Neuroscience 233 (2013) 72–85

INCREASED VULNERABILITY OF *PARKIN* KNOCK DOWN PC12 CELLS TO HYDROGEN PEROXIDE TOXICITY: THE ROLE OF SALSOLINOL AND *N*M-SALSOLINOL

YANG SU, [†] JINYAN DUAN, [†] ZHENGXIN YING, YING HOU, YANYAN ZHANG, RUI WANG AND YULIN DENG *

School of Life Science, Beijing Institute of Technology, Beijing, China

Abstract—Dopamine-derived neurotoxins, 1-methyl-4-phenyl-1,2,3,4-tetrahydroisoquinoline (salsolinol) and 1(*R*), 2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (*N*M-salsolinol) are the two most possible 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-like endogenous neurotoxin candidates that involved in the pathogenesis of Parkinson's disease (PD). The levels of endogenously synthesized salsolinol and *N*M-salsolinol are increased in the cerebrospinal fluid (CSF) of PD patients. Both of them lead to neurotoxicity in dopaminergic cells by inhibiting mitochondrial electron transport chain. To study the role of salsolinol and *N*M-salsolinol in Parkin deficiency-induced dopaminergic cell damage, we determined the cellular level of oxidative stress, the formation of salsolinol and *N*M-salsolinol, the level of mitochondrial damage and cell viability with/without the presence of exogenous H_2O_2 using differentiated dopaminergic PC12 cells. Our data show that *parkin* knock down elevates cellular oxidative stress, salsolinol and *N*M-salsolinol levels, which are responsible for the higher cell mortality in Parkin-deficient cells upon exposure to exogenous H_2O_2. The level of mitochondrial membrane potential loss, cristae disruption and the release of cytochrome *c* increased significantly along with the increased level of salsolinol and *N*M-salsolinol, whereas compared to *parkin* knock down cells in the presence of H_2O_2, the mitochondrial damage and higher cell mortality were both diminished when the levels of salsolinol and *N*M-salsolinol was reduced. The results not only indicate the elevated level of salsolinol and *N*M-salsolinol, but also reveal the potential role of salsolinol and *N*M-salsolinol in *parkin* knock down-induced cell vulnerability. We assume that *parkin* deficiency is the trigger of excessive oxidative stress, elevated endogenous neurotoxin levels and mitochondrial damage, which eventually results in cell death of dopaminergic cells.

*Corresponding author. Address: School of Life Science, Beijing Institute of Technology, 5 South Zhongguancun Street, Haidian District, Beijing 100081, China. Tel/fax: +86-10-68914607.
E-mail address: deng@bit.edu.cn (Y. Deng).
[†] These authors contributed equally to this work.

Abbreviations: AR-JP, autosomal recessive juvenile Parkinson's disease; COMT, catechol-*O*-methyltransferase; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; MAO, monoamine oxidase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MTT, methylthiazol tetrazolium; NAC, *N*-acetylcysteine; *N*M-salsolinol, 1(*R*),2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline; PD, Parkinson's disease; ROS, reactive oxygen species; salsolinol, 1-methyl-4-phenyl-1,2,3,4-tetrahydroisoquinoline; TH, tyrosine hydroxylase.

Key words: *parkin*, salsolinol, *N*M-salsolinol, endogenous, eurotoxinsoxidative stress, mitochondrial damage.

