Resveratrol Oligomers from *Vitis amurensis* Attenuate β-Amyloid-Induced Oxidative Stress in PC12 Cells

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Oxidative damage induced by β-amyloid (Aβ) is closely associated with the hallmark pathologies of Alzheimer’s disease (AD) and may play a critical role in the development of AD. In this study, the protective effects of vitisin A and heyneanol A, resveratrol oligomers isolated from *Vitis amurensis* Rupr. (Vitaceae), against Aβ-induced oxidative cell death were investigated using rat pheochromocytoma (PC12) cells. Exposure of PC12 cells to the Aβ (20 μM) for 24 h resulted in neuronal cell death, whereas pretreatment with vitisin A or heyneanol A at the concentration range of 5—50 μM reduced Aβ-induced cell death. In addition, Aβ-induced elevation of reactive oxygen species generation, the primary cause of Aβ-induced oxidative stress, was attenuated by treatment of vitisin A or heyneanol A (10, 25, 50 μM). Aβ-treated cells also displayed characteristic features of apoptosis such as induction of DNA fragmentation and caspase-3 activation, but vitisin A and heyneanol A (10, 50 μM) significantly suppressed these events. These results suggest that vitisin A and heyneanol A prevent Aβ-induced neurotoxicity through attenuating oxidative stress induced by Aβ, and may be useful as potential preventive or therapeutic agents for AD.

**Key words** β-amyloid; Alzheimer’s disease; vitisin A; heyneanol A; resveratrol oligomer; *Vitis amurensis*; reactive oxygen species

Alzheimer’s disease (AD), a neurodegenerative disorder of the cortex and hippocampus, is associated with a selective loss of cholinergic neurons and characterized by cognitive and memory deficiencies. The presence of intracellular neurofibrillary tangles and extracellular amyloid deposits in the brain are the hallmarks of AD. The principal component of amyloid deposits found in the plaques is β-amyloid (Aβ).1,2) The progressive accumulation of Aβ peptide is widely believed to initiate the pathogenesis of AD and to trigger a cascade of events such as neurotoxicity, oxidative injury, and inflammatory response that contribute to the progression of AD.2,3)

There is mounting evidence that oxidative stress plays a primary role in the pathogenesis of AD. Aβ facilitates the generation of free radicals causing the peroxidation of membrane lipids, and the increased production of reactive oxygen species (ROS) results in cellular damage.3) Moreover, several studies have suggested that oxidative stress precedes Aβ deposition.3,4) This vicious cycle between Aβ deposition and free radical generation eventually leads to neuronal damage, cognitive dysfunction, and various behavioral and psychological symptoms in AD patients.6) Therefore, considerable efforts have been made to search for the antioxidants that reduce Aβ-induced oxidative stress in AD. Among them, much attention has been drawn to polyphenols that are natural substances obtained from plants, fruits, and vegetables.

*Vitis amurensis* Rupr. (Vitaceae), a wild-growing grape, is widely distributed in Japan, China, and Korea. Its fruits have been used as the raw materials for juice and wine in Korea, and its roots have been used as traditional medicine for the treatment of cancer and various pains. In recent studies, it has been reported that the roots of *V. amurensis* possess anti-inflammatory and anti-tumor activity and contain structurally diverse resveratrol oligomers.5,6) Resveratrol (trans-3,5,4’-trihydroxystilbene), a naturally occurring polyphenol mainly found in grapes and red wine, has been spotlighted as potential therapeutic agents for several pathological disease. Many studies demonstrated that resveratrol prevents the cognitive deficits as well as the oxidative stress in the treatment of neurodegenerative diseases such as AD.9,10) This representative nonflavonoid polyphenol has been purported to provide many beneficial effects, mainly attributable to its powerful antioxidant capability and its neuroprotective action is also principally related to this property.11,12) However, the neuroprotective effect of resveratrol oligomers has not been studied yet in spite of their diverse biological activities including antioxidative and anti-inflammatory activities.7,13) In a preliminary study (unpublished data), we found that the extract of the roots of *V. amurensis* protected rat pheochromocytoma (PC12) cells against Aβ-induced cytotoxicity. On the basis of these facts, it is expected that the resveratrol oligomers from *V. amurensis* may have potential as novel therapeutic agents for AD. In this study, we isolated vitisin A and heyneanol A from *V. amurensis* as active principles using a bioassay-linked fractionation and evaluated their protective effects against Aβ-induced neurotoxicity.

