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Article

Activation of the PERK Branch of the UPR as a Strategy for Improving Outcomes in Acute Ischemic Stroke

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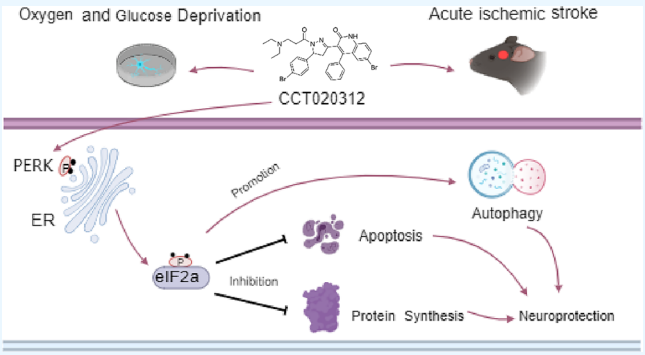
ABSTRACT: Brain ischemia disrupts endoplasmic reticulum (ER) dynamics, causes ER stress, and triggers the unfolded protein response (UPR). During the UPR, protein kinase RNA-like ER kinase (PERK) phosphorylates eIF2α, shutting down global protein synthesis, inhibits protein synthesis, and provides neuroprotection during acute ischemic stroke. Herein, middle cerebral artery occlusion/reperfusion (MCAO/R) and PERK neuron-specific deletion conditional knockout mice were employed to observe the function and mechanisms of PERK. CCT020312, a novel selective PERK activator, specifically activates PERK and provides neuroprotection both in vivo and in vitro stroke models. Additionally, CCT020312 enhanced neuronal survival and cerebral microvessels and decreased the level of astrogliosis in acute ischemic stroke mice. Furthermore, in vivo experiments demonstrated that CCT020312 not only prevented apoptosis but also enhanced the PERK/p-eIF2α/LC3-II autophagy signaling pathway in MCAO/R mice. In conclusion, our study supports the potential therapeutic value of targeting PERK in acute ischemic stroke, offering a promising strategy for enhancing stroke outcomes through the modulation of protein synthesis and the autophagy pathway.



INTRODUCTION

Acute ischemic stroke is a major artery cerebrovascular disease that causes significant economic losses per year. However, tissue plasminogen activator (tPA) within 3−4.5 h of stroke onset and mechanical thrombectomy were approved treatments for stroke patients.[1] However, tPA treatment, especially when delayed, is accompanied by intracranial hemorrhage and hemorrhagic transformation.[2] Although mechanical thrombectomy retrieved occlusive clots from brain vessels, recanalization was still unsolved in certain patients with reperfusion injury.[2] Therefore, discovering new therapeutic targets is crucial for stroke recovery.

Ischemic stroke disrupts endoplasmic reticulum (ER) function, results in ER stress, and activates the unfolded protein response (UPR).[3] Importantly, the function of the UPR is to maintain cellular homeostasis by triggering apoptosis, an important homeostatic process that, when compromised, can lead to the accumulation of misfolded proteins.[4] Three canonical branches of the UPR, controlled by different ER stress sensor proteins—protein kinase RNA-like ER kinase (PERK), ATF6, and IRE1—have been proposed.[5] Activation of PERK induces the phosphorylation of eukaryotic initiation factor 2-alpha (eIF2α), whereas eIF2α phosphorylation suppresses global protein translation, reducing ER protein load and stress.[6,7] In our previous work, we used the transient middle cerebral artery occlusion (tMCAO) model in PERK-conditional knockout (cKO) mice to confirm that PERK provided neuroprotection in acute ischemic stroke. We also showed salubrinal inhibited eIF2α dephosphorylation, improving stroke outcome by suppressing protein synthesis.[3] However, direct PERK activation in ischemic strokes has rarely been reported.

Recently, UPR-specific compounds have been identified,[8,9] providing preclinical research resource for ischemic stroke.[10] The novel compound CCT020312 was first reported as a selective PERK activator in a study that found it prevented proliferative activity in human colon cancer cells and chemosensitizing activity in U2OS human osteosarcoma cells.[11] Following these findings, several studies reported that CCT020312 may represent a novel treatment for use in cancer therapy, such as for breast cancer, colorectal cancer, or glioblastoma.[12−14] In line with this, we found that CCT020312 reduced the tumor size and prolonged survival in glioblastoma

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in vitro and in vivo (unpublished). Furthermore, CCT020312 was also reported to mitigate tau pathology in Alzheimer's disease (AD) and extend survival in Huntington's disease.[15,16] Systemic PERK knockout mice experience severe bone loss, and antioxidant N-acetylcysteine (NAC) was found to prevent the expression of phosphorylated PERK, osteoclast-related proteins, and autophagy-related proteins. More importantly, CCT020312 was found to reverse the inhibitory effect of NAC and inhibit inflammation-mediated osteoporosis in ovariectomized rats.[17,18]

Recently, our work confirmed that overexpression of BAG3 activated the autophagy pathway and inhibited apoptosis, providing neuroprotection 24 h poststroke, whereas LC3-II-dependent autophagy was still robust 24 h poststroke.[19] Therefore, although PERK-dependent eIF2α phosphorylation was increased 1 h after stroke and disappeared 6 h poststroke, we hypothesized that CCT020312 administration may enhance the PERK/p-eIF2α/LC3-II pathway in acute ischemic stroke and provide neuroprotection via the autophagy pathway. Furthermore, inhibition of apoptosis and suppression of protein synthesis are potential mechanisms that underlie CCT020312's short- and long-term neuroprotective effects in ischemic stroke. In conclusion, our study demonstrates for the first time that CCT020312 directly activates PERK, providing neuroprotective effects with influences on short- and long-term stroke outcomes. The enhancement of neuronal survival, increased neurogenesis, and decreased astrogliosis further underscore the potential therapeutic value of targeting PERK in acute ischemic stroke.

