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Prevention of Cyclophilin D-Mediated mPTP Opening Using Cyclosporine-A Alleviates the Elevation of Necroptosis, Autophagy and Apoptosis-Related Markers Following Global Cerebral Ischemia-Reperfusion

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Abstract The mitochondrial permeability transition pore (mPTP) is a complex channel of the inner membrane, the opening of which leads to mitochondrial swelling and dissipation of mitochondrial membrane potential (MMP). Here, we aimed to evaluate the role of the cyclophilin D (CypD) as a prominent mediator of mPTP, on necroptosis and autophagy as well as apoptosis, beyond the global cerebral ischemia-reperfusion (I/R) injury. We showed that while cerebral I/R injury is accompanied by loss of MMP, mitochondrial swelling and programmed cell death, pretreatment with cyclosporine-A (CsA) as a potent inhibitor of CypD, led to partial but significant reduction in necroptosis markers, RIP1 and RIP3 as well as activity of glutamate-ammonia ligase (GLUL) and glutamate dehydrogenase 1 (GLUD1), downstream enzymes of RIP3. Administration of CsA also partially decreased autophagy associated proteins. Furthermore, we demonstrated that Bax/Bcl-2 ratio as well as caspase-3 activation, as the executioner of apoptosis, noticeably decreased by CsA pretreatment. Taken together, our results suggest that the CypD alongside the apoptosis regulation plays a partial role in inducing necroptosis and autophagy.

Keywords Autophagy · Apoptosis · Necroptosis · mPTP · Cyclosporine-A · Cerebral ischemia-reperfusion

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Introduction

Reperfusion of cerebral blood supply after global cerebral ischemia, which results from disruption in blood flow to whole or a large part of the brain, paradoxically invokes tissue insult (known as cerebral ischemia reperfusion (I/R) injury) and leads to neural death in vulnerable brain areas particularly hippocampal neurons (Pulsinelli 1985; Hossmann 1998; Alexandrov 2010). Opening of the mitochondrial permeability transition pore (mPTP), a mega-channel complex in the inner mitochondrial membrane, is thought to play a key role in I/R-induced cell death (Kalogeris et al. 2012; Elrod and Molkentin 2013). The mPTP opening upon persistent stimulation such as I/R injury disrupts the permeability barrier of the inner mitochondrial membrane and leads to mitochondrial swelling, dissipation of mitochondrial membrane potential, depletion of ATP, and a transient increase in reactive oxygen species (ROS) generation (Zorov et al. 2000; Halestrap et al. 2004; Halestrap 2009). Whereas the molecular structure of the mPTP still remains elusive, it has been proposed that cyclophilin D (CypD), a member of the immunophilin family of peptidyl-prolyl cis-trans isomerases (PPIase), is a bona-fide component of the pore (Elrod and Molkentin 2013) and acts as a crucial positive regulator in opening of this channel (Schinzel et al. 2005). Pharmacological and genetic inhibition of CypD, which results in reduced infarction size following focal cerebral I/R injury, supports the notion that the mPTP opening occurs following the transient cerebral ischemia (Matsumoto et al. 1999; Schinzel et al. 2005; Wu et al. 2006).

The neural cell death following cerebral ischemia was thought to occur by necrosis, but accumulating studies have revealed that cell death in many neurons will undergo through programmed processes like apoptosis and autophagy (Mehta et al. 2007; Kalogeris et al. 2012). Moreover, while necrosis has been formerly regarded as an unregulated form of cell death, recent evidence indicates that necrotic cell death may

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be executed through signal transduction pathways (Vandenabeele et al. 2010). Necroptosis, initiated by formation of complex containing receptor interacting protein kinase-1 (RIPK1) and RIPK3, is the best-characterized form of regulated necrosis. Present understanding suggests the importance of mitochondrial events in pathological necroptosis (Vandenabeele et al. 2010). Whereas genetic studies have suggested that the mPTP opening is predominantly involved in necrosis (Baines et al. 2005; Nakagawa et al. 2005; Schinzel et al. 2005), it is unclear whether it plays a role as a contributing factor in induction of necroptosis. In addition, it has been proposed that the mPTP opening could also be considered as a mechanism involved in apoptosis, since osmotic swelling due to mPTP opening may lead to the rupture of the outer membrane, and release of proapoptotic proteins from mitochondria (Halestrap 2009). Moreover, opening of the mPTP might also stimulate autophagy to eliminate abnormal mitochondria (Elmore et al. 2001; Rodriguez-Enriquez et al. 2004).

