Mitochondrial Dysfunction and Oxidative Damage in parkin-deficient Mice*

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Loss-of-function mutations in parkin are the predominant cause of familial Parkinson’s disease. We previously reported that parkin−/− mice exhibit nigrostriatal deficits in the absence of nigral degeneration. Parkin has been shown to function as an E3 ubiquitin ligase. Loss of parkin function, therefore, has been hypothesized to cause nigral degeneration via an aberrant accumulation of its substrates. Here we employed a proteomic approach to determine whether loss of parkin function results in alterations in abundance and/or modification of proteins in the ventral midbrain of parkin−/− mice. Two-dimensional gel electrophoresis followed by mass spectrometry revealed decreased abundance of a number of proteins involved in mitochondrial function or oxidative stress. Consistent with reductions in several subunits of complexes I and IV, functional assays showed reductions in respiratory capacity of striatal mitochondria isolated from parkin−/− mice. Electron microscopic analysis revealed no gross morphological abnormalities in striatal mitochondria of parkin−/− mice. In addition, parkin−/− mice showed a delayed rate of weight gain, suggesting broader metabolic abnormalities. Accompanying these deficits in mitochondrial function, parkin−/− mice also exhibited decreased levels of proteins involved in protection from oxidative stress. Consistent with these findings, parkin−/− mice showed decreased serum antioxidant capacity and increased protein and lipid peroxidation. The combination of proteomic, genetic, and physiological analyses reveal an essential role for parkin in the regulation of mitochondrial function and provide the first direct evidence of mitochondrial dysfunction and oxidative damage in the absence of nigral degeneration in a genetic mouse model of Parkinson’s disease.

Parkinson’s disease (PD) is the second most prevalent neurodegenerative disease. Clinical manifestations of PD include postural instability, bradykinesia, resting tremor, and rigidity. Neuropathologically, the disease is characterized by the selective degeneration of the dopaminergic neurons in the substantia nigra (1). The etiology of PD is still unknown, although clinical and experimental evidence implicate the involvement of mitochondrial dysfunction (2, 3) and oxidative stress (4, 5). Analysis of mitochondria isolated from idiopathic PD patients showed inhibited capacity of NADH-ubiquinone reductase, complex I of the mitochondrial electron transport chain, and increased production of reactive oxygen species (ROS) (6). Similar changes have been seen in autopsy cases of patients with presymptomatic PD, suggesting that mitochondrial dysfunction and oxidative stress precede clinical manifestations (7).

Exposure to selective neurotoxins, including paraquat and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), has been linked to either increased risk of PD or chemically induced parkinsonism (8, 9). These compounds have been shown experimentally to decrease mitochondrial function and selectively inhibit the activity of complex I (10). In vitro chemical inhibition of complex I results in reduced oxidative phosphorylation and increased mitochondrial generation of ROS, similar to what was observed in mitochondria from PD patients (11–13). Pathological examinations of PD brains have revealed increases in protein and lipid byproducts of ROS, including protein carbonyls and 4-hydroxynonenal (4HNE) (14, 15). Furthermore, 4HNE forms adducts with and inhibits the activities of the D1 dopamine (DA) receptor and the DA transporter (DAT), suggesting that lipid peroxides may contribute to the disruption of DA signaling (16, 17). Cultured dopaminergic neurons have been shown to exhibit enhanced sensitivity to paraquat and MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) as well as ROS (18). These findings suggest that mitochondrial dysfunction and accompanying ROS generation could be a common mechanism for the selective loss of substantia nigra neurons and the nigrostriatal DA signal in PD (19).

In addition to the more prevalent, idiopathic form, a subset of PD patients exhibits familial inheritance patterns. Large numbers and varieties of autosomal recessively inherited mutations in parkin are the predominant cause of familial PD (20). Initially described as juvenile-onset, atypical parkinsonism lacking Lewy bodies, subsequently identified cases are often clinically and pathologically indistinguishable from early onset familial or sporadic PD, including the presence of Lewy bodies in a single case (21–23). We have recently reported that the loss of parkin function in mice results in nigrostriatal dysfunction, as evidenced by increased extracellular dopamine concentra-

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tion in the stratum, reduced synaptic excitability in the striatal neurons, and behavioral deficits in paradigms that are sensitive to alterations in the nigrostriatal pathway (24). Despite measurable differences in nigrostriatal function in parkin−/− mice (24), no reduction in the number of dopaminergic neurons was observed in two independently generated parkin−/− mice (24, 25).

