

REVIEW

Cerebral ischemia and the unfolded protein response

Donald J. DeGracia*[†] and Heather L. Montie*

*Department of Physiology, and the [†]Center for Molecular Medicine and Genetics², Wayne State University School of Medicine, Detroit, Michigan, USA

Abstract

We review studies of endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) following cerebral ischemia and reperfusion (I/R). The UPR is a cell stress program activated when misfolded proteins accumulate in the ER lumen. UPR activation causes: (i) a PERK-mediated phosphorylation of eIF2 α , inhibiting protein synthesis to prevent further accumulation of unfolded proteins in the ER and (ii) upregulation of genes coding for ER-resident enzymes and chaperones and others, via eIF2 α (p), and ATF6 and IRE1 activation. UPR-induced transcription increases capacity of the ER to process misfolded proteins. If ER stress and the UPR are prolonged, apoptosis ensues. Multiple forms of ER stress have been observed following brain I/R. The UPR

following brain I/R is not isomorphic between *in vivo* I/R models and *in vitro* cell culture systems with pharmacological UPR induction. Although PERK and IRE1 are activated in the initial hours of reperfusion, total PERK decreases, ATF6 is not activated, and there is delayed appearance of UPR-induced mRNAs. Thus, multiple damage mechanisms associated with brain I/R alter UPR expression and contribute to a pro-apoptotic phenotype in neurons. Insights resulting from these studies will be important for the development of therapies to halt neuronal death following brain I/R.

Keywords: Brain ischemia and brain reperfusion, CHOP, IRE1, PERK, protein synthesis inhibition, unfolded protein response.

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It has been known for three decades that protein synthesis inhibition (PSI) occurs in brain neurons during reperfusion following global and focal ischemia (Hossmann 1993). The first identified molecular mechanisms of PSI were traced to signaling modifications in the eukaryotic initiation factors (eIF) that control global rates of protein synthesis: eIF2 and eIF4 (DeGracia *et al.* 2002). This raised the question as to how cerebral ischemia and reperfusion (I/R) lead to signaling modifications in the translational machinery of the neuron. An independent line of investigation revealed that depletion of endoplasmic reticulum (ER) Ca²⁺ stores, a form of ER stress, caused PSI (Brostrom and Brostrom 1998). This observation was brought to bear on PSI following brain I/R, with the suggestion that ER stress is one of the cellular pathologies induced by ischemia (Paschen 1996). Subsequently, a set of interlinked molecular pathways activated in response to ER stress have been described, and collectively these pathways are referred to as the unfolded protein response (UPR; Kaufman 1999). The purpose of the present review is to critically evaluate studies showing that ER stress and UPR activation occur following brain I/R. A major result of these studies is that expression of the UPR is not

isomorphic between *in vivo* I/R models and *in vitro* cell culture systems where the UPR is pharmacologically induced. Although PERK and IRE1 are activated during initial reperfusion, total PERK levels decrease, ATF6 is not activated, and there is a delay in appearance of UPR-induced mRNAs following brain I/R. These observations indicate that

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Address correspondence and reprint requests to Dr D. J. DeGracia, Department of Physiology, Wayne State University, 3125 Scott Hall, 540 East Canfield Ave, Detroit, MI 48201, USA.

E-mail: ddegraci@med.wayne.edu

Abbreviations used: ATF, activating transcription factor from the basic leucine zipper family; BCAA, bilateral carotid artery occlusion; CHOP, C/EBP homology protein; eIF, eukaryotic initiation factor; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum associated protein degradation; ERSE, endoplasmic reticulum stress response element; GRP78, glucoseregulated protein 78; I/R, ischemia and reperfusion; IRE1 α , inositol requiring ER transmembrane RNase α isoform; NOS, nitric oxide synthase; PERK, PKR-like ER eIF2 α kinase; PKR, RNA-dependent protein kinase; PSI, protein synthesis inhibition; SERCA, sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase; SNAP, S-nitroso-N-acetyl-penicillamine; UPR, unfolded protein response; XBP-1, x-box binding protein.

the multiple damage mechanisms associated with brain I/R alter the expression of the UPR and contribute to a proapoptotic phenotype in neurons.

