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Mitochondrial membrane potential decrease caused by loss of PINK1 is not due to proton leak, but to respiratory chain defects

Taku Amo [a,1], Shigeto Sato [b], Shinji Saiki [b], Alexander M. Wolf [a], Masaaki Toyomizu [c], Clement A. Gautier [d], Jie Shen [d], Shigeo Ohta [a], Nobutaka Hattori [b,*]

[a] Department of Biochemistry and Cell Biology, Institute of Development and Aging Sciences, Graduate School of Medicine, Nippon Medical School, 1-396 Kosugi-cho, Nakahara-ku, Kawasaki 211-8533, Japan
[b] Department of Neurology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
[c] Animal Nutrition, Life Sciences, Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai 981-8555, Japan
[d] Center for Neurologic Diseases, Brigham and Women's Hospital, Program in Neuroscience, Harvard Medical School, Boston, MA 02115, USA

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ABSTRACT

Mutations in *PTEN-induced putative kinase 1* (*PINK1*) cause a recessive form of Parkinson's disease (PD). PINK1 is associated with mitochondrial quality control and its partial knock-down induces mitochondrial dysfunction including decreased membrane potential and increased vulnerability against mitochondrial toxins, but the exact function of PINK1 in mitochondria has not been investigated using cells with null expression of PINK1. Here, we show that loss of PINK1 caused mitochondrial dysfunction. In PINK1-deficient ($PINK1^{-/-}$) mouse embryonic fibroblasts (MEFs), mitochondrial membrane potential and cellular ATP levels were decreased compared with those in littermate wild-type MEFs. However, mitochondrial proton leak, which reduces membrane potential in the absence of ATP synthesis, was not altered by loss of PINK1. Instead, activity of the respiratory chain, which produces the membrane potential by oxidizing substrates using oxygen, declined. H_2O_2 production rate by $PINK1^{-/-}$ mitochondria was lower than $PINK1^{+/+}$ mitochondria as a consequence of decreased oxygen consumption rate, while the proportion (H_2O_2 production rate per oxygen consumption rate) was higher. These results suggest that mitochondrial dysfunctions in PD pathogenesis are caused not by proton leak, but by respiratory chain defects.

Introduction

Parkinson's disease (PD) is a neurodegenerative disease characterized by loss of dopaminergic neurons in the substantia nigra. Mitochondrial dysfunction has been proposed as a major factor in the pathogenesis of sporadic and familial PD (Abou-Sleiman et al., 2006). In particular, the identification of mutations in *PTEN-induced putative kinase 1* (*PINK1*) has strongly implicated mitochondrial dysfunction owing to its loss of function in the pathogenesis of PD (Valente et al., 2004). PINK1 contains an N-terminal mitochondrial targeting sequence (MTS) and a serine/threonine kinase domain (Valente et al., 2004). PINK1 kinase activity is crucial for mitochondrial maintenance via TRAP phosphorylation (Pridgeon et al., 2007). Loss of PINK1 function induces increased vulnerability to various stresses (Exner et al., 2007; Haque et al., 2008; Pridgeon et al., 2007; Wood-Kaczmar et al., 2008). However, silencing of PINK1 has only been partial and only one study has been performed to assess mitochondrial functions in steady and artificial states with complete ablation of PINK1 expression (Gautier et al., 2008).

Several studies have shown that PINK1 acts upstream of parkin in the same genetic pathway (Clark et al., 2006; Park et al., 2006) and co-overexpressed PINK1 and parkin both co-localized to mitochondria (Kim et al., 2008). Overexpression of PINK1 promotes mitochondrial fission (Yang et al., 2008). Fission followed by selective fusion segregates dysfunctional mitochondria and permits their removal by autophagy (Twig et al., 2008). PINK1 loss-of-function decreases mitochondrial membrane potential (Chu, 2010) and the PINK1–parkin pathway is associated with mitochondrial elimination in cultured cells treated with the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which causes mitochondrial depolarization (Geisler et al., 2010; Kawajiri et al., 2010; Matsuda et al., 2010; Narendra et al., 2008, 2010; Vives-Bauza et al., 2010). However, the exact mechanism underlying the mitochondrial depolarization induced by PINK1 defects leading to mitochondrial autophagy has not been examined in detail.

Abbreviations: $\Delta\psi$, mitochondrial membrane potential; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MEFs, mouse embryonic fibroblasts; PD, Parkinson's disease; PINK1, PTEN-induced putative kinase 1; ROS, reactive oxygen species; TMRM, tetramethylrhodamine methyl ester; TPMP, triphenylmethylphosphonium.

* Corresponding author. Fax: +81 3 5800 0547.
E-mail address: nhattori@juntendo.ac.jp (N. Hattori).
[1] Present address: Department of Applied Chemistry, National Defense Academy, 1-10-20 Hashirimizu, Yokosuka 239-8686, Japan.

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Here, we describe a detailed characterization of mitochondria in PINK1-deficient cells. We show that PINK1 deficiency causes a decrease in mitochondrial membrane potential, which is not due to proton leak, but to respiratory chain defects.

Materials and methods

PINK1 knock-out mouse embryonic fibroblasts (MEFs)

PINK1 knock-out MEFs were prepared and cultured as described previously (Matsuda et al., 2010). Mouse embryonic fibroblasts (MEFs) were derived from E12.5 embryos containing littermate 4 mice of each genotype. Embryos were mechanically dispersed by repeated passage through a P1000 pipette tip and plated with MEF media containing DME, 10% FCS, 1× nonessential amino acids, 1 mM L-glutamine, penicillin/streptomycin (invitrogen). The $\psi 2$ cell line, an ecotropic retrovirus packaging cell line, was maintained in Dulbecco's modified Eagle medium (DMEM, Sigma) with 5% fetal bovine serum and 50 μg/ml kanamicin. Transfection of the $\psi 2$ cells with pMESVTS plasmids containing an SV40 large T antigen was performed by lipofection method according to the manual provided by the manufacturer (GIBCO BRL). Five micrograms of the plasmids was used for each transfection. Transfectants were selected by G418 at the concentration of 0.5 mg/ml, and 10 clonal cell lines were established. The highest titer of 5×10^4 cfu/ml was obtained for the conditioned medium of a cell line designated $\psi 2$SVTS1. 10^6 MEFs were plated onto a 10-cm culture dish and kept at 33 °C for 48 hours. Then medium was replaced with 2 ml supplemented with polybrene-supplemented medium conditioned by the $\psi 2$SVTS1 cells at confluency for 3 days. Infection was continued for 3 hours, and the medium was replaced with a fresh one. The infected MEFs were cultured at 33 °C until immortalized cells were obtained.

We confirmed that the differences we detected in this study were due to the PINK1 deficiency, not to artificial effects by immortalization, by measuring cellular respiration rates of not immortalized MEFs from other littermates (Supplemental figure). The respiration rates of not immortalized MEFs were slightly slower than those of immortalized MEFs, but the differences between PINK1$^{+/+}$ and $^{-/-}$ MEFs were consistent (Fig. 2A).

Cell growth

Cells were seeded in 12-well plates at density of $3\sim6\times10^3$ cells/well and incubated in DMEM high glucose medium (4.5 g/l glucose and 1 mM sodium pyruvate) supplemented with 10% fetal bovine serum. After a day, the medium was replaced with DMEM glucose-free medium supplemented with 1 g/l galactose, 1 mM sodium pyruvate and 10% fetal bovine serum (DMEM galactose medium) at 37 °C in an incubator with a humidified atmosphere of 5% CO_2. Cells were trypsinized and live cells were assessed by trypan blue dye exclusion.

Mitochondrial morphological changes

Cells were seeded in 6-well plates at 2.0×10^5/well and incubated in DMEM high glucose medium (4.5 g/l glucose and 1 mM sodium pyruvate) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. After a day, the medium was replaced with DMEM glucose-free medium supplemented with 1 g/l galactose, 1 mM sodium pyruvate and 10% fetal bovine serum (DMEM galactose medium) at 37 °C in an incubator with a humidified atmosphere of 5% CO_2. 24 hours later, cells were fixed and immunostained with anti-Tom20 antibody to visualize mitochondria according to a protocol as previously described (Kawajiri et al., 2010). All images were obtained using an Axioplan 2 imaging microscope (Carl Zeiss, Oberkochen, Germany).

Cellular ATP levels

Intracellular ATP levels were determined by a cellular ATP assay kit (TOYO B-Net, Tokyo, Japan) according to the manufacturer's instructions using a Lumat LB9507 luminometer (Berthold Technology, Bad Wildbad, Germany).

Membrane potential

Fluorescence images were recorded using a multi-dimensional imaging workstation (AS MDW, Leica Microsystems, Wetzlar, Germany) with a climate chamber maintained at 37 °C. Fluorescence was quantified with a CCD camera (CoolSnap HQ, Roper Scientific, Princeton, NJ) using a 20× objective. Cells were stained for 1 hour with a non-quenching concentration (20 nM) of tetramethylrhodamine methyl ester (TMRM) in a 96-well plate. The cell-permeable cationic dye TMRM accumulates in mitochondria according to the Nernst equation. Nuclei were stained with 250 nM Hoechst 34580. Mitochondrial TMRM fluorescence was integrated in a 40-μm diameter circular area around the nucleus, and the minimum fluorescence in this area was subtracted as background fluorescence.

Cell respiration

Cell respiration was measured at 37 °C using the Oxygen Meter Model 781 and the Mitocell MT200 closed respiratory chamber (Strathkelvin Instruments, North Lanarkshire, United Kingdom). Cells were cultured in DMEM with 4.5 g/l of glucose supplemented with 10% FBS. Cells were then trypsinized and resuspended in Leibovitz's L-15 medium (Invitrogen) at density of 8.0×10^6 cells/ml. The oxygen respiration rate was measured under each of the following three conditions: basal rate (no additions); State 4 (no ATP synthesis) [after addition of 1 μg/ml oligomycin (Sigma)], uncoupled [after addition of 3 μM FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Sigma)] using Strathkelvin 949 Oxygen System. After sequential measurements, the endogenous respiration rate was determined by adding 1 μM rotenone + 2 μM myxothiazol.

