DJ-1 UPREGULATES GLUTATHIONE SYNTHESIS DURING OXIDATIVE STRESS AND INHIBITS A53T α-SYNUCLEIN TOXICITY*

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Running title: DJ-1 regulates glutathione synthesis and Hsp70

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DJ-1 is the third gene which has been linked to Parkinson’s disease. Mutations in the DJ-1 gene cause early-onset PD with autosomal recessive inheritance. To clarify the mechanism of DJ-1 protection, we have overexpressed the gene in cultured dopaminergic cells which were then subjected to chemical stress. In the rat dopaminergic cell line, N27, and in primary dopamine neurons, overexpression of wild-type DJ-1 protected cells from death induced by hydrogen peroxide and 6-hydroxydopamine. Overexpressing the L166P mutant DJ-1 had no protective effect. By contrast, knocking down endogenous DJ-1 with antisense DJ-1 rendered cells more susceptible to oxidative damage. We have found that DJ-1 improves survival by increasing cellular glutathione levels through an increase in the rate-limiting enzyme, glutamate cysteine ligase. Blocking glutathione synthesis eliminated the beneficial effect of DJ-1. Protection could be restored by adding exogenous glutathione. Wild-type DJ-1 reduced cellular reactive oxygen species and reduced the levels of protein oxidation caused by oxidative stress. By a separate mechanism, overexpressing wild-type DJ-1 inhibited the protein aggregation and cytotoxicity usually caused by A53T human α-synuclein. Under these circumstances, DJ-1 increased the level of heat shock protein 70 but did not change the glutathione level. Our data indicate that DJ-1 protects dopaminergic neurons from oxidative stress through upregulation of glutathione synthesis and from the toxic consequences of mutant human α-synuclein through increased expression of heat shock protein 70. We conclude that DJ-1 has multiple specific mechanisms for protecting dopamine neurons from cell death.

Parkinson’s disease (PD)1 is a common neurodegenerative disease characterized by the loss of dopaminergic neurons in the substantia nigra and by the presence of intracellular inclusions called Lewy bodies (LBs) (1, 2). The etiology of PD may involve both genetic and environmental factors (3). In recent years, several genes linked to PD have been discovered. α-Synuclein was the first gene identified whose mutations, A53T and A30P, cause autosomal dominant forms of PD (4, 5). Importantly, aggregated α-synuclein has been found to be a major component of LBs (6). Mutations in a second gene, Parkin, cause early-onset PD with autosomal recessive inheritance (7). Parkin is an E3 ubiquitin ligase controlling protein degradation through the ubiquitin-proteasome system (8).

DJ-1 is the third PD gene identified and is linked to early-onset disease with autosomal recessive inheritance (9). Eleven different mutations have been found in the DJ-1 gene including missense, truncation, and splice-site mutations as well as large deletions, suggesting that loss of DJ-1 function leads to neurodegeneration (9-11). DJ-1 is a 189 amino acid protein with multiple functions (12). DJ-1 interacts with H-ras to increase cell transformation (13). DJ-1 is a regulatory subunit of an RNA-binding protein complex (14). Through binding to PIASxα, the transcriptional inhibitor of androgen receptor, DJ-1 can positively regulate transcription of the androgen receptor (15). Of most interest, DJ-1 has been identified as a hydroperoxide-responsive protein that becomes more acidic following oxidative stress, suggesting that it may function as an antioxidant protein (16, 17).

To study the role of DJ-1 protein in dopaminergic cells, we have overexpressed wild-type (WT) DJ-1 and L166P mutant DJ-1 in a rat
dopaminergic cell line (N27 cells) and in rat dopamine neurons from embryonic mesencephalic cultures (18, 19). Our results show that overexpression of WT DJ-1 can protect dopamine neurons from oxidative stress, while knockdown of endogenous DJ-1 renders cells more susceptible to oxidative damage. We have found that DJ-1 protects cells from oxidative stress by upregulating cellular glutathione (GSH) levels. By a separate mechanism, overexpression of WT DJ-1 inhibits A53T human α-synuclein toxicity through upregulation of heat shock protein 70 (Hsp70).

Materials And Methods

cDNA cloning, mutagenesis, and adenovirus construction - The human wild-type DJ-1 cDNA was amplified by PCR from an EST clone. The point mutation to produce L166P was performed using a PCR based mutagenesis. To knock down rat endogenous DJ-1 protein, we designed and screened small interfering RNA (siRNA) to rat DJ-1 using a siRNA Target Finder program (genscript.com). A 75-bp synthesized DNA fragment encoding DJ-1 siRNA was cloned into the same vector as WT DJ-1 (termed as anti-sense DJ-1, “AS DJ-1”). All constructs were verified by DNA sequencing. We used the Ad-Easy system to create adenoviruses expressing human WT DJ-1, human L166P DJ-1, and rat AS DJ-1. We have previously described the creation of adenoviruses expressing WT and A53T human α-synuclein (18, 19).

N27 cell culture, adenovirus transduction, and drug treatments - Cells from the dopaminergic cell line N27 were cultured in 24-well plates in RPMI 1640 medium with 10% fetal bovine serum, 2 mM L-glutamate, and 100 U/ml penicillin and streptomycin (18, 19). To transduce cells, adenoviruses at a concentration of 200 plaque forming units (pfu)/cell were mixed with medium and added to cultures for 24 hr. Under these conditions, more than 80% of cells expressed transgene 24 hr later. For α-synuclein plus DJ-1 co-transduction, each adenovirus was used at 200 pfu/cell. Two days after adenovirus transduction, cells were incubated with drugs at various doses for 24 hr: H2O2 (25-100 μM, Sigma), 6-hydroxydopamine (6-OHDA, 10-80 μM, RBI), and proteasome inhibitor lactacystin (0.1-1.0 μM, Sigma).