The unfolded protein response

The ER lumen is a 'factory' performing post-translation modification on nascent secreted and transmembrane proteins. Nascent peptides undergo several ATP and Ca^{2+} -dependent post-translational modifications in the ER compartment: disulfide bond formation, N-linked glycosylation, controlled proteolysis, and chaperone-mediated formation of final conformation (Kaufman 1999). There is also a quality control system for detecting and eliminating improperly processed proteins, ER-associated protein degradation (ERAD), where the misfolded peptide is transported in reverse through the translocon, ubiquitinated, and degraded in the proteasome (Brodsky and McCracken 1999). As each of these processes is critical to proper peptide folding, their disruption results in accumulation of misfolded proteins in the ER lumen.

Eukaryotic cells have evolved a set of molecular pathways for coping with ER stress, collectively termed the UPR (Fig. 1). The UPR is activated when there is disruption of any of the above-mentioned processes, or when the ER is depleted of Ca^{2+} or adenosine triphosphate (ATP; Kaufman 1999). There is an evolutionary development of the UPR

pathways from yeast to metazoans (Harding *et al.* 2002). However, as this review is concerned with the UPR in brain, we will focus exclusively on the metazoan UPR. The UPR is carried out by three ER-transmembrane effector proteins: PERK, IRE1, and ATF6. All three effectors bind the ER chaperone GRP78 on their ER luminal domains, where GRP78 acts to repress their activity (Bertolotti *et al.* 2000; Shen *et al.* 2002). Accumulation of misfolded proteins in the ER lumen provides a mass action effect for the dissociation of GRP78 from the three effectors, allowing their activation.

When activated, PERK, IRE1, and ATF6 carry out the two main functions of the UPR (Ron and Harding 2000). First, protein synthesis is inhibited to prevent further accumulation of improperly processed proteins in the ER lumen. PERK phosphorylates the alpha subunit of eIF2 [eIF2 α ; phosphorylated form eIF2 α (P)], causing PSI by reducing the amount of initiator tRNA delivered to the ribosome (DeGracia *et al.* 2002). Second, the mammalian UPR involves upregulation of at least 18 genes (Okada *et al.* 2002), which can be broadly broken into two categories (Oyadomari and Mori 2004). One category involves ER chaperone and ER enzyme genes (e.g. GRP78, PDI) induced via parallel activation of ATF6 and IRE1 α . The other category of genes, discussed below, derives from eIF2 α phosphorylation and can be expressed independent of the UPR (Harding *et al.* 2000).

With GRP78 dissociation, ATF6 translocates to the Golgi membrane where proteases S1P and S2P cleave off the 50-kDa cytosolic domain (Haze *et al.* 1999). The 50-kDa cytosolic ATF6 translocates to the nucleus and binds the ERSE (ER stress response element) promoter sequence contained in genes for ER-resident proteins (Roy and Lee 1999) and upregulates their transcription.

Activated IRE1 α (the ubiquitous isoform) is an mRNA endoribonuclease with one known substrate: the mRNA coding for the transcription factor XBP-1 (Yoshida *et al.* 2001; Calfon *et al.* 2002; Lee *et al.* 2002). Active IRE1 α cleaves a 26 nt 'non-classical intron' from *xbp-1* mRNA in a reaction homologous to tRNA transcript processing (Gonzalez *et al.* 1999). The cleaved *xbp-1* mRNA is ligated by tRNA ligase. This cleavage and ligation operation, called 'processing', results in an *xbp-1* mRNA that is more efficiently translated to produce a stable 54 kDa protein product (Yoshida *et al.* 2001; Calfon *et al.* 2002). The 54-kDa XBP-1 protein is an ERSE-binding transcription factor with overlapping binding specificity to ATF6 (Lee *et al.* 2003). Further, ATF6 itself induces *xbp-1* transcription (Yoshida *et al.* 2001). Hence, it is thought that ATF6 represents a fast track to UPR-mediated transcription as it requires only cleavage and nuclear translocation, whereas IRE1 α provides a longer UPR transcriptional response, as it requires translation of XBP-1. Another important function of active IRE1 α is that it has been shown to be involved in activation of ER-resident caspase 12 (Yoneda *et al.* 2001).

