Aged Garlic Extract Reduces ROS Production and Cell Death Induced by 6-Hydroxydopamine through Activation of the Nrf2-ARE Pathway in SH-SY5Y Cells

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ABSTRACT

Many degenerative or pathological processes, such as aging, cancer and coronary heart disease, are related to reactive oxygen species (ROS) and radical-mediated reactions. We examined the effectiveness of aged garlic extract (AGE), a garlic preparation rich in water-soluble cysteinyl moieties, for protection of cells from ROS produced by 6-hydroxydopamine (6-OHDA) using human neuroblastoma SH-SY5Y cells. Concomitant treatment of cells with AGE (2 and 4 mg/ml) showed the dose-dependent protective effect on the cell death induced by 6-OHDA. In addition, the AGE treatment significantly suppressed the increase of ROS generation by 6-OHDA. Furthermore, the protective effect of AGE was accompanied by activation of the nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) pathway and the increase of mRNAs of heme oxygenase-1 and NAD(P)H quinone oxidoreductase 1. These two enzymes are important in the cellular antioxidant system. These results indicated that AGE protected cells from ROS damage by not only capturing ROS directly but also activating the cellular antioxidant system by stimulating antioxidant gene expression via the Nrf2-ARE pathway. The present study suggested that AGE may be useful for prevention and treatment of cell damage caused by ROS.

Keywords: Aged Garlic Extract (AGE); 6-OHDA; ROS; Nrf2-ARE Pathway; SH-SY5Y Cells

1. Introduction

Garlic (*Allium sativum* L.) has been used for the prevention and treatment of a variety of diseases including heart disease and cancer [1]. Among the various garlic preparations available, aged garlic extract (AGE) preparation employs a unique manufacturing process, involving mild and long extraction up to 20 months that leads to enrichment of water-soluble cysteinyl moieties and reduction of harsh agents and the garlic odor [2]. AGE is also less irritating and has little toxic side effects compared to the raw garlic [3]. AGE and its components have been shown to possess pharmacologically beneficial activities, such as anti-stress [4], anti-fatigue [5], immunomodulatory activities [6] as well as cardiovasculo- [7-9] and hepatoprotective properties [10]. Moreover, AGE has been shown to possess superior antioxidant properties compared to the raw garlic, which may be important for its pharmacological effects [11]. In our previous study we have demonstrated that AGE enhances neurite outgrowth of PC12 cells by its scavenging effect on reactive oxygen species (ROS) [12].

Although oxygen is essential to many living organism for the production of energy to fuel biological processes, oxygen-centered free radicals and other ROS produced excessively can cause cell damage and death. The human body has both enzymatic and non-enzymatic antioxidant systems to protect cellular molecules against ROS-induced damage [13]. However, these innate defenses may not be sufficient if oxidative stress is severe or continuous. There is an increasing body of evidence suggesting that many degenerative or pathological processes, such as aging, cancer and coronary heart disease, are related to ROS and radical-mediated reactions [14,15]. Antioxidant agents can reduce oxidative damage to cells, defend against cancer-causing agents and help lower the risk of cardiovascular disease, diabetes and some forms of dementia including Alzheimer’s disease [16,17]. In our series of survey to find active compounds that stimulate nerve cells, we have shown that fullerene derivatives produce the enhancing effect on neurite outgrowth by scavenging cytotoxic ROS [18]. Recently Baati et al. have reported that the repeated oral administration of fullerene extends the lifespan of rats by attenuation of the
oxidative stress [19].

6-Hydroxydopamine (6-OHDA) is one of potent neurotoxins and induces cell death associated with ROS generation [20]. It has been reported that treatment with 6-OHDA causes the activation of c-JUN N-terminal kinase and caspases, DNA fragmentation and apoptosis in various cell types [21-23].

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a basic leucine zipper transcription factor, and forms heterodimers with the small Maf proteins and binds to the antioxidant response element (ARE). Nrf2 plays a critical role in the constitutive and inducible expression of numerous detoxifying and antioxidant genes, including heme oxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase 1 (NQO1), through activation of the ARE in the regulatory region of the gene [24-27]. Nrf2 binds to Keap1, a cytoplasmic cysteine riched protein, under normal conditions. But when cells undergo certain adverse conditions such as oxidative stress, Nrf2 is liberated from Keap1, and allowed to translocate into the nucleus and bind to ARE [28,29]. Therefore, the Nrf2-Keap1 complex is thought to be a cytoplasmic sensor system for oxidative stress.