MATERIALS AND METHODS

Animals. Male C57BL/6J mice aged around 10−12 weeks were obtained from the Guangdong Medical Laboratory Animal Center (Guangdong, China) and acclimated in a warm environment prior to experimental procedures. All procedures were approved by the Administration Committee of Experimental Animals in Guangdong Province (No. 202200103) and the Ethics Committee of Shenzhen University.

Conditional *PERK* knockout (*Perk*$^{f/f}$, C57BL/6 background, catalog: 02171) mice were provided by Cyagen Biosciences (Soochow, China). We crossed *Perk*$^{f/f}$mice with Camk2a-Cre mice (catalog: C001015) under the control of the neuron-specific Camk2a promoter and obtained *Perk*$^{f/f}$; Camk2a (Perk-cKO) mice.[3] *Perkk*$^{f/f}$ mice were used as controls. The detailed information is provided in the Supplement Materials.

Focal Ischemic Stroke. Male C57BL/6J mice (10−12 weeks) were anesthetized with isoflurane and maintained at 37.0 °C by a heating pad (R&D, Shenzhen, China) during the entire procedure. The left common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed. Next, the regional cerebral blood flow was monitored using laser Doppler flowmetry (Moor Instruments, Devon, UK). Thereafter, a monofilament (R&D, Shenzhen, China) was inserted into the ICA via the CCA, and the artery was blocked for 30 min. Thereafter, the filament was removed and mice were back to homecages. Animals with brain hemorrhage and those that did not reduce cerebral blood flow were excluded. Animals excluded from analysis are listed in Table S1.

Drug Administration. CCT020312 was purchased from MedChemExpress (New Jersey, United States) and prepared. Mice were treated with vehicle or CCT020312 (1−5 mg/kg) by an investigator blinded to genotype via an intraperitoneal injection in 200 μL at 1 h poststroke. For the long-term assay, CCT020312 was administered for 1 week.

Neurologic Scores. The mice were blindly evaluated to assess neurological impairments according to a 48-point scoring system.[3,20] In brief, general status (0−12), simple motor deficits (0−14), complex motor deficits (0−8), and sensory deficits (0−14) were scored, where 0 = no deficits and 48 = maximal deficits.

Infarct Volume. Mice were sacrificed 24 h poststroke. Brains were immediately cut into 1 mm thick sections and stained with 2,3,5-triphenyltetrazolium chloride. Images of the infarct area were captured and analyzed using a Fiji ImageJ.

Rotarod. Sensorimotor functions were assessed weekly using a Rotarod test. Mice were trained for three consecutive days before surgery. The rods were set to rotate and accelerate 4−40 rpm within 300 s. The maximum time for each trial was 300 s.

Open Field Test. The open field test was used to assess anxiety-like behavior and general exploratory behavior per week until poststroke.[20] Briefly, mice were put in the center of the apparatus, consisting of a square area surrounded by white walls (35 × 35 × 35 cm), and the total distance and time spent in the central area were recorded and analyzed using EthoVision software.

SUnSET. To analyze protein synthesis in vivo, we developed a new approach based on the surface sensing of translation (SUnSET) method.[3,21] The SUnSET technique assesses protein synthesis using the nonradioactive compound puromycin (HY-B1743A, MedchemExpress). Puromycin can be incorporated into polypeptides during translation because of its structural analogue of aminoacyl tRNAs. The newly synthesized polypeptides containing puromycin can then be evaluated by Western blotting using a puromycin antibody (antipuromycin antibody, MABE343, Sigma-Aldrich). In vitro tests were performed using brain slices. Mouse brain slices were rapidly collected and placed in artificial cerebrospinal fluid (ACSF; pH 7.4, R24037, Yuanye, Shanghai). Ipsilesional slices (300 μm thick) were prepared, placed in 6-well plates, and incubated with ACSF buffer for 2 h at 37 °C. The slices were then lysed for Western blot analysis.

Immunohistochemistry. Mice were perfused with 0.9% ice-cold saline under deep anesthesia, followed by 4% paraformaldehyde perfusion. Thereafter, brains were transferred to series of solutions with gradually increasing sucrose for 48−72 h. Thereafter, brain samples were cut into 20 μm thick brain slices obtained using a CryoStar NX50 Cryostat (Waltham, MA, USA) and stored at −20 °C. The immunochemistry was performed using a standard protocol. The primary antibodies anti-NeuN, anti-GFAP, anti-Iba1, and anti-CD31 (ab104224, ab7260, ab15690, and ab28364, Abcam, Cambridge, UK) were used, and the corresponding secondary antibodies (A32742, A11008, Thermo Fisher Scientific) were employed. Images were captured using a fluorescence microscope (LSM 800, Zeiss, Germany).

Western Blot. Ipsilesional cortex and striatum tissues were harvested and homogenized in RIPA lysis buffer containing 1% protease and phosphatase inhibitors. The lysates were centrifuged, and the supernatant was collected. The protein concentrations were measured, and equal amounts of protein from each sample were loaded onto an SDS-PAGE gel. The Western blots were performed using a standard protocol.

