Brain ischemia and reperfusion activates the eukaryotic initiation factor 2α kinase, PERK

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Abstract
Reperfusion after global brain ischemia results initially in a widespread suppression of protein synthesis in neurons, which persists in vulnerable neurons, that is caused by the inhibition of translation initiation as a result of the phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2α). To identify kinases responsible for eIF2α phosphorylation [eIF2α(P)] during brain reperfusion, we induced ischemia by bilateral carotid artery occlusion followed by post-ischemic assessment of brain eIF2α(P) in mice with homozygous functional knockouts in the genes encoding the heme-regulated eIF2α kinase (HRI), or the amino acid-regulated eIF2α kinase (GCN2). A 10-fold increase in eIF2α(P) was observed in reperfused wild-type mice and in the HRI−/− or GCN2−/− mice. However, in all reperfused groups, the RNA-dependent protein kinase (PKR)-like endoplasmic reticulum eIF2α kinase (PERK) exhibited an isomorph mobility shift on SDS–PAGE, consistent with the activation of the kinase. These data indicate that neither HRI nor GCN2 are required for the large increase in post-ischemic brain eIF2α(P), and in conjunction with our previous report that eIF2α(P) is produced in the brain of reperfused PKR−/− mice, provides evidence that PERK is the kinase responsible for eIF2α phosphorylation in the early post-ischemic brain.

Keywords: brain ischemia and reperfusion, eIF2α, GCN2, HRI, PERK, PKR.

Neuronal protein synthesis is inhibited in the post-ischemic brain (Krause and Tiffany 1993) as a result of a rapid and large increase in the phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2α) (Burd et al. 1994; DeGracia et al. 1996) that occurs initially in neurons throughout the entire brain (Page et al. 2000), but persists in vulnerable neurons (DeGracia et al. 1997). A complex including eIF2-bound GTP delivers the first methionine for all new peptide synthesis, and this GTP is hydrolyzed during translation initiation (Merrick 1992). The nucleotide exchange protein, eIF2B, is responsible for removing GDP from eIF2 at the end of an initiation cycle, but eIF2 phosphorylated on the α-subunit [eIF2α(P)] sequesters eIF2B and thus obstructs subsequent rounds of translation initiation.

There are four known eIF2α kinases (GCN2, HRI, PKR and PERK) that, either individually or in combination, could mediate post-ischemic eIF2α phosphorylation. Previous work showed that the RNA-dependent eIF2α kinase (PKR) is not required for post-ischemic eIF2α phosphorylation (DeGracia et al. 1999). The heme-regulated eIF2α kinase (HRI) coordinates globin synthesis with heme availability in erythroid cells. Only a limited quantity of HRI is present in brain (Pal et al. 1991; Crosby et al. 1994; Mellor et al. 1994), but its activation by transition metal ions (Hurst et al. 1987) characteristic of brain reperfusion (O’Neil et al. 1996) renders HRI a possible candidate for mediating eIF2α phosphorylation. The amino acid-regulated eIF2α kinase GCN2 is expressed in mammalian brain (Sood et al. 2000), and the potential accumulation of uncharged tRNAs consequent to lowered ATP levels in ischemic neurons could activate GCN2. The PKR-like endoplasmic reticulum eIF2α kinase (PERK, also known as PEK – pancreatic eIF2α kinase) is most highly expressed in pancreas but is also expressed in brain (Shi et al. 1998). PERK is activated by the
endoplasmic reticulum (ER) unfolded protein response (UPR; Harding et al. 1999; Kaufman 1999), which has been suggested to occur during brain ischemia and reperfusion (Paschen and Doutheil 1999). We show here that inactivating mutations of HRI or GCN2 do not attenuate the levels of post-ischemic brain eIF2α(P), as has been previously shown for the PKR mutation (DeGracia et al. 1999). However, mobility shifts of PERK indicating its activation are observed concurrently with phosphorylation of eIF2α during early reperfusion, supporting the hypothesis that PERK activation is responsible for phosphorylation of eIF2α in the early reperfused brain.