Mitochondrial respiration and membrane potential

Mitochondria were prepared from cultured MEFs as previously described (Amo and Brand, 2007). Mitochondrial oxygen consumption with 5 mM succinate as a respiratory substrate was measured at 37 °C using a Clark electrode (Rank Brothers, Cambridge, United Kingdom) calibrated with air-saturated respiration buffer comprising 0.115 M KCl, 10 mM KH_2PO_4, 3 mM HEPES (pH 7.2), 2 mM $MgCl_2$, 1 mM EGTA and 0.3% (w/v) defatted BSA, assumed to contain 406 nmol atomic oxygen/ml (Reynafarje et al., 1985). Mitochondrial membrane potential ($\Delta\psi$) was measured simultaneously with respiratory activity using an electrode sensitive to the lipophilic cation $TPMP^+$ (triphenylmethylphosphonium) (Brand, 1995). Mitochondria were incubated at 0.5 mg/ml in the presence of 80 ng/ml nigericin (to collapse the pH gradient so that the proton motive force was expressed exclusively as $\Delta\psi$) and 2 μM rotenone (to inhibit complex I). The $TPMP^+$-sensitive electrode was calibrated with sequential additions of $TPMP^+$ up to 2 μM, then 5 mM succinate was added to initiate respiration. Experiments were terminated with 2 μM FCCP, allowing correction for any small baseline drift. $\Delta\psi$ was calculated from the distribution of $TPMP^+$ across the mitochondrial inner membrane using a binding correction factor of 0.35 mg protein/μl. Respiratory rates with 4 mM pyruvate + 1 mM malate as a substrate in State 3 (with 0.25 mM ADP) and State 4 (with 1 μg/ml oligomycin) were determined using the Oxygen Meter Model 781 and the Mitocell MT200 closed respiratory chamber (Strathkelvin Instruments).

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Modular kinetic analysis

To investigate differences in oxidative phosphorylation caused by PINK1 knock-out, we applied a systems approach, namely modular kinetic analysis (Amo and Brand, 2007; Brand, 1990). This analyzes the kinetics of the whole of oxidative phosphorylation divided into three modules connected by their common substrate or product, $\Delta\psi$. The modules are (i) the reactions that produce $\Delta\psi$, consisting of the substrate translocases, dehydrogenases and other enzymes and the components of the respiratory chain, called 'substrate oxidation'; (ii) the reactions that consume $\Delta\psi$ and synthesize, export and dephosphorylate ATP, consisting of ATP synthase, the phosphate and adenine nucleotide translocases and any ATPases that may be present, called the 'phosphorylating system'; and (iii) the reactions that consume $\Delta\psi$ without ATP synthesis, called the 'proton leak' (Brand, 1990). The analysis reports changes anywhere within oxidative phosphorylation that are functionally important but is unresponsive to changes that have no functional consequences. Comparison of the kinetic responses of each of the three modules to $\Delta\psi$ obtained using mitochondria isolated from PINK1$^{+/+}$ and PINK1$^{-/-}$ MEFs would reveal any effects of PINK1 on the kinetics of oxidative phosphorylation. Oxygen consumption and $\Delta\psi$ were measured simultaneously using mitochondria incubated with 80 ng/ml nigericin and 4 μM rotenone. Respiration was initiated by 5 mM succinate. The kinetic behavior of a '$\Delta\psi$-producer' can be established by specific modulation of a $\Delta\psi$-consumer and the kinetics of a consumer can be established by specific modulation of a $\Delta\psi$-producer (Brand, 1998). To measure the kinetic response of proton leak to $\Delta\psi$, the State 4 (non-phosphorylating) respiration of mitochondria in the presence of oligomycin (0.8 μg/ml; to prevent any residual ATP synthesis), which was used solely to drive the proton leak, was titrated with malonate (up to 8 mM). In a similar way, State 4 respiration was titrated by FCCP (up to 1 μM) for measurement of the kinetic response of substrate oxidation to $\Delta\psi$. State 3 (maximal rate of ATP synthesis) was obtained by addition of excess ADP (1 mM). Titration of State 3 respiration with malonate (up to 1.1 mM) allowed measurement of the kinetics of the $\Delta\psi$-consumers (the sum of the phosphorylating system and proton leak). The coupling efficiencies of oxidative phosphorylation were calculated from the kinetic curves as the percentage of mitochondrial respiration rate at a given $\Delta\psi$ that was used for ATP synthesis and was therefore inhibited by oligomycin. Note that any slip reactions will appear as proton leak in this analysis (Brand et al., 1994).

Mitochondrial ROS production

Mitochondrial ROS production rate was assessed by measurement of H_2O_2 generation rate, determined fluorometrically by measurement of oxidation of Amplex Red to fluorescent resorufin coupled to the enzymatic reduction of H_2O_2 by horseradish peroxidase using a spectrofluorometer RF-5300PC (Shimadzu, Kyoto, Japan). The H_2O_2 generation rate was measured in non-phosphorylating conditions (= State 4) using either pyruvate/malate or succinate as respiratory substrates. Mitochondria were incubated at 0.1 mg/ml in respiration buffer. All incubations also contained 5 μM Amplex Red, 2 U/ml horseradish peroxidase and 8 U/ml superoxide dismutase. The reaction was initiated by addition of 5 mM succinate or 4 mM pyruvate + 1 mM malonate and the increase in fluorescence was followed at excitation and emission wavelengths of 560 and 590 nm, respectively. Appropriate correction for background signals and standard curves generated using known amounts of H_2O_2 were used to calculate the rate of H_2O_2 production in nmol/min/mg mitochondrial protein. The percentage free radical leak, which is a measure of the number of electrons that produce superoxide (and subsequently H_2O_2) compared with the total number of electrons which pass thorough the respiratory chain, was calculated as the rate of H_2O_2 production divided by the rate of O_2 consumption (Barja et al., 1994).

Statistics

Values are presented as means ± SEM except Fig. 2D, in which error bars indicate SD. The significance of differences between means was assessed by the unpaired Student's *t*-test using Microsoft Excel; *P* values < 0.05 were taken to be significant.

Results

Cell growth and mitochondrial morphology

In general, cultured cells gain their energy mostly from glycolysis. Therefore, cells deficient in respiratory function can grow in normal medium, although possibly at a slower rate, relying predominantly on glycolysis (Hofhaus et al., 1996). Actually, ρ^0 cells, which lack mitochondrial DNA completely, can grow producing energy exclusively through glycolysis (King and Attardi, 1989). On the other hand, galactose metabolism via glycolysis is much slower than glucose metabolism (Reitzer et al., 1979). Therefore, cells in galactose medium are forced to oxidize pyruvate through the mitochondrial respiratory chain for energy required for growth. Consequently, cells with defects in their mitochondrial respiratory chains show growth impairments in galactose medium. To evaluate this phenomenon is also observed in our cells, we examined growth retardation by addition of mitochondrial complex I inhibitor, rotenone (Fig. 1A). In glucose medium, 10 nM rotenone had only a slight effect on the growth of PINK1$^{+/+}$ MEFs and slower growth was observed even in the presence of 100 nM rotenone. However, in the galactose medium, 10 nM rotenone significantly inhibited the growth of PINK1$^{+/+}$ MEFs and 100 nM roteone completely arrested the growth. Therefore, we could confirm that the growth impairment of our cells in the galactose medium was due to mitochondrial respiratory chain defects.

PINK1 acts upstream of parkin, regulating mitochondrial integrity and function; therefore, loss of PINK1 is considered to affect mitochondrial functions. To assess the mitochondrial functions of PINK1$^{-/-}$ MEFs, growth capability in a medium in which galactose replaced glucose was examined. As shown in Fig. 1B, PINK1$^{-/-}$ MEFs appeared to show clear growth impairments in the galactose medium, whereas PINK1$^{+/+}$ MEFs grew slightly slower than in the glucose medium.

No differences of mitochondrial morphology between PINK1$^{+/+}$ and $^{-/-}$ MEFs in the glucose medium were detected (Fig. 1C), consistent with the previous report (Matsuda et al., 2010). However, in the galactose medium, mitochondria of the PINK1$^{-/-}$ MEFs were more fragmented compared to the PINK$^{+/+}$ MEFs (Fig. 1C). This is consistent with previous reports, which found mitochondrial morphological changes were more pronounced when PINK1 knock-down HeLa cells were grown in low-glucose medium (Exner et al., 2007) and human PINK1 homozygous mutant fibroblast in galactose medium (Grünewald et al., 2009). In these cells, mitochondrial morphological changes were associated with the mitochondrial functional impairment.