Cell viability by MTT assay - Twenty-four hours after drug treatment, methylthiazol tetrazolium (MTT, Sigma) was added to culture medium (final concentration at 0.4 mg/mL) and incubated for two hours. Culture medium was removed and precipitates were dissolved in 0.04 M HCl in isopropanol. Cell viability was measured by spectrometry at OD590 as described (18, 19).

Primary rat mesencephalic culture, adenovirus transduction, and drug treatments - Rat ventral mesencephalon from embryonic day 15 was mechanically dissociated and cultured in 24-well plates in F12 medium with 10% fetal bovine serum, 2 mM L-glutamate, and 100 U/ml penicillin and streptomycin (18). Two days later, adenoviruses were used to transduce cells at a concentration of 400 pfu/cell. Because AS DJ-1 transduced cells could not be imaged with antibodies to DJ-1, the reporter construct GFP adenovirus at 200 pfu/cell was always co-transduced with AS DJ-1 adenovirus. Control cells were transduced with GFP adenovirus at 200 pfu/cell. Adenoviruses were incubated in culture medium for two days. Three days after adenovirus transduction, cells were exposed to drugs at three doses for 24 hr: H2O2 (0, 50, and 100 μM), 6-OHDA (0, 50 and 100 μM), dopamine (0, 50 and 100 μM), and lactacystin (0, 0.5 and 1.0 μM).

Immunocytochemistry - N27 cells and rat primary cultures were fixed in 4% paraformaldehyde for 30 min and processed for immunocytochemistry as described (18, 19). The antibodies included mouse anti-human DJ-1 (1:500, Stressgen); rabbit anti-DJ-1 (recognizes both human and rat DJ-1, 1:300, Chemicon); mouse anti-α-synuclein (1:500, Transduction laboratory); rabbit anti-tyrosine hydroxylase (TH, 1:150, Pel Freez). Green fluorescence (FITC) and Texas-Red conjugated secondary antibodies were used for double labeling of cells.

Apoptosis in primary dopamine neurons - Following DJ-1/TH or GFP/TH dual immunostaining, cells were stained with nuclear dye Hoechst 33258 (10 μg/mL, Molecular Probes). We examined transduced dopamine neurons (i.e. DJ-1/TH or GFP/TH dual positive cells) and identified apoptotic cells with nuclear
condensation or fragmentation. Approximately 150–200 transduced dopamine cells from three wells were assessed for each condition and the rates of apoptosis were determined. Experiments were repeated three times for each condition.

**Measurement of intracellular glutathione (GSH) levels** - N27 cells were scraped and collected by centrifugation. The cell pellet was resuspended in 1 ml of PBS containing 1 mM EDTA and homogenized. The supernatant was deproteinized by 10% metaphosphoric acid (Sigma) and collected for determining total GSH levels according to manufacturer’s protocol (Glutathione assay kit, Cayman Chemical, Ann Arbor, MI). A duplicate culture with the same treatments was used to determine total cell number by MTT assay. The total GSH levels were expressed as nmol/mg of protein and then as the percentage of control.

**Inhibition of GSH synthesis and treatment with exogenous GSH** - GSH is synthesized by glutamate cysteine ligase (GCL) and glutathione synthetase (GS). Buthionine sulfoximine (BSO) is an irreversible inhibitor of GCL. To deplete intracellular glutathione, N27 cells were pretreated with 200 μM DL-buthionine sulfoximine (BSO, Sigma) for 24 h before adenovirus transduction. BSO was maintained during and after adenovirus transduction until GSH assay. To test if exogenous GSH could reverse BSO inhibition of DJ-1 function, GSH (100 μM, Sigma) was added to culture medium during the adenoviral transduction.

**GSH synthesis enzyme activity assays** - N27 cells were collected, homogenized and centrifuged at 12,000 g for 30 min. The GCL and GS enzyme activities were determined by the formation of ADP using a coupled assay with pyruvate kinase and lactate dehydrogenase. The GCL reaction mixture (final volume, 200 μl) contained 100 mM Tris-HCl buffer (pH 8.2), 20 mM MgCl₂, 150 mM KCl, 10 mM L-glutamate, 10 mM L-cysteine, 5 mM ATP, 2 mM EDTA, 0.2 mM NADH, 2 mM phosphoenolpyruvate, 2 U pyruvate kinase, and 2 U lactate dehydrogenase. The GS reaction mixture contained 100 mM Tris-HCl buffer (pH 8.2), 20 mM MgCl₂, 50 mM KCl, 10 mM ATP, 2 mM EDTA, 5 mM glycine, 5 mM L-γ-glutamyl-L-α-amino butyrate, 0.2 mM NADH, 0.5 mM phosphoenolpyruvate, 2 U pyruvate kinase, and 2 U lactate dehydrogenase. The reaction was initiated by adding 100 μg protein extract, and the absorbance at 340 nm was monitored. Specific activity is expressed as units/mg of protein and then as the percentage of control.