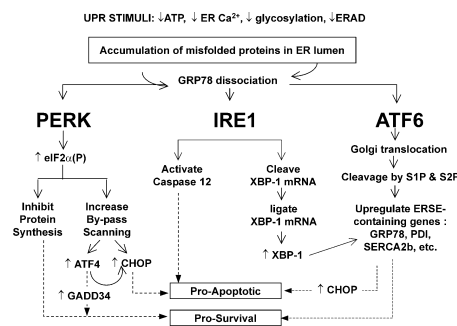


Fig. 1 Diagram of the UPR network. Several inputs (stimuli) induce ER stress including depletion of ER Ca^{2+} , decreased ATP, and inhibition of disulfide bond formation, glycosylation, or ERAD. These stimuli activate the three UPR effectors. PERK, an eIF2 α kinase, phosphorylates eIF2 α leading to inhibition of protein synthesis, but also to the paradoxical increased translation of ATF4 and CHOP transcription factors. ATF4 contributes to the induction of the eIF2 α (p) phosphatase targeting subunit GADD34. Active IRE1 α cleaves a 26 nt sequence from *xbp-1* mRNA, and the tRNA ligase-ligated transcript leads to enhanced translation of XBP-1 protein, a transcription factor for ER-resident enzymes and chaperones. Active IRE1 α is also involved in caspase 12 activation. ATF6 activation involves Golgi translocation and cleavage of its 50 kDa cytosolic domain to produce an active transcription factor for ER-resident enzymes and chaperones. The UPR can lead to a pro-survival or a pro-apoptotic phenotype.

Gene transcription mediated by eIF2 α phosphorylation occurs in parallel with that of ATF6 and XBP-1. Phosphorylated eIF2 α increases translation of rare mRNAs containing upstream open reading frames by a process termed bypass scanning (Dever *et al.* 1995). The mRNA coding for the transcription factor ATF4 has been shown to be translated by this means (Harding *et al.* 2000). ATF4 induces genes for lipid and amino acid biosynthesis, among others (Harding *et al.* 2002). Phosphorylation of eIF2 α is also linked to induction of GADD34, a protein phosphatase-1 targeting subunit that plays a role in dephosphorylating eIF2 α (P) and ending UPR-induced PSI (Novoa *et al.* 2001).

UPR activation also leads to transcription and translation of CHOP (GADD153). The *chop* promoter is complex, and both ATF4 and XBP-1/ATF6 can contribute to *chop* transcription (Ma *et al.* 2002). Appearance of *chop* mRNA is a hallmark of ER stress and a marker of UPR activation. CHOP is a transcription factor (Ubeda *et al.* 1999), and its expression is generally associated with apoptosis (Zinszner *et al.* 1998; McCullough *et al.* 2001).

Therefore, by downregulating translation and upregulating transcription of ER-resident enzymes and chaperones, the UPR acts primarily to protect against ER stress. However, sustained activation of UPR effectors can lead to an apoptotic phenotype via CHOP transcription and translation, and by caspase 12 activation. It is this 'double edged sword' feature of the UPR that has made it an attractive model as a contributor to cell death following brain I/R (DeGracia *et al.* 2002). If indeed cerebral ischemia causes ER stress and activates the UPR, the resultant molecular cascades could contribute to either cell survival or cell death. Emerging evidence supports this hypothesis. We first discuss evidence of ER stress and then summarize results pertaining to activation of the UPR following brain I/R.

ER stress following brain ischemia and reperfusion

The observation of polysome disaggregation and PSI reported by Kleihues and Hossmann (1971) is now interpreted as an indicator of ER stress (Paschen 1996). Electron microscopy revealed alterations of ER and Golgi morphology in reperused neurons where these lost their 'flattened pancake' appearance and became rounded vesicles (Petito and Pulsinelli 1984). We showed accumulation of lipid peroxidation products in these structures (White *et al.* 1993). The most recent microscopy work has shown accumulation of protein aggregates in reperused neurons that persisted in vulnerable CA1 pyramidal neurons but dissipated in the more resistant dentate gyrus granular cells (Hu *et al.* 2001). Hu *et al.* (2000) has also shown accumulation of Triton X-100-insoluble, highly ubiquitinated proteins, which correlates with the presence of the aggregates, suggesting a defect in protein degradation.

