agarose were all obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Protease inhibitors were puchased from Sigma, and all the other chemicals were of reagent grade.

Animals, surgical procedures and experimental design

Incomplete forebrain ischaemia was induced in adult Wistar rats (mean body weight of 250 g) by the standard four-vessel occlusion model [22], as modified by Schmidt Kastner et al. [23]. Electroencephalograms and neurological investigations were performed to verify ischaemic severity, and the Ethics Committee at the Institute of Neurobiology approved the experiments. Briefly, on day one both vertebral arteries were irreversibly occluded by coagulation through the alar foramina after anaesthesia with ketamine and xylozine (100 mg and 15 mg/kg of body weight respectively; intraperitoneal injection). On day two both common carotid arteries were occluded under anaesthesia with 2.5% halothane in a mixture of O_2/N_2O (3:7); 2 min before the carotids were occluded for 30 min by means of small atraumatic clips the halothane was omitted from the mixture. A microthermistor placed deep in the ear monitored normothermic conditions (37 °C) that were maintained with a homeothermic blanket. Sham-control animals were prepared in the same way, but the carotids were not occluded. Animals were decapitated after 30 min of ischaemia, or after 30 min of ischaemia followed by 15–30 min or 2–6 h of reperfusion. The brains were rapidly removed and processed.

Extract preparations

Fresh neocortex and hippocampus obtained under the different experimental conditions were dissected and homogenized 1:2 (w/v) with buffer A [50 mM Hepes/KOH (pH 7.55), 140 mM potassium acetate, 4 mM magnesium acetate, 2.5 mM dithiothreitol (DTT), 0.32 M sucrose, 2 mM benzamidine, 1 mM EDTA, 2 mM EGTA, 10 μg/ml pepstatin A, 1 μg/ml leupeptin and 1 μg/ml antipain]. The postmitochondrial supernatant (PMS) was obtained by centrifugation at 11000 ***g*** for 10 min at 4 °C, and was kept at −70 °C until used. Salt-washed supernatant (SK) was obtained by tissue (frozen) homogenization (1:4; w/v) in buffer B [20 mM Tris/HCl (pH 7.6), 5 mM magnesium acetate, 50 mM KCl, 1 mM EDTA, 2 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 mM benzamidine, 100 mM NaF, 20 mM sodium β-glycerophosphate, 20 mM sodium molybdate and 0.2 M sucrose]. After centrifugation at 500 ***g*** for 10 min the SK was obtained as described elsewhere [15]. Whole lysates from hippocampus, dissected into a CA1-enriched fraction and the remaining hippocampus (RH; containing CA3 and dentate gyrus), were prepared by tissue homogenization in 50 mM Tris/HCl (pH 7.4), 50 mM KCl, 1 mM EDTA, 1 mM PMSF, 2 mM benzamidine, 2 mM EGTA, 50 mM NaF, 20 mM sodium β-glycerophosphate, 20 mM sodium molybdate and 1% (v/v) Nonidet P40. Protein determination was performed as described previously [24]. For hippocampal extracts two rats per sample were needed. Statistical analysis between sham-control and ischaemic animals was calculated by ANOVA followed by Dunnett's or Student's *t* tests.

Polysome profile determination

PMS fractions prepared from fresh hippocampus and thawed only once (an absorbance at 260 nm of 3 units) were loaded on to a 25–55% sucrose gradient containing 20 mM Tris/HCl (pH 7.6), 3 mM magnesium acetate and 100 mM KCl. Ultracentrifugation was performed using a Beckman rotor SW 60 at 40000 rev./min (164000 ***g***) for 90 min. The ultraviolet absorption of the gradient was automatically recorded at 254 nm with a density gradient fractionator coupled to an Uvicord detector (LKB Produkter AB, Bromma, Sweden). The profiles were highly reproducible and the relative abundance of heavy polysomes and the appearance of 60 S and 40 S subunits in a given profile were used for interpretations. All of the procedure was performed at 4 °C.

