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

Naoki Tajiri,1,2 Cesar V. Borlongan1 & Yuji Kaneko1

1 Center of Excellence for Aging & Brain Repair, Department of Neurosurgery and Brain Repair, University of South Florida Morsani College of Medicine, Tampa, FL, USA
2 School of Physical Therapy & Rehabilitation Sciences, University of South Florida Morsani College of Medicine, Tampa, FL, USA

SUMMARY

Aims: Hypoxic-ischemia alters mitochondrial membrane potential (Δψm), 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 Δψm 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.

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

Keywords
Cytochrome c; Mitochondrial DNA; Mitochondrial membrane potential (Δψm); Mitochondrial permeability transition pore; Oxygen glucose deprivation/reperfusion injury.
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]. Ca2+ accumulation contributes to neuronal function; however, excessive mitochondrial Ca2+ overload causes a severe reduction in mitochondrial membrane potential (∆Ψm), 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. Cyclopamine 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 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 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 × 10⁴ 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 transporter-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) to be retained within live cells, including an intense uniform green fluorescence and ethidium homodimer (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 fluorescence 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

**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 fluorescence 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 mm², n = 10) to reveal the cell viability. To precisely calibrate the cell viability, the values were standardized form fluorescence 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®M 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 EC₅₀ concentration of CsA that gives half-maximal response, was calculated from the equation, 

\[
\frac{A + [B-A]/(1 + \alpha/EC_{50})^h}{}
\]

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 Δψm: Tetramethylrhodamine Methyl Ester (TMRM)**

For the measurement of Δψm, 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 × 10⁴ cell/well) in 400 µL Neural medium containing 2 nm 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 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 EC50 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), Δψm 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.
and 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/10^6 cells versus 41.8 ± 2.03 pmoles/min/10^6 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 Δψm (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 Δψm, 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, followed 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.
(2-fold higher) without altering the hexokinase activity and the Δψm; (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
I spontaneously releasing ROS, a hallmark biochemical feature of oxidative stress [48]. A cell death mechanism characterized by the collapse of ΔΨm, 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.

Acknowledgments

CVB is supported by NIH NINDS 1RO1NS071956-01, James and Esther King Foundation for Biomedical Research Program, SanBio Inc., Celgene Cellular Therapeutics, KMPHC, and NeuralStem Inc. NT is supported by Byrd Institute Small Grants Program (BRD 220). The funders have no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We thank Ms. Sussannah Kaelber for her technical assistance in journal formatting of this manuscript.

Conflict of Interest

The authors declare no conflict of interest.