Parkin has been reported as an E3 ubiquitin-protein ligase (26). Previous reports described several substrates for parkin-mediated ubiquitylation (27). It has been suggested that the loss of parkin function results in aberrant accumulation of mediators of mitochondrial dysfunction, protection of monoaminergic neurons against proteasomal dysfunctions, and kainic acid-induced toxicity (30). It was shown that parkin−/− mice (24, 25), no reduction in the number of dopaminergic neurons was observed in two independently generated parkin−/− mice (24, 25).

In the substantia nigra (28). However, steady-state levels of p53 have been postulated to confer toxicity upon dopaminergic neurons (29). Previous reports described several substrates for parkin-mediated ubiquitylation (27). It has been suggested that the loss of parkin function results in aberrant accumulation of mediators of mitochondrial dysfunction, protection of monoaminergic neurons against proteasomal dysfunctions, and kainic acid-induced toxicity (30). It was shown that parkin−/− mice (24, 25), no reduction in the number of dopaminergic neurons was observed in two independently generated parkin−/− mice (24, 25).

Recent evidence has also suggested a role for parkin in the protection of monoaminergic neurons against proteasomal dysfunction, α-synuclein overexpression-mediated cell death (29), and kainic acid-induced toxicity (30). It was shown that parkin is localized in mitochondria and inhibits mitochondria-dependent cell death (31). Other studies demonstrate that overexpression of mutant parkin elevates cellular markers of oxidative stress, whereas overexpression of wild-type parkin results in reduced levels of these markers (32). These observations are consistent with findings from parkin-null flies, which exhibit altered mitochondrial morphology and degeneration of tissues such as wing flight muscles and spermatocytes (33). These results raised the possibility that parkin may be involved in mitochondrial function. Based on these observations, we hypothesized that lack of parkin function may cause impairment of mitochondrial function in parkin−/− mice.

To determine whether a lack of parkin causes changes in protein abundance and/or modification, we conducted a nonbiased proteomic analysis of the ventral midbrain of parkin−/− and wild-type mice. Using a well established method for two-dimensional analysis of brain lysates (34), we were able to detect ~8000 discrete protein spots from extracts of the ventral midbrain of parkin−/− and wild-type mice. Comparative analysis of 10 pairs of wild-type and parkin−/− brain samples revealed reproducible, quantitative changes of fifteen protein spots by silver staining. Subsequent mass spectrometric (MS) analysis revealed that these 15 spots represented 14 distinct proteins, 13 of which exhibited decreases in abundance in brains of parkin−/− mice and 1 additional protein which exhibited altered electrophoretic mobility, consistent with differential post-translational modification. Eight of these proteins were involved in either oxidative phosphorylation or antioxidant activities. Consistent with these findings, parkin−/− mice exhibited decreases in oxidative phosphorylation, weight gain, and antioxidant capacity as well as increased ROS-mediated tissue damage, suggesting an essential role for parkin in regulating normal respiratory function of mitochondria as well as in the protection of cells from oxidative stress.

**MATERIALS AND METHODS**

**Mice**—Mice bearing a germline disruption of exon 3 of the parkin gene were generated as previously described (24). Mice used for all studies of the proteomic analysis were in the hybrid background of C57BL/6 and 129/Sv. Mice used for proteomic studies were the 129/Sv inbred strain.

