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Induction of Sirtuin1 Activity in SH-SY5Y Cells by Cyanidin-3-O-Glucocide Induced

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Abstract

An increase in oxidative stress plays a key role in neurotoxicity induction and cell death, which leads to neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease. Cyanidin-3-glucoside (C3G) is a common anthocyanin and shows antioxidant activity in neuronal cells. Silent information regulator 2-related protein 1 (Sirt1) regulates antioxidant and anti-inflammatory effects. However, the effects of C3G on Sirt1 in neuronal cells remain unclear. This study evaluated the effect of C3G on Sirt1 expression and activity in human neuroblastoma (SH-SY5Y) cells. In the study, C3G increased the expression of Sirt1 and Sirt1 activity in SH-SY5Y cells. Additionally, C3G increased the expression of nuclear factor erythroid 2-related factor 2, a vital transcription factor for regulating the expression of antioxidant genes, as well as antioxidant enzymes such as superoxide dismutase and catalase. Moreover, C3G protected SH-SY5Y cells from oxidative stress. These results suggest that C3G decreased oxidative stress-induced cell injury by increasing the expression of Sirt1 and other antioxidant factors. Therefore, C3G might merit further investigation for use in attenuating the progress of neurodegenerative diseases.

Keywords

Cyanidin-3-O-Glucocide, Sirtuin1, Anti-Senescence, Antioxidant, Oxidative Stress, Nerve Cells

1. Introduction

The causes of Parkinson's disease (PD) and Alzheimer's disease (AD) are still unknown although several environmental and genetic risk factors have been suggested [1]. AD and PD are characterized by dysfunction, damage and death of neurons, resulting in the progressive deterioration of the structure and cogni-

tive functions in brain [2]. PD and AD are diseases associated with aging, in which oxidative stress has been implicated. An increase in oxidative stress is responsible for inducting neurotoxicity and cell death, which can lead to neurodegenerative diseases such as PD and AD. Reducing neuronal cell death could help control the onset and progression of these diseases.

Recent *in vivo* studies have highlighted the neuroprotective effects of dietary anthocyanins. Cyanidin-3-glucoside (C3G) is a common anthocyanin found in black rice, black bean, purple potato, and many colorful berries [3]. Some studies have reported that C3G protected neuronal cell death in human neuroblastoma cells from reactive oxygen species (ROS), apoptosis and inflammation [4] [5] [6] [7]. These reports indicate that C3G has an antioxidant capacity and may also protect cells from oxidative stress involved in neurodegenerative diseases. This finding suggests that C3G has potential for treatment of neurodegenerative diseases like PD and AD.

Silent information regulator 2-related protein 1 (Sirt1) is universally conserved in eukaryotes, and it was recently shown to regulate lifespan extension in budding yeast and nematodes [8]. It was reported that the yeast Sirt1 protein is a NAD^+-dependent histone deacetylase playing a crucial role in transcriptional silencing, genome stability, and longevity [8]. Sirt1 regulates inflammation, aging, mitochondrial biogenesis, stress resistance, cellular senescence, and apoptosis/autophagy [9] [10] [11] [12]. A study using postmortem brains reported that Sirt1 protein levels were reduced in all brain regions in PD and AD [13]. Sirt1 is a potential candidate for redox modulation as its activity is regulated by NAD^+, and it is sensitive to the redox state [14] [15]. Sirt1 also regulates the burden of cellular oxidative stress and concomitant toxicity [16] [17] [18]. However, the effects of C3G on Sirt1 in neuronal cells are unclear. This study investigated the effect of C3G on Sirt1 expression and activity in human neuroblastoma (SH-SY5Y) cells.