In the present study, we examined the protective effect of AGE on the cell death induced by 6-OHDA and its ability to activate antioxidant systems in cultured SH-SY5Y cells. Our results indicated that AGE effectively inhibited the cell death induced by ROS through capturing ROS directly and also inducting antioxidant gene expression via the Nrf2-ARE pathway. Therefore AGE may be useful for the prevention and treatment of diseases associated with ROS.

2. Materials and Methods

2.1. Materials

6-OHDA, phenylmethylsulfonyl fluoride, leupeptin, DMEM (low glucose) with L-glutamine and Ham’s F-12 with L-glutamine were purchased from Wako Pure Chemicals (Osaka, Japan). Fetal bovine serum (FBS) with L-glutamine were purchased from Wako Pure Chemicals (Osaka, Japan). Fetal bovine serum (FBS) with L-glutamine was obtained from Takara Bio Inc. (Shiga, Japan). Anti-Nrf2 antibody was obtained from Abcam (Tokyo, Japan). 1-(4-Amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU or nimustine hydrochloride) was a gift from Daiich Sankyo Co., Ltd. (Tokyo, Japan).

2.2. Preparation of AGE

AGE was prepared as reported [12] and supplied by Wakunaga Pharmaceutical Co. Ltd. (Hiroshima, Japan). Briefly, garlic cloves (Allium sativum L.) grown under controlled organic conditions without the use of chemical fertilizers, herbicides or pesticides were sliced and soaked in an aqueous ethanol solution and extracted/aged up to 20 months at room temperature. AGE was obtained by removing ethanol from the AGE extract under in vacuo.

2.3. Cell Culture and Treatment

The human neuroblastoma SH-SY5Y cells were purchased from ATCC (Manassas, VA, USA). SH-SY5Y cells were cultured in DMEM/F-12 medium supplemented with 10% FBS, penicillin (100 U/ml) /streptomycin (100 μg/ml) in a humidified 5% CO₂ atmosphere at 37°C. In general, cells were seeded on a 96-well multi-dish (2.5 × 10⁴ cells/well) and incubated for 24 h before use. The cytotoxicity of 6-OHDA, MPP⁺ and ACNU was assessed by treating cells for 24 h, 72 h and 48 h, respectively.

2.4. Cell Viability

MTT assay [30] was used to determine the cell viability. Briefly, after the medium was replaced with the one containing MTT (0.25 mg/ml), the cell culture was continued for 5 h. After the medium containing MTT was removed and a 10% sodium dodecyl sulfate (SDS) solution in H₂O/DMF = 1/1 (v/v) was added, the culture dishes were then left standing overnight at room temperature. Optical absorption was measured at 550 nm. Cell viability was expressed as % of the control.

2.5. Measurement of ROS Generation

ROS generation was measured according to the method of Keston et al. [31] using the 24-well multi-dishes. Briefly, DCB-FDA (100 μM) dissolved in 500 μl of the medium without serum (ns-medium) was added to cell culture (2.6 × 10⁴ cells/well) and cells were incubated for 60 min. After cells were washed twice with ns-medium, the fresh ns-medium with or without AGE was added. After 30 min incubation, 6-OHDA was added and cells were cultured for 1 h. An equal volume of RIPA buffer (includes 100 mM Tris-HCl (pH 8.0), 300 mM NaCl, 1% sodium deoxycholate, 0.2% SDS and 2% Triton X-100) was added and the fluorescence was measured (excitation at 490 nm and emission at 530 nm). In order to avoid the
2.6. Expression of mRNAs of HO-1, NQO1 and Nrf2

After cells were treated with AGE (4 mg/ml) for 24 h, total RNA was extracted with RNAiso. cDNA was synthesized using the PrimeScript RT reagent kit. The real-time PCR using SYBR Green detection system was performed on an Applied Biosystems 7500 real-time PCR system (Applied Biosystems Japan Ltd.). Relative mRNA levels were quantified using the comparative CT method with Applied Biosystems 7500 System Software. The mRNA values were normalized against the average values of GAPDH. The primers used for real-time PCR were as follows; 5'-AGAGCTGCCAGCAAGGCTG-3' (forward) and 5'-ACCAGCAGCTGGGCTCTGT-3' (reverse) for HO-1, 5'-ATGTATGACAAAGGACCCTTC-C-3' (forward) and 5'-TCCCTTGCAGAGAGTACATG-G-3' (reverse) for NQO1, 5'-ACACGGTCCACAGCTCCCACTGC-3' (forward) and 5'-TGATCAAATCCA-TGTCTTG-3' (reverse) for Nrf2, 5'-GCCITCCGTGTCCCCAGTCG-3' (forward) and 5'-CAATGCCAGCCC-CAGCGTCA-3' (reverse) for GAPDH.

