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Oxidative stress induction of DJ-1 protein in reactive astrocytes scavenges free radicals and reduces cell injury

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Abbreviations: ANOVA, analysis of variance; ASK1, apoptosis signal regulating kinase 1; C46, C53 and C106, cysteine residues at amino acid numbers 46, 53 and 106, respectively; DAB, 3,3′-diaminobenzidine; DMEM, Dulbecco’s modified Eagle’s medium; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; Daxx, death-domain-associated protein; ESR, electron spin resonance; FCS, fetal calf serum; GFAP, glial fibrillary acidic protein; GST, glutathione S-transferase; GST-DJ-1, glutathione S-transferase-tagged recombinant human DJ-1 protein; H2O2, hydrogen peroxide; HIPK1, homeodomain-interacting protein kinase 1; ICA, internal carotid artery; MAP2, microtubule-associated protein-2; MCAO, middle cerebral artery occlusion; MR, magnetic resonance; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide; Nrf2, nuclear factor erythroid 2-related factor; *OH, hydroxyl radical; PB, phosphate buffer; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline containing 0.3% Triton X-100; PSF, pyrimidine tract-binding protein-associated splicing factor; ROS, reactive oxygen species; SEM, standard error of the mean; TH, tyrosine hydroxylase; TTC, 2,3,5-triphenyltetrazolium chloride; pl, isoelectric point

Key words: DJ-1, release, astrocytes, focal ischemia, oxidative stress sensor, neuroprotection

Astrocytes, one of the predominant types of glial cells, function as both supportive and metabolic cells for the brain. Under cerebral ischemia/reperfusion-induced oxidative conditions, astrocytes accumulate and activate in the ischemic region. DJ-1 has recently been shown to be a sensor of oxidative stress in living cells. However, the function of astrocytic DJ-1 is still unknown. In the present study, to clarify the effect of astrocytic DJ-1 protein under oxidative insult, we used a focal ischemic rat model that had been subjected to middle cerebral artery occlusion (MCAO) and reperfusion. We then investigated changes in the distribution of DJ-1 in astrocytes, DJ-1 release from cultured astrocytes, and the effects of recombinant DJ-1 protein on hydrogen peroxide (H2O2)-induced death in normal and DJ-1-knockdown SH-SY5Y cells and on in vitro scavenging of hydroxyl radicals (*OH) by electron spin resonance spectrometry. At 24 h after 2-h MCAO and reperfusion, an infarct lesion was markedly observed using magnetic resonance imaging and 2,3,5-triphenyltetrazolium staining. In addition, reactive astrocytes enhanced DJ-1 expression in the penumbral zone of the ischemic core and that DJ-1 protein was extracellularly released from astrocytes by H2O2 in vitro primary cultures. Although DJ-1-knockdown SH-SY5Y cells were markedly vulnerable to oxidative stress, treatment with glutathione S-transferase-tagged recombinant human DJ-1 protein (GST-DJ-1) significantly inhibited H2O2-induced cell death. In addition, GST-DJ-1 protein directly scavenged *OH. These results suggest that oxidative stress induces the release of astrocytic DJ-1 protein, which may contribute to astrocyte-mediated neuroprotection.

Introduction

DJ-1, which was discovered as a novel oncogene product in collaboration with activated small GTP-binding protein ras,1 has recently been identified as the causative gene of familial Parkinson’s disease PARK7.2 DJ-1 has many functions, such as transcriptional factor, molecular chaperone, etc. 3-6 X-ray crystallographic and biological analyses have shown that wild-type DJ-1 protein forms a homodimer. In addition, these analyses have also shown that L166P-mutation in DJ-1 protein, which was first found in a PARK7 patient, disrupts dimer formation, resulting in a loss of function.7-11 Furthermore, the cysteine residues in DJ-1 protein are oxidized, and the isoelectric point (pl) of DJ-1 is shifted to be more acidic.12 Thus, DJ-1 protein may play an important role in oxidative stress-mediated disorders.

