Neuroscience 232 (2013) 90–105

DECREASED PARKIN SOLUBILITY IS ASSOCIATED WITH IMPAIRMENT OF AUTOPHAGY IN THE NIGROSTRIATUM OF SPORADIC PARKINSON'S DISEASE

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Abstract—Parkinson's disease (PD) is a motor disorder that involves death of dopaminergic neurons in the substantia nigra *pars compacta*. Parkin is an autosomal recessive gene that is mutated in early onset PD. We investigated the role of parkin and autophagic clearance in postmortem nigrostriatal tissues from 22 non-familial sporadic PD patients and 15 control samples. Parkin was insoluble with altered cytosolic expression in the nigrostriatum of sporadic PD. Parkin insolubility was associated with lack of degradation of ubiquitinated proteins and accumulation of α-Synuclein and parkin in autophagosomes, suggesting autophagic defects in PD. To test parkin's role in mediating autophagic clearance, we used lentiviral gene transfer to express human wild type or mutant parkin (T240R) with α-Synuclein in the rat striatum. Lentiviral expression of α-Synuclein led to accumulation of autophagic vacuoles, while co-expression of parkin with α-Synuclein facilitated autophagic clearance. Subcellular fractionation showed accumulation of α-Synuclein and tau hyper-phosphorylation (p-Tau) in autophagosomes in gene transfer models, similar to the effects observed in PD brains, but parkin expression led to protein deposition into lysosomes. However, parkin loss of function mutation did not affect autophagic clearance. Taken together, these data suggest that functional parkin regulates autophagosome clearance, while decreased parkin solubility may alter normal autophagy in sporadic PD.

Key words: α-Synuclein, autophagy, striatum, parkin, Parkinson disease.

INTRODUCTION

Parkinson's disease (PD) is predominantly sporadic, but some disease-causing mutations suggest a genetic component in disease pathogenesis. Rare mutations in a number of genes are associated with familial forms of PD (Gasser, 2009). Dominantly inherited mutations in leucine-rich repeat kinase 2 (LRRK2) and α-Synuclein cause late onset PD. Genome-wide association studies suggest that naturally occurring sequence variants in α-Synuclein and LRRK2, as well as Tau, constitute an increased risk for late onset sporadic PD (Martin-Villalba et al., 2001; Healy et al., 2004; Cookson and Bandmann, 2010). PD is characterized by death of dopaminergic neurons in the substantia nigra (SN) (Kuhn et al., 2006; Benner et al., 2008; Reynolds et al., 2008) and formation of inclusions known as Lewy bodies (LBs), which primarily contain aggregated α-Synuclein (Spillantini et al., 1997, 1998a,b; Wakabayashi et al., 1997; Goedert, 1999, 2001; Spillantini and Goedert, 2000; Takeda et al., 2000; Trojanowski and Lee, 2003; Lundvig et al., 2005). Mutations in autosomal recessively inherited genes like PARK-2, PTEN-induced kinase-1 (PINK1) and DJ-1, lead to early onset Parkinsonism (Kitada et al., 1998; Lucking et al., 2000; Cookson and Bandmann, 2010). Parkin is an E3 ubiquitin ligase involved in degradation of misfolded proteins (Shimura et al., 2000). Parkin is known to mediate selective autophagy of dysfunctional mitochondria (Narendra et al., 2008; Park et al., 2009; Geisler et al., 2010; Vives-Bauza et al., 2010), while α-Synuclein was suggested to impair autophagy (Winslow et al., 2010; Winslow and Rubinsztein, 2011). Normal autophagy is a multi-step process that involves generation of the phagophores, formation of autophagosomes, which fuse with endosomes to form amphisomes or with lysosomes to form autophagolysosomes (Kovács et al., 1982; Iwata et al., 2005; He and Klionsky, 2009). Changes in autophagy are recognized in neurodegeneration, where accumulation of autophagosomes are characterized by un-degraded autophagic vacuoles in neurons (Kegel et al., 2000; Nixon et al., 2005, 2008; Yang et al., 2007; Mizushima et al., 2008; Winslow and Rubinsztein, 2008).