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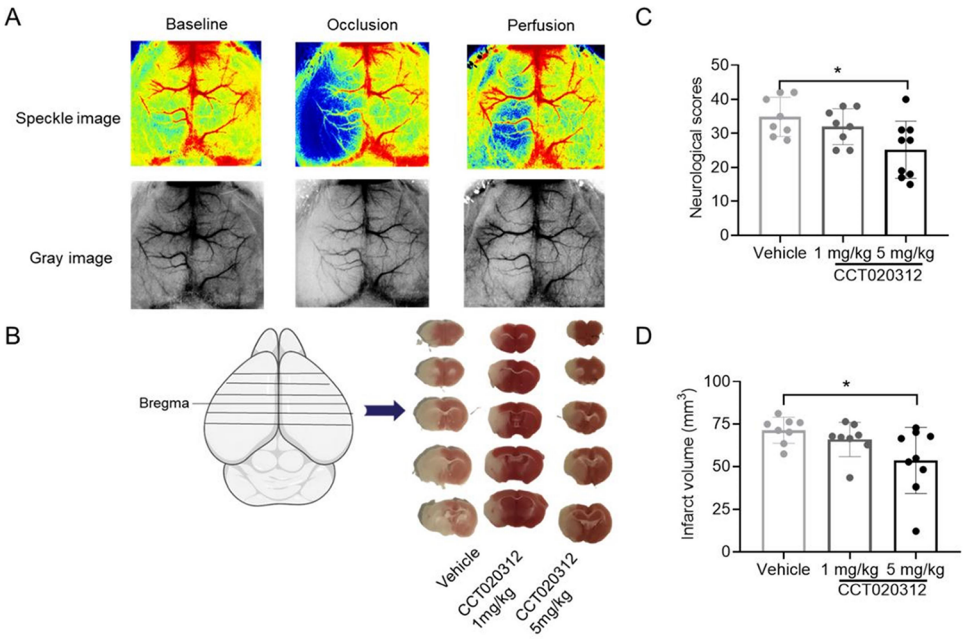

Figure 1. Postacute delivery of CCT020312 improves stroke outcomes. MCAO was performed on mice using filament occlusion (n = 8−9 per group), and they were then treated with either vehicle or CCT020312 via intraperitoneal injection 1 h poststroke. Cerebral blood flow was detected and screened using laser speckle contrast images. Neurological function and infarct volume were evaluated using neurological scores and (2,3,5-triphenyltetrazolium chloride) TTC staining at 24 h poststroke. (A) Cerebral blood flow was screened by laser speckle contrast imaging and gray images before stroke, during occlusion, and poststroke in tMCAO. (B) TTC staining for the vehicle- and CCT020312-treated groups. (C, D) Neurological scores and infarct volumes for the vehicle- and CCT020312-treated groups. Results are expressed as the mean ± SD; tMCAO, transient middle cerebral artery occlusion; MCAO, middle cerebral artery occlusion; *p < 0.05, **p < 0.01, and ***p < 0.001 vs vehicle.

Primary antibodies (PERK, p-eIF2α, P62, LC3II, β-tubulin, and β-actin) and the appropriate secondary antibodies HRP-linked antirabbit or mouse IgG were used; all of these antibodies were purchased from Cell Signaling Technology, Inc. Finally, the membranes were developed by using a chemiluminescence solution, and the bands were visualized. The data were analyzed using ImageJ software.

Cell Lines and Cell Cultures. Human neuroblastoma (SY5Y) cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Thermo Fischer Scientific) supplemented with 10% fetal bovine serum and 100 U/mL penicillin/streptomycin (Life Technologies). Oxygen-glucose deprivation (OGD) was induced in a hypoxia chamber (1% O_2, Toepffer Lab Systems, Göppingen, Germany) with a glucose-free medium (Life Technologies). For treatment with the PERK activator CTT020312, a concentration of 2 μg/mL was used.

Real-Time Apoptosis and Necrosis Assay. To measure apoptotic and necrotic events, we used the RealTime-Glo Annexin V Apoptosis and Necrosis assay (no. A1011; Promega, MD, U.S.A.) following the manufacturer's protocol. Briefly, 5×10^4 SY5Y cells/well were seeded in a black 96-well plate. Addition of the detection reagents to the cells 20 h later defines time point t = 0. At each chosen time point, green fluorescence intensity ($\lambda_{ex}/\lambda_{em}$ = 485 nm/520 nm) and luminescence intensity were measured with the fluorescence microplate reader FLx800 (Biotek, Winooski, VT, U.S.A.) over a time period of 72 h to quantify apoptosis and necrosis, respectively.

Statistical Analysis. The core stroke outcome was infarct volume; therefore, we used it to evaluate different group sizes and performed the calculation using Power Analysis and Sample Size (PASS) 2021 software.

Levene's test was used to verify normality for homogeneity of variance assessments. The Kruskal−Wallis tests were employed to analyze the behavioral data, followed by Dunn's post hoc tests and expressed as means ± SD. All other data were compared by one-way analysis of variance (ANOVA) with Dunnett's T3 post hoc tests and are shown as mean ± SD. A p-value less than 0.05 indicated statistical significance.

RESULTS

Delivery of CCT020312 Poststroke Enhanced Stroke Outcomes. To evaluate the effects of direct activation of PERK on the outcome of acute ischemic stroke, CCT020312 was administrated by intraperitoneal injection to sham surgery mice 1 h poststroke. The level of p-eIF2α, which is downstream of PERK, was assessed, and we found that CCT020312 directly activated PERK and increased p-eIF2α levels 1−6 h after administration. The levels returned to the normal level 12−24 h after administration, compared with the vehicle group (Figure S1).

The tMCAO model was employed to evaluate the effects of CCT020312 on ischemic stroke. Figure 1A shows that cerebral blood flow dropped and was restored after reperfusion. In the tMCAO groups, we found that treatment with CCT020312 at 5 mg/kg led to significantly decreased infarct volume, as staining by TTC (Figure 1B). Furthermore, neurological functions were enhanced, as determined by neurological scores, in 5 mg/kg CCT020312-treated tMCAO mice compared with groups treated with the vehicle and low-dose (1 mg/kg) (Figure 1C,D). In the 4-week long-term study, mice treated with CCT020312 did not show differences in body weight within all three groups (Figure S2A). Although the mice treated with 5 mg/kg CCT020312 traveled further distances than vehicle- and low-dose-treated mice in the OPT,