Materials and methods

Production of polyclonal anti-PERK antibody and of transfection-over-expressed PERK in mouse embryo fibroblasts (MEFs) have been previously described (Harding et al. 1999). Positive and negative controls were generated in MEFs with and without treatment with thapsigargin, an agent that causes ER stress, activates PERK and promotes eIF2α phosphorylation (Harding et al. 1999). We have characterized the antibody specific for Ser51 phosphorylated eIF2α (DeGracia et al. 1997), which for this study was purchased from Biosource International. All other chemicals were reagent grade.

The GCN2-knockout mice (GCN2−/−) were developed from 129SvEvTac/C57BL/6 mice by replacing the 5-kb DNA fragment containing the three exons encoding kinase domain of GCN2 mRNA and protein are absent in GCN2 homozygous knockout mice. The HRI knockout mice (HRI−/−) were developed from 129SvEvTac/C57BL/6 mice by replacing the 5-kb DNA fragment containing the three exons encoding kinase catalytic domain, from Vlb to X, of HRI with the neomycin gene under the control of the promoter of phosphoglycerate kinase (Han et al., submitted to Cell). There is neither HRI nor HRI-kinase activity in reticulocytes from the homozygous knockout mice (Han et al. 2000). Neonatal PERK knockout mice exhibit grossly dysregulated insulin and glucose homeostasis and are not suitable for ischemia studies (Harding et al. 2001).

All animal experiments were approved by the respective Institutional review boards and were conducted following the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Forebrain ischemia was produced by bilateral carotid artery occlusion (BCAO; Murakami et al. 1998). Mice weighing 25–30 g were anesthetized with ketamine (45 mg/kg intraperitoneally) and placed supine on a temperature-controlled operating surface. A midline neck incision was made between the manubrium and the mandible, and tracheostomy was performed using a 20-gauge (1.1 mm) catheter for positive pressure mechanical ventilation with a tidal volume of 10 ml/kg at a rate of 90 breaths per minute and positive-end expiratory pressure of 3-cm H2O. A warming pad and overhead lamp were used to maintain rectal temperature at 37.0 ± 0.5°C for the entire experimental period. Both common carotid arteries (CCA) were carefully freed from their sheaths (avoiding the vagus nerve) and then transiently clamped with micro-aneurysm clips for 20 min prior to 10-min reperfusion. Non-ischemic controls were anesthetized as above.

Forebrains were separated from the brainstem and cerebellum, and were sonicated on ice in 1: 5 v/w of ice-cold 50 mM HEPES (pH 7.5), 140 mM potassium acetate, 4 mM magnesium acetate, 2.5 mM dithiothreitol, 50 mM NaF, 5 μM okadaic acid, 0.23 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin and 7 μg/mL pepstatin A. The forebrain supernatants (FS) were prepared by centrifuging the homogenate at 12 000 g for 15 min at 4°C. The protein concentration was determined by the Folin phenol reagent method, and the FS were frozen in dry ice and ethanol and stored at −80°C until used. SDS–PAGE and immunoblotting were performed as previously described (DeGracia et al. 1996, 1997). Primary antibody dilutions for immunoblotting were 1: 750 anti-eIF2α(P) and 1: 10 000 anti-PERK. Relative band densities were determined utilizing the BioImage Intelligent Quantifier v3.0.

PERK immunoprecipitation (IP) was as previously described (Harding et al. 1999). The FS containing 2 mg protein were taken to 1% Triton X-100 and precleared with 1 μL of an unrelated antibody (lamin A/C; Santa Cruz Biototechnology) plus 20 μL Protein A-Sepharose beads (Zymed Laboratories). Precleared supernatants were incubated overnight at 4°C with end-over-end rotation with 15 μL Protein A-Sepharose beads prebound with 1 μL PERK antiserum. Proteins were eluted from the beads, subjected to 6% SDS–PAGE, electroblotted transferred to nitrocellulose and immunoblotted with PERK antiserum as above.