Assessments of mitochondrial functions at the cellular level

Because PINK1$^{-/-}$ MEFs showed severe growth impairments in the galactose medium, the mitochondrial functions of these cells were assessed at the cellular level. First, cellular respiration rates were measured (Fig. 2A). The basal respiration rate was significantly reduced in PINK1$^{-/-}$ cells compared with that in PINK1$^{+/+}$ cells (11.13 ± 0.71 versus 14.36 ± 1.01 nmol O/min/10^6 cells; $p<0.05$; $n=5$ independent experiments), consistent with previous reports using partial knock-down of PINK1 expression (Gandhi et al., 2009; Liu et al., 2009). Oligomycin inhibits ATP synthase, resulting in non-phosphorylating respiration. FCCP uncouples oxidative phosphorylation, leading to maximum respiration rates. In both conditions, the

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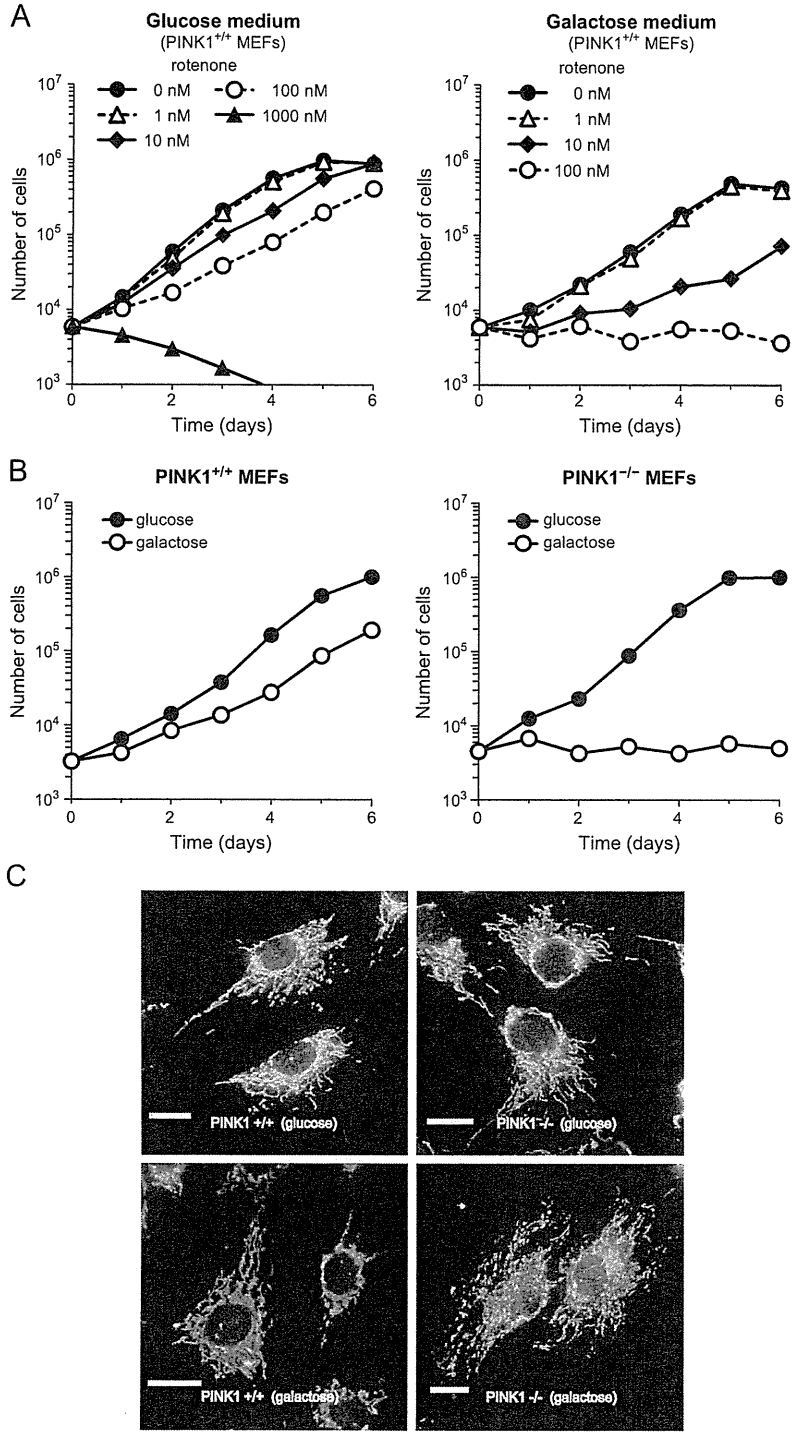

Fig. 1. (A) Growth retardation of PINK1$^{+/+}$ MEFs by mitochondrial complex I inhibitor, rotenone in glucose or galactose medium. Closed circles with solid line, 0 nM rotenone; open triangles with dashed line, 1 nM rotenone; closed diamonds with solid line, 10 nM rotenone; open circles with dashed line, 100 nM rotenone; closed triangles with solid line, 1000 nM rotenone. Cells grown in 12-well plates were trypsinized and live cells were assessed by trypan blue dye exclusion. (B) Growth curves of PINK1$^{+/+}$ and $^{-/-}$ MEFs. Closed symbols (*glucose*), growth curve for cells grown in DMEM containing 4.5 g/l glucose and 1 mM sodium pyruvate; open symbols (*galactose*), growth curve for cells grown in DMEM lacking glucose and containing instead 1.0 g/l galactose and 1 mM sodium pyruvate. Cells grown in 12-well plates were trypsinized and live cells were assessed by trypan blue dye exclusion. (C) Mitochondrial morphology of PINK1$^{+/+}$ and $^{-/-}$ MEFs. After incubating cells with the glucose or galactose medium for 24 hours, cells were fixed and immunostained with anti-Tom20 antibody to visualize mitochondria. Scale bar, 20 μm.

PINK1$^{-/-}$ cells respired significantly slower than the PINK1$^{+/+}$ cells (1.76 ± 0.13 versus 2.95 ± 0.27 ($p<0.01$; $n=5$ independent experiments) and 16.44 ± 1.80 versus 23.50 ± 1.18 nmol O/min/10^6 cells ($p<0.05$; $n=5$ independent experiments), respectively).

The main function of mitochondria is ATP synthesis via oxidative phosphorylation. ATP levels under basal conditions were significantly reduced in PINK1$^{-/-}$ MEFs (Fig. 2B), as reported previously for dissociated PINK1$^{-/-}$ mouse neurons (Gispert et al., 2009) and PINK1

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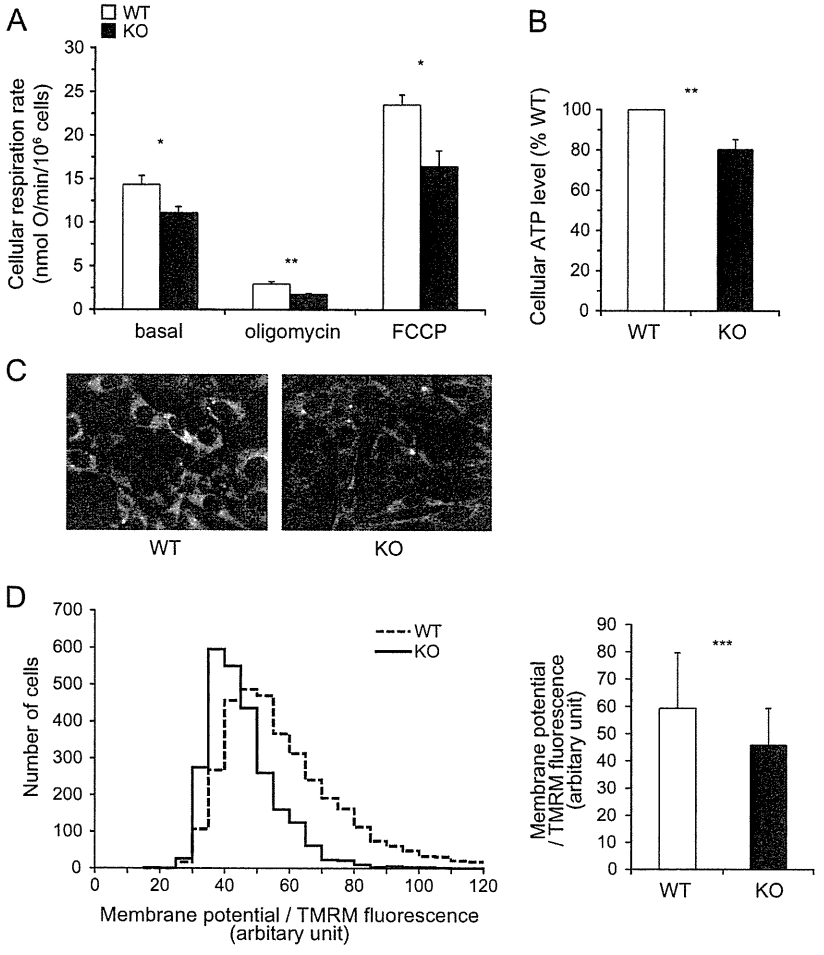

Fig. 2. Mitochondrial functions assessed at the cellular level. Open bars, PINK1$^{+/+}$ MEFs; closed bars, PINK1$^{-/-}$ MEFs. (A) Cell respiration rate of PINK1$^{+/+}$ and $^{-/-}$ MEFs. The oxygen respiration rate was measured at density of 8.0×10^6 cells/ml under each of the following three conditions: basal rate (no additions); State 4 (no ATP synthesis) [after addition of 1 μg/ml oligomycin], uncoupled [after addition of 3 μM FCCP]. After sequential measurements, the endogenous respiration rate was determined by adding 1 μM rotenone +2 μM myxothiazol. Error bars indicate SEM ($n=5$ independent experiments). (B) Cellular ATP levels. Data were normalized based on cell numbers and expressed as the percentage of the level in PINK1$^{+/+}$ cells. Error bars indicate SEM ($n=4$ independent experiments). (C) Live cell images of PINK1$^{+/+}$ and $^{-/-}$ MEFs with TMRM fluorescence. (D) Mitochondrial membrane potential evaluated by live cell imaging of TMRM fluorescence. *Left panel*, the distribution of TMRM fluorescence from 3537 PIINK1$^{+/+}$ and 2566 PINK1$^{-/-}$ cells from 12 wells per cell type; *right panel*, the average value of TMRM fluorescence per cell. Error bars indicate SD. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

siRNA knock-down PC12 cells (Liu et al., 2009). Mitochondrial membrane potential was also measured by live cell imaging of TMRM fluorescence. Typical images were shown in Fig. 2C. The histogram shows the distribution of TMRM fluorescence from 3537 PINK1$^{+/+}$ cells and 2566 PINK1$^{-/-}$ cells from 12 wells per cell type and the bar graph indicates the mean ± SD of TMRM fluorescence per cell (Fig. 2D). According to the Nernst equation, the ratio of TMRM fluorescence would translate into, on average, 6.88 mV lower mitochondrial membrane potential in the PINK1$^{-/-}$ cells if the plasma membrane potentials were not different between PINK1$^{+/+}$ and $^{-/-}$ cells. Mitochondrial membrane potential decrease was also showed previously in PINK1 knock-down HeLa cells (Exner et al., 2007) and in stable PINK1 knock-down neuroblastoma cell lines (Sandebring et al., 2009).

