Post-treatment with Posiphen Reduces Endoplasmic Reticulum Stress and Neurodegeneration in Stroke Brain

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Article

Post-treatment with Posiphen Reduces Endoplasmic Reticulum Stress and Neurodegeneration in Stroke Brain

Seong-Jin Yu, Kuo-Jen Wu, Eunkyung Bae, Yu –Syuan Wang, Chia-Wen Chiang, Li-Wei Kuo, Brandon K. Harvey, Nigel H. Greig, and Yun Wang

SUMMARY

Acetylcholinesterase (AChE) inhibitors have protective and anti-inflammatory actions against brain injury, mediated by nicotinic α7 cholinergic receptor activation. The use of AChE inhibitors in patients is limited by systemic cholinergic side effects. Posiphen, a stereoisomer of the AChE inhibitor Phenserine, lacks AChE inhibitor activity. The purpose of this study is to determine the protective effect of Posiphen in cellular and animal models of stroke. Both Posiphen and Phenserine reduced glutamate-mediated neuronal loss in co-cultures of primary cortical cells and microglia. Phenserine-, but not Posiphen-, mediated neuroprotection was diminished by the nicotinic α7 receptor antagonist methyllycaconitine. Posiphen antagonized NMDA-mediated Ca ++ influx, thapsigargin-mediated neuronal loss and ER stress in cultured cells. Early post-treatment with Posiphen reduced ER stress signals, IBA1 immunoreactivity, TUNEL and infarction in the ischemic cortex, as well as neurological deficits in stroke rats. These findings indicate that Posiphen is neuroprotective against stroke through regulating Ca ++ i and ER stress.

INTRODUCTION

Stroke is a major brain disease worldwide, being the second leading global cause of death in the past decade (2000–2012; www.who.int/mediacentre/factsheets/fs310/en/index.html). Tissue plasminogen activator (tPA) is the only US FDA-approved pharmacological therapy for acute ischemic stroke. tPA dissolves occluding blood clots at a very early stage of stroke, but its effectiveness is limited by a narrow therapeutic time window of 3 h. Less than 3% of patients with stroke receive tPA, because they do not arrive at a hospital early enough for treatment (Barber et al., 2001; Reed et al., 2001). It is thus important to develop new therapies for stroke.

Cholinergic mechanisms are involved in various models of neurodegeneration including stroke (Pavlov and Tracey, 2005; Vijayaraghavan et al., 2013). Acetylcholine (ACh) ameliorated glutamate or N-methyl-D-aspartate (NMDA)-mediated toxicity in neuronal cells (Wehrwein et al., 2004; Dajas-Bailador et al., 2000) and mitigated cell death and inflammation via the cholinergic anti-inflammatory pathway (Vijayaraghavan et al., 2013; Pavlov and Tracey, 2005). The hydrolysis of ACh is enhanced after stroke (Ben et al., 2010), which results in reduced ACh levels (Haba et al., 1991; Tanaka et al., 1994) and a rise in the ACh metabolite choline in brain (Scremin and Jenden, 1989). Pretreatment with selective acetylcholinesterase (AChE) inhibitor Phenserine (Greig et al., 2005) reduced activated caspase 3, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and volume of infarction in the rat focal middle cerebral artery occlusion (MCAo) model (Chang et al., 2017). However, the efficacy of AChE inhibitor treatment depends on cholinergic integrity as seen in patients with early Alzheimer’s disease (Richter et al., 2018) and the use of AChE inhibitors can be limited by the systemic cholinergic adverse effects (Colombres et al., 2004; Doody, 2003; Snape et al., 1999).

Posiphen (Pos), also known as (+)-Phenserine, a stereoisomer of Phenserine that cannot generate either Phenserine or its metabolites, has also been reported to increase cell survival (Lilja et al., 2013), protect against neurodegeneration in animal models of Alzheimer’s disease, and reduce neuroinflammation (Yu et al., 2013; Maccecchini et al., 2012; Teich et al., 2018). Unlike Phenserine, Posiphen is devoid of cholinesterase (ChE) inhibition activity or cholinergic side effects (Klein, 2007). Posiphen also does not bind to muscarinic or nicotinic cholinergic receptors (Lahiri et al., 2007; Lecca et al., 2019). As such, it appears to...
be distinct from various other Alzheimer’s disease drugs and potential treatments, such as Phenserine, that reduce degeneration through multiple mechanisms that include the cholinergic anti-inflammatory pathway (Pavlov et al., 2009; Han et al., 2017). The potential protective action against stroke has not been reported for Posiphen.

The endoplasmic reticulum (ER) is essential for the maintenance of intracellular protein function. The ER controls the folding of newly synthesized proteins into their mature conformation and the transport of matured/secreted proteins to other compartments within the cells. The ER also serves as the primary intracellular reservoir for calcium, and reducing ER Ca\(^{2+}\) causes ER stress. We previously reported a method to monitor ER calcium homeostasis through the use of secreted ER calcium—monitoring proteins (SERCaMPs) in SH-SYSY cells (Henderson et al., 2014). This construct also includes the reporter Gaussia luciferase (GLuc) thus forming GLuc-SERCaMP (Chen et al., 2019). In cells expressing GLuc-SERCaMP, the ER stressor thapsigargin (Tg) or methamphetamine induces secretion of GLuc (Chen et al., 2019). Systemic application of Tg increased GLuc release into plasma in rats receiving an intrahepatic injection of AAV-GLuc-SERCaMP (Wires et al., 2017). These data suggest that GLuc-SERCaMP is a useful pharmacological tool for monitoring ER stress in cell culture or in vivo.

Several genetically encoded Ca\(^{2+}\) indicators (GECIs) have been developed to monitor intracellular Ca\(^{2+}\) (Ca\(^{2+}\)) in neurons and non-neuronal cells (Mao et al., 2008; Shigetomi et al., 2010; Tian et al., 2009). gCaMP is a commonly used GECI probe, which consists of a single circularly permuted green fluorescent protein (GFP), calmodulin (CaM), and an M13 fragment from myosin light-chain kinase. Binding of Ca\(^{2+}\) to CaM induces conformational changes of gCaMP and results in increased fluorescence intensity in cells (Nakai et al., 2001). We previously delivered gCaMP5 to cultured neuronal cells using an adeno-associated viral (AAV) vector (Akerboom et al., 2012). We demonstrated that methamphetamine enhanced Ca\(^{2+}\) in real time in neurons, which was antagonized by MK801, Mg\(^{2+}\), or the ryanodine receptor (RyR) inhibitor dantrolene.

In the present study, we examined the neuroprotective actions of Phenserine and its non-cholinergic stereoisomer Posiphen in cellular and rodent models of stroke. Both Posiphen and Phenserine reduced glutamate-mediated neurodegeneration. Posiphen selectively mitigated thapsigargin (Tg)-mediated neuronal death and the release of ER stress markers in cells over-expressing GLuc-SERCaMP as well as the NMDA–mediated increase in Ca\(^{2+}\). Early post-ischemia treatment with Posiphen reduced the calcium-binding adaptor molecule 1 (IBA1) immunoreactivity, TUNEL activity, brain infarction, and the expression of ER stress markers in the lesioned cortex in stroke rats. These data suggest that Posiphen reduces ischemic neuronal injury through regulating Ca\(^{2+}\), and ER stress.

