Published in final edited form as:

Neurochem Res. 2012 June ; 37(6): 1261–1266. doi:10.1007/s11064-011-0662-4.

Pathobiochemical effect of acylated steryl- β -glucoside on aggregation and cytotoxicity of α -synuclein

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Abstract

Objective—Cycad seed consumption by the native islanders of Guam is frequently associated with high rates of Amyotrophic Lateral Sclerosis-Parkinsonism Dementia Complex (ALS-PDC); furthermore, accompanying pathological examination often exhibits α -synuclein inclusions in the neurons of the affected brain. Acylated steryl- β -glucoside (ASG) contained in cycad seeds is considered as causative environmental risk factor. We aimed to investigate whether ASG influences aggregation and cell toxicity of α -synuclein.

Methods—To understand whether ASG is a causative factor in the development of ALS-PDC, soybean-derived ASG was tested for its effect on *in vitro* aggregation of α -synuclein using Thioflavin-T. ASG was also tested to determine whether it modulates α -synuclein cytotoxicity in yeast cells. In addition, we determined whether an interaction between ASG and α -synuclein occurs in the plasma membrane or cytoplasm using three factors: GM1 ganglioside, small unilamellar vesicles, and ATP.

Results—In the present study, we found that ASG-mediated acceleration of α -synuclein aggregation is influenced by the presence of ATP, but not by the presence of GM1. ASG accelerated the α -synuclein aggregation in the cytoplasm. ASG also enhanced α -synuclein-induced cytotoxicity in yeast cells.

Conclusions—This study demonstrated that ASG directly enhances aggregation and cytotoxicity of α -synuclein, which are often observed in patients with ALS-PDC. These results, using assays that replicate cytoplasmic conditions, are consistent with the molecular mechanism that cell toxicity is caused by intracellular α -synuclein fibril formation in neuronal cells.

Background

ALS-Parkinsonism Dementia Complex (ALS-PDC) is a unique neurodegenerative disease. It is remarkable that ALS and PDC, either in isolation or in combination occur with a high frequency in the Chamorro people of Guam, Kii Peninsula of Japan, and west New Guinea (1-4). The etiology of ALS-PDC has led to a growing awareness of the involvement of purely genetic and/or environmental factors. A potential environmental factor that has been considered to be involved in the etiology of ALS-PDC is the consumption of traditional foods made from flour of the cycad seed (*Cycas micronesica*), which has often been used as a primary foodstuff, especially during periods of famine (3-5). Strong epidemiological data links disease development to cycad seed consumption. Cycad seeds are harvested, cut open to expose the starchy endosperm, sliced into 'chips', and then washed for periods of up to 10 days. Components of cycad seeds, particularly, steryl- β -glucosides including acylated steryl- β -glucoside (ASG in Fig. 1) and beta-N-methylamino-L-alanine (BMAA) have been focused

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upon as to their neurotoxicity (6, 7). ASG and BMAA have been considered the principal components of cycad neurotoxin, a conclusion that is supported by a feeding experiment in CD1 mice (8, 9).

However, BMAA induces acute toxicity in mice, making it difficult to explain the overall symptoms of ALS-PDC. Moreover, it has been shown that cycads were not used for food in two other high-incidence epicenters: Kii Peninsula and west New Guinea. In addition, ASG is generally found in many other edible plants in addition to cycads, and is consumed as a primary ordinary foodstuff all over the world; hence a particular genetic background of patients cannot be ignored in an etiological study of ALS-PDC(8). Thus, we hypothesize that ASG might be an environmental risk factor contributing to the pathogenesis of ALS-PDC, which is caused or affected both by genetic and environmental component. PDC in Guam is characterized by a marked loss of neurons and deposition of numerous neurofibrillary tangles (NFTs) in the neocortex, hippocampus, amygdale, and brainstem (10). Significant α -synuclein deposition is observed in the amygdalae. These direct observations of the intracerebral pathology of ALS-PDC led us to examine whether ASG may induce α -synuclein aggregation. Protein misfolding of α -synuclein causes α -synuclein fibril formation in the cytoplasm of neuronal cells, subsequently leading to neurotoxicity of progressing severity (11). To demonstrate the potential relationship between ASG and α -synuclein fibril formation in the cytoplasm, we first tested for ASG's effect on aggregation of normal human α -synuclein by the use of an assay system replicating the *in vitro* conditions of a human subject. We then examined the growth of transformed yeast cells by α -synuclein in the presence or absence of ASG.

