RESEARCH COMMUNICATION

Phosphorylation of the α subunit of initiation factor 2 correlates with the inhibition of translation following transient cerebral ischaemia in the rat

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Rats were subjected to the standard four-vessel occlusion model of cerebral transient ischaemia (vertebral and carotid arteries) for 15 and 30 min. After a 30 min recirculation period, protein synthesis rate, initiation factor 2 (eIF-2) and guanine nucleotide exchange factor (GEF) activities, and the level of phosphorylation of the α subunit of eIF-2 (eIF-2 α) were determined in the neocortex region of the brain from sham-operated controls and ischaemic animals. Following reversible cerebral ischaemia, the protein synthesis rate, as measured in a cell-free system, was significantly inhibited (70%) in the ischaemic animals. eIF-2 activity, as measured by its ability to form a ternary complex, also decrease parallel to the decrease in protein synthesis. As eIF-2 activity was assayed in the presence of Mg²⁺ and GTP-

regenerating capacity, the decrease in ternary-complex formation indicated the possible impairment of GEF activity. Since phosphorylated eIF-2 [eIF-2(α P)] is a powerful inhibitor of GEF, the levels of phosphorylated eIF-2 α were determined, and an increase from 7% phosphorylation in sham control rats to 20% phosphorylation in 15 min and 29% phosphorylation in 30 min in ischaemic rats was observed, providing evidence for a tight correlation of phosphorylation of eIF-2 α and inhibition of protein synthesis. Moreover, GEF activity measured in the GDP-exchange assay was in fact inhibited in the ischaemic animals, proving that protein synthesis is impaired by the presence of eIF-2(α P), which blocks eIF-2 recycling.

INTRODUCTION

Relatively little effect on the integrity of the protein-synthesizing apparatus has been observed during cerebral ischaemia itself. Neither the enzymes found as a part of the cell sap, nor ribosomes 'frozen' as a consequence of the energy failure, lose their activity [1–3].

The post-ischaemic recirculation phase, which follows reversible cerebral ischaemia, is characterized by considerable changes in protein-synthesis machinery. Re-introduction of the oxygenated blood inhibits protein synthesis transiently in ischaemia-resistant areas such as the neocortex and the CA3 region of the hippocampus, whereas in ischaemia-vulnerable areas, such as the striatum or the CA1 region, inhibition is persistent [4–8]. Inhibition of protein synthesis is associated with polysome disaggregation into monosomes and subunits, suggesting that polypeptide chain initiation is stopped or slowed in relation to elongation and termination. Disaggregated polysomes were found by analysing polysomal profiles [2,3,9–11]. The reason for polysome disaggregation has not yet been fully clarified, but the possibility of reversing the inhibition of [¹⁴C]leucine incorporation into proteins *in vitro* by intact eukaryotic initiation factor 2 (eIF-2) and/or a high concentration of GTP suggests that, as in reticulocyte lysates, eIF-2 activity might be decreased during the early phases of recirculation [12,13].

The initiation of protein synthesis depends on eIF-2, since it is required to prime each 40 S ribosomal subunit for every round of translation. Phosphorylation of the α subunit of eIF-2 (eIF-2 α) by the haem-controlled inhibitor (HCI) correlates with the inhibition of initiation rates; this mechanism is fully established in rabbit

reticulocyte lysates. eIF-2 phosphorylated in its α subunit [eIF-2(α P)] is not inactive as such, but it cannot be recycled by guanine nucleotide exchange factor (GEF) after its release as an eIF-2(α P)·GDP complex ([14]; reviewed in [15–17]). This mechanism may also operate in other eukaryotic cells (cultured cell lines or animal tissues) that have been deprived of serum, starved of amino acids, subjected to heat stress etc. [15–17], but characterization of the eIF-2 α kinase(s) has not been successful, with the exceptions of the double-stranded-RNA-activated inhibitor (DAI), which is an interferon-induced kinase [18], and of protein kinase GCN2 from yeast [19]. Direct evidence of decreased activity of eIF-2 and GEF after transient cerebral ischaemia was found by Hu and Wieloch [8]; however, their data neither confirmed eIF-2 α phosphorylation, nor proved an alternative mechanism.