Cyclosporine-A (CsA), an immunosuppressive agent, has been proposed to block the mPTP by binding to CypD. CsA acts by inhibiting the PPIase activity of CypD, results in the dissociation of CypD from adenine nucleotide translocator (ANT), which is localized in the inner membrane, and acts as a structural component of the mPTP (Griffiths and Halestrap 1991; Woodfield et al. 1998; Crompton 1999). It has been demonstrated that CsA provides a certain degree of protection to the rat brain from stroke (Osman et al. 2011). We hypothesize that the neuroprotective effect of CsA against cerebral I/R injury may be associated with prevention of different modes of I/R-induced cell death. Thus, the present study aimed to clarify the importance of CypD in induction of necroptosis, autophagy, and apoptosis beyond the global cerebral I/R by intracerebroventricular (i.c.v.) administration of CsA.

Material and Methods

Animals

Adult male Wistar rats (weighing from 250 to 300 g) were housed in a standard animal room on a 12-h light/dark cycle with a controlled temperature and humidity, and given *ad libitum* access to food and water. All experimental procedures were approved according to the Ethics Committee of Shahid Beheshti University of Medical Sciences in accordance with the international guidelines for animal experiments (No. 80–23, revised 1996).

Experimental Design

In the first step and in order to determine the most effective dose of CsA in inhibition of the mPTP, rats were randomly divided into four groups, as follows: (i) sham-vehicle group, (ii) I/R-vehicle group, (iii) I/R- 0.5 μM CsA group, and (iv) I/R- 1 μM CsA group. For drug administration, CsA (C1832, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) and administered by i.c.v injection at the volume of 1 μL, 15 min before the onset of global cerebral ischemia in I/R- 0.5 μM CsA and I/R- 1 μM CsA groups. The equal volume of vehicle (DMSO) was administered by i.c.v injection in vehicle groups. To evaluate the effect of CsA on different groups, the mitochondrial swelling and mitochondrial membrane potential (MMP) were measured.

In the next step, rats were randomly assigned into the following three experimental groups: (i) sham-vehicle group, (ii) I/R-vehicle group, and (iii) I/R-CsA (effective dose). Preliminary analyses determined that there were no significant differences between sham-vehicle group and sham groups that received CsA; therefore, their data were pooled. Experimental animals were sacrificed after 24 h, and the amount of necroptosis, apoptosis, and autophagy associated proteins and also necroptosis enzymes activity were measured in hippocampus.

Surgery

Transient global cerebral ischemia was induced using the 4-vessel occlusion (4VO) model as described previously (Pulsinelli and Brierley 1979). Briefly, animals were anesthetized with intraperitoneal injections of chloral hydrate (350 mg/kg, i.p.). Then, both vertebral arteries were occluded by electrocoagulation through the alar foramina, and the common carotid arteries were exposed, and a sterile string looped around each artery without interrupting arterial blood flow. Electroencephalogram (EEG) electrodes were placed bilaterally at the skull on the parietal cortex to assure to induce successful 4VO. After 24 h, global cerebral ischemia was initiated by clamping the common carotid arteries using atraumatic aneurysm clips for 30 min, under the anesthesia as described from the first day. Those rats were only selected for the experiments if their EEG was flattened during the ischemia (Diler et al. 2002; Mohagheghi et al. 2013a, b). During surgery and recovery, body temperature was monitored by rectal probe (Citizen-513w) and maintained at 37 ± 0.5 °C using surface heating. Sham-operated animals underwent the same procedure except of carotid and vertebral occlusion.

For i.c.v. administration of CsA, the experimental rats were placed in a stereotaxic apparatus, the scalp was incised and retracted, and the holes were drilled in the skull with a dental burr. CsA was administered into the right lateral cerebral ventricle 15 min prior to the onset global cerebral ischemia with a total volume of 1 μL at a speed of 0.5 μL/min using a Hamilton syringe. Stereotaxic coordinates for i.c.v. injection were as follows: anterior-posterior −0.8 mm (relative to

bregma), lateral +1.5 mm (from midline), and dorso-ventral −3.8 mm (from the skull surface) (Paxinos and Watson 2007).

Mitochondrial Preparation

Experimental animals were decapitated by CO_2 asphyxiation, and the brains promptly were removed and hippocampi were excised from the brain. Mitochondria were isolated from hippocampi of different groups by differential centrifugation with employing the isolation buffer (containing sucrose, Tris, and EDTA) based on the procedure described previously (Clark and Nicklas 1970). Protein concentrations of samples were assessed by the colorimetric method of Bradford, using bovine serum albumin (BSA), as standard (Bradford 1976). All of the procedures were performed on ice (4 °C). Mitochondria was freshly prepared for functional measurements and were used within 4 h.