There is direct evidence of ER stress following brain I/R. Parsons *et al.* (1997) showed a decrease in SERCA (sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase) activity in microsomes from ischemic brain. Further studies (Parsons *et al.* 1999) showed an uncoupling of SERCA Ca²⁺ transport from ATP hydrolysis. Kohno *et al.* (1997), using Ca²⁺-oxalate-pyruantimonate staining, were able to detect disappearance of Ca²⁺ deposits in the ER of reperused hippocampal CA1 neurons. Significantly, this effect could be avoided by pre-treatment with the nitric oxide synthase (NOS) inhibitor N ω -nitro-L-arginine (L-NNA). The results of Parsons *et al.* (1997, 1999) are consistent with Kohno *et al.* (1997), and together indicate dysfunction of ER Ca²⁺ handling following brain I/R. The relative dearth of studies addressing this issue invites further work. Does ER Ca²⁺ depletion affect all neuronal populations, or is it confined to specific brain regions? What is the time course for this phenomena in different brain regions? Is there a strong correlation between ER Ca²⁺ depletion and SERCA dysfunction? How do ER Ca²⁺ levels correlate with protein synthesis inhibition?

In conclusion, current studies suggest that multiple causes of ER stress occur in neurons following cerebral I/R: depletion of ER Ca²⁺, aggregation of proteins, decreased protein degradation, and accumulation of lipid peroxidation products in ER and Golgi structures. This presents a situation substantially different from cell culture studies of ER stress and the UPR, where single pharmacologic agents are used to induce one specific form of ER stress. It is therefore of interest to ask if multiple forms of ER stress cause a more intense UPR, or are there unexpected synergies that modify the pattern of UPR expression when multiple forms of stress converge on the ER simultaneously? Paschen *et al.* (2001) investigated the simultaneous effect of ER stress by thapsigargin (a SERCA inhibitor that leads to ER Ca²⁺ depletion) and oxidative stress via H₂O₂. Pre-exposure of primary neuronal cultures to H₂O₂ followed by thapsigargin completely blocked transcription of *grp78*, *grp94*, and *chop* mRNAs. This result indicates that multiple stresses interfere with the execution of the UPR program. Continued work is needed in this area.

Expression of the UPR network following brain reperfusion

The UPR can be viewed as a chemical network (Fig. 1), with inputs (ER stress), convergent nodal points (the three effectors), and outputs (PSI, gene transcription products, survival or apoptotic phenotypes). Clearly then, characterization of the UPR in the reperused brain, and establishment of causality, necessitates evaluation of each of these network components. To date, different aspects of the UPR network have been characterized in specific studies.

A well characterized aspect of UPR expression following brain reperfusion revolves around PSI. It is now established

that neuronal PSI in the initial hours of reperfusion is due to a large increase in PERK-mediated eIF2 α phosphorylation (reviewed in DeGracia *et al.* 2002). Following global ischemia, PSI initially encompasses the entire brain, but translation does not recover in vulnerable brain regions such as hippocampal CA1 (Hossmann 1993). There is a growing concern that this persistent PSI is not related to the UPR. Levels of eIF2 α (P), but not eIF2 α , fall by 6–8 h reperfusion (Althausen *et al.* 2001; Kumar *et al.* 2003). Studies have shown rises in both mRNA levels (Doutheil *et al.* 1999; Jin *et al.* 2001) and protein levels (Imai *et al.* 2002; Garcia *et al.* 2004) of GADD34 during the initial hours of reperfusion, which is expected to contribute to eIF2 α (P) dephosphorylation. In spite of eIF2 α (P) dephosphorylation, PSI persists (Althausen *et al.* 2001; Kumar *et al.* 2003). The levels of eIF4E and eIF4G, the initiation factors responsible for delivering the mRNA to the ribosome during translation initiation, have also been shown to decrease following several hours of reperfusion (Martin de la Vega *et al.* 2001; Mengesdorf *et al.* 2002), indicating that persistent PSI is due to multiple causes (Garcia *et al.* 2004).

