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2 DYSREGULATION OF PARKIN IN THE SUBSTANTIA NIGRA OF *DB/DB* 3 AND HIGH-FAT DIET MICE

4 **R. KHANG,** [a] **C. PARK** [b,c] **AND J.-H. SHIN** [a,c*]

5 [a] *Division of Pharmacology, Department of Molecular Cell*
6 *Biology, Samsung Biomedical Research Institute,*
7 *Sungkyunkwan University School of Medicine, Suwon, Republic of*
8 *Korea*

9 [b] *Division of Biochemistry and Molecular Biology, Department of*
10 *Molecular Cell Biology, Samsung Biomedical Research Institute,*
11 *Sungkyunkwan University School of Medicine, Suwon, Republic of*
12 *Korea*

13 [c] *Mass Spectrometry, Research Core Facility, Samsung*
14 *Biomedical Research Institute, Sungkyunkwan University School*
15 *of Medicine, Suwon, Republic of Korea*

16 **Abstract—Parkinson's disease (PD) is characterized by selective loss of dopaminergic neurons in the substantia nigra (SN). Epidemiological evidence has suggested a link between type 2 diabetes and PD, although the mechanisms remain largely unknown. We applied LC–MS/MS-based pattern analysis to investigate altered proteomes in the SN of *db/db* mice (db-SN) and high-fat diet mice (HFD-SN), revealing that the level of mitochondrial proteins has changed in the SN of diabetic mice compared to that of control mice. Since mitochondrial proteins were robustly altered in db-SN and HFD-SN, we performed immunoblot analysis to monitor the level of parkin, PINK1 (phosphatase and tensin homolog-induced putative kinase 1) and DJ-1 that were directly involved in mitochondrial dynamics. As a result, PINK1 and DJ-1 level was unchanged, whereas a significant loss of parkin was found in db-SN and HFD-SN, leading to the accumulation of parkin-interacting substrate (PARIS) and the reduction of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). Interestingly, these alterations were reversed by the administration of metformin, one of most frequently prescribed anti-hyperglycemic agents. The slight loss of dopaminergic neurons was found in chronic HFD-SN that was restored by metformin. Taken together, our data suggest that the dysregulation of Parkin–PARIS–PGC-1α pathway by metabolic malregulation may contribute to the pathogenesis of PD and metformin might exert a neuroprotective effect on PD via the restoration of parkin.** **© 2015 Published by Elsevier Ltd. on behalf of IBRO.**

Key words: Parkinson's disease, parkin, PARIS, type 2 diabetes, metformin.

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INTRODUCTION 18

Parkinson's disease (PD) is the most common movement disorder and is characterized by selective and massive loss of dopaminergic neurons (DA) in the substantia nigra pars compacta (SNpc) (Savitt et al., 2006). PD is characterized by a series of classic motor symptoms, including resting tremor, rigidity of the skeletal muscles of the face and hands, bradykinesia, and postural instability (Savitt et al., 2006). Several PD-associated genes, including *parkin*, *α-synuclein*, *leucine-rich repeat kinase 2* (*LRRK2*), *DJ-1*, *PINK1*, and *ATP13A2* have been identified, and an investigation of their biology has shed light on the pathogenesis of PD. Mutation of parkin, an E3 ubiquitin ligase, results in autosomal recessive juvenile Parkinsonism (Kitada et al., 1998). In sporadic PD, post-translational modification of parkin by nitrosative, oxidative, dopaminergic stress, and c-Abl lead to loss of the catalytic activity of parkin, resulting in the accumulation of toxic substrates and neuronal death (Dawson and Dawson, 2014).

Type 2 diabetes (T2D), the most common type of diabetes, is characterized by peripheral insulin resistance and impaired insulin secretion from pancreatic β-cell insulin in response to hyperglycemia (Lu and Hu, 2012). An increasing number of epidemiological studies have focused on the relationship between diabetes and the risk of PD (Lu and Hu, 2012; Santiago and Potashkin, 2013). A recent prospective study following 51,552 Finnish men and women with no history of PD at the baseline showed that T2D is associated with an increased risk of PD (Hu et al., 2007). This observation was strongly supported by the results of another study of 1565 PD patients showing that the risk of PD is 40% higher in diabetic PD patients than in non-diabetic patients (Xu et al., 2011), suggesting that there may be a common physiological pathway between PD and T2D (Schernhammer et al., 2011).

*Correspondence to: J.-H. Shin, Division of Pharmacology, Department of Molecular Cell Biology, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon, Gyeonggi-do 440-746, Republic of Korea. Tel: +82-031-299-6192; fax: +82-031-299-6209.
E-mail address: jshin24@skku.edu (J.-H. Shin).

Abbreviations: AIMP2, aminoacyl-tRNA synthetase complex-interacting multifunctional protein 2; CTX, cerebral cortex; Chow-SN, SN of chow-diet mice; DA, dopaminergic neurons; FBP1, far-upstream element (FUSE) binding protein 1; GO, gene ontology; HFD, high-fat diet; HRP, horseradish peroxidase; IR-SH, insulin-resistant SH-SY5Y cells; PARIS, parkin interacting substrate; PBS, phosphate-buffered saline; PD, Parkinson's disease; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PINK1, phosphatase and tensin homolog-induced putative kinase 1; SN, substantia nigra; STR, striatum; STRAP, software tool for researching annotations of proteins; T2D, type 2 diabetes; TH, tyrosine hydroxylase; WT-SN, SN of wild-type littermate mice.

