

PERK (Protein Kinase RNA-Like ER Kinase) Branch of the Unfolded Protein Response Confers Neuroprotection in Ischemic Stroke by Suppressing Protein Synthesis

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Background and Purpose—Ischemic stroke impairs endoplasmic reticulum (ER) function, causes ER stress, and activates the unfolded protein response. The unfolded protein response consists of 3 branches controlled by ER stress sensor proteins, which include PERK (protein kinase RNA-like ER kinase). Activated PERK phosphorylates eIF2 α (eukaryotic initiation factor 2 alpha), resulting in inhibition of global protein synthesis. Here, we aimed to clarify the role of the PERK unfolded protein response branch in stroke.

Methods—Neuron-specific and tamoxifen-inducible PERK conditional knockout (cKO) mice were generated by cross-breeding Camk2a-CreERT2 with *Perk*^{fl/fl} mice. Transient middle cerebral artery occlusion was used to induce stroke. Short- and long-term stroke outcomes were evaluated. Protein synthesis in the brain was assessed using a surface-sensing-of-translation approach.

Results—After tamoxifen-induced deletion of *Perk* in forebrain neurons was confirmed in PERK-cKO mice, PERK-cKO and control mice were subjected to transient middle cerebral artery occlusion and 3 days or 3 weeks recovery. PERK-cKO mice had larger infarcts and worse neurological outcomes compared with control mice, suggesting that PERK-induced eIF2 α phosphorylation and subsequent suppression of translation protects neurons from ischemic stress. Indeed, better stroke outcomes were observed in PERK-cKO mice that received postischemic treatment with salubrinal, which can restore the ischemia-induced increase in phosphorylated eIF2 α in these mice. Finally, our data showed that post-treatment with salubrinal improved functional recovery after stroke.

Conclusions—Here, we presented the first evidence that postischemic suppression of translation induced by PERK activation promotes recovery of neurological function after stroke. This confirms and further extends our previous observations that recovery of ER function impaired by ischemic stress critically contributes to stroke outcome. Therefore, future research should include strategies to improve stroke outcome by targeting unfolded protein response branches to restore protein homeostasis in neurons.

Visual Overview—An online [visual overview](#) is available for this article. (*Stroke*. 2020;51:1570-1577. DOI: 10.1161/STROKEAHA.120.029071.)

Key Words: animals ■ mice ■ neurons ■ neuroprotection ■ proteostasis

Cerebral ischemia/reperfusion injury can drastically impact protein synthesis in the brain. Kleihues and Hossmann¹ first reported that protein synthesis is severely suppressed during early reperfusion after complete forebrain ischemia. When reperfusion is extended, protein synthesis recovers substantially in the cerebral cortex, which is relatively resistant to forebrain ischemia. Follow-up studies found a close relationship between neuronal survival and the capacity of post-ischemic neurons to restore the translational machinery.² Therefore, a better understanding of protein synthesis in the postischemic brain is of pivotal importance.³

It has long been established that postischemic inhibition of protein synthesis is caused by a block of translation at the initiation process.⁴ Translation initiation is controlled by the phosphorylation state of a variety of initiation factors among which the alpha subunit of eIF2 (eukaryotic initiation factor 2) plays a central role.³ In the physiological state, only a small fraction of eIF2 α is phosphorylated. A variety of severe forms of cellular stress trigger massive phosphorylation of eIF2 α , which then suppresses the GDP/GTP exchange factor and thereby blocks the initiation process of translation, leading to suppression of global protein synthesis. Indeed, levels of

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eIF2 α phosphorylation (p-eIF2 α) were found to be increased after transient brain ischemia^{5,6}; however, the underlying mechanisms were not known.

We hypothesized, >20 years ago, that postischemic shutdown of translation results from impairment of endoplasmic reticulum (ER) function caused by ischemic stress, because neurons react to transient ischemia and to conditions associated with ER stress in a very similar way.⁷ ER stress activates the unfolded protein response (UPR), which comprises 3 adaptive stress response pathways.⁸ These 3 UPR branches are mediated by stress sensor proteins located in the ER membrane: ATF6 (activating transcription factor 6), IRE1 (inositol-requiring enzyme-1), and the PERK (protein kinase RNA-like ER kinase). Importantly, upon activation, PERK specifically phosphorylates eIF2 α .⁹ Over the past decades, a large body of evidence has accumulated to support our hypothesis. Many studies have shown that protein homeostasis (proteostasis) in brain cells is disrupted after either global or focal brain ischemia (ischemic stroke), which results in ER stress and subsequent UPR activation, including the PERK UPR branch.^{10–15} Recently, we demonstrated that activation of the ATF6 or IRE1 UPR branch is neuroprotective in experimental stroke.^{12,13} Thus, only the role of the PERK pathway in stroke outcome remains largely unknown. Here, we aimed to determine this role by using, for the first time in experimental stroke, a PERK conditional knockout (cKO) mouse model in which deletion of *Perk* in forebrain neurons was induced by tamoxifen. We provide evidence that activation of the PERK UPR branch in experimental stroke is neuroprotective, which is due to PERK-induced p-eIF2 α and subsequent suppression of global protein synthesis.

Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request. Detailed Methods are available in the [Data Supplement](#).

Animals

Animal experiments were approved by the Duke University Animal Care and Use Committee. C57Bl/6, *Perk*-cKO (*Perk*^{fl/fl}, C57Bl/6 background), and Cre driver Camk2a-CreERT2 mice (C57Bl/6 background) were purchased from The Jackson Laboratory (Maine). To generate experimental mice with inducible deletion of *Perk* in forebrain neurons, we crossed *Perk*^{fl/fl} mice with Camk2a-CreERT2 mice expressing the tamoxifen-inducible CreERT2 under the control of the neuron-specific Camk2a promoter and obtained *Perk*^{fl/fl};Camk2a-CreERT2 (PERK-cKO) mice and *Perk*^{fl/fl} mice as controls. CAG-SUMO mice were generated previously in our laboratory.¹⁶

Animal Surgery

Animal surgeries were performed on male mice (2–4 months old). The online tool Quickcalcs was used to randomize animals to experimental groups. Transient middle cerebral artery occlusion was performed as described before.¹⁷ Transient forebrain ischemia was induced by bilateral common carotid artery occlusion.¹⁴

Neurological Function and Infarct Volume

The 48-point scoring system, rotarod, tight rope, open field tests, and infarct volumes were used to evaluate stroke outcomes. All evaluations were performed in a blind fashion.

Drug Administration

Tamoxifen (20 mg/kg) was dosed intraperitoneally once daily for 5 days. Salubrinol (1 mg/kg) was administered via intraperitoneal injection.

In Vivo Surface Sensing of Translation

Analysis of protein synthesis in vivo was performed using an approach based on the surface-sensing-of-translation (SUNSET) technique.¹⁸

Western Blotting and Quantitative Reverse Transcription Polymerase Chain Reaction

Western blotting and quantitative reverse transcription polymerase chain reaction (qRT-PCR) were performed using our standard protocols.^{14,15}

Statistical Analysis

The primary outcome for the stroke experiments was infarct volume, which was used to determine group sizes. Statistical analyses were assessed by unpaired Student *t* test when comparing 2 groups, except for Western blot, qRT-PCR, and neurological score data, which were performed using the Mann-Whitney *U* test. To compare >2 groups, 2-way ANOVA with post hoc Holm-Sidak correction for multiple comparisons was performed. Data were presented as median or mean \pm SD. *P* < 0.05 was considered significant, while 0.05 < *P* < 0.1 was considered a statistical trend.

Results

Stroke Outcome Was Worse After Deleting *Perk* in Forebrain Neurons

To delete *Perk* in forebrain neurons in an inducible fashion, we used a Camk2a-CreERT2 driver mouse line. To verify its CreERT2 expression pattern, we first crossed the driver mice with our CAG-SUMO mouse line,¹⁶ in which the mCherry reporter is expressed upon Cre-mediated recombination, to generate Camk2a-CreERT2;CAG-SUMO mice. After treating these mice with tamoxifen for 5 days, we observed strong mCherry fluorescence in forebrain neurons (Figure I in the [Data Supplement](#)), as expected.

Next, we crossed Camk2a-CreERT2 with *Perk*^{fl/fl} mice to generate *Perk*^{fl/fl};Camk2a-CreERT2 (PERK-cKO) mice in which *Perk* can be deleted in most excitatory forebrain neurons after tamoxifen treatment (Figure 1A). To characterize this new mouse line, adult PERK-cKO and littermate control (*Perk*^{fl/fl}) mice were treated with tamoxifen for 5 days. Three weeks later, deletion of *Perk* exons in the genome was verified by PCR using brain DNA samples (Figure 1B). qRT-PCR analysis on RNA samples confirmed a marked decrease in *Perk* mRNA levels in PERK-cKO mouse brains (Figure 1C). Therefore, for the following experiments, PERK-cKO and control mice were subjected to this tamoxifen treatment regime (ie, 5 days intraperitoneal injection followed by 3 weeks recovery). To evaluate the effect of PERK deficiency on p-eIF2 α in the poststroke brain, after tamoxifen treatment, control and PERK-cKO mice were then subjected to 30 minutes middle cerebral artery occlusion and 1 hour reperfusion, and the brain cortex samples were evaluated by Western blotting. As expected, PERK protein levels were less in PERK-cKO versus control mice (contralateral samples; Figure 1D). Of note, levels of GCN2 (general control non-repressible 2) and PKR (protein kinase R), other 2 eIF2 α

