

PARKINSON'S DISEASE GENE PRODUCT, PARKIN, HAS ALTERNATIVE AND REVERSIBLE FUNCTIONS, BOTH AS AN E3 LIGASE AND A REDOX MOLECULE.

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Abstract – We have recently shown that Parkin reacts with and reduces H_2O_2 , a main component in mitochondrial oxidative stress, and forms Parkin dimer. In this *in vitro* study, we indicated that H_2O_2 leads to Parkin autoubiquitination as well as dimerization, and reduces E3 ligase activity in the ubiquitin-proteasome system. However, the antioxidant Dithiothreitol (DTT) decomposes polyubiquitinated Parkin dimers to the monomer level and reactivates Parkin's E3 ligase activity, resulting in the increase of polyubiquitination of the substrate. Thus, Parkin protein has alternative and reversible functions as an E3 ligase and as a redox molecule to reduce oxidative stress. In other words, Parkin has a unique recycling system.

Keywords - Parkin; Parkinson's Disease; Mitochondria; Oxidative Stress; E3 Ligase; Ubiquitination; Proteasome; Hydrogen Peroxide; Auto-ubiquitination

I. INTRODUCTION

Parkin was identified as the first-ever recessively inherited Parkinson's disease (PD) gene by Kitada and his colleagues in 1998 [1]. Parkin protein was first characterized as an E3 ligase in 2000. This hypothesis illustrates that Parkin ubiquitinates its substrate and degrades it in the ubiquitin-proteasome system [2]. Furthermore, in 2010, several groups reported that Parkin ubiquitinates its substrate on the surface of mitochondria and degrades depolarized mitochondria through autophagosomes [3].

On the other hand, in parallel with the development of the Parkin-E3 ligase theory, other evidence has also been reported by a variety of researchers; Parkin has a protective function from oxidative stress and maintains mitochondrial quality. This hypothesis is also linked to the classical pathological theory of sporadic-type PD. Parkin translocates mitochondria and protects against oxidative stress [4], while the molecular mechanism by which it does so has not yet been elucidated enough. Furthermore, the mutual relationship between the two principal theories has not yet been clarified.

We have recently reported that Parkin reacts with H_2O_2 directly and reduces H_2O_2 [5], which is a main component of reactive oxygen species (ROS) in mitochondria along with superoxide anion. After reaction with H_2O_2 , Parkin generates its dimer, however, which is decomposed to Parkin monomer again by antioxidant. Our next question is whether or not Parkin regains E3 ligase activity when Parkin dimer returns into its monomer via an antioxidant. In this study, our data will provide an answer to this question, and we believe that our data also will provide the clue to make clear the mutual relationship between the two theories.

II. MATERIAL & METHODS

***In vitro* Parkin autoubiquitination assay:** *In vitro* Parkin autoubiquitination assay was performed by "Parkin Auto-

Ubiquitination kit (BostonBiochem; Cat. # K-105)" following the company's instructions. Briefly, a 20 μ l reaction mixture was incubated for 55 min at 37°C, including E1 enzyme (final 100 nM), UbcH7 (E2 enzyme, 5 μ M), His₆-Parkin (1.5 μ M), Biotin-Ubiquitin (100 μ M), Reaction Buffer (final 1 mM Mg-ATP, 50 mM Hepes pH 7.5, 50 mM NaCl, 1 mM DTT), both with and without H_2O_2 (final 2 nM) or/and Dithiothreitol (DTT, final 100 mM). The reaction was terminated by 6X SDS Loading Buffer for Western blot. Thus, in this experiment, Parkin proteins were exposed to four sets of oxidants, H_2O_2 or/and antioxidant, DTT: final concentration, 1) no H_2O_2 , 2) 2 nM H_2O_2 , 3) 100 mM DTT, for each for 55 min incubation at 37°C, and 4) 2 nM H_2O_2 for 55 min + 100 mM DTT for the next 5 min. Each reaction mixture was loaded on an SDS-PAGE gel for Western blot analysis. Anti-Parkin polyclonal antibody (Cell Signaling) was used as a primary antibody (1:1000~5000 dilution), and anti-rabbit IgG antibody was used as a secondary antibody (1:10000 dilution).

Fas associated factor 1 (FAF1) polyubiquitination assay

by Parkin: This experiment was performed in the same manner as the Parkin autoubiquitination assay, except that 90 ng of FAF1 protein (GST-FAF1, Abnova), one of Parkin's substrates [6], was added to the reaction mixture. Parkin proteins were exposed to four sets of oxidants, H_2O_2 or/and antioxidant, DTT: final concentration, 1) no H_2O_2 , 2) 2 nM H_2O_2 , 3) 100 mM DTT, each for 60 min incubation at 37°C, and 4) 2 nM H_2O_2 for 60 min + 100 mM DTT for the next 40 min. Each reaction mixture was loaded on an SDS-PAGE gel for Western blot. Anti-Parkin antibody (Cell Signaling), anti-ubiquitin antibody (Millipore), and anti-FAF1 antibody (Abcam) were utilized in the Western blot analysis.