Nuclear factor erythroid 2-related factor 2 (Nrf2) plays a central role in regulating the expression of antioxidant genes, including superoxide dismutase (SOD) and catalase [19] [20] [21] [22]. Nrf2 is retained in the cytoplasm as an inactive complex with its cytosolic repressor, Kelch-like ECH associated protein-1 (Keap1). The dissociation of Nrf2 from Keap1 is important for its nuclear translocation, followed by binding to antioxidant response elements (ARE) and activation of cytoprotective genes such as SOD and catalase [20] [22] [23]. Therefore, Nrf2 is necessary for the maintenance of redox homeostasis. In this study, we also examined the effect of C3G on Nrf2 expression in SH-SY5Y cells.

2. Experimental

2.1. SH-SY5Y Cell Culture and Treatment with C3G

SH-SY5Y cells were purchased from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). Cells were grown to 80% - 90% confluence in DMEM containing 10% fetal bovine serum (FBS), L-glutamine (4 mM), penicillin (100 U/ml) and strep-

tomycin (100 mg/ml) at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air and passaged by trypsinization.

Before treating the cells with C3G (Tokiwa phytochemical Co., Ltd., Chiba, Japan), the culture medium was replaced with DMEM containing 2% FBS as serum may include antioxidants, chelators of transition metal ions, and high-density lipoproteins [24]. C3G (10 - 100 mM) was subsequently added to the medium.

2.2. Cell Viability

Cell viability was assessed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS assay) from Promega (Madison, WI, USA). Briefly, SH-SY5Y cells in 96-well plates were treated with C3G (0 - 200 μM) for 24 h or were pretreated with C3G (50 μM) for 4 h and subsequently exposed to H_2O_2 or 6-hydroxydopamine (6-OHDA) for 24 h, respectively. 6-OHDA causes dopaminergic neurodegeneration in experimental models of PD via an oxidative stress-mediated process [25]. After treatment with C3G, the medium containing detached cells was removed. Cells remaining on the 96-well plates were washed with DMEM (FBS-free) and incubated with fresh DMEM (100 ml) and MTS assay solution (10 μl) at 37˚C for 60 min. The produced MTS formazan was measured at 490 nm with a Bio-Rad Model 680 microplate reader (Hercules, CA, USA). Additionally, the viability of SH-SY5Y cells was assessed by measuring lactate dehydrogenase (LDH) release. After treating SH-SY5Y cells in 12-well plates with C3G (50 μM) and 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) for 24 h, aliquots of the medium were taken to measure the activity of LDH released from cells. AAPH is a free-radical-generating azo compound [26]. The remaining intracellular LDH was released by adding 0.1% Triton X-100 in phosphate-buffered saline at pH 7.4. LDH activity was measured spectrophotometrically on the basis of an increase in absorbance at 340 nm with 60 mM lithium lactate in 0.3 M diethanolamine buffer (pH 9.0) after the reaction was initiated by adding 3 mM (final concentration) NAD^+. Released LDH activity was expressed as a percentage of total LDH activity (in the medium and in the remaining cells).

2.3. Measurement of Sirt1, Nrf2, Superoxide Dismutase 1, and Catalase *m*RNA Levels

Quantitative RT-PCR analysis was used to measure mRNA levels of Sirt1, Nrf2, superoxide dismutase 1 (SOD1), and catalase. Total RNA from cells treated with C3G (0 - 100 μM) was extracted with RNAspin Mini (GE Healthcare Life Sciences, Buckinghamshire, England) according to the manufacturer's protocol. mRNA expression was quantified by real-time RT-PCR using a 7500 Fast Real-Time PCR system (Life Technologies, Applied Biosystems, Foster City, CA, U.S.A.) and the One Step TB Green® PrimeScript™ PLUS RT-PCR kit (TaKaRa Bio, Shiga, Japan). The following sense and antisense primers, respectively, were used: Sirt1, 5_-TGC CGG AAA CAA TAC CTC CAC CTG-3_ and 5_-ACA GAC ACC CCA GCT CCA GTT -3_; Nrf2, 5_-TCA GCC AGC CCA GCA CAT CC

-3_ and 5_-TCT GCG CCA AAA GCT GCA TGC-3_; SOD1, 5_-TGG CCG ATG TGT CTA TTG AA-3_ and 5_-GGG CCT CAG ACT ACA TCC AA -3_; catalase, 5_-GCC TGG GAC CCA ATT ATC TT-3_ and 5_-GAA TCT CCG CAC TTC TCC AG-3_; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5_-TTG ATT TTG GAG GGA TCT CG-3_ and 5_-AAC TTT GGC ATT GTG GAA GG-3. mRNA levels were acquired from the value of the threshold cycle (Ct) of Sirt1, Nrf2, SOD1, or catalase mRNA and normalized to that of GAPDH. Relative mRNA levels were compared and expressed as a percentage of control levels.