Determination of the state of phosphorylation of eIF2α, eIF4E and 4E-BP1

SK fractions (75 μg) from sham-control and ischaemic animals were resolved in horizontal isoelectric focusing (IEF) slab gels and analysed by protein immunoblotting as described previously [5,15]. Stained eIF2 and eIF4E bands were scanned and quantified using an image analyser equipped with a Diversity One software package (PDI, Huntington Station, NY, U.S.A.). The phosphorylation status of 4E-BP1 was determined in SK fractions (100 μg) from sham-control and ischaemic animals resolved by SDS/PAGE [15% (w/v) polyacrylamide] and analysed by protein immunoblotting performed with a commercial antibody against 4E-BP1 (1:1000 dilution). eIF4G levels were determined

in PMS fractions (50 μg) resolved in 7.5 % (w/v) polyacrylamide gels and analysed by protein immunoblotting with a mixture of polyclonal antibodies raised against the N- and C-terminal fragments of eIF4GI (1:2000 dilution). At least duplicate analyses from each animal were run.

p70^{S6K} assay

Kinase activity was measured by an immune complex kinase assay. Briefly, aliquots of SK (duplicates) adjusted to 30 μg of protein were diluted in sample buffer (40 μl) and incubated for 4 h at 4 °C with 8 μl of p70^{S6K} antibody and for 1 h with 12.5 μl of Protein A/G PLUS Agarose. The immune complex was collected by centrifugation and washed three times with kinase assay buffer [40 mM Tris/HCl (pH 7.0), 10 mM magnesium acetate and 1 mM DTT]. p70^{S6K} activity was assayed by incubating the immunoprecipitate at 30 °C in 50 μl of kinase assay buffer supplemented with 0.2 mM S6 peptide (RRRLSSLRA; where single-letter amino-acid notation has been used) and [γ-^{32}P]ATP (100 μmol/litre; 0.1 Ci/mmol). The reaction contents were spotted on to phosphocellulose filter paper (Whatman P-81) and washed three times with 75 mM phosphoric acid prior to counting in a scintillation counter. Previous experiments have shown that under these conditions, the incorporation of ^{32}P into the S6 peptide is linear and proportional to the amount of protein in the assay. The results are expressed as pmol of phosphate incorporated/mg of protein.

7-Methylguanosine triphosphate (m^{7}GTP)–Sepharose chromatography

eIF4E, identified by its ability to cross-link to the mRNA 5′-cap structure, can be subsequently purified by affinity chromatography on a cap-containing matrix [19]. For the isolation of eIF4E, PMS fractions from control and ischaemic rats (adjusted to 150 or 500 μg for eIF4G–eIF4E and 4E-BP1–eIF4E respectively) were incubated with 30 μl of m^{7}GTP–Sepharose (Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.) for 30 min at 4 °C. The beads were washed three times with buffer A containing 0.1 mM GTP. Proteins were eluted with SDS sample buffer and then subjected to electrophoresis in 7.5 % (w/v) polyacrylamide gels (eIF4G) or 15 % (w/v) polyacrylamide gels (4E-BP1). eIF4G and 4E-BP1 levels in the complex were detected by immunoblot analysis and quantified as described above for the individual factors.

RESULTS

Polysome profiles

Disaggregation of heavy polysomes (five or more) into lighter polysomes or ribosomal subunits, and depressed protein synthesis, indicate that deregulation of protein synthesis is being exerted at the level of initiation of polypeptide chains. Experiments performed with cortex extracts from ischaemic animals have shown an extremely high degradation of polysomes at short reperfusion times and slight recovery after 3 h of reperfusion [9,10]. We ran polysome profiles from hippocampus extracts at different reperfusion times as previously described [25]. As shown in Figure 1 there was a clear-cut decrease in the heavy polysomes and an increase in the lighter species (80 S and 60 S) in extracts reperfused for 30 min compared with controls. After reperfusion for 4–6 h the amount of ribosomal subunits partially decreased and a very slight increase in heavy polysomes was observed.

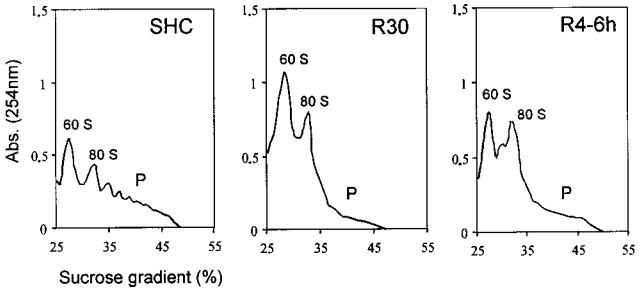

Figure 1 Polysome profiles corresponding to hippocampus from sham-control and ischaemic rats

PMS fractions from sham-control (SHC) and ischaemic animals were analysed on an exponential (25–55%) sucrose gradient as described in the Materials and methods section. Abs., absorbance; P, polyribosomes; R30, 30 min of ischaemia followed by 30 min of reperfusion; R4-6h, 30 min of ischaemia followed by 4–6 h of reperfusion.