The issue of reperfusion-induced PSI is important as there has been increased application of gene expression technology to reperfused brain (e.g. DNA microarray analysis). Such studies have revealed that reperfused brain expresses a number of repair programs via altered gene transcription, including upregulation of heat shock proteins, antioxidant enzymes, ATP conservation, etc. (Schmidt-Kastner *et al.* 2002). However, none of these programs can be effected without protein synthesis. This consideration leads to a significant question about reperfusion-induced PSI: is it protective or detrimental? This issue has recently been discussed in detail in the context of the protein synthesis requirements of apoptosis versus those of the UPR (Paschen 2003). In the scope of the present article, the concern revolves around the observation that UPR-induced PSI is clearly a protective, but temporary, response. PSI must abate to allow translation of the majority of UPR-induced transcripts (Yoshida *et al.* 2003). That multiple causes contribute to PSI following brain I/R, indicates that viewing PSI as a homogeneous phenomena will not be helpful. UPR-mediated PSI may be protective to reperfused brain by helping to abate ER stress, but prolonged, non-UPR-mediated PSI would be expected to be deleterious and prevent the successful execution of endogenous cerebral genetic responses to I/R injury. Thus, characterizing all factors contributing to PSI following brain I/R will be important for developing rational pharmacologic interventions, and each factor is expected to have its specific role in neuronal survival or death.

A major indicator of UPR activation following brain I/R is the observation of PERK activation (Kumar *et al.* 2001; Hayashi *et al.* 2003, 2004). PERK is activated completely by 10-min reperfusion following 10-min global ischemia

(Kumar *et al.* 2003). We ruled out the possibility of activation of multiple eIF2 α kinases during I/R by showing wild-type levels of eIF2 α phosphorylation in HRI, GCN2, and PKR knockouts (DeGracia *et al.* 1999; Kumar *et al.* 2001). Following cardiac arrest, we observed loss of PERK by 90-min reperfusion (Kumar *et al.* 2003). We showed PERK to be an *in vitro* calpain substrate (Kumar *et al.* 2003), but we found no evidence of calpain activation in the animals in which PERK was degraded (unpublished observation). Hayashi *et al.* (2003), following 5-min bilateral carotid artery occlusion (BCAO), quantitated only phosphorylated PERK levels, but their data show substantially decreased total PERK levels, even at 2 days reperfusion. Thus, the studies that have measured total PERK levels indicate PERK is being lost during reperfusion. As UPR-induced PSI is generally protective, loss of PERK would be expected to be detrimental if there is sustained ER stress in the reperfused brain. Because there is an \sim 25-fold increase in eIF2 α (P) by 90-min reperfusion (DeGracia *et al.* 1996), that eIF2 α (P) levels persist after PERK is lost in the first several hours of reperfusion indicate that the rate of eIF2 α (P) dephosphorylation is slower than the rate of PERK loss.

What causes PERK activation? The study by Hayashi *et al.* (2003) showed decrease PERK activation and decreased oxidative damage to ER membranes in SOD-overexpressing rats compared to wild-type, indicating a role for free radicals in PERK activation. Consistent with this observation, when we performed brief BCAO on eNOS and nNOS knockout mice, PERK activation and eIF2 α (P) formation were blocked in the knockouts, but not in the wild-type mice (Figs 2a and b). Further, SNAP administration to neuroblastoma cells also activated PERK (Fig. 2b). It has been shown that NO induces eIF2 α (P) and PSI (Kim *et al.* 1998), as well as inactivation of SERCA and depletion of ER calcium stores (Doutheil *et al.* 2000). Thus, PERK activation following brain I/R appears to involve, at minimum, NO and free radical production.