Cerebral ischemia, one of the leading causes of death and long-lasting disability, results from a transient or permanent reduction in...
cerebral blood flow in a major brain artery.\textsuperscript{13,14} The production of reactive oxygen species (ROS) has been implicated in reperfusion injury after cerebral ischemia, and antioxidant enzymes are considered to be among the major mechanisms by which cells counteract the deleterious effect of ROS after cerebral ischemia.\textsuperscript{15,16} Previous studies have reported that activated astrocytes were accumulated in the boundary zone (penumbra) of the ischemic core in the striatum.\textsuperscript{17,18} However, the function of DJ-1 protein in astrocytes remains unclear.

In the present study, to clarify the effect of astrocytic DJ-1 protein under massive oxidative insult, we examined changes in the distribution of DJ-1 in astrocytes in the in vivo ischemic brain, DJ-1 release from cultured astrocytes, and the effects of recombinant DJ-1 protein on hydrogen peroxide (H\textsubscript{2}O\textsubscript{2})-induced death in normal and DJ-1-knockdown SH-SY5Y cells and on in vitro scavenging of hydroxyl radicals (\textsuperscript{•}OH).

**Results**

**Alteration of DJ-1 distribution in ischemic rat brains.** To analyze changes in DJ-1 in the brain under oxidative stress, we used an ischemic rat model that had been subjected to middle cerebral artery occlusion (MCAO) and reperfusion. First, we investigated MCAO-induced injury using magnetic resonance (MR) imaging. In sham-operated rats, an infarct lesion was not detected as MR signal on T2-weighted MR images. At 24 h after 2-h MCAO and reperfusion, an infarct lesion, as represented by an area of hyperintensity (white), was markedly observed in the ipsilateral cortex and striatum (Fig. 1A). Similar neurodegeneration was observed on staining with 2,3,5-triphenyltetrazolium chloride (TTC) (Fig. 1B). Also, microtubule-associated protein-2 (MAP2)- and tyrosine hydroxylase (TH)-immunoreactivities of the ipsilateral striatum were markedly decreased in ischemic rats (Fig. 1C and D).

![Image of MRI and TTC staining](image)

**Intensive DJ-1 immunoreactive astrocytes after MCAO and reperfusion.** To examine the change in DJ-1 in astrocytes in ischemic rats, we performed double-staining using antibodies against
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GFAP and DJ-1. At 24 h after reperfusion, GFAP-immunopositive astrocytes were activated and DJ-1-immunoreactivity was markedly increased in the boundary zone of the ischemic core in the striatum, compared to the contralateral side (Fig. 2).

**Evaluation of H$_2$O$_2$-induced cell death.** To analyze the effects of DJ-1, we used in vitro primary cultured astrocytes and neuroblastoma SH-SYSY cells. In the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay, although massive cell death of SH-SYSY cells was caused by H$_2$O$_2$ in a concentration-dependent manner, astrocytes were relatively resistant to H$_2$O$_2$-induced cell death (Fig. 3A). To determine the endogenous DJ-1 protein level in astrocytes and SH-SYSY cells, we performed western blotting using anti-DJ-1 antibody. The amount of DJ-1 protein in astrocytes was about six-fold greater than that in SH-SYSY cells (Fig. 3B).

In addition, we measured the amount of DJ-1 protein, which was secreted under oxidative conditions from astrocytes into cultured media. In normal cultured media, released DJ-1 protein was undetectable. In contrast, DJ-1 protein was gradually and significantly released by exposure to H$_2$O$_2$ in a concentration-dependent manner (Fig. 4).

**Protective effects of recombinant DJ-1 protein in SH-SYSY cells.** To clarify the function of released DJ-1 protein, we further examined the effects of recombinant human DJ-1 protein on H$_2$O$_2$-treated SH-SYSY cells (Fig. 5). In the present study, we generated DJ-1-knockdown SH-SYSY cells, in which the endogenous expression of human DJ-1 was suppressed by approximately 74% (Fig. 5B). In normal SH-SYSY cells, cell death slightly occurred with 25 μM H$_2$O$_2$ (Figure 5A and C). In contrast, the same concentration (25 μM) of H$_2$O$_2$ caused marked and significant cell death in DJ-1-knockdown cells. Simultaneous treatment with 5 μM glutathione S-transferase-tagged recombinant human DJ-1 protein (GST-DJ-1), but not glutathione S-transferase (GST), rescued DJ-1-knockdown cells to the level in normal cells (Fig. 5A and D). In addition, a concentration-dependent curve of H$_2$O$_2$-induced cell death was further shifted to the right by treatment with GST-DJ-1, but not GST (Fig. 5A).