2.7. Preparation of Nuclear Extract

SH-SY5Y cells were treated with tBHQ (50 μM) for 3.5 h or with AGE (4 mg/ml) for 1, 3 or 6 h in 100-mm dishes. Nuclear extracts were prepared using the method of Hara et al. with slight modifications [32]. Briefly, treated cells were washed once with ice-cold phosphate-buffered saline, and scraped off in a buffer A (including 20 mM HEPES (pH 7.8), 15 mM KCl, 2 mM MgCl2, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin). The cell suspension was allowed to stand for 15 min, and then cells were lysed for 5 min after addition of 0.2% Nonidet P-40. The cell lysates were centrifuged for 5 min at 800 g, and the pellets were resuspended in buffer B (including 20 mM HEPES (pH 7.8), 0.4 M NaCl, 2 mM MgCl2, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 25% glycerol), followed by gentle shaking for 30 min. After the suspension was centrifuged for 20 min at 20,000 g, the supernatant was stored at −80°C. All procedures were performed at 4°C. Protein concentrations in nuclear extracts were determined using a modified Bradford method [33].

2.8. Western Blotting

Nuclear protein extracts (10 μg protein/each sample) were electrophoresed in a 7.5% SDS-polyacrylamide gel, and then the protein bands were transferred onto a PVDF membrane. The membrane was incubated with anti-Nrf2 antibody (1:200) or anti-Lamin B1 antibody (1:1000), followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:1000). Proteins were visualized using the ECL plus western blotting detection system (GE Healthcare Japan).

2.9. Statistical Analysis

The data are expressed as the mean ± S.D. Statistically significant differences were determined using Student’s t-test or one-way ANOVA followed by Bonferroni’s multiple comparison test. Differences at p < 0.05 were considered to be significant.

3. Results

3.1. Concomitant Treatment of AGE Suppressed the Cell Death Induced by 6-OHDA

Three test compounds, 6-OHDA, MPP+ and ACNU, that induce cytotoxicity in nerve cells were chosen and the protective effect of AGE (2 and 4 mg/ml) on cell death induced by these compounds was examined. 6-OHDA is a potent neurotoxin and induces cell death associated with ROS generation [20], whereas MPP+ is an inducer of Parkinsonism and inhibitor of mitochondrial respiration chain [34] and ACNU is an alkylating anticancer drug used for the therapy of brain cancer [35]. The cytotoxicity of each compound in SH-SY5Y cells was examined and the dose-response curve of each compound was obtained. As shown in Figure 1, 6-OHDA caused 50% cell death at a concentration, 105 μM as previously reported [20]. The results in Figure 2 showed that the concomitant treatment of cells with 2 or 4 mg/ml of AGE significantly reduced the extent of cell death induced by 100 or 150 μM of 6-OHDA, respectively. We next examined whether AGE inhibited the cell death induced by MPP+ and ACNU. As shown in Figures and 4, respectively, AGE gave no protective effect on cell death caused by MPP+ and ACNU. These results indicated that among three test compounds examined, the protective effect of AGE on cell death was only observed in the case of 6-OHDA that produced ROS.

3.2. AGE Suppressed ROS Generation Induced by 6-OHDA

In order to determine whether AGE suppressed ROS generation induced by 6-OHDA, we examined the intracellular level of ROS using a redox-sensitive fluorescence reagent, DCFH-DA [31]. As shown in Figure 5, addition of 6-OHDA at 100 and 200 μM increased ROS production in SH-SY5Y cells, whereas concomitant treat-
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Figure 1. Cytotoxicity of 6-OHDA against SH-SY5Y cells. SH-SY5Y cells were exposed to the indicated concentrations of 6-OHDA for 24 h. Cell viability was assessed using MTT assay as described in the Materials and Methods. The data are expressed as mean ± S.D. (n = 6).

Figure 2. Effect of AGE on 6-OHDA-induced cytotoxicity. SH-SY5Y cells were treated with AGE (2 or 4 mg/ml) for 30 min, and then the cells were exposed to 6-OHDA (100 or 150 μM) for 24 h. Cell viability was assessed using MTT assay as described in the Materials and Methods. The data are expressed as mean ± S.D. (n = 5 - 6). **Denotes a significant difference compared with the corresponding value of the system without AGE (p < 0.01) as evaluated by Bonferroni’s multiple comparison test after one-way ANOVA.