Determination of the MMP

Mitochondrial membrane potential was measured by determination of mitochondrial uptake of cationic fluorescence probe Rhodamine 123. The mitochondrial fractions were incubated with Rhodamine 123 dye in MMP assay buffer (Baracca et al. 2003). The Rhodamine123 fluorescence was monitored using fluorescence spectrophotometer at the excitation and emission wavelengths of 490 and 535 nm, respectively.

Determination of Mitochondrial Swelling

Mitochondrial swelling activity was measured based on a method described previously (Zhao et al. 2010). Briefly, the isolated mitochondria were incubated in swelling buffer, and the absorbance of mitochondrial suspension was monitored spectrophotometrically at 540 nm.

Immunoblot Analysis

Hippocampi were lysed using ice-cold lysis buffer and protein concentration was determined using the Bradford method (Bradford 1976). Immunoblotting was used to measure the protein level of Bcl2, Bax, caspase-3, RIP1, caspase-8, LC3, Beclin-1 and β-actin (Cell Signaling Technology, Beverly, MA, USA), and RIP3 (Santa Cruz Biotechnology, Dallas, TX, USA). After incubation with horseradish peroxidase-coupled secondary antibody (Cell Signaling Technology, Beverly, MA, USA), immunoreactive bands were visualized by enhanced chemiluminescence (ECL) kit (Amersham Bioscience, Piscataway, USA). Relative density of the bands was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

GLUL Enzyme Assay

Glutamate-ammonia ligase (GLUL) activity was assayed based on a method described by Kingdon et al. (1968). In this assay, the ADP produced during glutamine synthesis is converted into ATP by reaction with phospho(enol) pyrovate catalyzed by pyruvate kinase, and the pyruvate thus produced is reduced by NADH in the presence of lactate dehydrogenase. The overall reaction is measured spectrophotometrically by following the change in absorbance at 340 nm, due to the oxidation of NADH.

GLUD1 Enzyme Assay

Glutamate dehydrogenase 1 (GLUD1) activity was measured by the method described by Doherty (1970). In this assay, oxidation of the reduced co-enzyme NADH was measured spectrophotometrically by monitoring the absorbance at 340 nm in the presence of reaction mixture consisted of potassium phosphate buffer (pH 7.0), NH_4Cl, 2-Oxo-glutarate, and samples.

Statistical Analysis

Statistical analysis of the results was carried out using the GraphPad Prism (version 5, GraphPad Software Inc., San Diego, CA, USA). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. Each value indicates the mean ± S.E.M. from at least four independent experiments (biological replicates) and three replicates (technical replicates). A P value of <0.05 was considered statistically significant ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

Results

Effect of Different Concentrations of CsA on Mitochondrial Swelling and MMP in Global Cerebral I/R

CsA has been shown to possess neuroprotective properties through its ability to block the mPTP (Osman et al. 2011). However, it might have a neurotoxic effect at high concentrations in brain ischemia (Murozono et al. 2004). Using different concentrations of CsA in this investigation showed that doses higher than 1 μM caused an increase in the mortality rate in ischemic group (data not shown). Therefore, precise assessment of the effects of CsA for selecting the most protective dosage was the first critical step. Accordingly, we measured the mitochondrial swelling and MMP in the presence of different concentrations of CsA. As shown in Fig. 1a, although mitochondrial swelling decreased in 0.5 μM CsA-treated rats, this decline became significant at 1 μM CsA