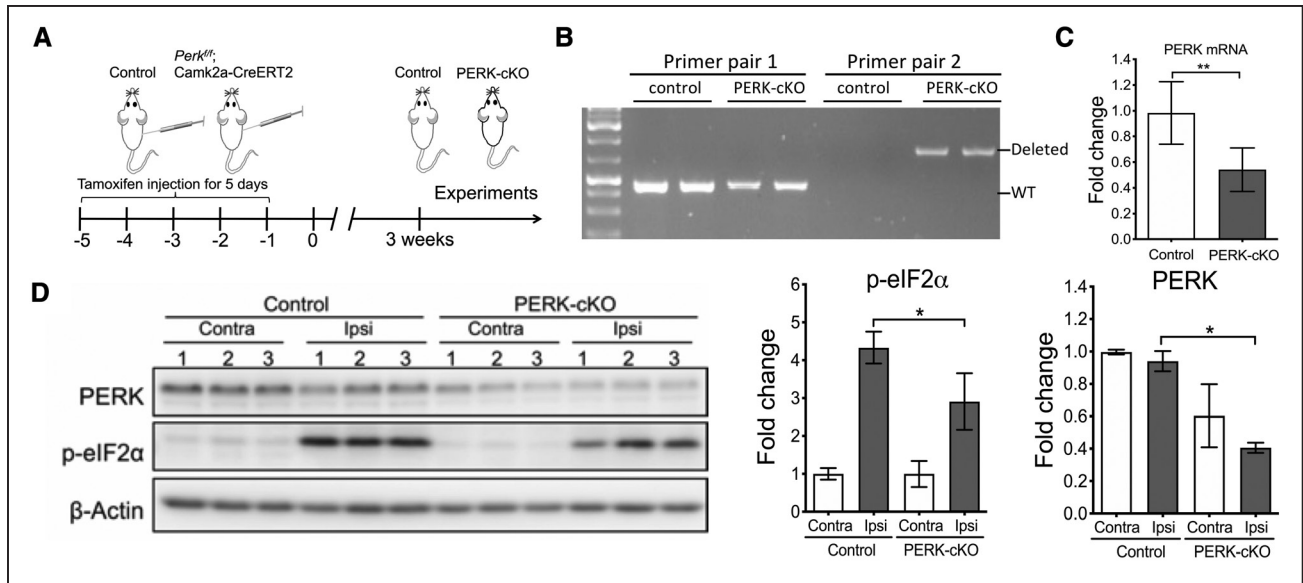


Figure 1. Characterization of PERK (protein kinase RNA-like ER kinase) conditional knockout (cKO) mice. **A**, Schematic depicting the timeline for the generation of conditional *Perk* deletion mice for the subsequent experiments. *Perk*^{fl/fl};Camk2a-CreERT2 (PERK-cKO) mice were identified by polymerase chain reaction (PCR) genotyping and treated with tamoxifen daily for 5 d. Three weeks later, mice were used for experiments. **B**, Verification of *Perk* deletion in the brain of PERK-cKO. PCR analysis on brain DNA samples was used to confirm deletion of *Perk* exons in the brain of PERK-cKO mice. **C**, Quantitative reverse transcription PCR (qRT-PCR) analysis on brain RNA samples was used to confirm deficiency in *Perk* mRNA expression in PERK-cKO mouse brains (n=6). **D**, Western blot analysis. Control and PERK-cKO mice were subjected to 30 min middle cerebral artery occlusion (MCAO) and 1 h reperfusion. Brain cortex samples from the contralateral (Contra) and ipsilateral (Ipsi) hemispheres were collected and evaluated by Western blotting. Intensities of each band were measured and normalized to β -actin (n=3). The mean values in Contra samples of control mice were set to 1.0. Data are presented as mean \pm SD. p-eIF2 α indicates phosphorylated eukaryotic initiation factor 2 alpha; and WT, wild-type. * P <0.05, ** P <0.01.

kinases in the brain, were not significantly changed by *Perk* deficiency (Figure II in the [Data Supplement](#)). Consistent with previous studies,¹² levels of p-eIF2 α were markedly increased at 1 hour reperfusion after stroke in control mice. By comparison, in PERK-cKO mice, the increase in levels of p-eIF2 α was significantly lower after stroke (Figure 1D), indicating that this activation primarily involved the PERK branch.

Our next step was to determine the effect of PERK deficiency on stroke outcome. We first confirmed that control and PERK-cKO mice exhibited similar body weight and cerebral blood flow response to the transient middle cerebral artery occlusion procedure (Figure IIIA and IIIB in the [Data Supplement](#)). We also analyzed spontaneous locomotor activity and found no significant difference between the genotypes (Figure IIIC in the [Data Supplement](#)). For the first stroke experiment, we examined the short-term outcome during the first 3 days after stroke. Compared with control mice, PERK-cKO mice exhibited significantly worse performance on neurological scoring and the tight rope test and had larger infarct volumes (control, 53.4 \pm 12.1 mm³, versus PERK-cKO, 81.0 \pm 14.4 mm³; P <0.001; Figure 2A and 2B). To assess long-term functional stroke outcomes, behavioral outcomes were evaluated each week for 3 weeks after stroke. Compared with control mice, PERK-cKO mice consistently performed worse on motor coordination tests (ie, rotarod and tight rope tests), and it appeared that functional recovery in PERK-cKO mice was less evident than in control mice over 3 weeks after stroke (Figure 2C). PERK-cKO mice also traveled less distance in the open field test (Figure 2C). Taken together, these

data indicated that posts ischemic activation of the PERK UPR branch is neuroprotective in experimental stroke.

