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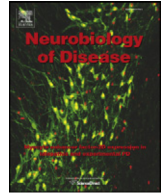

Increased 14-3-3 phosphorylation observed in Parkinson's disease reduces neuroprotective potential of 14-3-3 proteins

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ABSTRACT

14-3-3 proteins are key regulators of cell survival. We have previously demonstrated that 14-3-3 levels are decreased in an alpha-synuclein (αsyn) mouse model of Parkinson's disease (PD), and that overexpression of certain 14-3-3 isoforms is protective in several PD models. Here we examine whether changes in 14-3-3 phosphorylation may contribute to the neurodegenerative process in PD. We examine three key 14-3-3 phosphorylation sites that normally regulate 14-3-3 function, including serine 58 (S58), serine 184 (S184), and serine/threonine 232 (S/T232), in several models of PD and in human PD brain. We observed that an increase in S232 phosphorylation is observed in rotenone-treated neuroblastoma cells, in cells overexpressing αsyn, and in human PD brains. Alterations in S58 phosphorylation were less consistent in these models, and we did not observe any phosphorylation changes at S184. Phosphorylation at S232 induced by rotenone is reduced by casein kinase inhibitors, and is not dependent on αsyn. Mutation of the S232 site affected 14-3-3θ's neuroprotective effects against rotenone and 1-methyl-4-phenylpyridinium (MPP^+), with the S232D mutant lacking any protective effect compared to wildtype or S232A 14-3-3θ. The S232D mutant partially reduced the ability of 14-3-3θ to inhibit Bax activation in response to rotenone. Based on these findings, we propose that phosphorylation of 14-3-3s at serine 232 contributes to the neurodegenerative process in PD.

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Introduction

Disruption of 14-3-3 protein expression and function has been recently implicated in Parkinson's disease (PD) pathogenesis. The 14-3-3 proteins are a highly conserved family of proteins found throughout the evolutionary scale and are implicated in many cellular functions, including transcription, metabolism, and apoptosis (Dougherty and Morrison, 2004; Mackintosh, 2004). This protein family, which includes seven isoforms in mammals, is a key regulator of cell death and acts to promote cell survival through inhibition of many known pro-apoptotic factors (Porter et al., 2006; Masters and Fu, 2001). 14-3-3s have been shown to interact with several key proteins implicated in PD, including alpha-synuclein (αsyn), parkin, and leucine-rich repeat kinase 2 (LRRK2) (Dzamko et al., 2010; Li et al., 2011; Nichols et al., 2010; Ostrerova et al., 1999; Sato et al., 2006; Xu et al., 2002). 14-3-3s are a key hub of dysregulated proteins in a transcriptional analysis of PD patients (Ulitsky et al., 2010). 14-3-3s show homology to αsyn and coimmunoprecipitate with αsyn in normal brain (Ostrerova et al., 1999; Xu et al., 2002). Coimmunoprecipitation of 14-3-3s with αsyn is increased in the substantia nigra (SN) of PD brains (Sato et al., 2006; Xu et al., 2002), a predominant region involved in PD, and 14-3-3s colocalize with αsyn in Lewy Bodies (Berg et al., 2003; Kawamoto et al., 2002). Four isoforms have been shown to colocalize with αsyn in Lewy bodies in human PD, including 14-3-3ε, γ, θ, and ζ (Berg et al., 2003). We have previously shown that expression of several 14-3-3 isoforms is decreased with overexpression of wildtype human αsyn in neuroblastoma cells or transgenic mice (Yacoubian et al., 2008, 2010; Ding et al., 2013). Changes in 14-3-3θ and other isoforms are observed at the mRNA level in both the substantia nigra and cortex of an αsyn mouse model (Yacoubian et al., 2008, 2010). 14-3-3s are also key interactors of wildtype LRRK2, and several PD-associated LRRK2 mutants have been shown to be unable to bind 14-3-3s (Dzamko et al., 2010; Li et al., 2011; Nichols et al., 2010).