**Effects of recombinant DJ-1 protein on 'OH trapping in vitro.** To clarify the direct scavenging effect of recombinant DJ-1 protein against ROS, such as 'OH, we further performed electron spin resonance (ESR) analysis using the spin-trapping agent 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) (Fig. 6). As an internal reference, Mn$^{2+}$ signal was detected as two peaks at both edges (Fig. 6A–D). Although no marked signal was detected in the absence of Fe$^{2+}$, major four peaks with an intensity ratio of 1:2:2:1 appeared at the mid-section between the Mn$^{2+}$ signal, in the presence of H$_2$O$_2$ and Fe$^{2+}$ (Fig. 6B). This characteristic quartet signal was almost completely suppressed by thiourea (data not shown), a specific 'OH scavenger, suggesting that the quartet signal indicates a DMPO-OH spin adduct. On the other hand, H$_2$O$_2$-induced DMPO-OH signal was slightly reduced by GST, and markedly reduced by GST-DJ-1 (Fig. 6).

**Discussion**

Oxidative stress is defined as an imbalance between ROS generation and the anti-oxidant capacity of a cell. The production of ROS, which occurs immediately and massively after focal ischemia and reperfusion, is followed by delayed post-ischemic inflammation.
and apoptosis, and these events are involved in the progression and expansion of neurodegeneration.\(^{21,22}\) We observed severe neuronal damage in the striatum and cerebral cortex at 24 h after 2-h MCAO and reperfusion (Fig. 1). We then performed a noninvasive evaluation of the infarct area, using a 7T Unity Inova MR scanner, and detected the infarct lesion, as represented by an area of hyperintensity (white), in the ipsilateral cortex and striatum. Subsequently, we examined MAP2- and TH-immunoreactivities and confirmed that these immunoreactivities corresponded to the results of MR imaging. It has been shown that severe neuronal damage leads to a strong and delayed activation of astrocytes and produces neurotrophic factors.\(^{17}\) In this study, we also observed the accumulation of reactive astrocytes in the penumbral region of the ischemic core in the ipsilateral striatum and cerebral cortex (Figs. 1 and 2). Recently, it has been reported that DJ-1 protein was increased in the serum or plasma of patients with breast cancer, melanoma, familial amyloidotic polyneuropathy, stroke and Parkinson’s disease. Therefore, oxidative stress may be related to the onset of these disorders.\(^{23-27}\)

In the present study, we found that DJ-1 protein was actually released from astrocytes into the culture medium without cell death (Fig. 4). In addition, the amount of secreted DJ-1 protein was increased by exposure to H\(_2\)O\(_2\) in a concentration-dependent manner. Thus, oxidative stress activates astrocytes and induces DJ-1 secretion.

In addition, astrocytes were more resistant to H\(_2\)O\(_2\)-induced cell death than SH-SYSY cells, due to the higher expression of DJ-1 protein (Fig. 3), while DJ-1-knockdown SH-SYSY cells were more susceptible to exposure to H\(_2\)O\(_2\). H\(_2\)O\(_2\)-induced cell death was significantly inhibited by simultaneous treatment with recombinant GST-DJ-1 protein, but not by GST-tag protein alone (Fig. 5). From these observations, we consider that astrocytes, which are rich in DJ-1 protein, are resistant to oxidative stress; and that DJ-1 protein...
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have suggested that H₂O₂ is produced by mitochondrial dysfunction, and •OH is easily generated in the presence of Fe²⁺ (ref. 28). •OH is one of most potent neurotoxic factors under ischemic insult and also causes protein oxidation. In the present study, •OH, which was generated in vitro by H₂O₂ and Fe²⁺, was directly scavenged partially by GST alone and markedly by GST-DJ-1 protein. A cysteine residue is oxidized from a reduced form (-SH) to sulphenation (-SOH), sulphination (-SO₂H) and sulphonation (-SO₃H), in order of oxidative development. Thus, the oxidation of cysteine residues contributes to the direct scavenging of •OH. However, since GST alone did not inhibit the H₂O₂-induced cell death of SH-SY5Y cells, the scavenging of •OH by cysteine residues (cysteine oxidation) by itself may be insufficient to give a neuroprotective effect.