Assessments of mitochondrial functions using isolated mitochondria

To further analyze mitochondrial functions, we measured the kinetics of oxidative phosphorylation using isolated mitochondria from PINK1$^{+/+}$ and $^{-/-}$ MEFs. Fig. 3 shows the kinetics of the three modules of oxidative phosphorylation using succinate as a respiratory substrate (complex II-linked respiration). Fig. 3A shows the kinetic response of substrate oxidation to its product, $\Delta\psi$. The substrate oxidation kinetic curve for PINK1$^{-/-}$ cells was clearly shifted lower compared with that for PINK1$^{+/+}$ cells, indicating that the loss of PINK1 caused mitochondrial respiratory chain defects. Fig. 3B shows the kinetic response of proton leak to its driving force, $\Delta\psi$, and Fig. 3C shows the kinetic response of the ATP phosphorylating pathway to its driving force, $\Delta\psi$. Both kinetic curves for PINK1$^{+/+}$ and $^{-/-}$ MEFs (open and closed symbols, respectively) were overlapping, implying that there were no significant differences in those modules.

We also independently measured the mitochondrial oxygen consumption rate using pyruvate/malate as a respiratory substrate instead of succinate to check complex I. Modular kinetic analysis using pyruvate/malate is technically difficult for the following reasons: (1) the oxygen consumption rate with pyruvate/malate is much slower than succinate respiration; and (2) there are no competitive inhibitors of complex I-linked respiration, such as malonate for succinate respiration. As shown in Fig. 4A, the respiration rates in State 3 and 4 with pyruvate/malate of isolated mitochondria from PINK1$^{-/-}$ cells (closed symbols) were significantly slower than those of PINK1$^{+/+}$ cells (open symbols), as in the case of succinate respiration (Fig. 4B; data derived from the kinetic curves in Fig. 3).

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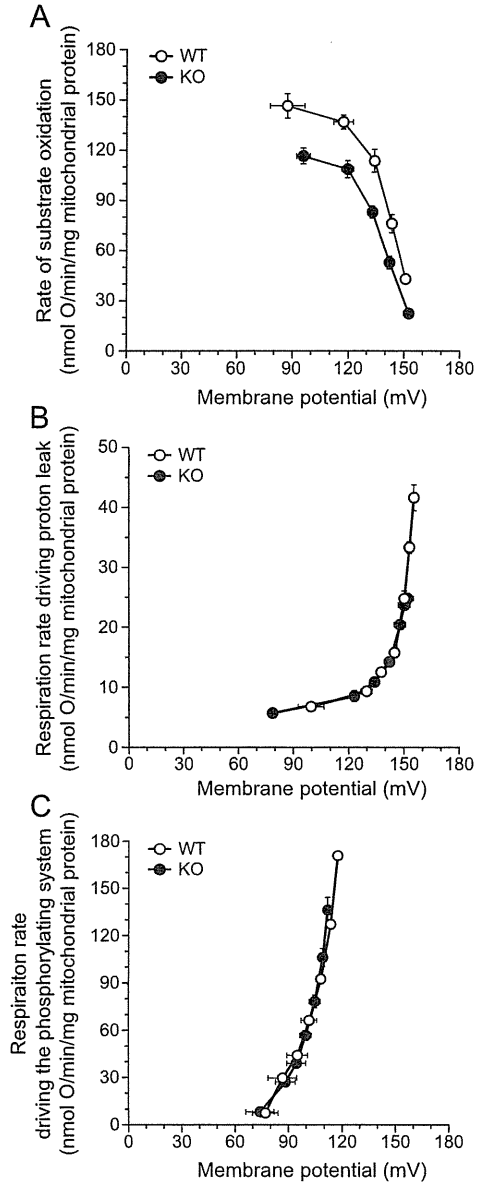

Fig. 3. Modular kinetic analysis of oxidative phosphorylation in mitochondria isolated from PINK1$^{+/+}$ and $^{-/-}$ MEFs. Modular kinetic analysis of the kinetic responses to membrane potential, $\Delta\psi$, of respiration driving (A) substrate oxidation ($\Delta\psi$ titrated with uncoupler, FCCP, starting in State 4), (B) proton leak ($\Delta\psi$ titrated with malonate, starting in State 4) and (C) the phosphorylating system, calculated by subtracting respiration driving proton leak from respiration driving the $\Delta\psi$-consumers ($\Delta\psi$ titrated with malonate starting in State 3; not shown) at each $\Delta\psi$. Open symbols, PINK1$^{+/+}$ MEFs; closed symbols, PINK1$^{-/-}$ MEFs. Error bars indicate SEM ($n=4$ independent mitochondrial preparations).

Mitochondrial ROS production

Mitochondrial ROS production rate was assessed by measurement of the H_2O_2 generation rate. Mechanisms of mitochondrial ROS production were well described elsewhere (Fig. 1 of Lambert et al., 2010). Pyruvate and malate generate NADH, which induced forward electron transport and generate ROS mainly from complex I and III. For pyruvate/malate respiration, the basal H_2O_2 generation rate (measured in the absence of respiratory chain inhibitors) was not different between PINK1$^{+/+}$ and $^{-/-}$ mitochondria (Fig. 4C). The addition of antimycin A and further addition of rotenone, which inhibited forward electron transport at complex III and I, respectively, enhanced H_2O_2 generation. During succinate respiration in the absence of respiratory chain inhibitors, ROS are generated mainly from the quinine binding site of complex I due to reverse electron flow from coenzyme Q to complex I. For succinate respiration, H_2O_2 generation rate in the absence of reparatory chain inhibitors was higher in PINK1$^{+/+}$ mitochondria than in PINK1$^{-/-}$ mitochondria, but the difference was not significant (Fig. 4D). The addition of rotenone, which blocks reverse electron flow from coenzyme Q to complex I, attenuated H_2O_2 generation.

Figs. 4 C and D show a tendency for PINK1$^{+/+}$ mitochondria to generate more ROS than PINK1$^{-/-}$ mitochondria. However, their respiration rates were remarkably different (Figs. 4A and B). Therefore, we calculated the percentage free radical leak, which is the fraction of molecules of O_2 consumed that give rise to H_2O_2 release by mitochondria (free radical leak) during either pyruvate/malate or succinate State 4 respiration (Figs. 4E and F). For pyruvate/malate respiration, mitochondria isolated from PINK1$^{-/-}$ cells had higher proportion of H_2O_2 generation than PINK1$^{+/+}$ mitochondria. During succinate respiration without respiratory inhibitors, PINK1$^{-/-}$ mitochondria had also higher proportion of free radical leak mainly from complex I due to reverse electron flow from coenzyme Q to complex I. Because the differences disappeared with addition of rotenone, which inhibit reverse electron flow, ROS generation enhanced by loss of PINK1 was mostly from complex I.

Discussion

We produced an *in vitro* model of Parkinson's disease, immortalized PINK1$^{-/-}$ MEFs. Previously, impairment of mitochondrial respiration was observed in the brains of PINK1$^{-/-}$ mice (Gautier et al., 2008). PINK1$^{-/-}$ MEFs clearly showed a phenotype of mitochondrial dysfunctions, which is consistent with PD pathogenesis. This phenotype was apparent in a cell growth experiment using medium containing galactose instead of glucose (Fig. 1B). Mitochondrial fragmentation was observed when PINK1$^{-/-}$ MEFs grew in the galactose medium (Fig. 1C), which was consistent with previous reports (Exner et al., 2007; Grünewald et al., 2009). Our results have unveiled that the PINK1$^{-/-}$ MEF line could be a potential PD model, presenting growth retardation due to decreased mitochondrial respiration activity. Thus, the PINK1$^{-/-}$ MEFs are a useful tool for evaluating the role of PINK1 in mitochondrial dysfunction and relevant to PD.

In PINK1$^{-/-}$ MEFs, mitochondrial membrane potential was decreased compared with that in littermate wild-type MEFs (Figs. 2C and D), as reported previously for PINK1 knock-down HeLa cells (Exner et al., 2007) and stable PINK1 knock-down neuroblastoma cell lines (Sandebring et al., 2009). This is a key event during elimination of mitochondria. Mitochondrial fission followed by selective fusion segregates damaged mitochondria, which decreases their membrane potential, and permits their removal by autophagy (Twig et al., 2008). The PINK1–parkin pathway is thought to have a crucial role in this mitochondrial elimination mechanism (Geisler et al., 2010; Kawajiri et al., 2010; Matsuda et al., 2010; Narendra et al., 2008, 2010; Vives-Bauza et al., 2010). To clarify what caused the decrease in mitochondrial membrane potential, we performed a modular kinetic analysis using isolated mitochondria (Fig. 3). This analyzes the kinetics of the whole of oxidative phosphorylation divided into three modules connected by their common substrate or product, mitochondrial membrane potential ($\Delta\psi$). The modules are include one $\Delta\psi$-producer (substrate oxidation) and two $\Delta\psi$-consumers (phosphorylating system and proton leak) (Brand, 1990). To decrease $\Delta\psi$, the $\Delta\psi$-producer should be down-regulated and/or $\Delta\psi$-consumers should be up-regulated. As cellular ATP levels were decreased compared with those in littermate wild-type MEFs (Fig. 2B), it is unlikely that the phosphorylating system is up-regulated. Indeed, the kinetics of the phosphorylation module were not altered (Fig. 3C). The other $\Delta\psi$-consumer, proton leak,