**RESULTS**

**Posiphen and Phenserine Elicit Neuroprotection in Primary Cortical Cells and Microglia Co-cultures**

We first examined Posiphen- and Phenserine-mediated neural protection in a mixed primary cortical cell and BV2 microglia co-culture. A high dose (100 µM) of glutamate (Glu) was used to generate neurodegeneration and inflammation (Wu et al., 2017; Choi et al., 1987) and to simulate overflow of elevated glutamate during cerebral ischemia (Shen et al., 2005). Glutamate significantly reduced the neuronal marker Microtubule-Associated Protein 2 (MAP2; Figures 1A and 1C, p < 0.001), associated with an elevation in ionized calcium-binding adapter molecule 1 (IBA1) immunoreactivity (Figures 1A and 1D, p < 0.001). Both responses were significantly mitigated by 15 µM Phenserine (MAP2, p < 0.001; IBA1: p = 0.005) or by Posiphen (MAP2, p < 0.001; IBA1: p = 0.05) as seen in Figures 1C and 1D (timeline of experiment: Figure 1B). Co-treatment with the nicotinic α7 receptor antagonist methyllycaconitine (MLA, at 500 nM) significantly attenuated Phenserine- (Figure 1E, Glu + Phe versus Glu + Phe + MLA, p = 0.004), but not Posiphen- (Glu + Pos versus Glu + Pos + MLA, p = 0.980; Glu versus Glu + Pos + MLA, p < 0.001), mediated protection, suggesting a non-cholinergic protective action of Posiphen and a cholinergic component for the neuroprotective actions of Phenserine.

**Posiphen Reduces Glutamate Neurotoxicity in Primary Cortical Neuronal Cultures**

Since inflammation mainly occurs in the neuron + astrocyte/microglia co-culture, we next examined the protective effect of Posiphen and Phenserine 15 µM in primary cortical neuronal culture in the absence of microglia. Posiphen selectively antagonized glutamate-mediated neuronal loss (Figures 2A and 2B,
Glu versus Glu + Posiphen, *p < 0.001). A mild, but not significant, improvement in MAP2 immunoreactivity was found after Phenserine treatment (Glu versus Glu + Phe, *p = 0.107). A significant difference was also found between Glu + Pos and Glu + Phe (*p = 0.001, Figure 2B). Glutamate-mediated apoptosis was examined by TUNEL labeling (Figure 2C). Posiphen, but not Phenserine, significantly reduced glutamate-enhanced TUNEL activity in the neuronal culture (Figure 2D, *p < 0.001).

(+)-N8-NorPosiphen is a major metabolite of Posiphen found in brain and plasma after systemic administration of Posiphen (Teich et al., 2018). (+)-N8-NorPosiphen, similar to Posiphen, does not bind to cholinergic receptors and has no anti-AChE activity in peripheral erythrocytes (Yu et al., 2013). Treatment with (+)-N8-NorPosiphen (15 µM) significantly antagonized glutamate-mediated loss of MAP2 immunoreactivity in primary neuronal culture (*p < 0.001, F(3,20) = 29.986, one-way ANOVA + Newman-Keuls [NK] test, Figure 2E), suggesting (+)-N8-NorPosiphen is an active metabolite of Posiphen.
Posiphen Alters Intracellular Ca
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We previously measured glutamate-mediated increase in intracellular Ca++ signal in primary cortical neurons overexpressing an intracellular Ca++ probe, gCaMP5, by AAV serotype 1 (Yu et al., 2016). Utilizing this approach we examined NMDA-mediated changes in Ca++ in primary cortical cultures. Cultured cells were infected with AAV1-gCaMP5 on DIV5. Intracellular Ca++, as indicated by a change in intracellular green fluorescence, was monitored on DIV 10. Representative interactions between Posiphen, Phenserine, and NMDA are shown in the captured live images (Figure 3B). NMDA at 100 nM induced a rapid increase in intracellular Ca++ as indicated by increasing intracellular green fluorescence (Figure 3B, left lower panel). Peak fluorescence occurred within 3–6 s after treatment. Posiphen or Phenserine (15 μM) was given to cells 4 min before the application of NMDA (timeline, Figure 3A). Pretreatment with Posiphen (Figure 3B, middle panels), but not Phenserine (Figure 3B, right panels), suppressed

Figure 2. Posiphen, but Not Phenserine, Antagonized Glutamate-Mediated Neuronal Loss and TUNEL Labeling in Primary Cortical Neuronal Culture
(A and C) Representative photomicrographs demonstrate that Posiphen antagonized glutamate-mediated (A) loss of MAP2 immunoreactivity and (C) an increase in TUNEL labeling.
(B) Posiphen, but not Phenserine, significantly antagonized glutamate- (100 μM) mediated neuronal loss (*p < 0.001, Glu versus Glu + Posiphen; p = 0.107, Glu versus Glu + Phenserine, F3,34 = 33.168, one-way ANOVA + NK test, n = 7 in each group).
(D) Posiphen significantly antagonized glutamate-mediated increased TUNEL activity (*p < 0.001, Glu versus Glu + Posiphen; p = 0.212, Glu versus Glu + Phenserine, F3,26 = 47.867, one-way ANOVA + NK test, n = 6–9 in each group).
(E) (+)-N8-NorPosiphen (15 μmol/L), a metabolite of Posiphen with no anti-AChE activity, also antagonized glutamate-mediated loss of MAP2 immunoreactivity (*p < 0.001, F3,20 = 29.986, n = 6 in each group). “n” represents the number of replicates. Data are represented as mean ± SEM. Scale bar: 100 μm in (A) and 50 μm in (C).

Posiphen Alters Intracellular Ca++
We previously measured glutamate-mediated increase in intracellular Ca++ signals in real time in primary cortical neurons overexpressing an intracellular Ca++ probe, gCaMP5, by AAV serotype 1 (Yu et al., 2016). Utilizing this approach we examined NMDA-mediated changes in Ca++ in primary cortical cultures. Cultured cells were infected with AAV1-gCaMP5 on DIV5. Intracellular Ca++, as indicated by a change in intracellular green fluorescence, was monitored on DIV 10. Representative interactions between Posiphen, Phenserine, and NMDA are shown in the captured live images (Figure 3B). NMDA at 100 nM induced a rapid increase in intracellular Ca++ as indicated by increasing intracellular green fluorescence (Figure 3B, left lower panel). Peak fluorescence occurred within 3–6 s after treatment. Posiphen or Phenserine (15 μM) was given to cells 4 min before the application of NMDA (timeline, Figure 3A). Pretreatment with Posiphen (Figure 3B, middle panels), but not Phenserine (Figure 3B, right panels), suppressed
Figure 3. Posiphen Reduced NMDA-induced Ca++ in Primary Cortical Neuronal Culture Overexpressing gCaMP5

The calcium probe gCaMP5 was overexpressed in the primary cortical neurons through AAV infection.

