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Effect of Alteration of Glutathione Content on Cell Viability in α-Synuclein-Transfected SH-SY5Y Cells

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Abstract

It is well known that α-synuclein (αS) plays an important role in the pathogenesis of Parkinson's disease (PD). Moreover, oxidative stress is also thought to be an important factor in PD due to induction of dopaminergic neuronal cell death by free radicals and enhancement of αS fibrillation by oxidized stress. In the present study, to clarify the role of glutathione (GSH), an intracellular antioxidant, on the molecular mechanism of αS-induced cell injury, we examined the effects of L-buthionine-SR-sulfoximine (BSO), a GSH synthase inhibitor, with or without N-acetyl-L-cysteine (NAC), a source of GSH, on αS-induced cell injury in human neuroblastoma SH-SY5Y cells. Treatment with BSO significantly reduced the cell viability of both empty-vector- and αS-transfected SH-SY5Y cells in a dose-dependent manner ($p < 0.01$), although the ratio of αS-induced reduction of cell viability in α-syn-transfected cells was much greater than that in empty-vector-transfected cells. Moreover, BSO significantly reduced the intracellular total GSH level in both types of transformant cells. However, NAC significantly prevented BSO-induced reduction of both cell viability and GSH level in the αS-transfected cells. These findings suggest that GSH plays an important role in αS-induced cell injury by reducing cell viability.

Keywords

α-Synuclein, L-Buthionine-SR-Sulfoximine, N-Acetyl-L-Cysteine, Glutathione, SH-SY5Y Cells, Parkinson's Disease

1. Introduction

Parkinson's disease (PD) is a progressive, mainly sporadic neurodegenerative

disorder that is characterized by severe motor symptoms including uncontrollable tremor, slowness of movements, and rigidity. This pathology is characterized by loss of dopaminergic neurons in the substantia nigra pars compacta and the presence of cytoplasmic inclusions known as Lewy bodies (LB) [1]. Alpha-synuclein (*α*S) is a small protein of 140 amino acids with strong links to several neurodegenerative diseases such as PD and is well known as a major component of LB [2] [3]. Moreover, oxidative stress, including the reactive oxygen or nitrogen species generated in the enzymatic oxidation [4] or auto-oxidation of an excess amount of dopamine (DA) [5], is thought to play an important role in dopaminergic (DAergic) neurotoxicity [6]. DAergic neuron-specific oxidative stress has been a recent focus [7] because DA and its metabolites containing 2 hydroxyl residues exert toxicity in DAergic neuronal cells, primarily due to the generation of highly reactive DA quinone (DAQ) or DOPA quinone [7]. To control the harmful effects of oxidative stress, efficient detoxification pathways in various cells have been identified. Of the various antioxidant systems in the brain, the glutathione (GSH) system is particularly important in controlling cellular redox states and is the primary defense mechanism for the removal of peroxide from the brain [8] [9]. In addition, GSH also has quinone-quenching activities against DA- or L-DOPA-induced neurotoxicity [10]. In the present study, we examined the effects of L-buthionine-SR-sulfoximine (BSO), a typical GSH synthase inhibitor, with or without N-acetylcysteine (NAC), a source of GSH, on *α*S-induced cell injury in human neuroblastoma SH-SY5Y cells, to clarify the role of GSH on the molecular mechanism of *α*S-induced cell injury.

2. Materials and Methods

2.1. Construction of Expression Vectors

Human *α*S cDNA was cloned by reverse transcription polymerase chain reaction (RT-PCR) amplification and then was ligated into a plasmid vector. Templates for use in the PCR were generated by reverse transcription of total RNA isolated from human neuroblastoma SH-SY5Y cells by the acid guanidinium-phenol-chloroform method using *α*S gene-specific primers [11]. To construct the *α*S cDNA expression vector, a fragment of *α*S cDNA was ligated into pIRES-EGFP (Clontech, Palo Alto, CA) using the calcium phosphate method (Lipofectin® Reagent: Invitrogen, Carlsbad, CA). For selection, 300 μl/ml geneticin disulfate (Sigma, St. Louis, MO) was added to the culture.

