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Protective effect of ε-viniferin on β-amyloid peptide aggregation investigated by electrospray ionization mass spectrometry

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ABSTRACT

Abnormal β-amyloid peptide accumulation and aggregation is considered to be responsible for the formation and cerebral deposition of senile plaques in the brains of patients with Alzheimer's disease (AD). Inhibition of the formation of β-amyloid (Aβ) fibrils would be an attractive therapeutic target for the treatment of AD. Resveratrol and its derivatives exhibit a broad range of pharmacological properties such as protection against cardiovascular diseases and cancers, as well as promoting antiaging effects. We reported previously that ε-viniferin glucoside (VG), a resveratrol-derived dimer, strongly inhibits Aβ (25-35) fibril formation in vitro. In this study, we investigated the effects of VG on the aggregation of the full-length peptides (Aβ (1-40) and Aβ (1-42)) and on the β-amyloid-induced toxicity in PC12 cells. VG inhibited Aβ cytotoxicity and the non-covalent complex between VG and Aβ was observed by electrospray ionization mass spectrometry.

1. Introduction

Alzheimer's disease (AD), a progressive neurodegenerative disease of the elderly[1], is characterized by the abundance of protein deposits in neurons that trigger neuronal degeneration. These deposits result in the intracellular accumulation of phosphorylated tau protein (Pτ) and extracellular accumulation of amyloid β-peptides (Aβ).[2] Pτ and Aβ accumulation leads to the deposition of neurofibrillary tangles and amyloid plaques, respectively, which promote pro-inflammatory responses and activate the neurotoxic pathway, leading to the dysfunction and death of brain cells.[3,4]

Despite recent progress in symptomatic therapy, an effective therapeutic approach that interferes directly with the neurodegenerative process in AD, especially the accumulation of Aβ, is still unavailable.[5] Recently, numerous studies have suggested that a wide range of polyphenols may have neuroprotective effects both in vitro and in vivo.[6–15] Among these polyphenols, resveratrol and particularly its derivatives have demonstrated promising neurodegenerative activities.[8–11,13–15] Some of these studies suggest that polyphenols exert protective effects through their ability to scavenge reactive oxygen species.[6–10] Furthermore, other reports indicate that polyphenols could prevent the development of AD, not only by scavenging reactive oxygen species but also by directly inhibiting the formation of Aβ fibril deposits in the brain.[12–15] Indeed, pathologic and biochemical studies suggest that fibrillar Aβ are reactive oxygen species generators, whereas monomeric (soluble) Aβ acts as a natural antioxidant that prevents the neuronal cell death due to oxidative stress.[16–18] Polyphenols are capable of inhibiting fibril formation in vitro by forming a complex with Aβ. This mechanism, which is unrelated to oxidative conditions, is highly relevant for designing future inhibitors as therapeutic agents for the treatment of AD.

Nevertheless, the exact mechanisms of anti-amyloidogenic activities are unclear. Mass spectrometry allows the monitoring of non-covalent complexes between polyphenols and proteins.[19–22] For example, a complex between Aβ and oleuropein could be observed by means of electrospray ionization mass spectrometry (ESI–MS).[21,22]

In a previous article, we reported the effect of resveratrol and its oligomers against Aβ (25-35) fibril formation.[14] Among the compounds tested, ε-viniferin glucoside (VG) exhibited the strongest inhibitory activity of Aβ (25-35) aggregation in vitro. From these results, the inhibitory properties of VG on both Aβ (1-40) and Aβ (1-42) aggregation were verified. Second, the protective effects of VG from Aβ (25-35), Aβ (1-40) and Aβ (1-42) toxicity of VG on PC12 cells was investigated. Third, in order to better understand the formation mechanism of the Aβ complex, the non-covalent complex between VG and Aβ (1-40) was monitored by electrospray ionization mass spectrometry.

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2. Results and discussion

2.1. VG effects on Aβ-induced aggregation and toxicity

Using an original routine in vitro assay with UV–visible measurements, we recently reported that stilbenoids, which are resveratrol-type compounds, inhibit the aggregation of the Aβ (25-35) peptide.[13,14] In this previous work, we used for practical reasons the Aβ (25-35) fragment, which retains the properties of neurotoxicity and aggregation of the entire peptide.[24,25] Among all the compounds tested, the dimeric stilbenoid VG (Fig. 1) exhibited an extremely efficient inhibition of Aβ (25-35) aggregation. Strong inhibition of Aβ fibril formation by VG led to a very low IC_{50} of less than 1 μM.[14] Since the full-length Aβ (1-40) and Aβ (1-42) peptides are more biologically relevant, we used the same methodology to test the inhibitory properties of VG against full-length peptide Aβ fibrilization (Table 1). VG inhibited the fibrillization of both peptides with the same order of magnitude as Aβ (25-35).