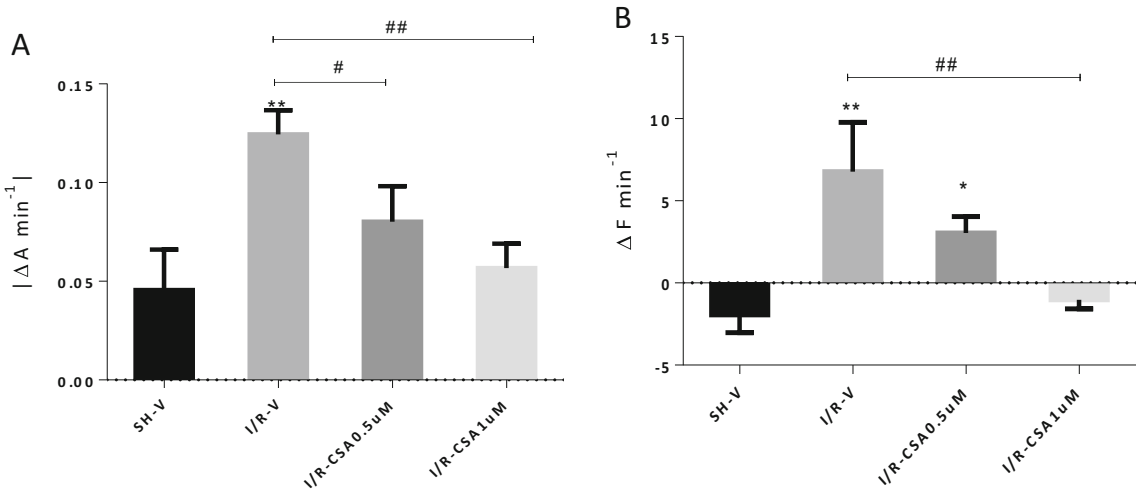

Fig. 1 The protective effect of CsA (0.5 and 1 μM) on mitochondrial swelling and MMP following transient global cerebral ischemia. **a** Mitochondrial swelling was measured by determination of absorbance at 540 nm. **b** MMP was measured by fluorescence spectrophotometer using Rhodamin 123 dye ($\lambda_{ex/em} = 490/535$ nm). All values are expressed the mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$ *vs*. sham-vehicle group; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ *vs*. I/R-vehicle rats. *SH-V* sham-vehicle, *I/R-V* ischemia/reperfusion-vehicle, *I/R-CsA* ischemia/reperfusion-cyclosporine-A

concentration compared to I/R-vehicle group ($P < 0.01$). Furthermore, administration of 1 μM CsA, 15 min before the onset of global cerebral ischemia, significantly increased MMP compared with the untreated group ($P < 0.01$) (Fig. 1b). Therefore, 1 μM CsA could block the mPTP totally, leading to better recovery of mitochondrial function. This is reflected by reversing the induced mitochondria swelling and loss of MMP.

Necroptosis-Associated Proteins Were Partially Attenuated by Administration of CsA

Activation of RIP1 and RIP3 is a crucial requisite in induction of necrosome (Giampietri and Starace 2014). Our pilot data showed a maximum increase of RIP1 and RIP3 in hippocampal region at 24 h after global cerebral ischemia, highlighting the increased possibility of necrosome formation for triggering the necroptotic signaling. Therefore, we chose this time point to evaluate the effect of the CsA in necroptotic cell death. As shown in Fig. 2b, c, the administration of CsA 15 min prior the onset of global cerebral ischemia resulted in significant reduction in amount of RIP1 and RIP3 compared to untreated group ($P < 0.05$).

CsA Administration Reduced Activities of GLUL and GLUD1 Enzymes

GLUL is a cytosolic enzyme that catalyzes the condensation of glutamate and free ammonia to glutamine. GLUD1 is a mitochondrial matrix enzyme that converts glutamate to α-ketoglutarate (Vandenabeele et al. 2010). It has been shown that these enzymes are downstream of RIP3 (Zhang et al. 2009). To further unravel the effect of the CsA on necroptosis, the activity of GLUL and GLUD1 enzymes were determined using biochemical assays. As depicted in Fig. 3a, b, activity of GLUL and GLUD1 enzymes increased significantly in I/R-vehicle rats compared to the basal levels in sham group. However, CsA pretreatment diminished activities of GLUL and GLUD1 in comparison to I/R-vehicle group ($P < 0.05$).

Administration of CsA Partially Decreased Autophagy Associated Proteins

Beclin-1 (mammalian ortholog of the yeast Atg6) that is essential for autophagosome formation is used as a marker of autophagy activation (Wei et al. 2012). In addition, an increase in the LC3-II/LC3-I ratio is also the hallmark of autophagy and correlates with an increased number of autophagosomes (Kabeya et al. 2000). To study the effect of mPTP on autophagy-related proteins, we measured the level of Beclin-1 and LC3-II/LC3-I ratio in ischemic rats in the presence or absence of CsA. As presented in Fig. 4, there was a significant increase in the amount of Beclin-1 and LC3-II/LC3-I ratio in ischemic group relative to sham group. However, there was a significant decrease in LC3-II/LC3-I ratio in CsA-treated rats relative to the I/R-vehicle animals (Fig. 4b). In addition, as can be seen in Fig. 4c, Beclin-1 was also reduced in CsA-administrated rats compared to the untreated rats ($P < 0.05$).