Publication History

Manuscript Received : 29 July 2016
Manuscript Accepted : 7 August 2016
Revision Received : 20 August 2016
Manuscript Published : 31 August 2016

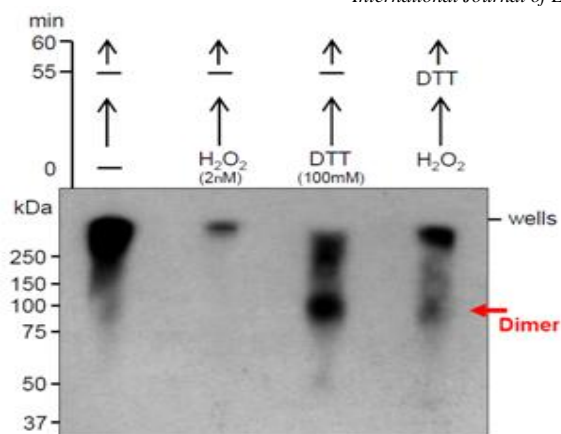


Figure 1. *In vitro* Parkin autoubiquitination assay (1)

III. RESULTS

Various types of Parkin autoubiquitination have been reported, importantly those that influence E3 ligase activity. Thus, this study investigated first how oxidative stress, H_2O_2 , affects Parkin autoubiquitination (Figure 1 and 2). In Figure 1, 2nM H_2O_2 induces high molecular weight (HMW) of Parkin-autoubiquitination aggregations. An antioxidant, DTT, leads to long vertical smear bands from dimer level to HMW of Parkin polyubiquitin chains. Interestingly, 100 mM DTT changes the HMW of Parkin-autoubiquitination aggregations caused by H_2O_2 into long vertical smear bands including the

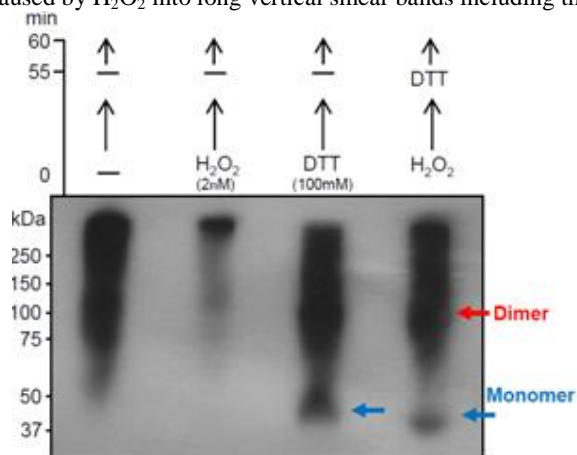


Figure 2. *In vitro* Parkin autoubiquitination assay (2): The film for the WB analysis was exposed and developed under different conditions, as shown in Figure 1, from the points of primary antibody (Ab) dilution and exposure time.

dimer level. Figure 2 shows smaller Parkin polyubiquitin chains at the monomer level. These figures demonstrate that H_2O_2 stress facilitates Parkin autoubiquitination. However, an antioxidant, DTT, reduces the HMW of Parkin polyubiquitin chains to the Parkin dimer or monomer level.

Next, using FAF1 with a GST tag, a Parkin substrate, we applied a similar approach to the substrate polyubiquitination assay to investigate how oxidative stress, H_2O_2 , affects substrate polyubiquitination and E3 ligase activity. Figure 3

shows that 2 mM H_2O_2 induced a very low-intensity band of FAF1 polyubiquitin chains (*), suggesting that H_2O_2 reduces Parkin's E3 activity. However, after exposure to H_2O_2 , 100 mM DTT transforms this low-intensity band to a higher intensity (**). DTT likely reactivates the disrupted Parkin.

Figure 4 shows that, when the same membrane was used for the immunoblot by anti-Parkin antibodies, H_2O_2 induces a HMW for Parkin-polyubiquitination aggregations, but DTT also decomposes this aggregation and generates various sizes of polyubiquitinated Parkin including the monomer level.

Figure 5 shows that, when the same membrane was used for the immunoblot by anti-ubiquitin antibodies, H_2O_2 generates a HMW of polyubiquitin complex bands. Interestingly, after exposure to H_2O_2 , DTT transforms them into long vertical smear bands including about 100 kDa level (see and compare * and **). Taken together, these results suggest that H_2O_2 also facilitates Parkin-polyubiquitination aggregations and reduces E3 ligase activity, resulting in decreased FAF1 polyubiquitination. However, DTT decomposes polyubiquitinated Parkin dimer and generates Parkin monomer, which regains E3 ligase activity and facilitates FAF1 polyubiquitination to be degraded properly.