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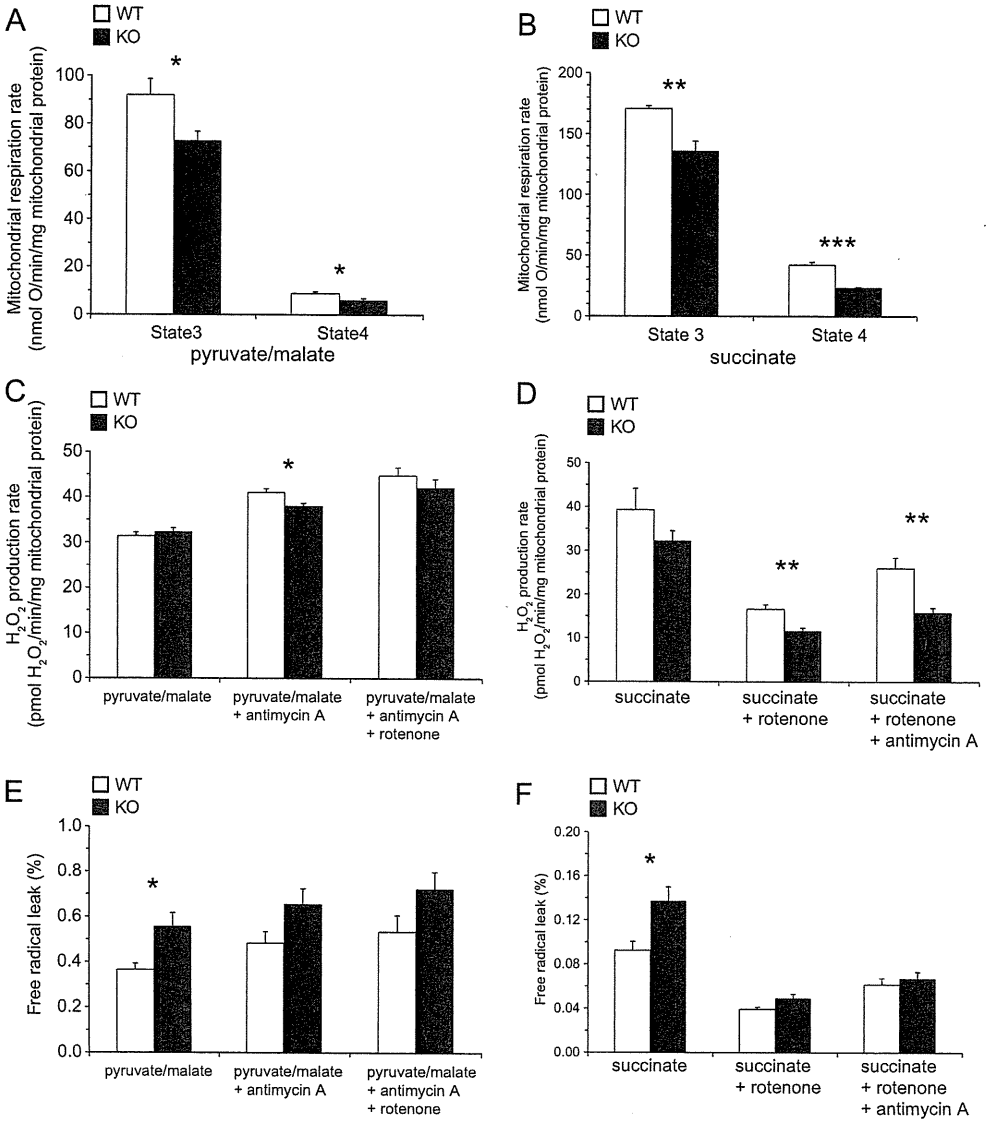

Fig. 4. Oxygen consumption rate and H_2O_2 production rate of mitochondria isolated from PINK1$^{+/+}$ and $^{-/-}$ MEFs. Open bars, PINK1$^{+/+}$ MEFs; closed bars, PINK1$^{-/-}$ MEFs. (A) State 3 and State 4 respiration rate of mitochondria with pyruvate/malate as a respiratory substrate. (B) State 3 and State 4 respiration rate of mitochondria with succinate as a respiratory substrate. Data were derived from the results of modular kinetic analysis (Fig. 3). State 3 respiration rates were the kinetic start points of the $\Delta\psi$-consumers (the sum of the phosphorylating system and proton leak). State 4 respiration rates were average values of the respiration rates at the kinetic start points of substrate oxidation and proton leak. (C, D) Mitochondrial H_2O_2 production rate with pyruvate/malate (C) or succinate (D) as a respiratory substrate. (E, F) Percentage free radical leak (FRL) for State 4 respiration with pyruvate/malate (E) or succinate (F) as a respiratory substrate. Error bars indicate SEM ($n=5$ and 4 independent mitochondrial preparations for pyruvate/malate and succinate respiration, respectively). *$P<0.05$; **$P<0.01$; ***$P<0.001$.

which partially dissipates the membrane potential without ATP synthesis, was also not changed (Fig. 3B). Therefore, the decrease in membrane potential caused by loss of PINK1 is likely to have been caused only by lower activity of the $\Delta\psi$-producer, substrate oxidation (Fig. 3A). This is the first report showing that mitochondrial membrane potential decrease caused by loss of PINK1, which is the key event for the following mitochondrial elimination, was not due to proton leak, but to respiratory chain defects. We used only succinate (a complex II-linked substrate) as a respiratory substrate in the modular kinetic analysis for technical reasons. However, complex I-linked respiration (pyruvate/malate) was also decreased in PINK1$^{-/-}$ MEFs like succinate respiration (Fig. 4A).

The mitochondrial respiration rates in State 4 were decreased in PINK1$^{-/-}$ MEFs, and consequently, the proportions of free radical leak were significantly higher in PINK1$^{-/-}$ MEFs than in PINK1$^{+/+}$ MEFs (Figs. 4E and F). Because the differences disappeared with addition of rotenone (complex I inhibitor, which inhibits reverse electron flow from coenzyme Q to complex I), ROS generation enhanced by loss of PINK1 was mostly from complex I. These results are partially consistent with those in previous reports, suggesting that MPTP and rotenone induce neuronal cell death by inhibiting complex I activity, leading to a PD-like phenotype (Dauer and Przedborski, 2003; Jackson-Lewis and Przedborski, 2007; Trojanowski, 2003).

In this study, we developed an *in vitro* PD model, the PINK1$^{-/-}$ MEF line, and established the experimental conditions for cell growth to detect mitochondrial dysfunction. This is the first report showing that complete ablation of PINK1 causes a decrease in mitochondrial membrane potential, which is not due to proton leak, but to respiratory chain defects.

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Review Article

Genetic Mutations and Mitochondrial Toxins Shed New Light on the Pathogenesis of Parkinson's Disease

Shigeto Sato and Nobutaka Hattori

Department of Neurology, Juntendo University School of Medicine, Tokyo 113-8421, Japan

Correspondence should be addressed to Nobutaka Hattori, nhattori@juntendo.ac.jp

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The cellular abnormalities in Parkinson's disease (PD) include mitochondrial dysfunction and oxidative damage, which are probably induced by both genetic predisposition and environmental factors. Mitochondrial dysfunction has long been implicated in the pathogenesis of PD. The recent discovery of genes associated with the etiology of familial PD has emphasized the role of mitochondrial dysfunction in PD. The discovery and increasing knowledge of the function of PINK1 and parkin, which are associated with the mitochondria, have also enhanced the understanding of cellular functions. The PINK1-parkin pathway is associated with quality control of the mitochondria, as determined in cultured cells treated with the mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP), which causes mitochondrial depolarization. To date, the use of mitochondrial toxins, for example, 1-methyl-4-phynyl-tetrahydropyridine (MPTP) and CCCP, has contributed to our understanding of PD. We review how these toxins and familial PD gene products are associated with and have enhanced our understanding of the role of mitochondrial dysfunction in PD.

1. Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, affecting 1% of the population above the age of 60. The classical form of the disease is characterized clinically by rigidity, resting tremor, bradykinesia, and postural instability. In addition to these four cardinal symptoms, many nonmotor symptoms frequently appear in PD, such as cognitive impairment, hallucinations, delusion, behavioral abnormalities, depression, disturbances of sleep and wakefulness, loss of smell, pain, and autonomic dysfunctions such as constipation, hypotension, urinary frequency, impotence, and sweating. The pathological hallmarks of PD are the preferential loss of dopaminergic neurons of the substantia nigra (SN) pars compacta and formation of Lewy bodies. Exposure to environmental factors inducing mitochondrial toxin like1-methyl-4-phynyl-tetrahydropyridine (MPTP) produces selective degeneration of dopaminergic neurons in SN and results in an irreversible Parkinsonism [1–3]. The active metabolite of MPTP, 1-methyl-4-phenylpyridinium ion (MPP+), is an inhibitor of complex I, and it accumulates in dopaminergic neurons because it is actively transported via dopamine transporter (DAT) [4–6]. The inhibition of the electron transport induces oxidative damage by increasing the formation of reactive oxygen species (ROS) and leads to further mitochondrial dysfunction [7]. These findings were supported by evidence of oxidative damage including an increase in lipid peroxide [8], decrease in glutathione [9], increase in hydroxynonenal-modified proteins [10], and increase in 8-hydroxy-deoxy guanine [11] in SN. ROS impair mitochondrial proteins, further aggravating mitochondrial function. Ultimate outcomes are dissipation of mitochondrial membrane potential and the release of cytochrome c into the cytoplasm and activation of the apoptotic cascade. A biochemical link between MPTP toxicity and Parkinsonism was confirmed with the finding of low levels of complex I in the SN, skeletal muscle, and platelets in patients with PD [12, 13]. In contrast, it remains unknown whether this systemic deficiency of complex I is crucially related to dopaminergic cell loss in PD. Rats administered rotenone (an inhibitor of complex I) developed neuronal degeneration and formation of synuclein-positive

inclusions; however, the degree of complex I inhibition was not severe enough to induce brain mitochondrial dysfunction [14]. Although inhibition of complex I and production of free radical result in increased oxidative stress, it remains unclear whether such dysfunction is a primary or a secondary process in the pathogenesis of the disease.

