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DJ-1 ameliorates ischemic cell death *in vitro* possibly via mitochondrial pathway

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Abstract

DJ-1 is an important redox-reactive neuroprotective protein implicated in regulation of oxidative stress after ischemia. However the molecular mechanism, especially the mitochondrial function, by which DJ-1 protects neuronal cells in stroke remains to be elucidated. The aim of this study was to reveal whether DJ-1 translocates into the mitochondria in exerting neuroprotection against an *in vitro* model of stroke. Human neural progenitor cells (hNPCs) were initially exposed to oxygen–glucose deprivation and reperfusion injury, and thereafter, DJ-1 translocation was measured by immunocytochemistry and its secretion by hNPCs was detected by enzyme-linked immunosorbant assay (ELISA). Exposure of hNPCs to experimental stroke injury resulted in DJ-1 translocation into the mitochondria. Moreover, significant levels of DJ-1 protein were secreted by the injured hNPCs. Our findings revealed that DJ-1 principally participates in the early phase of stroke involving the mitochondrial pathway. DJ-1 was detected immediately after stroke and efficiently translocated into the mitochondria offering a new venue for developing treatment strategies against ischemic stroke.

Keywords

Stroke; Mitochondria; Stem cells; Cell death; Neuroprotection

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 the insult to the infarcted core, and subsequently the formation of an ischemic penumbra, which over a sub-acute period remains as salvageable neural tissue thereby amenable to therapeutic intervention. Secondary cell death processes, including oxidative stress, can further exacerbate cell death in the penumbra limiting neurorestoration. Oxidative stress has been implicated in the pathogenesis of central nervous system damage in different neurodegenerative disorders including Alzheimer's disease and Parkinson's disease (PD). DJ-1 is a multifunctional redox-sensitive protein that mediates neuroprotection by dampening mitochondrial oxidative stress (Canet-Aviles et al., 2004), molecular chaperoning of PD-aggregating protein α -synuclein (Dawson and Dawson, 2003), stimulating antiapoptotic and antioxidative gene expression (Clements et al., 2006; Fan et al., 2008), and facilitating the prosurvival Akt while suppressing apoptosis signal-regulating kinase (ASK1) pathways (Gorner et al., 2007; Junn et al., 2005; Yang et al., 2005). It also acts as a positive regulator of androgen receptor-dependent transcription (Canet-Aviles et al., 2004; Dawson and Dawson, 2003). DJ-1 is localized both in the cytoplasm and nucleus, is translocated to the mitochondria of a variety of mammalian cells by oxidative stress or mitogen stimulation (Canet-Aviles et al., 2004), and is secreted into the serum under pathologic conditions such as breast cancer and melanoma (Tsuboi et al., 2008; Waak et al., 2009). Accumulating evidence has implicated the role of mitochondria in abrogating free radical generation (Nakamura and Lipton, 2010), which served as impetus for us to determine whether translocated DJ-1 in the mitochondria might attenuate mitochondrial injury or reduce the mitochondrial reactive oxygen species (ROS) production. We hypothesized that in addition to DJ-1 acting as an intracellular defense system 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. Our long-standing interest in translating stem cell therapy from the laboratory to the clinic guided us to use human neural progenitor cells (hNPCs) as a platform to examine whether DJ-1 translocated into the mitochondria and secreted ex-tracellularly under hypoxic-ischemic condition.

Materials and methods

Cell culture and oxygen-glucose deprivation (OGD)