**Semi Quantitative RT-PCR analysis** - We used semi quantitative RT-PCR to determine the mRNA levels of GCL, GS, and Hsp70. Total RNA was isolated from cultures using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. One μg of total RNA was used to synthesize first strand cDNA in 20 μl of reaction with a RT-PCR kit (Invitrogen). For each 50 μl PCR reaction, 2 μl of cDNA was used with gene specific primers (primer sequences available upon request). PCR conditions were optimized and linear amplification range was determined for each primer pair by varying annealing temperature and cycle numbers. We also amplified β-actin as internal control. Ten μl of PCR product was separated on 1.2 % agarose gel, and image density was analyzed using ImageJ software. Levels of mRNAs were expressed as the ratio to β-actin and then as the percentage to control.

**Measurement of intracellular ROS levels** - Intracellular ROS levels were measured by flow cytometry in cells loaded with the redox-sensitive dye DCFH-DA (Molecular Probes). Approximately 1 x 10⁶ N27 cells were incubated in the dark for 30 min at 37 °C with 10 μM DCFH-DA, harvested, and resuspended in the PBS. Fluorescence was recorded on FL-1 channel of FACScan® (Becton Dickinson).

**Western blotting** - Cells were incubated in a lysis buffer (0.5 mM EDTA, 150 mM NaCl, 50 mM Tris, 0.5% Nonidet P-40, pH 7.4) for 30 min at 4 °C and centrifuged for 10 min at 13,000 X g, and supernatants were collected. Protein concentration was determined using the BCA method. Fifty μg of total protein was separated on 12% SDS-PAGE gel and processed for Western blotting using the following antibodies: mouse anti human DJ-1 (1:4,000, Stressgen), rabbit anti-pan DJ-1 (1:5,000, Chemicon), mouse anti-α-synuclein (1:4,000, Transduction Laboratories), rabbit anti Hsp70 (1:3,000, Chemicon), and mouse anti β-actin (1:5000, Chemicon). The Western blotting was detected with a chemiluminescent detection kit (NEN Life Sciences).
Detection of protein carbonyls - Cells were collected by scraping, and proteins were lysed in lysis buffer. Soluble protein was used to determine protein carbonyls by the Oxyblot protein oxidation kit according to manufacturer’s protocol (Chemicon). Briefly, 10 μg (per 10 μl) of soluble protein was incubated for 15 min with an equal volume of 12% SDS and 2 volumes of 2,4-dinitrophenylhydrazine (DNPH), and then with 1.5 volumes of neutralization solution to stop the reaction. The samples were spotted on nitrocellulose membrane, dried and cross-linked with UV Cross-linker (Stratagene). Membranes were incubated with blocking buffer (PBS with 3% bovine serum albumin, 0.01% sodium azide, and 0.2% Tween-20) for 30 min at room temperature, exposed to rabbit anti-DNPH protein antibody (1:4000) for 1 hr, followed by goat anti-rabbit IgG coupled to horseradish peroxidase (1:10,000) for 1 hr at room temperature. Membranes were washed after each step in PBS with 0.2% Tween-20 and visualized with a chemiluminescent detection kit (NEN Life Sciences). Dot blots were scanned and intensities of the dots were quantified with NIH Image J by measuring density in a preset circle of the same size. To confirm equal protein loading for dot blots, same amount of protein (10 μg) was separated by 10% SDS-PAGE and detected by Western blotting using anti β-actin antibody (1:5000, Chemicon).

Statistics - All experiments were repeated at least three times. Data were analyzed using multivariate ANOVA and the Fisher LSD post hoc test. Significance was set at p<0.05. Values are shown as mean ± SEM.

RESULTS

Overexpression of human DJ-1 protects N27 cells from oxidative stress - We used adenoviral vectors to induce DJ-1 overexpression in N27 cells. Two days after adenovirus transduction, more than 80% of cells had strong expression of human DJ-1 in cytoplasm (Fig. 1A, B). Cells transduced with L166P DJ-1 had weaker expression of human DJ-1 compared to WT DJ-1 (Fig. 1C). Using a pan DJ-1 antibody to detect both human and endogenous rat DJ-1, we found endogenous expression of rat DJ-1 in untransduced N27 cells (control, Fig. 1D). After transduction with human WT DJ-1, the expression of total DJ-1 was significantly enhanced (Fig. 1E). By contrast, the expression of total DJ-1 was significantly reduced in N27 cells transduced with AS DJ-1 (Fig. 1F), indicating the successful knockdown of endogenous rat DJ-1. We used Western blotting to confirm the expression levels of human DJ-1 as well as total DJ-1 in N27 cells (Fig. 1G). Quantitative data show that cells transduced with human WT DJ-1 had about four times the total DJ-1 of control cells while cells transduced with L166P DJ-1 had twice the levels of control. Knockdown of endogenous DJ-1 with AS DJ-1 produced levels which were 20% of control (Fig. 1H).

We analyzed cell viability in N27 cells which had been transduced with WT DJ-1, L166P DJ-1, and AS DJ-1 for two days and treated with H2O2, 6-OHDA, and lactacystin for 24 hr. We found that cells overexpressing WT DJ-1 had significantly better cell viability than untransduced control or GFP-transduced cells after oxidative treatments (*p<0.05, **p<0.01, n=12, Fig. 1 I, J). This protective effect was not present in L166P DJ-1 transduced cells (p>0.1 compared to control, n=12). Reducing endogenous DJ-1 with AS DJ-1 led to significantly worse cell viability than control (p<0.05, n=12), indicating that endogenous DJ-1 protects against oxidative stress. Overexpression of WT DJ-1 did not protect N27 cells against cell toxicity caused by the proteasome inhibitor lactacystin (data not shown), indicating that the action of DJ-1 has some metabolic specificity.

