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Oxidative insults induce DJ-1 upregulation and redistribution: Implications for neuroprotection

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Abstract

Oxidative stress is implicated in the pathogenesis of central nervous system damage in neurodegenerative diseases as well as in normal aging. Parkinson's disease (PD) is one of the most common age-related neurodegenerative diseases caused by both environmental and inherited factors. DJ-1 mutations were recently identified in familial PD. The aim of this study was to elucidate the effects of the neurotoxins rotenone and 6-hydroxydopamine that lead to intracellular reactive oxygen species (ROS) on DJ-1 expression levels and intracellular distribution. The sensitivity to oxidative insults induced by rotenone, 6-hydroxydopamine and hydrogen peroxide of transfected human neuroblastoma cells that were engineered to have increased or decreased DJ-1 levels was also examined. Overexpression of DJ-1 resulted in increased cellular resistance to these insults and reduced intracellular ROS. Contrary effects were achieved when DJ-1 levels were reduced by siRNA. Exposure of naïve neuroblastoma cells to rotenone or 6-hydroxydopamine induced upregulation of DJ-1 mRNA and protein levels. Pretreatment with an antioxidant abolished these changes, implying that the upregulation of DJ-1 resulted from oxidative stress. Neurotoxins exposure not only induced upregulation of DJ-1, but also induced cellular redistribution of DJ-1 manifested by translocation of DJ-1 into the mitochondria. These results may imply that DJ-1 plays an important role in the neuronal defense mechanism against oxidative insults.

Keywords: Parkinson's disease; Rotenone; 6-Hydroxydopamine; DJ-1; Oxidative stress; Mitochondria

1. Introduction

Oxidative stress and mitochondrial dysfunction are implicated in the pathogenesis of central nervous system (CNS) damage in neurodegenerative diseases as well as normal aging (Lin and Beal, 2006). Parkinson's disease (PD) is a progressive neurodegenerative disease affecting over 1% of the population above the age of 65 (Hoehn and Yahr, 1967). PD is a multifactorial disease caused by both genetic and environmental factors (Litvan et al., 2007). The major pathological hallmark of PD is degeneration of the dopaminergic neurons in the substantia nigra pars compacta. Data from human post-mortem brain tissues and from experimental models indicate that reactive oxygen species (ROS) and decrements in mitochondrial complex I activity are important in the pathogenesis of PD (Zhang et al., 2000).

Although most patients suffering from PD have a sporadic disease, several genetic causes have been identified in the past decade. An increasing number of genes that cause inherited forms of PD have provided the opportunity for new insights into the mechanisms at the basis of the disease. These genes include alpha-synuclein, parkin, PINK1, dardarin (LRRK2), UCHL1 and DJ-1 (Hardy et al., 2006). DJ-1 deletions and point mutations have been found worldwide, and loss of functional protein was shown to cause autosomal recessive PD (Abou-Sleiman et al., 2004; Bonifati et al., 2003). DJ-1 was first described a decade ago (Nagakubo et al., 1997). It encodes a small 189 amino acid protein that is ubiquitously expressed and highly conserved in diverse species (Bai et al., 2006). The high expression of DJ-1 in the CNS is not confined to a single anatomical or functional system (Bader et al., 2005; Shang et al., 2004). Within the substantia nigra, however, DJ-1 is localized in both neuronal and glial cells, and selective

Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; 6-OHDA, 6-hydroxydopamine; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetyl cysteine; PD, Parkinson's disease; ROS, reactive oxygen species.

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enrichment of DJ-1 was recently found in striatal neuronal processes in humans and in non-human primates, suggesting a distinct role for DJ-1 in the striato-nigral system (Olzmann et al., 2007). Furthermore, several studies reported that DJ-1 knockdown renders cells more susceptible to oxidative stress (Taira et al., 2004; Yokota et al., 2003; Zhou and Freed, 2005). Yokota et al. (2003) reported that cell death with hydrogen peroxide exposure was dramatically rescued by over-expression of wild-type DJ-1, while Taira et al. (2004) reported that DJ-1 knockdown rendered neuroblastoma cells more susceptible to hydrogen peroxide-induced cell death.

DJ-1 has several isoforms with different isoelectric points (p*I*) (Bandopadhyay et al., 2004; Choi et al., 2006; Kinumi et al., 2004). Under oxidative conditions DJ-1 undergoes p*I* shift into more acidic isoforms. This p*I* shift is caused by the oxidation of cysteine and methionine residues in DJ-1 (Choi et al., 2006; Kinumi et al., 2004). Post-mortem studies of brain samples taken from sporadic PD patients found that the acidic isoforms of DJ-1 are more abundant in PD brains as compared to controls (Bandopadhyay et al., 2004; Choi et al., 2006). Moreover, elevated levels of DJ-1 were reported in the cerebrospinal fluid (CSF) of sporadic PD patients (Waragai et al., 2006). These studies imply that DJ-1 has a role not only in selective inherited cases but also in the more common sporadic disease.

Loss-of-function DJ-1 mutations are linked to the degeneration of dopaminergic neurons and PD. Rotenone and 6-hydroxydopamine are neurotoxins that elicit parkinsonian phenotypes in rodents and are commonly used to induce in vitro and in vivo models of PD. These toxins induce ROS production and oxidative stress. Therefore, we hypothesized that decreasing DJ-1 levels by siRNA for DJ-1 may predispose neuroblastoma cells to toxins-induced cell death, while overexpression of DJ-1 may have a protective effect against these neurotoxins. We therefore aimed to elucidate, using a cellular model, the role of DJ-1 in the cellular response to these neurotoxins that cause oxidative stress.