Apoptosis Associated Proteins Were Attenuated by CsA Administration

To determine the effect of CsA on apoptosis, we measured the level of apoptotic associated proteins in the experimental

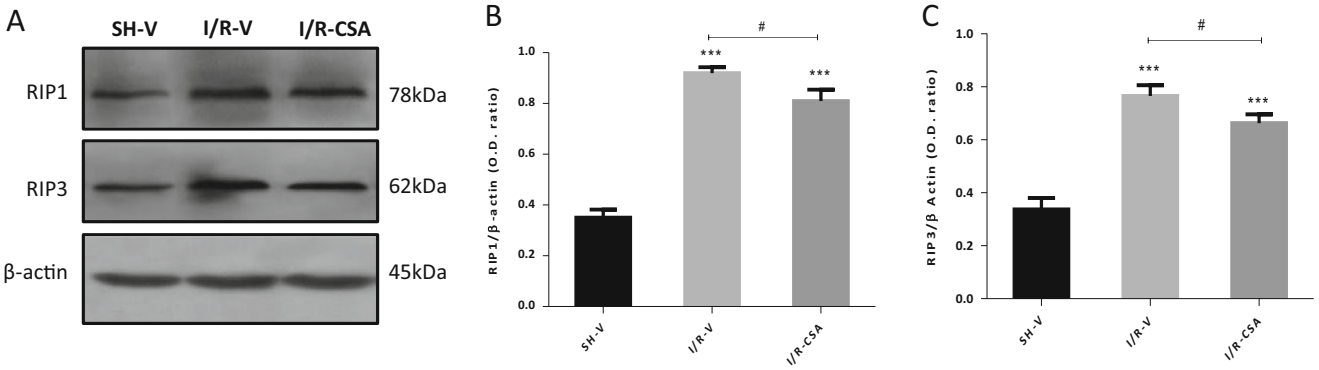

Fig. 2 The protein level of necroptosis-related proteins in the hippocampus after transient global cerebral ischemia in experimental groups. Representative immunoblots of RIP1, RIP3, and β-actin (**a**). Densitometric analysis of RIP1 bands (**b**) and RIP3 bands (**c**) normalized against β-actin. The ordinate of the bar graphs is expressed as the optical density ratio to β-actin. All values are represented the mean ± SEM. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ *vs.* sham-vehicle group, $^{\#}P < 0.05$; *vs.* I/R-vehicle rats. *SH-V* sham-vehicle, *I/R-V* ischemia/reperfusion-vehicle, *I/R-CsA* ischemia/reperfusion-cyclosporine-A

groups. As shown in Fig. 5, cleaved caspase-3 and Bax/Bcl-2 ratio elevated in ischemic rats which received DMSO relative to sham group. However, the level of these markers decreased significantly in I/R-CsA group compared to the I/R-vehicle groups. Additionally, since caspase-8 may act as a negative regulator possibly by promoting the cleavage of both RIP1 and RIP3, we measured caspase-8 protein level in experimental groups. As depicted in Fig. 5d, cleaved caspase-8 declined significantly in I/R-CsA group compared to the I/R-vehicle rats ($P < 0.05$).

Discussion

The mPTP is shown to contribute to ischemic neuronal cell death (Schinzel et al. 2005), and CypD, a crucial component of the mPTP, is proposed to regulate necrosis (Nakagawa et al. 2005). However, it is not clear to what extent CypD contributes to programmed cell death including necroptosis, autophagy, and apoptosis. Here, we showed that inhibition of CypD by CsA partially attenuated the necroptosis-related proteins and enzymes, alongside with autophagic markers, and markedly decreased the apoptosis-related proteins (Fig. 6).

Necroptosis is triggered by the interaction of RIP1 with RIP3, resulting in a so-called necrosome complex that is crucial for the initiation of necroptosis. Although the downstream events of necrosome are not fully understood, the activation of certain metabolic enzymes such as GLUD1 and GLUL enzymes has been characterized (Zhang et al. 2009; Vandenabeele et al. 2010; Nikseresht et al. 2015). Herein, we observed that the mitochondrial swelling and collapse of MMP occurred during the elevation of necroptosis-associated proteins and enzymes activity induced by global cerebral I/R injury. In this line, the swollen mitochondria have been observed using electron microscopy in embryonic fibroblasts treated with TNFα/zVAD-fmk, as an inducer of necroptosis (Karch et al. 2015). Simultaneous loss of MMP during necroptosis has been also reported in previous studies (Degterev et al. 2005; Sawai 2014; Zhang et al. 2016). However, there are some reports showing that MMP transiently increases during the early phases of necroptosis (Vanden Berghe et al. (2010) and some showing that loss of MMP is