2.2. Western Blot Analysis for Confirmation of the *α*S Expression in SH-SY5Y Cells

Total cell lysates from transformant SH-SY5Y cells were prepared with 10 μg/ml phenylmethylsulfonyl fluoride in ice-cold RIPA buffer [phosphate-buffered saline (pH 7.4), 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)], and analysis using the total cell lysate was performed as described previously [12]. The blots were incubated with rabbit polyclonal antibody for *α*S

(Santa Cruz Biotechnology, Santa Cruz, CA), then reacted with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Chemicon, Temecula, CA). After a washing with 20 mM Tris-buffered saline containing 0.1% Tween 20, the blots were developed using an ECL Western blotting detection system (Amersham, Buckinghamshire, UK) according to the protocol provided by the manufacturer.

2.3. Cell Culture

In the investigations performed in the present study, with the exception of the determination of cell viability, human neuroblastoma SH-SY5Y cells were treated with BSO (final conc. 50 - 500 μM for 144 h) with or without NAC (final conc. 100 - 1000 μM for 144 h), which were all purchased from Sigma. For the preparation of the GSH samples, SH-SY5Y cells were grown for 144 h in the presence or absence of BSO with or without NAC. BSO and NAC were dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in the culture medium was maintained at ≤0.01%. Human neuroblastoma SH-SY5Y (ATCC cell bank; catalogue number CRL-2266: Rockville, MD) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. For the experiments, the cells were plated onto 96-well plates at a density of 4.0×10^4 cells/ml (SH-SY5Y) in order to assess cell viability. In addition, the cells were plated separately onto 6-well plates at 2.0×10^5 cells/ml in order to determine the GSH content. All cultures were maintained at 37˚C in a gas mixture of 5% - 95% CO_2-air. The SH-SY5Y cells were grown for 2 - 3 days before being used in experiments.

2.4. Cell Viability

The cell viability in each well of the 96-well plate was determined by quantitative colorimetric assay using WST-1 [12], a modification of the standard MTT assay. Viability was expressed as a percentage of the cell viability from each vehicle-treated control culture in the normal-medium-exposed group.

2.5. Glutathione (GSH) Content

GSH levels were determined using the enzymatic recycling method with some modifications [12]. Briefly, SH-SY5Y transformant cells were homogenized in 0.1-M phosphate buffer (pH 7.4); then 10% trichloroacetic acid was added in an equivalent volume to the cellular homogenates. Acid extracts were mixed with 0.01 M phosphate buffer (pH 7.4, 580 μL), NADPH (4 mM, 50 μL) and glutathione reductase (6 U/mL, 100 μL) and incubated for 5 min at 37˚C. 3-Carboxy-4-nitrophenyl disulfide (10 mM, 50 μL) was added just before the absorbance was read. The formation of 2-nitro-5-thiobenzoic acid was measured at 450 nm for 6 min. Total GSH was determined from a standard curve constructed using known amounts of GSH.

2.6. Statistical Analysis

Data are presented as the means ± SEM. Differences between groups were tested for statistical significance using one-way analysis of variance (ANOVA) followed by the post hoc Tukey-Kramer Multiple Comparisons test. P-values <0.05 were considered to indicate a statistically significant difference.

3. Results

Expression of *α*S proteins was detected in both *α*S-transfected and empty vector-transfected SH-SY5Y cells, although that in *α*S-transfected cells were stronger than that in control cells (Figure 1). BSO induced a reduction of cell viability in *α*S-transfected SH-SY5Y cells; in particular, treatment with BSO (100 μM) for 144 h resulted in markedly reduced cell viability in *α*S-transfected cells compared with the control cells (Figure 2; $p < 0.01$). However, treatment with NAC (100 - 1000 μM) dose-dependently prevented the BSO-induced reduction of cell viability in *α*S-transfected cells (Figure 3). BSO induced a reduction of GSH content in *α*S-transfected SH-SY5Y cells, but treatment with NAC prevented the BSO-induced reduction of GSH contents (Figure 4).