2.4. Assay of Sirt1 Activity

After SH-SY5Y cells were treated with C3G, the cells were washed with DPBS and collected in microtubes. The cell pellets were lysed in 0.1% Triton X-100 in phosphate-buffered saline at pH 7.4 as lysis buffer containing protease inhibitors and sonicated on ice. The lysate was centrifuged at 12,000 rpm for 10 min at 4˚C. The supernatant was used as the cell lysate. Sirt1 activity was measured using the Sirt1 Activity Assay Kit (Abcam, Cambridge, England) according to the manufacturer's protocol. The cell lysate was incubated with an anti-Sirt1 antibody (Abcam, Cambridge, England). After that, Protein G Sepharose 4 Fast Flow beads (20 μL of 50% bead slurry) (GE Healthcare, Buckinghamshire, UK) were added. The lysate was centrifuged at 5000 rpm for 1 min at 4˚C. The pelleted beads were washed with lysis buffer and once with Sirt1 assay buffer (50 mM Tris-HCl (pH 8.8), 0.5 mM DTT). Fluorescence intensities were quantified using GloMax (Promega KK, Madison, USA). Released Sirt1 activity was expressed as a percentage of control levels.

2.5. Measurement of Sirt1, Nrf2, SOD1, and Catalase Protein Levels

Sirt1, Nrf2, SOD1, and catalase protein levels were analyzed by Western blotting. After treatment with C3G (0 - 100 μM), SH-SY5Y cells were washed with DPBS and lysed in protease inhibitor-containing radioimmunoprecipitation assay buffer (Pierce, Rockford, IL, USA). The lysate was centrifuged at 10,000 ×g for 15 min, and 20 μg of protein in the supernatant was resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The separated proteins were blotted onto a nitrocellulose or PVDF membrane. The membrane was incubated with primary antibodies for Sirt1, Nrf2, SOD1, catalase or β-actin, and horseradish peroxidase-conjugated secondary antibodies. Chemiluminescence was detected with an ECL Plus western blot detection kit (GE Healthcare, Buckinghamshire, UK). Protein expression in each sample was determined by normalizing target band intensity to β-actin band intensity. Band intensities were quantified using ImageJ software.

2.6. Other Procedures

Protein concentrations were determined by the Bradford method using bovine

serum albumin as the standard.

2.7. Statistical Analysis

All experiments were performed independently a minimum of three times. Data were combined and expressed as means ± standard deviation. Statistical significance between the two groups was evaluated using Student's t-test or the Scheffe test after one-way or two-way analysis of variance with Tukey's post-hoc test. A *P* value of <0.05 was considered significant.

3. Results

3.1. Effect of C3G on the Viability of SH-SY5Y Cells

SH-SY5Y cells were treated with C3G at the indicated concentrations for 24 h. Cell viability estimated by the MTS assay is shown in Figure 1. Treatment with C3G at 10, 50, and 100 μM had no effect on cell viability, but a significant reduction in cell viability was seen at C3G concentrations of 200 μM (data not shown). Thus, for the following experiments, we used C3G at 0 - 100 μM, which did not affect cell viability.

3.2. Effect of C3G on Sirt1 Expression in SH-SY5Y Cells

We examined the effect of C3G on Sirt1 expression levels. Figure 2 demonstrates that treatment with C3G increased both mRNA and protein levels of Sirt1. Treatment with 10, 50, and 100 μM C3G resulted in an increase of 2.5, 2.5, and 3.0 folds of Sirt1 mRNA. In addition, a modest increase of 1.1, 1.2, and 1.3 folds at the level of protein.