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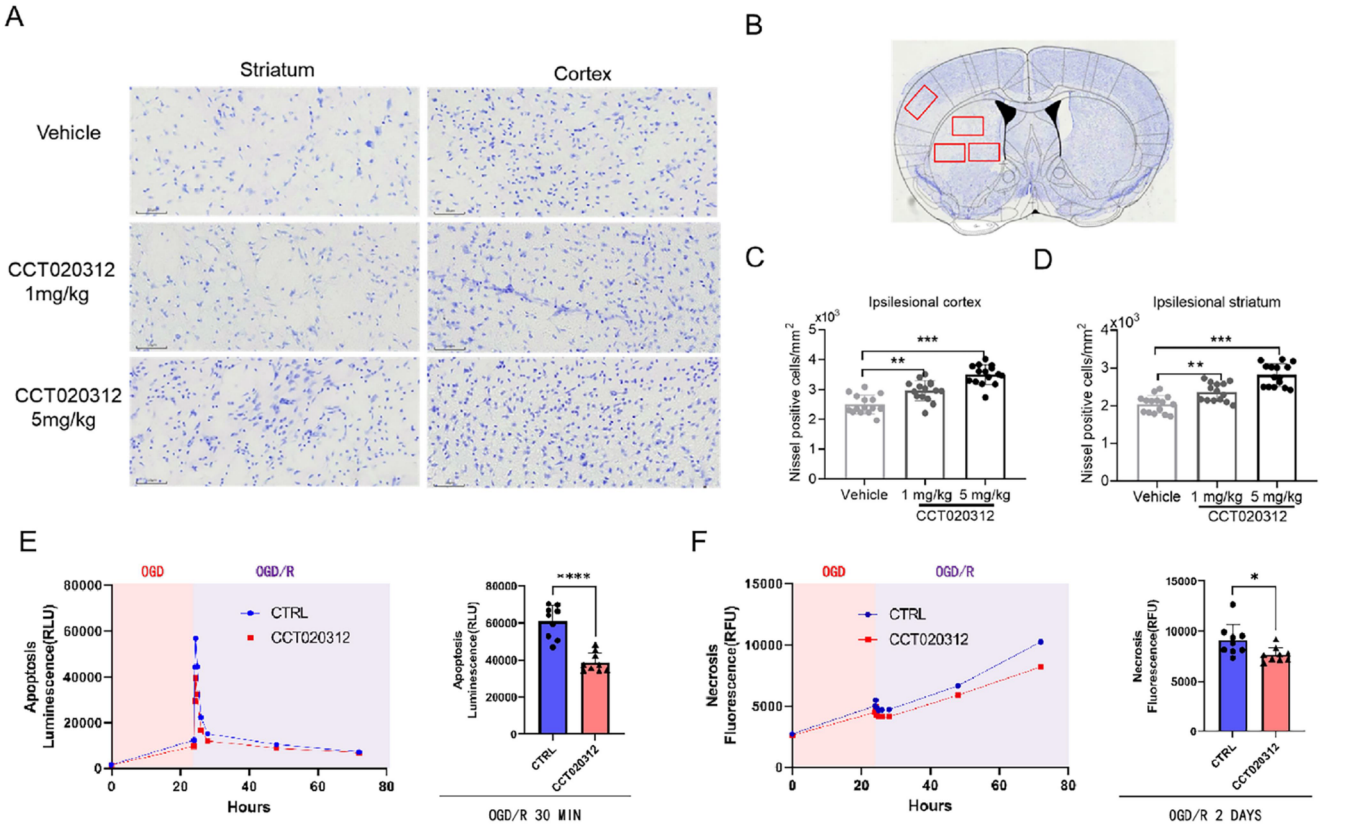

Figure 2. Postacute delivery of CTT020312 prevented apoptosis. MCAO was performed on mice using filament occlusion, and then they were treated with vehicle or CCT020312 1 h poststroke. Brain slices were obtained 24 h after perfusion (n = 6). (A) Nissl staining, (B) region of interest, and (C, D) quantitative analysis in the ipsilesional striatum and cortex. MCAO, middle cerebral artery occlusion. (E, F) Continuous detection of cell death using an in vitro stroke model in SY5Y cells. (E) Apoptotic death was recorded by measurement of luminescence generated by the binding of phosphatidylserine (PS) on the outer cell membrane leaflet to a NanoLuc Binary Technology (NanoBiT) luciferase. The time point 30 min after OGD/R was further analyzed. (F) Necrotic death was detected in SY5Y cells by the measurement of fluorescence emitted by a nonmembrane-permeable dye after cytosolic double-strand DNA binding. The time point 2 days after OGD/R was further analyzed. Data are processed in triplicate, of which the mean values are formed. A representative result is shown. Data are mean ± SD values. Evaluated by multiway ANOVA followed by Bonferroni tests. *p < 0.05, **p < 0.01, and ***p < 0.001 vs vehicle.

there were no differences among the three groups (Figure S2C). However, our results indicated that CCT020312 enhanced mice coordination 1 week after stroke, and consistently improved motor activation was observed for 4 weeks (Figure S2B).

Delivery of CCT020312 Poststroke Reduced Cell Apoptosis. To investigate the potential neuroprotective mechanisms of CCT020312, brain slices were obtained from tMCAO mice treated with or without CCT020312 for 24 h. As expected, more apoptotic Nissl-stained cells were observed in the vehicle- or low-dose-treated mice; however, there were more surviving neuronal cells in the 5 mg/kg CCT020312 group (Figure 2A). Additionally, normal sharp demarcations were observed in the CCT020312 group, while the vehicle and low-dose groups showed cytosol condensation (Figure 2B). Furthermore, in both the ipsilesional cortex and the striatum, there were significantly more survival cells in the 5 mg/kg CCT020312 group compared to the control and low-dose groups (Figure 2C,D). Moreover, double staining with anti-CHOP and TUNEL assay also indicated that CCT020312 may prevent cell apoptosis (Figure S3).

To further analyze the impact of CCT020312 on cell death in vitro, we performed a Real-Time-Glo Annexin V Apoptosis and Necrosis assay in a stroke model using the neuroblastoma cell line SY5Y (Figure 2E,F). In line with our in vivo data, the activation of PERK with the compound CCT020312 drastically reduced the Reox-mediated apoptotic peak 30 min after the OGD (Figure 2E) and decreased the Reox-mediated necrotic event 2 days after the OGD (Figure 2F).