Expression of WT DJ-1 protects rat primary dopamine neurons from oxidative stress - Embryonic rat mesencephalic cultures were transduced with adenovirus containing WT DJ-1, L166P DJ-1, or AS DJ-1 for three days, followed by treatment with H2O2, 6-OHDA, and lactacystin for 24 hr. About 25-30% of total cells were positive for DJ-1 after immunostaining with human DJ-1 antibody in WT and L166P DJ-1 transduced cultures (Fig. 2A, B). There were very few DJ-1 positive cells in AS DJ-1 transduced cultures (Fig. 2C). In transduced dopamine neurons (i.e., DJ-1/TH or GFP/TH double-labeled cells), we found that overexpression of WT DJ-1 protected cells from apoptosis induced by oxidative stress (Fig. 2D-F), while L166P DJ-1 did not protect (Fig. 2G-I). Using Western blotting
with human DJ-1 and pan DJ-1 antibodies, we confirmed overexpression of human WT DJ-1 and the knockdown of rat endogenous DJ-1 (Fig. 2J, K).

We analyzed apoptotic cell death in dopamine neurons. Quantitative data show that dopamine cells transduced with WT DJ-1 had significantly fewer apoptotic cells after oxidative stress than did control cultures (*p<0.05, n=12, Fig. 2L, M). In contrast, knocking down endogenous DJ-1 with AS DJ-1 led to significantly more apoptotic dopamine cells than seen in control cultures treated with oxidizing agents (*p<0.05, n=12, Fig. 2L, M). As we found with N27 cells, cell toxicity caused by the proteasome inhibitor lactacystin was not prevented by overexpression of WT DJ-1 (data not shown).

**DJ-1 protects N27 cells against oxidative damage by upregulating intracellular GSH levels** - Since overexpression of WT DJ-1 can protect dopaminergic cells from oxidative stress, we measured the intracellular levels of the reducing agent GSH in N27 cells with or without 24 hr exposure to 50 μM H2O2. Without H2O2 treatment, there were no significant differences in GSH levels between cells overexpressing DJ-1 and control cells (p>0.1, n=9, Fig. 3A). With H2O2 treatment, we found that control cells showed a fall in GSH levels while cells overexpressing WT DJ-1 could sustain normal GSH concentrations (*p<0.05, n=9, Fig. 3A). Cells overexpressing AS DJ-1 had levels of GSH which were even lower than controls (*p<0.05, n=9, Fig. 3A). Cell viability was directly linked to GSH levels with WT DJ-1 treated cells having significantly greater viability than control or AS DJ-1 treated cells (*p<0.05, n=9, Fig. 3B). Blocking GSH synthesis with BSO significantly reduced intracellular GSH levels, even in WT DJ-1 transduced cells, indicating that DJ-1 acts through GSH synthesis to increase GSH levels rather than by reducing breakdown of GSH (Fig. 3A). Even in cells transduced with WT DJ-1, BSO treatment led to cell death from oxidative stress (Fig. 3B). When transduced cells were supplemented with exogenous GSH (200 μM) with BSO also present, intracellular GSH levels rose and cell viability recovered to near control levels in all transduced groups (Fig. 3A, B).

We went on to assay the activity of two GSH synthetic enzymes. We found that WT DJ-1 significantly increased GCL enzymatic activity under oxidative stress (*p<0.05 compared to control, n=9, Fig. 3C), while GS enzymatic activity was not changed in any transduced groups (p>0.1, n=9, Fig. 3C). Furthermore, we found that WT DJ-1 significantly increased mRNA levels for GCL after oxidative stress (**p<0.01 compared to control without H2O2, n=9, Fig. 3D). The levels of mRNA for GCL were also increased in control, GFP and L166P DJ-1 treated cells, although to a lesser extent than WT DJ-1 (*p<0.05 compared to control without H2O2, n=9, Fig. 3D). By contrast, treatment with AS DJ-1 produced no increase in GCL mRNA, suggesting that a certain amount of endogenous DJ-1 is required to increase GCL mRNA. The GS mRNA levels were not changed after H2O2 exposure in any treatment group (data not shown). Because GCL is the rate-limiting enzyme in GSH synthesis, increased GCL transcription and enzymatic activity caused increased synthesis of GSH. The results indicate that DJ-1 protects cells against oxidative stress by increasing biosynthesis of intracellular GSH.

**DJ-1 reduced ROS and protein oxidation levels in N27 cells after oxidative stress** - Since WT DJ-1 increased GSH levels, we examined other cellular oxidation products. We used the fluorescent sensor DCF to measure cellular ROS by flow cytometry. Sample images of ROS are shown in Fig. 4A-D. Results showed that expression of WT DJ-1 significantly reduced total cellular ROS levels after oxidative stress compared to control, while AS DJ-1 significantly increased ROS levels (both **p<0.01, n=9, Fig. 4E). The L166P DJ-1 had no effect on ROS. We have also examined protein oxidation after oxidative stress. A sample blot is shown in Fig. 4F. The protein carbonyl dot blots showed that WT DJ-1 significantly reduced protein oxidation compared to control (**p<0.01, n=8, Fig. 4F, G), while AS DJ-1 further increased protein oxidation (*p<0.05, n=8, Fig. 4F, G). Transduction with L166P DJ-1 showed no protection against protein oxidation compared to control (p>0.1, n=8, Fig. 4F, G).