3.3. Effect of C3G on Sirt1 Activity in SH-SY5Y Cells

We then examined the effect of C3G on Sirt1 activity in SH-SY5Y cells. Figure 3 demonstrates that treatment of C3G dose-dependently increased Sirt1 activity.

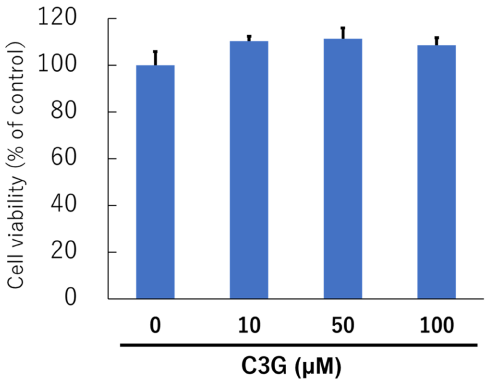

Figure 1. Effect of cyanidin-3-glucoside on viability of human neuroblastoma cells. Human neuroblastoma (SH-SY5Y) cells were treated with cyanidin-3-glucoside (C3G) at the indicated concentrations for 24 h. Cell viability estimated by MTS assay. Values are means ± standard deviations of six experiments. *Significant difference compared with the control ($P < 0.05$).

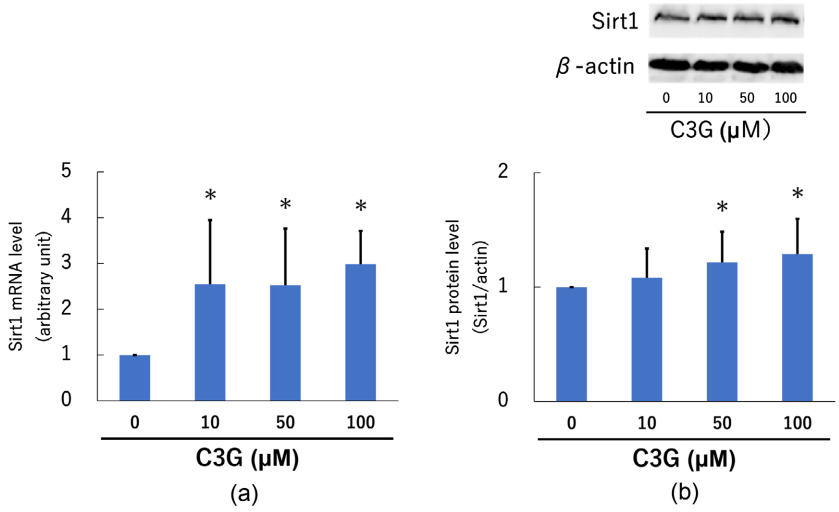

Figure 2. Effect of cyanidin-3-glucoside on silent information regulator 2-related protein 1 mRNA and protein levels in human neuroblastoma cells. (a) Silent information regulator 2-related protein 1 (Sirt1) mRNA levels. Human neuroblastoma (SH-SY5Y) cells were treated with 10 - 100 μM cyanidin-3-glucoside (C3G) for 2 h. Values are means ± standard deviations (SD) of four experiments. (b) Sirt1 protein levels. SH-SY5Y cells were treated with 10 - 100 μM C3G for 24 h. Values are means ± SD of five experiments. *Significant difference compared with the control ($P < 0.05$).

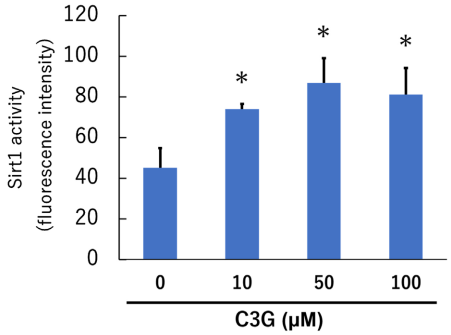

Figure 3. Effect of cyanidin-3-glucoside on silent information regulator 2-related protein 1 activity in human neuroblastoma cells. Human neuroblastoma cells were treated with cyanidin-3-glucoside at the indicated concentrations for 24 h. Silent information regulator 2-related protein 1 activity estimated by immunoprecipitation method. Values are means ± standard deviations of six experiments. *Significant difference compared with the control ($P < 0.05$).