CCT020312 Administration Poststroke Promoted Neuronal Survival, Reduced Peri-Infarct Astrogliosis, and Increased Brain Capillary Density. To evaluate the neurological changes induced by CCT020312, neuronal survival in the striatum was evaluated using NeuN staining. We found that there were significantly more NeuN positive cells in the 5 mg/kg CCT020312 group compared with the 1 mg/kg CCT020312- and vehicle-treated groups (Figure 3A,C). Moreover, peri-infarct astrogliosis, evaluated by GFAP immunohistochemistry in the striatum, was significantly lower in the 5 mg/kg CCT020312 group compared with the 1 mg/kg and vehicle groups, 24 h poststroke (Figure 3A,D). Microglial activation, defined by Iba1 immunoreactivity, was not influenced by CCT020312 at either dose (Figure 3B,E). Finally, brain capillary density, detected using CD31 immunohistochemistry, was significantly increased in the 5 mg/kg CCT020312 group at 24 h poststroke, compared with the 1 mg/kg and vehicle groups (Figure 3B,F).

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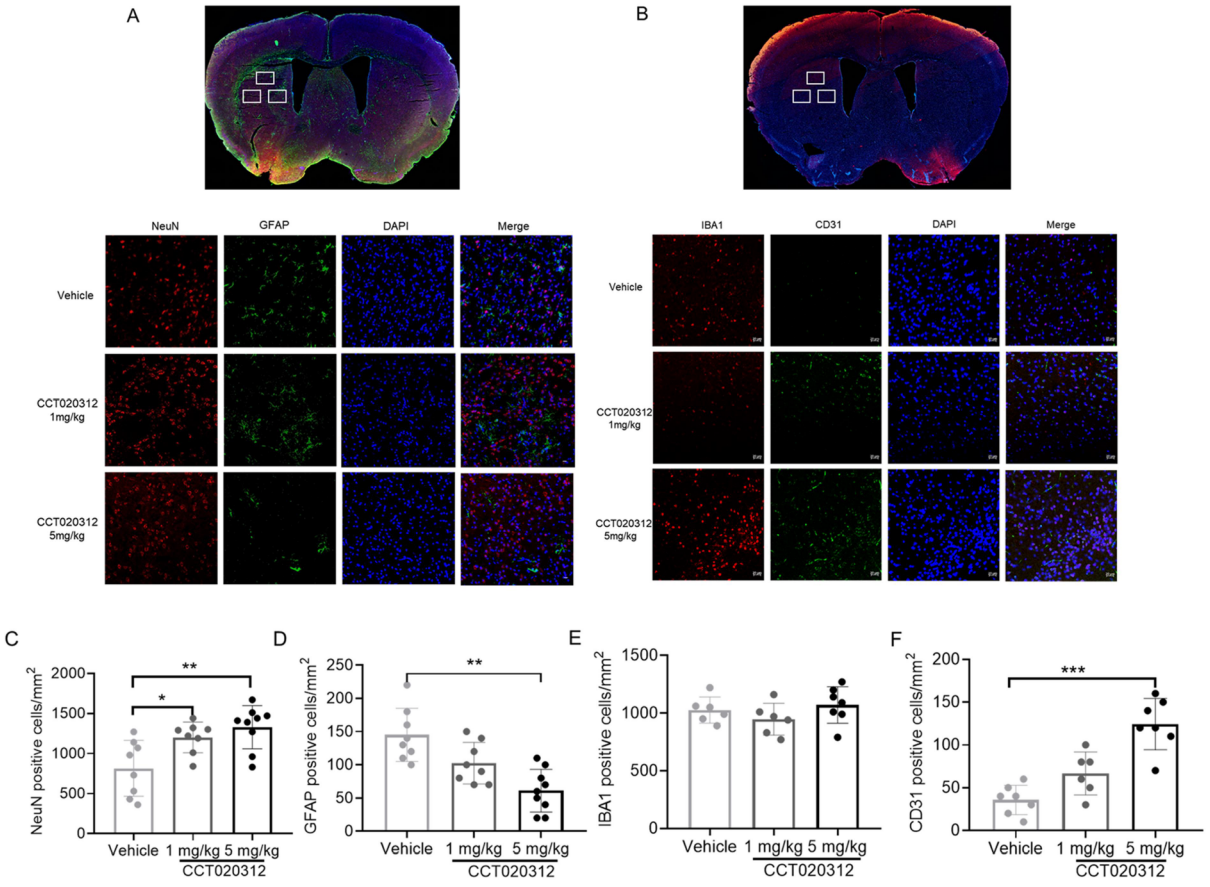

Figure 3. Postacute delivery of CTT020312 treatment enhanced neuron survival and microvessel density and decreased astrogliosis. Mice were subjected to a 30 min occlusion, and then vehicle (saline) or CCT020312 was administrated via intraperitoneal injection 1 h poststroke. Brain sections were harvested at 24 h poststroke and stained for (A, C, and D) NeuN and GFAP and (B, E, and F) Iba1 and CD31. Scale bar is 100 μm (n = 6 per group).

Delivery of CCT020312 Prevented Global Protein Translation in Ischemic Stroke. To investigate the potential mechanisms of CCT020312 in ischemic stroke, SUnSET, a method previously established by our group, was employed to measure global protein translation in vitro.[3] As expected, eIF2α phosphorylation was increased poststroke (Figure 4B), while treatment with CCT020312 significantly increased p-eIF2α and decreased protein synthesis, as determined by labeling with puromycin in a dose-dependent manner in acute ischemic stroke brain slices (Figure 4B–D).