With respect to the transcriptional effectors of the UPR, ATF6 is not activated by 4 h of reperfusion following 10 min global brain ischemia (Kumar *et al.* 2003). We observed IRE1 α to undergo a modification consistent with its activation at 90 min reperfusion (Kumar *et al.* 2003). However, we did not detect 54 kDa XBP-1, CHOP or ATF4 proteins by 4 h reperfusion. Paschen *et al.* (2003), reported processing of *xbp-1* mRNA at 1–2 h reperfusion following global and focal brain ischemia, and reported detection of 54 kDa XBP-1 at 6 h reperfusion following focal, but not global, ischemia. Tajiri *et al.* (2004) also showed processing of *xbp-1* mRNA by 2 h reperfusion following 15 min BCAO in mouse. Hence, IRE1 α is activated and *xbp-1* mRNA is processed in the initial hours of brain reperfusion. Activation of IRE1 α would also be expected to lead to caspase 12 activation. Indeed, evidence of caspase 12 activation has been presented following both transient (Shibata *et al.* 2003) and permanent

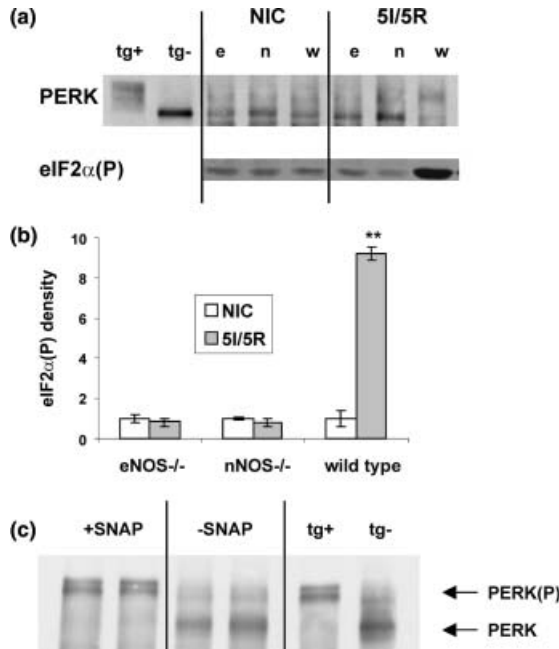


Fig. 2 (a) Following 5 min bilateral carotid occlusion (BCAO) and 5 min reperfusion, PERK is not activated nor is eIF2 α phosphorylated in eNOS (e) and nNOS (n) knockout mice compared to wild type C57Bl6 (w) mice. Therefore, NO production may be a causal event in PERK activation. PERK activation is determined by an upward mobility shift following sodium dodecyl sulfate polyacrylamide gel electrophoresis (Kumar *et al.* 2001). (b) There is no change in eIF2 α (P) levels between non-ischemic controls (\square , NIC) and 5 min BCAAO ischemia and 5 min reperfusion (\blacksquare , 5I/5R) in eNOS and nNOS knockout mice, although there is a 10-fold increase in eIF2 α (P) in the 5I/5R wild-type mice. ($n = 3$ per group, $**$ ANOVA $p = 3 \times 10^{-10}$). (c) Treatment of neuroblastoma 104 (NB104) cells with 1 mM SNAP for 3 h activated PERK. In both (a) and (c), tg⁺ and tg⁻ are thapsigargin-treated and -untreated, respectively, NB104 cells, used as controls for PERK activation.

(Mouw *et al.* 2003) focal ischemia, but has not yet been measured in a global model of brain I/R.

What of the UPR-induced mRNAs? In Paschen *et al.* (2003), levels of *grp78*, *grp94*, and *chop* (*gadd153*) transcripts showed no more than twofold increase by 6 h reperfusion, whereas *hsp-70* showed roughly a 600-fold increase. Previous results from this group showed a sixfold increase in *chop* mRNA at 4–8 h reperfusion in the hippocampus, but not cerebral cortex, following 30 min 4-vessel occlusion global ischemia (Paschen *et al.* 1998). Following 15 min BCAAO in mice, Tajiri *et al.* (2004) showed appearance of *chop* and *grp78* mRNA at 6 h reperfusion that peaked at 12–24 h reperfusion. A third study showed maximal levels of *grp94* transcript at 12 h reperfusion following BCAAO (Kim *et al.* 2003). In comparison, activation of the UPR by thapsigargin in primary neuronal culture caused a 10-fold increase in *grp78* and

chop mRNA at 3 h post-treatment (Mengesdorf *et al.* 2001). Hence, the appearance of UPR-induced mRNA takes several hours longer to manifest in the reperfused brain compared to pharmacologically manipulated cell culture systems.