Materials and Methods

Materials

The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO): 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC); normal human α -synuclein (recombinant, expressed in *Escherichia coli*, N-terminal histidine-tagged); and Thioflavin-T (Th T). Acylated steryl- β -glucoside (derived from soybean) was purchased from Matreya. GM1 ganglioside was purified from bovine brain according to a procedure we previously established (12).

Preparation of small unilamellar vesicles (SUVs) by sonication

SUVs (GM1-SUV and SUV) including GM1/DPPC and DPPC alone were prepared by sonication following previously published methods (13). Briefly, GM1/DPPC (molar ratio 1:1, unless otherwise noted) and DPPC alone were prepared at 10-20 mg/mL lipid per mL solvent (chloroform/methanol (2:1, v/v) by sonication. The solvent was evaporated using a dry nitrogen or argon stream in a fume hood. The lipid film was thoroughly dried to remove residual organic solvent by subjecting the vial or flask to a vacuum pump overnight. The thin film was hydrated in 100 mM NaCl and 20 mM Tris buffer (pH 7.4) with gentle mixing. Hydrated samples were then sonicated at 4 °C with a probe sonicator for 4 min of 10 sec pauses following 10 sec sonication periods. Prior to assay, SUVs were mixed with monomeric α -synuclein. The SUV/ α -synuclein mixture was adjusted by a 10:1 mass ratio of lipid to protein.

Analysis of α -synuclein fibril formation

To induce fibrillization (14), α -synuclein, (80 μ M), protein was incubated in either the presence (20 μ g/mL) or absence of ASG in KHM buffer (40 mM HEPES-KOH, pH 7.4; 150 mM KCl; 20 mM MgCl₂; 1 mM dithiothreitol) plus ATP (10 mM) and an ATP regeneration system (20 mM creatine phosphate and 0.001 mg/mL creatine kinase) (15). The sampling

took place for time periods varying from 0–48 hours at 37°C with 80 rotations per minute on a Mini-rotator (Glas-Col, LLC, Terre Haute, IN). Every 8 hours, reactions were supplemented with a fresh ATP regeneration system to maintain ATP levels.

Fibril amount was determined by ThT fluorescence. ThT in 50 mM glycine (pH 8.5) was added to reach final concentrations of 0.25 μ M α -synuclein and 10 μ M ThT. Fluorescence at 485 nm was measured after excitation at 450 nm (16).

Effect of ASGs on α -synuclein cytotoxicity

Saccharomyces cerevisiae W303 (MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100) was transformed using one of two plasmids. The first, plasmid p2HGP, is an empty vector (control) while the second, p423GPD α -synuclein-YFP, allows for consistent expression of α -synuclein-YFP under the GPD promoter (17). The plasmid transformed cells were streaked onto histidine (SC-H)-free synthetic medium and incubated for 3 days at 30°C. ASG was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 5 mg/mL, and 100 μ L of the solution was streaked onto SC-H plates to achieve a final concentration of 20 μ g/mL. To address the toxicity of α -synuclein with ASG, cells were grown on SC-H for 1 day at 30°C then resuspended in water at 10^7 cells/mL by adjusting to 1.0 value at OD₆₀₀. The cell suspensions were serially diluted (1:10), and 4 μ L of each dilution was spotted onto 2 plates of different media (SC-H and SC-H including 20 μ g/mL ASG) and incubated for 2 days at 30°C.

Statistical analysis

Unless otherwise stated, results are presented as mean $\pm$ standard error. Assays were performed in triplicate. The experimental data were analyzed using one-way ANOVA, followed by Tukey's multiple comparison test and Dunnett's test. Statistical analyses were performed using the GraphPad Prism 5.0 software package (GraphPad, San Diego, CA, USA).