(A) Timeline of drug treatment. Posiphen or Phenserine (15 μM) was given (blue arrow) 4 min before administration of NMDA (100 nM, red arrow, time 0). Typical real-time intracellular Ca++ concentration (Ca++i) fluorescence images were taken at 5 s before and 5 s (black arrows) after NMDA administration.

(B) Administration of NMDA triggered a rapid increase in intracellular Ca++ as indicated by increasing intracellular green fluorescence at 5 s after NMDA administration (left lower panel), compared with the image taken 5 s before drug treatment (left upper panel). Pretreatment with Posiphen (middle panels), but not Phenserine (right panels), suppressed NMDA-mediated increased intracellular Ca++ signals. Scale bar, 100 μm.

(C) The intensity of fluorescence (% maximal response) was analyzed in each well every second. Administration of NMDA (red arrow at time 0) significantly increased Ca++i, as compared with the response before NMDA administration (n = 13). Pretreatment with Posiphen (n = 8) significantly antagonized NMDA-mediated increases in Ca++i (Pos + NMDA versus NMDA: p < 0.001, two-way ANOVA + NK test). Significant reductions in Ca++i after Posiphen treatment occurred between 1 and 17 s after administration of NMDA (**p = 0.001–0.047, two-way ANOVA + NK test). Treatment with Phenserine (n = 8) did not alter NMDA-mediated Ca++i (Phe + NMDA versus NMDA: p = 0.619).

(D) The peak Ca++i was significantly suppressed after Pos treatment (**p < 0.001). "n" represents the number of replicates. Data are represented as mean ± SEM.
NMDA-mediated intracellular Ca^{++} signals. The temporal changes in intensity of intracellular gCaMP5 fluorescence was analyzed in each well every second from 0 to 30 s (Figure 3C). NMDA significantly increased Ca^{++} fluorescence intensity (p < 0.01, n = 13). Pretreatment with Posiphen (n = 8), but not Phenserine (n = 8), significantly attenuated NMDA-induced increases in Ca^{++}i (Pos + NMDA versus NMDA: p < 0.001; Pos + NMDA versus Phe + NMDA: p < 0.001; Phe + NMDA versus NMDA: p = 0.619, Figure 3C). The reductions in Ca^{++}i after Posiphen treatment were statistically significant between 1 and 6 s after administration of NMDA (p = 0.001–0.036, two-way ANOVA + NK test) and Glu-mediated secretion of GLuc (E). Posiphen significantly reduced Tg (*p < 0.001) and Glu-mediated gLuc activity. (A), (B), (C), n = 6–8; (D), (E), n = 4–8 in each group). “n” represents the number of replicates. Data are represented as mean ± SEM.

Posiphen Reduces ER Stress in Cultured SH-SY5Y and Primary Cortical Cells

As disturbance of intracellular and ER Ca^{++} homeostasis can lead to ER stress (Krebs et al., 2015; Williams et al., 2013), we next examined the interaction of Posiphen with the ER stressor Tg in primary cortical cell cultures. Treatment with Tg (500 nM) significantly reduced MAP2 immunoreactivity (Figure 4A and 4B, p < 0.001). This response was partially antagonized by Posiphen (Figure 4A, p = 0.026), but not by Phenserine (each 15 μM) (Figure 4B, q = 2.673, one-way ANOVA on rank + NK test).

Tg-mediated ER stress was also examined in SH-SY5Y cells expressing GLuc-SERCaMP, a reporter for ER stress and proteostasis (Trychta et al., 2018) that is triggered by ER calcium depletion (Henderson et al., 2014). Treatment with Tg significantly increased luciferase (GLuc) activity in media collected 48 h after...
drug treatment (Figure 4C, p = 0.002, Tg versus veh). Co-treatment with Posiphen effectively blocked Tg-induced GLuc activity (p < 0.001). Phenserine also reduced the Tg-induced GLuc activity (Tg versus Phe + Tg, p = 0.001, Figure 4C) but was less effective than Posiphen (Pos + Tg versus Phe + Tg, p = 0.002, Figure 4C).

Tg-mediated GLuc-SERCaMP release was also examined in primary cortical cells expressing GLuc-SERCaMP on DIV13 (Figures 4D and 4E). Tg or glutamate significantly increased the secretion of GLuc at 8 or 48 h post-treatment, respectively (Figure 4D, p = 0.002; E: p < 0.001). Both Tg and glutamate responses were antagonized by Posiphen (Figure 4D, Tg + Pos versus Tg, p = 0.001; Figure 4E, Glu + Pos versus Glu, p = 0.016). Phenserine did not significantly alter Tg- (p = 0.153, Figure 4D) or glutamate-mediated secretion of GLuc (Figure 4E).

Neuroprotective Action of Posiphen in Stroke Rats

Pretreatment with Phenserine has been reported to reduce brain infarction volume (Chang et al., 2017). Phenserine and Posiphen were first examined here for stroke protection activity using standard triphenyl tetrazolium chloride (TTC) staining. Animals received a 60-min MCAo on day 0. Phenserine (1 mg/kg/day, i.p.) or Posiphen (25 mg/kg/day, i.p.) was administered daily as a post-treatment for 4 days (from day 1 to day 4) after MCAo. Both Posiphen and Phenserine significantly reduced brain infarction on day 5 (p < 0.01, n = 10, Figure 5). Phenserine is dose limited at 25 mg/kg by its cholinergic actions, whereas Posiphen is not and can hence be administered at this and higher doses (Lahiri et al., 2007). All animals receiving Posiphen (25 mg/kg) tolerated the dose well prior to TTC staining. The protective effect of Posiphen was further examined by MRI T2WI in 16 rats (Figure 6A). Posiphen (25 mg/kg/day, i.p.) or vehicle was administered twice, at 1 h and 1 day after MCAo. An increase in T2WI signal intensity was found in the cortex on the lesioned side 2 days after MCAo. Typical T2WIs from two rats receiving Posiphen or vehicle are illustrated in Figure 6A1. Post-treatment with Posiphen significantly reduced brain infarct size (p < 0.001, Figure 6A2). Two neurological tests were used to examine behavioral improvement in 24 stroke rats. In an elevated body swing test, Posiphen treatment significantly reduced body asymmetry in 20 trials (*p = 0.0014, vehicle, n = 11, Posiphen, n = 13, t test, Figure 6A3). Posiphen also significantly attenuated neurological deficits, examined by Bederson’s neurological test, in stroke rats (p = 0.001; vehicle, n = 11, Posiphen, n = 13, Mann-Whitney rank-sum test, Figure 6A3).
Figure 6. Post-treatment with Posiphen Reduced Neurodegeneration in Stroke Rats

Adult rats received a 60-min MCAo. Posiphen (25mg/kg, i.p.) or veh was given 30 min after the MCAo and the following day (days 0 and 1).