2. Materials and methods

2.1. Materials

The following reagents used were: tri-reagent (Sigma, St Louis, MO, USA); pIRES2-acGFP1 plasmid (Chemicon, Temecula, CA, USA); rabbit anti-DJ-1 (Chemicon, Temecula, CA); mouse anti-beta actin (Sigma, St Louis, MO, USA); mouse anti human mitochondria (Chemicon, Temecula, CA); Alexa 568-conjugated goat anti-rabbit (Molecular Probes, Invitrogen, Eugene, OR, USA); horseradish peroxidase conjugated goat anti mouse and goat anti rabbit (Sigma, St Louis, MO, USA); goat anti mouse biotinilated antibodies (Molecular Probes, Invitrogen, Eugene, OR, USA) and alexa-488 conjugated streptavidin (Molecular Probes, Invitrogen, Eugene, OR, USA); 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St Louis, MO, USA); Hoechst 33258 (Sigma, St Louis, MO, USA); Super Signal West Pico Chemiluminescent substrate (Pierce Biotechnology, Rockford, IL, USA); BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA); random primer (Invitrogen, Carlsbad, CA); Sybr green PCR master mix (Applied Biosystems, Warrington, UK); RNase inhibitor (RNAguard, Amersham Pharmacia biotech); Super Script II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA, USA); Rotenone (ICN Biomedical, Aurora, Ohio); 6-hydroxydopamine (Sigma, St Louis, MO, USA); *N*-acetyl cysteine (Sigma, St Louis, MO, USA); H_2DCFDA (Sigma, St Louis, MO, USA); Dulbecco's Modified Eagle's Medium (DMEM) (Biological Industries Israel Beit Haemek Ltd., Kibbutz Beit Haemek, Israel); fetal calf serum (FCS) (Biological Industries Israel Beit Haemek Ltd., Kibbutz Beit Haemek, Israel); diethyl pyrocarbonate (DEPC) water (Biological Industries Israel Beit Haemek Ltd., Kibbutz Beit Haemek, Israel); Complete protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany); geneticin (G418) (Gibco, Invitrogen). Other chemicals were purchased from Sigma Chemicals Co., St. Louis, MO, USA.

2.2. Cell culture and treatments

Human neuroblastoma SH-SY5Y and M17 cells were obtained from the American tissue Type Culture Collection (ATCC, Rockville, USA). Cells were grown under sterile conditions as monolayer in DMEM medium supplemented with 10% heat-inactivated fetal calf serum (FCS), gentamicin (50 mg/ml), and glutamine (5 mM) in a 5% CO_2 humidified atmosphere at 37 °C. The medium was routinely changed every 4 days, and cells were passaged every 8 days. All experiments were performed on cells near confluence.

Neuroblastoma cells were exposed to H_2O_2 (0–75 μM; Sigma), rotenone (0–1 μM; ICN Biomedical), or 6-hydroxydopamine (6-OHDA) (0–100 μM; Sigma), for 24 h in order to produce reactive oxygen species (ROS) formation. In some of the experiments, cells were pretreated with the antioxidant *N*-acetyl cysteine (5 mM NAC; Sigma), 1 h prior to exposure to the toxins and during the toxins exposure. Toxins solutions were prepared immediately before applying them to the cells. Rotenone was dissolved in DMSO, 6-hydroxydopamine was dissolved in ice cold 0.9% saline containing 0.2% ascorbic acid and protected from light.

2.3. Cellular transfections

The coding region of human wild-type DJ-1 cDNA was subcloned into pIRES2-acGFP1 plasmid (BD Biosciences, Clontech) (pGFP-DJ-1). In order to achieve overexpression of DJ-1, SH-SY5Y neuroblastoma cells were stably transfected with the plasmid containing wild-type DJ-1. We used naïve neuroblastoma cells as well as cells stably transfected with the empty vector as controls.

Decreased expression of DJ-1 was achieved by stable transfection with pSilencer2.1-U6 plasmid (Ambion) containing siRNA for DJ-1. For targeting human DJ-1 (GGTCATTACACCTACTCTGAGAATCGT), the loop sequence (TTCAAGAGA) flanked by the sense and antisense siRNA sequence

was inserted immediately downstream of U6 promoter in pSilencer2.1-U6 plasmid, according to the instructions of the manufacturer. As negative controls, neuroblastoma cells were transfected with pSilencer2.1-U6 negative control (siRNA-control; Ambion).

Transfections were performed using the lipofectamine 2000 reagent (Invitrogen). In order to achieve stable transfections we treated the transfected cells with geneticin (G418) for a month. Stable transfection was verified by measuring DJ-1 mRNA and protein levels using real-time PCR and Western blotting, respectively.

2.4. Protein extraction and western blotting

Protein extraction and Western blotting were done as previously described (Lev et al., 2006). Briefly, neuroblastoma cells were trypsinized, centrifuged and resuspended in lysis buffer to prepare whole-cell lysates. Cell debris was removed by centrifugation at 20,000 × *g* for 15 min at 4 °C. Protein concentration was determined by the BCA method (Pierce). Twenty-five micrograms of total protein cell lysate were separated by 12% SDS-PAGE gels and transferred to a nitrocellulose membrane. The membranes were probed with rabbit anti-DJ-1 antibody (1:5000; Chemicon Laboratories), and mouse anti beta-actin (1:10,000; Sigma), followed by horseradish peroxidase conjugated secondary antibody (1:10,000; Sigma) and developed with the Super Signal West Pico Chemiluminescent substrate (Pierce).

2.5. RNA isolation

Total RNA was isolated from cultured neuroblastoma cells using a commercial reagent TriReagent™ (Sigma) and the manufacturer's recommended procedure. The amount of RNA was determined spectrophotometrically using the ND-1000 spectrophotometer (NanoDrop). RNA quality was verified by measuring the OD260/OD280 ratio. RNA was stored at −80 °C until used.

2.6. cDNA synthesis and real-time quantitative PCR

cDNA synthesis and real-time quantitative PCR were done as previously described (Lev et al., 2006). Briefly, first-strand cDNA synthesis was carried out in a final reaction volume of 20 μl containing 1 μg of the total RNA, random primer (1.3 μM, Invitrogen) in diethyl pyrocarbonate (DEPC) water at a total volume of 10 μl. After incubation in 70 °C for 10 min and cooling to 4 °C for 10 min, the following reagents were added to a final concentration of: 1× buffer supplied by the manufacturer, 10 mM DTT, 20 μM dNTPs, 20 U of RNase inhibitor (RNAguard, Amersham Pharmacia Biotech) and 10 U of the enzyme RT-superscript II (Invitrogen) reverse transcriptase. RT reaction was performed at 25 °C for 10 min, 42 °C for 2 h followed by 70 °C for 15 min and 95 °C for 15 min. Samples were stored at −20 °C until used.