**MATERIALS AND METHODS**

**Isolation of Vitisin A and Heyneanol A** The roots of *V. amurensis* were purchased from herbal market in Korea and the voucher specimen is deposited in Herbarium of College of Pharmacy, Seoul National University. Dried plant material was refluxed with 70% methanol for 3 h and this methanolic extract was successively partitioned with CHCl₃ and BuOH. The BuOH fraction was found to have the protective effect on Aβ-induced cell damage in MTT assay (data not shown). Thus, the BuOH-soluble portion was chromatographed over a silica gel column (250 g, 7×40 cm; Merck, Art 9385, Germany) using a CHCl₃:MeOH (7:1) mobile phase, and five fractions were obtained. From the most active fraction 4, two major compounds were isolated using semi-preparative
HPLC method with CH₃CN–H₂O (30 : 70) mobile phase and ODS column (RP-18, 10 µm, 250×10 mm, Alltech, U.S.A.). Based on the spectral data, the chemical structures of the compounds were identified as vitisin A and heyneanol A, respectively (Fig. 1).

**Materials**

β-Amyloid peptide (Aβ₂₅₋₃₅), resveratrol, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and 2,7-dichlorofluoroscein diacetate (H₂DCF-DA) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). RPMI 1640 medium and fetal bovine serum were provided from Gibco BRL (Grand Island, NY, U.S.A.). Aβ₂₅₋₃₅ stock solution of 1 mM was prepared in deionized distilled water and stored at −20 °C. The stock solution was diluted to desired concentration immediately before use. Each test compound (vitisin A, heyneanol A and resveratrol) was dissolved in DMSO with the concentration of 50 mM and further diluted with PBS. The final concentration of DMSO was 0.1%, which did not affect cell viability.

**Cell Culture and Treatment**

PC12 cells obtained from the Korean Cell Bank Line (KCLB, Seoul, Korea) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 units/ml of penicillin G (Gibco BRL Life Technologies) and 50 µg/ml streptomycin at 37 °C in a humidified 95% air/5% CO₂ incubator. For assays except for caspase-3 activation, the cells were plated at a density of 2×10⁵ cells/well in 96-well plates and incubated for 24 h. To assess caspase-3 activation, PC12 cells were plated at a density of 10⁴ cells/culture dish (60 mm Ø). At the end of incubation period, cells were pretreated with test compound and incubated for 1 h. After the pretreatment period, Aβ₂₅₋₃₅ solution (final concentration, 20 µM) was added to culture medium and incubated for 24 h. Controls were not treated with test compound and Aβ₂₅₋₃₅.

**Determination of Cell Viability**

Cell viability was measured by quantitative colorimetric assay with MTT. Briefly, after treatment for 24 h, MTT solution (final concentration, 500 µg/ml) was added and cells were incubated at 37 °C for 4 h. Supernatants were then aspirated off and formazan crystals were dissolved with DMSO. Absorbance at 560 nm was measured with a microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

**Measurement of Intracellular ROS**

The intracellular ROS production was measured using a non-fluorescent H₂DCF-DA by the method of Wang and Joseph (16) with slight modification. Cells were incubated with 5 µM H₂DCF-DA for the last 30 min of the incubation with Aβ₂₅₋₃₅. Then cells were washed with PBS and the relative levels of fluorescence were quantified using a fluorescence spectrophotometer (TECAN, Switzerland) with an excitation wavelength at 485 nm and an emission wavelength at 535 nm.

**Quantification of DNA Fragmentation**

DNA fragmentation was quantified using the Cell Death Detection ELISA plus kit (Roche Molecular Biochemicals, Germany). After 24 h incubation with Aβ₂₅₋₃₅ and/or test compound, the cells were lysed for 30 min at room temperature. Lysate was centrifuged at 200 g for 10 min and the supernatant 20 µl was transferred into the streptavidin-coated plate. Eighty microliters of freshly prepared immunoreagent was added to each well and incubated for 15 min until the color development is sufficient for photometric analysis. The absorbance at 405 nm (reference wavelength 490 nm) was measured using a microplate reader. DNA fragmentation was expressed as the enrichment factor using the following equation:

\[
\text{enrichment factor} = \frac{\text{absorbance of the sample}}{\text{absorbance of the control}}
\]

sample: cells treated with Aβ or/and test compound,
control: cells without Aβ treatment