**Two-dimensional Gel Electrophoresis and Mass Spectrometry**—Protein samples for two-dimensional gel electrophoresis were prepared from the dissected ventral midbrain (including the substantia nigra) of each of the 10 pairs of parkin−/− and wild-type mice as previously described (35) with the following modifications. The solutions used for extraction were 100 mM phosphate buffer, pH 7.1 (0.2 M KCl, 20% w/v 4% w/v 3-[3-chloro-3-dimethylammonio]-1-propane-sulfonate) (A), protease inhibitor solution I (1 Complete™ tablet Roche Applied Science) dissolved in 2 ml of buffer A (B), and protease inhibitor solution II (1.4 μM pepstatin A and 1 mM phenylmethylsulfonyl fluoride in ethanol) (C). The frozen tissue was transferred into a mortar placed in a liquid nitrogen bath. An aliquot of 1.25 parts w/v of tissue was added to 2 ml of buffer A and protease inhibitor solution II were added to the tissue and ground to a fine powder. The resulting powder was filled into a 2-ml microtube, quickly thawed, supplied with 0.034 parts of glass beads, and then sonicated in an ice-cold water bath 6 times for 10 s with intervals of 1 min 50 s. The homogenate was stirred 30 min in the presence of 0.025 parts w/v of 1 M EGTA (Merck). 6.5 μl of a 1 mg/ml DTT dilution solution were added, and stirring was continued for an additional 30 min. The protein extract was supplied with 0.1 parts w/v of amphotyle mixture Servalyte pH 2–4 (Serva, Heidelberg, Germany) and stored at −80 °C or analyzed immediately.

Proteins were separated by large two-dimensional gels as described previously (34, 35). Briefly, the gel format was 40 cm (isoelectric focusing) × 30 cm (SDS-PAGE, 15%) × 0.75 mm. The amount of the protein sample applied to the gel was 5 μl (60 μg/μl). For sample comparisons brain extracts from each pair of parkin−/− mice and control mice were run and stained in parallel. High sensitivity silver staining was used to visualize protein patterns (35). Two-dimensional gels were evaluated visually, and changes of spots were considered with respect to variation in the presence or absence, quantity, and spot position. Protein spots found to be reproducibly altered in parkin−/− versus wild type were evaluated with the Proteomweaver imaging software Version 2.1 (Definiens, Munich, Germany). Although the mice we used are in a homogenous genetic background (129Sv inbred strain), we still observed individual variations. Protein alterations confirmed in more than six pairs of mice were scored. All 10 parkin−/− mice investigated were affected in at least 7 of 14 proteins, and 5 mice were affected in more than 12 proteins. Data were analyzed by Student’s t test.

For protein identification using MS, 18-μl (60 μg/μl) samples were electrophoresed on 1.5-mm gels and stained with MS-compatible silver stain or colloidal Coomassie Brilliant Blue G-250. Protein spots of interest were excised from gels and subjected to in-gel trypsin digestion without reduction or alkylation. Tryptic fragments were analyzed by a combination of matrix-assisted laser desorption ionization time-of-flight and liquid chromatography/electrospray ionization ion trap MS. The mass spectra were analyzed using Protein Prospector (MS-Fit) and Sequest Version 3.1 software.

**Mitochondrial Respiration**—Mice were euthanized by CO2 inhalation, and tissues were rapidly dissected on ice. Brains were removed, and striata were isolated as described previously (24). Striata from 2 mice of each genotype were pooled for mitochondrial isolation. Tissue samples were homogenized in 1 ml of buffer A (120 mM NaCl, 5 mM Tris, 2 mM EGTA, pH 7.4, at 4 °C) with 5 strokes of a Teflon Dounce. Samples were centrifuged for 3 min at 2000 × g to remove nuclei and tissue particles. Supernatants were collected and centrifuged for 10 min at 12,000 × g to pellet mitochondria and synaptosomes. The crude pellet was resuspended in 10 ml of buffer A with the addition of 0.02% w/v of digitonin to disrupt synaptosomal membranes and release trapped mitochondria. The resuspended pellet was centrifuged for 10 min at 12,000 × g to pellet mitochondria, which was resuspended in 100 μl of buffer A, and protein content was determined by BCA assay (Pierce). Mitochondria were resuspended at a final concentration of 0.4 mg/ml protein in 0.5 ml of buffer B (120 mM KCl, 3 mM HEFES, 1 mM EGTA, 2 mM KH2PO4, pH 7.4) with 15% w/v of bovine serum albumin and assayed for respiration using an excess of 8 mM glutamate, 8 mM malate (complex I), 4 mM succinate (complex II), or 0.4 mM N,N,N’,N’-tetramethylphenediylenediamine (TMPD)/1 mM ascorbate (complex III/IV) as electron donors. ADP was added in limiting amounts (14 μM), and state 3 respiration was measured. After depletion of ADP, state 4 respiration was determined. Measurements were made in the absence of a proton gradient. Mitochondrial respiration was determined using a platinum electrode with a 1-mL buffer chamber (DM-10, Rank Bros., Ltd., UK). Due to intra-day variations in respiratory rates, samples were represented as a percentage of wild-type state 3 respiration for succinate. Data were analyzed by unpaired Student’s t test.