Ischemia-Induced Suppression of Protein Synthesis in the Brain Was Diminished After Deleting *Perk* in Forebrain Neurons

To determine mechanistically whether modulation of protein synthesis is responsible for worse stroke outcome in PERK-cKO mice, we adopted a novel approach known as SUNSET, which is based on the incorporation of puromycin into proteins followed by Western blotting using puromycin-specific antibodies.¹⁸ The SUNSET method was first verified in brain slices in vitro (Figure IVA in the [Data Supplement](#)). Then, we found that intracerebroventricular but not intravenous injection of puromycin can be used to label newly synthesized proteins in the brain (Figure IVB and IVC in the [Data Supplement](#)), indicating that puromycin cannot cross the blood-brain barrier. Finally, we performed a dose- and time-response analysis of puromycin incorporation in the brain after intracerebroventricular injection (Figure IVD and IVE in the [Data Supplement](#)). Based on the data, for the following experiments, puromycin (20 μ g/per mouse) was administered by intracerebroventricular injection, and 2 hours later, brain tissue samples were collected for Western blotting analysis. To clarify the effects of brain ischemia/reperfusion on protein synthesis, a transient forebrain ischemia model was used because this model allowed us to obtain relatively homogeneous ischemia-affected tissue samples, thus minimizing the effects of confounding factors such as infarct sizes that would be encountered in a stroke model.