**DJ-1 inhibits A53T human α-synuclein toxicity and aggregation in N27 cells** - We have shown previously that overexpression of A53T mutant human α-synuclein in N27 cells leads to protein aggregation and apoptotic cell death (18). In the current study, N27 cells were transduced...
with adenovirus expressing A53T human α-synuclein and co-transduced with WT DJ-1, L166P DJ-1, or AS DJ-1. Cells which expressed only human A53T α-synuclein frequently had large, α-synuclein-positive cytoplasmic aggregates and apoptotic nuclei (Fig. 5A-E). By contrast, when WT DJ-1 was co-expressed with A53T α-synuclein, cells had fewer aggregates and less apoptosis (Fig. 5F-I). Quantitatively, we found that cells expressing WT DJ-1 had significantly improved cell viability with reduced α-synuclein aggregation compared with control (**p<0.01, n=12, Fig. 5J-L). By contrast, cells expressing AS DJ-1 had significantly fewer viable cells, higher apoptotic rates, and greater α-synuclein aggregation compared to control (*p<0.05, n=12, Fig. 5J-L).

To test if GSH is involved in DJ-1 mediated α-synuclein toxicity, we examined intracellular GSH levels and assayed GCL and GS enzyme activity. There were no differences in GSH levels, GCL and GS enzyme activity between DJ-1 transduced cells and other treatments (p>0.1, n=9, Fig. 5M-O).

**DISCUSSION**

Mutations in the DJ-1 gene have been associated with an autosomal recessive form of PD. The role of DJ-1 in the pathogenesis of PD is not well understood. In our study, we have found that DJ-1 can prevent dopamine cell death caused by two distinctly different metabolic challenges through two different protective mechanisms. During oxidative stress, expression of DJ-1 increased glutathione synthesis by increasing both the transcription and enzymatic activity of the rate-limiting enzyme, GCL. In response to A53T mutant α-synuclein overexpression, DJ-1 activated the molecular chaperone protein Hsp70 which blocks α-synuclein aggregation and toxicity.

Glutathione is the most abundant cellular thiol and plays a central role in maintaining cellular redox status and protecting cells from oxidative injury (20). In many cell types, depletion of glutathione results in oxidative stress and increased cytotoxicity, whereas elevation of intracellular glutathione levels protects cells from oxidative damage (21, 22). We observed that increased glutathione was specifically linked to improved survival of both primary dopamine neurons and a dopamine cell line. Protection from oxidative damage by glutathione was demonstrated by reduced ROS and reduced levels of oxidized proteins in DJ-1 treated dopamine cells. Knocking down endogenous DJ-1 with anti-sense DJ-1 reduced cellular glutathione levels and rendered dopaminergic cells more susceptible to oxidative stress.

We have found that the DJ-1 upregulates cellular glutathione through increasing GCL mRNA levels and GCL enzymatic activity during oxidative stress. The increase in GCL mRNA is positively correlated to total DJ-1 levels in the cells. WT DJ-1 produced higher levels of total DJ-1 compared to GFP or L166P DJ-1 controls, which led to greater increases in GCL mRNA than seen with other treatments. Transcription of GCL is regulated by many different molecules (23,24), so that cells may be able to maintain relatively stable
levels of GCL mRNA even when DJ-1 levels were reduced by AS DJ-1. Although GCL mRNA levels were not affected by AS DJ-1 treatment, the GCL enzymatic activity was significantly reduced during oxidative stress. In fact, there were uniform reductions of GCL enzymatic activity in WT DJ-1, GFP, L166P DJ-1 or Control cells after oxidative stress, and the extent of reduction was similar to treatment with AS DJ-1. It is possible that the normal GCL enzymatic function is impaired under oxidative conditions. Our results indicate that AS DJ-1 treatment blocks the increase in GCL mRNA needed to sustain GCL activity, GSH levels, and cell viability.

For the L166P mutant DJ-1, mutant levels doubled the endogenous concentration of native DJ-1. It is known that the mutation blocks the dimerization ability of DJ-1. The monomer form is more rapidly degraded (25,26). That phenomenon likely accounts for the lower protein concentrations we have observed despite similar concentrations of infecting viral particles. Because the function of DJ-1 depends on being dimerized (25-27), the lack of protection after L166P DJ-1 is probably related to its inability to dimerize.

Others have noted that DJ-1 has anti-oxidant activity (28-31). Studies on DJ-1 knockout mice have found various motor/behavioral and pharmacological deficits (32,33). DJ-1 null mice are more sensitive to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment and oxidative stress (34). Moreover, WT mice overexpressing DJ-1 are resistant to MPTP toxicity (34). In a recent study, DJ-1 has been found to sequester the cell death protein Daxx in the nucleus and prevent Daxx-induced apoptosis after oxidative stress (35). In Drosophila, inhibition of DJ-1 function results in increased ROS, hypersensitivity to oxidative stress, degeneration of dopamine neurons, and impairment in phosphatidylinositol 3-kinase/Akt signaling pathways (36). Our experiments are the first to show that upregulation of glutathione synthesis is an important mechanism by which DJ-1 protects against oxidative damage. Because we have seen that DJ-1 does not protect against proteasome inhibitor-induced cell death, our results suggest that the anti-oxidant effects of DJ-1 are specific.