Initiation factor levels and phosphorylation status

We analysed the eIF2α phosphorylation status in hippocampus extracts from sham-control and ischaemic animals (Figure 2A). Changes in the eIF2α(P) levels in the hippocampus resembled those already reported in the cortex [26], although the recovery to control values was faster in the hippocampus (reperfusion for

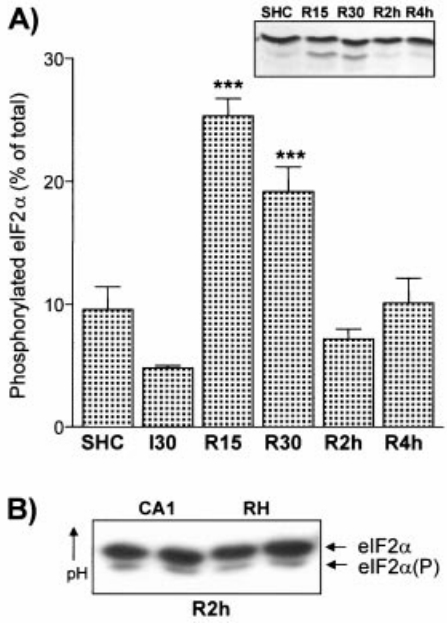

Figure 2 Phosphorylated eIF2α levels corresponding to hippocampus after ischaemia and reperfusion

(**A**) SK fractions from sham control (SHC) and reperfused animals were subjected to IEF and protein immunoblot analysis as described in the Materials and methods section, and bands were stained with alkaline phosphatase reagents. Inset: representative Western blot. I30, 30 min of ischaemia; R15, R30, R2h and R4h, 30 min of ischaemia followed by 15 min, 30 min, 2 h or 4 h of reperfusion respectively. Values represent the means $\pm$ S.E.M. for 6–10 different animals. Statistical significance between sham-control and ischaemic animals was calculated by ANOVA followed by Dunnett's test. *** $P < 0.001$. (**B**) Lysates from CA1 and RH subfields were analysed as described above, and were developed using the ECL® reagent.

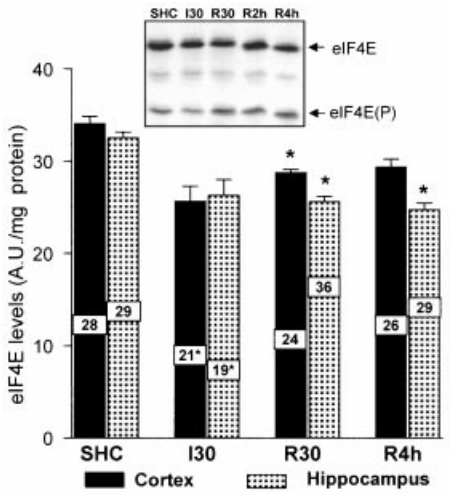

Figure 3 Total and phosphorylated eIF4E levels after ischaemia and reperfusion

SK fractions from sham-control and reperfused animals were subjected to IEF and protein immunoblot analysis as in Figure 2. Inset: representative Western blot from cortex extracts. Values represent the means ± S.E.M. for 6–10 different animals. Numbers within the bars correspond to the percentage of eIF4E(P) over the total. Statistical significance was determined as described in Figure 2, except that the Student's *t* test was used. $^{*}P < 0.05$. A.U., arbitrary units.

2 h) than in the cortex (reperfusion for 4–6 h). eIF2α(P) levels were also determined in whole lysates from the hippocampal subfields (reperfusion for 2 h) to analyse possible differences between vulnerable (CA1-enriched fraction) and more resistant (RH fraction) subfields. Results using the ECL® detection method, which intensifies bands, are shown in Figure 2(B). No differences in the percentage of eIF2α(P) were found between CA1 and RH.