We previously found that recombinant GST-DJ-1 protein was taken up into dopaminergic neurons after intranigral microinjection and inhibited neural death in the in vivo brain of Parkinson's disease-model rats. Recent studies have reported that DJ-1 inhibits various apoptotic pathways, such as pyrimidine tract-binding protein-associated splicing factor (PSF), apoptosis signal regulating kinase 1 (ASK1), death-domain-associated protein (Daxx), and/or the reduction of homeodomain-interacting protein kinase 1 (HIPK1). In addition, DJ-1 induces anti-oxidative episodes mediated by the stabilizing nuclear factor erythroid 2-related factor (Nrf2), which is a master regulator of antioxidant gene responses. Interestingly, DJ-1 oxidation at C106 is necessary for DJ-1 to exert anti-apoptotic and molecular chaperoning responses, and massive oxidation may cause its inactivation. In brief, SOH- and SO₂H-oxidation at C106 in DJ-1 protein may be active forms, while SO₃H-peroxidation may cause loss-of-function and an inactive form. Based on these observations, we consider that oxidized DJ-1 protein after MCAO and reperfusion may induce anti-apoptotic responses and result in compensatory neuroprotection.

In conclusion, since astrocytes are endogenously rich in DJ-1 protein, these glial cells may be relatively resistant to oxidative stress compared to neurons. In addition, astrocytic DJ-1 protein was secreted by exposure to H₂O₂. Furthermore, recombinant GST-DJ-1 protein directly trapped •OH and inhibited H₂O₂-induced cell death. These results suggest that astrocytic DJ-1 protein may be useful for the development of novel therapeutic targets in various oxidative stress-mediated disorders, including cerebral ischemia.

Materials and Methods

Preparation of recombinant human DJ-1 protein and anti-DJ-1 antibody. Recombinant proteins, such as GST and GST-DJ-1, were expressed in and purified from Escherichia coli as described previously. The preparation and specificity of a rabbit polyclonal DJ-1 antibody have been described previously. Rat model of focal cerebral ischemia. Wistar rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The animals were acclimated to and maintained at 23°C under a 12-h light/dark cycle (lights on 08:00-20:00 hours). Rats were housed in standard laboratory cages and had free access to food and water throughout the study period. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocols were approved by the Committee for Animal Research at Kyoto Pharmaceutical University.

Figure 6. Direct •OH scavenging of recombinant GST-DJ-1 protein. Traces show typical spectra of DMPO-OH spin adducts generated from the vehicle control (Control, A) or H₂O₂/Fe²⁺, in the absence (H₂O₂, B) or presence of GST (GST, C) and GST-DJ-1 (DJ-1, D). (E) The amount of •OH was semiquantitatively measured as the formation of DMPO-OH spin adducts by ESR spectrometry, after the reaction for exactly 1 min. Each value is the mean±SEM of three determinations, based on H₂O₂/Fe²⁺ as 100% (B). Significance (Bonferroni/Dunn post hoc comparisons after ANOVA): **p < 0.01, ***p < 0.001 vs. signal intensity of H₂O₂/Fe²⁺, and †p < 0.05 vs. signal intensity of H2O2/Fe2+ with GST.

released from astrocytes may contribute to this neuroprotection. However, since cerebral ischemia/reperfusion causes neuronal death, astrocytic release of DJ-1 may be a compensatory neuroprotective response.