2. Involvement of Two Mitochondrial Toxic Pathways in Synuclein, DJ-1, and Parkin Mice Model

Several mutations of the synuclein gene (SNCA) at the *PARK1* locus induce autosomal dominant Parkinsonism. Three missense mutations: A53T [15], A30P [16], and E46K [17], duplications [18–21], and triplications [22, 23] of SNCA have so far been described. Triplications are associated with Parkinsonism and dementia, and the age of onset is younger than the other mutations, and the neuropathological changes are those of diffuse Lewy body disease. Regarding the pathogenesis of *PARK1*-linked PD, accumulation of normal synuclein is likely to predispose nigral neurons for protofibril formation. Toxicity associated with increased synuclein expression is an important cellular event that enhances the genetic predisposition to sporadic PD. At present, indirect evidence suggests a relationship between synuclein and oxidative stress, including protein carbonylation and lipid peroxidation. Furthermore, synuclein-deficient mice were found to have striking resistance to MPTP-induced degeneration of dopaminergic neurons, and this resistance appeared to be related to failure of the toxin itself. Interestingly, there was dissociation in the resistance between MPTP- and rotenone-induced cell vulnerability of synuclein-null dopaminergic neurons [24]. This result suggests that MPTP associates with synuclein through another pathway independent of complex I inhibition (mitochondrial dysfunction), to finally induce dopaminergic cell death. Several mutations of the DJ-1 gene at the *PARK7* locus induce autosomal recessive Parkinsonism [25]. Clinical phenotype is characterized by an onset in the midthirties, good levodopa response, and slow disease progression. Several lines of evidence suggest that it plays a role in the oxidative stress response [26, 27]. Subcellular localization studies have shown DJ-1 to be present in the cytosol, mitochondria, and nucleus [26, 28, 29]. Junn et al. [30] showed that in response to oxidative stress, some of the DJ-1 protein is translocated from its major cytosolic pool to mitochondria and nucleus. DJ-1 null mice are vulnerable to MPTP [31]. On the other hand, Thomas et al. [32] reported that the susceptibility of SN to MPTP in mice is independent of parkin activity. In short, the absence of parkin does not seem to increase the vulnerability of dopaminergic neurons to MPTP intoxication. Another study also found that oxidative stress, including MPTP, altered parkin solubility, causing parkin aggregation, thereby suggesting parkin dysfunction as a pathogenic mechanism of sporadic PD [33].

3. Functional Interplay between PINK1 and Parkin to Maintain Mitochondrial Integrity

Many mutations of the parkin gene at the *PARK2* locus induce autosomal recessive Parkinsonism [34–38]. The usual age of onset is between 20 and 40 years. Clinical features consist of dystonia and sleep benefit, which are also characteristic symptoms. Despite affected patients responding well to levodopa, they soon develop motor fluctuations. Conversely, mutations of the PINK1 (PTEN-induced kinase 1) gene at the *PARK6* locus induce autosomal recessive Parkinsonism. The age of onset is slightly delayed relative to *PARK2*, that is, from 32 to 48 years [39]. The affected patients show levodopa-responsive Parkinsonism. PINK1 contains an N-terminal mitochondrial targeting signal and a highly conserved serine/threonine kinase domain, and many missense and nonsense mutations have been reported at the kinase domain [40–44]. In particular, the identification of PINK1 mutations has strongly implicated mitochondrial dysfunction in the pathogenesis of PD [40]. The activity of PINK1 kinase is crucial for mitochondrial maintenance via TRAP phosphorylation [45]. The loss of PINK1 function results in increased vulnerability to various stresses [46–48]. Drosophila models have demonstrated that PINK1 and parkin ensure stable mitochondrial function. Parkin null mutants show severe mitochondrial pathology associated with reduced lifespan, apoptosis, and muscle degeneration [49]. While the PINK1 mutant phenotype can be rescued by parkin gene overexpression [50, 51], the converse does not occur, suggesting that parkin acts downstream of PINK1 in a common pathway to maintain mitochondrial integrity. PINK1 loss-of-function results in reduced mitochondrial membrane potential [52], and the PINK1-parkin pathway is associated with mitochondrial elimination in cultured cells treated with the mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP), which causes mitochondrial depolarization [53–58]. The exact mechanism underlying CCCP-induced mitochondrial depolarization, leading to mitochondrial autophagy, has been examined in detail. At steady state, parkin is localized throughout the cytosol but not in the mitochondria. However, parkin was rapidly recruited into the mitochondria when HeLa cells were treated with CCCP [55]. Furthermore, PINK1 recruits parkin from the cytoplasm to the low-membrane potential mitochondria, resulting in the mitochondrial degradation. Interestingly, the ubiquitin-ligase activity of parkin is repressed in the cytoplasm at steadystate; however, PINK1-dependent mitochondrial localization triggered by mitochondrial depolarization liberates the potential enzymatic activity of parkin. While CCCP is well described, its mitochondrial toxic effects provide new insights on the functional interplay between PINK1 and parkin.

4. Accumulation of PINK1 in Damaged Mitochondria

PINK1 is localized in both the mitochondria [40, 59] and the cytoplasm [55, 60]. Treatment with CCCP results in gradual accumulation of PINK1 and translocation of the cytoplasmic

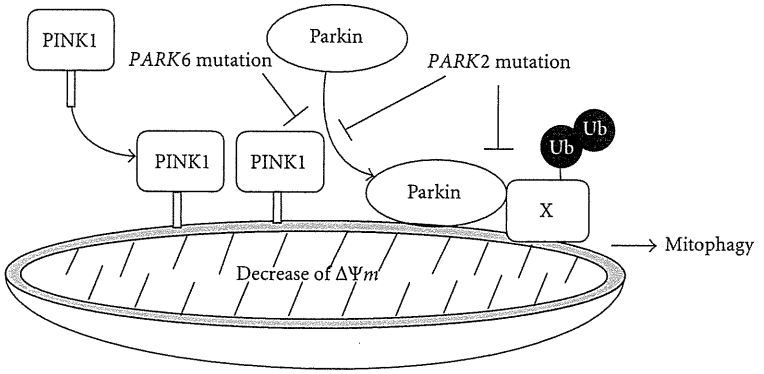

FIGURE 1: Schematic representation of PINK1-parkin-mediated mitophagy. In damaged mitochondria, PINK1 and parkin regulate mitochondrial elimination by inducing mitophagy. Under steady state, PINK1 is cleaved and degraded rapidly in the mitochondria. This process may be inhibited by the mitochondrial depolarization, resulting in PINK1 accumulation in the mitochondria. This accumulation is a crucial signal for parkin recruitment to the mitochondria. Parkin is presumed to ubiquitinate substrate (X), resulting in the induction of mitophagy.

PINK1 to the mitochondria. The subcellular localization of PINK1 is regulated by the mitochondrial membrane potential. Such accumulation may be the first trigger of PINK1-related parkin recruitment. Co-overexpression of PINK1 and parkin results in their colocalization in the mitochondria [61]. Even when these cells were not treated with CCCP, overexpression of PINK1 was associated with translocation of parkin to the cells, together with their mitochondrial aggregation.

Moreover, overexpression of both PINK1 and parkin in the cells resulted in the complete disappearance of the mitochondria. These results suggest that both PINK1 and parkin are indispensable for mitochondrial elimination and that accumulation of PINK1 in the mitochondria results in recruitment of parkin to the mitochondria even in the absence of CCCP [54].

5. PINK1 Kinase Activity Is Essential for Translocation of Parkin

PINK1 is composed of an atypical N-terminal mitochondrial targeting signal and transmembrane domain, kinase domain in the middle, and a conserved C-terminal domain, and deletion of the N-terminal amino acids abolished the mitochondrial localization of PINK1 [62]. Among other mutations, G309D, L347P, and G409V are associated with reduction in PINK1-kinase activity, and a C-terminal domain deletion mutant is associated with PINK1 dysfunction [63, 64]. The G309D/L347P/G409V mutants preserved mitochondrial localization, though their mitochondrial elimination was less compared to cells expressing both the wild-type PINK1 and parkin. When introduced into PINK1-deficient cells, the mutants were unable to complement the localization of parkin [55]. These results indicate that targeting the kinase activity and mitochondrial distribution of PINK1 is important for the mitochondrial recruitment of parkin (Figure 1).

6. PINK1 Deficiency Itself Causes Respiratory Chain Defects

Impaired mitochondrial respiration was observed in the brain of PINK1 null mice [65] although the mechanism linking PINK1 to mitochondrial membrane potential remains to be determined. Amo et al. [66] reported depletion of the mitochondrial membrane potential and cellular ATP levels (~80%) in PINK1-deficient mouse embryonic fibroblasts (MEFs) compared with those in littermate wild-type MEFs. However, loss of PINK1 did not alter mitochondrial proton leak, which reduces the membrane potential in the absence of ATP synthesis. Instead, the authors reported reduced activity of the respiratory chain, which produces the membrane potential by oxidizing substrates using oxygen. The H_2O_2 production rate by PINK1 null mitochondria was lower due to low oxygen consumption rate, while the proportion (H_2O_2 production rate per oxygen consumption rate) was higher. These results suggest that mitochondrial dysfunction in PD is not caused by proton leak, but by a defective respiratory chain. Furthermore, rate of free radical leak was significantly higher in PINK1-deficient MEFs than in wild-type MEFs. Because the differences disappeared with the addition of rotenone (inhibitor of complex I, which inhibits reverse electron flow from coenzyme Q to complex I), conceivably ROS generation enhanced by loss of PINK1 was mostly from complex I. With regard to PINK1-related PD, ROS may be an important factor. The above may also explain why cytoplasmic PINK1 protects neurons against MPTP [47]. Inhibition of complex I itself is associated with increased ROS production [67]. These results are at least in part consistent with those of previous studies, suggesting that MPTP and rotenone induce neuronal cell death by inhibiting complex I activity, leading to a PD-like phenotype [68–70] (Figure 2).

