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PARKIN, PARKINSON DISEASE GENE PRODUCT, DIRECTLY REDUCES HYDROGEN PEROXIDE (MITOCHONDRIAL OXIDANT), AND FORMS DIMERIZATION REVERSIBLY.

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Abstract – The current central dogma of Parkin function is that the E3 ligase Parkin ubiquitinates its substrate on the surface of damaged mitochondria and removes them to maintain mitochondrial quality. However, recent pathological and biochemical evidence confirmed that Parkin has a protective function from oxidative stress, especially in mitochondria. The relationship between the two hypotheses has not yet been clear. In this short report, we show; First, Parkin reacts with H_2O_2 directly and reduces it, and turns into Parkin dimer. Furthermore, supplied antioxidant changes dimer into monomer again. These suggest that Parkin is also a redox molecule in mitochondria as well as E3 ligase outside mitochondria.

Keywords - Parkin; PINK1; DJ-1; Familial Parkinson's Disease; Mitochondria; Oxidative Stress; E3 Ligase; Hydrogen Peroxide; Dimerization

I. INTRODUCTION

Mutations in recessively inherited Parkinson's disease (PD) genes, *parkin*, *PINK1*, and *DJ-1* manifest young-onset typical parkinsonian symptoms [1]. The relationship between three genes has been elucidated for years, and it has been reported that they are indispensable for maintenance of mitochondrial quality and protection from oxidative stress. It is widely affirmed from the *in vitro* studies using uncouplers that the cooperation of Parkin and PINK1 induce "mitophagy" of damaged mitochondria; PINK1 on the depolarized mitochondria recruits Parkin, and then Parkin, an E3 ligase, ubiquitinates mitochondrial surface proteins and as a result, induces mitophagy and maintains mitochondrial quality [2]. Thus, loss of PINK1 or Parkin may cause accumulation of depolarized abnormal mitochondria in cells.

Nevertheless, no evidence of abnormal mitochondrial accumulation has been reported so far. On the other hand, the molecular mechanism of protection from oxidative stress by Parkin, and the relationship between its E3 ligase and antioxidant function, are also still unclear. In this short report, we first initiated the characterization of Parkin as a redox molecule which reacts with Hydrogen peroxide, one of the major mitochondrial free radicals as well as a superoxide anion.

II. MATERIAL & METHODS

H_2O_2 Assay and Peroxidase Assay: Hydrogen peroxide (H_2O_2) and peroxidase activity were determined by measuring fluorescence intensity of Amplex® Red reagent following the company's instructions (Molecular Probes). The tag of MBP (Maltose Binding Protein)-Parkin protein was utilized for the above assays. In an H_2O_2 assay, four amounts of each protein of MBP-Parkin, alpha-Synuclein (SNCA), and MBP were reacted in 60 µl mixtures with H_2O_2 (final 5.0 µM) for 30 min at 37°C. Then, 50 µl of each reacted sample was mixed with 50 µl of the Amplex Red reagent/HRP working solution and incubated for 30 min at 37 °C. Fluorescence at Ex560 nm and Em590 nm was measured. Of note, the final concentration of H_2O_2 of each component was two-fold lower (2.5 µM) by mixing with the same volume of reaction buffer. Similarly, a peroxidase assay was performed by directly mixing 50 µl of each protein, four amounts of which were prepared, with 50 µl of the Amplex Red reagent/H_2O_2 working solution for 30 min at 37 °C, and the fluorescence was determined.

Reversible Oxidation of Purified Parkin Protein: c-Myc-Parkin proteins were utilized to expose to five sets of oxidant, H_2O_2 or/and antioxidant, Dithiothreitol (DTT): final concentration, 1) 0.2 mM H_2O_2, 2) 2 mM H_2O_2, 3) 100 mM DTT, for each for 5 min incubation at room temperature, 4) 0.2 mM H_2O_2 for 5 min + 100 mM DTT for next 5 min, 5) 2 mM H_2O_2 for 5 min + 100 mM DTT for next 5 min. Each 100 ng protein was loaded on an SDS-PAGE gel for Western blot analysis. Anti-Parkin antibody (Cell Signaling) was used as a primary antibody (1:1000 dilution), and anti-rabbit IgG antibody was used as a secondary antibody (1:10000 dilution).

III. RESULTS

The previous investigation by Meng *et al*. [3] reported that mass spectrometry analysis detected that H_2O_2 treatment in recombinant GST-Parkin changes specific cysteine residues of Parkin to sulfonated cysteine residues, resulting in perturbation of its E3 activity. From the opposite perspective, this suggests that Parkin has a potentially vigorous antioxidant function that provides protons via its cysteine residues. To confirm that at the molecular level, we treated H_2O_2 directly with purified Parkin protein. Parkin cleared H_2O_2 in a dose-dependent manner (Fig. 1).

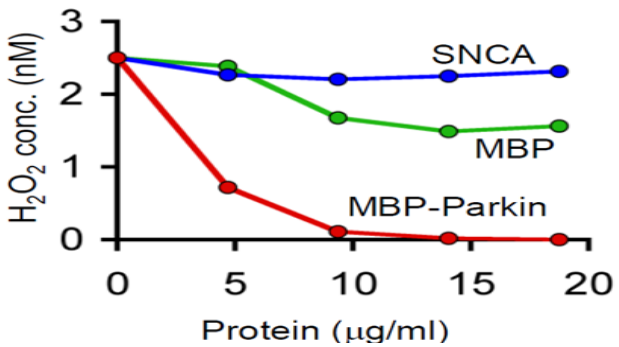

Figure 1. H_2O_2 Assay. Parkin reacts with H_2O_2 and reduces H_2O_2.