When dopamine neurons were subjected to a different metabolic stress, overexpression of the protein A53T α-synuclein, the protein aggregated and killed the cells (18, 37-39). The protein aggregation of A53T human α-synuclein is initiated by the formation of oligomers and protofibrils (40). In dopamine neurons co-expressing DJ-1, α-synuclein protein aggregation was reduced and cell survival was improved without any change in glutathione synthesis or glutathione concentration. Instead, we found that the concentration of Hsp70 was increased. These beneficial effects of DJ-1 were not seen with the PD-associated mutant form of DJ-1, L166P. Similar to GCL, the increase in Hsp70 mRNA and protein depended on total DJ-1 levels. WT DJ-1 treatment resulted in higher levels of total DJ-1, and hence led to a greater increase in Hsp70 compared to GFP, L166P DJ-1, or control cells. Interestingly, A53T α-synuclein alone or in combination with GFP or L166P DJ-1 induced a significant increase in Hsp70. That increase was blocked when native DJ-1 was reduced by AS DJ-1. Others have shown that increased Hsp70 can reduce α-synuclein protein aggregation and toxicity in Drosophila (41,42), mouse (43,44), and cell culture models (45,46). Hsp70 preferentially binds and stabilizes α-synuclein protofibrils which results in inhibition of larger α-synuclein aggregates and cell toxicity (47). Recent studies by Shendelman et al. suggested that DJ-1 has redox-dependent chaperone activity (48). Under oxidized conditions, DJ-1 protein can function as a molecular chaperone to prevent abnormal protein aggregation of α-synuclein and of neurofilament light subunits (48). Our results are the first to show that DJ-1 triggers upregulation of Hsp70 and thereby reduces α-synuclein toxicity. Furthermore, we have found that DJ-1 can upregulate Hsp70 and inhibit α-synuclein protein aggregation and toxicity without a change in glutathione metabolism and in the absence of oxidizing conditions. Therefore, DJ-1 can act directly or indirectly to modify chaperone protein activity.

Several studies have identified DJ-1 as a transcriptional regulator. DJ-1 forms regulatory subunits in a large RNA-binding protein complex which is known to play an important role in the post-transcriptional control of gene expression (14). By directly binding to PIASxα, the transcriptional inhibitor of the androgen receptor,
DJ-1 positively regulates the transcription of the receptor (15). A recent study found that DJ-1 binds to nuclear proteins p54nrb and pyrimidine tract-binding protein-associated splicing factor (PSF), two multifunctional regulators of transcription and RNA metabolism (49). As a result, DJ-1 can function as a transcriptional co-activator to regulate the expression of neuroprotective genes (49). In *Drosophila*, DJ-1 has been found to be a key negative regulator of tumor suppressor PTEN (50). In our study, we have seen that DJ-1 upregulates transcription of GCL during oxidative stress and upregulates transcription of Hsp70 after overexpression of A53T α-synuclein. These results show that DJ-1 has multiple mechanisms for protecting cells.

Others have shown that DJ-1 can self-oxidize by forming cysteine-sulfinic acid under oxidative conditions and thereby shifting its pI from 6.1 to 5.8 (28,51). Mutant L166P DJ-1 is unstable and cannot form dimers (25,26). Because L166P cannot dimerize and does not protect against oxidative damage, it is likely that dimer formation is necessary for the anti-oxidant effects of DJ-1. In Figure 7, we propose a model mechanism by which DJ-1 may protect cells from oxidative stress and from A53T α-synuclein aggregation. After oxidative stress, it is likely that the oxidized form of DJ-1 dimer gains the ability to regulate transcription of GCL which leads to an increase in GSH levels. With A53T α-synuclein treatment, perhaps a different dimer form of DJ-1 upregulates Hsp70 mRNA and protein, and eventually reduces α-synuclein aggregation and toxicity.

Both oxidative stress and abnormal α-synuclein aggregation have been implicated in the pathogenesis of PD (52). Our findings that DJ-1 has dual roles as an anti-oxidant and as an inhibitor of α-synuclein aggregation may provide clues as to why patients with DJ-1 gene mutations developed early-onset PD. Our studies on cultured dopamine neurons provide molecular mechanisms for the critical role that DJ-1 plays in protecting dopamine neurons from oxidative stress and A53T mutant α-synuclein aggregation and toxicity.

REFERENCES


**FOOTNOTES**

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The abbreviations used are: BSO, buthionine sulfoximine; DA, dopamine; GSH, glutathione; 6-OHDA, 6-hydroxydopamine; Hsp70, heat shock protein 70; GCL, glutamate cysteine ligase; GS, glutathione synthetase; LBs, Lewy bodies; MTT, methylthiazoletetrazolium; PD, Parkinson’s disease; ROS, reactive oxygen species; TH, tyrosine hydroxylase; WT, wild-type.*
Fig. 1. DJ-1 protects N27 cells from oxidative stress-induced death. **A-C**, N27 cells were transduced with adenovirus expressing WT DJ-1 (**A, B**) or L166P DJ-1 (**C**) and stained with anti-human DJ-1 antibody. High levels of WT DJ-1 were expressed in transduced cells. L166P DJ-1 treated cells had weaker expression of DJ-1 than WT DJ-1. The mutant form did not lead to cell loss. **D-F**, N27 cells were transduced with empty (**D**), WT DJ-1 (**E**), or AS DJ-1 (**F**) adenovirus and stained with pan DJ-1 antibody which recognizes both human and rat DJ-1. Low endogenous levels of DJ-1 are found in N27 cells (**D**). Transduction with WT DJ-1 leads to overexpression (**E**). AS DJ-1 treatment reduces endogenous DJ-1 (**F**). **G**, Western blotting confirms DJ-1 overexpression in N27 cells while DJ-1 is knocked down by adenovirus containing AS DJ-1 as detected by human DJ-1 and pan DJ-1 antibodies. β-actin was used as a loading control. **H**, quantitative data from three independent experiments (n=6) show the magnitude of DJ-1 overexpression after WT DJ-1 transduction (400%) and of DJ-1 down regulation in AS DJ-1 treated cells (20%) compared to control. **I-J**, N27 cells were transduced with GFP, WT DJ-1, L166P DJ-1 or AS DJ-1 and subjected to H₂O₂ (**I**) or 6-OHDA (**J**). Cell viability was determined by MTT assays. The figure shows that WT DJ-1 protects cells from death induced by either form of oxidative stress (n=12). By contrast, knocking down endogenous DJ-1 with AS DJ-1 reduces cell viability after oxidative stress (n=12). Results are shown as mean ± SEM. *p<0.05, **p<0.01. Bar = 5 μm.