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disease that is characterized by degeneration of dopaminergic neurons in the substantia nigra of the midbrain (Scott and Netsky, 1961; Olasode, 2001). It is clinically defined by a core set of progressive movement disorders (Fox et al., 2011). Although 90–95% of PD cases have no genetic basis, approximately 5–10% of PD are caused by inherited mutations (Mizuno et al., 2006). During the past few years, several genes that cause hereditary forms of PD have been identified (Kitada et al., 1998; Vila and Przedborski, 2004; Mizuno et al., 2006). The identification of these genes has provided tremendous insights into the pathogenesis of PD. Among the familial PD, *parkin* mutations are the most common causes of autosomal recessive PD, including the autosomal recessive juvenile PD (AR-JP) (Lucking et al., 2000; Hattori and Mizuno, 2004; Hedrich et al., 2004; Bonifati, 2012). Studies show that Parkin belongs to a family of proteins with conserved ubiquitin-like domain and functions as a ubiquitin E3 protein ligase (Shimura et al., 2000; Chaugule et al., 2011). However, it has been reported that the concentrations of some Parkin substrates which are also key proteins for PD pathogenesis, such as CDCrel-1, synphilin-I and α-synuclein are not altered in *parkin*-null mice (Goldberg et al., 2003; Hattori and Mizuno, 2004). These findings indicate that the function of Parkin is not limited to an E3 ligase in the ubiquitin–proteasome system. Recently, a *Drosophila* model with inactivated ortholog of human *parkin* showed muscle degeneration and mitochondrial pathology (Darios et al., 2003; Greene et al., 2003), which suggests a physiological role of Parkin at mitochondrial levels. Although the role of Parkin in mitochondrial protection has been identified (Darios et al., 2003; Mitsui et al., 2008; Narendra et al., 2008; Koh and Chung, 2010), the mechanism of participation of Parkin deficiency in PD is not yet completely clear.

The relationship between mitochondrial dysfunction and PD has been suggested in numerous studies (Kosel et al., 1999; Zhu and Chu, 2010). The earliest clue came from the observation that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), an inhibitor of

0306-4522/12 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2012.12.045

mitochondrial complex I, exposure causes PD-like syndrome in humans (Davis et al., 1979; Langston and Ballard, 1983). Additional evidence showed that rotenone, another mitochondrial complex I inhibitor, also induces pathological features of PD in rats (Betarbet et al., 2000; Perier et al., 2003). The finding that MPTP and other chemicals cause PD-like syndrome in humans stimulates the searching for endogenous toxins that have a similar structure or function as MPTP. Therefore, several dopamine-derived alkaloids have been proposed as candidates of endogenous neurotoxins such as 1-methyl-4-phenyl-1,2,3,4-tetrahydroisoquinoline (salsolinol), 1(*R*), 2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (*N*M-salsolinol),tetrahydropapaveroline and β-carbolines (Naoi et al., 2002a; Mravec, 2006). Among these alkaloids, salsolinol and *N*M-salsolinol are considered as the two most possible neurotoxin candidates that involved in the pathogenesis of PD (Naoi et al., 2000) since endogenously synthesized salsolinol and its derivatives are increased in the CSF of PD patients (Naoi et al., 1998). In brain, salsolinol can be endogenously synthesized from dopamine and acetaldehyde by salsolinol synthase or Pictet–Spengler reaction (Naoi et al., 2004; Mravec, 2006). Thus, it is not surprising that high concentrations of salsolinol and its metabolites have been found in basal ganglia, especially in the striatum and substantia nigra which are rich in dopamine (Musshoff et al., 1999, 2000). Similar to MPTP or 6-OHDA, salsolinol and *N*M-salsolinol also lead to neurotoxicity in dopaminergic cells by inhibiting mitochondrial electron transport chain (Storch et al., 2000; Mravec, 2006). Specifically, *N*M-salsolinol, which is synthesized from salsolinol by *N*-methyltransferase, is considered as a stronger neurotoxin than salsolinol and is capable of activating mitochondria-initiated apoptotic cascade in dopaminergic cells (Akao et al., 2002; Jantas et al., 2008).