(A1) Representative T2WI from two stroke rats receiving with vehicle (upper) or Posiphen (lower). Scale bar, 10 mm.

(A2) The area of infarction was averaged in each millimeter in stroke rats receiving vehicle (n = 8) or Posiphen (n = 8). Posiphen treatment significantly reduced brain infarct size (*p < 0.001, two-way ANOVA + NK test).
Animals were euthanized and perfused on day 3 for TUNEL and IBA1 immunohistochemical assays. TUNEL activity was quantified and averaged in three consecutive brain sections at the level of the anterior commissure for each animal (Figure 6 B1). Posiphen significantly reduced TUNEL-positive cell density (Figure 6 B2, p = 0.048, veh n = 3; Pos, n = 5, t test).

Enhanced IBA1 immunoreactivity was found in the peri-lesioned cortical area in stroke animals receiving vehicle (Figure 7A1). Microglia exhibited a ramified morphology in the non-lesioned cortex (Figure 7A1, left panel, inset), whereas rounded or ameboid microglial cells were found in the lesioned cortex in stroke animals receiving vehicle (Figure 7A1, middle panel, inset). Posiphen reduced IBA1 immunoreactivity (Figure 7A1) as well as morphological evidence of activation of microglia in the peri-lesioned area (Figure 7A1, right panel, inset). Partially ramified microglia were also found in the peri-lesioned area in animals receiving Posiphen (Figure 7A1, right panel, inset). The averaged IBA1 optical density in the peri-lesioned zone was significantly reduced by Posiphen (p < 0.01, Figure 7A2). The reduction of IBA1 activity by Posiphen was also confirmed by western blot analysis (Figure 7B1). Cortical brain tissue was collected from eight rats on day 2. Posiphen significantly reduced IBA1 protein expression in the lesioned cortex (Figure 7B2, p < 0.01).

Detection of ER Stress in Stroke Brain

The ability of Posiphen to mitigate ER stress was next examined using GLuc-based SERCaMP. A total of 10 rats received intracerebral administration of AAV-GLuc SERCaMP 2 weeks before MCAo. Posiphen (n = 5) or vehicle (n = 5) was given i.p. 1 h and 1 day after a 30-min MCAo. Animals were anesthetized 60 min after the second dose of Posiphen for an in vivo imaging system (IVIS) scanning. The GLuc substrate Coelenterazine (100 µg/150 µL) was administered intravenously through the tail vein. A minimal GLuc luminescence signal was detected before injection of Coelenterazine (Figure 8A). Enhanced GLuc activity was found 1 min after substrate administration, gradually declining over 10 min. Posiphen significantly attenuated GLuc activity (Figure 8B, p = 0.007). No GLuc activity was found in control animals without MCAo. Cerebral tissues were collected after IVIS scanning for GLuc immunostaining. GLuc immunoreactivity was found in the cortex near the AAV injection sites (Figure 8C). As seen in Figure 8D, GLuc was expressed mainly in neuronal cells. Cerebral cortical tissues were also collected from 12 rats at 1 day after a 30-min MCAo (vehicle, n = 6; Posiphen, n = 6) and 5 non-stroke rats for the ER stress marker BiP protein analysis (Figures 8E and 8F). Posiphen (n = 6) significantly reduced the expression of BiP in the cortex on the lesioned side (p = 0.017, Figures 8E and 8F).

Another set of animals (nonstoke, n = 8, stroke + veh, n = 8, stroke + Posiphen, n = 8) was used to examine ER stress markers by qRTPCR. Cortical tissue was collected 2 days after a 60-min MCAo. BiP and Chop, but not Perk, were significantly upregulated in the cortex on the ischemic side (Figure 8G, BiP, p = 0.007, Chop, p = 0.006). Post-treatment with Posiphen reduced expression of BiP (p = 0.025) and marginally attenuated Chop expression (p = 0.056). In the contralateral cortex, expression of Chop or BiP was not altered by MCAo or Posiphen treatment (data not shown). Perk, however, was significantly upregulated in the contra-lateral cortex after MCAo (data not shown).

DISCUSSION

We characterized the protective effect of Posiphen in neuronal cultures and experimental animals. Posiphen reduced the glutamate-mediated loss of MAP2-ir, the glutamate-induced increased TUNEL staining, and the glutamate-induced microglia activation in primary neuronal cultures. Parallel neuroprotective effects were seen in animals, since early post-stroke treatment with Posiphen significantly improved behavioral function, reduced brain infarction, and suppressed expression of inflammatory and ER stress markers in stroke rats. Thus, Posiphen is a potent non-cholinergic neuroprotective agent against ischemic stroke in animals.

CNS cholinergic activity is altered by stroke. For example, ACh levels in hippocampus are decreased following stroke in rats (Haba et al., 1991; Tanaka et al., 1994). The binding of the cholinergic ligands, [3H]quinuclidinyl...
benzilate or pirenzepine, to muscarinic receptors was found reduced up to 14 days after transient forebrain ischemia in the gerbil (Onodera et al., 1987; Hirata et al., 1992). Nicotinic ACh receptors (alpha 7R) were significantly down-regulated after hypoxia-ischemia injury in the neonatal mouse brain (Hua et al., 2014). Recent studies support a cholinergic anti-inflammatory pathway modulating cell death and inflammation during cerebral ischemia (Pavlov and Tracey, 2005; Vijayaraghavan et al., 2013). The non-selective cholinesterase inhibitor rivastigmine (formerly known as ENA-713), which increases ACh in the synaptic cleft and facilitates cholinergic transmission, reduced ischemia-mediated pyramidal cell loss in the hippocampal CA1 region (Tanaka et al., 1994). Furthermore, elevated total ChE hydrolytic activity has been shown to correlate with the expression of inflammatory markers and cytokines in patients with stroke (Ben et al., 2010), suggesting that hydrolysis of ACh by elevated ChE reduces ACh availability to influence inflammation. We found that the AChE inhibitor Phenserine antagonized glutamate-mediated neuronal death and microglia activation in a mixed neuron/microglia culture; Phenserine-mediated neuroprotection was partially antagonized by methyllycaconitine, indicating its protection was, in part, mediated through the nicotinic a7 receptor. This finding is also supported by a recent study showing that treatment with the nicotinic receptor a7 agonist PNU-282987 reduced expression of activated caspase-3 in stroke brain and reduced neurological deficits (Duris et al., 2011). Phenserine, likewise, significantly reduced brain infarction size in stroke rats (Figure 5). This result cross-validates and extends a prior study demonstrating that pretreatment with Phenserine (1 mg/kg, single dose) reduces stroke volume in rats (Chang et al., 2017). Importantly, Phenserine was administered after ischemic injury in the present study. Mechanisms underpinning neuroprotective actions of Phenserine appear to be cholinergically as well as non-cholinergically mediated.