Because of 14-3-3s' anti-apoptotic role, we have previously hypothesized that disruption of 14-3-3s in PD could lead to the activation of cell death pathways that are normally inhibited by 14-3-3s. In support of this hypothesis, we have shown that overexpression of 14-3-3θ, ε, or γ reduced cell loss in response to the Parkinsonian toxins rotenone and 1-methyl-4-phenylpyridinium (MPP^+) in dopaminergic cell culture, while other isoforms showed variable effects (Yacoubian et al., 2010). Human 14-3-3θ and the *Caenorhabditis elegans* 14-3-3 homologue *ftt-2* also reduced cell loss in transgenic *C. elegans* that

Abbreviations: αsyn, alpha-synuclein; CK, casein kinase; GFP, green fluorescent protein; LDH, lactate dehydrogenase; LRRK2, leucine-rich repeat kinase 2; MPP^+, 1-Methyl-4-phenylpyridinium; n.s, not significant; PD, Parkinson's disease; PBS, phosphate-buffered saline; SN, substantia nigra; M17, SK-N-BE(2)-M17.

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overexpresses αsyn (Yacoubian et al., 2010). The neuroprotective effect of 14-3-3θ against rotenone toxicity is dependent on the inhibition of the pro-apoptotic factor Bax (Slone et al., 2011).

In this study, we evaluate whether altered phosphorylation of 14-3-3s may contribute to the dysfunction of 14-3-3s in PD. A well-recognized mechanism for regulating 14-3-3 function is phosphorylation of 14-3-3s at three conserved phosphorylation sites: serine 58 (S58), serine 184 (S184), and serine/threonine 232 (S/T232) (Aitken, 2002, 2006). S58 phosphorylation, found in all isoforms except 14-3-3σ and θ, has been shown to regulate dimerization (Powell et al., 2003; Woodcock et al., 2003). Phosphorylation at S184, found in 14-3-3β, ε, σ, and ζ, regulates ligand interactions (Sunayama et al., 2005; Tsuruta et al., 2004; Yoshida et al., 2005). Phosphorylation at both S58 and S184 has been linked to the release of pro-apoptotic factors and cell death (Sunayama et al., 2005; Tsuruta et al., 2004; Yoshida et al., 2005; Zhou et al., 2009). Least understood is phosphorylation at S/T232, found in 14-3-3θ and ζ (Clokie et al., 2005; Dubois et al., 1997). It may regulate ligand binding as the C-terminal loop can fold back into the peptide-binding pocket (Obsilova et al., 2004).

Kulanthingal et al. have previously demonstrated in a proteomics study that alterations in 14-3-3 phosphorylation are observed in neuroblastoma cells overexpressing αsyn (Kulathingal et al., 2009). Which phosphorylation sites and which isoforms are involved have not been fully examined, nor the consequences of such phosphorylation changes in PD models. In this study, we examine which phosphorylation sites are altered and the consequences of such changes. Specifically, we test whether any of the three key phosphorylation sites are altered in cells treated with rotenone, in cells that conditionally overexpress αsyn, and in human PD brains. We observe that changes in phosphorylation are observed at two of these phosphorylation sites, S58 and S232, and that phosphorylation at S232 reduces the neuroprotective effect of 14-3-3θ. These findings suggest that increased 14-3-3 phosphorylation observed in PD may promote neurodegeneration in PD.

Material and methods

Materials

Rotenone and MPP+ were purchased from Sigma (St. Louis, MO). 5,-6-Dichloro-1-β-ᴅ-ribofuranosyl-1H-benzimidazole (DRB) was purchased from Enzo Life Sciences (Farmingdale, NY) and 4-(4-(2,3-dihydrobenzo[1,4]dioxin-6-yl)-5-pyridin-2-yl-1H-imidazol-2-yl) benzamide (D4476) was obtained from EMD Millipore/Biosciences (Billerica, MA). Primary antibodies that were used include mouse monoclonal antibody against 14-3-3θ (Abcam, Cambridge, MA), rabbit polyclonal antibody against pan 14-3-3 isoforms (Abcam), rabbit polyclonal antibody against serine 232 14-3-3θ (Abcam), rabbit polyclonal antibody against serine 58 14-3-3ζ (Abcam), sheep polyclonal antibody against serine 184 14-3-3 (Enzo Life Sciences), mouse monoclonal antibody against V5 (Life Technologies, Grand Island, NY), mouse monoclonal 6a7 antibody against activated Bax (Sigma), rabbit polyclonal antibody against Bax (Cell Signaling, Danvers, MA), mouse monoclonal against cyclophilin D (EMD Biosciences), rabbit polyclonal antibody against cleaved caspase 3 (Cell Signaling), or mouse monoclonal antibody against α-tubulin (Sigma). Secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA).

