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DJ-1 expression modulates astrocyte-mediated protection against neuronal oxidative stress

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Abstract

DJ-1 deficiency is a cause of genetic Parkinson's disease (PARK7 PD). In sporadic PD, however, DJ-1 is abundantly-expressed in reactive astrocytes. This may represent a compensatory protective response. In initial support of this hypothesis, we have shown *in vitro* that DJ-1 over-expressing astrocytes protect neurons against rotenone-induced death. Rotenone, a pesticide linked to increased PD risk, can stimulate oxidative stress. This process is implicated in PD pathogenesis. Since DJ-1 can enhance antioxidant systems, we hypothesized that augmenting its expression in astrocytes would protect co-cultured neurons against oxidative stress. We report here that DJ-1 over-expressing astrocytes were significantly more protective against rotenone-induced neuronal thiol oxidation than wild-type astrocytes in neuron-astrocyte co-cultures. DJ-1 knock-down astrocytes, on the other hand, were significantly impaired in their capacity to protect neurons against thiol oxidation. Each of these findings was replicated using astrocyte conditioned media on neuron-enriched cultures. Thus, DJ-1 modulated, astrocyte-released soluble factors must be involved in the mechanism. This is the first demonstration that the manipulation of a PD-causing gene in astrocytes affects their ability to protect neurons against oxidative stress.

Keywords

Astrocyte; glia; rotenone; oxidative stress; thiol; Parkinson disease

Introduction

Parkinson's disease (PD) brains show deficient mitochondrial Complex I activity and accelerated oxidative stress (Schapira et al. 1989; Giasson et al. 2002). Occupational exposure to Complex I inhibitors, including the pesticide rotenone, has been epidemiologically-linked with elevated PD risk (Ascherio et al. 2006; Brown et al. 2006; Gash et al. 2008; Tanner et al. 2011). Rotenone causes oxidative stress and experimental parkinsonism (Betarbet et al. 2000; Cannon et al. 2009; Panov et al. 2005). It is therefore reasonable to consider toxicant-induced Complex I inhibition and oxidative stress as potential contributors to PD pathogenesis.

DJ-1 was linked to PD when deletions in its gene (*PARK7*) were discovered to cause familial disease (Bonifati et al. 2003). We have demonstrated that DJ-1 is abundantly-expressed in PD astrocytes (Rizzu et al. 2004). We have also shown that astrocytic DJ-1 over-expression protects co-cultured neurons against rotenone-induced death, that astrocytic

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DJ-1 knock-down impairs this neuroprotective capacity, and that each process involves astrocyte-released soluble factors (Mullett and Hinkle 2009, 2011).

DJ-1 promotes antioxidant mechanisms (Blackinton et al. 2009; Liu et al. 2008; Aleyasin et al. 2007; Zhou and Freed 2005), and astrocytes release antioxidant molecules (Sukumari-Ramesh et al. 2010; Hirrlinger et al. 2002; Garg et al. 2009; Ishida et al. 2006; Vargas et al. 2006). Therefore, we hypothesized that (i) DJ-1 over-expression would enhance the capacity of astrocytes to protect co-cultured neurons against rotenone-induced oxidative stress, (ii) DJ-1 knock-down would impair the capacity of astrocytes to protect against neuronal oxidative stress, and that (iii) the mechanism in each case would involve astrocyte-released soluble factors.

Materials and Methods

Cell cultures

Primary cultures were generated as we have previously reported (Mullett and Hinkle 2009, 2011). Astrocytes were prepared from postnatal day 1 CD1 mouse cerebral cortex by dissociating tissues into Neurobasal media (Life Technologies, Grand Island, NY) containing 10% fetal calf serum (FCS, Hyclone, Logan, UT) and antibiotic-antimycotic (ABAM, Life Technologies). The plating density was 7.3×10^4 trypan blue-excluding cells/cm². The cultures were maintained in Dulbecco's modified Eagle media (DMEM)/F12 (Sigma, St. Louis, MO)/10% calf serum (CS, Hyclone)/ABAM and contained ~97% glial fibrillary acidic protein immunoreactive (GFAP+) astrocytes.

Neurons were prepared from embryonic day 15 (E15) cortex by plating 4.5×10^4 trypan blue-excluding cells/cm² in Neurobasal/10% FCS/ABAM onto previously-transfected astrocyte monolayers (contact co-cultures) or coverslips (neuron-enriched cultures). The cells were maintained in Neurobasal/1X B27 (Life Technologies)/0.5 mM GlutaMAX (Life Technologies)/ABAM until treatments commenced on astrocyte day in vitro (DIV) 20/ neuron DIV 6. These preparations contained >97% microtubule associated protein 2 immunoreactive (MAP2+) neurons.