To correlate the fibrillization inhibition properties of VG with the protective effect against Aβ toxicity, the polyphenol-Aβ incubates were dosed onto PC12 cells. These cells were used as a suitable model for studying neurotoxic agents like stilbenoids.[10] The results of the MTT viability assay are shown in Figure 2, where the active fragment Aβ (25-35) and both full-length peptides were tested. VG alone did not significantly affect the viability in the untreated cells, with the maximum concentration of 10 μM, and did not affect MTT reduction alone (result not shown). As previously reported by Jang et al.[10], submicromolar concentrations of both Aβ peptides were sufficient to induce metabolic interruptions in PC12 cells. Incubation of PC12 cells with 10 μM Aβ (25-35), Aβ (1-40), and Aβ (1-42) for 24 h decreased the cell viability compared to control to 33%, 30%, and 46%, respectively (Fig. 2). VG had a strong protective effect against cell death induced by both of the Aβ peptides. In the presence of 10 μM VG, the viability of cells treated with Aβ (25-35), Aβ (1-40), and Aβ (1-42) for 24 h was 80%, 79%, and 87%, respectively. The data do not show a significant difference between cells treated by Aβ (25-35) or full-length peptides and VG. Thus, VG protected PC12 cells from Aβ-induced toxicity.

Much attention has focused on the relationship between apoptosis and oxidative stress in AD. Concerning stilbenoids, Bastianetto et al. showed that red wine polyphenols such as resveratrol are capable of both protecting and rescuing cultured rat hippocampal cells against nitric oxide-induced toxicity.[8] Jang et al. suggested that resveratrol oligomers such as vitisin A prevent Aβ-induced neurotoxicity by attenuating the oxidative 'stress' induced by Aβ in PC12 cell cultures.[11] In our study, VG inhibited Aβ fibril formation from fresh Aβ. Moreover, cell culture experiments with PC12 cells showed that VG reduced Aβ-induced damage. Thus it might be reasonable to speculate that this polyphenol could prevent the

Table 1
Inhibition of Aβ (25-35), Aβ (1-40) and Aβ (1-42) fibril formation

Peptide	Inhibition (%)			
	Resveratrol		ε-viniferin glucoside	
	5 μM	10 μM	5 μM	10 μM
Aβ (25-35)	–	63 ± 8	–	93 ± 3
Aβ (1-40)	22 ± 7	52 ± 10	59 ± 7	97 ± 13
Aβ (1-42)	–	61 ± 16	70 ± 2	89 ± 9

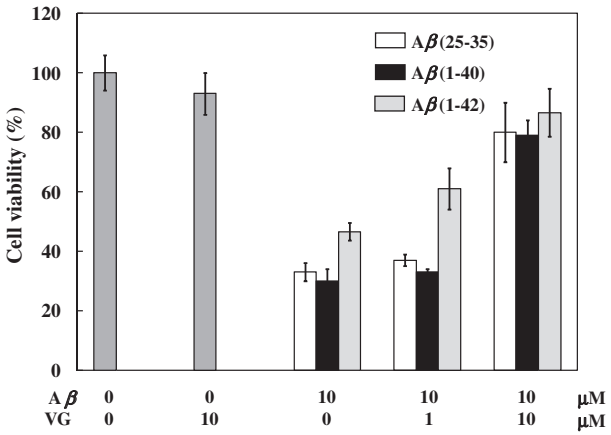

Figure 2. Protective effects of ε-viniferin glucoside on Aβ (25-35)-, Aβ (1-40)- and Aβ (1-42)- induced cell death. The experiment was repeated three times.

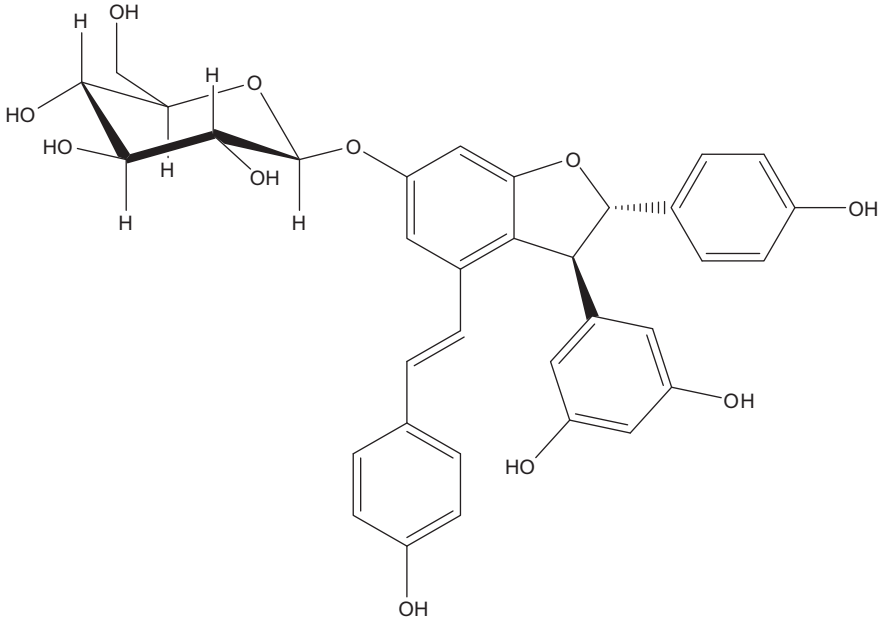

Figure 1. Molecular structure of ε-viniferin glucoside (VG).