To determine the role of parkin and its association with baseline autophagy in sporadic PD, we analyzed human postmortem nigrostriatal tissues via fractionation to determine protein solubility and investigated the effects of parkin on autophagic clearance in lentiviral gene transfer animal models. We sought to determine whether lentiviral expression of α-Synuclein affects autophagy

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Abbreviations: AD, Alzheimer's disease; ANOVA, analysis of variance; ARJPD, autosomal recessive juvenile PD; CMA, chaperon-mediated autophagy; DAPI, 4',6-diamidino-2-phenylindole; DLB, dementia with LB; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ELISA, enzyme-linked immunosorbent assay; GFAP, glial fibrillary acid protein; HDAC6, histone deacetylase 6; HDAC6, histone deacetylase 6; LBs, Lewy bodies; LC3, microtubule-associated light chain protein-3; LRRK2, leucine-rich repeat kinase 2; PBS, phosphate buffered saline; PD, Parkinson's disease; PMD, postmortem dissection; p-Tau, tau hyper-phosphorylation; SD, standard deviation; SN, substantia nigra; STEN buffer, Salt, Tris, EDTA, NP40; TH, tyrosine hydroxylase.

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http://dx.doi.org/10.1016/j.neuroscience.2012.12.018

and if parkin activity reverses α-Synuclein effects. We previously generated animal models expressing lentiviral α-Synuclein and found that parkin expression decreases α-Synuclein levels in the absence of ubiquitination (Burns et al., 2009; Khandelwal et al., 2010). Here we tested whether parkin expression regulates α-Synuclein clearance via autophagic degradation *in vivo*.

EXPERIMENTAL PROCEDURES

Human postmortem brain tissues – Human postmortem caudate and midbrain regions from 22 PD patients and 15 age-matched control subjects were obtained from Johns Hopkins University brain bank. The age, sex, stage of disease and postmortem dissection (PMD) are summarized for each patient in Tables 1 and 2. The cause of death is not known. To extract the soluble fraction of proteins, 0.5 g of frozen brain tissues were homogenized in 1 × Salt, Tris, EDTA, NP40 (STEN buffers) (50 mM Tris (pH 7.6), 150 mM NaCl, 2 mM EDTA, 0.2% NP-40, 0.2% Bovine Serum Albumin (BSA), 20 mM phenylmethanesulfonylfluoride and protease and phosphatase cocktail inhibitor), centrifuged at 10,000*g* for 20 min at 4 °C, and the supernatants were collected. All samples were then analyzed by enzyme-linked immunosorbent assay (ELISA) (see below) or Western blot using 30 μg of protein. To extract the insoluble fraction, the pellet was re-suspended in 4 M urea solution and centrifuged at 10,000*g* for 15 min, and the supernatant was collected and 30 μg of protein was analyzed by Western blot. Western blots were quantified by densitometry using Quantity One 4.6.3 software (Bio Rad). Densitometry was obtained as arbitrary numbers measuring band intensity. Data were analyzed as mean ± standard deviation (SD), using Two-tailed *t*-test ($P < 0.02$) and analysis of variance (ANOVA), Neumann–Keuls with multiple comparisons ($P < 0.05$) to compare PD and control groups.

Immunohistochemistry on slides from human patients was performed on 30-μm-thick paraffin-embedded brain slices de-paraffinized in Xylenes 2 × 5 min and sequential ethanol concentration, blocked for 1 h in 10% horse serum and incubated overnight with primary antibodies at 4 °C. After 3 × 10 min washes in 1 × phosphate buffered saline (PBS), the samples were incubated with the secondary antibodies for 1 h at RT, washed 3 × 10 min in 1 × PBS. Parkin was immunoprobed (1:200) with mouse anti-parkin (PRK8) antibody that recognizes a.a. 399–465 (Signet Labs, Dedham, MA) or rabbit polyclonal (1:200) anti-parkin (AB5112) antibody that recognizes a.a. 305–323 (Millipore) and counterstained with 4',6-diamidino-2-phenylindole (DAPI). Map 2 was probed (1:300) with mouse monoclonal antibody (Pierce). Glial fibrillary acid protein (GFAP) was probed (1:200) with mouse (GA5) Mouse mAb #3670 (Cell Signaling) or (1:200) rabbit polyclonal (ab4674) antibody (Abcam). Tyrosine hydroxylase (TH) was probed (1:100) with rabbit polyclonal (AB152) antibody (Millipore) and counterstained with DAB.