**Electron Microscopy**—Wild-type and parkin−/− mice were euthanized by CO2 inhalation and transcardially perfused with 20 ml of...
phosphate-buffered saline followed by 10 ml of fresh 2.5% glutaraldehyde plus 2.5% formaldehyde in 100 mM cacodylate buffer. Brains were removed and post-fixed in the above fixative for an additional 18 h at 4 °C. Brains were washed in phosphate-buffered saline and cut into 1-mm thick coronal sections, and striata were dissected and processed for standard electron microscopy by epon embedding with osmium tetraoxide fixation and uranyl acetate counterstaining. Mitochondrial number and morphology were determined in images from 2–5 different fields from 2 mice per genotype by an investigator blind to the genotype.

Body Weight Measurements—Body weights were measured in parkin−/− and wild-type mice at regular intervals beginning 10 days after weaning. Weights were analyzed by two-way analysis of variance fol-
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Protein alterations in parkin−/− mice

| Protein alterations in mitochondrial OXPHOS | Spot number | Accession number | Frequency of alteration | Quantitative changes |
|--------------------------------------------|-------------|------------------|------------------------|----------------------|
| Pyruvate dehydrogenase E1α/β               | B6          | P35487           | 7                      | −57.2 ± 15.4         |
| NADH-ubiquinone oxidoreductase 24-kDa subunit | A5          | Q9D6J6           | 7                      | +41.4 ± 10.9         |
| NADH-ubiquinone oxidoreductase 30-kDa subunit | A7          | Q9DCT2           | 8                      | −9.1 ± 2.4           |
| Cytochrome c oxidase, subunit Vb            | A2          | P12075           | 7                      | −48.7 ± 11.7         |

Proteins involved in oxidative stress

| Protein alterations in mitochondrial OXPHOS | Spot number | Accession number | Frequency of alteration | Quantitative changes |
|--------------------------------------------|-------------|------------------|------------------------|----------------------|
| Peroxiredoxin 2                            | A3          | Q61171           | 8                      | −26.8 ± 7.4          |
| Peroxiredoxin 6                            | A9          | D08709           | 6                      | −28.6 ± 8.8          |
| Peroxiredoxin 1                            | B2          | P35700           | 8                      | −27 ± 5.1            |
| Lactoylglutathione lyase                    | A4          | Q9CPU0           | 9                      | −20.0 ± 4.7          |

Other Proteins

| Protein alterations in mitochondrial OXPHOS | Spot number | Accession number | Frequency of alteration | Quantitative changes |
|--------------------------------------------|-------------|------------------|------------------------|----------------------|
| Hypothetical protein DJ37E16.5 (novel protein similar to nitrophenylphosphatasases from various organisms) | A8          | Q9UGY2           | 8                      | −22.0 ± 8.1          |

% SWISS-PROT/TrEMBL accession number.

Quantitative changes shown as the mean ± S.E. All alterations are statistically significant by Student’s t test (p < 0.05).

RESULTS

Proteomic Analysis of parkin−/− Mice—Because parkin is an E3 ubiquitin ligase (26), we anticipated that loss of parkin would result in accumulation of its substrates, which may in turn cause nigrostriatal dysfunction and nigral degeneration. To identify the proteomic difference between parkin−/− and wild-type mice, we used large-gel two-dimensional electrophoresis (34) to separate proteins in the ventral midbrain of each of the 10 pairs of parkin−/− and wild-type mice at ~8 months of age. Proteins were resolved in the first dimension by their isoelectric point (pI) on a 40-cm tube gel using carrier ampholytes and subsequently resolved in the second dimension by their molecular weight on 30 × 30-cm SDS-PAGE gels. After silver staining, we detected reproducible, specific changes in 15 of ~8000 discrete spots between the genotypes (Fig. 1). Contrary to our expectations, the staining intensity of all but one of these 15 protein spots was decreased in parkin−/− mice. Isolation of protein spots from these gels followed by tryptic digestion and subsequent matrix-assisted laser desorption ionization and electrospray ionization MS provided the identification of these proteins (Table I). Fourteen of the 15 spots represented distinct proteins, whereas one protein was detected twice in 2 adjacent spots (A5 and A6) with varying pl, suggesting a post-translational modification. Changes in protein spot intensity that were confirmed in more than six pairs of parkin−/− and wild-type mice were scored (Table I).

