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## Brain ischemia and reperfusion activates the eukaryotic initiation factor 2 $\alpha$ 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  $\alpha$ -subunit of eukaryotic initiation factor 2 (eIF2 $\alpha$ ). To identify kinases responsible for eIF2 $\alpha$  phosphorylation [eIF2 $\alpha$ (P)] during brain reperfusion, we induced ischemia by bilateral carotid artery occlusion followed by post-ischemic assessment of brain eIF2 $\alpha$ (P) in mice with homozygous functional knockouts in the genes encoding the heme-regulated eIF2 $\alpha$  kinase (HRI), or the amino acid-regulated eIF2 $\alpha$  kinase (GCN2). A 10-fold increase in eIF2 $\alpha$ (P) was observed in reperused wild-type mice and in

the HRI $-/-$  or GCN2 $-/-$  mice. However, in all reperused groups, the RNA-dependent protein kinase (PKR)-like endoplasmic reticulum eIF2 $\alpha$  kinase (PERK) exhibited an isoform 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 eIF2 $\alpha$ (P), and in conjunction with our previous report that eIF2 $\alpha$ (P) is produced in the brain of reperused PKR $-/-$  mice, provides evidence that PERK is the kinase responsible for eIF2 $\alpha$  phosphorylation in the early post-ischemic brain.

**Keywords:** brain ischemia and reperfusion, eIF2 $\alpha$ , GCN2, HRI, PERK, PKR.

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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  $\alpha$ -subunit of eukaryotic initiation factor 2 (eIF2 $\alpha$ ) (Burda *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 guanine nucleotide exchange protein, eIF2B, is responsible for removing GDP from eIF2 at the end of an initiation cycle, but eIF2 phosphorylated on the  $\alpha$ -subunit [eIF2 $\alpha$ (P)] sequesters eIF2B and thus obstructs subsequent rounds of translation initiation.

There are four known eIF2 $\alpha$  kinases (GCN2, HRI, PKR and PERK) that, either individually or in combination, could mediate post-ischemic eIF2 $\alpha$  phosphorylation. Previous work showed that the RNA-dependent eIF2 $\alpha$  kinase (PKR) is not required for post-ischemic eIF2 $\alpha$  phosphorylation (DeGracia *et al.* 1999). The heme-regulated eIF2 $\alpha$  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 eIF2 $\alpha$  phosphorylation. The amino acid-regulated eIF2 $\alpha$  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 eIF2 $\alpha$  kinase (PERK, also known as PEK – pancreatic eIF2 $\alpha$  kinase) is most highly expressed in pancreas but is also expressed in brain (Shi *et al.* 1998). PERK is activated by the

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**Abbreviations used:** BCAA, bilateral carotid artery occlusion; ER, endoplasmic reticulum; eIF2, eukaryotic initiation factor 2; FS, forebrain supernatant; GCN2, general control non-derepressible-2; HRI, heme-regulated inhibitor; IP, immunoprecipitation; MEF, mouse embryo fibroblast; PERK, PKR-like ER kinase; PKR, RNA-dependent protein kinase; tg, thapsigargin.

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 eIF2 $\alpha$ (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 eIF2 $\alpha$  during early reperfusion, supporting the hypothesis that PERK activation is responsible for phosphorylation of eIF2 $\alpha$  in the early reperused 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 eIF2 $\alpha$  phosphorylation (Harding *et al.* 1999). We have characterized the antibody specific for Ser51 phosphorylated eIF2 $\alpha$  (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 deleting 413 nucleotides from exon 12 that encodes essential regions of the kinase catalytic domain. 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 domains, from VIIb 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 H<sub>2</sub>O. A warming pad and overhead lamp were used to maintain rectal temperature at 37.0  $\pm$  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 w/v 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  $\mu$ M okadaic acid, 0.23 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/mL aprotinin, 10  $\mu$ g/mL leupeptin and 7  $\mu$ g/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  $-80^{\circ}\text{C}$  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-eIF2 $\alpha$ (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  $\mu$ L of an unrelated antibody (lamin A/C; Santa Cruz Biotechnology) plus 20  $\mu$ L Protein A-Sepharose beads (Zymed Laboratories). Precleared supernatants were incubated overnight at 4°C with end-over-end rotation with 15  $\mu$ L Protein A-Sepharose beads prebound with 1  $\mu$ L PERK antiserum. Proteins were eluted from the beads, subjected to 6% SDS-PAGE, electroblot transferred to nitrocellulose and immunoblotted with PERK antiserum as above.