Results

Time course for α -synuclein fiber formation

ATP is needed for α -synuclein aggregation in the cytoplasm. This necessity is illustrated by our finding that the presence of ATP remarkably enhanced α -synuclein aggregation (Fig. 2A), while in the absence of ATP, α -synuclein aggregation showed only beginning levels of kinetics, even after 48 hours incubation. ASG showed no effect on α -synuclein aggregation. On the other hand, the presence of ATP increased α -synuclein aggregation up to 140 % as compared with aggregation in the absence of ATP. This finding implicates that ASG-induced aggregation in the cytoplasm causes enough fibril formation to lead to cytotoxicity. In contrast, SUV affected α -synuclein aggregation, but ASG did not influence α -synuclein aggregation in the presence of SUV (Fig. 2B). Additionally, GM1-SUV remarkably suppressed α -synuclein aggregation as compared with SUV; however, ASG-induced α -synuclein aggregation did not occur in the presence of GM1-SUV. This finding implies that ASG is not effective in regions or circumstances, such as the plasma membrane, in which GM1 is located abundantly.

Various factors involved in the plasma membrane and cytoplasm were examined as to their effect on ASG-induced α -synuclein aggregation (Fig. 3A). The presence of ATP or ATP plus SUV affected ASG-induced α -synuclein aggregation with a significant statistical difference ($*p < 0.01$). ASG-induced aggregation was enhanced further by the presence of ATP and SUV as compared with only ATP. Furthermore, to determine whether the interaction between various cytoplasmic factors and ASG-induced α -synuclein aggregation

occurs in a time-dependent manner, α -synuclein incubation was extended up to 96 hrs (Fig. 3B). After 48 hrs of incubation, the aggregation plateaued at 72 and 96 hrs. In summarizing these findings, there is no intrinsic difference between α -synuclein aggregation in either the plasma membrane or cytoplasm. Our experiment was able to reconstitute conditions of both compartments. The result was interpreted by varying degrees of aggregation by appearance. ASG-induced α -synuclein aggregation was independent of additive factors after 96 hrs incubation (Fig. 3B). This finding suggests that ASG enhances α -synuclein aggregation but does not change the ultimate endpoint level of α -synuclein aggregates.

ASG-induced α -synuclein's cytotoxicity

The α -synuclein-expressing cells displayed a marked reduction in cell growth compared with vector-expressing cells on the control plate (Fig. 4). On the ASG plate, 20 μ g/mL of ASG-treatment caused α -synuclein-expressing cells to exhibit a remarkable decreased growth as compared with non-treated cells on the control plate.

Discussion

α -Synuclein was originally found as the precursor of the non-A β component of amyloid in brains from Alzheimer's disease (AD) patients. Subsequently, α -synuclein was found in fibril inclusions as components of Lewy bodies in Parkinson's disease and multiple system atrophy (18, 19). Under normal conditions, the native structure of α -synuclein is an unfolded monomer reversibly equilibrated to α -helix form. This form of α -synuclein allows the molecule to associate with the surface phospholipids of vesicle membranes. Both this membrane interaction and an increased concentration of α -synuclein can facilitate the formation of α -synuclein dimers in the membrane or in the cytoplasm. Through dimerization, α -synuclein adopts a β -sheet secondary structure, and the membranes associate with α -synuclein monomers or other dimers, leading to oligomer formation. These oligomers in turn lead to fibril formation and are deposited as amyloid in Lewy bodies causing Lewy neuritis (11).

Consumption of a certain compound in cycad seeds traditionally used for food is assumed to be the risk factor responsible for ALS-PDC being a unique neurological disorder found in natives of Guam (1-5). Steryl glucosides such as ASG have been proposed as the cycad neurotoxin of ALS-PDC (20). Recently, various biological activities of ASG have been reported. These activities, namely anti-diabetic (21, 22), analgesic (23), and anti-hemolytic (24) activities as well as association with macrophage activation (25), implicate ASG as having potential medical use. However, when considering the side effects of ASG, it is important for us to know the relevance of ASG to the ALS-PDC disease state.