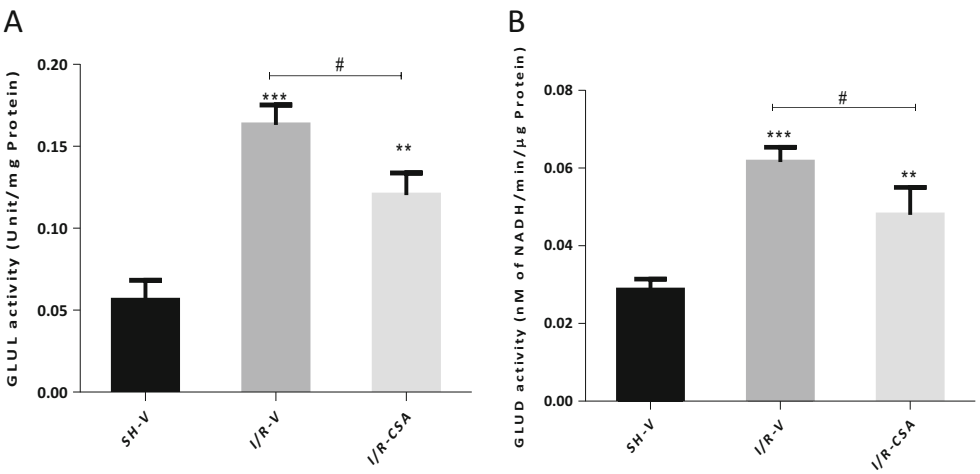

Fig. 3 Effect of CsA on GLUL and GLUD1 enzyme activities in the hippocampus after transient global cerebral ischemia in experimental groups. Values are mean ± SEM ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$ *vs.* sham-vehicle group, $^{\#}P < 0.05$; *vs.* I/R-vehicle rats). *SH-V* sham-vehicle, *I/R-V* ischemia/reperfusion-vehicle, *I/R-CsA* ischemia/reperfusion-cyclosporine-A

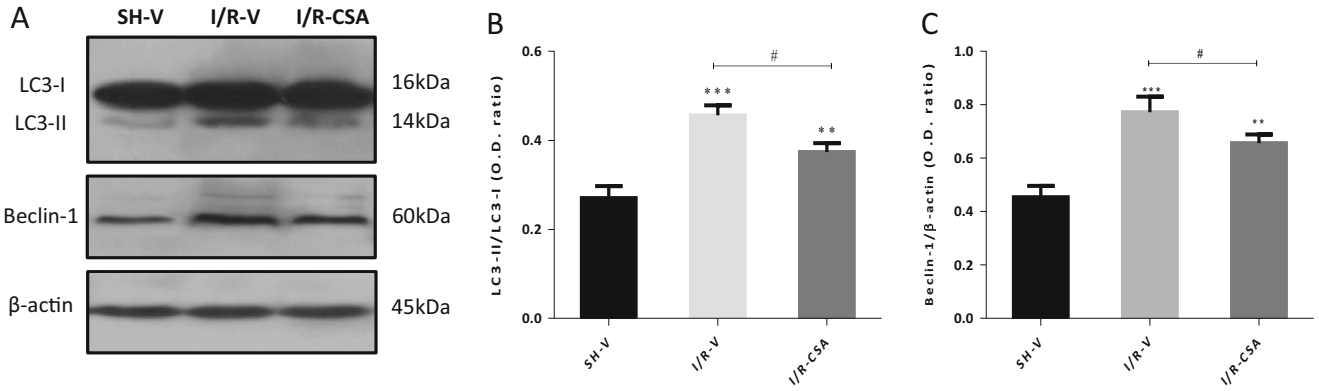

Fig. 4 The amount of apoptosis-related proteins in the hippocampus after transient global cerebral ischemia in experimental groups. Representative immunoblots of Bcl-2, Bax, caspase-3, caspase-8, and β-actin (**a**). Densitometric analysis of cleaved caspase-3 bands (**b**). Bax-to-Bcl-2 bands (**c**) and cleaved caspase-8 bands (**d**) normalized against β-actin. The ordinate of the bar graphs is expressed as the optical density ratio to β-actin. Values are mean ± SEM (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ *vs*. sham-vehicle group, $^{\#}P < 0.05$; *vs*. I/R-vehicle rats). *SH-V* sham-vehicle, *I/R-V* ischemia/reperfusion-vehicle, *I/R-CsA* ischemia/reperfusion-cyclosporine-A

not detectable prior to the final stages of cell death induced by TNFα/zVAD (Temkin et al. 2006; Tait et al. 2013). It seems that changes in the MMP in necroptotic cells could be different in early and late stages and may also depend on the inducing factor of cell death.