Real-time quantitative reverse transcription polymerase chain reaction (PCR) of the desired genes was performed in an ABI Prism 7700 sequence detection system (Applied Biosystems) using Sybr green PCR master mix (Applied Biosystems) and the following primers: GAPDH sense: CGA CAG TCA GCC GCA TCT T, GAPDH antisense: CCA ATA CGA CCA AAT CCG TTG; DJ-1 sense: CAT GAG GCG AGC TGG GAT TA, DJ-1 antisense: GCT GGC ATC AGG ACA AAT GAC. GAPDH gene, which served as an internal control, is a valid reference 'housekeeping' gene for transcription profiling, which has also been used for qPCR experiments in previous studies.

For quantification of DJ-1 and GAPDH mRNA, real time quantitative PCR (qPCR) was performed in triplicates. qPCR amplification was performed on the target gene, i.e. DJ-1, and the reference gene, GAPDH, for each sample, in separate wells of the same PCR reaction plate, which also showed a standard curve for each gene amplified and no template controls (NTC). Optimal experimental parameters (hybridization temperature, elongation time, and primers' concentration) were determined for each primer pair. A single peak in melting curve analysis assessed the specificity of the PCR product for each gene.

PCR was performed in a total volume of 20 μl containing 1 μl of the above-described cDNA, 1 μl each of the 3′ and 5′ primers (at a final concentration of 500 nmol/l each), 10 μl of AbsoluteTM QPCR SYBR® Green ROX Mix and 8 μl of DEPC water. The amplification protocol was 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min each. Quantitative calculations of the gene of interest (DJ-1) versus GAPDH were done using the ddCT method.

2.7. Immunocytochemistry and confocal microscopy analysis

Cells were plated on cover slips and treated with rotenone or 6-hydroxydopamine for 24 h. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 and were then incubated in a blocking solution followed by 1-h incubation with rabbit anti-DJ-1 antibody (1:1000; Chemicon Laboratories) at room temperature. After washing with PBS, the cells were incubated with fluorescent Alexa 488-conjugated goat anti-rabbit antibodies (1:1000; Molecular Probes) for 1 h at room temperature.

Staining for human mitochondria was done by incubation with mouse anti human mitochondria (1:100, Chemicon Laboratories) at 4 °C overnight, followed by incubation with goat anti mouse biotinilated antibodies (1:200; Molecular Probes, Invitrogen) and alexa-568-conjugated streptavidin (1:200; Molecular Probes, Invitrogen).

Cover slips were inverted onto microscope slides and confocal images were taken with a Zeiss LSM 510 META confocal microscope. All images were taken with a 63′-oil objective, and the settings were similar for all images. Controls were incubated with secondary antibodies in the absence of primary antibody.

2.8. Cell viability

The MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay: cells were plated in 96-well

plates and viability was evaluated 24 h after toxin exposure. MTT solution was added to each well (reaching a final concentration of 0.5 mg/ml) followed by incubation at 37 °C for 2 h. The medium was then removed and the formazan crystals were dissolved in DMSO. Absorbance was determined at 564 nm in a microplate reader. Cell viability was evaluated in triplicates for each treatment. All experiments were repeated at least three times.

The changes in nuclear morphology of apoptotic cells were investigated by labeling the cells with the nuclear stain Hoechst 33258 and examining them under fluorescent microscopy. After being treated with rotenone or 6-hydroxydopamine for 24 h, the cells were fixed with cold 70% ethanol, and incubated with Hoechst 33258 (10 μg/ml) for 15 min at 37 °C in the dark, and then the nuclear morphology was observed under a fluorescence microscope (Olympus, bx52). Cells that exhibited reduced nuclear size, chromatin condensation, intense fluorescence, and nuclear fragmentation were considered as apoptotic.

2.9. Measurement of intracellular reactive oxygen species (ROS)

Neuroblastoma cells cultured with increasing concentrations of rotenone or 6-hydroxydopamine for 24 h were harvested, and suspended in PBS. 50,000 cells were placed in each well in a black 96-well plate. The generation of ROS was measured using H_2DCFDA, which is incorporated into the cells and cleaved into fluorescent DCF in the presence of ROS. 10 μM H_2DCFDA (Sigma) was added to the cell suspension, and the cells were incubated in the dark at 37 °C for 30 min. DCF fluorescence was measured by FLUOstar spectrofluorometer microplate reader at 520 nm. The generation of ROS was quantitatively assayed by the increase in DCF fluorescence. Each experiment was repeated at least three times in triplicates.

2.10. Statistical analysis

Statistical analysis was done using the SPSS software. Comparison between two groups was performed using Student's *t*-test, while multiple comparisons between more than two groups was analyzed by one-way ANOVA and post hoc tests by LSD. Data evaluated for the effects of two variables was analyzed using two-way ANOVA. Results are presented by mean ± standard deviation. Statistical significance is defined as *p*-value of ≤ 0.05.

3. Results

3.1. Overexpression of DJ-1 confers cellular resistance to oxidative insults

We hypothesized that decreasing DJ-1 levels by siRNA for DJ-1 may predispose neuroblastoma cells to toxins-induced cell death, while overexpression of DJ-1 may have a protective effect. To test this hypothesis, we generated cells over-expressing DJ-1 or expressing siRNA for DJ-1 thereby

