CNS Neuroscience & Therapeutics

ORIGINAL ARTICLE

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Cyclosporine A Treatment Abrogates Ischemia-Induced Neuronal Cell Death by Preserving Mitochondrial Integrity through Upregulation of the Parkinson's Disease-Associated Protein DJ-1

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Keywords

Cytochrome c; Mitochondrial DNA; Mitochondrial membrane potential ($\Delta\psi$m); Mitochondrial permeability transition pore; Oxygen glucose deprivation/reperfusion injury.

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SUMMARY

Aims: Hypoxic-ischemia alters mitochondrial membrane potential ($\Delta\psi$m), respiratory-related enzymes, and mitochondrial DNA (mtDNA). Drugs acting on mitochondria, such as cyclosporine A (CsA), may reveal novel mitochondria-based cell death signaling targets for stroke. Our previous studies showed that Parkinson's disease-associated protein DJ-1 participates in the acute endogenous neuroprotection after stroke via mitochondrial pathway. DJ-1 was detected immediately after stroke and efficiently translocated into the mitochondria offering a new venue for developing treatment strategies against stroke. Here, we examined a molecular interaction between CsA and mitochondrial integrity in the *in vitro* acute stroke model of oxygen glucose deprivation/reperfusion (OGD/R) injury with emphasis on DJ-1. **Methods:** Primary rat neuronal cells (PRNCs) were exposed to OGD/R injury and processed for immunocytochemistry, ELISA, and mitochondria-based molecular assays to reveal the role of DJ-1 in CsA modulation of mitochondrial integrity. **Results:** Administration of CsA before stroke onset (24 h pre-OGD/R) afforded significantly much more robust neuroprotective effects than when CsA was initiated after stroke (2 h post-OGD/R), revealing that CsA exerted neuroprotection in the early phase of ischemic stroke. CsA prevented the mitochondria-dependent cell death signaling pathway involved in cytochrome c (Cyt *c*)-induced intrinsic apoptotic process. CsA preserved cellular ATP content, but not hexokinase activity under hypoxic conditions. CsA prevented both mtDNA decrement and $\Delta\psi$m degradation after reperfusion, and enhanced secretion of DJ-1 in the mitochondria, coupled with reduced oxidative stress. **Conclusion:** These observations provided evidence that CsA maintained mitochondrial integrity likely via DJ-1 upregulation, supporting the concept that mitochondria-based treatments targeting the early phase of disease progression may prove beneficial in stroke.

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Introduction

Stroke is characterized by neural tissue death due to deprivation of oxygen, glucose, and other nutrients that results from a reduction in blood flow to the brain. Disease progression with stroke primarily involves a primary insult characterized by an infarcted core, and subsequently the formation of an ischemic penumbra, which over a subacute period remains as salvageable neural tissue, thereby amenable to therapeutic intervention [1–3]. Secondary cell death processes, including oxidative stress, can further exacerbate cell death in the penumbra limiting neurorestoration [1–3]. Oxidative stress has been implicated in the pathogenesis of many central nervous system (CNS) disorders, including Alzheimer's disease and Parkinson's disease (PD) [4–7].

The mitochondria are the energy factories of the cells and play essential roles in energy metabolism, including electron transport and adenosine triphosphate (ATP) synthesis, and an important source of reactive oxygen species (ROS) which at abnormally

high levels can cause cellular damage [8]. Mitochondrial biogenesis is a tightly regulated metabolic process in healthy cells controlled by the mitochondrial DNA (mtDNA). Damage to the energetic integrity of the mitochondria accompanies adult ischemia, with aberrant opening of the mitochondrial permeability transition pore (MPTP), a core mediator of ischemic cell death [9–13]. Ca^{2+} accumulation contributes to normal neuronal function; however, excessive mitochondrial Ca^{2+} overload causes a severe reduction in mitochondrial membrane potential ($\Delta\psi$m), and triggers the aberrant opening of the MPTP and membrane permeability leading to the release of apoptotic proteins, such as cytochrome c (Cyt *c*) and apoptosis-inducing factor (AIF), from the mitochondrial inner membrane space [14,15]. To this end, inhibition of MPTP may afford neuroprotective effects. Cyclosporine A (CsA) has been shown to be a potent inhibitor of MPTP opening in animal models of CNS disorders, including stroke, traumatic brain injury, and Parkinson's disease (PD) [10–13,15–19]. CsA acts through binding with cyclophilin–calcineurin (CN) complex to suppress cytokine gene expression and block of T lymphocyte action. CsA is the treatment of choice for immunosuppression in organ and neural transplantation [10,17,20]. The inhibition of CN can result in the inhibition of nitric oxide synthase activation, free radical formation [10,21], or the mitochondria-induced cell death [10,22,23]. However, to date, the exact mechanism of CsA in exerting neuroprotection remains not fully understood.