In order to determine the role of mPTP in necroptotic signaling, we used CsA for inhibiting CypD, as a gatekeeper of mPTP. CypD inhibition by CsA partially alleviated the levels of RIP1 and RIP3 and also activities of GLUL and GLUD1 enzymes following global cerebral I/R injury. In this regard, the findings from in vitro and in vivo studies revealed that genetic or pharmacological inhibition of CypD increases cell viability against necroptosis inducers (He et al. 2009; Tischner et al. 2012; Karch et al. 2015; Zhao et al. 2015). An in vitro study showed that deletion of the gene encoding CypD in mouse embryonic fibroblasts did not significantly changed the amount of RIP1 and RIP3 (Karch et al. 2015). The cause of contradictory results might lie in the difference of tissue

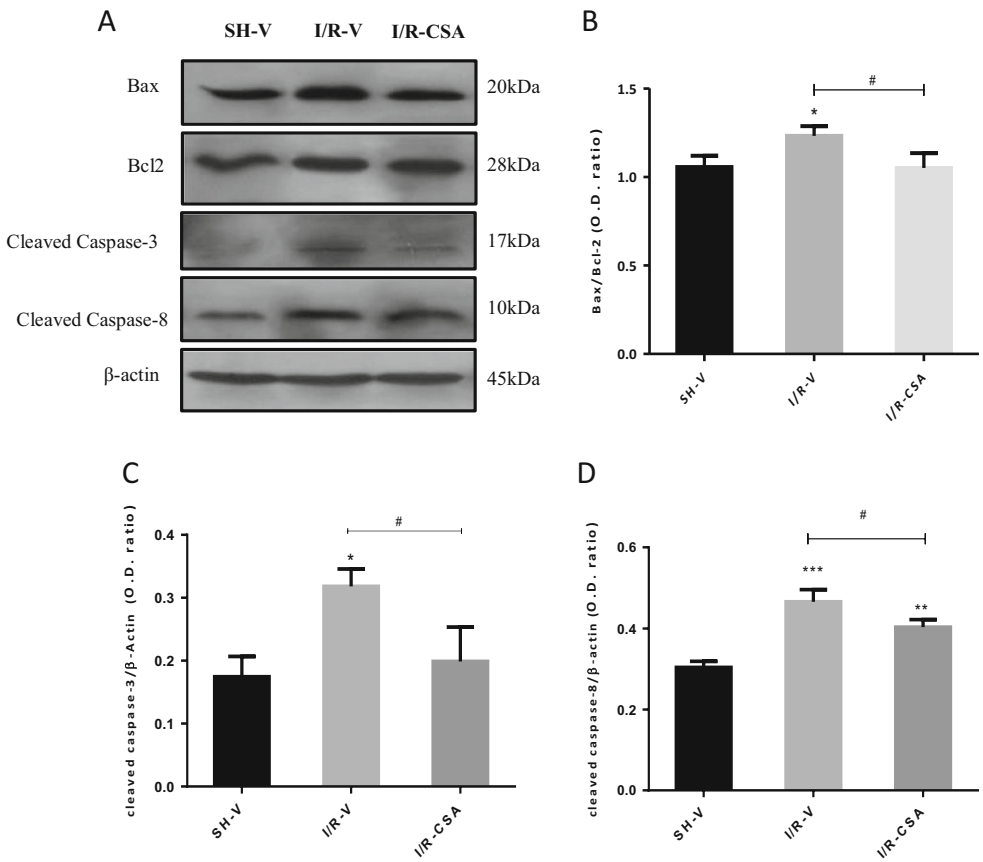

Fig. 5 The protein level of autophagy-related proteins in the hippocampus after transient global cerebral ischemia in experimental groups. Representative immunoblots of LC3, Beclin-1 and β-actin (**a**) and densitometric analysis of LC3-II-to-LC3-I bands (**b**) and Beclin-1 (**c**) normalized against β-actin. The ordinate of the bar graphs is expressed as the optical density ratio to β-actin. Values are mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ *vs*. sham-vehicle group, $^{\#}P < 0.05$; *vs*. I/R-vehicle rats). *SH-V* sham-vehicle, *I/R-V* ischemia/reperfusion-vehicle, *I/R-CsA* ischemia/reperfusion-cyclosporine-A