References

1. Heiss WD. Ischemic penumbra. Evidence From functional imaging in man. J Cereb Blood Flow Metab 2000;20: 1276–1293.
2. Warach S. Measurement of the ischemic penumbra with MRI. It's about time. Stroke 2003;34:2313–2334.
3. Lu HH. A new penumbra: Transitioning from injury into repair after stroke. Nat Med 2008;14:497–500.
4. Dawson TM, Dawson VL. Molecular pathways of neurodegeneration in parkinson's disease. Science 2005;308:619–822.
5. Nakamura T, Itohno SA. Redox regulation of mitochondrial fission, protein misfolding, synaptic damage, and neuronal cell death: Potential implications for alzheimer's and parkinson's diseases. Apoptosis 2010;15:1354–1363.
6. Ariga H, Takahashi-Niki K, Kato I, Maita H, Niki T, Iuchi-Ariga SM. Neuroprotective function of dj-1 in parkinson's disease. Osid Med Cell Leng 2013;2013:685920.
7. Takahashi-Niki K, Inafune A, Michitani N, et al. Dj-1-dependent protective activity of dj-1-binding compound no. 23 against neuronal cell death in mptp-treated mouse model of parkinson's disease. J Pharmacol Sci 2013;127:305–310.
8. Achatma G, Sussex R, Feng L, et al. Novel role of p53 in neuroprotection. J Pharmacol Exp Ther 2009;329:1363.
9. Borlongan CV, Yu G, Matsukawa N, et al. Acute functional effects of cyclosporine-a and methylprednisolone treatment in adult rats exposed to transient ischemic stroke. J Cereb Blood Flow Metab 2005;26:1503–1512.
10. Luke D, Burns J, Bae EC, van Loon H, Borlongan CV. A review of laboratory and clinical data supporting the safety and efficacy of cyclosporin in a traumatic brain injury. Neurosurgery 2011;68:1172–1185; discussion 1185–1176.
11. Borlongan CV, Stahl CE, Elmer E, Keep MF, Watanabe S. Cyclosporine a, but not fk 506, protects against cell death following acute cardiac ischemia-reperfusion injury. FEBS Lett 2000;529:73–79.
12. Butcher SP, Henshall DC, Teramura Y, Iwaoaki K, Starkey J. Neuroprotective actions of fk506 in experimental stroke: In vivo evidence against an antiepticotic mechanism. J Neurosci 1997;17:6939–6946.
13. Ferberg H, Ferrand-Drake M, Bengtsson F, Halestrap AP, Wieloch T. Cyclosporin a, but not b, 506, protects mitochondria and neurons against hypoglycemic damage and implicates the mitochondrial permeability transition in cell death. J Neurosci 1998;18:5115–5139.
14. Uchino H, Elmer E, Uchino K, et al. Amelioration of cyclosporin a induced brain damage in transient forebrain ischemia in the rat. Brain Res 1998;812:216–226.
15. Randopadhyay N, Kingsbury AE, Cookson MR, et al. The expression of dj-1 (park7) in normal human cns and idiopathic parkinson's disease. Mol Neurobiol 2003;25:101–108.
16. Lu SC. Regulation of glutathione synthesis. Mol Aspects Med 2009;30:42–59.
17. Neubig RR, Spedding M, Kenakin T, Christopoulos A, Mouradian MM. Interaction of dj-1 with daxx inhibits cell death following acute cardiac ischemia-reperfusion injury. Glii Dis 2004;11:1083–1102.
18. Halestrap AP. What is the mitochondrial permeability transition pore? J Mol Cell Cardiol 2009;46:621–831.
19. Osman MM, Lulic D, Glover L, et al. Cyclosporine-a as a neuroprotective agent against stroke: Its translation from laboratory research to clinical application. Neuropeptides 2011;45:359–368.
20. Borlongan CV, Emterich D, Hofer BJ, Bartus RT. Bradykinin receptor agonist facilitates low-dose cyclosporine-a protection against 6-hydroxydopamine neurotoxicity. Brain Res 2002;965:211–220.
21. Borlongan CV, Yu G, Matsukawa N, et al. Acute functional effects of cyclosporine-a and methylprednisolone treatment in adult rats exposed to transient ischemic stroke. J Cereb Blood Flow Metab 2005;26:1503–1512.
22. Friberg H, Ferrand-Drake M, Bengtsson F, Halestrap AP, Wieloch T. Cyclosporin a, but not fk 506, protects against cell death following acute cardiac ischemia-reperfusion injury. FEBS Lett 2000;529:73–79.
23. Uchino H, Elmer E, Uchino K, et al. Amelioration of cyclosporin a induced brain damage in transient forebrain ischemia in the rat. Brain Res 1998;812:216–226.
24. Borlongan CV. Kainic acid-induced golgi complex fragmentation/dispersal shifts the proteolysis of reelin in primary rat neuronal cells: An in vitro model of early stage epilepsy. Mol Neurobiol 2016;53:1874–1883.
25. Kon K, Kim JS, Uchiyama A, Jaeschke H, Lammers J. Lysosomal iron mobilization and induction of the mitochondrial permeability transition in acetaminophen-induced toxicity to mouse hepatocytes. Toxicol Sci 2010;117:101–108.
26. Tanaka A. Parkin-mediated selective mitochondrial neuroprotection in experimental ischemic stroke. BMC Neurosci 2009;10:126.
27. Bell E, Cao X, Mobli JA, et al. Rapamycin has a deleterious effect on min-a cells and rat and human islets. Diabetes 2003;52:2711–2719.
28. Kaneko Y, Sullivan R, Dailey T, Yale FL, Tajiri N, Borlongan CV. Kinetic acid-induced golgi complex fragmentation/dispersal shifts the proteolysis of reelin in primary rat neuronal cells: An in vitro model of early stage epilepsy. Mol Neurobiol 2016;53:1874–1883.
29. Clements CM, McNally RS, Comti BJ, Mak TW, Ting JP. Dj-1, a cancer- and parkinson's disease-associated protein, stabilizes the antioxidant transcriptional master regulator nrf2. Proc Natl Acad Sci USA 2006;103:15901–15906.
30. Junn E, Taniguchi H, Jeong BS, Zhao X, Ichijo H, Mouzannar MM. Interaction of dj-1 with daxx inhibits apoptotic signal-regulating kinase 1 activity and cell death. Proc Natl Acad Sci USA 2005;102:9691–9696.
31. Canet-Aviles RM, Wilson MA, Miller DW, et al. The parkinson's disease protein dj-1 is neuroprotective due to cysteine-sulfuric acid-driven mitochondrial localization. Proc Natl Acad Sci USA 2004;101:9103–9108.
32. Tsuoda Y, Mimutomo R, Ishikawa S, Matsumoto K, Iguchi-Ariga SM, Ariga H. Dj-1, a casseve gene product of a familial form of parkinson's disease, is secreted through microdomains. FEBS Lett 2008;582:246–252.
33. Matsukawa N, Yonoshita T, Han K, et al. Therapeutic targets and limits of minocycline neuroprotection in experimental ischemic stroke. BMC Neurosci 2009;10:126.
34. Bell E, Cao X, Mobli JA, et al. Rapamycin has a deleterious effect on min-a cells and rat and human islets. Diabetes 2003;52:2711–2719.
35. Kaneko Y, Sullivan R, Dailey T, Yale FL, Tajiri N, Borlongan CV. Kinetic acid-induced golgi complex fragmentation/dispersal shifts the proteolysis of reelin in primary rat neuronal cells: An in vitro model of early stage epilepsy. Mol Neurobiol 2016;53:1874–1883.
36. Kon K, Kim JS, Uchiyama A, Jaeschke H, Lammers J. Lysosomal iron mobilization and induction of the mitochondrial permeability transition in acetaminophen-induced toxicity to mouse hepatocytes. Toxicol Sci 2010;117:101–108.
37. Lu SC. Regulation of glutathione synthesis. Mol Aspects Med 2009;30:42–59.
38. Neubig RR, Spedding M, Kenakin T, Christopoulos A, Mouradian MM. Interaction of dj-1 with daxx inhibits cell death following acute cardiac ischemia-reperfusion injury. Glii Dis 2004;11:1083–1102.
42. Bande MF, Santiago M, Blanco MJ, et al. Serum dj-1/park 7 is a potential biomarker of choroidal nevi transformation. *Invest Ophthalmol Vis Sci* 2012;53:62–67.