The majority of the proteins altered in parkin−/− mice are functionally implicated in either mitochondrial respiration (subunits of pyruvate dehydrogenase and mitochondrial complexes I and IV) or oxidative stress (peroxiredoxin (PRDX) 1, 2, and 6 and lactoylglutathione lyase), indicating a connection between loss of parkin expression and mitochondrial and/or antioxidant deficiencies. The 24-kDa subunit of complex I was shifted to a more acidic pI in the parkin−/− brain (Fig. 1E, spots A5 and A6), indicating the protein had undergone a differential post-translational modification in the parkin−/− mouse brain. Based on the nature and degree of the pl shift from spot A6 to A5, possible modifications included oxidation or nitration of the protein. These modifications have been shown previously to appear in cells and tissues after exposure to oxidative stress (36–38). The alteration of both 24- and 30-kDa subunits suggested a general impairment of complex I in parkin−/− mice. Furthermore, spot B1 (subunit Vb of complex IV) was also down-regulated in parkin−/− mice, suggesting additional alterations in complex IV of the mitochondrial respiratory chain. The remaining proteins include those regulating the cytoskeleton (profilin II and laasp-1), vesicular transport (vacuolar protein sorting-29), and inhibiting aggregation of misfolded proteins (α-crystallin).

parkin−/− Mice Exhibit Reduced Mitochondrial Respiration—Previous reports suggest that PD may be linked to mitochondrial dysfunction (39). The biochemical changes detected in mitochondrial markers in the ventral midbrain of parkin−/− mice (Fig. 1) suggested possible mitochondrial deficiency in these mice. To address this issue directly, we examined the metabolic capacity of mitochondria isolated from the striatum
of parkin−/− mice and wild-type controls by assaying for state 3 and state 4 respiration using substrates for complex I (glutamate/malate), complex II (succinate), and complex III/IV (TMPD/ascorbate). State 3 respiration measures the capacity of mitochondria to metabolize oxygen and the selected substrate in the presence of a limiting quantity of ADP, which is a substrate for complex V, the ATP synthase. In contrast, state 4 respiration occurs when all ADP is exhausted and measures respiration associated with proton leak across the inner mitochondrial membrane and represents a “basal-coupled” rate of respiration for each metabolic substrate.

Consistent with reductions in several units of complexes I and IV, we observed reduced rates of State 3 respiration (wild type versus parkin−/−: 75.6 ± 13.1 versus 62.8 ± 11.2, 147.7 ± 17.3 versus 110.3 ± 11.1, and 366.6 ± 72.2 versus 282.2 ± 59.7 nmol of O₂/min/mg of protein for complex I, II, and III/IV, respectively; data are the mean ± S.E.), indicating a reduced capacity of mitochondrial metabolism. Representative traces for succinate-mediated respiration are shown in Fig. 2, A and B. Due to intra-day variations in absolute respiratory rate, data were analyzed as a fraction of the wild-type state 3 respiration for succinate (Fig. 2C). Consistent, statistically significant reductions were seen in mitochondria from parkin−/− mice using substrates that enter the electron transport chain at complex I (glutamate/malate), complex II (succinate), and complex III/IV (TMPD/ascorbate), suggesting an overall reduction in respiratory capacity. Measurement of state 4 respiration using either succinate or TMPD/ascorbate similarly exhibited a significant decrease in capacity in parkin−/− mice (Fig. 2D). The comparable magnitude of reduction in both state 3 and 4 respiration further supported the indication of reduced mitochondrial capacity for electron transport in parkin−/− mice. Respiratory control ratio (ratio of state 3:state 4 respiration) provides a measure of the efficiency of coupling of the electron transport chain. The respiratory control ratio (Fig. 2E) for all substrates was similar in parkin−/− and wild-type mice, indicating that the relative efficiency of metabolic coupling between the complexes of the electron transport chain is unchanged. The absence of a change in the respiratory control ratio indicates that the decrease in metabolism was likely due to a reduction in capacity (rather than efficiency) of the electron transport chain.