It is not doubtful that ROS generation is harmful to the cells, but the process of cell death is supposed to be slow. The crucial point is how inhibition of complex I

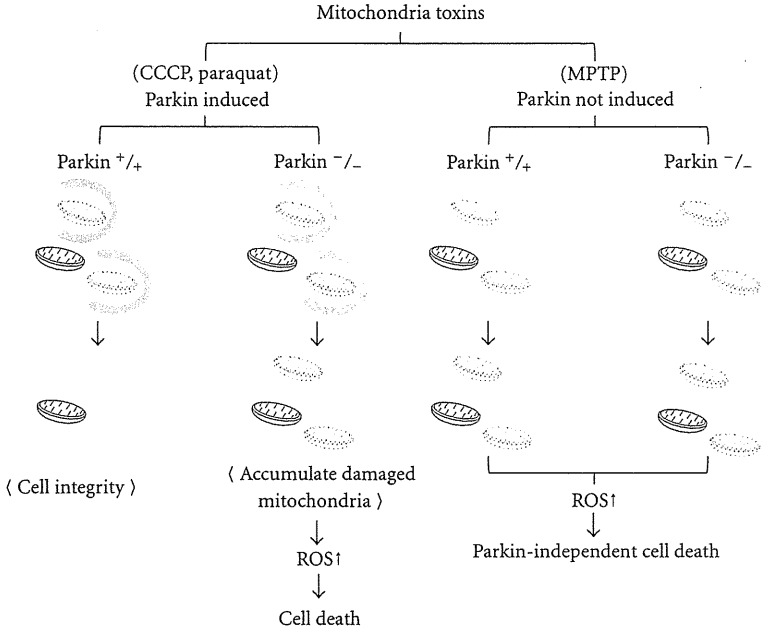

FIGURE 2: Two mechanisms of mitochondrial toxicity and parkin function. The effect of mitochondrial toxicity is different between CCCP and MPTP. Treatment with CCCP recruits parkin to the mitochondria resulting in mitophagy to keep mitochondrial integrity. Parkin deficiency is associated with accumulation of damaged mitochondria and accelerated cell death. Treatment with MPTP does not necessarily induce parkin. Parkin may be the sensor of damage-adaptive autophagy.

affects mitochondrial dysfunction including mitochondrial depolarization. Considering that the onset of *PARK6* (at 32–48 years) is slightly delayed relative to that of *PARK2* [39], some cases of PINK1 mutation might not affect parkin recruitment and thus maintain at least part of mitochondrial integrity. This may explain the late onset of *PARK6*. On the other hand, parkin did not translocate into the mitochondria when cells were treated with MPTP (our unpublished data). This finding means that inhibition of complex I does not necessarily induce low membrane potential. Further research is needed to investigate two independent pathogenic mechanisms related to MPTP and CCCP (Figure 2).

7. Conclusion

Cell death of dopaminergic neurons is due to a combination of exogenous stress and genetic predisposition. The discovery of PD genes has provided important insight including an understanding of PINK1-parkin mediated mitophagy. Furthermore, mitochondrial toxins provided crucial clues: (1) CCCP directly affects mitochondrial dysfunction and induces mitophagy; (2) MPTP toxicity seems to alter ROS generation rather than mitochondrial depolarization. The effects of mitochondrial toxins do not seem to be a one-way manner. The information is available for understanding the pathogenesis in PD. Here, we touched on the fringes of molecular mechanisms of PINK1-parkin-mediated mitophagy. Further research will elucidate how this quality control system applies to neurons.

Abbreviations

CCCP: Carbonyl cyanide m-chlorophenylhydrazone
DAT: Dopamine transporter
MEFs: Mouse embryonic fibroblasts
MPTP: 1-methyl-4-phynyl-tetrahydropyridine
PD: Parkinson's disease
PINK1: PTEN-induced putative kinase1
ROS: Reactive oxygen species
SN: Substantia nigra.

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Digest シリーズ

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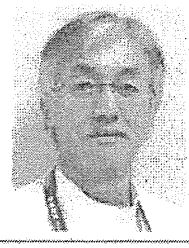
New gene discovery from one patient



坪井　義夫（つぼい　よしお）
1986年千葉大学医学部卒業。'97年より福岡大学神経内科所属。2000年から3年間米国Mayo Clinic留学後，'05年准教授，'11年より教授。研究テーマ：臨床神経学，運動障害学，特にパーキンソン病，レビー小体型認知症，前頭側頭型認知症，レストレスレッグズ症候群，プリオン病。

坪井　義夫

福岡大学医学部 神経内科学教室

Key Words: Perry症候群，家族性パーキンソン病，ダイナクチン，TDP-43

■はじめに

家族性パーキンソニズムは，1990年代後半に至るまでの神経学史において，稀な疾患とみなされ，遺伝型，臨床症候，画像所見，病理所見の特徴を記述し，分類学的試みが行われるにとどまり，病態解明には至らなかった。近年遺伝子学の発達により，この分野がパーキンソン病病態解明の足がかりとして，一気に神経科学分野でのトピックスに躍り上がった。その発端はαシヌクレイン遺伝子変異の発見であったと思われるが，これはイタリアのContursi家系の地道な臨床研究が結実した結果である。この蛋白が孤発性パーキンソン病脳にみられるLewy小体の構成蛋白であることから，この蛋白の機能解析，凝集の意義について研究が盛んになり，現在，凝集物の前段階であるαシヌクレインオリゴマーに細胞毒性があることが判明し，そこをターゲットとした新規治療薬の開発までに至っている。

また，αシヌクレイン蛋白凝集は細胞から細胞へ伝達するプリオン病の感染様式に類似するといったパーキンソン病の病勢進展におけるプリオン仮説まで登場している。ここにもうひとつ重要な蛋白が，パーキンソニズムの病態に関わる可能性が見えてきた。Perry症候群の研究から見出された，微小管を通じて細胞内の物質輸送に関わるダイナクチンである。

■Perry症候群と家族性のパーキンソン病研究

1975年にカナダのPerryらが，常染色体優性遺伝の家族性パーキンソニズムにうつ，体重減少，中枢性低換気を伴う疾患を初めて報告した。その後，類似の症候を呈するもう1家系がカナダに存在することが判明した。その後，約10年間忘れられていたが，1988年に米国，1992年にフランスより同様家系の報告があり，フランス家系の報告者，LechevalierによりPerry症候群（Perry-Purdy症候群）と名付けられた。これらの報告は，その臨床経過があまりにも特徴的で，他の家族性パーキンソニズムとは独立した疾患であることを報告者は確信していた。さらに1993年英国から報告されたのち，この疾患はさらに10年間空白の時期を迎える。

■始まりは一人の患者さん

私は1999年に43歳，若年性のうつ症状を呈した

■Yoshio Tsuboi, M.D., Ph.D.
Professor
Department of Neurology, Fukuoka University

患者のコンサルトを受けたが，その時の最初の記述が以下のようである。

「身長163 cm, 体重63 kg。軽度仮面様顔貌で精神活動の低下（無気力）がみられる。四肢の筋緊張がやや亢進し，Westphal現象が陽性，下肢はやや痙性が疑われる。歩行は腕の振りが欠如している。」

この当時の症状をまとめると，軽度のパーキンソン症候群があるも，目立つ症状は無気力であった。ちなみにベックのうつ病調査表では正常範囲であった。頭部のMRIでも異常はみられず，軽度パーキンソニズムに対してL-DOPAを内服し，軽度の改善をみたとの記載がある。

その後この患者は，仕事に対する意欲がなくなり，他の精神科病院に入院し抗うつ剤による治療を受けるが，そこで明らかなパーキンソニズムの悪化を示した。この状態は先の入院中に軽度錐体外路症状がみられたことから，薬剤誘発性パーキンソニズムではなく，薬剤によりパーキンソニズムが顕在化したものと解釈したほうが良いと思われる。その後薬剤の中断でいったん軽快するも，以前より明らかに進行した状態を呈していた。2000年X月に精神科病院に入院中の朝に，意識障害で発見された。その時の動脈血液ガス分析ではPCO2が72 Torrと高値で，救急病院に搬送され，CO2ナルコーシスと判断された。その時は人工呼吸器管理で意識は回復し，呼吸器からの離脱も可能であった。さらに当院に転院したが，驚いたことに，体重が初回入院時の63kgから38kgまで減少していた。その間の食事摂取に関しては，それ以前と大きく変わっていなかったものと考えられた。当然，この低換気に関して諸検査が行われたが，心機能，肺機能に異常はなく，Perryの原著にもある中枢性換気不全という病態が考えられた。このパーキンソニズム，うつ，体重減少，中枢性低換気はPerry症候群の4徴候といえる。この時点で我々はこの家系が本邦に未だ報告のないPerry症候群であると確信した。

さらに原著の記載以外に我々の臨床的検討から様々なことがわかってきた。まずL-DOPAに対する効果は良い点，しかしすぐにその効果が減弱し，薬の効果が血中濃度と主に変動するウエアリング・オフ現象，ジスキネジアの早期出現などで，うつはあまり強くなく中核症状は無気力（apathy）であること，抗精神病薬の薬剤感受性が強いことなどであった。

■パーキンソン病遺伝学黎明期

この間1997年以来に家族性パーキンソン病の研究はいつの間にか黎明期を迎えていた。この年にCuntursi家系からαシヌクレイン遺伝子の異常が発見され，臨床遺伝研究が盛んになった。さらに前頭側頭型認知症パーキンソニズムでもタウ遺伝子変異が発見。その後さらに拍車がかかり，常染色体劣性パーキンソニズムで*Parkin*, *DJ-1*, *PINK1*遺伝子の変異が見つかり，常染色体優性型においても新たに，*LRRK2*, *SCA2*遺伝子の関与が相次いで判明した（表）。この時に，私はフロリダのMayo Clinic Jacksonvilleに籍をおき，時の神経内科医のWszolek先生と遺伝学者のFerrer先生と共に，Perry症候群の臨床病理学的研究，および原因遺伝子の探求を目的に，国際共同研究が2001年に始まった。