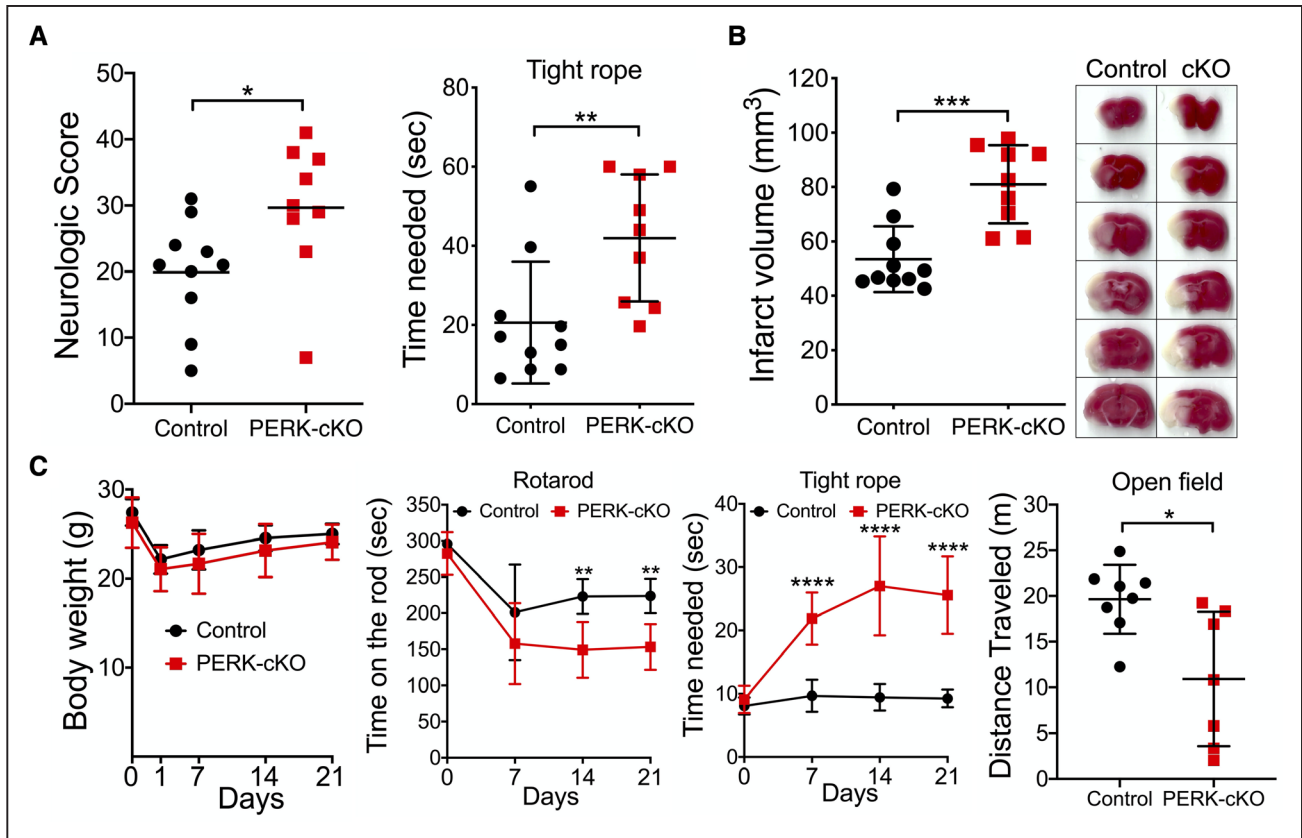


Figure 2. Deletion of *Perk* (protein kinase RNA-like ER kinase) in forebrain neurons worsens both short- and long-term outcomes after ischemic stroke. **A** and **B**, Short-term stroke outcomes (3 d). PERK conditional knockout (cKO) and littermate control mice ($n=9-10/\text{group}$) were subjected to 30 min middle cerebral artery occlusion (MCAO). After 24 h reperfusion, mice were evaluated for neurological deficit scores and by tight rope test (**A**). On day 3 after stroke, infarct volumes were measured (**B**; shown are representative TTC-stained brain slices). **C**, Long-term stroke outcomes (3 wk). PERK-cKO and control mice ($n=7-8/\text{group}$) were subjected to 30 min MCAO. Body weight was monitored over time. Tight rope and rotarod tests were evaluated presurgery (day 0) for baselines and then weekly for 3 wk. The open field test was performed on day 21 after stroke. Horizontal bars represent median value of neurological scores, and other data are presented as mean \pm SD. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

Wild-type mice were subjected to sham or 15 minutes forebrain ischemia, and puromycin was injected intracerebroventricular at 15 minutes reperfusion. Two hours later, mice were euthanized, and hippocampal samples were analyzed by Western blotting using puromycin- and p-eIF2 α -specific antibodies. Transient forebrain ischemia triggered a marked increase in p-eIF2 α and a substantial block of puromycin incorporation into proteins, indicating postischemic inhibition of global protein synthesis (Figure 3A). These results largely agreed with previous studies³ and thus validated our new in vivo approach to assess protein synthesis in the brain.

It has been shown that neuronal *Perk* deletion does not modify global translation in the brain slice under physiological state.¹⁹ To investigate the effects of neuronal *Perk* deletion on postischemic protein synthesis, we then subjected control and PERK-cKO mice to 15 minutes forebrain ischemia followed by puromycin injection at 15 minutes reperfusion. As expected, postischemic activation of p-eIF2 α was significantly less pronounced in PERK-cKO than in control mice. Consequently, a higher level of puromycin incorporation, reflecting less suppression of protein synthesis, was observed in PERK-cKO mice (Figure 3B). To further substantiate the link between less activation of p-eIF2 α and higher protein synthesis observed in PERK-cKO mice, we used salubrinal—a

potent inhibitor of dephosphorylation of p-eIF2 α ²⁰ that can increase levels of p-eIF2 α in the brain, as confirmed by our Western blotting (Figure V in the Data Supplement). Indeed, the marked effects of *Perk* deletion on postischemic p-eIF2 α and protein synthesis were largely reversed in salubrinal-treated PERK-cKO mice (Figure 3C), indicating that salubrinal can rescue impaired PERK signaling in PERK-cKO mice. Together, these data demonstrated the primary role of the PERK branch in p-eIF2 α -induced suppression of protein synthesis in the postischemic brain.

Suppression of Protein Synthesis Induced by p-eIF2 α Is Neuroprotective in Stroke

Our results above suggest that PERK-mediated p-eIF2 α suppresses protein synthesis in the postischemic brain, which is neuroprotective in experimental stroke. If this is the case, administering salubrinal in PERK-cKO mice to increase p-eIF2 α would be expected to rescue the worse stroke outcome observed in PERK-cKO mice (Figure 2). To test this, we performed stroke on PERK-cKO mice and treated the knockout mice with vehicle or salubrinal at 30 minutes reperfusion. The worse stroke outcome in PERK-cKO mice was indeed reversed by salubrinal treatment, as treated PERK-cKO mice had a trend ($P=0.08$) of better neurological scores and significantly smaller infarct volumes (vehicle,



Figure 3. PERK (protein kinase RNA-like ER kinase) conditional knockout (cKO) mice exhibit protein synthesis suppression in the brain to a lesser extent than control mice after brain ischemia/reperfusion. Mice were subjected to sham surgery or 15 min forebrain ischemia. After 15 min reperfusion, puromycin was administered, and 2 h later, hippocampal samples were collected for Western blot analysis. **A**, Suppression of protein synthesis after brain ischemia/reperfusion. Levels of newly synthesized proteins labeled with puromycin were markedly lower in the postischemic brains than in the sham brains ($n=3$). **B**, The marked postischemic suppression of protein synthesis was largely nullified by salubrinal (Sal) treatment ($n=7$). **C**, The effect of *Perk* deletion on postischemic suppression of protein synthesis was largely nullified by salubrinal (Sal) treatment ($n=7$). Data are presented as mean \pm SD. KO indicates knockout; and p-eIF2 α , phosphorylated eukaryotic initiation factor 2 alpha. * $P<0.05$.

70.6 \pm 10.9 mm³, versus salubrinal, 42.2 \pm 16.8 mm³; mean \pm SEM $P<0.01$; Figure 4). Together, these results strongly support our hypothesis that postischemic activation of the PERK UPR branch is neuroprotective in experimental stroke and that this neuroprotection is provided by PERK-induced activation of p-eIF2 α and corresponding suppression of protein synthesis.