Another PD gene, DJ-1, which is well known as an antioxidant and redox molecule, has already been shown to have a similar result using the same experiment [4].

Next, we performed a peroxidase assay of purified Parkin, but Parkin revealed no peroxidase activity, suggesting that the Parkin molecule reacts with H_2O_2 directly through its cysteine residues, not as a catalytic enzyme (Fig. 2).

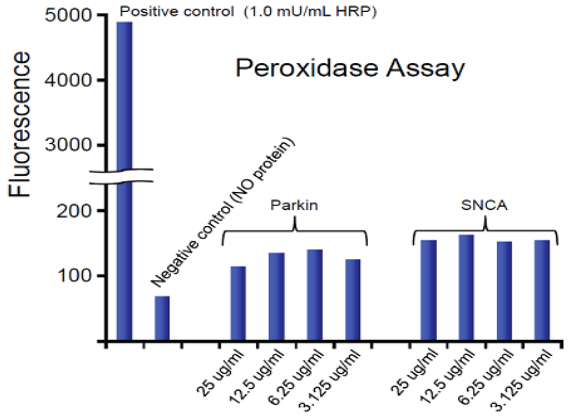

Figure 2. Peroxidase Assay. Parkin has no peroxidase activity.

To observe structural changes of Parkin protein by oxidative stress, we performed Western blot analysis of purified Parkin protein using anti-Parkin antibodies (Fig. 3). Parkin generates a dimer with H_2O_2 dose dependency. DTT, an antioxidant, reduces Parkin's dimerization. Interestingly, this dimerization treated with H_2O_2 is decomposed by DTT and returns to a Parkin monomer.

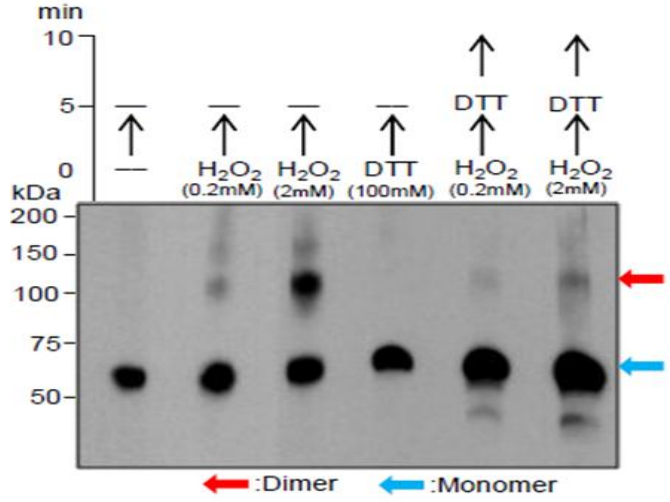

Figure 3. Reversible Oxidation of Purified Parkin Protein.

IV. DISCUSSIONS

As far as we know, there are no pathological reports that support abnormal mitochondrial accumulation, although there are many reports of autopsy cases lacking *parkin* or *PINK1* genes. Their knockout mouse models also revealed normal numbers and normal morphology, a fact confirmed by several groups [5, 6]. Although we do not deny the central principle at all, there may be several compensatory pathways that trigger mitophagy in addition to the Parkin/PINK1 pathway. By contrast, increased iron accumulation in the substantia nigra (SN) was confirmed in an autopsy case lacking the *parkin* gene, suggesting abundant reactive oxygen species (ROS) in the SN [7]. Although a mitophagy hypothesis of Parkin is not clearly able to explain a highly selective neuronal loss of SN and locus coeruleus because mitophagy is a universal phenomenon of all living cells, the lack of Parkin as an antioxidant will adequately explain the loss of dopaminergic neurons, where abundant ROS were generated from dopamine and its metabolites, with which Parkin reacts directly [8]. The synergistic effect of the loss of two functions of Parkin may accurately describe Parkin's real pathology.

In normal cellular metabolism, mitochondrial electron transport generates the formation of superoxide anion and subsequently H_2O_2 [9]. H_2O_2 increases in concentration under certain physiological and pathophysiological conditions and can oxidatively modify cellular components [9]. It is well known that Parkin translocates into mitochondria and has an antioxidant effect [10]. However, the molecular mechanism has not yet been sufficiently elucidated. Our study suggests that Parkin translocates into mitochondria and reacts with H_2O_2 and reduced H_2O_2. When Parkin is oxidized by H_2O_2, it generates dimer at the molecular level. Interestingly, Lavoie *et al.* [11] reported that Parkin generates its polymerization and results in a high molecular weight (HMW) of Parkin aggregation at the cell culture level. Our result suggests that this HMW of Parkin is not simply polymerization but is receiving some other modification including Parkin auto-polyubiquitination [12] at the cell culture level.

As for whether the Parkin function in mitochondria is merely a reaction with H_2O_2 or has other extensive functions as a local redox molecule, further research will likely be necessary.

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