Parkinson's disease-associated protein DJ-1 is a multifunctional redox-sensitive protein that mediates neuroprotection by dampening mitochondrial oxidative stress [6,7,24–28], molecular chaperoning of PD-aggregating protein a-synuclein [4], stimulating antiapoptotic and antioxidative gene expression [29] and facilitating the prosurvival Akt while suppressing apoptosis signal-regulating kinase (ASK1) pathways [30]. DJ-1 is localized both in the cytoplasm and nucleus, translocates to mitochondria of a variety of mammalian cells by oxidative stress or mitogen stimulation [31], and is secreted into the serum under pathologic conditions such as breast cancer and melanoma [32]. Our previous studies demonstrated a novel observation of DJ-1 translocation into the mitochondria after oxygen glucose deprivation/reperfusion (OGD/R) injury in human neural progenitor cells (hNPCs) and primary rat neuronal cells (PRNCs), which opens new avenues of research and therapeutic development targeting DJ-1 for rescuing stroke and other neurological disorders characterized by rampant mitochondrial deficits [25–27]. Accumulating evidence has implicated the role of mitochondria in abrogating free radical generation [5] which served as impetus for us to determine whether DJ-1 translocated into the mitochondria might attenuate mitochondrial injury or reduce the mitochondrial ROS production in neurological disorders [25–27].

We hypothesized that in addition to DJ-1 acting as an intracellular therapeutic molecule against oxidative stress, the protein also functions as an extracellular signaling molecule, thereby allowing coordination between neighboring neuronal cells via paracrine and/or autocrine cues. Here, we tested whether CsA stimulated DJ-1 secretion in neuronal cells. The aims of this study were to explore the neuroprotective mechanism of CsA in PRNCs under hypoxic-ischemia condition using an *in vitro* acute stroke model of OGD/R injury, in particular focusing on the role of neuronal mitochondria in cell death signaling and as a therapeutic target for stroke.

Materials and Methods

Cell Culture and OGD/R

PRNCs were obtained from BrainBits (E18 rat cortex; Springfield, IL, USA). According to the protocol, cells (4×10^4 cells/well) were suspended in 200 μL Neural Medium (NbActive 4; BrainBit) containing 2 mM L-glutamine and 2% B27 in the absence of antibiotics and grown in Poly-L-Lysine-coated 96-well plates (354516; BD Biosciences, Franklin Lakes, NJ, USA) at 37°C in humidified atmosphere containing 5% carbon dioxide in 40% of the neuron and 60% astrocyte cell population (determined immunocytochemically using vesicular glutamate trransporter-1). After 5-day culturing (approximately cell confluence of 70%), PRNCs were exposed to OGD/R as described previously with few modifications [26,33]. The cells were initially exposed to OGD/R medium (glucose-free Dulbecco's Modified Eagle Medium, Gibco, Life Technologies, Waltham, MA, USA), then placed in an anaerobic chamber (Plas-Labs, Inc., Lansing, MI, USA) containing 95% nitrogen and 5% carbon dioxide for 15 min at 37°C, and finally, the chamber was sealed and incubated for 90 min at 37°C (hypoxic-ischemic condition). Control cells were incubated in same buffer containing 5 mM glucose at 37°C in a regular CO_2 (5%) incubator (normoxic condition). OGD/R was terminated by adding 5 mM glucose to medium, and cell cultures re-introduced to the regular CO_2 incubator (normoxic condition) at 37°C for 2 h, of which period represented a model of "reperfusion."

Administration of CsA

For pre-stroke administration of CsA (Paddock Laboratories, Inc., Minneapolis, MN, USA), cells were pre-treated with CsA 24 h before stroke onset (24 h pre-OGD/R), while for post-stroke regimen, cells were exposed to CsA at 2 h after stroke (2 h post-OGD/R). PRNCs were subjected to OGD/R for 90 min, followed by a 2-h reperfusion period under normoxic condition.

Measurement of Cell Viability: Calcein-AM Fluorescence Dye

Measurement of cell viability was performed by both fluorescent live/dead cell assay and trypan blue exclusion method [34,35]. This dye is ferrous iron sensitive [36]. A two-color fluorescence cell viability assay was performed by Calcein-AM (L3224; Invitrogen, Waltham, MA, USA) to be retained within live cells, including an intense uniform green fluorescence and ethidium homodimer (EthD-1) to bind the nuclei of damaged cells (bright red fluorescence). After 2-h reperfusion, the PRNCs were incubated with 2 μM Calcein-AM and 4 μM EthD-1 for 45 min at room temperature in darkness according to the manufacturer's instructions. Green fluorescence of the live cells was measured by the Gemini EX florescence plate reader (Molecular Device, Sunnyvale, CA, USA), excitation at 485 nm and emission at 538 nm. In addition, trypan blue (15250-061, Gibco, Life Technologies) exclusion method was conducted and mean viable cell counts

were calculated in four randomly selected areas (1 mm^2, n = 10) to reveal the cell viability. To precisely calibrate the cell viability, the values were standardized form florescence intensity and trypan blue data [26,35].