Human and rat DJ-1 protein have three cysteine residues at amino acid numbers 46, 53 and 106 (C46, C53 and C106, respectively), and C106 is the most sensitive to oxidative stress. On the other hand, GST-tag protein involves four cysteine residues. Recent studies
Male Wistar rats weighing 260–300 g were used in the present study. Focal cerebral ischemia was induced by the intraluminal introduction of a nylon thread as described previously. Briefly, animals were anesthetized with 4% halothane (Takeda Pharmaceutical, Osaka, Japan) and maintained on 1.5% halothane using a facemask. After a midline neck incision was made, 20 mm of a 4-0 nylon thread with its tip rounded by heating and coated with silicon (Xantopren M; Heraeus Kulzer, Hanau, Germany) was inserted into the left internal carotid artery (ICA) as far as the proximal end using a globular stopper. The origin of MCA was then occluded by a silicon-coated embolus. Anesthesia was discontinued, and the development of right hemiparesis with upper limb dominance was used as the criterion for ischemic insult. After 120 min of MCAO, the embolus was withdrawn to allow reperfusion of the ischemic region via the anterior and posterior communicating arteries. Body temperature was maintained at 37–37.5°C with a heating pad and lamp during surgery. In the sham operation, a midline neck incision was made to expose the arteries, but the nylon thread was not inserted into the carotid artery.

MR imaging. Rats were subjected to a sham-operation or an operation on the MCAO. After 24 h, MR images of the rat heads were acquired with a 7 T Unity Inova MR scanner (Varian, Palo Alto, CA). A T2-weighted MR sequence was used with an acquisition repetition time of 2,000 ms, echo time of 40 ms, 35 x 35-mm2 field of view, and slice thickness of 1 mm. During the MR sessions, spontaneously breathing animals were anesthetized with 1.5% isoflurane in 50% O2 and 50% air.

TTC staining. At 24 h after MCAO, brains were removed and cut into 2-mm-thick coronal sections. These sections were immersed in a 2% solution of TTC (Wako Pure Chemical Industries, Osaka, Japan) in saline at 37°C for 20 min, and then fixed in 4% paraformaldehyde in 100 mM phosphate buffer (PB) at 4°C.

Immunohistochemistry. Rats were deeply anesthetized with pentobarbital (100 mg/kg, i.p.) and perfused through the aorta with 150 mL of 10 mM phosphate-buffered saline (PBS), followed by 300 mL of a cold fixative consisting of 4% paraformaldehyde in 100 mM PB. After perfusion, brains were quickly removed and post-fixed for two days with 4% paraformaldehyde in 100 mM PB and then transferred to 15% sucrose solution in 100 mM PB containing 0.1% sodium azide at 4°C. The brain samples were cut into 20-μm thick slices using a cryostat and collected in 100 mM PBS containing 0.3% Triton X-100 (PBS-T). The free-floating sections were treated with 0.3% H2O2 in PBS-T to eliminate endogenous peroxidase activity. After several washes, sections were incubated for three days at 4°C with the following primary antibodies: MAP2 (dilution, 1:3,000; Sigma, St. Louis, MO) and TH (1:3,000; Sigma); mouse monoclonal antibodies against GFAP (1:3,000; Chemicon International, Temecula, CA); rabbit polyclonal antibodies against DJ-1 (1:10,000). After several washes, sections were incubated with biotinylated antibodies against mouse or rabbit IgG (1:2,000; Vector Laboratories, Burlingame, CA) for 2 h at room temperature. The sections were then incubated with 1:4,000 avidin peroxidase (ABC Elite kit, Vector Laboratories) for 1 h at room temperature. All of the sections were washed several times with PBS-T after each incubation, and labeling was then revealed by 3,3’-diaminobenzidine (DAB; Dojindo Laboratories, Kumamoto, Japan), with nickel ammonium, which yielded a dark blue color.

