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Research Paper

ATP Maintenance via Two Types of ATP Regulators Mitigates Pathological Phenotypes in Mouse Models of Parkinson's Disease

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ABSTRACT

Parkinson's disease is assumed to be caused by mitochondrial dysfunction in the affected dopaminergic neurons in the brain. We have recently created small chemicals, KUSs (Kyoto University Substances), which can reduce cellular ATP consumption. By contrast, agonistic ligands of ERRs (estrogen receptor-related receptors) are expected to raise cellular ATP levels via enhancing ATP production. Here, we show that esculetin functions as an ERR agonist, and its addition to culture media enhances glycolysis and mitochondrial respiration, leading to elevated cellular ATP levels. Subsequently, we show the neuroprotective efficacies of KUSs, esculetin, and GSK4716 (an ERRγ agonist) against cell death in Parkinson's disease models. In the surviving neurons, ATP levels and expression levels of α-synuclein and CHOP (an ER stress-mediated cell death executor) were all rectified. We propose that maintenance of ATP levels, by inhibiting ATP consumption or enhancing ATP production, or both, would be a promising therapeutic strategy for Parkinson's disease.

1. Introduction

The human brain constitutes only 2–3% of the total body weight but monopolizes about 15% of the total blood flow, and it consumes about 20% of the total oxygen inhaled by the lungs. Simply put, the brain needs a lot of energy, namely ATP, for its functions and survival. Indeed, a decrease in ATP production in certain area(s) of the brain or the central nervous system can lead to neuronal cell death in the affected area(s) and thus cause ischemic or degenerative neuronal diseases.

Parkinson's disease is the second-most frequent neurodegenerative disease, with an incidence of approximately one per 1000 individuals. Parkinson's disease is caused by gradual cell death of dopaminergic neurons in the substantia nigra, and therapeutic chemicals or drugs that prevent this cell death are currently not available. In the early 1980s, certain numbers of drug users near San Francisco were observed manifesting Parkinson's disease phenotypes (Langston et al., 1983). The examination clarified that they suffered from accidental intoxication by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which was a contaminant in the drug they used. Later, it was found that 1-methyl-4-phenylpyridinium (MPP+), a metabolite of MPTP, but not MPTP itself, is specifically incorporated into dopaminergic neurons in the substantia nigra, where it inhibits mitochondrial complex I, suppresses ATP production, and ultimately kills the dopaminergic neurons in vivo, not only in humans but also in other animals, e.g. mice and rats (Dauer and Przedborski, 2003; Davis et al., 1979; Langston et al., 1983; Ramsay et al., 1991). These results have strongly implicated mitochondrial dysfunction or ATP decrease as a pathological mechanism in Parkinson's disease. Since then, MPTP has been widely used to experimentally create animal models of Parkinson's disease.

The most well-known, if not invariable, pathological hallmark of Parkinson's disease is the presence of Lewy bodies, protein aggregates composed of α-synuclein, in dopaminergic neurons in the affected brain region (Dauer and Przedborski, 2003; Halliday et al., 2014; Polymeropoulos et al., 1997; Spillantini et al., 1997). Similar α-synuclein aggregates are also observed in cortical neurons in dementia with Lewy bodies and in glial cells in multisystem atrophy (Dauer and Przedborski, 2003; Halliday et al., 2014). These neurodegenerative diseases are collectively referred to as "α-synucleinopathies" (McCann et al., 2014). From a genetic point of view, 18 genetic loci have been linked to familial Parkinson's disease, and are named PARK1 to PARK18 (Klein and Westenberger, 2012; Lin and Farrer, 2014). PARK1 encodes α-synuclein itself (Klein and Westenberger, 2012; Lin and Farrer, 2014; Polymeropoulos et al., 1997). PARK2, PARK6, and PARK17 encode Parkin, PINK1, and VPS35, respectively (Kitada et al., 1998; Klein

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and Westenberger, 2012; Lin and Farrer, 2014; Sharma et al., 2012; Valente et al., 2004; Vilariño-Güell et al., 2011; Zimprich et al., 2011). It is noteworthy that both Parkin and PINK1 collaboratively function to maintain mitochondrial function (Pickrell and Youle, 2015), and that VPS35 also operates to maintain mitochondrial function (Tang et al., 2015; Wang et al., 2016). Furthermore, it has been shown that genetic manipulation to maintain mitochondrial functions renders mice resistant to MPTP-induced Parkinson's disease (Hasegawa et al., 2016a; Mudò et al., 2012). These lines of evidence again indicate that dysfunctional mitochondria and ATP decrease are underlying factors in the etiology of Parkinson's disease, and suggest a potential link between the production of α-synuclein aggregates and ATP decrease.

In addition to Parkinson's disease, abnormal protein aggregates are also observed in several other neurodegenerative disorders, e.g. Alzheimer's disease, amyotrophic lateral sclerosis (ALS), polyglutamine diseases, etc., suggesting that common mechanisms underlie these disorders (Kakizuka, 1998). During long-term analyses of the molecular bases of neuronal cell death in neurodegenerative disorders (Ikeda et al., 1996; Kakizuka, 1998; Kawaguchi et al., 1994), we found that ATP consumption by valosin-containing protein (VCP) was profoundly involved in neurodegenerative phenotypes (Higashiyama et al., 2002; Manno et al., 2010). It is notable that *VCP* mutations have been implicated in IBMPFD (inclusion body myopathy with Paget disease of bone and frontotemporal dementia) (Watts et al., 2004) and rare cases of familial ALS (Johnson et al., 2010). In our analyses, all examined mutated VCP proteins had elevated ATPase activities, and the relative increase in activity levels appeared to be correlated with the severity of the clinical phenotypes (Manno et al., 2010). From these results, we hypothesized that specific inhibitors of VCP ATPase activity might ameliorate the disease phenotypes of familial VCP diseases as well as neuronal cell death in other diseases.