Sirt1 activity was increased by 45.2, 74.1, 86.9, and 81.2 folds in fluorescence intensity by 0, 10, 50, and 100 μM C3G, respectively.

3.4. Effect of C3G on Nrf2 Expression in SH-SY5Y Cells

We examined the effect of C3G on Nrf2 expression levels. **Figure 4** demonstrates that C3G increased the mRNA and protein levels of Nrf2. Treatment with 10, 50, and 100 μM C3G increased Nrf2 mRNA levels by 2.9, 2.7, and 3.0 folds. Treatment with C3G resulted in a modest increase of 1.2, 1.3, and 1.3 folds in protein levels, which did not increase at higher doses of C3G.

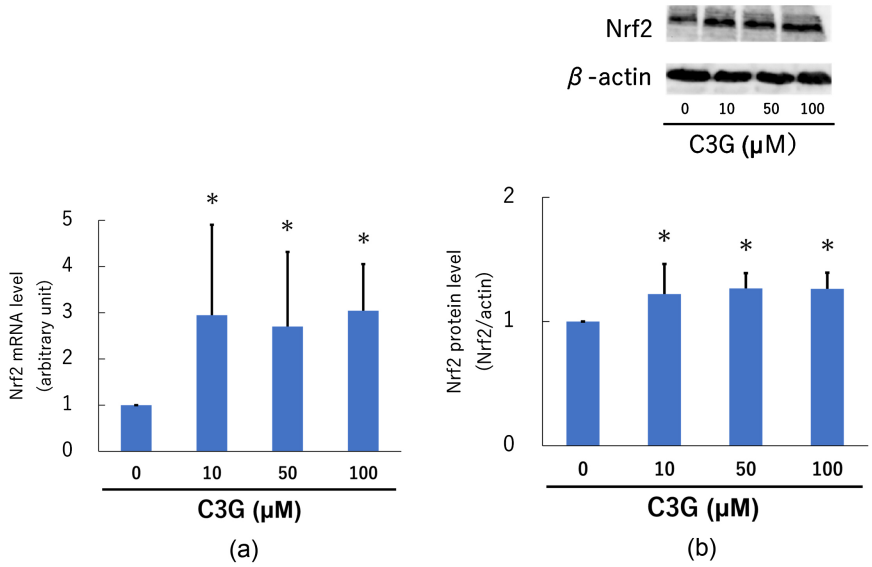

Figure 4. Effect of cyanidin-3-glucoside on nuclear factor erythroid 2-related factor 2 mRNA and protein levels in human neuroblastoma cells. (a) Nuclear factor erythroid 2-related factor 2 (Nrf2) mRNA levels. Human neuroblastoma (SH-SY5Y) cells were treated with 10 - 100 μM cyanidin-3-glucoside (C3G) for 2 h. Values are means ± standard deviations (SD) of four experiments. (b) Nrf2 protein levels. SH-SY5Y cells were treated with 10 - 100 μM C3G for 24 h. Values are means ± SD of five experiments. *Significant difference compared with the control ($P < 0.05$).

3.5. Effect of C3G on SOD1 and Catalase Expression in SH-SY5Y Cells

We then examined the effect of C3G on SOD1 and catalase expression levels. Figure 5 demonstrates that C3G increased the mRNA and protein levels of SOD1 and catalase. The SOD1 and catalase mRNA levels were increased by 1.8, 1.6, and 1.6 and 23.6, 25.5, and 27.1 folds after treatment with 10, 50, and 100 μM C3G, respectively. The SOD1 and catalase protein levels were modestly increased by 1.1, 1.3, and 1.2 and 1.2, 1.5, and 1.3 folds by 10, 50, and 100 μM C3G, respectively.