Posiphen is a stereoisomer of Phenserine with no direct cholinergic (i.e., binding to cholinergic receptors) or indirect cholinergic (i.e., AChE inhibition) activity (Yu et al., 2013). Posiphen has been shown to lower amyloid-beta precursor protein (APP) level in cultured human neuroblastoma cells and in mouse brain (Lahiri et al., 2007). Posiphen, similar to Phenserine, suppresses phytohemagglutinin-induced interleukin-1 mRNA expression in cultured human peripheral blood mononuclear cells (Yu et al., 2013). In a small non-randomized clinical study, Posiphen was found to reduce inflammatory markers (i.e., MCP-1, Complement C3) in the CSF of patients with mild cognitive impairment (Maccecchini et al., 2012). These data suggest that Posiphen has neuroinflammatory modulatory effects in the CNS. We demonstrated here that Posiphen selectively mitigated glutamate-induced neurotoxicity in neuronal cultures. A similar protective response was found after treatment with (+)-N8-NorPosiphen. (+)-N8-NorPosiphen is a major metabolite of Posiphen.
Figure 8. Posiphen Suppressed ER Stress in Stroke Brain

(A) Posiphen (25mg/kg, i.p.) reduced release of ER stress marker GLuc-SERCaMP from the lesioned brain at 1 day after a 30-min MCAo. Adult rats received intracerebral administration of AAV-GLuc SERCaMP 2 weeks before the MCAo. GLuc activity was determined by IVES after administration of Coelenterazine (i.v. through the tail vein). Peak GLuc activity was found 1 min after injection of Coelenterazine in a stroke rat receiving veh (left panel, blue color) and was reduced by Posiphen (right panel).
found in brain and plasma after systemic administration of Posiphen (Macccechini et al., 2012; Teich et al., 2018). (+)-N8-NorPosiphen, similar to Posiphen, does not bind to cholinergic receptors and is devoid of AChE inhibitory activity (Yu et al., 2013). Notably, the protective effect of Posiphen was not altered by the nicotinic α7 receptor antagonist methyllycaconitine, supporting the premise that Posiphen induces protection through non-cholinergic mechanisms.

Intracellular Ca++ homeostasis plays an important role in regulating cell signaling, function, and death. Ischemic/hypoxic injury facilitates glutamate release and increased Ca++ levels in the cytoplasm. Blocking NMDA receptors inhibits the inward flow of calcium ions and reduces neuronal injury (Lin et al., 2019). We previously demonstrated that MK801 reduced Glu-activated Ca++ influx in neuronal cells expressing the Ca++ probe gCaMP5 (Yu et al., 2016). To characterize the direct neuroprotective actions of Posiphen, we evaluated intracellular Ca++ homeostasis using the same technique. Posiphen, but not Phenserine, significantly antagonized the NMDA-mediated increase in intracellular Ca++. As increasing intracellular Ca++ further activates apoptosis, we found that Posiphen treatment reduced TUNEL labeling in cultured neuronal cells and in stroke brain.

An imbalance in cytosolic Ca++ can lead to ER stress. The high Ca++ in ER is preserved by Ca++ regulator proteins (Sacro ER Ca++ ATPase protein or SERCA, Ryanodine receptor or RyR, and inositol triphosphate receptor) on the ER membrane (Raffaello et al., 2016). Tg inhibits SERCA and elicits ER stress as a consequence of the change in cytosolic and ER Ca++ (Giorgi et al., 2016). Similarly, methamphetamine can induce ER stress (Chen et al., 2019) and increase cytoplasmic Ca++ (Yu et al., 2016) in primary neuronal cultures. Co-treatment with the RyR antagonist dantrolene, which regulates ER stress (Li et al., 2003), inhibits the methamphetamine-mediated increase in Ca++ (Yu et al., 2016). In this study, we found Posiphen not only inhibited Ca++ but also selectively suppressed Glu and Tg-mediated Glu-SERCaMP release and Tg-mediated neuronal death in cell culture. Furthermore, Posiphen, but not Phenserine, significantly inhibited Glu or Tg-mediated ER stress in SH-SY5Y cells and primary neurons, as determined by Glu-SERCaMP release (Henderson et al., 2014). These data hence support the neuroprotective action of Posiphen through inhibition of ER stress in cell culture. Ischemic brain injury also leads to ER stress and can induce further damage in vivo. In this study, we overexpressed Glu-SERCaMP in the cerebral cortex by AAV infection. Glu immunoreactivity was mainly located in NeuN (+) cells in the cerebral cortex. We demonstrated that ischemia causes release of Glu in the lesioned cortex, determined by IVIS analysis, suggesting that this ER stress marker was mainly released from neuronal cells. Post-treatment with Posiphen reduced Glu release from the ischemic brain. The reduction of ER stress by Posiphen was further validated by the downregulation of the ER stress markers BiP and Chop in the ischemic cortex, suggesting that Posiphen reduces ER stress in ischemic brain. As a consequence of the reduction of ER stress, we found that animals treated with Posiphen had a smaller brain infarction, determined by T2W MRI imaging. In accordance with our in vitro cell culture study, Posiphen also reduced TUNEL labeling, morphological activation of microglia, and IBA1 expression in the stroke brain and augmented normal behavioral function in stroke rats.

In conclusion, we demonstrated that Posiphen is neuroprotective against excitatory amino acid and ischemia-mediated brain injury through regulating Ca++ and ER stress. Early post-treatment with Posiphen reduces neurodegeneration, inflammation, and neurological deficits in stroke animals. Posiphen is...
currently under clinical trials for Alzheimer’s disease (https://clinicaltrials.gov/ct2/show/NCT02925650). It has been reported that people with Alzheimer’s disease have a higher risk of stroke (Tolppanen et al., 2013). A high incidence of dementia was also found in the year after a major stroke (Pendlebury and Rothwell, 2019). Posiphen may be a useful therapeutic agent to prevent or treat comorbidity of stroke and dementia, while devoid of cholinergic side effects in patients.

Limitations of the Study
Ischemic brain injury activates a series of time-dependent pathophysiological responses. Some of these reactions occur shortly after stroke, whereas others can be activated at a much later stage. This study focused on the protective effect of Posiphen at the early phase after injury. We demonstrated that Posiphen reduces injury-mediated Ca++i, ER stress, apoptosis in cell culture and in a rat model of stroke. Future studies will need to focus on studying the interaction of Posiphen with delayed degenerative responses and neural repair after ischemic brain injury. Additional experiments will be needed to determine the protective effect of (+)-N8-NorPosiphen, a major metabolite of Posiphen, in stroke brain.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.100866.