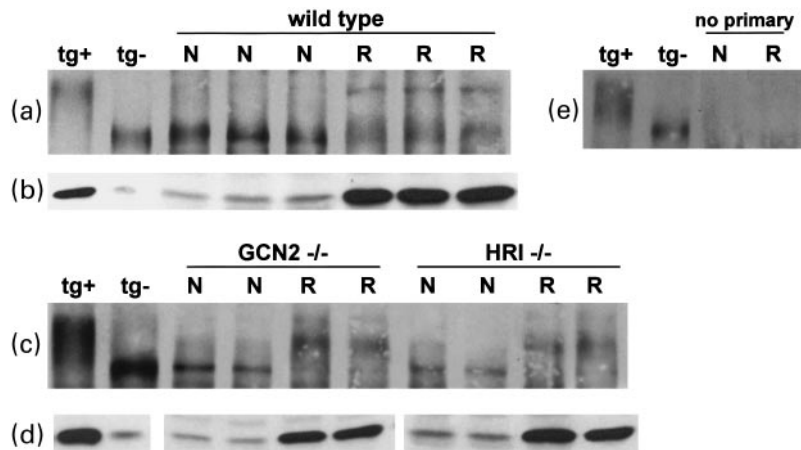
## Results

Ischemia and reperfusion induced a mean 10-fold increase in eIF2 $\alpha$ (P) in all animal groups (Figs 1b and d), and levels of eIF2 $\alpha$ (P) in HRI $^{-/-}$  and GCN2 $^{-/-}$  following ischemia and reperfusion were indistinguishable from those seen in the wild type. Note that there is very little eIF2 $\alpha$ (P) in untreated MEFs [thapsigargin- (tg-)] in contrast to the strong response to thapsigargin (tg+).

The activity of the eIF2 $\alpha$  kinase PERK correlates with its phosphorylation status, and the phosphorylated active forms of PERK have reduced mobility on SDS-PAGE (Harding *et al.* 1999, 2000). In lysates from reperused brain there was a shift in immunoprecipitated PERK mobility from a higher mobility species, consistent with the inactive hypophosphorylated form of the protein, to a lower mobility form comigrating with the hyperphosphorylated-activated forms of the kinase present after treatment of MEFs with thapsigargin (Figs 1a and c). Immunoblots from non-ischemic mice show a single signal that comigrates with inactive PERK. In contrast, all reperused groups show loss of the high-mobility, inactive, PERK species and acquisition of a lower mobility species 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 eIF2 $\alpha$  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 eIF2 $\alpha$  is caused by phosphatase inactivation during ischemia/reperfusion, DeGracia *et al.* (1999) found no reduction in eIF2 $\alpha$ (P) phosphatase activity in reperused brain homogenates. An alternative mechanism that could be involved in the phosphorylation of



**Fig. 1** Immunoblots for PERK (a and c) and eIF2 $\alpha$ (P) (b and d). Western blots were prepared using brain lysates from either non-ischemic controls (N) or following 20-min BCAA and 10 min reperfusion (R) in GCN2 $^{-/-}$ , HRI $^{-/-}$  and C57BL/6 wild-type mice. GCN2 and HRI deletions had no effect on eIF2 $\alpha$  phosphorylation induced by brain ischemia and reperfusion. There is very little eIF2 $\alpha$ (P) and activated PERK in untreated MEFs (tg $^{-}$ ) in contrast to the strong response to thapsigargin (tg $^{+}$ ), which induces the mobility shift of PERK activation (a and c) and concurrent eIF2 $\alpha$  phosphorylation (b and d). In reperfused brain, there is loss of the inactive form of PERK with a concurrent shift to a lower mobility form comigrating with activated PERK, and concurrent with large increases in eIF2 $\alpha$ (P) (see text). Control immunoprecipitations were carried out in the absence of PERK antiserum and produced no signal in the region of PERK mobility (E).

eIF2 $\alpha$  is inactivation (by deglycosylation) of an inhibitor p78 that normally binds eIF2 and precludes eIF2 $\alpha$  phosphorylation (Datta *et al.* 1989). However, Owen *et al.* (2001) found no reperfusion-induced loss of total or glycosylated p78 in western blots or immunohistochemical staining in vulnerable neurons. Therefore, we sought evidence for involvement of an eIF2 $\alpha$  kinase activated by ischemia/reperfusion.

There are four known mammalian eIF2 $\alpha$  kinases: GCN2, HRI, PKR and PERK. HRI $^{-/-}$  and GCN2 $^{-/-}$  mice are shown here to have levels of post-ischemic eIF2 $\alpha$  phosphorylation indistinguishable from wild-type levels, thus adding these two eIF2 $\alpha$  kinases to PKR (DeGracia *et al.* 1999) in the list of kinases that do not have an essential role in eIF2 $\alpha$  phosphorylation during early reperfusion. By contrast, the eIF2 $\alpha$  kinase PERK is shown here to be in its activated form during reperfusion and is correlated with eIF2 $\alpha$  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 Ca $^{2+}$  depletion, but also lowered ATP levels or altered ER redox state (Bertolotti *et al.* 2000). Paschen and Doutheil (1999) have suggested that ER Ca $^{2+}$  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 eIF2 $\alpha$  kinases beyond the four listed above. These observations build a strong circumstantial case for PERK as the eIF2 $\alpha$  kinase mediating the inhibition of protein synthesis during early reperfusion.

Further studies will be needed to delineate the roles of the eIF2 $\alpha$  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 eIF2 $\alpha$  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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