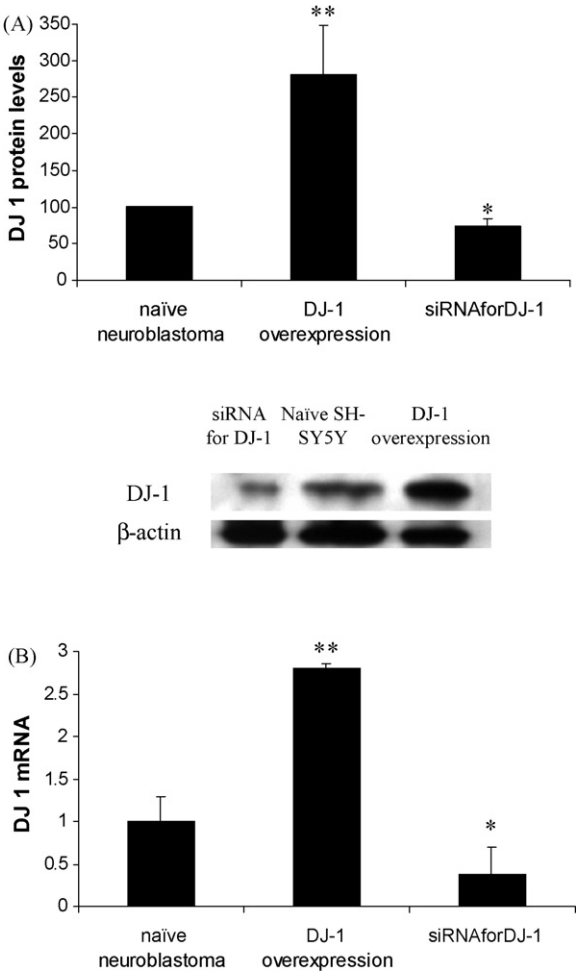

Fig. 1. DJ-1 expression levels in transfected cells. (A) Western blot analysis of DJ-1 protein expression levels in naïve SH-SY5Y neuroblastoma cells, cells overexpressing DJ-1 and cells transfected with siRNA for DJ-1. A representative Western blot is shown blow the graph. (B) Quantitative real-time PCR analysis of DJ-1 mRNA levels in the transfected cells. Error bars indicate mean ± S.D. $*p < 0.05$ and $**p < 0.001$. Each experiment was repeated three times.

decreasing DJ-1 levels. Stable transfection inducing overexpression of DJ-1 was verified by an increase in DJ-1 protein (281% ± 66% of control levels, Fig. 1A) and mRNA levels (279.9% ± 5% of control levels, Fig. 1B). Transfection with siRNA for DJ-1 resulted in decreased DJ-1 expression (to 74.4% ± 9.99% of DJ-1 protein, as quantified by Western blot and 51.9% ± 10.1% of DJ-1 mRNA as quantified by real time PCR, Fig. 1A and B). These cells had similar growth rates and maintained normal morphological features similar to naïve neuroblastoma SH-SY5Y cells.

Rotenone and 6-hydroxydopamine are commonly used neurotoxins that elicit experimental models of PD. These toxins induce ROS production and oxidative stress induction. The toxic insults were implicated by exposure of neuroblastoma cells, SH-SY5Y and M17, to increasing concentrations of 6-hydroxydopamine and rotenone. In order to demonstrate that these neurotoxins induce oxidative insults, we also exposed neuroblastoma cells to increasing doses of hydrogen peroxide and evaluated the effect of DJ-1 levels on cellular viability and

intracellular ROS accumulation with exposure to a pure oxidative insult.

Cellular viability of human neuroblastoma cells was evaluated, using the MTT assay and Hoechst 33258 nuclear morphology, with exposure to increasing doses of rotenone, 6-hydroxydopamine or H_2O_2. Under all three toxic conditions, a dose-dependent decrease in cell viability was observed by both methods. Overexpression of DJ-1 restored cell viability after exposure to toxins, as compared to mock-transfected cells and to naïve control cells (Fig. 2). Decreasing DJ-1 levels by siRNA led to increased vulnerability to toxins exposure, as compared to naïve cells and to cells transfected with pSilencer2.1-U6 negative control (siRNA-control; Ambion). Overexpression of DJ-1 conferred a similar protective effect when neuroblastoma cells were exposed to hydrogen peroxide, as compared to rotenone or 6-hydroxydopamine (Fig. 2), implying that DJ-1 conferred resistance to the neurotoxins by protecting the cells from oxidative insults.

3.2. Correlation of intracellular ROS and DJ-1 expression levels

To verify whether the protection conferred by DJ-1 overexpression is related to the level of intracellular ROS, we measured intracellular ROS production using the fluorescent DCF method (in which the measured fluorescence correlates with the amount of intracellular ROS). Overexpression of DJ-1 decreased intracellular ROS with exposure to rotenone, 6-hydroxydopamine, or H_2O_2 (Fig. 3). No significant increase in intracellular ROS was observed by DJ-1 knockdown alone, probably due to the modest knockdown used. However, reduced DJ-1 levels, achieved through siRNA knockdown of DJ-1, resulted in increased intracellular ROS when the cells were exposed to the toxins (Fig. 3), indicating the central role of DJ-1 in the protection against toxic exposure. Therefore, overexpression of DJ-1 not only confers resistance to oxidative insults but also decreases intracellular ROS induced by these toxins.

3.3. Oxidative stress induces upregulation of endogenous DJ-1

Since we observed that overexpression of DJ-1 confers decreased intracellular ROS and increased resistance to the toxic effects of rotenone and 6-hydroxydopamine, we wanted to examine whether exposure to these neurotoxins affects the level of the endogenous DJ-1 protein. Indeed, exposure of naïve neuroblastoma cells to increasing concentrations of rotenone or 6-hydroxydopamine led to the upregulation of DJ-1, as indicated by the elevated levels of both DJ-1 mRNA and protein levels. Exposure to rotenone or to 6-hydroxydopamine led to a dose dependent increase in DJ-1 protein levels that were up to fourfold higher than those at baseline (Fig. 4A and B).