The severity, the intraintra-ischaemic temperature and the duration of the ischaemia are apparently the main factors influencing the reversibility of the initiation block. The importance of renewing protein synthesis for the complete functional and metabolic recovery of brain during the post-ischaemic period is evident from the finding that neuronal necrosis is preceded by persistently decreased protein synthesis in selectively vulnerable regions of the brain [6,20]. The functional recovery of brain has only been observed in those cases in which protein synthesis was restored [2,21].

The present paper gives evidence that the α subunit of eIF-2 becomes phosphorylated after 30 min of post-ischaemic recirculation, and that the level of this phosphorylation correlates perfectly with the level of protein-synthesis inhibition produced during the same 30 min recirculation period. Also, it provides

Abbreviations used: eIF-2, eukaryotic initiation factor 2; eIF-2 α , the α subunit (38 kDa) of eIF-2; eIF-2(α P), the phosphorylated form of eIF-2; GEF, guanine nucleotide exchange factor, also named eIF-2B; HCI, haem-controlled inhibitor; PMS, postmitochondrial supernatant; S-100K, salt-wash postmicrosomal supernatant; Met-tRNA_i, initiation form of methionyl-tRNA; IEF, isoelectric focusing.

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evidence that phosphorylation of eIF-2 α is probably the primary cause of the inhibition of GEF activity, and consequently of protein-synthesis initiation.

MATERIALS AND METHODS

Induction of ischaemia

Male Wistar rats (mean body weight 250 g) were used. The standard four-vessel occlusion model [22], as subsequently modified [23], was used to produce incomplete forebrain ischaemia. The experiments were approved by the ethical committee at Hospital Ramón y Cajal. Briefly, on the first day, after anaesthesia with sodium pentobarbital (50 mg/kg body wt., intraperitoneally), both vertebral arteries were irreversibly occluded by coagulation through the alar foramina, and, on the second day, anaesthesia was induced by placing the rat in a jar with 2.5% halothane in a mixture of O₂/N₂O (3:7), after which both common carotid arteries were occluded for 15 or 30 min by small atraumatic clips. The severity of ischaemia was judged by neurological investigation. Normothermic conditions were monitored using a thermistor in the ear and maintained at approx. 37 °C by placing the animals on a heating pad under a light bulb. After ischaemia, both clips were removed from the common carotid arteries. Sham control animals were prepared in the same way, but the carotids were not occluded. Animals were killed immediately at the end of a 30 min recirculation period, their neocortex was dissected, and subcellular fractions were prepared and kept at -80 °C until use. Protein determination was as described by Bradford [24].

Translation assays *in vitro*

The protein synthesis rate was determined in a cell-free system validated for the brain [25,26]. Cerebral neocortex was homogenized 1:2 (w/v) with buffer H (50 mM Hepes/KOH, pH 7.55, 140 mM potassium acetate, 4 mM magnesium acetate, 2.5 mM dithiothreitol and 0.32 M sucrose) and centrifuged at 11 000 *g* for 10 min at 4 °C to obtain the postmitochondrial supernatant (PMS). The complete reaction system in a final volume of 50 μ l contained 50 mM Hepes/KOH, pH 7.55, 150 mM potassium acetate, 5 mM magnesium acetate, 2.5 mM dithiothreitol, 0.32 M sucrose, 1 mM ATP, 0.75 mM GTP, 20 mM phosphocreatine, 150 μ g/ml creatine kinase, 50 μ M amino acids, 100 μ g of PMS and 5 μ Ci of [4,5-³H]leucine (25 μ M; 66 Ci/mmol). The reactions were carried out at 30 °C for 45 min and then placed in 1 ml of distilled water. The samples were incubated for 20 min at 37 °C after addition of 0.5 ml of 1 M KOH containing 2 mg/ml leucine, and the protein was precipitated with 25% trichloroacetic acid (containing 2 mg/ml leucine) and washed before filtration on a Whatman GF/C glass-fibre filter. The filters were counted for radioactivity after agitation for 60 min with Normascent 22 scintillation liquid.

eIF-2 assay

The GTP-dependent binding of eIF-2 factor to the initiation form of methionyl-tRNA (Met-tRNA_i) was measured in the PMS (30 μ g) as described by Martín et al. [27,28], in the presence of Mg²⁺ (1.5 mM) and GTP-regenerating capacity (2.5 mM phosphoenolpyruvate and 2 units/ml of pyruvate kinase). The results are expressed as pmol of Met-tRNA_i incorporated/mg of protein in a 10 min incubation.