4. Discussion

*α*S protein has been directly linked to PD-associated neurodegeneration, and in

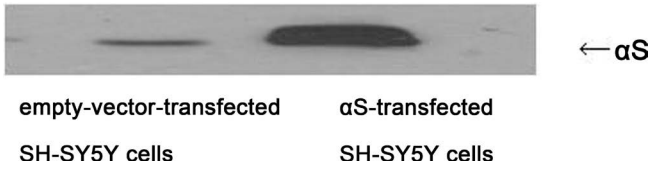

Figure 1. Immunoblot analysis of *α*S expression in stable transformant SH-SY5Y cells. Whole cell extracts from empty-vector-transfected SH-SY5Y cells and *α*S-transfected SH-SY5Y cells were analyzed Western blot with rabbit anti-*α*S antibody.

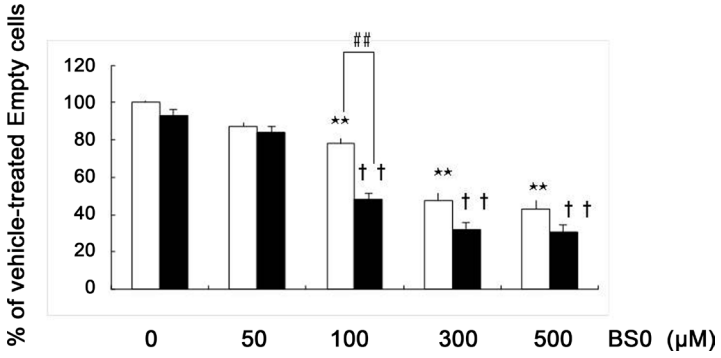

Figure 2. Effects of BSO on cell viability in transformant SH-SY5Y cells. SH-SY5Y cells were maintained in the presence or absence of BSO (final conc. 100 - 1000 μM) for 144 h, and then the effects of BSO on cell viability were assessed. Each column represents the mean % ±SEM of vehicle-treated empty-vector-transfected cells (n = 9 - 12). **p < 0.01 compared with vehicle-treated empty-vector-transfected cells. ††p < 0.01 compared with vehicle-treated *α*S-transfected cells. ##p < 0.01 compared with BSO 100 μM-treated Empty cells.

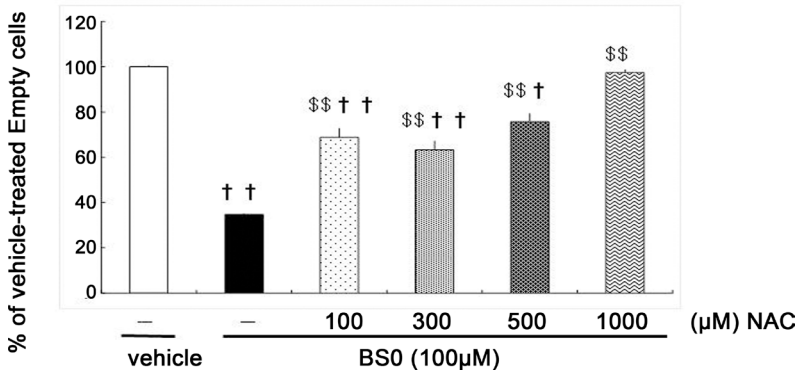

Figure 3. Effects of NAC on cell viability in *α*S-transfected SH-SY5Y cells. SH-SY5Y cells were maintained in the presence or absence of BSO (final conc. 100 μM) with or without NAC (final conc. 100 - 1000 μM) for 144 h, and then the effects of NAC on cell viability were assessed. Each column represents the mean % ±SEM of vehicle-treated Empty cells (n = 12 - 24). †p < 0.05, ††p < 0.01 compared with vehicle-treated *α*S-transfected cells. $$p < 0.01 compared with BSO 100 μM-treated *α*S-transfected cells.