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AUTHOR CONTRIBUTIONS
Y.W. conceived and designed the study, drafted the manuscript; S.-J.Y., K.-J.W., E.B., and Y.-S.W. carried out surgery, cell culture, behavioral, protein, immunohistochemical, PCR, and statistical analysis; C.-W.C. and L.-W.K. conducted image analysis; B.K.H. designed AAV for ER stress and Ca++ imaging; N.H.G. provided Posiphen, (+)-N8-NorPosiphen, and Phenserine. N.H.G. and B.K.H. offered critical insight and participated in article drafting.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Supplemental Information

Post-treatment with Posiphen Reduces Endoplasmic Reticulum Stress and Neurodegeneration in Stroke Brain

Seong-Jin Yu, Kuo-Jen Wu, Eunkyung Bae, Yu–Syuan Wang, Chia-Wen Chiang, Li-Wei Kuo, Brandon K. Harvey, Nigel H. Greig, and Yun Wang
Transparent Methods

Animals, AAV vectors, and materials: Adult female timed pregnant and male Sprague-Dawley rats (purchased from the BioLASCO, Taiwan) were used in this study. Experimental procedures followed the guidelines of the “Principles of Laboratory Care” (National Institutes of Health publication No. 86-23, 1996) and were approved by the National Health Research Institutes (Taiwan) Animal Care and Use Committee (Protocol No. 105079-A; 105080). Serotype 1 adeno-associated viral (AAV) vectors were constructed to express GFP (Luo et al., 2013), gCaMP5 (Yu et al., 2016), and GLuc-SERCaMP (Henderson et al., 2015) according to Howard et al with modification (Howard et al., 2008). Vectors were purified by CsCl gradient and titered by quantitative PCR. Viral titers are recorded as viral genome per milliliter. A viral aliquot was thawed, sonicated for 10 s in a 180z ultrasonic cleaner, and diluted to 10 times in PBS with 0.5 mmol/L MgCl2 (Life Technologies). To SH-SY5Y cells overexpressing SERCaMP described in Henderson et al (Henderson et al., 2014), half of their cell media was removed and, thereafter, 5 µL of virus was added to each well for 1hr at 37 °C. After 1hr, fresh media was added in the same volume to replace that previously removed from the cells. Glutamate, NMDA, methyllycaconitine, and Tg were purchased from Sigma-Aldrich (St. Louis, MO, USA). Posiphen, Phenserin, and Posiphen metabolites were synthesized as their water-soluble tartrate salts by the Intramural Research Program, National Institute of Aging, NIH according to Yu et al (Yu et al., 1998, Yu et al., 2013). Chemical characterization confirmed the structure of each, chiral purity and chemical purity (>99.5%).

Primary Cortical Culture: Primary cortical neuron (PCN) cultures (around 65% neurons +35% glia) were prepared from embryonic (E14–15) cortex tissues obtained from fetuses of timed pregnant Sprague-Dawley rats. After removing the blood vessels and meninges, pooled cortices were trypsinized (0.05 %; Invitrogen, Carlsbad, CA, USA) for 20 min at room temperature. After rinsing off trypsin with pre-warmed Dulbecco’s modified Eagle’s medium (Invitrogen), cells were dissociated by trituration, counted, and plated into 96-well (5.0x10^4/well) cell culture plates pre-coated with polyethyleneimine (Sigma-Aldrich). The culture plating medium consisted of neurobasal medium supplemented with 2 % heat-inactivated FBS, 0.5 mmol/L L-glutamine, 0.025 mmol/L L-glutamate and 2 % B27 (Invitrogen). Cultures were maintained at 37 °C in a humidified atmosphere of 5 % CO2 and
95 % air. The cultures were fed by exchanging 50 % of media with feed media (neurobasal medium) with 0.5 mM L-glutamate, and 2 % B27 with antioxidants supplement on days in vitro (DIVs) 3 and 5. On DIV 7, 10 and 12 cultures were fed with media containing B27 supplement without antioxidants (Invitrogen). Viral transductions were performed on DIV 5 in culture. On DIV 13, cultures were treated glutamate (100 μmol/L), thapsigargin (500 nmol/L), Posiphen (15 μmol/L), Phenerine (15 μmol/L) or vehicle.

**Primary rat cortical neuron (PCN) and microglia co-culture:** BV2 microglia were cultured separately, detached by 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA, Invitrogen), and centrifuged at 100 g for 5 min. BV2 cells were resuspended in the feeding media containing B27 supplement without antioxidants (-AO, from Invitrogen). The density of surviving cells was counted using a trypan blue assay; cells were plated on the PCN plated-wells at a concentration of 3.0x10^3/well on DIV 7. The co-cultures were fed with -AO media on DIVs 7 and 10. On DIV 10, cultures were treated glutamate (100 μmol/L) or vehicle. At 48 hr after drug treatment, cells were fixed 4% paraformaldehyde (PFA, Sigma-Aldrich) for 1 hr at room temperature.

**Immunocytochemistry:** After removing 4% PFA solution, cells were washed with PBS. Fixed cells were treated with blocking solution (2 % BSA, 0.1 % Triton X-100, and 5 % goat serum in PBS) for 1 hr. The cells were incubated for 1 day at 4 °C with a mouse monoclonal antibody against microtubule-associated protein 2 (MAP2; 1:500; Millipore, Billerica, MA) and ionized calcium-binding adaptor molecule 1 IBA1 (1:500; Wako, Richmond, VA, USA) and then rinsed three times in PBS. The bound primary antibody was visualized using AlexaFluor 568 goat anti-mouse secondary (Invitrogen). Images were acquired using a monochrome camera Qi1-mc attached to NIKON TE2000-E inverted microscope by blinded observers.

**TUNEL assay for cell culture:** Cultures were assayed for DNA fragmentation using a TUNEL-based method (In Situ Cell Death Detection Kit; Roche, Indianapolis, IN). Briefly, 4% PFA fixed cells were permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. To label damaged nuclei, 50 μL of the TUNEL reaction mixture was added to each sample and kept at 37° C in a humidified chamber for 60 min. Procedures for positive and negative controls were carried out as described in the manufacturer’s manual (Roche).
Controls consisted of not adding the label solution (terminal deoxynucleotidyl transferase) to the TUNEL reaction mixture. Images were acquired using a monochrome camera Qil-mc (Diagnostic Instruments, Inc., Sterling Heights, MI, USA) attached to a NIKON TE2000 inverted microscope (Nikon Instruments Inc., Melville, NY, USA). Data were analyzed using NIS-Elements AR 3.2 Software (Nikon Instruments).

**SH-SY5Y-GLuc-SERCaMP Cell culture:** SH-SY5Y-GLuc-SERCaMP were cultured in a 37°C humidified incubator with 5% CO₂ in DMEM (4.5 g/L D-GLucose) containing 2 mM GlutaMAX, 10% bovine growth serum (Sigma Aldrich), 10 U/mL penicillin (Thermo Fisher Scientific), and 10 μg/mL streptomycin (Thermo Fisher Scientific). Cells were plated at 5 x 10⁴ cells per well (100 μL volume). On the next day, media were exchanged into DMEM (4.5 g/L D-GLucose) containing 2 mM GlutaMAX, 1.5% BGS, 10 U/mL penicillin and 10 μg/mL streptomycin before 16-hr drug pre-treatment. Cells were incubated for 48 hr after adding drugs. Media was collected (5 μL) before and after drug treatment, at indicated time points, for enzymatic assay as described above.