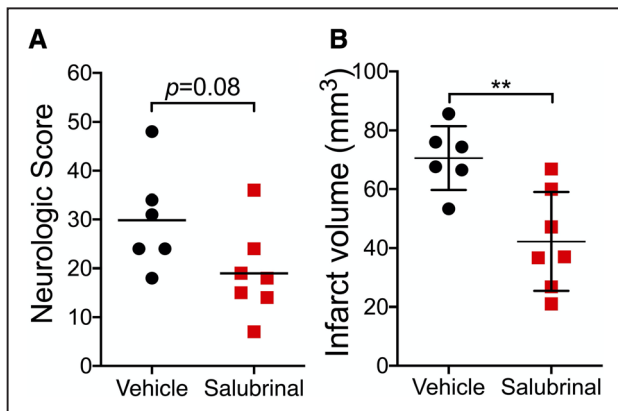


Figure 4. Post-treatment with salubrinal improves stroke outcome in PERK (protein kinase RNA-like ER kinase) conditional knockout (cKO) mice. PERK-cKO mice were subjected to 30 min middle cerebral artery occlusion (MCAO), and at 30 min reperfusion, vehicle or salubrinal was intraperitoneally injected. After 24 h reperfusion, animals were evaluated for neurological deficit scores (**A**) and then for infarct volume (**B**). Horizontal bars represent median value of neurological scores. Infarct volumes are presented as mean \pm SD ($n=6-7$ /group). ** $P<0.01$.

Finally, our data also indicate that salubrinal treatment could be a therapeutic approach suitable for translational studies in stroke. To initially assess the translational potential of salubrinal, we evaluated the effects of salubrinal on stroke outcome using C57Bl/6 mice. Stroke mice were dosed with vehicle or salubrinal at 30 minutes reperfusion. In the salubrinal-treated mice, performance on neurological scoring and on tight rope and rotarod tests at day 1 poststroke was markedly improved (Figure 5A through 5C), and infarct volumes were significantly reduced (vehicle, 68.5 \pm 12.6 mm³, versus salubrinal, 42.3 \pm 20.8 mm³; $P<0.05$; Figure 5D). Of note, although salubrinal treatment has been previously attempted in an experimental stroke study,²¹ this is the first experimental stroke study focused on neurological function—a measure of outcome most relevant for stroke patients—to show that salubrinal treatment is neuroprotective in ischemic stroke.

Discussion

The observation that transient brain ischemia triggers a short-term shutdown of translation was reported >40 years ago.^{1,4} However, the mechanisms that underpin ischemia-induced suppression of protein synthesis and its impact on the recovery of neurons from ischemic stress were not clarified for a long period of time. Although it has long been speculated that transient brain ischemia impairs ER function and consequently activates the PERK UPR branch, which increases