43. Vachon P, Beaudy F, Marier JJ, Ste-Marie L, Montgomery J. Cyclosporin a in blood and brain tissue following intra-carotid injections in normal and stroke-induced rats. *Brain Res* 2002;943:1–8.

44. Stoltzman CA, Peterson CW, Breen KT, Muoio DM, Billin AN, Ayer DE. Glucose sensing by mndoa: Mlx complexes: A role for hexokinases and direct regulation of thioredoxin-interacting protein expression. *Proc Natl Acad Sci USA* 2008;105:6912–6917.

45. Parone PA, Martinou JC. Mitochondrial fission and apoptosis: An ongoing trial. *Biochim Biophys Acta* 2006;1763:522–530.

46. Yang Y, Candidato-Jutil E, Thompson JJ, et al. Increased intracellular matrix metalloproteinase activity in neurons interferes with oxidative DNA repair in focal cerebral ischemia. *J Neurochem* 2010;112:134–149.

47. De Vos KJ, Grierson AJ, Ackerley S, Miller CC. Role of axonal transport in neurodegenerative diseases. *Annu Rev Neurosci* 2008;31:151–173.

48. Fulda S, Galluzzi L, Kroemer G. Targeting mitochondria for cancer therapy. *Nat Rev Drug Discov* 2010;9:447–464.

49. Reyes BC, Pappura V. Mitochondria modulate cal2 + -dependent glutamate release from rat cortical astrocytes. *J Neurosci* 2008;28:9682–9691.

50. Kriechbaumer G, Ruckerbauer S, Barbulla LF, et al. Reduced basal autophagy and impaired mitochondrial dynamics due to loss of parkinson’s disease-associated protein dj-1. *PLoS One* 2010;5:e9367.

51. Zorov DB, Filburn CR, Klotz LO, Zweier JL, Sollott SJ. Reactive oxygen species (ROS)-induced ROS release: A new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. *J Exp Med* 2000;192:1001–1014.

52. Osman C, Voelker DR, Langer T. Making heads or tails of phospholipids in mitochondria. *J Cell Biol* 2011;192:7–16.

53. Yam H, Pu XP. Expression of the parkinson’s disease-related protein dj-1 during neural stem cell proliferation. *Biof Pharw Biol* 2010;18:21–21.

54. Rubinsztein JD, White E. Autophagy and metabolism. *Science* 2010;330:1344–1348.

55. Barsoum MJ, Yuan H, Gerencser AA, et al. Nitric oxide-induced mitochondrial fission is regulated by dynamin-related gtpases in neurons. *EMBO J* 2006;25:3900–3911.

56. Sahin E, DrPitho RA. Axis of aging: Telomeres, protein and mitochondria. *Nat Rev Mol Cell Biol* 2012;13:395–404.

57. Ayaz S, Morandat S, El Kirat K. The potent antimarial peptide cyclosporin a induces the aggregation and permeabilization of sphingomyelin-rich membranes. *Langmuir* 2011;27:9465–9472.

58. Premkumar L, Dobaczewska MK, Riedl SJ. Identification of an artificial peptide motif that binds and stabilizes reduced human dj-1. *J Struct Biol* 2011;176:414–418.

59. Anderson PC, Daggett V. Molecular basis for the structural instability of human dj-1 induced by the h166p mutation associated with parkinson’s disease. *Biochemistry* 2008;47:9380–9393.

60. Qunigley PM, Korotkov K, Barcynx F, Hol WG. The 1.6-a crystal structure of the class of chaperones represented by escherichia coli hsp70 reveals a putative catalytic trid. *Proc Natl Acad Sci USA* 2010;107:3137–3142.