hNPCs were obtained from Neuromics. According to the protocol, cells (4×10^4 cells/well) were suspended in 200 μ L neural medium containing 2 mM L-glutamine and 10 ng/mL leukemia inhibitory factor in the absence of antibiotics and grown in Poly-L-Lysine-coated 96-well (BD) at 37 °C in humidified atmosphere containing 5% carbon dioxide. After 5 days culturing (approximately cell confluence of 70%), hNPCs were exposed to OGD, an *in vitro* stroke model as described previously with few modifications (Borlongan et al., 2010). The cells were initially exposed to OGD medium (116 mM NaCl, 5.4 mM KCL, 0.8 mM MgSO₄, 1 mM NaH₂PO₄, 26.2 mM NaHCO₃, 0.01 mM glycine, and 1.8 mM CaCl₂ pH 7.4), then placed in an anaerobic chamber (Plas Labs) containing nitrogen (95%) and carbon dioxide (5%) for 15 min at 37 °C, and finally the chamber was sealed and incubated for 90 min at 37 °C (hypoxic-ischemic condition). The control cells were incubated in the same buffer containing 5 mM glucose at 37 °C in a regular CO₂ (5%) incubator (normoxic condition). OGD was terminated by adding 5 mM glucose to the medium and cell cultures

were re-introduced to the regular CO₂ incubator (normoxic condition) at 37 °C for 2 h, of which period represented a model of “reperfusion”. To confirm that extracellular DJ-1 was secreted by hNPCs, anti-DJ-1 antibody (ratio 1:20; Abcam, ab76008) was added to the cell culture medium to capture extracellular DJ-1 during the reperfusion period.

Measurement of mitochondrial activity, cell viability, and oxidative stress

Following reperfusion, reduction of 3- (4, 5-dimethyl-2-thiazoyl)-2, 5-diphenyltetrazolium bromide (MTT) by cellular dehydrogenases was used as a measure of mitochondrial activity as previously described (Borlongan et al., 2010). In addition, trypan blue (0.2%) 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 after hypoxic–ischemic and normoxic condition. Briefly, within 5 min after adding trypan blue, we digitally captured under microscope (× 200) ten pictures (approximately 100 cells/picture) for each condition, then randomly selected five pictures, and counted the number of cells for each individual treatment condition (normoxic, hypoxic–ischemic, and hypoxic–ischemic + DJ-1 antibody). Normalized cell viability was calculated from the following equation: viable cells (%) = [1.00 – (Number of blue cells / Number of total cells)] × 100. Since glutathione (GSH) has been validated as an antioxidant component of oxidative defense system in the eukaryotic cell (Lu, 2009), and that increased intracellular GSH level provides a measure of toxicological response precluding cell death (Lu, 2009), we performed GSH assay using the manufacturer's protocol for GHS-Glo^{TM} Glutathione Assay kit (Promega).

Measurement of extracellular DJ-1 concentration

Following reperfusion, the DJ-1 concentration of cell supernatant was measured by CircuLex DJ-1/PARK-7 ELISA Kit (MBL International Corporation, CY-9050) according to the manufacturer's instructions (Bande et al., 2012). Absorbance from each sample was measured using a Synergy HT plate reader (Bio-Tex) at dual wavelengths of 450/540 nm.

Immunocytochemical analysis

hNPCs (8×10^4 cell/well) in 400 µL neural medium in Poly-L-Lysine 8-chamber (BD) were fixed in 4% paraformaldehyde for 20 min at room temperature after OGD or non-OGD treatment. After blocking reaction with 5% normal goat serum (Vector), the cells were incubated overnight at 4 °C with rabbit monoclonal anti-DJ-1 (1:100; Abcam, ab76008) and mouse monoclonal anti-ATP synthase (mitochondrial) β-chain (1:200; Cell Signaling Technologies, 05-709) with 5% normal goat serum. The sections were incubated in secondary antibody of goat anti-rabbit IgG-Alexa 488 (green) and Alexa 405 (blue)/594 (red) anti-mouse IgG (1:1000; Molecular Probes). For visualization of mitochondrial membrane potential, hNPCs were incubated with 100 nM MitoTracker Red (Invitrogen, M7512) for 30 min before cell fixation, washed with PBS (Narendra et al., 2008; Tanaka et al., 2010), and performed immunocytochemical analysis. Cells were processed for Hoechst 33258 (bisBenzimideH 33258 trihydrochloride, Sigma) immunostaining, and subsequently embedded with mounting medium. Immunofluorescent images were visualized using Zeiss Axio Imager Z1. Control experiments were performed with the omission of the primary antibodies yielding negative results.