Measurement of Oxidative Stress: Glutathione (GSH) Activity

As glutathione has been validated as an antioxidant component of oxidative defense system in eukaryotic cell [25,26,37] and that increased total intracellular glutathione level provides a measure of toxicological response precluding cell death [25,26,37], we performed glutathione assay using manufacturer's protocol for GHS-Glo™ Glutathione Assay Kit (V6911; Promega, Madison, WI, USA). The optical density of solubilized purple formazan was measured at 570 nm on a Synergy HT plate reader (Bio-Tex, Inc., Houston, TX, USA). The value of EC_{50}, concentration of CsA that gives half-maximal response, was calculated from the equation, $y = A + [(B-A)/(1 + (x/EC_{50})^h)]$, where y is the observed value, A is minimal value, B is maximal value, x is the concentration of CsA, and the Hill coefficient (h, 1.0) gives the largest absolute value of the slope of the curve [38].

Measurement of mtDNA Stability: Picogreen Fluorescence Intensity

Effect of CsA with mtDNA stability measured by Picogreen fluorescence (P11496; Invitrogen) intensity [39]. To quantitating, the degree of mtDNA depletion within living cells was performed by Picogreen according to the manufacturer's instructions. After 2-h reperfusion, the PRNCs were incubated with Quant-iT Picogreen dsDNA reagent for 5 min at room temperature in darkness. The green fluorescence of the live cells was measured by the Gemini EX florescence plate reader (Molecular Device), excitation at 480 nm and emission at 520 nm.

Measurement of $\Delta\psi m$: Tetramethylrhodamine Methyl Ester (TMRM)

For the measurement of $\Delta\psi m$, PRNCs were incubated with 25 nM tetramethylrhodamine methyl ester (TMRM) (88065; Sigma-Aldrich, Fremont, CA, USA) for 45 min before completed reperfusion [39–41] and the fluorescence was measured by the Gemini EX florescence plate reader (Molecular Device), excitation at 549 nm and emission at 573 nm.

Measurement of ATP Levels

The measurement of ATP content was analyzed using ATP bioluminescence assay kit (11699709001; Roche Life Science, Indianapolis, IN, USA) according to the manufacturer's instructions. After 2-h reperfusion, the PRNCs were incubated with cell lysis reagent (Sigma, Fremont, CA, USA) and protease inhibitor cocktail (Sigma) to the samples for 5 min at room temperature in darkness. Luciferase reagent was added to the samples, and the luminescence of the live cells was measured by the Gemini EX florescence plate reader (Molecular Device).

Measurement of Hexokinase Activity: G6P

The measurement of hexokinase activity was measured by glucose 6 phosphate dehydrogenase assay kit (ab102529; Abcam, Cambridge, MA, USA) according to the manufacturer's instructions. After 2-h reperfusion, the PRNCs were incubated with reaction mix to the samples for 30 min at room temperature in darkness. Absorbance from each sample was measured in duplicate using a Synergy HT plate reader (Bio-Tex, Inc.) at wavelengths of 450 nm.

Measurement of Extracellular DJ-1 Concentration

The quantitative measurement of DJ-1/PARK-7 in PRNCs supernatant was analyzed using a CircuLex DJ-1/PARK-7 ELISA Kit (CY-9050; MBL International Corporation, Woburn, MA, USA) according to the manufacturer's instructions [42]. Under baseline conditions (without OGD/R conditions), dose range of 50 nM – 10 μM of concentration of CsA cell supernatant was measured with extracellular DJ-1 levels. Absorbance from each sample was measured in duplicate using a Synergy HT plate reader (Bio-Tex Inc.) at dual wavelengths of 450/540 nm.

Immunocytochemistry Analysis

PRNCs (8×10^4 cell/well) in 400 μL Neural medium containing 2 mM L-glutamine and 2% B27 in the absence of antibiotics and grown in Poly-L-Lysine 8 chamber (354632; BD Biosciences) were fixed in 4% paraformaldehyde for 20 min at room temperature after OGD/R or non-OGD/R treatment [26]. For Cyt *c*, mitochondria staining and for DJ-1, mitochondria staining, cells were blocked for 60 min at room temperature with 5% normal goat serum (50-062Z; Invitrogen, Camarillo, CA, USA) in PBS containing 0.1% Tween-20 (PBST) (Sigma-Aldrich). After blocking reaction, the cells were incubated overnight at 4°C with rabbit monoclonal anti-cytochrome c (1:250, ab76237; Abcam), mouse monoclonal anti-ATP synthase β-chain (Mitochondria) (1:200, 05-709; Cell Signaling Technology, Danvers, MA, USA), and rabbit monoclonal anti-DJ-1 (1:100, ab76008; Abcam) with 5% normal horse serum. The cells were washed five times for 10 min in PBST and then soaked in 5% normal goat serum in PBST containing corresponding secondary antibodies, goat anti-rabbit IgG-Alexa 488 (green; 1:1000, A11034; Invitrogen) and goat anti-mouse IgG-Alexa 594 (red; 1:1000, A11032; Invitrogen), for 90 min. Finally, cells were washed five times for 10 min in PBST and three times for 5 min in PBS, and subsequently embedded with mounting medium. Immunofluorescent images were visualized using Zeiss Axio Imager Z1 (Zeiss, Thornwood, NY, USA). Control experiments were performed with the omission of the primary antibodies yielding negative results.