DJ-1 knock-down and over-expression in astrocytes

Transfections with double-stranded anti-mouse DJ-1 siRNAs of sequence AGG CGC GGC TGC AGT CTT TAA (siDJ#2, Life Technologies) were used to knock down astrocyte DJ-1 to ~5% of endogenous levels (Mullett and Hinkle 2009). Non-silencing siRNAs of sequence AAT TCT CCG AAC GTG TCA CGT (siNS, Life Technologies) were used as transfection controls. The astrocytes were exposed to siRNAs over DIV 10–13. The siRNAs were then removed and the cells washed and allowed to recover overnight prior to E15 neuronal plating on astrocyte DIV 14.

Full-length mouse wild-type DJ-1 cDNA (mDJwt, NCBI ID BC002187, American Type Culture Collection)-containing pCMV-SPORT6 mammalian expression plasmid transfections were used to over-express DJ-1 in the astrocytes by ~2-fold (Mullett and Hinkle 2009). Vector plasmids were used as transfection controls. The astrocytes were exposed to the plasmids for 4 h on DIV 10. The plasmids were then removed and the cells washed and allowed to recover prior to E15 neuronal plating on astrocyte DIV 14.

The astrocytes were co-transfected with siRNAs + plasmids to produce transfection control (siNS + vector), DJ-1 knock-down (siDJ#2 + vector), or DJ-1 over-expressing (siNS + mDJwt) cells. This allowed for direct comparisons to be made with the appropriate transfection controls in place.

Experimental treatments

Neuron-transfected astrocyte co-cultures were exposed to rotenone (Sigma) 20 or 40 nM for 4 or 24 h. Rotenone required initial dilution into dimethyl sulfoxide (DMSO, Sigma) vehicle; treatments were then administered in Neurobasal/1X B27 antioxidant free/1X ABAM and compared to vehicle controls. For the astrocyte conditioned media experiments, 3 day media was collected from the transfected astrocytes and used to treat neuron-enriched cultures.

Quantification of neuronal thiol oxidation

Neuronal thiol oxidation was quantified as an indicator of oxidative stress using methods developed by the Greenamyre lab (Mastroberardino et al. 2008; Horowitz et al. 2011). Briefly, reduced/free thiols (-SH) were maleimide-labeled with 1 μ M Alexa Fluor 680 C₂ maleimide (Life Technologies) in 4% paraformaldehyde, 0.02% Triton X-100, and 1 mM N-ethylmaleimide (NEM, Sigma) in phosphate-buffered saline (PBS, pH 7.0) for 20 min at room temperature (RT). After washing the cells in PBS, oxidized/disulfide thiols (-S-S-) were reduced to free thiols using 5 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Sigma) in PBS for 20 min at RT. The newly-reduced free thiols were then maleimide-labeled with 1 μ M Alexa Fluor 546 C₅ maleimide (Life Technologies) in 1 mM NEM in PBS for 20 min at RT. 546-maleimide:680-maleimide emission ratios (SS/SH ratios) were then calculated selectively in MAP2+ neurons using a confocal microscope. High ratios represented a greater abundance of originally oxidized thiols, a signature of enhanced neuronal oxidative stress.

Statistical analysis

Independent cultures, transfections, and treatments were used for each experimental replicate. The data were analyzed by one way analysis of variance (ANOVA) followed by the Fisher test. The results were reported as significant when $p < 0.05$.

Results

We assessed the capacity of astrocytes to protect against neuronal thiol oxidation as a function of their DJ-1 expression level (Fig. 1). All data were collected at early time points (4 and 24 h) so that oxidative stress could be addressed in equal numbers of living neurons under conditions that are known to cause later cell death (Mullett and Hinkle 2009, 2011). Baseline differences were noted in neuronal thiol oxidation as a function of co-cultured astrocyte type in the 4 h no-rotenone group, but this was not seen in the 24 h no-rotenone group (Fig. 2). We concluded that the best overall interpretation was that no significant baseline differences existed.

Contact co-culture with DJ-1 over-expressing astrocytes significantly protected against rotenone-induced neuronal thiol oxidation relative to co-culture with transfection control or DJ-1 knock-down astrocytes (Fig. 2). DJ-1 over-expressing astrocyte conditioned media was also significantly more protective against rotenone-induced neuronal thiol oxidation than was transfection control or DJ-1 knock-down astrocyte conditioned media (Fig. 3).