Total eIF4E levels diminished during reperfusion in both regions, they reached control values after 4 h of reperfusion in the cortex and remained significantly lower in the hippocampus. The percentage of phosphorylated eIF4E [eIF4E(P)] significantly decreased after 30 min of ischaemia both in the cortex and the hippocampus, reaching control values after 4 h of reperfusion (Figure 3).

eIF2B activity

eIF2B activity was assayed by its capacity to exchange eIF2-bound [^{3}H]GDP with free GDP in PMS fractions prepared with fresh brain in buffer A (without protease inhibitors) as described previously [5,21]. eIF2B activity, to a great extent, paralleled eIF2α(P) levels, being significantly inhibited (60 %) after ischaemia for 30 min followed by reperfusion for 30 min, and reaching control values after reperfusion for 4 h (Figure 4).

p70^{S6K} activity

Different situations have been described where modifications in the phosphorylation status of ribosomal protein S6 correlate well with changes in protein synthesis rate [20]. p70^{S6K} is the cytoplasmic kinase that has been postulated to be responsible for controlling this process [20]. As shown in Figure 5, p70^{S6K} activity significantly decreased after ischaemia for 30 min, as well as after reperfusion for 30 min, in the cortex.

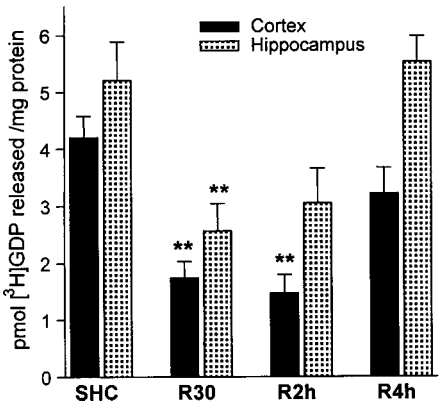

Figure 4 eIF2B activity after ischaemia and reperfusion

eIF2B activity assessed by its capacity to exchange GDP in a preformed binary complex eIF2–[^{3}H]GDP was assayed in PMS fractions from cortex and hippocampus. Values represent the means ± S.E.M. for 4–10 different animals. Statistical significance was determined as described in Figure 2, except that the Student's *t* test was used. $^{**}P < 0.01$. SHC, sham-control; R30, R2h and R4h, 30 min of ischaemia followed by 30 min, 2 h or 4 h of reperfusion respectively.

4E-BP1 phosphorylation status after ischaemia and reperfusion

4E-BP1 phosphorylation status was studied by analysing the different bands (α, β and γ) obtained from the extracts subjected to SDS/PAGE and protein immunoblotting (Figure 6A). Very disperse values among the different animals, especially for bands β and γ, were obtained (Figure 6B). Band α, which represents the completely unphosphorylated form, significantly increased following ischaemia for 30 min and decreased to control values after reperfusion for 30 min (Figures 6A and 6B). Conversely, band γ, which represents the most phosphorylated form, presented undetectable values after 30 min of ischaemia, significantly increased after reperfusion for 30 min to 2 h and reached control

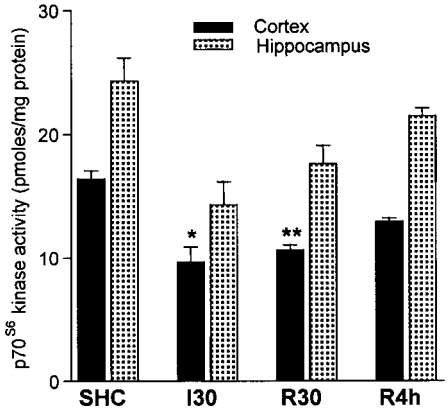

Figure 5 p70^{S6K} activity after ischaemia and reperfusion

The kinase activity was measured in SK fractions from sham-control (SHC) and ischaemic rats as described in the Materials and methods section. Results represent the means ± S.E.M. for 4–10 different animals. Statistical significance was determined as described in Figure 2. $^{*}P < 0.05$; $^{**}P < 0.01$. I30, 30 min of ischaemia; R30 and R4h, 30 min of ischaemia followed by 30 min or 4 h of reperfusion respectively.