3.6. Effect of C3G on Oxidative Stress in SH-SY5Y Cells

Finally, we examined whether C3G (50 μM) could protect SH-SY5Y cells from oxidative stress. The exposures to H_2O_2 and 6-OHDA resulted in approximately 40% and 80% cytotoxicity, respectively, as shown in Figure 6(a) and Figure 6(b). C3G reduced the H_2O_2- and 6-OHDA-induced cytotoxicity significantly. Additionally, exposure to AAPH resulted in approximately 50% cytotoxicity, as shown in Figure 6(c). C3G reduced the AAPH-induced cytotoxicity significantly.

4. Discussion

In this study, we evaluated the effect of C3G on Sirt1 expression and activity in SH-SY5Y cells. The present study demonstrated that C3G increased Sirt1

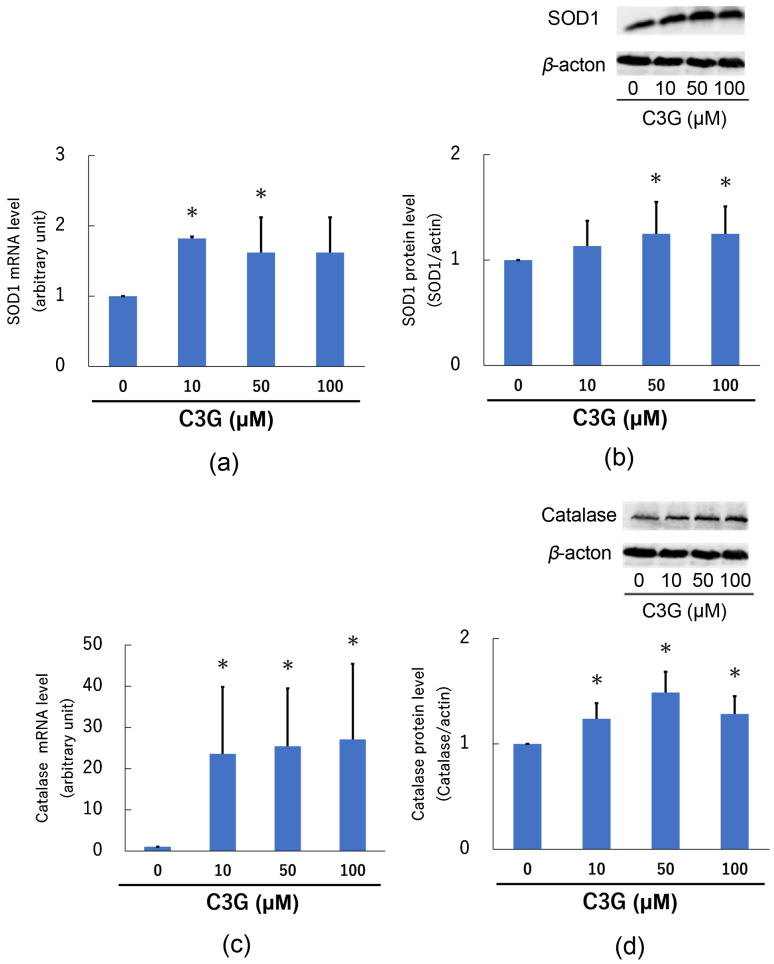

Figure 5. Effect of cyanidin-3-glucoside on superoxide dismutase 1 and catalase mRNA and protein levels in human neuroblastoma cells. (a) Superoxide dismutase 1 (SOD1) mRNA levels. Human neuroblastoma (SH-SY5Y) cells were treated with 10 - 100 μM cyanidin-3-glucoside (C3G) for 2 h. Values are means ± standard deviations (SD) of four experiments. (b) Catalase mRNA levels. SH-SY5Y cells were treated with 10 - 100 μM C3G for 2 h. Values are means ± SD of four experiments. (c) SOD1 protein levels. SH-SY5Y cells were treated with 10 - 100 μM C3G for 24 h. Values are means ± SD of five experiments. (d) Catalase protein levels. SH-SY5Y cells were treated with 10 - 100 μM C3G for 24 h. Values are means ± SD of five experiments. *Significant difference compared with the control ($P < 0.05$).