The core of our hypothesis is that ASG-induced α -synuclein aggregation causes cytotoxicity. A new finding in our study was that soybean-derived ASG stimulates aggregation of normal human α -synuclein (Fig. 2) and that subsequent increases in the cytoplasmic concentration of α -synuclein led to cell toxicity. Although the extracellular or plasma membrane functions of α -synuclein remain unknown, the cytoplasmic function of α -synuclein has been clarified. In most of the studies, increased intracellular α -synuclein led to increased aggregate localization and sympathetic nerve membrane binding. In the cytoplasm, ATP is known to be a factor for triggering α -synuclein aggregation (26). In our present experiment, ASG induced a remarkable enhancement of α -synuclein aggregation in the presence of ATP and SUV, but produced no effect on aggregation in the absence of ATP and SUV (Figs. 2a and 3a). Additionally, ASG-induced α -synuclein aggregation did not occur in the presence of GM1-SUV (Fig. 3A). Recently, GM1 was shown to be a mediator in certain essential signaling pathways of the nervous system and other tissues by interactions between GM1 and proteins. The interaction between GM1 and α -synuclein has

been focused on Parkinson's disease and shown in animal studies (27, 28). GM1 is localized mostly to a lipid raft within the plasma membranes. This raft associates with α -synuclein binding in a manner that promotes α -helical conformation and prevents pathological cytoplasmic fibrillation (29). Thus, we suggest ASG works by enhancement of α -synuclein aggregation in the cytoplasm.

In summary, the results of this study have demonstrated that ASG enhances *in vitro* α -synuclein aggregation and increased cell toxicity via cytoplasmic α -synuclein.

Acknowledgments

This work was supported by a project grant from NIH project grants (NS26994 and NS 11853) to Robert. K. Yu. Thanks are due to Ms. Dawn O'Brien for her editorial assistance.

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Fig. 1.
The chemical structure of acylated sterol β glucoside (ASG characterized by β -sitosterol glucoside). R represents acyl groups of several fatty acids (Reference # ?? Matreya, soybeans).

Fig. 2.

Effect of ASG on α -synuclein fibril formation in the presence of (A) 10 mM ATP or (B) GM1-SUV (or SUV). The mixture of α -synuclein (80 μ M) was incubated at 37°C for 48 hrs with 80 rotations per minute. In (A) and (B), 20 μ L of the aliquot was periodically taken from the incubating mixture, which was subsequently refilled to the same volume with either KMH buffer or ATP regeneration system (20 mM creatinine phosphate and 0.1 mg/mL creatine kinase) or only KMH buffer. The 20 μ L aliquot samples were dispensed into 96-well microtiter plates (white plate with clear bottom, Corning) and mixed with 80 μ L of 20 μ M Th T in 50 mM glycine buffer (pH 8.5). Th T fluorescence was quantitated by a Perkin-Elmer Victor V multilabel plate reader with excitation/emission filters set at 450 nm/485 nm.

Fig. 3.

(A) Effects of various intracellular and extracellular factors on ASG-induced α -synuclein aggregate formation. α -synuclein (80 μ M) was incubated at 37°C for 48 hours in KHM buffer plus one of the following: no additive; 10 mM ATP; 0.8 mM SUV; 10 mM ATP/0.8 mM SUV; 0.8 mM GM1-SUV; or 10 mM ATP/0.8 mM GM1-SU. Following incubation, samples were subjected to Th T fluorescence quantitation assay, as described in Methods and Materials. (B) Effect of incubation time of ASG-induced α -synuclein aggregation. The incubated mixture was prepared as described in part (A) but incubation time was extended from 48 hours to 96 hours.

Fig. 4. Effects of ASGs on the growth of serially diluted (1:10) yeast cells (strain W303 expressing p2HGDP and p423GDP α -synuclein genes), and 4 μ l of each dilution was spotted onto 2 plates of different media (SC-H and SC-H including 20 μ g/mL ASG) and incubated for 2 days at 30°C.