development of AD, not only by scavenging reactive oxygen species but also by directly inhibiting the deposition of Aβ fibrils in the brain by complex formation.

2.2. VG / Ab complex formation

However, the exact mechanisms of the anti-amyloidogenic activity of polyphenols are unclear. To investigate the mechanism of the anti-amyloidogenic activity of VG, ESI–MS was used to observe these non-covalent biomolecular complexes with the full-length peptide Aβ (1-40).

ESI mass spectrometric analysis of a Aβ (1-40) solution revealed a multiply charged ion envelope including signals at *m/z* 619.5 $[M+7H]^{7+}$, 722.4 $[M+6H]^{6+}$, 866.7 $[M+5H]^{5+}$, 1083.0 $[M+4H]^{4+}$ and 1443.5 $[M+3H]^{3+}$ in positive mode as previously observed,[21] and signals at *m/z* 720.3 $[M-6H]^{6-}$, 864.7 $[M-5H]^{5-}$, 1081.0 $[M-4H]^{4-}$ and 1441.2 $[M-3H]^{3-}$ in negative mode (Fig. 3a). ESI–MS analysis of VG gave an abundant MH^+ ion at *m/z* 617.0 and a weaker potassium adduct $[M+K]^+$ at *m/z* 655.0 in positive mode and a MH^- ion at *m/z* 615.1 in negative mode (Fig. 3b).

Since the prevalence of non-specific aggregation in the gas phase may be avoided by reducing the concentration of the interacting species in solution, the interactions were monitored at a relatively low concentration of 10 μM.[21] The ESI–MS of the Aβ (1-40) solution containing VG was studied by using solvent mixtures consisting of methanol/water and acetonitrile/water, since the importance of the solvent mixture in observing these complexes has been previously demonstrated.[21] The VG/Aβ complex was observed in both solvent mixtures methanol/water and acetonitrile/water, even when the organic solvent reached over 50%. Figure 3c presents the ESI–MS spectrum in negative mode of the Aβ solution containing VG (molar ratio 1:1, concentration 10 μM) using the solvent mixture methanol/water (35% methanol). Whereas the spectrum still exhibited ions due to the deprotonated peptide and VG alone, deprotoned VG/Aβ molecular complexes were clearly detected. Thus, the relatively intense peak at *m/z* 695.0 was attributed to the 2/1 complex $[Aβ+2VG-8H]^{8-}$ and the weaker peak at *m/z* 792.7 was attributed to the 2/1 complex $[Aβ+2VG-7H]^{7-}$. The VG/Aβ molar ratio excess of 5/1 provided identical results, confirming the specificity of the interaction and not any artifacts of the ESI process. Finally, experiments were performed with *p*-coumaric acid as negative control since a very weak inhibitory property on Aβ aggregation has already been shown.[26] *p*-coumaric acid gave an MH^+ ion at *m/z* 165.2 and a potassium adduct $[M+K]^+$ at *m/z* 203.1 in positive mode and a MH^- ion at *m/z* 163.1 in negative. The ESI–MS of the Aβ (1-40) solution containing *p*-coumaric acid (molar ratio 1:1, concentration 10 μM) was studied by using solvent mixtures methanol/water (35% methanol). No ion derived from the complex *p*-coumaric acid with Aβ could be detected.