Measurement of GEF activity

GEF activity was assayed by its capacity to exchange eIF-2-bound [³H]GDP with free GDP in PMS fractions from sham control and ischaemic animals. A binary complex of pure eIF-2 and [³H]GDP was formed (10 min, 30 °C) in a mixture containing 20 mM Hepes/Tris, pH 7.6, 100 mM KCl, 1 mM dithiothreitol, 0.5 mg/ml BSA, 1.6 μ M [³H]GDP (15.3 Ci/mmol) and brain eIF-2 (95% pure [29]). MgCl₂ was then added to a concentration of 1.5 mM, and the binary complex was stored on ice. The GEF assays were carried out at 30 °C in a final volume of 110 μ l, containing 20 mM Hepes/Tris, pH 7.6, 1 mM dithiothreitol, 100 mM KCl, 0.5 mg/ml BSA, 1.5 mM MgCl₂, 100 μ g of PMS, 500 μ M GDP and two different concentrations of eIF-2·[³H]-GDP (5 and 15 nM on average). After incubation for 2.5 min, 2 ml of wash buffer (20 mM Tris/HCl, pH 7.6, 100 mM KCl, 10 mM MgCl₂ and 2 mM dithiothreitol) was added, and the tubes were mixed and filtered through a nitrocellulose filter disc. The filters were washed in 3 $\times$ 2 ml of buffer and radioactivity was measured in a liquid-scintillation counter. Under these conditions the GDP exchange increases linearly with time up to 2.5 min of incubation, where more than 50% of the initial eIF-2·[³H]GDP is still present in the assay. The assays at the two different concentrations were performed with the same samples, on the same day, and repeated two or three times. One unit is defined as 1 pmol of [³H]GDP released/mg of protein in a 2.5 min incubation.

Determination of the state of phosphorylation of the eIF-2 α subunit

After killing, the cerebral neocortex from the rats was homogenized 1:4 (w/v) in 20 mM Tris/HCl, pH 7.6, containing 0.2 M sucrose, 1 mM dithiothreitol, 5 mM magnesium acetate, 50 mM KCl, 1 mM phenylmethanesulphonyl fluoride, 2 mM benzamidine, 1 mM EDTA, 2 mM EGTA, 100 mM NaF, 20 mM β -glycerophosphate and 20 mM sodium molybdate; the post-nuclear supernatant was obtained by centrifugation at 500 *g* for 10 min. Then 4 M KCl was added to give a final concentration of 0.55 M KCl, the mixture was stirred for 20 min at 4 °C and the postmicrosomal supernatant (S-100K) was then prepared by centrifugation at 200 000 *g* for 30 min. The supernatant was resolved in horizontal isoelectric-focusing slab gels (IEF) and analysed by protein immunoblot. Horizontal IEF was performed in a Multiphor II apparatus (Pharmacia/LKB) as described in [28]. A 10–20 μ l sample (up to 75 μ g of protein) was applied approx. 20 mm from the anode (it was found that this application closer to the pI for eIF-2 α gave better results, especially with relatively pure samples). The distance between the anode (10 mM H₃PO₄) and the cathode (20 mM NaOH) was 10 cm. The gels were pre-run for 30 min at 500 V, and samples were electrophoresed at 2000 V for 2 h with cooling (12–15 °C). Gels were electrophoretically transferred to polyvinylidene difluoride transfer membrane (DuPont) with a discontinuous buffer system: anode solution, pH 10.4 Tris (0.3 M), pH 10.4 Tris (0.1 M); cathode solution, pH 10.5, arginine (0.1 M), 0.01% (w/v) SDS; at 0.8 mA/cm² for 2 h. To identify the phosphorylated and the unphosphorylated forms of eIF-2 α , the pure factor was first phosphorylated with HCl (partially purified as described by Ranu and London [30]), then resolved by IEF, and the bands corresponding to eIF-2 α and eIF-2 α (P) protein were stained on immunoblots. Immunoblot analysis was performed with a polyclonal antiserum [31] purified by immunoaffinity [32] (1:100 dilution). Stained bands were scanned and quantified with an Image Analyzer (Discovery Series, PDI, New York, U.S.A.) equipped with a software package RFLPrint (PDI).