**Real-time epifluorescence measurement of gCaMP5 in cell culture:** Culture plates were placed on a motorized stage (Prior Scientific Inc.) of a Nikon TE2000 inverted microscope. Microscopic images were recorded (1 frame per second) from 10 sec before to 30 sec after drug treatment by an inverted microscope (ECLIPSE Ti2, Nikon, Melville, NY) described in Yu et al (Yu et al., 2016). The intensity of intracellular GFP fluorescence in each well was measured by the NIS Elements AR 5.11 Software.

**Gaussia luciferase secretion assay in culture:** For luciferase assays (Chen et al., 2019), 5 μl of culture medium was removed and transferred to an opaque-walled plate. GLuc substrate was PBS containing an additional 5 mM NaCl and 10 μM coelenterazine (Sigma-Aldrich). Coelenterazine stock solutions were prepared at 20 mM in acidified methanol (10 μl of 10 N HCl/1 ml of methanol) and stored at -80°C as single-use aliquots. The prepared substrate was incubated at room temperature for 30 min before use. Amount of luciferase was determined using a plate reader with an injector setup (Biotek Synergy HT, Winooski, VT) to immediately read the sample after injection. Typically, 100ul of substrate was injected into the well-containing cell culture medium. For secretion assays, vehicle controls were used in all experiments at concentrations equivalent to the drug treatments.
**Animal studies:** Adult male Sprague-Dawley rats were anesthetized with chloral hydrate (400 mg/kg). The right middle cerebral artery (MCA) was ligated (MCAo) with a 10-O suture and common carotids (CCAs) were clamped bilaterally by non-traumatic arterial clips to generate focal infarction in the cerebral cortex described in Luo et al (Luo et al., 2009). The suture on the MCA and arterial clips on CCAs were removed after 30-min (for the in vivo ER stress experiment) or 60-min (for infarct volume and behavior experiments) to generate ischemia for reperfusion injury (Luo et al., 2009). Core body temperature was monitored and maintained at 37°C. After recovery from the anesthesia, body temperature was maintained at 37°C using a temperature-controlled incubator. Two drug treatment protocols were used. (1) Posiphen (25 mg/kg/d, i.p.), Phenserine (1 mg/kg/d, i.p) or vehicle was administered to the animals after MCAo for 4 days. We and others have previously reported that the volume of infarction, measured directly by standard triphenyl tetrazolium chloride (TTC) staining, is affected by edema early after the MCAo (Lin et al., 1999, Lin et al., 1993). To reduce the confounding influence of brain edema, brain tissues were collected at 5 days after the MCAo for TTC staining. (2) Posiphen (25 mg/kg/d, i.p.) or vehicle was administered twice, at 1 hour and 1 day after 60-min MCAo. Brain infarction was measured non-invasively by MRI T2Wi. Animals were used for the behavioral assay afterward. Brain tissues were collected to determine the early biochemical changes after MCAo.

**Neurological test:** Two behavioral tests were used to analyze stroke behavior. (a) Body asymmetry was analyzed using an elevated body asymmetry test (Borlongan et al., 1998, Chang et al., 2002). Rats were examined for lateral movements/turning when their bodies were suspended 20 cm above the testing table by lifting their tails. The frequency of initial turning of the head or upper body contralateral to the ischemic side was counted in 20 consecutive trials. The maximum impairment in body asymmetry in stroke animals is 20 contralateral turns/20 trials. In non-stroke rats, the average body asymmetry is 10 contralateral turns/20 trials (i.e., the animals turn in each direction with equal frequency). (b) Neurological deficits were evaluated by the Bederson’s neurological test (Bederson et al., 1986).

**Magnetic resonance imaging:** A MRI experiment was performed on a 7T animal scanner (Biospec 70/30 AS, Bruker Biospin MRI, Ettlingen, Germany) with an actively shielded gradient (BGA-12-S, 670 mT/m, 175-ms rise time) described in Wu et al (Wu et al., 2015). A
linear volume resonator was used for RF pulse transmission and an actively decoupled surface coil was used for RF signal reception. The rats were anesthetized with isoflurane (3% induction and 1% maintenance in 30% O2/70% N2) and secured in a custom-made animal holder with dedicated water heated rat bed, maintaining the rat body temperature. The rate of respiration was monitored by a small animal monitoring unit (SAIl Inc., NY, USA).

T2-weighted imaging (T2WI) was performed on each rat on day 3 post-stroke. All image slices were acquired in a transverse view of rat brain to cover the whole brain. A fast spin-echo sequence, rapid acquisition with refocused echoes (RARE), was employed and sequence parameters were with a repetition time (TR) of 2742 ms, an echo time (TE) of 33 ms, slice thickness of 1 mm, 25 slices, a matrix size of 256 × 256, a number of excitations (NEX) of 4, and a field of view (FOV) of 25 mm × 25 mm. The acquisition time was approximately 6 min. For brain infarction measurement, the region-of-interests (ROIs) identified in hyper-intensity regions in T2WI were manually determined by two of the authors (CWC and LWK). The area of infarction was calculated by averaging ROIs in every two adjacent image slices of the brain from bregma +10 mm to bregma -14 mm. Lesion volume (LV) was calculated by the summation of total area infarction.

**In vivo delivery of AAV-GLuc-SERCaMP and imaging of GLucSERCaMP using the In Vivo Imaging System (IVIS):** Adult rats were anesthetized and were placed in a stereotaxic frame (Stoelting). AAV-gLuc-ASRTDL [2 µL of 5×10^9 viral genomes/µL per site] was delivered into 3 cortical sites in the distribution of the MCA. The stereotaxic coordinates were: AP 1.2 mm, ML 5 mm, DV -3.5 mm (site1); AP -0.3 mm, ML 5 mm, DV -3.5 mm (site 2); AP -1.8 mm, ML 5.5 mm, DV -3.5 mm (site 3) according to Airvaara et al (Airavaara et al., 2010). The rate of infusion (1 µL/min) was adjusted by a microprocessor controlled injector mounted to the stereotaxic frame (UMP4; World Precision Instruments, Sarasota, FL, USA). The needle remained in the brain for 2 min after the injection and then was slowly removed. After recovery from anesthesia, animals were housed in their home cages. At 2 weeks after viral infection, animals received a 30-min MCAo. One day after the MCAo, animals were anesthetized with isoflurane and then transferred to an IVS Lumina 2 Imaging System chamber (Caliper Life Sciences, Cheshire, United Kingdom). Coelenterazine (Regis Technologies, Morton Grove, IL) was given to the animals intravenously through the tail vein at a dose of 100 µg/150 µL/rat. GLuc fluorescence images in brains were acquired
using a CCD camera. The intensity of photons collected through IVIS was translated to false color images with strong fluorescence in yellow and was quantified by the imaging software.