Stereotaxic injection – Lentiviral constructs were used to generate the animal models as explained in (Burns et al., 2009; Khandelwal et al., 2010; Herman and Moussa, 2011). Stereotaxic surgery was performed to inject the lentiviral constructs into the striatum of 2-month old male Sprague–Dawley rats. $N = 8$ animals were used in each treatment. A total of 116 animals were used in these studies. All procedures were approved by the Georgetown University Animal Care and Use Committee (GUACUC).

Western blot analysis – To extract the soluble protein fraction, brain tissues were homogenized in 1 × STEN buffer, centrifuged at 10,000*g* for 20 min at 4 °C, and the supernatants containing the soluble fraction of proteins were collected. To extract the insoluble fraction the pellet was re-suspended in 4 M urea or 30% formic acid and adjusted to pH 7 with 1 N NaOH and centrifuged at 10,000*g* for 20 min at 4 °C, and the supernatant containing the insoluble fraction was collected and analyzed by Western blot. Total parkin was immunoprobed (1:1000) with PRK8 antibody as indicated (Burns et al., 2009) and phospho–parkin was probed (1:1000) with anti-Ser 378 antibodies (Pierce). α-Synuclein was probed with rabbit monoclonal (1:1000) antibody (Santa Cruz). Autophagy antibodies, including beclin-1 (1:1000), autophagy like gene (Atg)-7 (1:1000), Atg12 (1:1000) and LC3-B (1:1000), were used to probe according to autophagy antibody sampler kit 4445 (Cell Signaling, Inc.). Histone deacetylase 6 (HDAC6) was probed (1:500) using rabbit polyclonal anti-HDAC6 (Abcam). Rabbit polyclonal anti-SQSTM1/p62 (Cell Signaling Technology) was used (1:500). A rabbit polyclonal (Pierce) anti-LC3 (1:1000) and rabbit polyclonal (Thermo Scientific) anti-actin (1:1000) were used. LAMP-3 was probed (1:500) with rabbit polyclonal antibody (Aviva Systems). Rabbit anti-ubiquitin (Santa Cruz Biotechnology) antibody

Table 1. Description and clinical diagnosis of human PD patients and control subjects's tissues analyzed by Western blot and ELISA. PMD, post-mortem dissection

BRC #	diagnosis	Age	Sex	Race	PMD	Area
399	Control	79	F	W	24	Caudate
417	Control	80	F	W	6	Caudate
487	Control	73	M	W	22	Caudate
515	Control	62	M	W	19	Caudate
705	Control	73	M	W	9	Caudate
1277	Control	80	F	W	8	Caudate
2052	Control	79	M	W	16	Caudate
1690	PD	76	M	W	18	Caudate
1731	PD	77	M	W	16	Caudate
2140	PD with dementia	84	F	W	11	Caudate
2067	PD with dementia, cerebrovas. dis (NC)	76	M	W	19	Caudate
2019	PD with dementia, cerebrovas. dis	83	M	W	16.5	Caudate
1989	PD with dementia, LBD neocortical	84	M	W	5	Caudate
2074	PD, cerebrovascular disease	85	F	W	9	Caudate
1758	PD, DLB	81	M	W	11	Caudate
1948	PD, DLB	77	M	W	5	Caudate
1796	PD, Lewy body CHG limbic, porencephalic cyst	81	M	W	8.75	Caudate
1877	PD, Lewy body CHG neocortical	80	M	W	19	Caudate
1955	PD, Lewy body CHG neocortical	84	M	B	13	Caudate