A reduction in electron transport chain capacity is further confirmed by the significant reduction in respiratory rate for both succinate and TMPD/ascorbate after treatment with FCCP (Fig. 2F). FCCP treatment results in a collapse of the proton gradient across the inner mitochondrial membrane and allows mitochondrial respiration to proceed at the maximal capacity for the components of the electron transport chain without regard for either the capacity of complex V or proton leak. Thus, the decrease in mitochondrial respiration can be linked to a decrease in the capacity of the electron transport chain rather than a defect in ATP synthase capacity or function.

Mitochondrial Dysfunction Is Not Coupled with Alterations in Mitochondrial Morphology—The decreased abundance of...
several mitochondrial proteins and the altered mitochondrial respiratory chain activity prompted us to examine the morphology of striatal mitochondria from parkin−/− mice and wild-type controls. Previous studies indicate that parkin-null flies exhibit morphological changes in mitochondria of the wing muscle and spermatocytes (33). Electron microscopy of striatal sections revealed no apparent change in the total number or size of mitochondria present in parkin−/− mice (data not shown). Examination of 100 μm² of striatal area from two mice per genotype showed no gross alterations in mitochondrial morphology in parkin−/− mice (Fig. 3, A and B). Further examination at higher magnification (40,000×) showed that both parkin−/− and wild-type mitochondria had well formed cristae with no apparent swelling or separation of the inner and outer membranes (Fig. 3, C and D). These results suggest that reduced mitochondrial respiratory chain activity was not associated with detectable morphological changes in the mitochondria of parkin−/− mice.

parkin−/− Mice Exhibit Reduced Body Weight Gain—To determine whether the reduced mitochondrial respiratory capacity in the parkin−/− mouse results in a broad alteration in metabolic activity, we examined early weight gain in parkin−/− mice during the period immediately after weaning. Mice that were weaned at ~20 days of age were monitored for weight gain starting at ~30 days of age. Animals were weighed regularly for an additional 3 months. Data were pooled in either 10- or 20-day bins (±2 days). Over the course of the measurements, both male and female parkin−/− mice exhibited a significant decrease in the rate and total amount of weight gain (Fig. 4, A and B). Adult (6–12-month-old) male parkin−/− mice also exhibited significantly lower body weights (Fig. 4C).

Reduced Antioxidant Capacity and Elevated Oxidative Damage in parkin−/− Mice—The mitochondrial respiratory dysfunction in parkin−/− mice prompted us to investigate whether increased levels of oxidation products could be detected in these mice. Three of the proteins identified in our proteomic screen to be decreased in parkin−/− mice were small peroxide reductases, suggesting that the parkin−/− mouse may have a reduced ability to respond to the generation of ROS. Analysis of serum total antioxidant potential, defined as copper reducing equivalents, revealed that parkin−/− mice (n = 12) had reduced antioxidant capacity as compared with age-matched wild-type mice (n = 13) (5–12 months of age) (Fig. 5A).
Fig. 5. Reduced antioxidant capacity and increased ROS damage in parkin−/− mice. A, assays for serum antioxidant potential reveals a significant decrease in the total reducing capacity of parkin−/− mice (n = 12), as compared with wild-type mice (n = 13). *, p < 0.01 (Student’s t test). B, analysis of brain lysates from parkin−/− and wild-type mice at 3 and 18–20 months reveals that aged (18–20 month) parkin−/− mice exhibit increased levels of protein carbonyls. Incubation of the same blots with anti-actin antibody confirms equivalent loading of proteins in each lane. C–J, immunohistochemical analysis of parkin−/− and wild-type mice reveals an age-dependent increase in 4HNE immunoreactivity in the parkin−/− brain (D, F, and H) as compared with age-matched wild-type mice (C, E, and G). Comparable coronal sections of parkin−/− and control brains at 3 (C and D), 6 (E and F), and 18 (G and H) months of age are shown. I–L, higher power views of the boxed areas in G and H (I and J) or the boxed areas in I and J (K and L) show increased 4HNE immunoreactivity in parkin−/− mice (J and L). Arrows indicate 4HNE immunoreactivity in cell bodies. Scale bar, 200 μm.