**TTC staining of infarction volume.** Brains were removed, sliced into 2.0-mm thick sections, included in 2% wt/vol TTC, fixed in 4% wt/vol PFA, and then digitally scanned. The area of infarction was analyzed by observers blinded to treatment group (Shen et al., 2005, Tomac et al., 2002).

**Immunohistochemistry:** Animals were anesthetized and perfused transcardially with saline followed by 4% PFA in phosphate buffer (PB; 0.1 mol/L; pH 7.2), they were post-fixed for 18–20 hr and then transferred to 20% sucrose in 0.1 M PB for at least 16 hr. Serial sections of brains were cut at 30-μm thickness on a cryostat (model: CM 3050 S; Leica, Heidelberg, Germany). Brain sections were then incubated with primary antibodies against Gaussia luciferase (1:100; Cat: E8023S; New England Biolabs, MA, USA), GFAP (monoclonal 1:100; Cat: MAB360; Merck, NJ, USA), NeuN (monoclonal 1:100; Cat: MAB377; Merck, NJ, USA), or IBA1 (monoclonal 1:100, Chemicon, Billerica, MA, USA) at 4°C overnight. Sections were rinsed in 0.1 mol/L PB and incubated in Alexa Fluor 488 secondary antibody solution (1:500; Molecular Probes, Eugene, OR, USA). Control sections were incubated without the primary antibody. Brain sections were mounted on slides and coverslipped. Confocal analysis was performed using a Nikon D-ECLIPSE 80i microscope (Nikon Instruments, Inc., Tokyo, Japan) and EZ-C1 3.90 software (Nikon, Tokyo, Japan). The optical density of IBA1 immunoreactivity was quantified in three consecutive brain sections with a visualized anterior commissure in each animal. Six photomicrographs were taken along the perilesioned region per brain slices; IBA1 optical density was analyzed by NIS Elements AR 3.2 Software (Nikon) and was averaged in each brain for statistical analysis. All immunohistochemical measurements were performed by blinded observers.

**TUNEL labeling:** Apoptotic cell death was detected by terminal deoxynucleotidyl transferase (TdT), which catalyzes polymerization of labeled nucleotides to free 3’-OH DNA ends in a template-independent manner (TUNEL reaction), according to the manufacturer’s protocols
(In situ cell death detection kit, Cat. No. 11684795910, Roche). Brain sections were attached to a glass slide and were air dried at room temperature for 20 min. Sections were then washed with 0.1M PB for 30 min and incubated in permeabilization solution for 2 min on ice. 50 µl of TUNEL reaction solution was added to the samples, and incubated samples were placed in a humidified atmosphere for 60 min at 37°C in the dark. Sections were rinsed in 0.1M PB and were cover-slipped. For a negative control, sections were incubated with the same volume of label solution instead of the TUNEL reaction solution. Confocal analysis was performed using a Nikon D-ECLIPSE 80i microscope (Melville, NY, USA) and EZ-C1 3.90 software. The optical density of TUNEL positive cell was quantified in three consecutive brain sections with a visualized anterior commissure in each animal. Six photomicrographs were taken along the perilesioned region per brain slices; TUNEL positive optical density was analyzed by NIS Elements AR 3.2 Software (Nikon) and was averaged in each brain for statistical analysis.

**Western analysis:** Brain tissue was homogenized in RIPA lysis buffer (Cell Signal). The homogenate was centrifuged at 13200 rpm for 10 min at 4 °C, and the supernatant was collected. A bicinchoninic acid (BCA) protein assay was performed using bovine serum albumin to determine protein concentrations. The samples were diluted with RIPA buffer according to the BCA protein assay. Gels were transferred to a PVDF membrane after electrophoresis. The membranes were blocked in Odyssey blocking buffer (LI-COR) for 2 h or 5% milk (ECL) for 1 h at room temp. The blots were then probed with primary antibodies against ionized calcium-binding adapter molecule 1 (IBA1, 1: 1000, Wako) or immunoglobulin heavy chain binding protein (BiP, 1:1000, Cell signaling) at 4 °C for overnight, and actin (1:5000, Novus) at room temp for 1 h. The membrane was then incubated with an IRDye® 800CW Goat anti-Rabbit (1:2500 for IBA1, LI-COR), IRDye® 680LT Goat anti-Mouse (1:5000 for actin, LI-COR) or horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson lab) at room temp for 1 h, followed by washing with 0.1% Tween-20 (in TBS) five times for 5 min each. IBA1 and actin immunoreactivities were scanned by an infrared imaging system (Odyssey, LI-COR) and were quantified using Image
Studio Lite Ver 5.2 (LI-COR). The light emission signal of the BiP and actin was generated by using a Western Lightning Plus-ECL (PerkinElmer) and then displayed on X-ray film (Cat. No. NEF596, Kodak). ECL-Band intensity was quantified using Image J. Finally, the amount of IBA1 or BiP was normalized with actin on the same membrane.

**Quantitative Reverse Transcription –PCR (qRTPCR):** Brain cortical tissues were collected for qRT-PCR analysis at 2 days after the MCAo (Yu et al., 2017). An additional 8 non-stroke rats were used as the control group. Total RNAs were isolated by use of a RNeasy Mini Kit (Qiagen, #74106) and cDNAs were synthesized from 1ug total RNA by use of RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, #K1631). The TaqMan Gene Expression Assays primer for specifically detecting Rat Beta-actin (#Rn00667869_m1) and GAPDH (#Rn01775763_g1) were purchased from Thermo Scientific. Quantitative Real-Time PCR (qRT-PCR) was carried out using TaqMan Fast Advanced Master Mix (Life Technologies, #4444557) and Applied Biosystems 7500 Fast Real-Time PCR System. The expressions of PERK, CHOP, and BiP mRNA were measured by using SYBR (Luminaris Color HiGreen Low ROX qPCR Master Mix; ThermoScientific). The primers used were as follows:

PERK (GenBank Acc.), forward: 5'- GAAGTGGCAAGAGGAGATGG -3’ and reverse: 5’- GAGTGCCAGTCTGTGCTTT -3’; CHOP (GenBank Acc.), forward: 5’- ACCACCACACCTGAAAGCAG -3’, reverse: 5’- AGCTGGACACTGTCTCAAAG -3’; BiP (GenBank Acc.), forward: 5’- TCGACTTGGGGACCACCTAT -3’ and reverse: 5’- GCCCTGATCGTTGGCTATGA -3’. Expression and normalization of the target genes, PERK, CHOP, and BiP were calculated relative to the endogenous reference gene (Beta-actin and GAPDH averages) with a modified delta-delta-Ct algorithm that takes gene-specific amplification efficiency into account for accurate calculation. All experiments were carried out in duplicate.

**Statistics:** Data are presented as mean ± s.e.m. Unpaired t-test, 1 or 2-way ANOVA, 1 or 2-way ANOVA on rank were used for statistical comparisons, with a significance level of p<0.05. In the event of multiple comparisons, posthoc Newman-Keuls test (NK test) was performed.
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