Measurement of intracellular DJ-1 translocated into the mitochondria

Briefly, we digitally captured 20 pictures of immunocytochemically stained cells under the microscope ($\times 400$) (approximately 100 cells/picture) of each treatment condition (normoxic and hypoxic–ischemic), randomly selected ten pictures, counted the number of DJ-1 co-localized with mitochondria, then the value of DJ-1 that translocated into the mitochondria was calculated as follows: $\text{translocation (\%)} = [1.00 - (\text{Number of DJ-1 co-localized with mitochondria} / \text{Number of total mitochondria})] \times 100$.

Statistical analysis

The data were evaluated using One-way analysis of variance (ANOVA) followed by post hoc compromised t-tests. Statistical significance was preset at $p < 0.05$. Data are represented as means $\pm$ SD from quintuplicates of each treatment condition.

Results

OGD-reperfusion causes massive brain damage

To confirm the effect of hypoxic–ischemic condition on DJ-1, hNPCs were subjected to OGD. OGD for 90 min alone did not compromise cell survival as analyzed by trypan blue dye exclusion method and MTT assay, but the OGD-reperfusion insult (OGD then medium replenishment with 5 mM glucose and re-introduction to the normoxic condition for 2 h) significantly decreased cell viability ($F_{2, 18} = 138.289$, $p < 0.0001$) (Fig. 1A) and mitochondrial activity ($F_{2, 8} = 42.016$, $p < 0.0001$) (Fig. B). These observations of consistent cell death produced by our OGD-reperfusion model parallel recent *in vivo* evidence demonstrating that reperfusion causes massive brain damage and severely impairs neurological functions when blood supply returns to the lesion after a period of ischemia (Hayashi et al., 2009; Kahle et al., 2009; Waak et al., 2009; Yanagisawa et al., 2008).

hNPCs secrete DJ-1 after an experimental stroke insult

Next, we focused on characterizing oxidative damage, a major secondary cell death pathway subsequent to OGD-reperfusion phase (Lu, 2009; Pompella et al., 2003). Here, we measured the production of GSH that might render neuroprotective effects against ROS and also could modulate cell proliferation. The increase in GSH production in hNPCs after 2 h reperfusion following OGD was approximately twofold compared to normoxic conditions ($F_{2, 15} = 165.448$, $p < 0.0001$) (Fig. 1C), indicating that an endogenous repair mechanism, involving the elevation of intracellular GSH levels in response to cell death injury, was activated to protect hNPCs from oxidative damage. These results demonstrated that our *in vitro* OGD-reperfusion paradigm recapitulated a typical ischemic stroke seen in the *in vivo* model, with the latter similarly exhibiting a compensatory neuroprotection during the early phase of disease progression. Next, to reveal whether the therapeutic role of DJ-1 was accompanied by its extracellular secretion by hNPCs, we showed that treating the cell culture system with DJ-1 antibody exacerbated the OGD-induced reduction in cell viability and mitochondrial activity, despite further increasing GSH levels (Figs. 1A–C), implicating that the extracellular DJ-1, which was sequestered by the DJ-1 antibody ($F_{2, 6} = 423.034$, $p < 0.0001$) (Fig. 1D), was required for the observed neuroprotection.

DJ-1 translocates into the mitochondria under hypoxic–ischemic condition

An equally important finding here is the localization of DJ-1 in hNPCs as determined by immunofluorescent microscopy. First, the neuron cells were stained both with anti-DJ-1 antibody and anti-ATP synthase β -chain antibody under a normoxic condition (Figs. 2A–C). Employing the anti-ATP synthase β -chain antibody, of which antigen is localized in the mitochondrial inner membrane and linkages with mitochondrial complex I, the results (Figs. 2D–F) revealed that DJ-1 translocated into the mitochondrial inner membrane following the hypoxic–ischemic insult (Fig. 2F, represented by arrow heads). Higher magnification analyses further confirmed DJ-1 translocation into the mitochondria in hypoxic–ischemic (Figs. 2J–L), but not in normoxic condition (Figs. 2G–I).