In order to examine whether the increased DJ-1 protein levels resulted from upregulation of DJ-1 or from a decreased breakdown, we quantified DJ-1 mRNA levels using real-time PCR. We found that rotenone as well as 6-hydroxydopamine

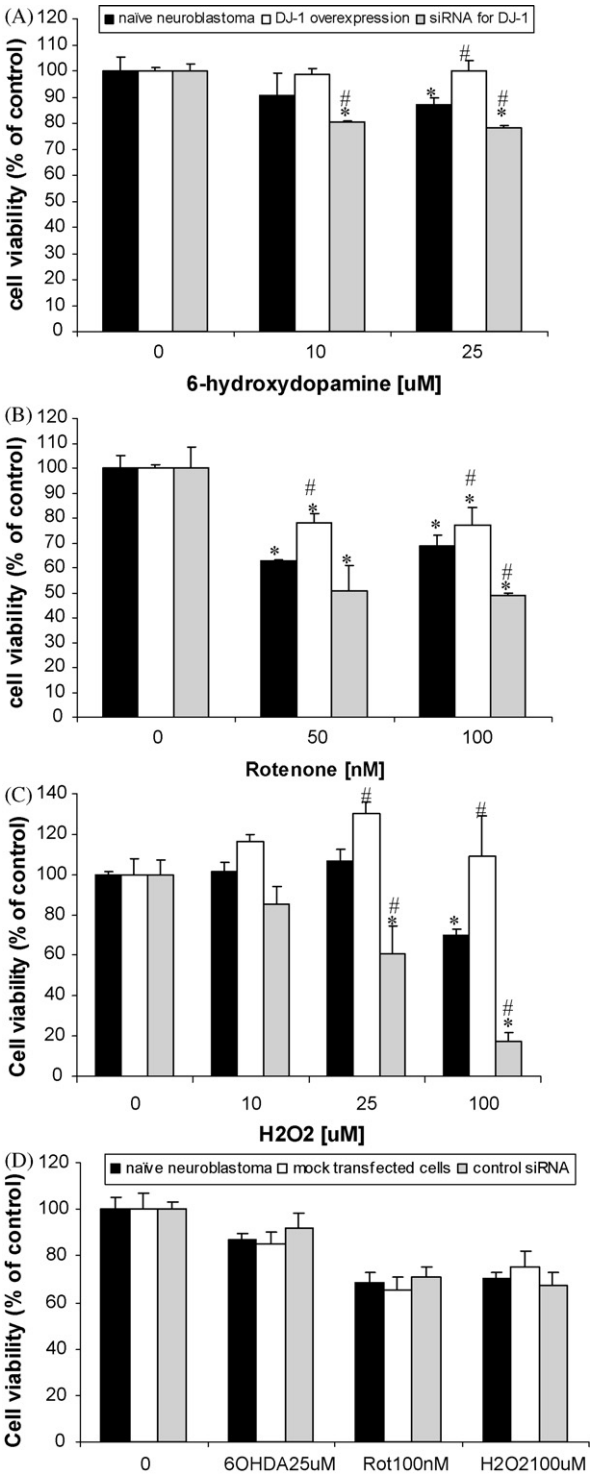

Fig. 2. Vulnerability of neuroblastoma cells to oxidative stress induced by toxins depends on DJ-1 expression levels. SH-SY5Y neuroblastoma cells were exposed to increasing doses of 6-hydroxydopamine (A), rotenone (B), or H_2O_2 (C) for 24 h. Toxins exposure resulted in a dose-dependent cell death. Overexpression of DJ-1 protected against 6-hydroxydopamine-induced (A), rotenone-induced (B), and H_2O_2-induced (C) cell death as measured by the MTT assay. Reduced DJ-1 expression by siRNA resulted in increased vulnerability to toxins exposure. Mock transfected cells and cells transfected with control siRNA did not statistically differ from naïve neuroblastoma cells in their vulnerability to these treatments (D). Error bars indicate mean ± S.D. $^{*}p < 0.05$ as compared to control. $^{\#}p < 0.05$ as compared to naïve neuroblastoma exposed to the same toxic dose. Each experiment was repeated three times in triplicates.