In the study of Tajiri *et al.* (2004), CHOP protein was observed in neuronal nuclei of the hippocampus and striatum by 24 h reperfusion, the degree of which correlated with the amount of apoptosis at 48 h reperfusion. Significantly, Tajiri *et al.* (2004) showed substantial attenuation of cell death in the hippocampus and striatum of CHOP homozygous knockout mice at 7 days following 15 min BCAAO. This result strongly supports the idea that CHOP induction plays a causal role in neuron death following brain I/R.

To summarize, during initial brain reperfusion, PERK is fully activated but its levels eventually decline, ATF6 is not activated, and IRE1 is activated and processes *xbp-1* mRNA. The effector functions of PERK, eIF2 α phosphorylation and PSI, occur immediately with reperfusion. The transcription of UPR-induced mRNAs is delayed by many hours in brain I/R, as compared to pharmacologic UPR induction in cell cultures.

The reasonable suggestion has been made that the delayed transcription of UPR-induced mRNAs is due to the severe block in translation during the initial hours of reperfusion, preventing the synthesis of 54 kDa XBP-1 protein needed to induce transcription (Paschen *et al.* 2003). However, XBP-1 is not the only transcription factor contributing to UPR-induced mRNA transcription. We suggest that a second contributor to delayed UPR transcription following brain I/R is lack of activation of ATF6 within the initial hours of reperfusion. ATF6 represents a rapid response to induce transcription during the UPR, as ATF6 activation is not protein synthesis-dependent (Haze *et al.* 1999). Further, ATF6 induces *xbp-1* mRNA to augment the IRE1-mediated transcriptional response (Yoshida *et al.* 2001). Thus, it is important to understanding why ATF6 is not activated following brain I/R. It is possible that free radical damage to ER membranes (White *et al.* 1993; Hayashi *et al.* 2003) precludes activation of ATF6 during reperfusion. By not expressing a complete transcriptional response involving both ATF6 and XBP-1, coupled to delayed translation of XBP-1, these factors may tip the scales in the reperfused brain towards pro-apoptotic transcription of CHOP and hence cell death, a notion supported by the results of Tajiri *et al.* (2004).

Conclusion

There is evidence for both ER stress and UPR activation following brain I/R. However, the time courses of UPR effector activation and expression of output are different from that seen in pharmacologic activation of the UPR in cell culture systems. Cerebral I/R is providing the first well-investigated example of

the UPR in an *in vivo*, clinically relevant pathological condition. The main lesson emerging from *in vivo* brain I/R studies is that multiple mechanisms are contributing to ER stress and likely limiting UPR-induced transcription. UPR expression following brain I/R is also expected to be modified by non-ER forms of I/R-induced cellular damage (White *et al.* 2000), including free radical production, proteolysis, alterations in kinase and phosphatase activity, and non-UPR-mediated PSI, all of which may converge on components of the UPR network and attenuate their activity.

In conclusion, further work should be directed towards: (i) continuing to characterize the time course and regional localization of UPR network components in the reperfused brain; (ii) continued studies into how other cell stresses and multiple forms of ER stress can alter the function of the UPR components; (iii) teasing apart the potentially multiple causes of ER stress occurring in the reperfused brain; (iv) delineating UPR and non-UPR contributions to PSI in the reperfused brain; and (v) determining why brain I/R activates PERK and IRE1 α , but not ATF6, and why PERK levels decrease. By clearly discerning these factors, it is expected that future therapies directed towards improving neuronal survival following brain I/R would be targeted towards enhancing the efficacy of the UPR, and other endogenous stress responses, and eliminating damage mechanisms that interfere with the successful execution of cell stress responses.

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