Figure 3. Effect of AGE on MPP+-induced cytotoxicity. The experimental procedures are the same as described in Figure 2 legend except that the exposure time to MPP+ was 72 h. The data are expressed as mean ± S.D. (n = 6).

Figure 4. Effect of AGE on ACNU-induced cytotoxicity. The experimental procedures are the same as described in Figure 2 legend except that the exposure time to ACNU was 48 h. The data are expressed as mean ± S.D. (n = 6).

3.3. AGE Increased the Expression of mRNAs of HO-1 and NQO1

With regard to the mode of protective action of AGE, two possibilities could be considered; 1) AGE itself captured and thus lowered ROS as already being reported [11,36,37] and/or 2) AGE activated the antioxidant response system in cells that can reduce the ROS level. As the representative antioxidant proteins, HO-1 and NQO1 were chosen and the effect of AGE on the level of their mRNAs was examined using real-time PCR. As shown in Figure 6, AGE significantly increased the level of mRNAs of HO-1 and NQO1.

3.4. AGE Induced the Nuclear Translocation of Nrf2

Nrf2 controls the expression of a cluster of antioxidant genes by binding to ARE that exists in the upstream of antioxidant genes [24-27,38]. Thus we examined the possibility that AGE activated the Nrf2-ARE pathway and the ARE-driven gene expression [39]. In this experiment, the effect of AGE (4 mg/ml) was examined during 1, 3 and 6 h treatment and tBHQ was used as a positive control to activate the Nrf2-ARE pathway [29,40]. As shown in Figure 7, AGE treatment induced the nuclear translocation of Nrf2 as early as 1 h and its effect persisted at 6 h. These findings suggested that AGE stimulated the expression of gene encoding HO-1 and NQO1 via the Nrf2-ARE pathway. The level of Nrf2 mRNA, however, was not increased by AGE (data not shown).

3.5. Pre-Treatment of AGE Suppressed the Cell Death Induced by 6-OHDA

Based on the above findings that AGE activated the cel-
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**Figure 5.** Inhibition of 6-OHDA-induced ROS production by AGE. SH-SY5Y cells were treated with AGE (4 mg/ml) for 30 min, and then the cells were further exposed to 6-OHDA (100 or 200 μM) for 1 h. ROS production was assayed as described in the Materials and Methods. The data are expressed as mean ± S.D. (n = 4). **Denotes a significant difference compared with the corresponding value of the system without AGE (p < 0.01) as evaluated by t-test.

**Figure 6.** Induction of mRNAs of HO-1 and NQO1 by AGE. SH-SY5Y cells were treated with AGE (4 mg/ml) for 1, 3 or 6 h. Total RNA was prepared, and then RT-PCR was performed as described in the Materials and Methods. The data are expressed as fold induction relative to the level at 0 h. (n = 3). **Denotes a significant difference compared with the 0 h value (p < 0.01) as evaluated by Bonferroni’s multiple comparison test after one-way ANOVA.

**Figure 7.** Nuclear translocation of Nrf2 by AGE. Nuclear extracts were prepared from SH-SY5Y cells treated with tBHQ (50 μM) for 3.5 h or AGE (4 mg/ml) for 1, 3 or 6 h. The extracts (10 μg) were subjected to Western blot analysis by using anti-Nrf2 and anti-lamin B1 antibodies.

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AGE pre-treatment also protected the cell death induced by 6-OHDA (Table 1). We found this protection was accompanied by the activation of the Nrf2-ARE pathway (Figures 6 and 7). The activation mechanism of AGE is so far not clear, but several possibilities are conceivable [39]. It is known that oxidative stress causes the liberation of Nrf2 from Keap1 in the Nrf2-Keap1 complex, followed by the translocation of Nrf2 into nucleus and activation of ARE [24-28]. AGE produced a low but significant level of ROS as shown in Figure 5, and this effect may have served as a trigger. AGE contains a variety of compounds, some of which can stimulate the pro-

Table 1. The effect of AGE-pre-treatment on 6-OHDA-induced cytotoxicity.¹

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4. Discussion

Using SH-SY5Y cells the protective effect of AGE was examined on the cytotoxicity induced by three test compounds, 6-OHDA, MPP⁺ and ACNU, that caused nerve cell death. Among three test compounds examined, the protective effect of AGE was only observed in the case of 6-OHDA that produced ROS (Figures 2 and 5). For the lower extent of cell damage induced by 100 μM of 6-OHDA, a lower level of AGE (2 mg/ml) was enough to provide the significant protection of cell viability, while a higher level of AGE (4 mg/ml) was required to counteract the effect of the higher concentration of 6-OHDA (150 μM). Previous studies showed that AGE possesses antioxidant activity [11] by scavenging ROS [11,36,37]. Our results shown in Figure 5 confirmed that AGE was capable of reducing the cellular ROS induced by 6-OHDA.