Fig. 2. DJ-1 protects rat primary dopamine neurons from cell death induced by oxidative stress. **A-C**, rat embryonic mesencephalic cultures were transduced with adenovirus containing WT DJ-1 (**A**), L166P DJ-1 (**B**), or AS DJ-1 (**C**) and stained with pan DJ-1 antibody. **D-F**, primary dopamine neurons transduced with WT DJ-1 were double stained with anti human DJ-1 (**D**) and TH (**E**) antibodies, and displayed normal nuclei (arrow in **F**, Hoechst staining). **G-I**, dopamine neurons transduced with L166P DJ-1 were positive for both human DJ-1 (**G**) and TH (**H**), but with condensed nuclei (arrowhead in **I**, Hoechst staining), indicating apoptotic cell death. **J**, Western blotting confirms DJ-1 overexpression in primary cultures while endogenous DJ-1 is knocked down by AS DJ-1. **K**, quantitative data (n=6) show the magnitude of DJ-1 overexpression after WT DJ-1 transduction (350%) and of DJ-1 down regulation in AS DJ-1 treated cells (30%) compared to control. **L-M**, rat primary mesencephalic cultures were transduced with various DJ-1 adenoviruses, followed by treatment with H₂O₂ (**L**) or 6-OHDA (**M**) for 24 hr. Apoptosis was determined by Hoechst staining in transduced dopamine neurons (i.e. DJ-1/TH double positive cells). Figures show that WT DJ-1 significantly reduces apoptotic cell death in primary dopamine neurons induced by oxidative stress, and AS DJ-1 significantly enhances cell death (n=12). Results are shown as mean ± SEM. *p<0.05, **p<0.01. Bar = 5 μm.

Fig. 3. DJ-1 increases GSH levels through increased GCL expression and activity. **A**, N27 cells were transduced with DJ-1 adenovirus and treated with 50 μM H₂O₂, 200 μM BSO, or 200 μM exogenous GSH for 24 hr. Total cellular GSH levels were measured and quantified as percentage of control. Expression of WT DJ-1 significantly increased GSH levels in cells after oxidative stress compared to controls (n=9), while knocking down endogenous DJ-1 with AS DJ-1 significantly reduced GSH levels (n=9). Treatment with BSO abolished the beneficial effect of WT DJ-1 (n=9). Adding exogenous GSH to the culture restored and improved cell viability under all culture conditions (n=9). **B**, cell viability was measured from duplicate cultures with the same treatment as (**A**). Data show cell viability is positively correlated to total cellular GSH levels (n=9). **C**, GCL and GS enzymatic activities were measured in protein extracts from DJ-1 transduced N27 cells with or without 50 μM H₂O₂ treatment. The figure shows...
that WT DJ-1 treated cells have significantly higher GCL activity under oxidative stress \( n=9 \), while GS activity is not changed after any treatment \( n=9 \). \( D \), semi quantitative RT-PCR analysis of GCL mRNA levels from DJ-1 transduced cells with or without 50 \( \mu \)M \( H_2O_2 \) treatment. A representative gel shows that the levels of GCL mRNA are significantly increased in WT DJ-1 transduced cells during oxidative stress. Quantitative data \( n=9 \) indicate that the levels for GCL mRNA are also significantly increased in Control, GFP and L166P DJ-1 treated cells after oxidative stress, although to a lesser extent than WT DJ-1. Treatment with AS DJ-1 did not change GCL mRNA \( n=9 \). Results are shown as mean \( \pm \) SEM. \* \( p<0.05 \), ** \( p<0.01 \).

**Fig. 4** DJ-1 reduces cellular levels of ROS and protein carbonyls in N27 cells. \( A-D \), N27 cells were transduced with adenovirus containing DJ-1 followed by incubation with 50 \( \mu \)M \( H_2O_2 \) for 24 hr. The ROS probe DCF-DA was added to cultures for 30 min. Sample images were taken from control and WT DJ-1 treated cells. DCF positive cells were measured by flow cytometry as shown in \( E \). WT DJ-1 significantly reduced ROS levels in cells \( \* \* \* p<0.01 \) compared to control, \( n=9 \), while AS DJ-1 significantly increased cellular ROS concentrations \( \* \* \* p<0.01 \) compared to control, \( n=9 \). \( F \), the amount of protein carbonyls was measured by Oxyblot kit. Equal amounts of sample \( (10-\mu g \text{ total protein}) \) were derivatized with 2,4-dinitrophenyhadrazine (DNPH) and spotted on nitrocellulose membrane. Dot blots were immunodetected by anti-DNPH antibody. Duplicate blots were used for each experiment and each experiment was done four times yielding 8 data points. A representative blot is show in \( F \). \( G \), Dot blots were scanned and image densities were measured by Image J software. Protein carbonyl levels are expressed as percentages of levels in control cells. Quantitative data indicate that expression of WT DJ-1 significantly reduced total protein carbonyls in cells after \( H_2O_2 \) treatment \( \* \* \* p<0.01 \) compared to control, \( n=8 \), while AS DJ-1 treatment significantly increased protein carbonyl levels \( \* \* \* p<0.05 \) compared to control, \( n=8 \). Results are expressed as mean \( \pm \) SEM. Bar = 25 \( \mu m \).