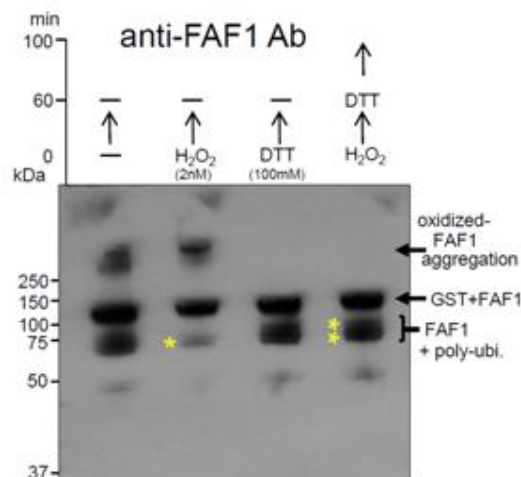


Figure 3. FAF1 Polyubiquitination Assay: WB Analysis using anti-FAF1 Ab.

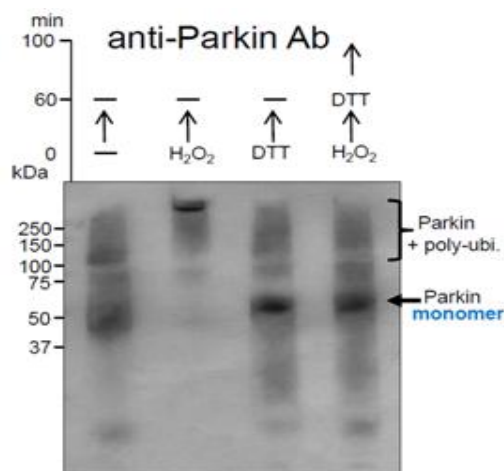


Figure 4. FAF1 Polyubiquitination Assay: WB analysis using anti-Parkin Ab.

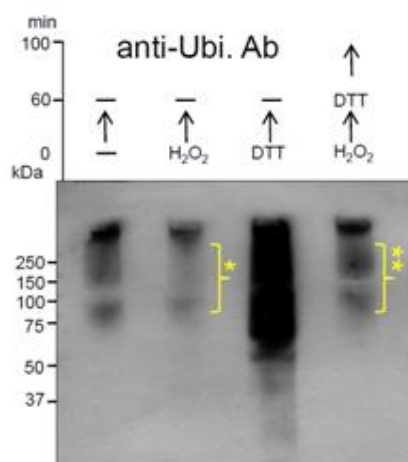


Figure 5. FAF1 Polyubiquitination Assay: WB Analysis using anti-ubiquitin Ab

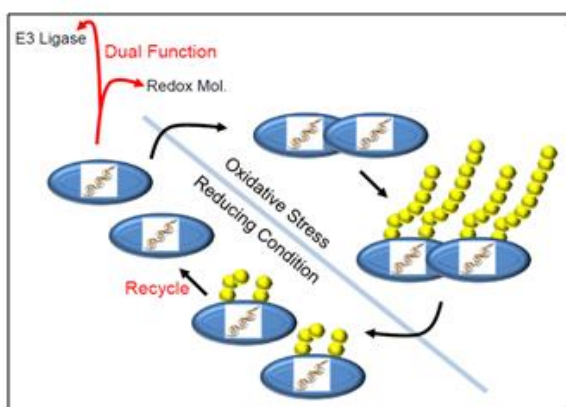


Figure 6. Schematic Model of a Unique Parkin Recycling System

IV. DISCUSSIONS

Previous investigations reported that Parkin itself is polyubiquitinated, which is called autoubiquitination. Various types of Parkin autoubiquitination have been reported; Parkin is likely capable of inducing monoubiquitination, multiple monoubiquitination, K48-linked polyubiquitination, and K63-linked polyubiquitination [7]. The ubiquitin-like domain of Parkin functions to inhibit its autoubiquitination [7].

Dongdong Yao et al. [8] showed that nitrosative stress leads to S-nitrosylation of wild-type Parkin and, initially, to a dramatic increase followed by a decrease in the E3 ligase-ubiquitin-proteasome degradative pathway. However, the initial increase in Parkin's E3 ligase activity leads to autoubiquitination of Parkin and subsequent inhibition of its activity, which would impair ubiquitination and clearance of Parkin substrates [8]. Thus, ROS may induce autoubiquitination and inhibit E3 ligase activity. Interestingly, this study showed that an antioxidant, DTT, shortens the polyubiquitin chains of Parkin to the monomer level.

Similarly, in FAF1 polyubiquitination assay, H_2O_2 also facilitates Parkin polyubiquitin chains and reduces E3 ligase activity, resulting in reduced FAF1 polyubiquitination. However, DTT decomposes Parkin autoubiquitination and

Parkin dimer to the monomer level, resulting in reactivating E3 ligase activity and facilitating FAF1 ubiquitination much more. It seems likely that such a similar unique Parkin recycling system, as shown in Figure 6, is actually working in the living cells, although antioxidants seem to be *in vivo* real objects like Glutathione. Further *in vivo* studies will likely be required to confirm this hypothesis.

ACKNOWLEDGMENT

We would like to express our sincere appreciation to Hirose-Zoen Co., Ltd., the Bickell Foundation, Parkinson Canada, Parkinson Research Consortium, and Ottawa Hospital Research Institute (especially, members in Schlossmacher Lab and Hakim Lab) for providing laboratory reagents, tools, scientific feedback, and financial support.

The authors declare that there are no conflicts of interest in the authorship or publication of this contribution.

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