Double-immunofluorescence staining. For double-immunofluorescence staining, sections were simultaneously incubated for three days at 4°C with the following primary antibodies: mouse monoclonal antibody against GFAP (1:1,000; Chemicon Int.) and rabbit polyclonal anti-DJ-1 antibody (1:5,000). Reactivity with primary antibody was detected with anti-mouse IgG antibody conjugated with Alexa Fluor 488 or anti-rabbit IgG antibody conjugated with Alexa Fluor 546 (each diluted 1:500; Molecular Probes, Eugene, OR) for 2 h at room temperature. Fluorescence was then detected using a laser scanning confocal microscope (LSM410, Carl Zeiss, Jena, Germany).

Cell culture and detection of extracellular DJ-1 protein. The human neuroblastoma cell line SH-SY5Y and rat primary astrocytes were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 50 μg/mL penicillin and 100 μg/mL streptomycin and kept at 37°C in humidified 5% CO2/95% air. Astrocytes were incubated in DMEM without FCS for 12 h and then in the presence or absence of H2O2 (at 0–100 μM) for an additional 24 h. After incubation, the media were centrifuged at 15,000 rpm at 4°C for 15 min to remove cells and debris, and transferred to a new tube. For the detection of extracellular DJ-1 protein, the collected media were subjected to western blotting with anti-DJ-1 antibody.

Establishment of DJ-1-knockdown cells. We established a DJ-1-knockdown SH-SY5Y cell line by a method that was essentially similar to that in mouse Flp-InT3 cells. In brief, the nucleotide sequence of the upper strand of the oligonucleotide used for construction of an siRNA vector targeting the human DJ-1 gene is 5’-GGATCCCGTCAAGGCCTGAGCCACATTGATAATCCGTTGCTGATGGCCAGGCTTTTTCCTTTTCCCCAAAAGCCTT-3’. After oligonucleotides corresponding to the upper and lower strands of DNA were annealed, they were inserted into BamHI-HindIII sites of pRNA-U6.1/Neo. These plasmids were transfected into human SH-SY5Y cells by the calcium phosphate precipitation method, and the cells were cultured in RPMI-1640 medium in the presence of 400 μg/mL G418 for 14 days. Cells that were resistant to the drug were then selected, and the intrinsic expression of DJ-1 was checked by western blotting with an anti-human DJ-1 antibody.

Measurement of cell viability. To evaluate cell viability, we performed MTT (Dojindo Laboratories) assay as an index of surviving cells. In living cells, MTT is converted to formazan, which has a specific absorption maximum. SH-SY5Y cells, in which endogenous DJ-1 expression was normal or knocked-down by siRNA vector, were seeded at 3 x 104 cells/well in a 96-microwell plate, and 12,500 μg/mL MTT was added to the drug were then selected, and the intrinsic expression of DJ-1 was checked by western blotting with an anti-human DJ-1 antibody.

Analysis of ESR. •OH formation was monitored by ESR spectrometry with DMPO (Labotec Ltd., Tokyo, Japan), a spin-trapping agent. In a final volume of 200 μL of 100 mM PB, recombinant protein such as GST-DJ-1 (5 μM) or GST (5 μM) was added to the reaction mixture containing diethylenetriamine pentaacetic acid (25 μM), FeSO4 (12.5 μM), H2O2 (1 μM) and DMPO.
(22.5 mM). These recombinant proteins and reagents were solubilized in Milli Q water. The reaction mixture was transferred to a flat quartz cuvette and placed in the cavity of an X-band JEOL RFR-30 Radical Analyzer system (JEOL Ltd., Tokyo, Japan). 

- OH, which was generated by Fenton's reaction between Fe²⁺ and H₂O₂, was trapped by DMPO, and the stable adduct DMPO-OH was measured exactly 1 min after the addition of DMPO. The Mn²⁺-derived split signal was used as the internal standard. Typical instrument settings were as follows: incident-microwave 4 mW, modulation-amplitude 0.03 mT, time-constant 0.10 s, and sweep rate 5 mT/min.

**Statistical analysis.** Results are presented as the mean ± standard error of the mean (SEM). The significance of differences was determined by an analysis of variance (ANOVA). Further post hoc comparisons were performed using Bonferroni/Dunn tests (Stat View; Abacus Concepts, Berkeley, CA).

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**References**