Statistics

The data were evaluated using ANOVA followed by *post hoc* Bonferroni's test. Statistical significance was preset at $P < 0.05$. Data are presented as mean ± SE from quintuplicates of each treatment condition.

Results

Pretreatment of CsA Enhances Neuroprotection Dose-Dependently

Administration of 10 μM CsA before 24 h pre-OGD/R afforded significantly much more robust neuroprotective effects compared to OGD/R-treated cells without CsA ($P < 0.01$) (Figure 1A). On the other hand, CsA was initiated after 2 h post-OGD/R has no inductive effect on cell survival ($P > 0.05$) (Figure 1A), suggesting that CsA facilitates the delayed intracellular neuroprotective signaling, but not acute signal transduction. We evaluated the dose–response of CsA efficiency (500 nM ~ 10 μM) during 24-h treatment, as half-life time of CsA is approximately within 24 h in rodents [43]. PRNCs viability significantly increased in administration of between 500 nM, 1 μM, and 10 μM ($P < 0.05$, $P < 0.05$, $P < 0.01$, respectively) (Figure 1B). These results indicate that effect of CsA occurs predominantly within the confined of concentrations and the time window.

Effects of CsA on Mitochondrial Activity of PRNCs after OGD/R

A cascade of hypoxic-ischemic cell death events may principally arise from a dysfunctional mitochondrial complex I or NADH dehydrogenase, culminating with aberrant accumulation of ROS, a hallmark biochemical feature of oxidative stress. The EC_{50} value of CsA on reducing the oxidative stress after OGD/R was approximately 10 μM ($P < 0.01$) (Figure 1C). Therefore, the following experiments were performed with treatment of 10 μM CsA during 24 h prior to OGD/R. Ten μM CsA significantly protected mtDNA degradation ($P < 0.01$) (Figure 1D), $\Delta\psi$m reduction ($P < 0.05$) (Figure 2A), and ATP decrement ($P < 0.05$) (Figure 2B) after OGD/R compared to without CsA. These data prompted us to examine whether CsA influenced ATP synthesis. ATP content was significantly different between CsA treatment at 24 h and non-treatment controls ($P < 0.05$) (Figure 2B). A hexokinase is an important enzyme which regulates phosphorylation of a hexose. G6P controls intercellular metabolic processes, such as glycolysis