**Fig. 5** DJ-1 inhibits A53T \( \alpha \)-synuclein protein aggregation and toxicity. \( A-E \), N27 cells were transduced with A53T \( \alpha \)-synuclein, followed by immunostaining with human \( \alpha \)-synuclein antibody. Overexpression of \( \alpha \)-synuclein in N27 cells is shown in \( A \), with many cells containing small \( \alpha \)-synuclein-positive aggregates (arrows in \( B, C, D \)). Most cells with \( \alpha \)-synuclein aggregates had apoptotic nuclei \( D, E \), long arrows indicate fragmented nuclei). \( F-I \), N27 cells were transduced with both A53T \( \alpha \)-synuclein and WT DJ-1, followed by \( \alpha \)-synuclein \( (F) \), DJ-1 \( (G) \) and Hoechst \( (H) \) staining. Figure \( I \) shows merged image from \( F, G, H \). These images show that expression of WT DJ-1 inhibits \( \alpha \)-synuclein aggregation and rescues N27 cells from A53T \( \alpha \)-synuclein induced toxicity. \( J-L \), quantitative data show cell viability \( (J) \), apoptosis rate \( (K) \), and the percentage of cells with \( \alpha \)-synuclein aggregates \( (L) \) in N27 cells with DJ-1 and A53T \( \alpha \)-synuclein dual treatments. Coexpression of DJ-1 and A53T \( \alpha \)-synuclein significa ntly increased cell viability by significantly reducing the apoptotic rate as well as \( \alpha \)-synuclein aggregation, as compared to control \( (n=12) \). Anti-sense DJ-1 treatment did the opposite, significantly reducing cell viability while increasing apoptosis and protein aggregation \( (n=12) \). Figures \( M-O \) present measurements of GSH levels \( (M) \), and enzymatic activity for GCL \( (N) \) and GS \( (O) \) in N27 cells after cotransduction with DJ-1 and A53T \( \alpha \)-synuclein. Data show that there are no changes in these three measurements even in WT DJ-1 treated cells \( (n=9) \). Results are shown as mean \( \pm \) SEM. \* \( p<0.05 \), \* \* \* \( p<0.01 \). Bar = 5 \( \mu m \).

**Fig. 6** DJ-1 inhibits A53T \( \alpha \)-synuclein aggregation by increasing Hsp70. \( A, D \), Western blot shows accumulation of \( \alpha \)-synuclein oligomers in N27 cells detected by \( \alpha \)-synuclein antibody after A53T
overexpression. Quantitative data \((D)\) show that coexpression of WT DJ-1 significantly reduced \(\alpha\)-synuclein oligomers compared to A53T \(\alpha\)-synuclein alone \((n=9)\), while AS DJ-1 significantly increased oligomers \((n=9)\). \(B, E,\) Western blot reveals that Hsp70 protein is significantly increased in WT DJ-1 treated cells compared to untreated control \((n=9)\). Cells expressing A53T \(\alpha\)-synuclein alone, or with GFP or L166P DJ-1 also show significant increases in Hsp70 \((n=9)\), although smaller than WT DJ-1. Cells treated with a combination of AS DJ-1 and A53T \(\alpha\)-synuclein failed to increase Hsp70 concentration \((n=9)\). \(C, F,\) a representative gel image and quantitative data from semi quantitative RT-PCR for Hsp70 mRNA shows parallel results with Hsp70 protein after all treatment \((n=9)\). Results are shown as mean ± SEM. *\(p<0.05\), **\(p<0.01\).

**Fig. 7** A proposed mechanism by which DJ-1 protects against oxidative stress and A53T \(\alpha\)-synuclein toxicity. Overexpression of human WT DJ-1 increases the total DJ-1 levels and thereby the DJ-1 dimer concentration. L166P mutant DJ-1 cannot dimerize and therefore has no effect on the concentration of DJ-1 dimer. By contrast, AS DJ-1 reduces endogenous DJ-1 monomer production and thereby reduces dimer levels. During oxidative stress, the DJ-1 dimer is oxidized that process may directly or indirectly enhance GCL mRNA expression. The increased GCL enzymatic activity and GSH levels lead to higher cell viability. When cells are exposed to A53T \(\alpha\)-synuclein, the DJ-1 dimer may undergo conformational changes which result in increased expression of Hsp70 mRNA and protein without changing glutathione metabolism. The molecular chaperone protein Hsp70 prevents oligomer and protofibril formation, eventually reducing cell toxicity.
**Figure 3**

**A**

GSH Levels (%)

- H2O2  |  H2O2  |  BSO+H2O2  |  BSO+H2O2 +GSH

**B**

Cell Viability (%)

- H2O2  |  H2O2  |  BSO+H2O2  |  BSO+H2O2 +GSH

**C**

Enzymatic Activity (%)

- H2O2  |  +H2O2  |  -H2O2  |  +H2O2

**D**

- GCL
- GS

**GCL mRNA (%)**

- H2O2  |  +H2O2

Legend:

- control
- GFP
- WT DJ-1
- L166P DJ-1
- AS DJ-1