Previously, we showed that PGC1β, a member of the PGC1 family, functions as a protein ligand specific for estrogen receptor-related receptors (ERRs) (Kamei et al., 2003). ERRs belong to the nuclear receptor superfamily and mediate transcription for mitochondrial biogenesis or enhancement of energy expenditure (Alaynick et al., 2007; Audet-walsh and Giguère, 2015; Dufour et al., 2007; Huss et al., 2004). Indeed, transgenic expression of PGC1β produced phenotypes of high-energy expenditure: PGC1β mice are big eaters but thin (Kamei et al., 2003). These results indicate that natural or synthetic ERR ligands could enhance ATP production.

Recently, we successfully created small chemicals that are able to suppress the ATPase activity of VCP (Ikeda et al., 2014). Under various stress conditions in cultured cells, the chemicals (KUSs) were able to significantly maintain cellular ATP levels, and consequently suppress ER stress and cell death (Ikeda et al., 2014; Nakano et al., 2016). In addition, KUSs showed significant efficacies in preventing retinal neuronal cell death in in vivo mouse models of retinitis pigmentosa, glaucoma, and ischemic retinal disease (Hasegawa et al., 2016b; Hata et al., 2017; Ikeda et al., 2014; Nakano et al., 2016). In this manuscript, we show that two classes of small chemicals, one for limiting ATP consumption, and the other for enhancing ATP production via ERRs, possess strong efficacies in maintaining ATP levels and in protecting neuronal cells from death in both in vitro cell culture and in vivo mouse models of Parkinson's disease.

2. Materials and Methods

2.1. Experimental Design

Our objectives were to evaluate the efficacy of ATP maintenance via two types of ATP regulators in MPTP-induced and rotenone-induced Parkinson's disease models. We investigated two classes of small chemicals, one for preventing ATP consumption by VCP (KUSs), and the other for enhancing ATP production via ERRs (esculetin). Their efficacies were first determined by assessing the inhibition of neuronal cell death, ATP levels, and ER stress (CHOP expression) in in vitro cell culture models of Parkinson's disease. We also examined their efficacies in in vivo mouse models of Parkinson's disease by monitoring behavior, ATP levels, and histology. Sample numbers and sizes, the composition of replicates, and doses of compounds were based on pilot experiments and prior knowledge of cultured cells or mouse experiments (Hasegawa et al., 2016b; Ikeda et al., 2014; Nakano et al., 2016). Randomization of animals in the study was based on initial assessments of locomotor activity with a rotarod test and body weight to ensure an equal distribution in each group. Group sizes were set to enable statistical analyses in detection of locomotor activity (more than five per group), histological analyses (more than three per group), or measurement of ATP levels in mouse brain (fourteen or fifteen per group). ATP levels were evaluated using two methods (classic luciferase activity-based methods and imaging of ATeam (Imamura et al., 2009; Nakano et al., 2011; Tsuyama et al., 2013). All rules were predefined in advance. All data were included. Cell culture experiments were performed more than three times. The final end point before sacrifice in mouse experiments was when Parkinson's disease symptoms fully appeared. All animal studies containing the final end points before sacrifice were approved by the Animal Care and Use Committee of Kyoto University.

2.2. Cell Culture

PC12 cells were cultured in low glucose Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque, Kyoto, Japan), supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA) and 5% horse serum (HS) (Sigma), and maintained at 37 °C in a humidified atmosphere of 10% CO_2. HEK293A cells were cultured in high glucose DMEM (Nacalai Tesque) supplemented with 10% FBS (Sigma), and maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. ERR Reporter Assay

HEK293A cells were cultured at 37 °C in high glucose DMEM (Nacalai Tesque), supplemented with 10% FBS (Sigma). Cells were transfected with plasmid DNA using Polyethylenimine "Max" (Polysciences Inc., Warrington, PA, USA). HEK293A cells were cultured in a 24-well plate, and co-transfected with 0.2 μg each of pTk-(GalRE) × 4-Luc and pCMX-β-galactosidase, along with 0.2 μg of one of the following: pCMX-Gal4 (as a control); pCMX-Gal4-hERRα; pCMX-Gal4-hERRβ; pCMX-Gal4-hERRγ. 24 h after transfection, KUS121, KUS187, esculetin, GSK4716, or GSK5182 were added to the culture medium, and cells were incubated for an additional 24 h. Then, whole cell lysates were prepared, and luciferase and β-galactosidase assays were carried out, as described previously (Saitou et al., 1994; Sasaoka et al., 2014). The observed luciferase activities were normalized by the β-galactosidase activities to compensate for different transfection efficiencies (Saitou et al., 1994; Sasaoka et al., 2014).

2.4. Assay of Neurotoxicity Induced by Mitochondrial Complex Inhibitors

1×10^5 PC12 cells were cultured in 6-well plates at 37 °C in DMEM supplemented with 10% FBS (Sigma) and 5% HS (Sigma). To stimulate differentiation, the culture medium was replaced with DMEM supplemented with 1% FBS, 1% HS, and 50 ng/ml NGF (Alomone Labs, Jerusalem, Israel), and cells were cultured for 24 h. During differentiation, KUSs and esculetin were included in test wells. Then mitochondrial respiratory chain complex inhibitors such as MPP + (75 μM) (Sigma), rotenone (10 nM) (Sigma), metformin (3 mM) (Sigma), antimycin (100 nM) (Sigma), and oligomycin (0.01 μg/ml) (Sigma) were added. After an additional incubation for 24 h, ATP assays and western blots were performed. In parallel assays, after 28 h, cell viability assays

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