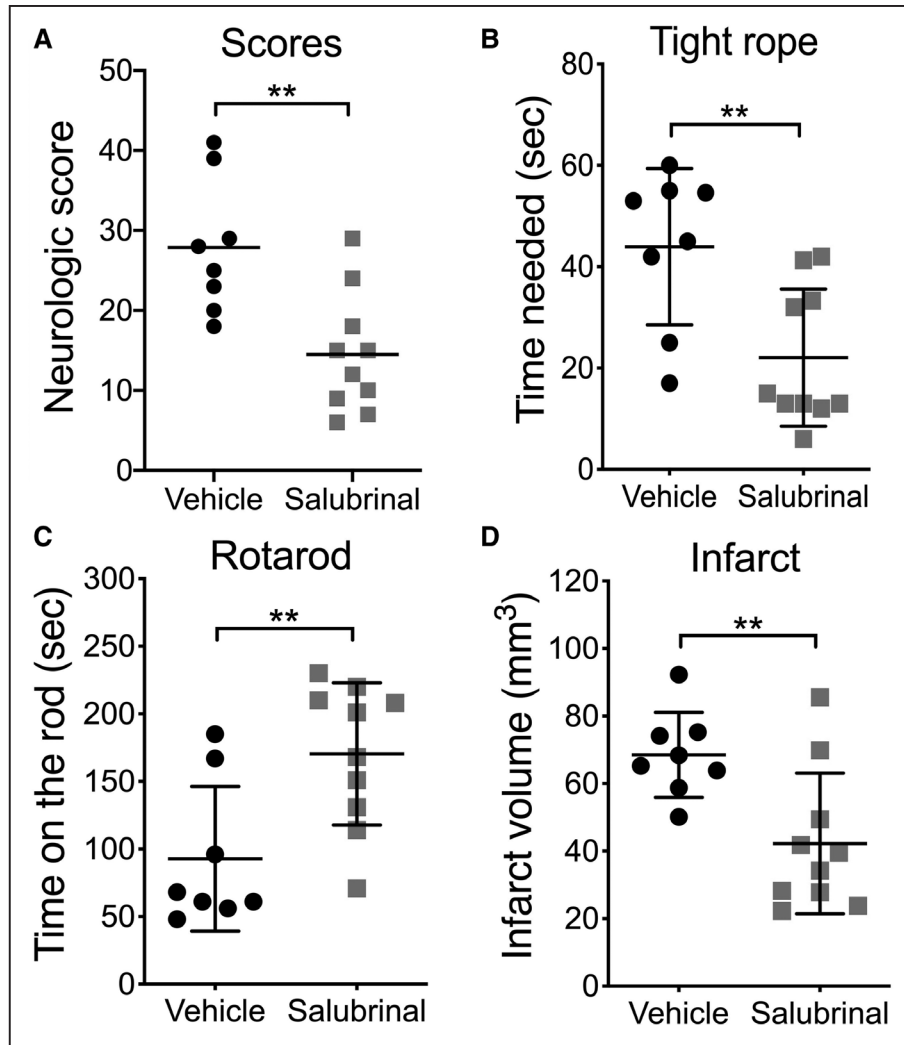


Figure 5. Stroke outcome in C57Bl/6 mice is improved after post-treatment with salubrial. C57Bl/6 mice ($n=8-10/\text{group}$) were subjected to 30 min middle cerebral artery occlusion (MCAO), and at 30 min reperfusion, vehicle or salubrial was intraperitoneally injected. After 24 h reperfusion, the animals were subjected to neurological scoring (A) and to tight rope (B) and rotarod (C) tests. D, On day 3 after stroke, infarct volumes were assessed. Horizontal bars represent median value of neurological scores, and other data are presented as mean \pm SD. * $P<0.05$, ** $P<0.01$.

p-eIF2 α , suppresses global protein synthesis, and helps neurons recover from ischemic stress, the causal relationships between these processes had not been established until now. Here, we have not only provided evidence for the link between the PERK/eIF2 α branch and suppression of protein synthesis in the poststroke brain but have also demonstrated for the first time that deletion of *Perk* in forebrain neurons worsens both short- and long-term stroke outcome, indicating that activation of the PERK UPR branch is neuroprotective in stroke. These findings, together with our previous stroke studies on the ATF6 and IRE1 UPR branches,^{12,13} support the notion that postischemic activation of the UPR helps neurons withstand ER stress caused by ischemia/reperfusion injury and thus represents a crucial endogenous prosurvival mechanism responsible for recovery of neurological function after stroke.

UPR is a highly conserved stress response that is activated when unfolded or misfolded newly synthesized proteins begin to accumulate in the lumen of the ER.^{8,22} Unfolded proteins are not functional, have low solubility, and form potentially

toxic aggregates. To restore ER function, the UPR is activated to increase the protein folding capacity (ATF6 and IRE1 branches), remove misfolded/unfolded proteins from the ER lumen (ATF6 and IRE1 branches), and inhibit translation to block new protein synthesis and thereby reduce ER workload (PERK branch). In the current study, we focused on the PERK UPR branch. Because we are particularly interested in the significance of UPR activation for recovery of neurons from ischemic stress, we decided to use PERK-cKO mice in which *Perk* was preferentially deleted in forebrain neurons. Further, since global *Perk* knockout mice have developmental defects and exhibit severe postnatal growth retardation,²³ to avoid potential effects of *Perk* deletion on brain development, we developed inducible PERK-cKO mice in which *Perk* was deleted by treating adult mice with tamoxifen.

It has been reported that the PERK UPR branch is activated after brain ischemia and that postischemic PERK activation is likely responsible for increased p-eIF2 α and consequently, suppression of translation.^{11,24} In one of these previous studies,

Owen et al²⁴ used mice in which *Perk* was globally deleted, except in pancreatic β -cells, and subjected them to cardiac arrest followed by resuscitation. However, protein synthesis was analyzed using an in vitro translation assay, rather than directly evaluated in the intact brain. Moreover, these animals were extremely sensitive to cardiac arrest and died shortly after resuscitation, thus precluding functional outcome analysis.²⁴

In the current study, we developed a new SUnSET-based approach to investigate the effect of brain ischemia on protein synthesis in vivo (Figure IV in the Data Supplement). Traditionally, brain ischemia-induced suppression of protein synthesis has been evaluated by autoradiography, which is based on radioactively labeled amino acids. The SUnSET technique uses puromycin, which is incorporated into newly synthesized proteins, and this incorporation can be evaluated by Western blot analysis using a puromycin-specific antibody. We have provided evidence that the SUnSET approach can be adapted to assess protein synthesis in the brain in vivo, as we showed here that puromycin incorporation sharply declined during early reperfusion after brain ischemia (Figure 3A), which is consistent with previous studies that used radioactive approaches.² Further, using this approach, we have shown that the decline was significantly less in PERK-cKO versus control mice (Figure 3B), and that this phenotype can be largely reversed by salubrinal treatment, thus establishing the link between the increase in PERK-mediated p-eIF2 α and inhibition of protein synthesis in the postischemic brain.