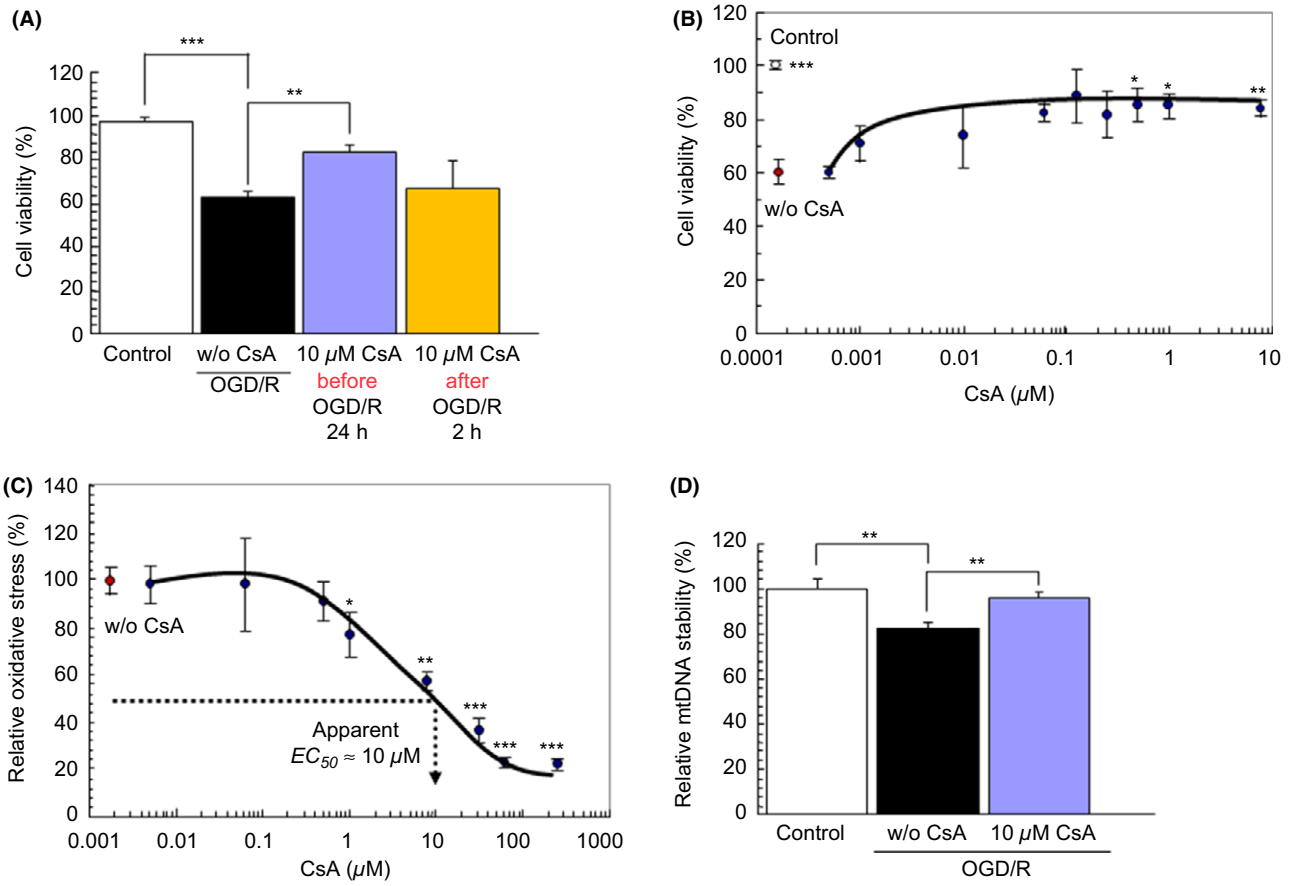

Figure 1 CsA possesses neuroprotective effects and attenuates the mtDNA degradation under hypoxic-ischemic condition. Administration of CsA before stroke onset (24 h pre-OGD/R) and CsA was initiated after stroke (2 h post-OGD/R). PRNCs were subjected to OGD/R for 90 min, followed by a 2-h reperfusion period under normoxic condition. Under hypoxic-ischemic condition, cell viability tested by Calcein-AM fluorescence dye (**A**). Cell viability dose-response curve (**B**). Effect of CsA with oxidative stress (**C**). Relative oxidative stress percentage (%) is calculated from the equation described in materials and methods. Effect of CsA with mtDNA stability measured by Picogreen fluorescence intensity (**D**). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus w/o CsA.

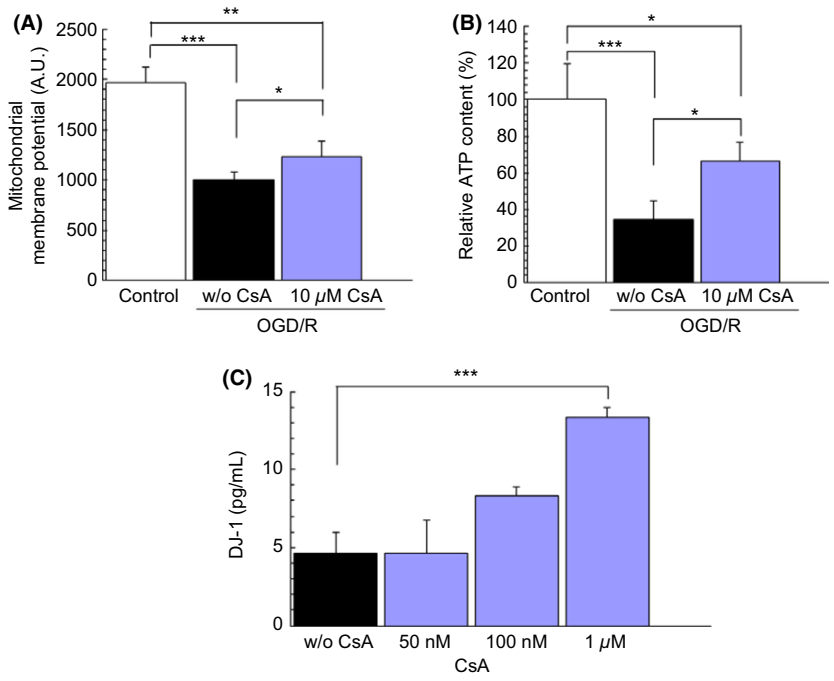

Figure 2 CsA enhances the secretion of DJ-1 and prevents the decline of both $\Delta\psi$m and cellular ATP. Administration of CsA before stroke onset (24 h pre-OGD/R). PRNCs were subjected to OGD/R for 90 min, followed by a 2-h reperfusion period under normoxic condition. Under hypoxic-ischemic condition, $\Delta\psi$m was measured by TMRM (**A**) and ATP levels were measured by ATP bioluminescence assay (**B**). Under baseline conditions (without OGD/R conditions), DJ-1 expression levels were detected by the CircuLex DJ-1/PARK ELISA kit (**C**). $^{*}P < 0.05$ $^{**}P < 0.01$, and $^{***}P < 0.001$.

and glycogen synthesis, and directly regulates thioredoxin-interacting protein expression [44]. Accordingly, we next investigated whether CsA increased the glucose transport activity. G6P activity was not significantly different between CsA treatment with 24 h and non-treatment controls (39.3 ± 1.00 pmoles/min/10^4 cells versus 41.8 ± 2.03 pmoles/min/10^4 cells, respectively, P value 0.324). Because ATP synthesis depends on the mitochondrial membrane potential, we subsequently tested whether CsA modified the membrane potential, and showed that CsA significantly protected the degradation of $\Delta\psi$m ($P < 0.05$) (Figure 2A). These data indicate that CsA did not influence glycolysis, but suppressed the abnormal accumulation of mitochondrial ROS in preventing damages to both complexes I and III.