Table 1 Protein synthesis rate and eIF-2 activity in cerebral neocortex from sham control and ischaemic rats

Protein synthesis was measured by the incorporation of [^{3} H]leucine into PMS from sham control and ischaemic animals, and eIF-2 activity was measured as its capacity to form the ternary complex in the same subcellular fractions as described in the Materials and methods section. (Abbreviations: I-15 R30, 15 min ischaemia, 30 min recirculation; I-30 R30, 30 min ischaemia, 30 min recirculation). Values represent means $\pm$ S.E.M. for 3–6 different animals. Statistical significance between sham control and ischaemic animals; *** P < 0.001; ** P < 0.01. Values in parentheses represent percentage inhibition.

	Protein synthesis (pmol of leucine/ mg of protein)	eIF-2 (pmol/mg of protein)
Sham control	17.40 $\pm$ 2.4	0.846 $\pm$ 0.09
I-15 R30	5.45 $\pm$ 0.8** (69)	0.439 $\pm$ 0.07***
I-30 R30	5.29 $\pm$ 0.5** (70)	0.206 $\pm$ 0.06***

RESULTS AND DISCUSSION

It is generally accepted that protein synthesis is inhibited during the period of recirculation which follows transient cerebral ischaemia. Protein synthesis was measured in a cell-free translation system to prove that under our experimental conditions inhibition was also produced. The cell-free system utilized has been validated for the brain and liver [25,26,33], and, in spite of its proven lower rate of synthesis, accurately reflects changes that occur *in vivo*. The system is inhibited by the initiation inhibitor aurintricarboxylic acid and initiates translation of endogenous as well as exogenous mRNA [25,26], proving that it is capable of efficient re-initiation. Furthermore, since synthesis occurs in the presence of a high concentration of exogenous amino acids, it is almost independent of changes in endogenous amino acid concentrations, and leucine incorporation is linear for up to 45 min of incubation [26]. As shown in Table 1, there is a significant decrease in protein synthesis in ischaemic animals.

eIF-2 activity was measured by its ability to form a ternary complex with GTP and Met-tRNA_i in the presence of Mg²⁺ and GTP-regenerating capacity. As shown in Table 1, eIF-2 activity decreases significantly in the ischaemic animals as compared with controls, and the inhibition is proportional to the length of the ischaemic period. The decrease in eIF-2 activity in this assay might indicate an impairment of GEF activity, due to the presence of eIF-2(α P) [14,34].

To determine the levels of eIF-2 α phosphorylation, S-100K samples were subjected to IEF. The fractions were prepared from fresh tissue in a relatively short period of time (3 h) in the presence of phosphatase inhibitors to avoid dephosphorylation of eIF-2(α P). The advantage of these samples over more crude fractions, such as homogenate or PMS, is that eIF-2 is more concentrated in them, and they are free of nucleic acids. As shown in Figure 1, our method clearly separates eIF-2(α P) from eIF-2 α . Quantification of bands shows that the amount of eIF-2(α P) increases with the length of the ischaemic period (Table 2) and parallel to the inhibition of eIF-2 activity and protein synthesis. The data after a 15 min period of ischaemia indicate an apparent increase in the total amount of eIF-2; nevertheless, given the paucity of this experimental group and the inherent unlikelihood that such a transient alteration could occur over the time period involved, this is not likely to be real.

The assay conditions used to measure GEF activity in this paper are very close to those designed by Rowlands et al. [35] to be sensitive to eIF-2 α phosphorylation. As shown in Table 3, at

Figure 1 Phosphorylation status of eIF-2 in cerebral neocortex from sham control and ischaemic rats

Salt-wash postmicrosomal supernatants (50 μ g) from sham control and ischaemic animals were subjected to IEF and protein immunoblot analysis as described in the Materials and methods section. Results of a typical experiment are shown in the Figure. Lane 1, 15 min ischaemia, 30 min recirculation; lane 2, eIF-2 phosphorylated with HCl; lane 3, sham control; lane 4, 30 min ischaemia, 30 min recirculation. Key: α , eIF-2 α ; α P, eIF-2(α P).