Next, following confirmation that DJ-1 translocated into the mitochondria of hNPCs after hypoxic–ischemic insult, we examined whether DJ-1 selectively translocated into the healthy mitochondria, damaged mitochondria, or both. Employing a chemical reagent MitoTracker, which accumulates on healthy mitochondria, DJ-1 was shown to translocate to the polarized mitochondria more than the depolarized mitochondria (Fig. 3), indicating that DJ-1 translocation is closely associated with preservation of functional mitochondria.

Finally, quantitative analyses showed that DJ-1 translocated into the mitochondria after the hypoxic–ischemic insult ($p < 0.01$) (Fig. 4), illustrating that DJ-1 may serve as a sensitive biomarker of early neuroprotection in response to acute hypoxic–ischemic injury, that can be detected extracellularly (Fig. 1D) and intracellularly (Fig. 3).

Discussion

The present study examined a molecular mechanism of DJ-1 in exerting neuroprotection against hypoxic–ischemic injury in a cell culture paradigm. We demonstrated a novel observation of DJ-1 translocation into the mitochondria after OGD in hNPCs, which opens new avenues of research and therapeutic development targeting DJ-1 for rescuing stroke and other neurological disorders characterized by rampant mitochondrial deficits. This study also provides evidence that DJ-1 was secreted extracellularly and that when the DJ-1 antibody is applied, DJ-1 secretion was sequestered as evidenced by ELISA. This blockade of DJ-1 resulted in reduction in mitochondrial activity and, eventually, cell viability under the OGD condition, altogether implicating the key role of mitochondrial translocation and extracellular secretion of DJ-1 in neuroprotection against stroke.

Translocation of DJ-1 into the mitochondria as a primary neuroprotective response

DJ-1 has been previously reported to be highly expressed in the nucleus and cytoplasm, and partially co-localized with mitochondria (Aleyasin et al., 2007; Kahle et al., 2009). Furthermore, DJ-1 has been implicated to play a pivotal role in the maintenance of mitochondrial complex I activity under oxidative stress without affecting the integrity of other mitochondrial electron transport complexes (Hayashi et al., 2009). A cascade of hypoxic–ischemic cell death events may consist of mitochondrial complex I spontaneously releasing ROS, a hallmark biochemical feature of oxidative stress. This initial stroke-induced ROS acts upon neighboring mitochondria precipitating mitochondrial permeability

transition pore opening, and generation of additional ROS. The wave of depolarization therefore represents successive permeability transition pore induction and ROS-induced ROS aberrant accumulation leading to apoptosis (Lu, 2009). The translocation of DJ-1 into the mitochondria may correspond to a primary neuroprotective response against stroke-associated oxidative stress in an effort to arrest the secondary cell death progression. Because mitochondrial complex I critically regulates oxidative stress and controls the ATP production in the eukaryotic cells, its dysfunction induces cell death (Foti et al., 2010; Taira et al., 2004). DJ-1 by translocating into the mitochondria achieves an efficient endogenous neuroprotection in mitigating mitochondrial defects.

Extracellular DJ-1 is a vital protein for hNPC survival against ischemic insults

That nuclear translocation of proteins may confer neuroprotection has been recently shown with other candidate therapeutic molecules in stroke (Gan et al., 2010; Pallast et al., 2010; Yang et al., 2008, 2009). The present data, therefore, strongly support the hypothesis that DJ-1 exerts significant control on both mitochondrial function and dynamic cell structure requiring a balance between fission and fusion. Our results also complement recent reports of DJ-1 targeting oxidative stress and inflammatory pathways (Aleyasin et al., 2007; Mullett et al., 2009; Yanagisawa et al., 2008). However, although we found increased levels of the antioxidant GSH at acute hypoxic-ischemic period, corresponding to DJ-1 mitochondrial translocation, the sequestration of extracellular DJ-1 with the antibody further led to GSH upregulation, but worsened cell viability and mitochondrial activity. These observations highlight the importance of DJ-1, in that while increased GSH levels have been widely shown as therapeutic against stroke, such neuroprotection is not recognized under conditions of DJ-1 depletion.