expression and Sirt1 activity levels in SH-SY5Y cells (**Figure 2** and **Figure 3**). The increase in Sirt1 expression by C3G may have resulted in increased Sirt1 activity. Sirt1 has previously been shown to inhibit apoptosis by regulating p53 and reducing oxidative stress by regulating antioxidant defenses via FOXO family members [27]. In addition, previous studies have indicated that Sirt1 was associated with inhibition of oxidative stress, neuronal survival, and brain plasticity [28]. These reports have suggested that the upregulation of Sirt1 could decrease oxidative stress and increase neuronal function. Taken together, C3G could decrease oxidative stress and increase neuronal function by increasing the expression and activity of Sirt1.

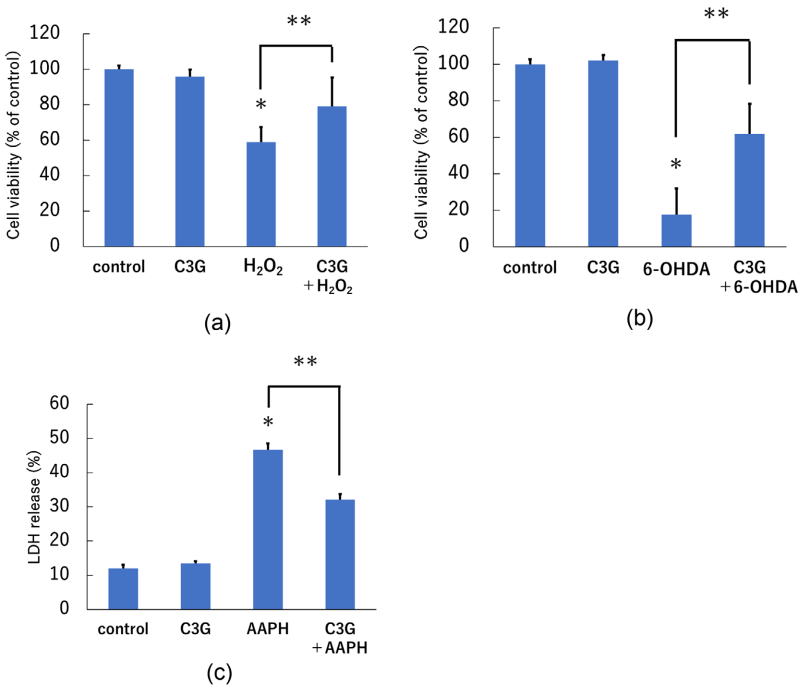

Figure 6. Effect of cyanidin-3-glucoside on oxidative stress in human neuroblastoma cells. Human neuroblastoma (SH-SY5Y) cells were pretreated with cyanidin-3-glucoside (C3G) (50 μM) for 4 h. Subsequently, the untreated or C3G-treated cells were exposed to 100 μM H_2O_2 (a), 200 μM 6-hydroxydopamine (b), and 10 mM 2,2'-azobis (2-amidinopropane) dihydrochloride (c) for 24 h. Values are means ± standard deviations of six experiments. *Significant difference compared with the control ($P < 0.05$). **Significant difference compared with the treatment with oxidizing agent alone ($P < 0.05$).