その後，それまで報告のあった6家系から5つの家系の主治医に連絡を取ることに成功し，その間，新たにハワイと本邦から未発表の家系が発見された。我々はなるべく詳細な臨床経過を集め検討したほか，すでに報告された家系の剖検脳組織をMayo Clinicに集め，さらにDNAサンプル（計8家系から17人の発症者を含む74人）も集約され，結果としてこの疾患の臨床病理的特徴と新たな遺伝子変異の検索が始まった。

■おわりに

比較的小さい家系が多かったPerry症候群において，責任遺伝子の検討作業は難航した。しかし，その後も発症者や家系内未発症者のDNAサンプルが徐々に集まり，責任遺伝子の連鎖解析はいくつかの染色体部位に絞られてきた。また2006年に，

表　パーキンソン病の原因遺伝子/染色体と臨床症候および病理所見

名称	遺伝型	染色体	蛋白	臨床症候	病理所見
PARK1&4	AD	4q21	SNCA	EOPD	Synucleinopathy
PARK 2	AR	6q25.2-27	Parkin	Juvenile and EOPD	
PARK 3	AD	2p13	unknown	LOPD	
PARK 5	AD	4p14	UCHL1	LOPD	
PARK 6	AR	1p35-36	PINK 1	EOPD	
PARK 7	AR	1p36	DJ-1	EOPD	
PARK 8	AD	12p12	LRRK2	LOPD	Synucleinopathy/Tauopathy
PARK 9	AR	1p36	ATP13A2	Kufor-Rakeb syndrome	
PARK 10	S	1p32	ELAV4	Not clear	
PARK 11	AD	2q36-q37	GIGYF2	LOPD	
PARK 12	S	Xq21-q25	unknown	Not clear	
PARK 13	AD	2p12	Omi/HTRA2	Not clear	
PARK 14	AR	22q13.1	PLA2G6	Adult onset dystonia-parkinsonism	
PARK 15	AR	22q12-13	FBXO7	Early onset parkinsonian-pyramidal syndrome	
SCA2	AD	12q24.1	Ataxin2	LOPD	Synucleinopathy
FTDP-17	AD	17q21-22	MAPT	FTDP-17	Tauopathy
Perry	AD	2p12-14	DCTN1	Perry syndrome	TDP-43 proteinopathy

AD：常染色体優性遺伝，AR：常染色体劣性遺伝，EOPD：若年発症パーキンソン病　S：孤発性
LOPD：高齢発症パーキンソン病，FTDP-17：17番染色体関連前頭側頭型認知症パーキンソニズム

TDP-43蛋白の異常な凝集が筋萎縮性側索硬化症および前頭側頭型認知症の中枢神経から発見されたが，これもPerry症候群の病態解明に偶然の発見を導いた。このように1人の患者さんから古くから知られていた疾患が再び注目され，遺伝学的，分子生物学的な偶然が重なり一気に新しい発見へと進むことができた。

News(学会情報)

●第45回日本臨床腎移植学会

開催日：2月1日（水）～3日（金）
代表者：服部　元史（東京女子医科大学教授）
会場：軽井沢プリンスホテルウエスト
テーマ：ハーモニー
事務局連絡先：東京女子医科大学腎臓小児科
TEL：03-3353-8111（内38317）
FAX：03-3359-4877
常設事務局URL：http://www.jscrt.jp/
開催案内URL：http://www.pcoworks.jp/jscrt45/

●第8回日本消化管学会総会学術集会

開催日：2月10日（金）～11日（土祝）
代表者：本郷　道夫（東邦大学病院教授）
会場：仙台国際センター，江陽グランドホテル
テーマ：「消化管学不楽是如何」
事務局連絡先：勁草書房コミュニケーション事業部
TEL：03-5840-6339
FAX：03-3814-6904
常設事務局URL：http://www.jpn-ga.jp/
開催案内URL：http://www.keisocomm.com/8jga/index.html

《トピックス》

1 パーキンソン病の基礎研究最前線：実地医家のための minimum requirement

船山 学*（ふなやま まなぶ） 富山弘幸**（とみやま ひろゆき）

ポイント

- パーキンソン病の原因遺伝子として *α-synuclein*, *parkin*, *PINK1*, *LRRK2* などがあるが，最近新規原因遺伝子 *VPS35* も報告され，分子遺伝学的研究は日進月歩である．
- *α-synuclein*, *LRRK2*, *GBA* は重要な感受性遺伝子であり，孤発性パーキンソン病の病態においても密接に関与している．
- ミトコンドリアの品質管理不全がパーキンソン病に関与している．
- パーキンソン病の基礎研究と日常診療の進歩のなかで，研究者および実地医家の双方の果たすべき役割がますます大きくなってきている．

キーワード アルファシヌクレイン，ベータグルコシダーゼ，エクソーム解析，次世代シークエンサー，ミトコンドリア，オートファジー・リソソーム

*順天堂大学大学院医学研究科 老人性疾患病態・治療研究センター　**順天堂大学医学部 脳神経内科/神経変性疾患病態治療探索講座

パーキンソン病の基礎研究は 90 年代後半から分子遺伝学的手法によってさまざまな病因遺伝子が単離され始めてから飛躍的に進み，正に日進月歩の勢いで次々に新しい重要な知見が明らかになってきている．本稿では基礎研究分野における最新の話題を中心に紹介する．

◉パーキンソン病の遺伝子研究

1997 年に *α-synuclein* 遺伝子変異が報告されて以来，家族性パーキンソン病の分子遺伝学的解析が進み，現在までに多くの遺伝子が単離されている（**表**）．また近年は，*α-synuclein* 遺伝子や *parkin* 遺伝子のようなメンデル遺伝型の原因遺伝子のほかに，パーキンソン病が発症しやすくなる発症感受性遺伝子もゲノムワイド関連解析の研究成果等から次々と報告されている（**表**）．

1．メンデル遺伝型原因遺伝子

家族性パーキンソン病はパーキンソン病の 5～10％を占めると考えられており，実地医家が病歴，家族歴を詳細に聴取し，家系図を記載していくことは大変重要である．

常染色体優性遺伝性の原因遺伝子として *α-synuclein*, *leucine-rich repeat kinase 2* (*LRRK2*) 等が報告されている．臨床像は中～高齢発症で典型的な孤発性パーキンソン病と同様の症例が多い．わが国においては *α-synuclein* の重複変異と *LRRK2* の点変異が報告されている．いずれも頻度はそれほど高くないが，地域差があり，地域集積性，遺伝性疾患が疑われる（もしくは否定できない）場合，前の世代も含め出身地を聴取することも遺伝子解析，診断上有用である．

表　パーキンソン病関連遺伝子の一覧

	染色体上の位置	遺伝子	遺伝形式
PARK1	4q22.1	*SNCA*	常優/感受性
PARK2	6q25.2-27	*parkin*	常劣
PARK3	2p13	unknown	常優
PARK4	4q22.1	*SNCA*（重複）	常優
PARK5	4p14	*UCH-L1*	常優
PARK6	1p36-p35	*PINK1*	常劣
PARK7	1p36	*DJ-1*	常劣
PARK8	12p11.2-q13.1	*LRRK2*	常優/感受性
PARK9	1p36	*ATP13A2*	常劣
PARK10	1p	unknown	感受性
PARK11	2q37.1	*GIGYF2*	常優
PARK12	Xq21-q25	unknown	感受性
PARK13	2p12	*HTRA2*	常優
PARK14	22q13.1	*PLA2G6*	常劣
PARK15	22p12.3	*FBXO7*	常劣
PARK16	1q32	unknown	感受性
—	4p16	*GAK*	感受性
—	6p21.3	*HLA-DRB5*	感受性
—	17q21.1	*MAPT*	常優/感受性
—	1q21	*GBA*	感受性
—	4p15	*BST1*	感受性

常染色体劣性遺伝性の原因遺伝子としては *parkin*，*PTEN induced putative kinase 1*（*PINK1*），*DJ-1*，*ATPase type 13A2*（*ATP13A2*），*phospholipase A2, group VI*（*PLA2G6*）等が報告されている．特に *parkin* はわが国から 1998 年に初めて報告された遺伝子で非常に重要である．*parkin* 遺伝子変異の頻度は常染色体劣性遺伝性のなかで約 50％と頻度が高く，若年発症の孤発例でも約 15％に認めたとする報告もある．したがって血族婚がある等劣性遺伝が疑われる患者，特に若年発症の患者については積極的に *pakin* 遺伝子検査を行うべきであろう．*PINK1* は臨床的には *parkin* 変異陽性例とほとんど変わりないが，若干発症年齢が高い印象がある．

若年性パーキンソニズムで最近注目されているのが *PLA2G6* である．*PLA2G6* は 2006 年に neurodegeneration with brain iron accumulation（NBIA）の原因遺伝子として報告されていたが，2009 年に若年性ジストニア-パーキンソニズム（*PARK14*）の原因遺伝子としても報告された．自験例では，認知症あるいは精神発達遅滞，または幻覚，妄想などの精神症状を呈する若年性パーキンソニズム患者 30 例について解析した結果，3 名に *PLA2G6* 遺伝子変異を認めた．これらの患者には既報とは異なりジストニアは認めず *PLA2G6* 陽性パーキンソニズムは臨床的に variable であるが，発症年齢，家族歴，認知症や精神症状の合併，大脳萎縮や鉄沈着のような MRI 所見など，臨床像をよく検討した後に変異解析をすれば比較的高頻度で変異を同定できる可能性が