Clinical implications of DJ-1 mitochondrial translocation and extracellular secretion

Finding strategies to facilitate DJ-1 mitochondrial translocation and to increase its extracellular secretion will have direct impact on treatment of stroke patients. Interestingly, altered expression of plasminogen activator inhibitor-1, which is the primary inhibitor of tissue-type plasminogen activators, has been shown to promote the apoptotic sequence involving cytochrome c release from mitochondria (Soeda et al., 2008). In addition, N-acetylaspartate is a small amino acid synthesized by neuronal mitochondria, which can be released in the extracellular space after reperfusion in animal models of brain ischemia and in serum of patients with acute ischemic stroke (Elting et al., 2004). Both these studies offer evidence on the critical role of mitochondria as a therapeutic target and biomarker for stroke. To this end, the present detection of DJ-1 translocation to the mitochondria and its secretion extracellularly may serve as a robust stroke therapeutic and biomarker.

In summary, whereas the present DJ-1 mitochondrial translocation and extracellular secretion were observed in cultured hNPCs, it is tempting to speculate that *in vivo* DJ-1 may similarly modulate the hNPC-rich neurogenic sites of lateral ventricle and hippocampal dentate gyrus. Along this line, we posit in agreement with recent reports (Aron et al., 2010; Gorner et al., 2007; Vasseur et al., 2009; Yan and Pu, 2010) that DJ-1 is a key protein for maintenance of hNPC survival in response to stroke and relevant brain insults.

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Fig. 1. OGD altered hNPC proliferation and DJ-1 secretion. Cultured hNPCs were subjected to OGD for 90 min, followed by a 2-hour reperfusion period under normoxic condition. Under hypoxic-ischemic condition, cell viability tests using trypan blue exclusion method (A) and MTT assay (B) revealed that the number of viable hNPCs significantly decreased, concomitant with increased GSH production (C) and detection of secreted DJ-1 (D). For panels C and D, both assays were normalized with cell number in each condition. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$. ND: non-detectable by the CircuLex DJ-1/PARK ELISA kit.

Fig. 2.

DJ-1 translocates into the mitochondrial inner membrane following hypoxic–ischemic insult. DJ-1 positive cells (A and D), mitochondria localization in hNPCs (B and E), and double-labeled DJ-1 and mitochondria (C and F). Arrow heads represent DJ-1 that translocated into the mitochondria following hypoxic–ischemic insult. Scale bars in A–F = 50 μ m. High magnification analysis of DJ-1 translocation into the mitochondria following hypoxic–ischemic insults (J–L). G–I were normoxic condition. DJ-1 positive cells (G and J), mitochondria localization in hNPCs (H and K), and double-labeled DJ-1 and mitochondria (I and L). Scale bars in G–L = 5 μ m.

Fig. 3.

DJ-1 selectively translocates to the electrochemically active mitochondria. For visualization of the mitochondrial membrane potential, cells were incubated with 100 nM MitoTracker (red) for 30 min before cell fixation (as described in the Materials and methods), and then immunostained for anti-DJ-1- (green) and anti-ATP synthase (mitochondrial) β -chain (blue). Depolarized (yellow arrows) and electrochemically active (white arrows) mitochondria are shown to indicate mitochondria that are impermeable and permeable to the dye MitoTracker, respectively. DJ-1 co-localized with electrochemically active mitochondria, but not with depolarized mitochondria. Scale bars = 5 μ m.

Fig. 4. Quantification of DJ-1 co-localization with mitochondria in hNPCs. Data were obtained from five independent experiments ($n > 750$ cells per condition). $**p < 0.01$.