Table 2 Quantification of the degree of phosphorylation of eIF-2 α in cerebral neocortex from control and ischaemic rats

Bands corresponding to eIF-2 α and eIF-2(α P), as shown in Figure 1, were quantified, and the results are expressed in A units/mm² per μ g of protein. Values represent means $\pm$ S.E.M. from 3–4 different animals. Statistical significance between control and ischaemic animals: ** P < 0.01. Values in parentheses represent eIF-2(α P) as a percentage of the total amount of eIF-2. Abbreviations: I-15 R30, 15 min ischaemia, 30 min recirculation; I-30 R30, 30 min ischaemia, 30 min recirculation.

	eIF-2(α P)	eIF-2(α + α P)
Sham control	2.26 $\pm$ 0.94 (7)	31.8 $\pm$ 4.1
I-15 R30	8.92 $\pm$ 2.6 (20)	47.9 $\pm$ 6.5
I-30 R30	10.8 $\pm$ 2.3** (28)	37.9 $\pm$ 5.2

Table 3 GEF activity in cerebral neocortex from sham control and ischaemic rats

GEF activity was measured by the GDP exchange assay in the PMS from sham control and ischaemic animals as described in the Materials and methods section. (Abbreviations: I-15 R30, 15 min ischaemia, 30 min recirculation; I-30 R30, 30 min ischaemia, 30 min recirculation). A unit of GEF activity represents 1 pmol of [^{3} H]GDP released/mg of protein in a 2.5 min incubation. Values represent means $\pm$ S.E.M. from 2–6 different animals. Values in parentheses represent % of control activity. Statistical significance between sham control and ischaemic animals; ** P < 0.01; * P < 0.05.

Substrate concn. ...	GEF activity (units)	
	5 nM	15 nM
Sham control	2.82 $\pm$ 0.24 (100)	4.45 $\pm$ 0.26 (100)
I-15 R30	1.06 $\pm$ 0.06** (38)	3.22 $\pm$ 0.40 (72)
I-30 R30	1.00 $\pm$ 0.34** (35)	3.15 $\pm$ 0.28* (71)

a low initial substrate concentration of 5 nM, inhibition of GEF activity was found in ischaemic animals in comparison with the sham control animals. With the use of a single concentration of eIF-2·[^{3} H]GDP in this assay, it is not possible to distinguish between decreased GEF activity due to endogenous eIF-2(α P) or another mechanism, such as a direct modification of GEF itself. Nevertheless, since eIF-2(α P) is a competitive inhibitor of GEF activity [35], by increasing the substrate concentration, a reversion of the inhibition should be expected. In fact, when a substrate concentration of 15 nM was used (Table 3), the inhibition was decreased almost to half, and this result strongly support the hypothesis that eIF-2(α P) is responsible for the inhibition of GEF activity.

In reticulocytes, phosphorylation of approx. 20–30% of all the eIF-2 α molecules is sufficient to bind and to inhibit GEF activity completely, since GEF is of relatively low abundance [36]. Our data show that, when 30% of the eIF-2 α molecules are phosphorylated, both GEF activity and protein synthesis are inhibited by only 60–70%, which may indicate a lower eIF-2/GEF ratio in brain tissue.

To our knowledge, our results are the first example of regulation of translational initiation, in brain tissue, at the level of eIF-2 recycling and dependent on eIF-2(α P) phosphorylation. They also constitute the second clear evidence of this mechanism in animal tissue, the other being the reported inhibition of translation in perfused liver under vasopressin treatment or amino acid deprivation [37–39]. Decreased phosphatase activity has been associated with phosphorylation of eIF-2 α in perfused liver deprived of a single essential amino acid [38], whereas activation of eIF-2 α kinases regulates phosphorylation of the protein in other systems [15,16]. Both kinase and/or phosphatase activities for eIF-2 factor have been described in the brain [40]; nevertheless the properties and regulation of these activities remain undefined.

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