PERK/p-eIF2α Influenced the Autophagy Pathway in Ischemic Stroke. To investigate the potential mechanisms, neuron-specific deletion of PERK (Perk-cKO) in the forebrain was established by crossing *Perk*$^{f/f}$ mice with Camk2a-Cre mice. Genotyping and Western blotting were confirmed, and PERK protein expression was also significantly decreased in Perk-cKO mice compared with the control mice (Figure S4). Since CCT020312 is a specific PERK activator and enhances stroke outcomes in wild-type mice, we considered whether it was dependent on the PERK/p-eIF2α pathway. As expected, Perk-cKO mice were treated with vehicle and CCT020312 1 h poststroke, and our results showed that CCT020312 did not affect the stroke outcomes, which were evaluated by neurological scores and infarct volume when PERK was deleted (Figure S5). Furthermore, to investigate the mechanism, Perk-cKO and control mice were subjected to 30 min of MCAO, and the injured tissues were collected 1 h poststroke for Western blotting. Our results showed that the autophagy marker LC3-II was significantly increased and the P62 expression was significantly decreased in Perk-cKO mice exposed to MCAO compared with control mice (Figure 5). In agreement with our in vitro data, LC3-II expression appeared to be related to PERK and was downstream of p-eIF2α. Several studies have confirmed that PERK/eIF2α is important for the activation of ER stress-related autophagy. The PERK/eIF2α pathway can promote the conversion of microtubule-associated protein 1 LC3-I to LC3-II, while LC3-I was transformed into LC3-II by cleavage of amino acids at the hydroxyl end, which activates the autophagy system.[22,23]

CCT020312 Enhanced the Autophagy Pathway In Vivo in Ischemic Stroke. We also tested whether CCT020312 activates autophagy in vivo. Perk-cKO mice failed to phosphorylate eIF2α and did not show an increased level of LC3-II expression. Therefore, *Perk*$^{f/f}$ mice were subjected to 30 min of MCAO, CCT020312 was administered 1 h poststroke, and the injured tissues were collected for Western blot. As expected, we found that p-eIF2α, as a downstream substrate of PERK, was activated by CCT020312, and meanwhile, the expression of the autophagy marker LC3-II was significantly increased, while that of P62 was dramatically decreased (Figure 6). Our results indicated that CCT020312 provides neuroprotection by enhancing the autophagy signaling pathway.

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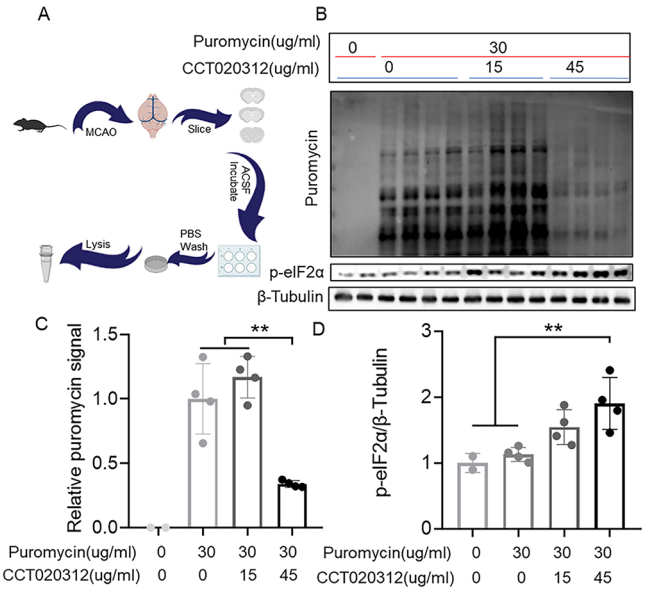

Figure 4. Postacute delivery of CTT020312 suppressed global protein synthesis. Mice were subjected to sham surgery or a 30 min MCAO occlusion. Brain slices (1 mm) were prepared and incubated with or without puromycin, and CCT020312 was added as indicated. Brain slice samples were collected for Western blot analysis. (A) Schematic diagram of the indicated procedure (created with MedPeer). (B) 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 = 2 or 4). (C, D) Puromycin labeling and p-eIF2 alpha expression in ispilesional and contralesional tissues in response to CCT020312 were measured, and the data are presented as the mean ± SD. MCAO, middle cerebral artery occlusion. *p < 0.05 vs vehicle.

DISCUSSION

Ischemic stroke triggers ER dysfunction, prompting the activation of the UPR to maintain protein homeostasis. Transgenic mice lines targeting the UPR branches (ATF6, IRE1, and PERK) have been instrumental in investigating their roles in ischemic stroke.[17,24,25] Building upon previous studies that demonstrated the neuroprotective effects of UPR branches in acute ischemic stroke models, our investigation focuses on the impact of CCT020312, a specific PERK activator. Herein, we found that CCT020312 enhanced stroke outcomes in both the short and long terms, as assessed by neurological scores and Rotarod testing. Furthermore, we found that CCT020312 inhibits neuronal apoptosis and enhances neuronal survival in acute phase stroke. Finally, our data showed that CCT020312 provided neuroprotection via suppressing global protein synthesis and enhanced the autophagy pathway mediated by PERK/p-eIF2α in acute ischemic stroke in vivo; thus, targeting protein translation could be a viable strategy for stroke therapy.

Forty years ago, protein synthesis was dramatically decreased during early perfusion using a forebrain ischemia model in cats.[26] Postischemic protein translation is regulated by multiple factors, including eIF2α, and its phosphorylation state plays a dominant role.[27] Four protein kinases that recognize stress induction and mediate protein synthesis signaling pathways during stress via eIF2 are GCN2, HRI, PKR, and PERK, which are responsible for amino acid deprivation, heme deprivation, viral infection, and misfolded proteins, respectively.[4] Given the high clinical importance of proteostasis in neurodegenerative diseases, industry and academia show a strong interest in the small molecules that target the UPR.[10] However, the direct activation of PERK has rarely been reported.