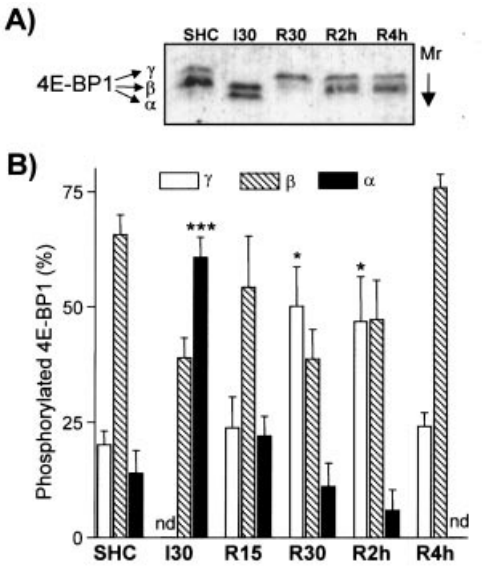

Figure 6 4E-BP1 phosphorylation status after ischaemia and reperfusion

SK fractions from the cortex of sham-control (SHC) and ischaemic rats were analysed by protein immunoblotting as described in the Materials and methods section. (**A**) Representative Western blot. α, unphosphorylated 4E-BP1, β and γ: phosphorylated 4E-BP1. (**B**) The different 4E-BP1 phosphorylation bands (α, β and γ) were quantified and the Figure shows the percentage of each band over the total. Values represent the means $\pm$ S.E.M. for 4–10 different animals. Statistical significance was determined as described in Figure 2. * $P < 0.05$; *** $P < 0.001$. I30, 30 min of ischaemia; R15, R30, R2h and R4h, 30 min of ischemia followed by 15 min, 30 min, 2 h or 4 h of reperfusion respectively; nd, no detectable levels.

values after reperfusion for 4 h. Similar results were obtained within the two regions. Total 4E-BP1 levels were significantly higher ($P < 0.05$) in the cortex than in the hippocampus (75.8 ± 10.3 versus 36.1 ± 5.9 arbitrary units/mg protein respectively), and did not change during ischaemia or reperfusion (results not shown).

eIF4G–eIF4E complex formation after ischaemia and reperfusion

We determined both eIF4G levels and eIF4G associated with eIF4E after m^{7}GTP–Sepharose chromatography, which reflects active eIF4F complex formation. As expected, 4E-BP1 binding to eIF4E was exclusively detected after 30 min of ischaemia in both the cortex and hippocampus, the amount of bound 4E-BP1 being lower in the hippocampus (Figure 7).

The specific eIF4G antibodies detected the expected band of 220 kDa corresponding to the initiation factor. This band

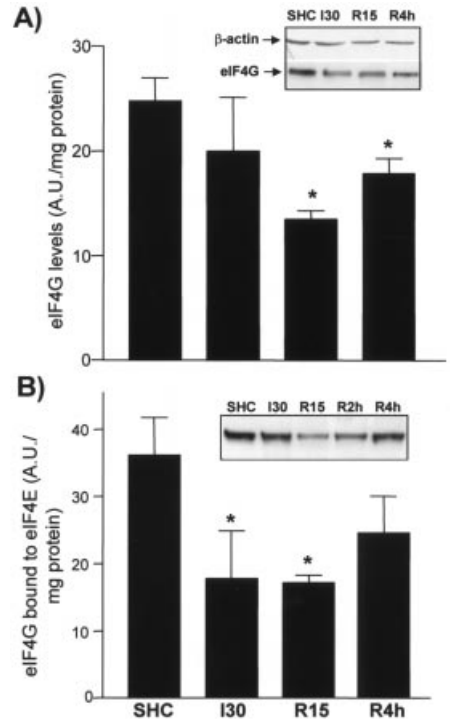

Figure 8 eIF4G levels and eIF4G–eIF4E complexes after ischaemia and reperfusion in the cortex

PMS fractions before (**A**) and after (**B**) m^{7}GTP–Sepharose chromatography from cortex were analysed by protein immunoblotting as described in the Materials and methods section. Values represent the means $\pm$ S.E.M. for 3–4 different animals. Insets: representative Western blots of eIF4G, including β-actin as a control of loading homogeneity (**A**) and eIF4G bound to eIF4E (**B**). Statistical significance was determined as described in Figure 2, except that the Student's t test was used. * $P < 0.05$. A.U., arbitrary units; SHC, sham-control; I30, 30 min of ischaemia; R15, R2h and R4h, 30 min of ischaemia followed by 15 min, 2 h or 4 h of reperfusion respectively.

diminished during ischaemia, remained significantly lower after reperfusion for 15 min and partially recovered following reperfusion for 4 h in the cortex (Figure 8A), and in the whole hippocampus (results not shown). eIF4F complex formation paralleled eIF4G levels, being significant lower following 30 min of ischaemia and reperfusion for 15 min and partially recovered after reperfusion for 4 h in the cortex (Figure 8B), and in the whole hippocampus (Figure 9C). No proteolytic fragments were recognized by the antibody in ischaemic samples from the cortex or the hippocampus.