This is, to the best of our knowledge, the first study to specifically clarify the role of PERK in ischemic stroke. Our data showed that deletion of *Perk* in neurons resulted in larger infarcts and worse neurological outcome after stroke. We also have provided evidence that PERK-mediated neuroprotection is mediated by p-eIF2 α and subsequent suppression of protein synthesis. Indeed, a neuroprotective effect similar to PERK activation was observed in PERK-cKO mice treated with salubrinal—a compound that blocks p-eIF2 α dephosphorylation resulting in an increase in p-eIF2 α levels and corresponding suppression of protein synthesis. Salubrinal almost completely reversed the larger infarcts observed in PERK-cKO versus control mice. Thus, postischemic activation of the PERK UPR branch is neuroprotective in experimental stroke.

It is our current understanding that transient brain ischemia impairs ER function, that is, the folding and processing of newly synthesized proteins, resulting in ER stress and activation of the UPR. ER stress is a potentially lethal situation for affected cells that requires immediate action including acute shutdown of translation to reduce the ER protein load (PERK branch). This happens immediately after brain ischemia as PERK activation, p-eIF2 α , and subsequent shutdown of translation are early postischemic stress responses.^{11,24} Thus, the postischemic activation of the PERK UPR branch could be a critical stress response that helps cells to survive ER stress conditions, as has been reported in a variety of cell culture studies.²⁵ The observation that PERK is the only eIF2 α kinase activated after brain ischemia further supports the neuroprotective role of the PERK UPR branch in experimental stroke.¹¹

Notably, in experimental subarachnoid hemorrhage—a pathological state associated with ischemic conditions—the PERK UPR branch is activated in a delayed fashion, and the

activation persists for at least 72 hours after subarachnoid hemorrhage. Under these experimental conditions, blocking PERK or downstream signals appears to be neuroprotective.²⁶ One explanation is that protein synthesis is required at the late stage for functional recovery of injured cells. Indeed, after brain ischemia, programmed recovery from translation suppression is required to translate stress messages, for example, prosurvival genes via activation of the ATF6 and IRE1 UPR branches, into the respective proteins to facilitate cellular recovery. This shift from programmed suppression to recovery of translation can be achieved by GADD34 (growth arrest and DNA damage-inducible protein 34), which helps to dephosphorylate p-eIF2 α and restore protein synthesis.²⁷ Our earlier observation that after short forebrain ischemia, GADD34 protein levels increase in the resistant cortex but not in the vulnerable hippocampal CA1 subfield further supports a mechanism whereby both acute PERK-induced block of translation and programmed recovery of translation are required to help neurons survive ischemia-induced ER stress.²⁸ Previous studies on the mechanisms underlying neuroprotection by pentobarbital suggest that the shift from programmed suppression to recovery of translation takes place between 2 and 8 hours post-ischemia. Pentobarbital that protects CA1 neurons from ischemic damage when applied shortly after ischemia, has no effect on the postischemic block of translational initiation 2 hours after ischemia. However, pentobarbital induces almost full recovery of translational initiation 8 hours after ischemia.²⁹

There are some limitations of this study to be noted. The salubrinal rescue experiment required a large number of age-matched male PERK-cKO mice. We used infarct volume as primary outcome to determine a practical sample size. However, this sample size appears to be underpowered for neurological score analysis. More, for the salubrinal treatment experiments, only male young mice were used. To further evaluate the therapeutic potential of salubrinal in stroke, female and aged animals, as well as other stroke models and species, should be considered in future studies.

With earlier and the current reports,^{12,13} we have now provided comprehensive information on the significance of neuronal activation of each UPR branch in experimental stroke. Indeed, forced activation of the ATF6 UPR branch results in smaller infarcts and better recovery of neurological function after ischemic stroke.¹² The IRE1 UPR branch is activated in experimental stroke, resulting in splicing of *Xbp1s* (X-box binding protein 1) mRNA and production of XBP1s protein¹⁰—a transcription factor that activates expression of proteins involved in the hexosamine biosynthetic pathway.³⁰ Hexosamine biosynthetic pathway provides the substrate for O-linked β -N-acetylation (O-GlcNAcylation) of proteins—a posttranslational modification that protects cells exposed to a variety of stress conditions.³⁰ Deletion of *Xbp1* in neurons worsens outcome in experimental stroke, while pharmacological boosting of O-GlcNAcylation results in better stroke outcome.¹³

Proteostasis—the dynamic equilibrium that generates functional proteins, maintains functional proteins, and eliminates nonfunctional proteins—is essential for cell survival. The UPR is a key element in the comprehensive cellular network that controls proteostasis.^{8,22,31} Experimental stroke is a severe form of metabolic stress that impairs most cellular pathways including ER-resident protein folding and maturation, and activation

of the UPR is conceivably critical for neuronal survival. This notion is corroborated by our earlier studies and this report. Boosting UPR branches in postischemic neurons could, therefore, be considered a promising strategy to restore neuronal function and improve stroke outcome. This could be of particular importance for the aged brain because aging is associated with a decline in the capacity to activate adaptive pathways to restore homeostasis impaired by ischemic stress.^{14,15,22,31} Given that UPR branches play different roles in restoring proteostasis impaired by ischemic stress, a combination treatment that boosts each UPR branch may provide an optimal approach to improve stroke outcome, even in elderly patients.

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Disclosures

None.

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