Cell culture

SK-N-BE(2)-M17 (M17) cells (ATCC, Manassas, VA) and SH-SY5Y cells (ATCC) were grown in Eagle's minimal essential media (MEM) and F12K media (ATCC) at 1:1 ratio supplemented with 10% fetal bovine serum (SAFC Biosciences, Lenexa, KS) and 1% penicillin/streptomycin (Life Technologies) at 37 °C with 5% CO2 in a humidified incubator.

Animals

Wildtype C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME) or Charles River Laboratories (Wilmington, MA). BAC wildtype and G2019S LRRK2 hemizygous transgenic mice (Melrose et al., 2010) obtained from Heather Melrose were backcrossed on a C57BL/6 background and were bred with wildtype C57BL/6 mice from Jackson labs (Bar Harbor, ME). The use of mice was supervised by the University of Alabama Animal Resources Program in accordance with the PHS policy on Humane Care and Use of Laboratory Animals. The mice were euthanized by CO_2 inhalation.

Western blot

The cells were spun down at 1500 g for 5 min, washed in PBS, and then sonicated for 10 s on ice in lysis buffer (150 mM NaCl, 10 mM Tris–HCl (pH 7.4), 1 mM EGTA, 1 mM EDTA, 0.5% NP-40, protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA), phosphatase inhibitor cocktail (Roche)), followed by centrifugation at 16,000 *g* for 10 min. Protein concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, IL, USA). Samples were boiled for 5 min in 4 × DTT sample loading buffer (0.25 M Tris–HCl (pH 6.8), 8% SDS, 200 mM DTT, 30% glycerol, bromophenol blue), resolved on 15% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. Blots were blocked in 5% non-fat dry milk in TBST (25 mM Tris–HCl (pH 7.6), 137 mM NaCl, 0.1% Tween 20) for 1 h, and then incubated overnight with primary mouse monoclonal antibody against 14-3-3θ (1:1000), rabbit polyclonal antibody against pan 14-3-3 isoforms (1:1000), rabbit polyclonal antibody against serine 232 14-3-3θ (1:1000), rabbit polyclonal antibody against serine 58 14-3-3ζ (1:1000), sheep polyclonal antibody against serine 184 14-3-3 (1:1000), mouse monoclonal antibody against V5 (1:5000), rabbit polyclonal against cleaved caspase 3 (1:1000), or mouse monoclonal antibody against α-tubulin (1:5000). After three washes in TBST, the blots were incubated with HRP-conjugated goat anti-mouse, anti-sheep, or anti-rabbit secondary antibody (Jackson ImmunoResearch) for 2 h and then washed in TBST six times for 10 min each. The blots were developed with the enhanced chemiluminescence method (GE Healthcare, Piscataway, NJ, USA).

Hippocampi from mouse brains were homogenized in a lysis buffer (175 mM NaCl, 50 mM Tris–HCl, pH7.4, 5 mM EDTA with protease inhibitor and phosphatase inhibitor cocktails) and sonicated for 10 s. After the addition of 1% Triton X-100, the lysates were then incubated on ice for 30 min and spun at 15,000 *g* for 1 h at 4 °C. The supernatant samples were resolved on SDS-polyacrylamide gels and analyzed by Western blotting as described above.

Bax oligomerization assay

After rotenone treatment, the cells were lysed by nitrogen cavitation and enriched for mitochondria as previously described (Slone et al., 2011). The mitochondrial-enriched pellet was solubilized in a 2% CHAPS buffer and then crosslinked with 1 mM ethylene-glycol-bis(succinic acid N-hydroxy-succinimide ester) (Sigma) as previously described (Slone et al., 2011). The protein samples were analyzed for Bax monomers and oligomers by Western blotting using a polyclonal antibody against Bax (1:1000).

Creation of doxycycline-inducible αsyn and GFP cell lines

Green fluorescent protein (GFP) or human wildtype αsyn was cloned into the tetracycline-inducible lentiviral construct pSLIK (Shin et al., 2006). The M17 cells were infected with the αsyn virus in the presence of 6 μg/ml polybrene (Sigma). At 72 h after infection, the infected cells were selected in the presence of G418 (Life Technologies). To induce GFP or αsyn expression, the cells were treated with doxycycline (EMD Millipore/Biosciences) at 2 μg/ml.

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