Although it has been shown that mitochondrial damage is involved in the pathology of PD, there is still controversy of whether the mitochondrial dysfunction should be considered primordial or secondary to other processes that eventually lead to neurodegeneration (Morais and De Strooper, 2010). When we are trying to answer this question, the interactions between oxidative stress, endogenous neurotoxin formation and mitochondrial dysfunction should be considered. Oxidative stress is also one of the major factors that contribute to dopaminergic cell degeneration in PD (Veech et al., 2000; Nunomura et al., 2007). Several biological markers of oxidative stress were elevated in the substantia nigra pars compacta in PD patients (Tobon-Velasco et al., 2010). As the oxidative stress level increased, lipid peroxidation-induced formation of acetaldehyde promotes the synthesis of salsolinol and *N*M-salsolinol in dopaminergic cells (Jamal et al., 2003a,b; Deng et al., 2008; Wang et al., 2008). Once the levels of salsolinol and *N*M-salsolinol are increased, they will induce strong mitochondria toxicity (Mravec, 2006) and subsequently elevate the cellular oxidative stress level *via* at least two confirmed mechanisms: release the reactive oxygen species (ROS) from damaged mitochondria (Naoi et al., 2002b; Wanpen et al., 2004) or impair the oxidative stress defense systems, such as glutathione peroxidase (GSH-Px) or superoxidase dismutase (SOD) (Wanpen et al., 2004; Kang, 2007). Because of the elevated cellular oxidative stress, the levels of salsolinol and *N*M-salsolinol could be further increased. Based on these facts above, it seems that oxidative stress, endogenous neurotoxin formation and mitochondrial damage may have a synergetic effect and play a role in dopaminergic cell death. However, this hypothesis is not completely confirmed yet.

The aim of the current study is to show the role of salsolinol and *N*M-salsolinol in Parkin deficiency-induced dopaminergic cell damage. Based on our results, we also tried to reveal the role of oxidative stress, endogenous neurotoxin formation and mitochondrial damage in the pathogenesis of AR-JP. The differentiated dopaminergic PC12 cells and *parkin* knock down cells were used in this study. We assumed that Parkin deficiency-induced mitochondrial dysfunction could be the trigger of salsolinol and *N*M-salsolinol accumulation in dopaminergic PC12 cells. The elevated salsolinol and *N*M-salsolinol could be responsible for the cell vulnerability of Parkin-deficient cells under the exogenous oxidative stress. Two inhibitors, *N*-acetylcysteine (NAC) and α-methyl-DL-tyrosine, were used to ensure the increase and the important role of salsolinol and *N*M-salsolinol in parkin knock down-induced cell vulnerability to H_2O_2 in PC12 cells.

EXPERIMENTAL PROCEDURES

Cell culture

All experiment protocols were approved by Beijing Institute of Technology Committee on Biosafety and Animal Care, and met the standards of international ethical guidelines for the care and use of animals and biological materials. Rat pheochromocytoma PC12 cell line was obtained from ATCC and differentiated by 50 ng/mL nerve growth factor (NGF, Sigma, NY, USA) according to methods described previously (Gunning et al., 1981; Schimmelpfeng et al., 2004). The differentiated PC12 cells were used in the following experiments. Cells were cultured in Dulbecco's modified Eagle's medium (Gibco, NY, USA) with 10% fetal bovine serum (FBS, Gibco) and 100 U/mL penicillin–streptomycin (Sigma) with 5% CO_2 and maximal humidity at 37 °C.

Plasmid construction

The plasmids used for RNAi experiments were constructed according to the instructions of pSilencer neo kit (Ambion, NY, USA). Briefly, the oligonucleotide sequences which encode anti-sense RNA that targets rat *parkin* gene from 262 to 280 nt were synthesized (Invitrogen, Shanghai, China). The sequences were 5′-gatccGAGTATCGTTCACATAGTAttcaaga gaTACTATGTGAACGATACTCtttttggaaa-3′ (forward) and 5′-agcttttccaaaaaaGAGTATCGTTCACATAGTAtctcttgaaTACTAT GTGAACGATACTCg-3′ (reverse). The two complementary oligonucleotides (forward and reverse) were annealed to form the double-stranded DNA inserts which contains the cohesive ends of *Bam*H I and *Hin*d III. The double-stranded DNA inserts were inserted into pSilencer vector between *Bam*H I and *Hin*d