**Measurement of Caspase-3 Activation**

Caspase-3 activity was measured with the fluorescence-based caspase-3 assay kit (Molecular probe, Eugene, OR, U.S.A.), in accordance with the protocol supplied by the manufacturer. The substrate for the assay is rhodamine 110 bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD-R110). After treatment with Aβ₂₅₋₃₅ in the presence or absence of test compound, the cells were harvested by centrifugation. The pellets were washed and lysed in 50 µl lysis buffer and subjected to a freeze-thaw cycle. The lysate was

*Fig. 1. The Chemical Structures of Resveratrol Oligomers Isolated from V. amurensis and Resveratrol*
Table 1. Protective Effects of Vitisin A, Heyneanol A and Resveratrol on Aβ-Induced Cell Death

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Vitisin A</th>
<th>Heyneanol A</th>
<th>Resveratrol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>60.2±2.2</td>
<td>60.2±2.2</td>
<td>60.2±2.2</td>
</tr>
<tr>
<td>5</td>
<td>67.7±1.0*</td>
<td>64.2±0.5*</td>
<td>62.9±0.5</td>
</tr>
<tr>
<td>10</td>
<td>76.8±1.9*</td>
<td>71.4±1.2*</td>
<td>68.6±1.4*</td>
</tr>
<tr>
<td>25</td>
<td>87.1±2.0*</td>
<td>77.7±2.2*</td>
<td>74.2±0.6*</td>
</tr>
<tr>
<td>50</td>
<td>95.3±2.3*</td>
<td>84.6±2.1*</td>
<td>79.1±2.9*</td>
</tr>
</tbody>
</table>

PC12 cells were treated with 20 μM Aβ in the absence or presence of test compounds at various concentrations for 24 h. Cell viability was measured using MTT reduction assay. Values are means±S.E. (n=3). * Significantly different from the group treated with Aβ alone (p<0.05).

RESULTS

Protective Effects of Vitisin A and Heyneanol A on Aβ-Induced Cell Death The cell viability was assessed by MTT reduction. Incubation of PC12 cells with Aβ (20 μM) for 24 h decreased the cell viability to 60% relative to control. Pretreatment of the cells with the test compound (vitisin A, heyneanol A and resveratrol) substantially reduced the cell death caused by Aβ in a concentration-dependent manner (Table 1). Vitisin A showed the strongest inhibitory activity on the Aβ-induced cell death and the next were in the decreasing order of heyneanol A and resveratrol. Significant protective effects were achieved starting from 10 μM (for resveratrol) and 5 μM (for vitisin A and heyneanol A) concentrations. At the corresponding concentration, heyneanol A increased the cell survival to a comparable extent as did the same concentration of resveratrol. However, the protective effect of vitisin A against Aβ-induced neurotoxicity significantly increased compared with that of resveratrol. With the concentration of 50 μM, viability of cells treated with resveratrol and vitisin A was 79% and 95%, respectively. Vitisin A or heyneanol A alone did not affect viability in untreated cells even with the concentration of 200 μM, but resveratrol showed cytotoxicity with the concentration of more than 100 μM (data not shown).

Effects of Vitisin A and Heyneanol A on Aβ-Induced ROS Accumulation The ROS level after Aβ exposure was measured by DCF fluorescence. Cells treated with 20 μM Aβ showed a significant increase (about three-fold) of intracellular ROS level compared with untreated cells (Fig. 2). The enhancement of ROS generation by Aβ was reduced by treatment with vitisin A or heyneanol A. At 50 μM, vitisin A and heyneanol A caused a 144% and a 94% reduction in ROS production induced by Aβ, respectively.

Effects of Vitisin A and Heyneanol A on Aβ-Induced DNA Fragmentation Figure 3 shows the enrichment factor of nucleosomes in the cytoplasm of cells treated with Aβ. Enrichment factor of cells treated with Aβ increased approximately 1.8-fold compared with untreated cells, which indicated the generation of mono- and oligonucleosomes in the cytoplasm of the apoptotic cells. However, treatment of PC12 cells with either vitisin A or heyneanol A significantly blocked DNA fragmentation in a dose-dependent manner. The enrichment factor was decreased to 1.17 and 1.31, by 50 μM of vitisin A and heyneanol, respectively.