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duction of ROS. Another possibility is that AGE contains some compounds that can react with and modify the SH group of cysteine residues of Keap1 molecule. It was reported that compounds such as sulforaphane and curcumin are metabolized to the active compounds that react with Keap1 to liberate Nrf2. Nrf2 then translocates and accumulates in nucleus and activates the Nrf2-ARE pathway [39,41,42]. AGE contains sulfide compounds such as diallyl sulfide, diallyl disulfide, diallyltrimethylsulfide, ajoene, S-allylcysteine (SAC) and S-allylmercaptocysteine (SAMC) [43] and these compounds can be a possible candidate that reacts with the SH group of cysteine residues. Recently, some of these compounds were shown to have the ability to induce the nuclear accumulation of Nrf2 and stimulate the production of antioxidant enzymes such as NQO1 and HO-1 [44-49]. The third possibility is that the activation mechanism of Nrf2-ARE pathway by AGE is mediated through some kinase systems [39]. In neuronal cells, the activation of phosphatidylinositol 3-kinase (PI3K)/Akt pathway is associated with the antioxidant system in cells [50,51]. Recently several compounds including carnosol and ginsenoside have been shown to induce the activation of the Nrf2-ARE pathway through the PI3K-Nrf2 system [50-53] or other systems [44,45,48].

Since AGE is known to contain a variety of sulfur compounds including SAC and SAMC [43], we examined the protective effect of those compounds against 6-OHDA (100 μM)-induced cytotoxicity in SH-SY5Y cells. As shown in Supplemental Figure 1, SAC showed the protective effect at doses ranging from 5 to 20 μg/ml. Since the concentration of SAC in 4 mg/ml of AGE is estimated to be approximately 2 μg/ml, it is possible that the protective effect of AGE may be partly due to SAC in combination with some other substances in AGE. On the other hand, SAC showed no protective effect on the cell viability even at the concentration as high as 100 μg/ml (data not shown). It is reported that both SAC and SAMC scavenge hydroxyl radical [11,54], but SAC is unable to capture superoxide anion radical [54]. On the other hand, we previously reported that SAC possesses the ability to scavenge superoxide anion radical [12]. If the main species of cytotoxic ROS produced by 6-OHDA is superoxide anion radical, the above results can be attributable to the action of SACM. Hara et al. showed that 6-OHDA-induced neurotoxicity is at least in part caused by superoxide [55], that is in line with our current view.

Garlic or its preparations have been reported to possess antioxidant activity [11,37,56-58]. In addition, antioxidant agents have been shown to modulate oxidative stress-induced changes in hypertension, atherosclerosis and diabetes [59,60]. Recently, Park et al. reported that the aged red garlic extract, another form of garlic extract preparation, reduced pulmonary inflammation through HO-1 induction [61]. Since some of those diseases are chronic, the long-term intake of antioxidant agents including garlic may be warranted for prevention and treatment of these diseases. However, it is not practical to use raw garlic because it has various side effects including anemia, weight loss and growth retardation [3,62]. AGE, on the other hand, can be tolerated for an extended period of intake without the side effect of raw garlic, and therefore could be a useful means of treating those diseases.

In conclusion, the present findings indicated that AGE protects cells from ROS-induced damage through both capturing ROS directly and stimulating antioxidant gene expression via the Nrf2-ARE pathway, and suggested that AGE may be useful for prevention and treatment of chronic diseases associated with ROS-induced cell injury.

5. Acknowledgements

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Supplemental Figure 1. Effect of SAMC on 6-OHDA-induced cytotoxicity. The experimental procedures are the same as described in the Figure 2 legend except that only 100 μM of 6-OHDA was used. The data are expressed as mean ± S.D. (n = 5 - 6). **Denotes a significant difference compared with the value of cell viability without SAMC (p < 0.01) as evaluated by t-test.