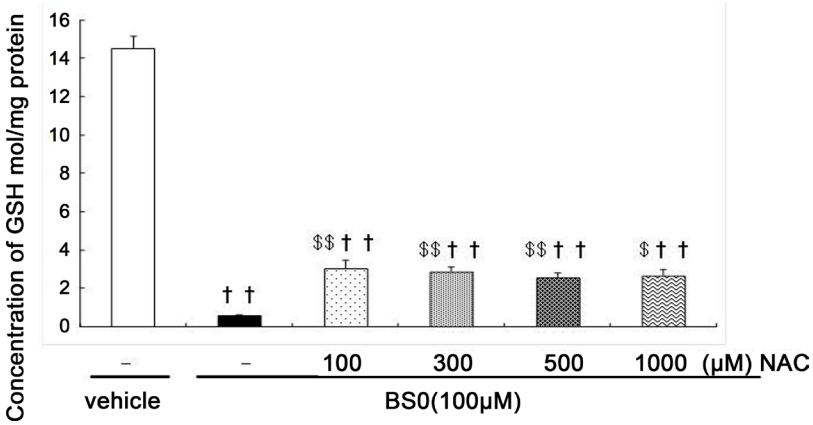

Figure 4. Effects of NAC on GSH content in *α*S-transfected SH-SY5Y cells. SH-SY5Y cells were maintained in the presence or absence of BSO (final conc. 100 μM) with or without NAC (final conc. 100 - 1000 μM) for 144 h, and then the effects of NAC on GSH content were assessed. Each column represents the mean nmol/mg ± SEM (n = 4 - 10). ††p < 0.01 compared with vehicle-treated *α*S-transfected cells. $$p < 0.01 compared with BSO 100 μM-treated *α*S-transfected cells.

patients exhibiting such neorodegeneration the *α*S protein is aberrantly folded, forming abnormal oligomers and amyloid fibrils within neurons or oligodendrocytes. Thus, the toxicity of *α*S is generally attributed to the formation of these toxic oligomers [3]. Oxidative conjugation of DA with *α*S inhibits the transition of *α*S from protofibrils to mature fibrils, leading to the potential accumulation of cytotoxic soluble protofibrils in DAergic neurons. The antioxidants have the ability to reverse the formation of these adducts, suggesting that catechol oxidation can contribute to the accumulation of *α*S protofibrils [6]. Moreover, the levels of GSH are lower in the SN early in PD, and this may contribute to mitochondrial dysfunction and oxidative stress, with the oxidative stress in turn increasing the accumulation of toxic forms of *α*S [13]. Several reports have sug-

gested that established risk factors such as oxidative stress, DA and *α*S, may in combination stabilize protofibrillar *α*S and promote the pathogenesis of PD [14]. However, the reason why the molecular basis of *α*S-induced cytotoxicity can be highly toxic to cells has remained unclear [3].

To examine the above hypotheses, we determined the effect of BSO on cell injury in the *α*S-transfected cells. BSO induced a reduction of both cell viability and total GSH content in *α*S-transfected SH-SY5Y cells; in particular, treatment with BSO (100 μM) for 144 h markedly reduced cell viability in *α*S-transfected cells compared with vector-transfected control cells. Thus, it could at least be concluded that GSH-related oxidative stress induced cell injury in *α*S-transfected cells. A previous report also suggested that *α*S aggregation disrupts mitochondria together with the enhanced oxidation [15]. In addition, we also hypothesized that supplementation with NAC would protect against *α*S-induced cytotoxicity. NAC (100 - 1000 μM) dose-dependently prevented the BSO-induced reduction of both cell viability and GSH content in *α*S-transfected cells, although the protective effects of NAC on BSO-induced reduction of GSH content were only partial. It is known that NAC not only raises GSH levels by acting as a cysteine donor in the synthesis of GSH, but also has several additional effects including direct antioxidant activity [16]. Systemic administration of NAC has been shown to increase brain levels of GSH in mice, reduce markers of oxidative damage, and increase brain synaptic mitochondrial complex I activity [17]. Oral treatment with NAC decreases *α*S levels in the brain and partially protects against loss of DAergic neurons associated with overexpression of *α*S in *α*S-overexpressing mice [13]. Moreover, manganese-induced *α*S oligomerization was also partially alleviated by pretreatment with GSH and aggravated by H_2O_2 pretreatment [18].

Considering these reports and our results regarding the partial effect of NAC on GSH content, the protective property of NAC may be mainly dependent on additional effects such as direct antioxidant activity. Thus, GSH would play important roles in *α*S-induced cytotoxicity, such as the reduction of cell viability, although further studies are required to clarify the molecular basis of *α*S-induced cytotoxicity.

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Conflict of Interest

The authors confirm that this article content has no conflict of interest.

Author Contribution

Ken-ichi Tanaka designed experiments, provided technical support, analyzed date and wrote manuscript; Kanako Sonoda co-designed and performed experiments; Masato Asanuma interpreted results and provided other related support.

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