CsA Stimulates the Secretion of DJ-1

The observed therapeutic benefits of CsA coincided with increased levels of DJ-1. Results revealed that there was a significant upregulation of DJ-1 at 1 μM of CsA compared to the baseline levels (without OGD/R conditions) ($P < 0.001$) (Figure 2C).

CsA Decreases the Release of Cyt c from Mitochondria

To establish an interaction between CsA and mitochondria in the *in vitro* acute stroke model of OGD/R injury with emphasis on DJ-1, we examined CsA and DJ-1 in the mitochondria at the level of immunocytochemical assays (Figure 3). At normoxic condition, Cyt *c* was retained within the mitochondria (Figure 3A–C). However, under hypoxic condition, the Cyt *c* was released outside the mitochondria (Figure 3D–F). Under hypoxic condition, treatment with 10 μM of CsA demonstrated that Cyt *c* was maintained within the mitochondria (Figure 3G–I).

CsA Enhances DJ-1 Translocation into the Mitochondria

To relate to this to the DJ-1 expression, we also analyzed the localization within the mitochondria (Figure 4). We found similar result, at normoxic condition, Cyt *c* was retained within the mitochondria (Figure 4A–C). However, under hypoxic condition, the Cyt *c* was released outside the mitochondria (Figure 4D–F). DJ-1 was co-localized with the mitochondria under hypoxic condition when treated with 10 μM of CsA (Figure 4G–I). These results suggest a direct interaction between DJ-1 and mitochondria in the observed CsA neuroprotection.

Discussion

This study reports that under hypoxic-ischemia condition, the $\Delta\psi$m, respiratory-related enzymes, and mitochondrial DNA deteriorate resulting in the aberrant accumulation of free radicals and ROS. CsA, traditionally considered a robust immunosuppressant drug, has been shown as a potent protective agent against neuronal cell death [10–13,15–19]. However, the molecular mechanism by which CsA interacts with mitochondrial membrane-associated proteins remains not fully understood. Using the *in vitro* acute stroke model of OGD/R injury, we examined here this interaction between CsA and the Parkinson's disease-associated protein DJ-1, which has been recently implicated in the regulation of mitochondrial integrity [6,7,25–28]. Administration of CsA before stroke onset (24 h pre-OGD/R), but not after stroke (2 h post-OGD/R), afforded significant neuroprotective effects compared to OGD/R-treated cells without CsA, characterized by the following cellular and molecular events: (1) CsA prevented the mitochondria-dependent apoptotic cell death associated with Cyt *c* release; (2) CsA protected cellular ATP decline

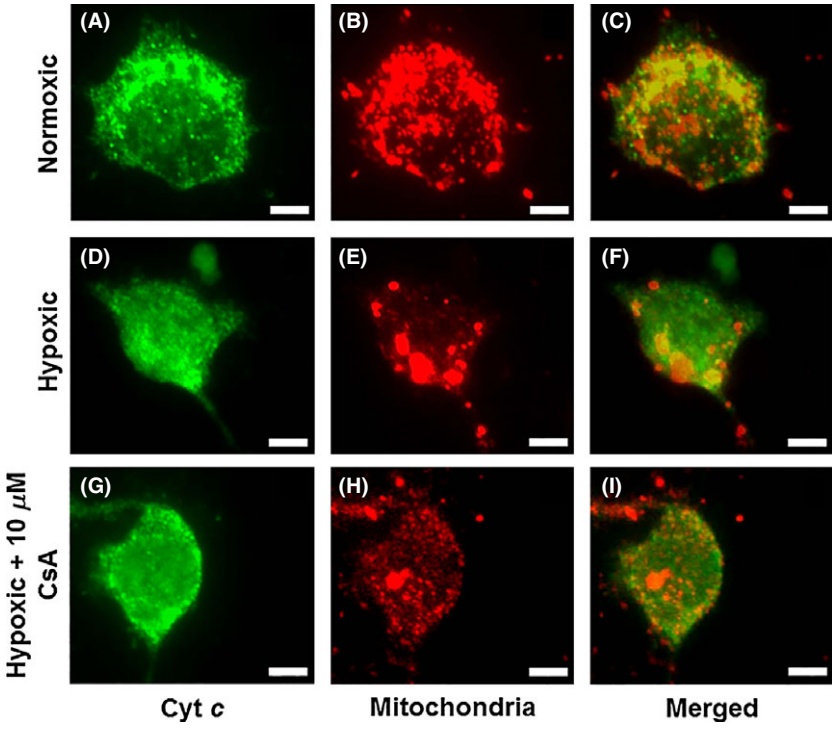

Figure 3 CsA decreases the releasing Cyt *c* from mitochondria, which turns on the caspases-induced apoptotic signal pathway. Cyt *c* (**A**, **D**, **G**) and Mitochondria (**B**, **E**, **H**) staining revealed an apparent maintained within the mitochondria under hypoxic condition when treated with 10 μM of CsA (**G**–**I**) compared to without CsA. Cyt *c* and Mitochondria double-positive cells are shown in panels: **C**, **F**, and **I**. Green: Cyt *c*, Red: Mitochondria. Scale bars = 5 μm.