To better understand protein synthesis in the brain, we previously established tamoxifen-induced and conditional PERK knockout mice.[3] Surprisingly, we found that PERK deletion in neurons decreased eIF2 phosphorylation but did not affect GCN and PKR expression.[3] Furthermore, we found that PERK deletion in neurons exacerbated brain injury and the potential mechanisms involved in suppressing global protein synthesis in a mouse MCAO model.[3] Notably, CCT020312 was first reported to be a selective PERK activator to prevent proliferative activity for cancer therapy, including breast cancer, colorectal cancer, and glioblastoma.[11,12−14;] We have observed that CCT020312 can kill glioma cells in vitro and in vivo and prolong survival in glioblastoma

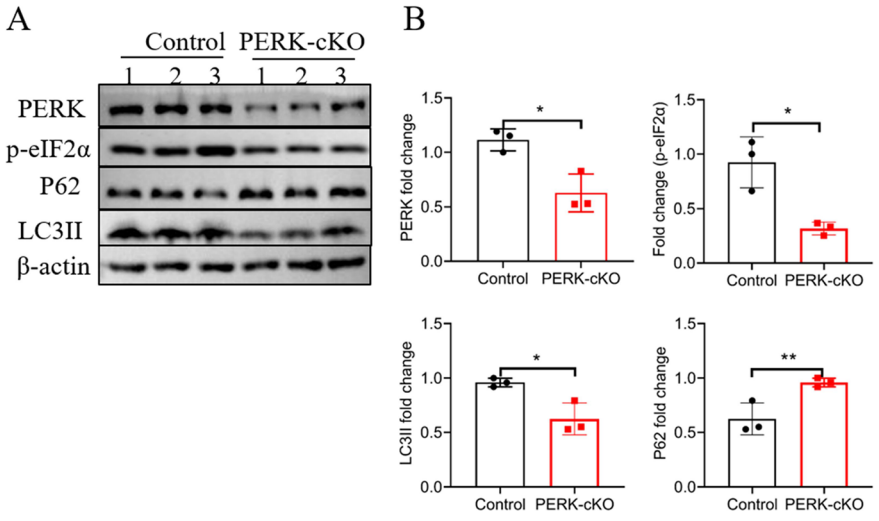

Figure 5. PERK mediated autophagy in ischemic stroke. Control or Perk-cKO mice were subjected to 30 min of MCAO. Ipsilesional cortex tissues were collected for Western blot analysis. (A) PERK, p-eIF2α, P62, and LC3-II were detected by Western blot. β-actin was used as a control for protein expression. (B) Quantities of PERK, p-eIF2α, LC3-II, and P62 in the ipsilesional tissues were measured (n = 3). Data are presented as mean ± SD. MCAO, middle cerebral artery occlusion. *p < 0.05 vs vehicle.

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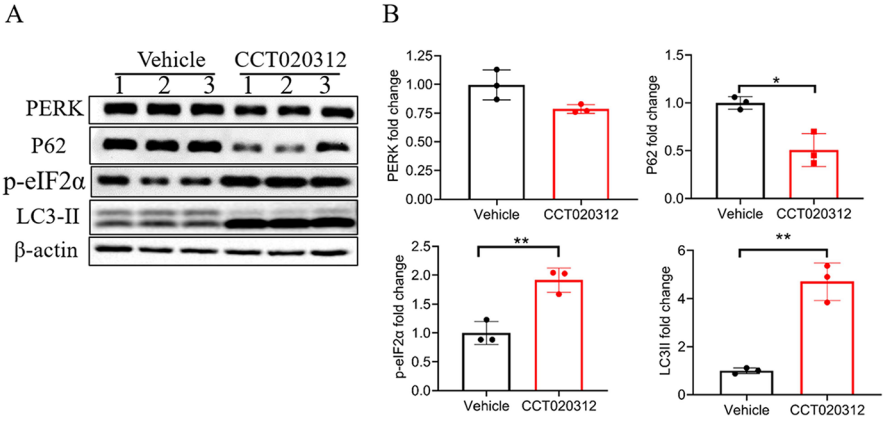

Figure 6. Postacute delivery of CCT020312 enhanced the PERK/p-eIF2α/LC3-II autophagy signaling pathway. Control or Perk-cKO mice were subjected to a 30 min MCAO and treated with CCT020312 1 h post MCAO. Brain tissues were collected for Western blot analysis. (A) PERK, p-eIF2α, eIF2α, LCII, P62, and Beclin were detected for Western blot. β-actin was used as a control for protein expression. (B) Quantities of PERK, p-eIF2α, LC3-II, and P62 in the ipsilesional and contralesional tissues were measured (n = 3). Data are presented as mean ± SD. MCAO, middle cerebral artery occlusion. *p < 0.05 vs vehicle.

(unpublished). Additionally, CCT020312 has been shown to mitigate tau pathology in neurodegenerative diseases such as AD and Huntington's disease.[15,16]

In a previous ischemic stroke study, activation of the PERK pathway and reversal of CTRP1 overexpression by CCT020312 alleviated the cell damage and neuronal injury induced by MCAO/R[28]; however, this study treated the rats with CCT020312 24 h before OGD and MCAO, whereas in our study, the mice were treated with CCT020312 at a dose of 5 mg/kg 1 h poststroke. Moreover, the administration methods were also different, as they administered CCT020312 through an intracerebroventricular injection, while we used intraperitoneal administration, which is more similar to that of previous studies[15−17] To confirm CCT020312 precisely activates PERK and its targets in the brain, we used the sham mice and intraperitoneal injection, then the brain tissues were collected at three time points from 1 to 6 h after administration. The Western blot results showed that p-eIF2α, which functions downstream of PERK, was significantly increased from 1 to 6 h; thereafter, it decreased and returned to normal at 24 h. Recently, our previous work confirmed that overexpression of BAG3 enhanced stroke outcomes by activating the autophagy pathway and inhibiting apoptosis even 24 h poststroke. Considering that the decreases in PERK/p-eIF2α 6 h after administration of CCT020312 returned to normal by 24 h, we thought that reinforcing PERK/p-eIF2α-dependent autophagy may enhance stroke outcomes.