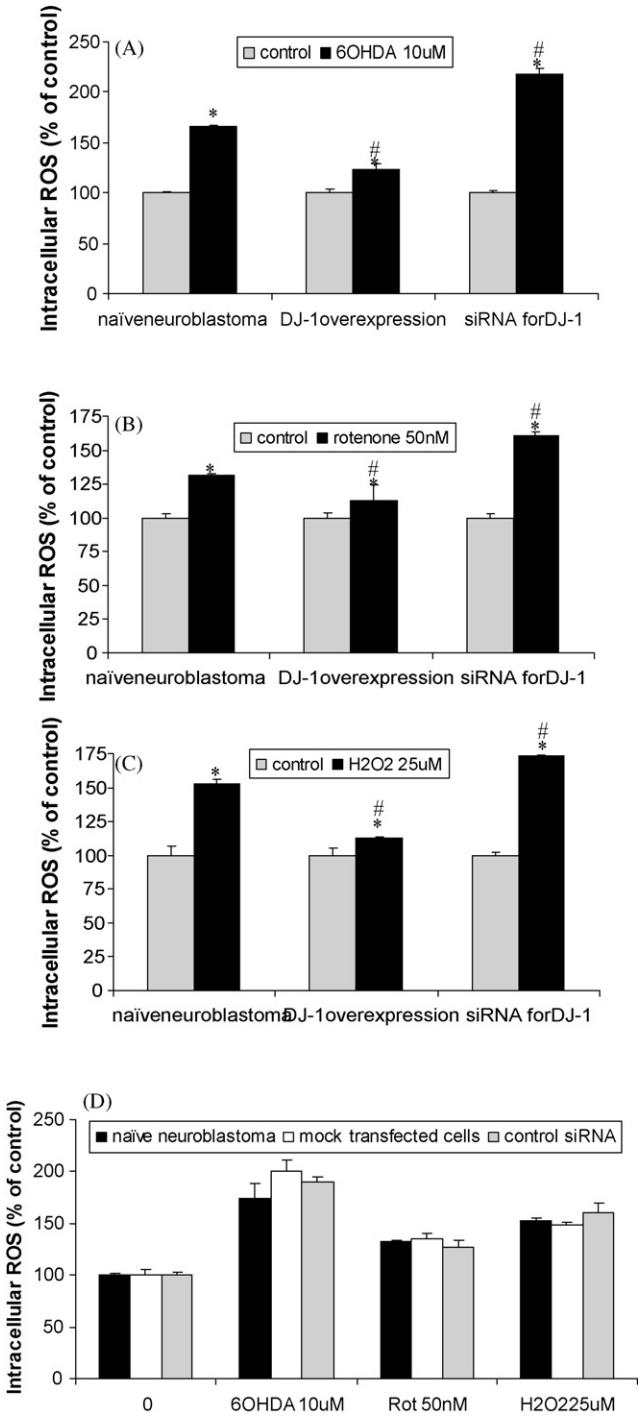

Fig. 3. DJ-1 overexpression decreases intracellular reactive oxygen species (ROS) induced by toxin exposure. DCF quantification of intracellular ROS indicates that exposure to 10 μM 6-hydroxydopamine (A), 50 nM rotenone (B), or to 25 μM H_2O_2 (C) results in accumulation of intracellular ROS in SH-SY5Y neuroblastoma cells. Overexpression of DJ-1 leads to decreased intracellular ROS with exposure to 6-hydroxydopamine (A), rotenone (B), as well as to H_2O_2 (C), while reduced DJ-1 expression by siRNA resulted in increased intracellular ROS with toxins exposure. Error bars indicate mean ± S.D. $^*p < 0.05$ as compared to control. $^{\#}p < 0.05$ as compared to naïve neuroblastoma exposed to the same toxic dose. Controls represent SH-SY5Y cells treated by serum free medium without toxin exposure. Intracellular ROS in cells exposed to vehicles was similar to cells treated with serum free medium (data not shown). Mock transfected cells and cells transfected with control siRNA did not statistically differ from naïve neuroblastoma cells in their intracellular ROS levels with exposure to these treatments (D). Each experiment was repeated three times in triplicates.

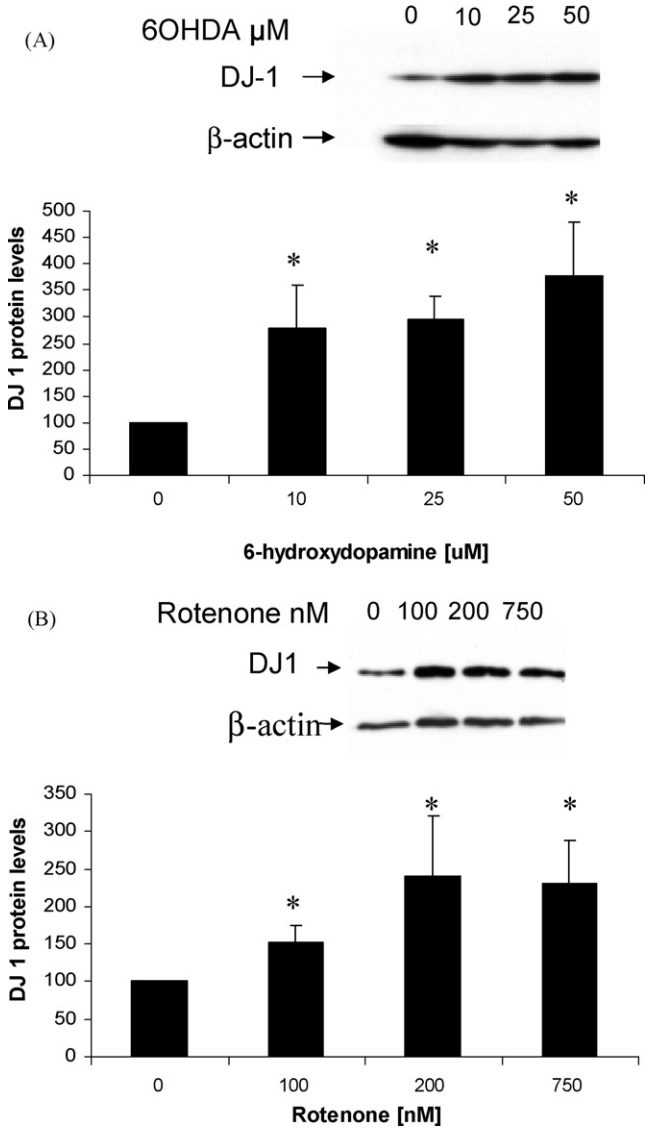

Fig. 4. Toxin exposure leads to upregulation of DJ-1 protein in naïve neuroblastoma cells. (A) Representative Western blot analysis demonstrating changes in DJ-1 protein levels 24 h after exposure of SH-SY5Y neuroblastoma cells to increasing doses of 6-hydroxydopamine. Quantification of DJ-1 protein levels as compared to beta-actin levels with exposure to increasing doses of 6-hydroxydopamine is shown in the graph. Error bars indicate mean ± S.D. $^*p < 0.05$. Each experiment was repeated three times in triplicates. (B) Representative Western blot analysis demonstrating changes in DJ-1 protein levels 24 h after exposure to increasing doses of rotenone. Quantification of DJ-1 protein levels as compared to beta-actin levels with exposure to increasing doses of rotenone is shown in the graph. Error bars indicate mean ± S.D. $^*p < 0.05$. Each experiment was repeated three times.

exposure led to a dramatic and dose dependent upregulation of DJ-1 mRNA (Fig. 5A and B). Elevation of DJ-1 mRNA was detected within 1 h after exposure to the toxic insult.

Pretreatment of the cells with the antioxidant *N*-acetyl cysteine (NAC) abrogated the observed upregulation of DJ-1 (Fig. 6). Treatment of neuroblastoma cells with NAC alone mildly decreased DJ-1 levels, due to a reduction in basal ROS (Fig. 6). This antioxidant effect implies that the upregulation of DJ-1 induced by these toxins indeed resulted from the oxidative stress.