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In this study, we utilized a proteomic approach to investigate changes of proteome in the SN of two T2D animal models, *db/db* (db-SN) and high-fat diet mice (HFD-SN). T2D models exhibited a loss of parkin, accumulation of parkin-interacting substrate (PARIS), and suppression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) in the SN. Furthermore, the anti-diabetic drug metformin restored levels of parkin and PGC-1α, suggesting that metabolic dysfunction might play a deleterious role in the pathogenesis of PD.

EXPERIMENTAL PROCEDURES

Animal experiments

All animal experiments were approved by the Sungkyunkwan University Ethics Committee, according to international guidelines. All efforts were made to minimize animal suffering and to reduce the number of animals used. All mice were maintained under a 12-h dark/12-h light cycle in air-controlled rooms and fed diet and water *ad libitum*. Experiments were performed with 3-month-old control ($Lepr^{+}/Lepr^{+}$ (WT)) and *db/db* ($Lepr^{db}/Lepr^{db}$ *mice*, protocol 000697; Jackson Laboratories, Bar Harbor, ME, USA) mice exhibiting severe obesity ($n = 10$) (Chen et al., 1996). Male C57BL/6J mice (Orient, Sungnam, Republic of Korea) were fed a chow-diet or high-fat diet (60% fat; Research diet, New Brunswick, NJ, USA) for 8 or 20 weeks ($n = 10$ per group) (Van Heek et al., 1997). Diabetic *db/db* and HFD mice were administrated clinical doses of metformin (400 mg/kg once daily for 2 weeks or indicated period) ($n = 3$) (Martin-Montalvo et al., 2013).

Sample preparation

Animals were perfused transcardially with phosphate-buffered saline (PBS) (pH 7.4) under pentobarbital anesthesia (50 mg/kg, intraperitoneal injection). Whole brains were removed and dissected to obtain SN, striatum (STR), and cerebral cortex (CTX). Brain tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 100× protease/phosphatase inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, USA) followed by three cycles of freezing/thawing. The concentration of the supernatant was determined by BCA assay.

LC–MS/MS, quantitative protein profiling, statistics and database searching

The lysate of db-SN or HFD-SN was loaded on 1DE-SDS–PAGE and total protein was visualized by colloidal Coomassie Blue (Novex, San Diego, CA, USA). Each lane was cut into 10 gel pieces and subsequently destained, reduced, alkylated and digested with modified sequencing grade trypsin (Sigma–Aldrich, St. Louis, MO, USA) as described (Shevchenko et al., 2006). We performed LC–MS/MS as described (Khang et al., 2014). Briefly, peptide mixtures were lyophilized, resuspended in 0.1% TFA, and injected in a Zorbox 300SB-C18 75-μm i.d. × 15-cm column (Agilent Technologies, Waldbronn, Germany). Peptides were separated by an UltiMate 3000 nano HPLC system (Dionex, Sunnyvale, CA, USA) and applied on-line to an LTQ (Thermo Finnigan, Waltham, MA, USA) ion-trap mass spectrometer. Spectra were collected in full-scan mode (350–1600 Da) followed by MS/MS scans of the five most intense ion peaks obtained from the full scan using dynamic exclusion criteria. LC/MS runs were analyzed using DeCyder MS software (version 2.0; GE Healthcare, Uppsala, Sweden). The relative abundance of each peptide in the respective gradient fraction was determined by peak integration. The threshold for differentially expressed proteins was defined as an at least 2-fold increase or decrease. MS/MS spectra were searched using MASCOTTM v2.3 (Matrix Science, London, UK). Proteins identified by multiple peptides with a significant MASCOT score ($p < 0.05$) were used for quantitative protein profiling.

In silico analysis of functional associations

Software tool for researching annotations of proteins (STRAP, http://www.bumc.bu.edu/cardiovascularproteomics/cpctools/; Boston University School of Medicine, Boston, USA) (Bhatia et al., 2009) was utilized to classify proteins into biological process, cellular component, and molecular function based on gene ontology (GO). A functional association network of proteins identified was generated using STRING 8.3 web server (http://string-db.org/) (Jensen et al., 2009).

Western blot

Protein samples (40 μg) were separated on SDS–PAGE (7.5–15%) (Mini Protean II, Bio Rad, Hercules, CA, USA) using the Laemmli sample buffer system (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and proteins were transferred to nitrocellulose membranes (Hybond ECL, Amersham, Amersham, UK). Membranes were blocked with 5% (w/v) non-fat dried milk in TBS-T for 3 h at room temperature and probed with primary antibodies overnight at 4 °C followed by the appropriate HRP (horseradish peroxidase)-conjugated secondary antibody for 1 h (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The primary antibodies used in this study were as follows: α-p-AKT (Ser473; Cell Signaling Technology, Danvers, MA, USA), α-AKT (Cell Signaling Technology, Danvers, MA, USA), α-Parkin (Cell signaling Technology, Danvers, MA, USA), α-PINK1 (Novus Biologicals, Littleton, CO, USA), α-DJ-1 (Santa Cruz biotechnology, Inc., Santa Cruz, CA, USA), α-FBP1 (BD Transduction Laboratories, San Jose, CA, USA), α-AIMP2 (Proteintech group, Inc., Chicago, IL, USA), α-PARIS (Merck Millipore, Billerica, MA, USA), α-PGC-1α (Calbiochem, Merck Millipore, Billerica, MA, USA), and α-actin-HRP (Abcam, Cambridge, UK). Bands were visualized with ECL-system reagents (Amersham, Amersham, UK). Band densities were quantified using NIH Image J and normalized to β-actin.

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