Based on this hypothesis, CCT020312, as a potential small compound, directly activates PERK and improves stroke outcomes as evaluated by neurological scores and Rotarod test outcomes in a mouse MCAO model. Considering that motor coordination is often damaged by ischemic stroke, the Rotarod is a robust tool for assessing neurological motor recovery in mice, and we found that CCT020312 improved stroke recovery in the short term for 24 h. Moreover, we found that the mice treated with CCT020312 for 1 week showed sustainably improved neurological behaviors in the long term, including behavioral outcomes on the Rotarod.

To investigate the neuroprotective mechanisms of CCT020312 in ischemic stroke, we employed the SUnSET method for measuring protein synthesis with the non-radioactive compound puromycin.[21] As expected, protein labeling with puromycin was detected in the vehicle groups, whereas no signal was detected in the ischemic group without puromycin. Importantly, the level of puromycin signal was dramatically decreased in a dose-dependent manner in the CCT020312 treatment groups. Moreover, the levels of p-eIF2α were also significantly higher in the 5 mg/kg CCT020312-treated group compared with those in the vehicle and low-dose groups. In line with our previous work, we found that CCT020312 enhanced stroke outcomes by suppressing protein synthesis. To the best of our knowledge, the inhibition of protein synthesis caused by PERK is essential for survival under an ischemic stroke because it reduces the ER workload. However, PERK-induced eIF2α phosphorylation appears to be a double-edged sword because it induces ER stress, while PERK activates transcription factor 4 (Atf4) mRNA, which promotes the expression of CHOP, a pro-apoptotic player in ER stress-induced cell death.[8,10] Indeed, the PERK branch plays a double-edged sword role, enhancing the survival in the early phase by reducing the ER overload and resulting in apoptosis in the late phase when ER homeostasis is in chaos.[10] Therefore, precise manipulation of the PERK branch of the UPR is of pivotal importance for stroke therapy. Our data support the fact that activation of the PERK branch of the UPR in neurons provides neuroprotective effects in acute phase stroke.[3,10] Notably, several small molecules have been reported to target the UPR branch, and more continue to be discovered due to the industry's need and academia's search for potential candidate drugs that target the UPR.[8,9] We previously used salubrinal to inhibit p-eIF2α dephosphorylation and showed that acute treatment with salubrinal is beneficial for stroke,[3] which is in line with a previous report.[29] Herein, based on our previous work, we found that CCT020312 directly enhanced PERK/p-eIF2α and enhanced short- and long-term stroke outcomes.

To investigate the potential mechanisms, Perk-cKO was established and was confirmed by genotyping and Western blotting. Several studies have reported that phosphorylated PERK/eIF2α activates autophagy and enhances the conversion of microtubule-associated protein 1 LC3-I to LC3-II.[22,23] In line with these reports, we found that LC3-II expression was significantly increased, while P62 expression was significantly decreased in Perk-cKO mice in acute ischemic stroke,

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compared with control mice. Our data indicated that phosphorylated PERK/eIF2a is vital for the expression of autophagy marker LC3-II. In a recent study, we confirmed that BAG3 overexpressed by AAV activated autophagy and inhibited apoptosis, providing neuroprotection 24 h after acute ischemic stroke. The autophagy pathway is still activated 24 h poststroke.[19] Therefore, considering that PERK/p-eIF2a is decreased 6 h poststroke, we thought CCT010312 may provide neuroprotection via reinforcement of the PERK/p-eIF2α-dependent autophagy pathway.

Therefore, MCAO was performed for 30 min in control mice, and then CCT020312 was administrated 1 h poststroke, the ipsilesional tissues were collected, and autophagy markers LC3-II and P62 were evaluated. As expected, our data showed that CCT020312 reinforced phosphorylated PERK/eIF2α and enhanced LC3-II expression while decreasing P62 expression, compared with control mice.

Notably, there are some limitations to our study. Only young male mice were evaluated. To further evaluate the therapeutic potential of CCT020312 in stroke, females and aged animals, as well as other stroke models and species, should be considered in future studies.

Ischemic stroke is caused by a lack of oxygen and energy, which impairs most cellular pathways, including ER-resident protein folding and maturation. Therefore, activation of the UPR is critical for handling ER-stress neuronal survival.[3] Focusing on the PERK branch, we employed Perk-cKO mice and two small molecular compounds, salubrinal and CCT020312, to evaluate the role of PERK in ischemic stroke. Based on our previous and current data, whether through direct or indirect means, activation of PERK plays an important role in neuroprotection in acute ischemic stroke, as it reduces global protein synthesis and the ER workload by activating autophagy in vivo. Therefore, the development of strategies targeting the PERK branch of the UPR represents an optimal approach to improving stroke outcomes.

CONCLUSIONS

CCT020312 shows promise as a therapeutic agent by activating PERK-p-eIF2α to enhance autophagy and reduce protein synthesis, offering neuroprotection in vitro and in vivo. However, further research is needed to address study limitations and confirm its efficacy across diverse species.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.5c00125.

Scheme S1, Perk-cKO mice model; Scheme S2, infarct volume measurements; Figure S1, verification of the in vivo effect of CCT020312 on eIF2α phosphorylation; Figure S2, CCT020312 improved the long-term stroke outcomes in acute mice ischemic stroke; Figure S3, delivery of CCT020312 poststroke reduced cell apoptosis; Figure S4, characterization of neuron-specific deletion of PERK-conditional knockout (cKO) mice; Figure S5, CCT020312 administration did not affect the stroke outcomes in Perk-cKO mice; and Table S1, animal use (PDF)

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X.L., D.L., L.Z., and L.M. contributed equally. Y.W. designed and conceptualized the study. The in vitro experiments were designed by Ulf Brockmeier. L.Z., X.L., Q.Y., L.M., D.L., Y.Y.,

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and X.Q. performed the experiments. Y.Z. and Ulf Brockmeier analyzed the data. Y.W. and G.H. wrote the manuscript.

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Notes

The authors declare no competing financial interest.

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