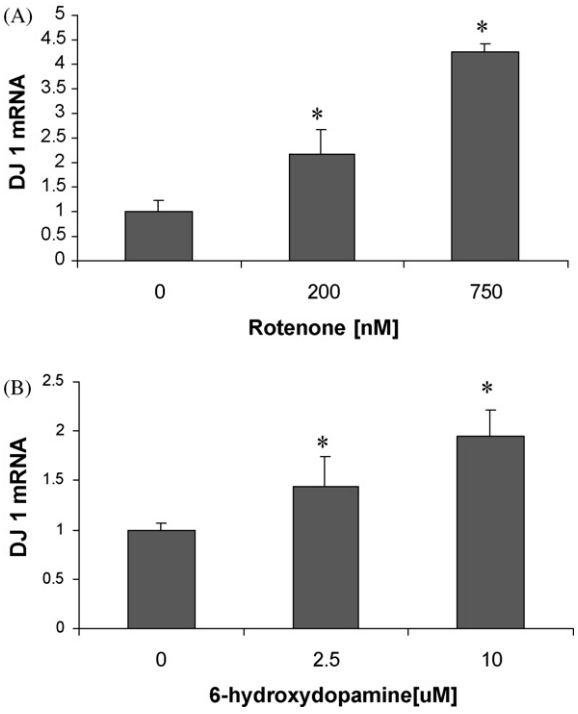

Fig. 5. Dose-dependent changes in DJ-1 mRNA levels after toxins exposure. (A) Quantitative real-time PCR demonstrated dose-dependent upregulation of DJ-1 mRNA of SH-SY5Y neuroblastoma cells within 1 h of rotenone exposure. (B) Similar changes in DJ-1 mRNA were demonstrated 1 h after 6-hydroxydopamine exposure. Error bars indicate mean ± S.D. $^{*}p < 0.05$. For quantification of DJ-1, GAPDH was used as housekeeping gene. Real time quantitative PCR (qPCR) was performed in triplicates. qPCR amplification was performed on the target gene, i.e. DJ-1, and the reference gene, GAPDH, for each sample, in separate wells of the same PCR reaction plate, which also showed a standard curve for each gene amplified and no template controls (NTC).

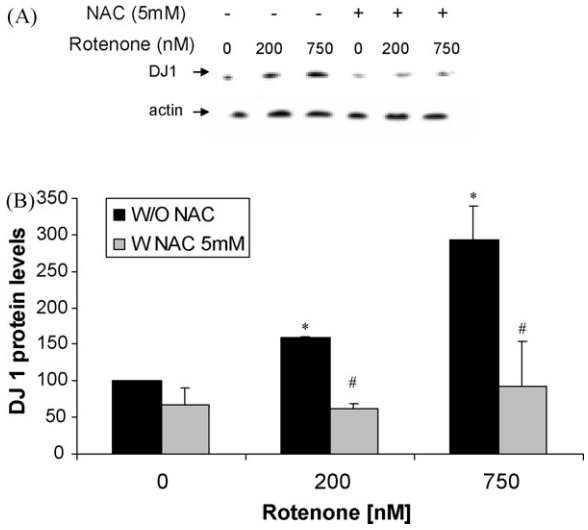

Fig. 6. Pre-treatment with antioxidant abolishes the elevation of DJ-1 levels. Representative Western blot analysis of DJ-1 expression levels of SH-SY5Y neuroblastoma cells in response to increasing levels of rotenone with and without pre-treatment with the antioxidant *N*-acetyl-cysteine (NAC) 5 mM. Quantification of DJ-1 protein levels as compared to beta-actin levels with exposure to increasing doses of rotenone with and without pretreatment with NAC is shown in the graph. Error bars indicate mean ± S.D. The experiment was repeated three times. The effect of NAC on DJ-1 expression was not statistically significant in cells not exposed to rotenone. In cells exposed to increasing levels of rotenone, pretreatment with NAC abolished elevation of DJ-1 levels and maintained DJ-1 levels similar to those of none exposed cells. $^{*}p < 0.05$ as compared to non-treated cells. $^{\#}p < 0.05$ as compared to naïve neuroblastoma exposed to the same toxic dose.

3.4. Oxidative insults lead to intracellular redistribution of DJ-1

Another facet of DJ-1 function in oxidative stress conditions is its intracellular distribution. At baseline, DJ-1 is present both in the cytoplasm and in the nucleus, as seen by immunocytochemical staining (Fig. 7A–C). Its predominant site is cytoplasmatic and diffused cytoplasmic staining for DJ-1 was observed (Fig. 7A–C).

Oxidative stress induced a visible increase in immunocytochemical staining for DJ-1, confirming the increased DJ-1 protein levels, that was also demonstrated by Western blot analysis (Fig. 4). Moreover, exposure to neurotoxins induced intracellular redistribution of DJ-1. Exposure to increasing doses of rotenone caused a shift of scattered cytoplasmatic DJ-1 into the mitochondria, in an apparent dose dependent manner (Fig. 7D–I). Exposure to 6-hydroxydopamine led to a similar shift of DJ-1 into the mitochondria (Fig. 7J–L). The redistribution of DJ-1 induced by these neurotoxins might serve as a part of the cellular defense mechanism.

4. Discussion

In this set of experiments we demonstrated that exposure to rotenone or 6-hydroxydopamine induces rapid upregulation of DJ-1 mRNA and protein levels in neuroblastoma cells. DJ-1 upregulation is related to oxidative stress induced by these toxins since pretreatment with an antioxidant abrogated DJ-1 upregulation. Overexpression of DJ-1 by stable transfection conferred increased resistance of neuroblastoma cells to oxidative insults induced by rotenone, 6-hydroxydopamine or hydrogen peroxide and reduced intracellular ROS induced by toxic exposure, while decreased DJ-1 levels by siRNA resulted in opposite effects. These results imply that DJ-1 has a role in the neural cellular defense mechanism against oxidative stress and explains the rapid upregulation of DJ-1 upon oxidative insult. Our results support previous findings that DJ-1 deficient mice are hypersensitive to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrindine (MPTP) (Kim et al., 2005) and that DJ-1alpha and DJ-1beta double knockout *Drosophila* flies display a striking sensitivity to environmental oxidative insults in vivo (Meulener et al., 2005; Yang et al., 2005).