Several lipid peroxides, especially 4HNE, are highly reactive, avidly form adducts with many proteins and have been detected in Lewy bodies (40). Immunohistochemical analysis of parkin−/− and wild-type mice at 3, 6, and 18 months revealed age-dependent increases in 4HNE immunoreactivity in the
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brain of parkin−/− mice as compared with wild-type controls (Fig. 5, C–L). At 3 months of age, neither parkin−/− nor wild-type mice showed significant immunoreactivity for 4HNE (Fig. 5, C and D). At 6 months, modest 4HNE immunoreactivity was detectable in brains of both parkin−/− and wild-type mice (Fig. 5, E and F). By 18 months, parkin−/− brains exhibit a marked increase in 4HNE immunoreactivity (Fig. 5, G–L). Higher magnification images show staining in cell bodies (Fig. 5L, arrows) in the parkin−/− brain. These findings demonstrated that, over the course of 18 months, parkin−/− animals experienced an increased incidence of ROS-mediated damage as compared with age-matched wild-type animals.

DISCUSSION

The identification of several genes linked to familial PD has provided new avenues for the investigation of the mechanism underlying the selective nigral degeneration. The first gene linked to familial PD was α-synuclein, in which missense and triplication mutations have been reported (41–43). The linkage of α-synuclein mutations to PD and the accumulation of insoluble α-synuclein in Lewy bodies provided a strong link between protein aggregation and PD pathogenesis. Similarly, mutations in parkin and UCH-L1, two proteins linked to the ubiquitin-proteasome pathway, supported a possible involvement of aberrant accumulation of insoluble protein substrate(s) in PD pathogenesis. However, most parkin-linked PD cases lack Lewy bodies, suggesting that parkin mutations may cause PD by mechanisms distinct from protein aggregation and Lewy body formation (22, 23). The association of parkin expression with cytoprotection in cultured cells (29) and flies (44), and its amelioration of mitochondrial dysfunction-induced cell death (31), provided tantalizing clues to an alternative mode of action, namely a regulatory function for parkin in mitochondrial activity.

In the present study we compared the proteome of the ventral midbrain of parkin−/− and wild-type mice and found specific and consistent decreases in the steady-state abundance of 13 proteins and altered electrophoretic mobility of 1 additional protein. Eight of these proteins are linked to mitochondrial respiration or detoxification of byproducts of mitochondrial respiration. Four proteins that are specifically reduced, i.e. the Ela subunit of pyruvate dehydrogenase, the 24- and 30-kDa subunits of complex I and subunit Vb of complex IV, are all directly involved in mitochondrial oxidative phosphorylation activity. An additional one, lactotry glutathione lyase, is part of a two-step enzymatic cascade to metabolize methylglyoxal to lactate (45, 46). Methylglyoxal is the non-enzymatic breakdown product of glycerol 3-phosphate and is linked to formation of advanced glycation end products. Accumulations of advanced glycation end product-modified proteins are present in Lewy bodies of PD patients (47). Three additional proteins showing selective and reproducible reductions, PRDX 1, 2, and 6, are small, thiol-dependent peroxidases. Members of the PRDX family have been linked to protection from ROS stresses, and mouse models of loss of PRDX proteins result in increased lipid peroxides, including 4HNE. These labile lipid peroxides may damage DAT, resulting in reduced DAT activity (17), leading to elevated extracellular DA, thereby exacerbating the oxidative stress. A recent paper shows that parkin is activated by ROS (65), suggesting that increased ROS mediated damage may result in further decreases of parkin activity in PD brains. Clinical reports indicate that heterozygous parkin mutations may represent a risk factor for late-onset PD in a subset of cohorts (66). This presents the hypothetical scenario of a vicious cycle where decreased levels of parkin result in mitochondrial dysfunction, which in turn lead to increased ROS formation and further inactivation of parkin and so forth. Our data provide compelling evidence for mitochondrial dysfunction and oxidative stress in the absence of nigral degeneration. This suggests that these events may be proximal in the cascade of the pathogenesis of both idiopathic and parkin-linked familial PD.

Acknowledgments—We thank Andrea Martins, Bettina Esch, Mary Wines, and Xiaoyan Sun for assistance.

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