Effects of Vitisin A and Heyneanol A on Caspase-3 Activation in Aβ-Treated PC12 Cells As shown in Fig. 4, Aβ treatment caused an increase of caspase-3 activity. There was about 2.1-fold induction of caspase-3 activity in cells treated with Aβ (compared with the control group), while pretreatment of vitisin A or heyneanol A resulted in a significant inhibition of this effect. The inhibitory effect of vitisin A on caspase-3 activity was stronger than that of heyneanol A. In particular, at the concentration of 50 μM, vitisin A and heyneanol A reduced the caspase-3 activity to 116% and...
that Aβ model of AD. Consistent with these findings, we confirmed that Aβ-induced apoptosis is mediated by free radicals.

Trials for the treatment of AD that target these biological mechanisms are currently being explored. In this study, Aβ caused a decrease in MTT reduction in PC12 cells, which was partly restored by addition of test compound. In protecting cells against Aβ toxicity, heyneanol A and resveratrol showed similar potency, while vitisin A was stronger than resveratrol. In particular, vitisin A and heyneanol A could retain efficiency in cells due to greater lipophilicity compared with resveratrol, and this may contribute the stronger activity of vitisin A and heyneanol A than resveratrol. These abilities of vitisin A and heyneanol A to reduce Aβ toxicity suggest that they may be beneficial for the treatment of AD. In light of this, the protective effects of vitisin A and heyneanol A against Aβ-induced neurotoxicity were evaluated on the aspects of oxidative stress.

Accumulating data from experimental and human studies indicate that oxidative stress is an important causative factor in the development and progression of AD.21,22) Pappolla et al.23) provided evidence that Aβ-induced neurotoxicity is mediated by free radicals in vitro and in a transgenic mouse model of AD. Consistent with these findings, we confirmed that Aβ stimulates ROS production and it is closely associated with Aβ-mediated neurotoxicity. As shown in Fig. 2, elevated ROS generation by Aβ was decreased in response to treatment of vitisin A or heyneanol A. From our results and those found in the literature, it can be explained that cytoprotective effects of vitisin A and heyneanol A may be attributed, at least in part, to their antioxidant properties. Moreover, these results provide evidence that vitisin A and heyneanol A could effectively prevent oxidative stress induced by Aβ.

Recently, many researches focus on the relationship between apoptosis and oxidative stress in AD. Aβ-induced ROS accumulation causes damage to neuronal membrane lipids, proteins and nucleic acids, and ultimately leads to apoptosis which is believed to play a critical role in cell loss during progression of AD.23) Some of the classical features of Aβ-induced apoptosis such as decreased cell viability, DNA condensation, and DNA fragmentation were detected in cultured hippocampal neurons.24) Another feature of apoptosis in AD is the activation of caspase-3. Harada and Sugimoto25) reported that Aβ-induced apoptosis in cultured rat cortical neurons accompanies caspase-3 activation, and the activated caspase-3 plays a role in the Aβ-induced apoptotic events. In line with these reports, the characteristic features of apoptosis such as DNA fragmentation and caspase-3 activation were seen in cells treated with Aβ in this study. These apoptotic features were attenuated by addition of vitisin A or heyneanol A, suggesting that these compounds exert protective effects against Aβ-induced cell death by affecting the execution phase of apoptosis.

Though both vitisin A and heyneanol A are resveratrol tetromers, their structures are quite different. The most prominent difference in their structures is the number of hydroxyl groups. Vitisin A has one more hydroxyl group compared with heyneanol A and the difference may have influence, among other factors, on their antioxidant activities. Because some antioxidants are known to attenuate Aβ-induced oxidative injury, the antioxidant properties of vitisin A and heyneanol A may also contribute to their protective effects.

In summary, the present study demonstrated that Aβ is capable of promoting PC12 cell apoptosis through an oxidative mechanism. However, vitisin A and heyneanol A reduced the Aβ-induced oxidative damage by inhibiting ROS production and eventually inhibited apoptotic cell death. Our findings suggest that vitisin A and heyneanol A from V. amurensis exhibit neuroprotective effects by attenuation of the biochemical alteration induced by Aβ and these compounds might be responsible for neuroprotection of V. amurensis. On these grounds, V. amurensis and its resveratrol oligomers may be promising candidates in the novel neuroprotective strategies for AD.

**REFERENCES**