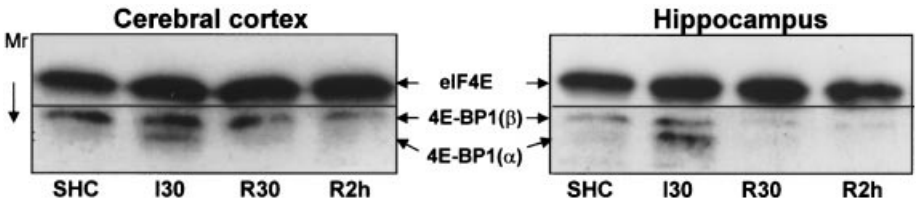

Figure 7 4E-BP1–eIF4E complexes after ischaemia and reperfusion

PMS fractions after m^{7}GTP–Sepharose chromatography from cortex and hippocampus were analysed by protein immunoblotting as described in the Materials and methods section. SHC, sham-control; I30, 30 min of ischaemia; R30 and R2h, 30 min of ischaemia followed by 30 min or 2 h of reperfusion respectively.

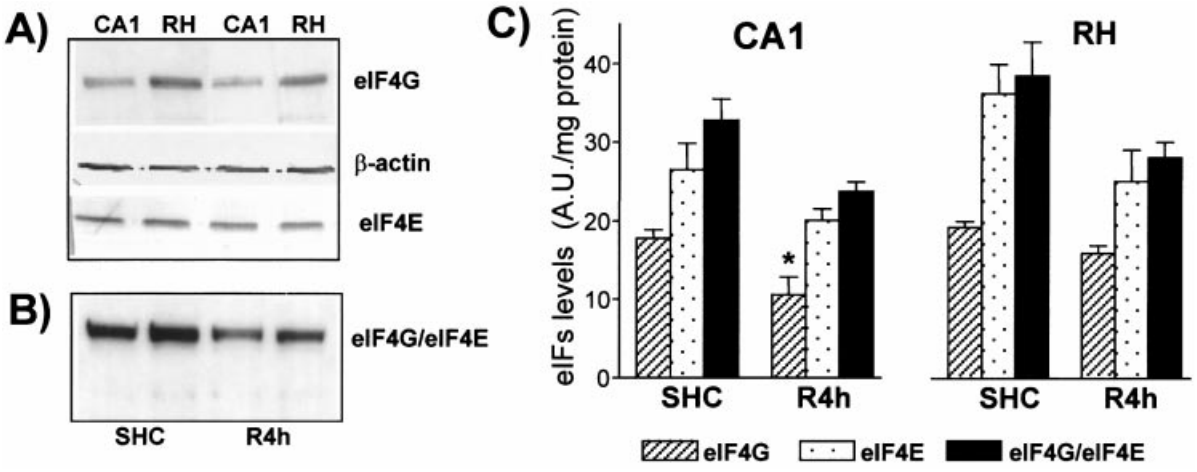

Figure 9 eIF4G and eIF4E levels, and eIF4G–eIF4E complexes, after ischaemia and reperfusion in the hippocampal subfields

PMS fractions before and after m^7GTP–Sepharose chromatography from CA1 and RH subfields were analysed by protein immunoblotting as described in the Materials and methods section. (**A** and **B**) Representative Western blots of eIF4G including β-actin as a control of loading homogeneity and eIF4G bound to eIF4E. (**C**) Values represent the means $\pm$ S.E.M. of 2–4 different animals. Statistical significance was determined as described in Figure 2, except that the Student's *t* test was used. * $P < 0.05$. A.U., arbitrary units; SHC, sham-control; I30, 30 min of ischaemia; R4h, 30 min of ischaemia followed by 4 h of reperfusion.

eIF4F complex formation as well as eIF4G and eIF4E levels were lower in the CA1 subfield compared with RH, and none of the three parameters reached control values following reperfusion for 4 h (Figures 9A, 9B and 9C).