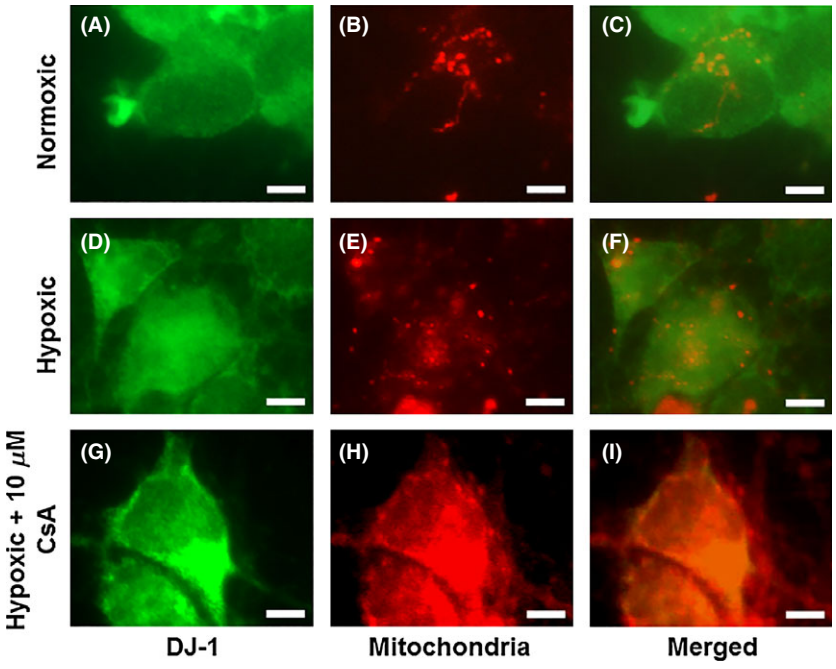

Figure 4 CsA enhances DJ-1 translocation into the mitochondria. DJ-1 (**A**, **D**, **G**) and Mitochondria (**B**, **E**, **H**) staining revealed an apparent maintained within the mitochondria under hypoxic condition when treated with 10 μM of CsA (**G**–**I**) compared to without CsA. DJ-1 and Mitochondria double-positive cells are shown in panels: **C**, **F**, and **I**. Green: DJ-1, Red: Mitochondria. Scale bars = 5 μm.

(2-fold higher) without altering the hexokinase activity and the $\Delta\psi$m; (3) blocked mtDNA degradation; and, interestingly, (4) enhanced secretion of DJ-1 in the mitochondria (Figure 5). This neuroprotection was achieved with the effective dose range of 500 nM – 10 μM of CsA.

CsA Protects the Opening of MPTP

Mitochondrial dysfunction is an important contributor to neurodegeneration [45,46], including stroke [47]. Hypoxic-ischemic cell death events may consist of mitochondria complex

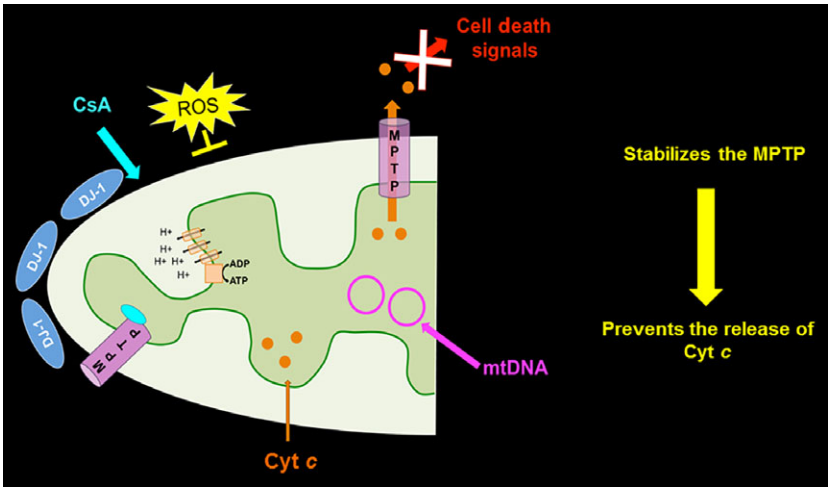

Figure 5 Schematic diagram of proposed neuroprotective mechanism of CsA and DJ-1 in mitochondria in stroke. This schematic diagram represents a speculative reparative mechanism underlying the both CsA and DJ-1 neuroprotective for mitochondria in stroke. Treatment of CsA upregulates DJ-1, stabilizes the MPTP and prevents the release of Cyt *c* from the mitochondria. The MPTP is thought to open in response to hypoxic-ischemia to the neuron cell. In combination, there is a reduction in mtDNA degradation and prevention of cellular ATP reduction.