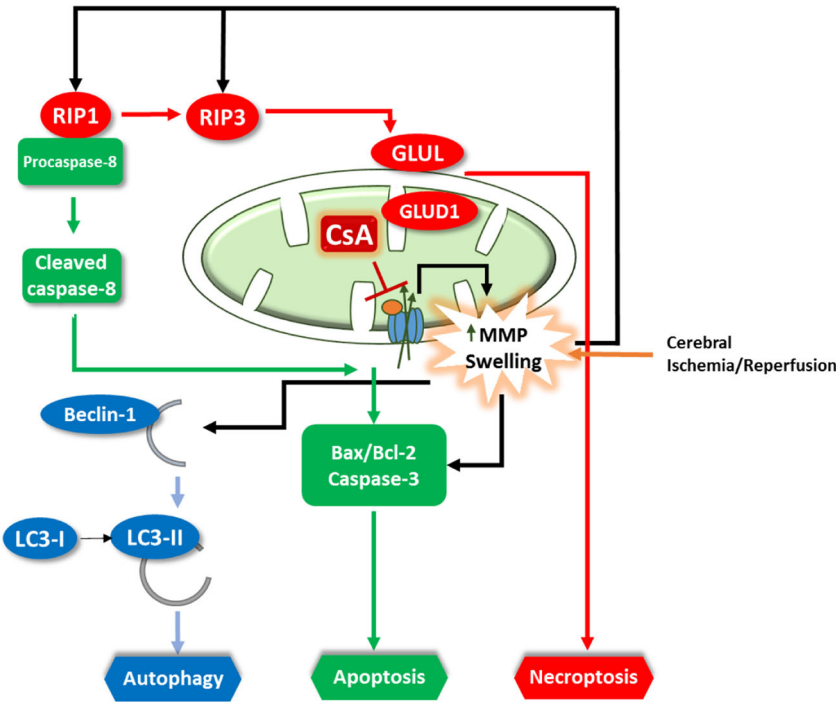

Fig. 6 Schematic representation of outcome of this study shows possible effects of mitochondrial permeability transition pore (illustrated in *black*), on necroptosis (*red*), apoptosis (*green*), and autophagy (*blue*) cell death pathways

contexts or indirect effects of immunosuppressive properties of CsA. However, cell survival observed in these studies and decreased activity of GLUL and GLUD1 enzymes shown in our study following inhibition of CypD all convey an upstream role of CypD in necroptosis regulation.

Considering the role of CypD and mPTP in autophagy, it has been previously shown that mPTP is involved in initiation of mitochondrial depolarization after autophagic stimulation in rat hepatocytes, and CsA prevents this depolarization and blocks autophagosome proliferation (Elmore et al. 2001). Accordingly, we found herein that administration of CsA partially decreases the amount of LC3-II/LC3-I and Beclin-1, and suggests that mPTP opening acts at least as a partial trigger of autophagy following cerebral I/R injury. We have also found that inhibition of autophagy attenuates the necroptosis-associated proteins and enzymes, suggesting that autophagy may act as an upstream signal for inducing necroptosis (submitted manuscript). Therefore, attenuation of necroptosis markers observed herein in response to CsA treatment could partly attributed to decrease in autophagy. However, decrease of autophagy may be also due to the alleviation of necroptosis, and the presence of positive feedback loop between autophagy and necroptosis reinforcing each other cannot be rule out.

Herein, we found that administration of CsA led to decrease in cleaved caspase-3 and-8 along with attenuation in Bax/Bcl-2 ratio. These findings are consistent with studies by Domanska-Janik et al. (2004), Li et al. (2009), and Yuen et al. (2011) who have suggested that CsA can attenuate apoptotic cell death induced by cerebral ischemia in vivo. While necroptosis and apoptosis are distinct processes of programmed cell death, there is cross-talk between them (Walsh 2014). It has been shown that under conditions that are insufficient to trigger apoptosis, the necroptotic cell death was induced (Giampietri and Starace 2014). Our very recent study has shown that apoptotic signaling decreased noticeably alongside with the peak time of necroptosis in cerebral I/R injury (submitted manuscript). Based on present findings, the suppression of apoptotic cell death using CsA in this time suggests that apoptotic cell death which occurred along with programmed necrosis was mostly related to mPTP opening. Therefore, CypD-mediated mPTP opening may also contribute to not only apoptosis but also necroptotic cell death in cerebral I/R injury.

Overall, our study indicates that CypD-mediated mPTP opening in global cerebral I/R injury is a common event leading to necroptosis, apoptosis, and autophagy, though its involvement seems to vary among different cell deaths.

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