The levels of Nrf2 mRNA and protein expression were increased after treatment with C3G (**Figure 4**). Recently, microRNAs (miRNAs) have been shown to be involved in the regulation of Nrf2 expression [29]. Therefore, miRNAs may be involved in the C3G-induced increased Nrf2 expression in SH-SY5Y cells. In addition, Nrf2 is one of the key oxidative stress regulators that induces transcription of a variety of genes by binding to the ARE [30]. Nrf2 activation upregulates the expression of a group of functionally diverse cytoprotective proteins, such as SOD, catalase, glutathione s-transferase, glutathione peroxidase, glutamate-cysteine ligase, and thioredoxin [19]. Although we did not examine whether C3G induces Nrf2 translocation into the nucleus, C3G-stimulated Nrf2 activation may be mediated by the dissociation of the Keap1-Nrf2 complex [31], thereby increasing the expression of SOD and catalase in our study (**Figure 5**). Moreover, recent studies have reported that Sirt1 could rapidly activate Nrf2 by decreasing acetylation of Nrf2 and that Sirt1 might be an upstream regulatory factor of the Nrf2 signal pathway [32] [33]. Therefore, C3G might decrease oxidative stress by inducing cytoprotective protein mediated by the Sirt1/Nrf2 pathway.

We performed experiments using H_2O_2, 6-OHDA, or AAPH to determine whether C3G may protect SH-SY5Y cells from oxidative stress. C3G clearly pro-

tected SH-SY5Y cells from toxicity induced by these oxidizing agents (**Figure 6**), indicating that C3G can counteract oxidative damage in cells. Mudo *et al.* reported that Sirt1 has a protective effect against the cytotoxicity of 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MTPT), which causes dopaminergic neurodegeneration, by increasing the expression of mitochondrial antioxidants manganese SOD and thioredoxin in an experimental model of PD [34]. Cyanidin reduced 1-methyl-4-phenylpyridinium^{+}-induced apoptosis of human neuroblastoma SH-SY5Y cells [4]. In addition, Tarozzi *et al.* demonstrated neuroprotective effects of C3G in SH-SY5Y cells on amyloid beta (25 - 35) oligomers, which are suggested to be responsible for synaptic dysfunction and/or neuronal loss in AD [35]. C3G reduced the level of ROS, which would otherwise induce cell death via the mitochondrial apoptotic pathway, indicating that C3G may have protective effects in AD [5]. These reports support our findings that C3G can increase antioxidant factors and protect neuronal cells. In addition, these findings suggest that C3G has beneficial properties that might be useful for treatments targeting at the development and progression of neurodegeneration caused by oxidative stress.

Our present data showed that C3G protected SH-SY5Y cells from oxidative stress. This may occur by increasing the expression and activity of Sirt1 and the expression of Nrf2 levels; however, since the effects on protein levels were modest, the induction of Sirt1 activity may also be driven by other mechanisms. However, since Sirt1 and Nrf2 control not only SOD1 and catalase genes but also the genes of many cytoprotective enzymes such as glutathione synthetase, glutathione peroxidase, thioredoxin, and heme oxygenase-1 [36] [37] [38], we speculated that the C3G-induced resistance to oxidative stress in SH-SY5Y cells is associated with the increased expression of other cytoprotective enzymes. This requires further investigation in the future.

The causes of neurodegenerative diseases are still under investigation although the loss of neurons has been suggested to cause the initiation and progression of neurodegenerative diseases [39]. Moreover, the levels of Sirt1 protein were reduced in all brain regions in neurodegenerative diseases [13]. Downregulation of Sirt1 protein and activity in disease groups could be associated with generalized neurodegeneration observed in these diseases and the associated synaptic and neuronal loss induced by chronic oxidative stress. Our study indicates that C3G could ameliorate the initiation and progression of neurodegenerative diseases if C3G also shows a protective effect on neuronal cells in a disease context.

In summary, we demonstrated that C3G increased Sirt1 expression and activity and Nrf2 expression. Nrf2 might, in turn, increase the expression of SOD1 and catalase, possibly resulting in enhanced resistance to oxidative stress.

5. Conclusion

Our data indicate that C3G induced the expression and activity of Sirt1 and the expression of Nrf2; thus, the decreased oxidative stress and increased antioxida-

tive factors might be mediated by the Sirt1/Nrf2 pathway. As oxidative stress is a key contributor to aging and neurodegenerative disease, C3G could be effective in preventing or attenuating the progress of neurodegenerative diseases.

Acknowledgements

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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