I spontaneously releasing ROS, a hallmark biochemical feature of oxidative stress [48]. A cell death mechanism characterized by the collapse of $\Delta\psi$m, which in turn triggers the aberrant disruption of the impermeability of the inner mitochondrial membrane, has been shown to accompany oxidative stress [25–27,49,50]. Initial stroke-induced ROS acts upon neighboring mitochondria, precipitating MPTP opening, and thereafter generating additional ROS [51]. The MPTP is thought to open in response to hypoxic-ischemia to the neuron cell [52]. Intracellular calcium and oxidative stress trigger a conformational change in the adenine nucleotide translocase (ANT) when associated with voltage-dependent anion channel (VDAC). This is facilitated by cyclophilin D (CypD), and blocked by CsA, which specifically binds to CypD. Of note, the initial accumulation of stroke-induced ROS may act upon neighboring mitochondria, thereafter precipitating the MPTP opening and generation of additional ROS. Once MPTP is breached, Cyt *c* is released from mitochondria to cytosol and induces the activation of apoptosis cascade events. Employing the anti-ATP synthase β-chain antibody, of which antigen is localized in the mitochondrial inner membrane and Cyt *c* antibody, the present results revealed that CsA prevents Cyt *c* releasing from mitochondria.

CsA Stimulates the Secretion of DJ-1 and Translocates into the Mitochondria

A major finding in the present study is the demonstration that neural cells secreted DJ-1, with significant upregulation of DJ-1 generated at 1 μM of CsA. This observation parallels similar reports documenting that breast cancer and melanoma tumor cells release DJ-1 to the serum *in vitro* and *in vivo* [53–55]. The elevated levels of extracellular DJ-1 following treatment with CsA, and the antibody sequestration of this secreted protein suggests an intimate involvement of DJ-1 in the initial endogenous neuroprotective process in response to stroke. Moreover, the translocation of DJ-1 into the mitochondria coinciding with reduced oxidative stress implies that DJ-1 may facilitate the molecular link between mitochondria and oxidative stress in establishing a potent neuroprotective pathway to halt the progression of secondary cell death inherent in stroke [25–27].

CsA Reduces mtDNA Degradation and Prevents of Cellular ATP Reduction

Maintenance of mitochondrial integrity has been suggested to be an important mechanism of extending lifespan, as decreased mitochondrial integrity, impaired ATP generation, and increased ROS levels have been implicated in aging [56]. To this end, understanding the role of CsA in mitochondrial integrity may reveal its neuroprotective action. CsA is a hydrophobic peptide drug, with its immunosuppressive activity closely associated with its binding to specific proteins of immune cells, such as CypD. Because of its hydrophobicity, CsA may interact with biological membranes, which may mediate its therapeutic effect [57]. DJ-1 protein stability and dimerization have been shown to be disrupted across the entire dimer interface, characterized by extended hydrophobic surfaces involved in dimer formation [58–60]. This significant increase in DJ-1 hydrophobic surface area [59] may contribute to its translocation into the mitochondria [25–27]. Our results suggest that CsA and DJ-1 may specifically interact within mitochondrial membranes, in that CsA facilitates DJ-1 translocation into the mitochondria allowing maintenance of mitochondrial integrity against ischemic cell death.

CsA Role in Apoptosis Associated with Cyt c Release

Cyt *c* induction during apoptosis involves (1) the binding of Cyt *c* to cardiolipin (CL) in the inner membrane of mitochondria (IM); (2) release of Cyt *c* upon complex-I-dependent oxidation of CL; (3) pro–caspase-8 (pro-8) binding to the outer membrane of mitochondria (OM), which leads oligomerization, and subsequently undergoes autocatalytic processing in a CL-dependent manner; (4) pro-8 cleaving Bid and forming truncated Bid (t-Bid), in turn facilitating the activation of Bax/Bak; and (5) phospholipid scramblase 3 (PLS3) allows export of CL to OM, mediating normal mitochondrial integrity [52]. In the present study, pretreatment of CsA upregulates DJ-1, stabilizes MPTP and prevents the release of Cyt *c* from the mitochondria. In tandem with this CsA stabilization of DJ-1 in the mitochondria, we found that there is a reduction in

mtDNA degradation and at the same time prevention of cellular ATP reduction. Preserving the integrity of the mitochondria via regulation of ATP production and Cyt *c* release is key to abrogating oxidative stress. CsA, via DJ-1 upregulation, preserves mitochondrial integrity and exerts antioxidative stress, demonstrating a novel neuroprotective mechanism for treatment of stroke and possibly other neurological disorders. These novel observations indicate that CsA- and DJ-1-neuroprotection amplify the maintenance of mitochondrial integrity and that mitochondria-based treatments targeting the early phase of disease progression may prove beneficial in stroke. Future studies will be required to use a DJ-1 knockdown or DJ-1 knockout cells into which CsA is administered. To translate this *in vitro* finding into an *in vivo* model, use a DJ-1 knockdown or knockout rodent model to further investigate the CsA-DJ-1 neuroprotective pathway in stroke.

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Conflict of Interest

The authors declare no conflict of interest.

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