DISCUSSION

The goal of research into ischaemia is to understand the mechanisms responsible for cell death. Experimental models of ischaemia include *in vitro* models, such as cell culture or brain slices, which constitute simple approximations to the *in vivo* situation. Nevertheless, the absence of blood flow as a variable and the absence of blood vessels in cell culture eliminate components that can be important in the damage process. For this reason we have used an *in vivo* model of incomplete forebrain ischaemia to determine the participation of several initiation factors in the ischaemia-induced inhibition of translation. The cortex and the hippocampus have been studied as being representative of resistant and vulnerable brain regions respectively. In addition, the hippocampus has been further dissected into CA1 (vulnerable) and RH (mostly resistant) subfields.

The findings obtained from the polysome profile results are in line with earlier results obtained in similar ischaemia models, and demonstrate that a block in the initiation step causes protein-synthesis depression in neuronal cells after reperfusion for 2–6 h [9]. Both our previous [15] and present results support eIF2B activity inhibition caused by eIF2α phosphorylation as being a major site of early post-ischaemic translational regulation. However, the protein synthesis rate, as measured in *in vivo* experiments, is still substantial after reperfusion for 2–6 h in the cortex and is profoundly inhibited in the hippocampus [1,7,8], whereas eIF2α(P) levels and eIF2B activity were already normalized at these time points. Moreover, data with the dissected hippocampus demonstrated that eIF2α(P) levels after reperfusion for 2 h in the CA1-subfield, with a more severely inhibited protein synthesis [7,8], were similar to that in RH subfields. Altogether these results indicate that another translational mechanism may be impaired, at least in the hippocampus, at longer reperfusion times.

Light and ultrastructural studies have shown high eIF2α(P) immunolabelling levels in the cytoplasm and nucleus of CA1 neurons after 10 min of reperfusion following 10 min of cardiac arrest in the rat, as well as a complete disappearance of cytoplasmic eIF2α(P) label in non-vulnerable neurons and intensification in cytoplasm and nuclei of CA1 after 1 h of reperfusion [18,27]. These findings have not been studied at longer reperfusion times, or reproduced in any other model. In our opinion, these immunocytochemistry studies are not supported by the biochemical studies achieved in the same model, which shows equal increased eIF2α(P) levels, both at 10 and 90 min reperfusion in PMS Western blots from ischaemic forebrains [16,18]. We understand that the observed eIF2α(P) dephosphorylation in non-vulnerable neurons and the eIF2α(P) accumulation in vulnerable neuron nuclei [27] should be reflected in Western blots of forebrain PMS (containing vulnerable, as well as non-vulnerable, cells and lacking nuclei) as a much decreased eIF2α(P) band after 90 min of reperfusion. In our ischaemia model the increased eIF2α(P) levels induced during reperfusion in the hippocampus resemble those found in the cortex [26]; in addition, we have not found differences between non-vulnerable or vulnerable regions after reperfusion for 2 h.

In conclusion our studies support the hypothesis that cytoplasmic eIF2α phosphorylation could be considered a classical stress-induced response, common to vulnerable and non-vulnerable cells. As a rule, the stress-induced repression of protein synthesis caused by eIF2α phosphorylation occurs in a few minutes and its recovery may require up to several hours [12,28]. In cultured cells, the ischaemia-induced translational repression caused by increased eIF2α phosphorylation occurs during the ischaemia period, and both parameters are normalized in the first hour of reperfusion [29]. However, as stated above, transient forebrain ischaemia represents a stress situation of extreme complexity, while translational repression is already induced during ischaemia, eIF2α phosphorylation is delayed until the reperfusion period, probably because the complete ATP depletion induced by ischaemia inactivates the eIF2α phosphorylation mechanisms.

Changes in gene and protein expression within the different models of global, as well as focal, ischaemia have been extensively reported [2,3,30]. Modifications in the activity or levels of factors implicated in mRNA recognition would induce selective translation of mRNAs, which could partially explain the different protein expression patterns between vulnerable and non-vulnerable cells. As far as we know we are the first to describe an ischaemia-induced modification in $p70^{S6K}$ activity. Interestingly, the decreased enzyme activity that was observed after ischaemia for 30 min, as well as after reperfusion for 30 min, was only significant in the cortex. This modification might induce protein ribosomal S6 dephosphorylation and consequently specifically inhibit the translation of mRNAs containing a 5′-TOP tract [20]. This early inhibition in $p70^{S6K}$ activity might positively participate in the recovery of protein synthesis in non-vulnerable cells, and warrants further investigation.