Alongside the upregulation of DJ-1, oxidative insults also led to intracellular redistribution of DJ-1. At baseline conditions, DJ-1 immunoreactivity was noted both in the cytoplasm and the nucleus, with predominant diffuse cytoplasmic staining. Oxidative insults led to an increased DJ-1 immunostaining with marked staining in the mitochondria. Canet-Aviles et al. (2004) reported that wild-type DJ-1 showed little specific localization to mitochondria. However, treatment with paraquat induced the localization of DJ-1 to mitochondria. Zhang et al. (2005) reported that about 25% of DJ-1 is located in the mitochondria; however, they failed to detect any increase

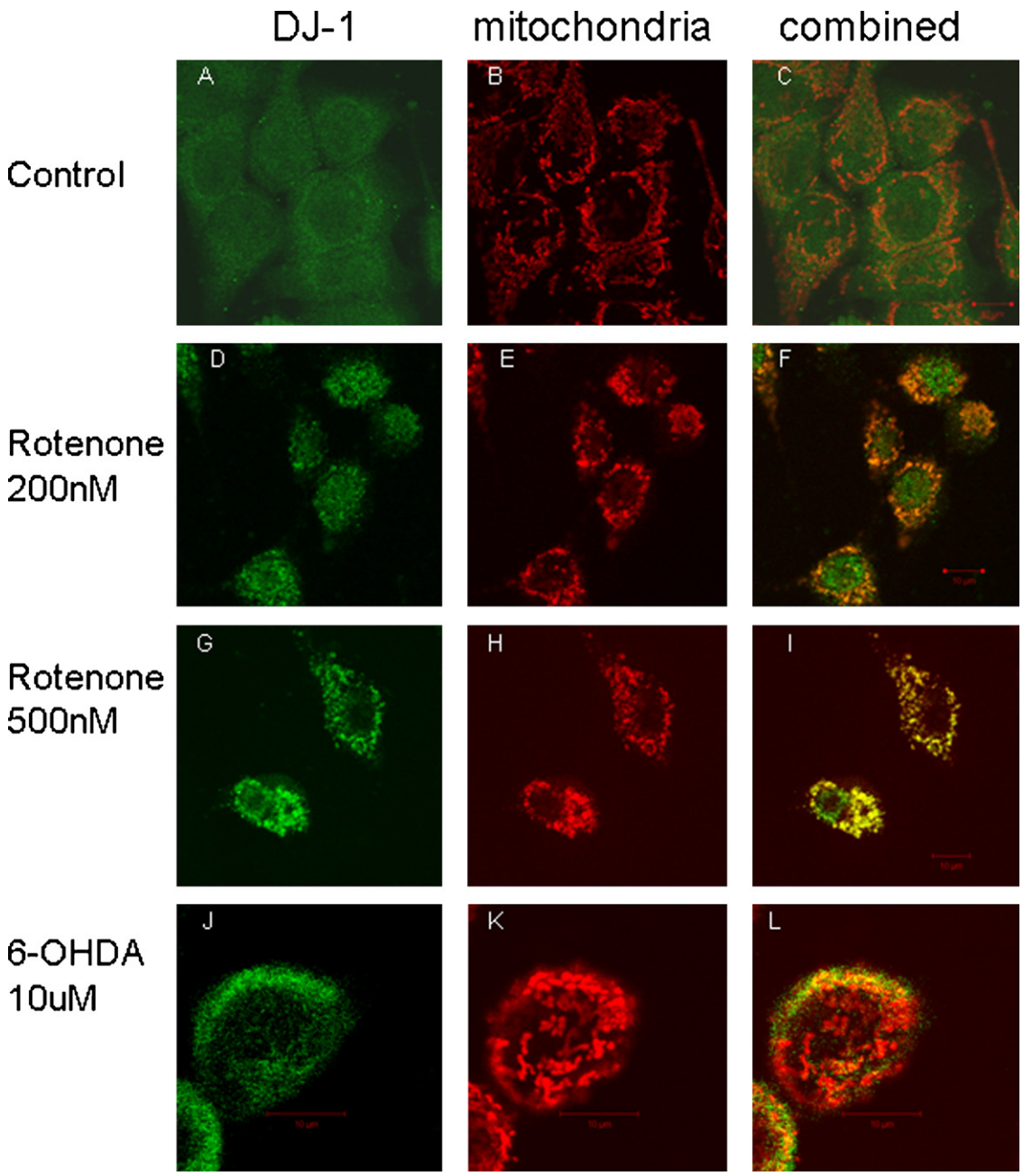

Fig. 7. Immunocytochemical analysis of DJ-1 protein intracellular distribution with exposure to rotenone and 6-hydroxydopamine. Immunocytochemical staining of SH-SY5Y neuroblastoma cells for DJ-1 (green, A) and human mitochondria (red, B) demonstrates that at basal conditions, DJ-1 is diffused in the cytoplasm and nuclei (C). With 24 h of exposure to 200 nM (D–F) or 500 nM (G–I) rotenone, the diffused cytoplasmic DJ-1 staining disappears and pronounced mitochondrial staining for DJ-1 is apparent. Similarly, after 24 h of exposure to 10 μM 6-hydroxydopamine mitochondrial staining for DJ-1 was apparent (J–L). Immunocytochemical staining was performed as described in Section 2. Staining was done in duplicates. In each experiment, specificity of the staining was verified by the absence of staining with second antibodies only, as well as by single immunocytochemical staining for each antibody alone. The experiment was repeated three times in duplicates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

in mitochondrial DJ-1 content after paraquat exposure. Our results support redistribution of DJ-1 into the mitochondria under oxidative stress conditions. The redistribution of DJ-1 induced by oxidative insults might serve as part of the cellular defense mechanism. Since the mitochondria have a central role in free radical generation, it is possible that the mitochondrial DJ-1 may have a role in preventing mitochondrial injury or decreasing mitochondrial ROS production. Another possible explanation is ROS scavenging by DJ-1 (Taira et al., 2004; Andres-Mateos et al., 2007). Taira et al. (2004) reported that DJ-1 eliminates hydrogen peroxide by protein oxidation, while it was shown recently that DJ-1 knockout mice have elevated mitochondrial hydrogen peroxide levels (Andres-Mateos et al., 2007).

In conclusion, upregulation and intracellular redistribution of DJ-1 were detected in response to several oxidative insults. Since overexpression of DJ-1 confers resistance to these toxic insults, we propose that these observed changes in DJ-1

expression and intracellular distribution might serve as a part of the neuronal cellular defense mechanism against oxidative insults. DJ-1 levels may serve as a biomarker for oxidative stress and aid in surveillance of disease activity. Moreover, developing methods to upregulate DJ-1 levels in cells in jeopardy may serve to protect them from further insults and enable the delay of disease progression.

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