Results

Ischemia and reperfusion induced a mean 10-fold increase in eIF2α(P) in all animal groups (Figs 1b and d), and levels of eIF2α(P) in HRI−/− and GCN2−/− following ischemia and reperfusion were indistinguishable from those seen in the wild type. Note that there is very little eIF2α(P) in untreated MEFs [thapsigargin− (tg−)] in contrast to the strong response to thapsigargin (tg+).

The activity of the eIF2α kinase PERK correlates with its phosphorylation status, and the phosphorylated active forms of PERK have reduced mobility on SDS–PAGE (Harding et al. 1996, 1997). Immunoblots from non-ischemic mice show a single signal that comigrates with activated PERK. There was no staining in the region of PERK in IPs lacking primary antiserum (e).

Discussion

Brain reperfusion following ischemia is associated with a rapid and large increase in neuronal eIF2α phosphorylation (Burda et al. 1994; DeGracia et al. 1996, 1997) and inhibits neuronal protein synthesis in the early post-ischemic brain (Sullivan et al. 1999). Although Burda et al. (1998) suggested that phosphorylation of eIF2α is caused by phosphatase inactivation during ischemia/reperfusion, DeGracia et al. (1999) found no reduction in eIF2α(P) phosphatase activity in reperfused brain homogenates. An alternative mechanism that could be involved in the phosphorylation of
eIF2α is inactivation (by deglycosylation) of an inhibitor p67 that normally binds eIF2 and precludes eIF2α phosphorylation (Datta et al. 1989). However, Owen et al. (2001) found no reperfusion-induced loss of total or glycosylated p67 in western blots or immunohistochemical staining in vulnerable neurons. Therefore, we sought evidence for involvement of an eIF2α kinase activated by ischemia/reperfusion.

There are four known mammalian eIF2α kinases: GCN2, HRI, PKR and PERK. HRI−/− and GCN2−/− mice are shown here to have levels of post-ischemic eIF2α phosphorylation indistinguishable from wild-type levels, thus adding these two eIF2α kinases to PKR (DeGracia et al. 1999) in the list of kinases that do not have an essential role in eIF2α phosphorylation during early reperfusion. By contrast, the eIF2α kinase PERK is shown here to be in its activated form during reperfusion and is correlated with eIF2α phosphorylation.

PERK is activated by ER luminal signals that involves altered interaction with the glucose-regulated protein 78 under conditions of ER stress, including not only ER Ca2+ depletion, but also lowered ATP levels or altered ER redox state (Bertolotti et al. 2000). Paschen and Drouhard (1999) have suggested that ER Ca2+ depletion during brain ischemia/reperfusion (Kohno et al. 1997) may cause ER stress. It is not known, however, whether this proposed mechanism plays a role in the activation of PERK that we observed here.

PERK is indispensable to translational regulation in ER-stressed cells (Harding et al. 2000), rendering it a good candidate for mediating the inhibitory effect of ischemia/reperfusion on neuronal protein synthesis. Unfortunately, the severe phenotype associated with the Perk−/− genotype precludes the study of these animals in the bilateral carotid occlusion experimental system (Harding et al. 2001). The sequencing of the human genome has not revealed other predicted eIF2α kinases beyond the four listed above. These observations build a strong circumstantial case for PERK as the eIF2α kinase mediating the inhibition of protein synthesis during early reperfusion.

Further studies will be needed to delineate the roles of the eIF2α kinases during extended reperfusion periods in vulnerable and non-vulnerable brain regions, and to verify that in vivo reduction of brain PERK levels by antisense or conditional knockout strategies will reduce reperfusion-induced eIF2α phosphorylation. This will allow the characterization of the contribution of post-ischemic cerebral protein synthesis inhibition to neuronal death, a crucial issue that has yet to be resolved (Lipton 1999; White et al. 2000).

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