During the ischaemic period we have found that eIF4E was dephosphorylated; however, at the present time the significance of eIF4E phosphorylation in the regulation of translation remains unclear. eIF4E is widely thought to be present at very low molar concentrations in most cell types. Unphosphorylated 4E-BP1α, which binds tightly to eIF4E, appears to inhibit eIF4E activity by preventing its association with eIF4G, thus impairing eIF4F formation and suppressing cap-dependent, but not cap-independent, translation [19,31,32]. Our results indicate that this is the case during ischaemia both in cortex and hippocampus; the decreased eIF4F formation observed during ischaemia is probably the result of both 4E-BP1 dephosphorylation and decreased eIF4G levels. As far as eIF4E and 4E-BP1 are concerned, a straight parallelism in the results obtained with the *in vivo* and *in vitro* ischaemia models is observed [33]. Nevertheless, eIF4E and eIF4G levels do not change during ischaemia and recirculation in the *in vitro* model [33], indicating that mechanisms inherent to the *in vivo* reperfusion could be implicated in the degradation of the factors.

To our knowledge, we are the first to describe a reperfusion-induced diminution in eIF4F complex formation both in the cortex and hippocampus. eIF4G degradation can be responsible for the decreased eIF4F formation during early reperfusion and consequently act concomitant to eIF2α phosphorylation in inhibiting overall translation. Calpain I activation has been characterized as the mechanism possibly implicated in eIF4G cleavage during ischaemia [6]. The decreased eIF4E and eIF4G levels could explain the diminution in eIF4F complex formation during late reperfusion; interestingly, eIF4F formation is lower in the CA1 subfield, which shows the strongest inhibition of protein synthesis *in vivo*. eIF4F formation not only regulates overall translation, but also selectively regulates mRNA translation. Decreased eIF4F formation like that found in the CA1-subfield might lead to a repression in the translation of the protein products of poorly translated mRNAs, such as those of growth and survival factors [19,32]. Further studies are necessary to prove whether or not the reperfusion-induced decrease in eIF4F formation is exclusively regulated by eIF4E and eIF4G levels, or whether other mechanism(s) are implicated as well.

Taken together, our findings suggest that the delayed and long-lasting recovery in global translation induced by eIF2α(P), along with the lower levels of eIF4F complex and the different mRNA pools in vulnerable and resistant cells [3,34], might induce a modification in the recovery programme of vulnerable neurons. Cell survival may be affected by the preferential translation of mRNAs encoding detrimental proteins and/or a reduced synthesis of short half-life machinery components, which could include eIF4E. The profound and irreversible inhibition of protein synthesis in vulnerable neurons, which could also be induced by reduced ATP levels [35], will disable them for either translating immediately early gene mRNAs involved in neuronal survival, or resynthesizing key enzymes and trophic factors, presumably leading to the delayed cell death. Experiments conducted to modify the stress response would help to establish the precise role of initiation factors in the delayed neuronal death.

Translation is one of the most complex cellular processes; other inhibitory mechanism(s) (whether or not they act in concert with those already described) could either remain operative or be induced exclusively in vulnerable cells at late reperfusion. The ischaemia-induced translational repression probably results from the summation of multiple altered regulatory sites, selectively impaired during the ischaemia and reperfusion periods. Any progression in the knowledge of the mechanisms implicated will provide potential targets for future therapeutic strategies directed to prevent pathophysiological events associated with the ischaemic brain.

This work was supported by Spanish PM97-0071 (Ministerio de Educación y Ciencia) and 00/0245 (Fondo de Investigaciones Sanitarias; FIS) grants, and a Slovakian, SK-VEGA 2/7231/20 grant. C. Martín de la Vega, C. Quevedo and M. E. Martín gratefully acknowledge receipt of support from the Ministry of Education, the Regional Government of Madrid and Health Ministry (FIS) respectively. We thank J. M. Martín Arias and M. I. Pérez Morgado for their editorial and technical assistance respectively. Likewise we are grateful to the late Dr E. C. Henshaw and Dr A. Nieto (Centro Nacional de Biotecnología, Campus Universidad Autónoma de Madrid, Canto Blanco, Madrid, Spain) for kindly providing anti-eIF2α and anti-eIF4GI antibodies respectively.

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Received 23 February 2001/11 April 2001; accepted 11 May 2001