Contact co-culture with DJ-1 knock-down astrocytes significantly enhanced rotenone-induced neuronal thiol oxidation relative to co-culture with transfection control and DJ-1 over-expressing astrocytes (Fig. 2). This result was not seen after 24 h exposure to 40 nM rotenone since the neurons co-cultured with transfection control astrocytes exhibited higher levels of thiol oxidation. DJ-1 knock-down astrocyte conditioned media was also significantly more permissive of rotenone-induced neuronal thiol oxidation than was transfection control or DJ-1 over-expressing astrocyte conditioned media (Fig. 3).

Discussion

This is the first demonstration that the manipulation of a PD-causing gene in astrocytes affects their capacity to protect neurons against rotenone-induced oxidative stress. This finding may be relevant to PD since oxidative stress is implicated in the mechanism of disease progression, and since rotenone exposure is linked to elevated PD risk (Tanner et al. 2011).

Reactive astrocytes abundantly-express DJ-1 in PD and other neurodegenerative diseases (Mullett et al. 2009; Rizzu et al. 2004; Bandopadhyay et al. 2004; Neumann et al. 2004). This may represent an attempt by astrocytes to protect themselves, and surrounding neurons, against disease progression (Mullett and Hinkle 2009). In initial support of this hypothesis, we have shown that DJ-1 over-expression in astrocytes enhances their neuroprotective capacity against rotenone and other pesticides *in vitro* (Mullett and Hinkle 2009, 2011).

We present here the first mechanistic data regarding neuroprotection in our *in vitro* system: DJ-1 over-expressing astrocytes, which model sporadic PD astrocytes, exhibited an enhanced capacity to protect co-cultured neurons against rotenone-induced oxidative stress. On the other hand, DJ-1 knock-down astrocytes, which model PARK7 PD astrocytes, were impaired in this capacity. Each of these findings was replicated by experiments using astrocyte conditioned media on enriched neuronal cultures.

Our data support the conclusion that astrocyte-released antioxidant molecules (or molecules that enhance antioxidant systems) may be involved in the neuroprotective mechanism. However, we have previously reported that supplementation with antioxidants such as glutathione, glutathione ethyl ester, and N-acetylcysteine does not protect neurons against rotenone-induced death in our model (Mullett and Hinkle 2011). Furthermore, although vitamin E supplementation does protect against neuronal death in this system, the neuroprotective disparity between DJ-1 knock-down and transfection control astrocytes remains (Mullett and Hinkle 2011). Therefore, although DJ-1 modulated, astrocyte-mediated neuroprotection against oxidative stress may be an important mechanistic *component* in our model, it cannot be the entire mechanism. Work is ongoing to identify the astrocyte-released factors and the other component neuronal pathways.

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Fig. 1.

Quantification of neuronal thiol oxidation as an indicator of neuronal oxidative stress. **a–c** The false-colored images represent SS/SH ratios assessed in MAP2+ neurons by confocal microscopy. High ratios (white-yellow) indicate high levels of neuronal thiol oxidation, whereas low ratios (blue-green) indicate low levels of neuronal thiol oxidation. Each image is from a neuron-astrocyte co-culture treated with 20 nM rotenone for 24 h. The DJ-1 expression status of each co-cultured astrocyte type is indicated below the figure. **d–f** MAP2+ (blue fluorescence) neuronal cell fields that correspond to the thiol oxidation images in a–c, respectively (boxes indicate the portions shown in the upper images). **g** Representative neuron-astrocyte co-culture showing the relative densities of each cell type at the time of assessments

Fig. 2.

DJ-1 over-expression in astrocytes protects contact co-cultured neurons against rotenone-induced oxidative stress. **a** Neuronal thiol oxidation, expressed as SS/SH ratios, was significantly reduced by co-culture with DJ-1 over-expressing astrocytes after 4 h of exposure to both 20 and 40 nM rotenone. Co-culture with DJ-1 knock-down astrocytes, on the other hand, allowed significantly more neuronal thiol oxidation under the same experimental conditions. **b** Similar results were seen when the co-cultures were exposed to the same doses of rotenone for 24 h. Asterisks (*) represent $p < 0.05$ for the indicated data vs. same dose/time transfection control, $n = 6$. All comparisons of same time DJ-1 over-expression vs. DJ-1 knock-down in the presence of rotenone were also significant

Fig. 3.

Conditioned media from DJ-1 over-expressing astrocytes protects neurons against rotenone-induced oxidative stress. Neuronal thiol oxidation, expressed as SS/SH ratios, was reduced when enriched neuronal cultures were exposed to 20 nM rotenone for 24 h in DJ-1 over-expressing astrocyte conditioned media (ACM). Under the same experimental conditions, DJ-1 knock-down ACM was more permissive of neuronal thiol oxidation. Asterisks (*) represent $p < 0.05$ for the indicated data vs. transfection control, $n = 4$. The comparison of DJ-1 over-expression vs. DJ-1 knock-down was also significant