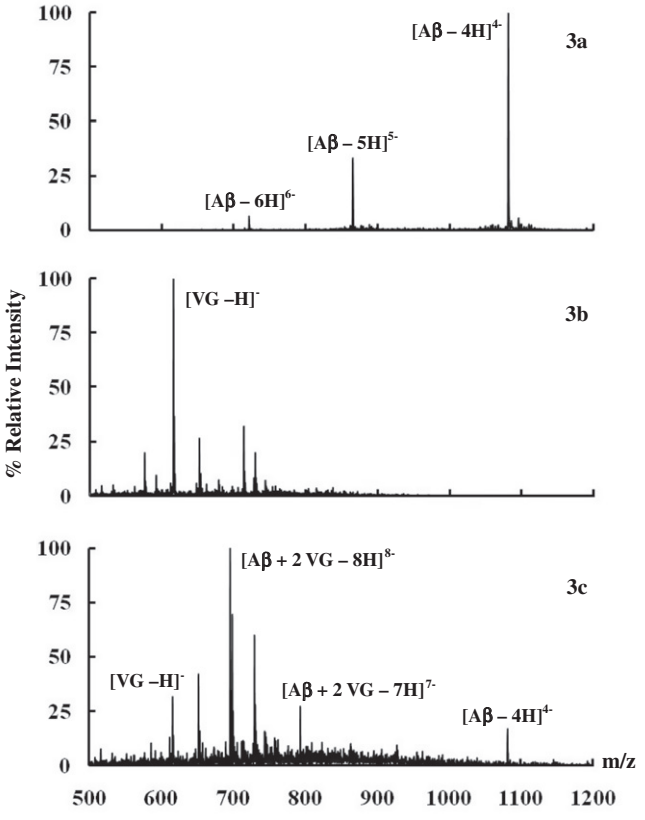

Figure 3. Electrospray ionization mass spectra of (**3a**) amyloid-β peptide Aβ (1-40) at 10 μM; (**3b**) ε-viniferin glucoside and (**3c**) the mixture of Aβ (1-40) and VG (1:1 molar ratio, 10 μM) in 35 % methanol.

Bazoti et al. studied the non-covalent interaction between Aβ (1-40) and oleuropein, a potent bioactive polyphenol, against AD.[21,22] They showed that Aβ (1-40) interacts with this polyphenol with 1/1 and 2 oleuropein/1 Aβ stochiometries, with the 1/1 non-covalent complex being the most important. In our study, we observed only the 2 VG/1 Aβ complex, the 1/1 complex not being detected at an intensity greater than the background. Thus, the specificity and the binding energies of the interaction between oleuropein/Aβ and VG/Aβ complexes could be quite different. However, these results confirm our previous hypothesis that the full-length peptide contains multiple binding sites within the peptide sequence.[26] Moreover, our data indicate that VG interacts with Aβ at an early stage in the polymerization process. The binding may lock the peptide conformation and thus prevent nucleation. Preliminary NMR experiments are in agreement with ESI–MS results with the observation between Aβ (1-40) and two VG molecules at the N and C termini of the peptide.[27] New experiments using NMR spectrometry will be conducted to extend the molecular knowledge of this interaction.

In conclusion, VG inhibits Aβ (25-35), Aβ (1-40) and Aβ (1-42) fibrillization in vitro and protects PC12 cells against Aβ-induced injuries. Apart from potential antioxidant activities, the inhibitory properties of VG on Aβ peptide aggregation need to be considered. This study confirms the utility of mass spectrometry for studying the non-covalent complexes between Aβ and polyphenols. Aβ (1-40) and VG interact non-covalently at 2 VG/1 Aβ stoichiometry, indicating that polyphenols could act on Aβ polymerization in the preliminary stages of aggregation. Therefore, VG may be a promising candidate for novel neuroprotective strategies for treating AD. Nevertheless, many studies have shown wide variability in the bioavailability and bioefficacy of the polyphenols.[28] Future studies evaluating both their beneficial and adverse effects in vivo need to be performed before the development of fortified foods or supplements containing pharmacologic doses of these compounds.

3. Material and methods

3.1. Material

VG was extracted from stem of Vitis vinifera using the protocol described by Delaunay et al.[23] Briefly, stilbenoid extracts were fractionated by column chromatography followed by centrifugal partition chromatography. VG was obtained by semipreparative high-performance liquid chromatography. Its structure was elucidated by one- and two-dimensional NMR analysis. Polyphenol

purity was controlled by HPLC–UV and HPLC–UV-ESI-MS spectroscopy.

*A*β (25-35), Aβ (1-40) and Aβ (1-42) peptides were purchased from Bachem (Torrance, USA) and EZBiolab Inc. (Carmel, USA), respectively. Peptides were used without further purification.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] horse serum and fetal bovine serum and *p*-coumaric acid were purchased from Sigma–Aldrich (St. Louis, USA). Dulbecco's modified Eagle's Medium (DMEM+GlutaMAX-1) was purchased from Invitrogen Ltd (Paisley, UK).

3.2. Antiaggregation assay

The detailed method for measuring the inhibitory activity on Aβ aggregation was given in a previous report.[13] Briefly, UV–visible measurements were used to search for inhibitors of Aβ fibril formation. Stock solutions of Aβ (25-35), Aβ (1-40) and Aβ (1-42) at 1 mM were prepared by solubilizing the lyophilized Aβ by briefly vortexing in sterile water at 4 °C, then by sonicating for 10 min. Polyphenols were solubilized in MeOH solution to a concentration of 1 mg/mL. Stock solutions were aliquoted and stored at −20 °C.

UV–visible spectrometry was performed on a Cary 300 bio UV–visible spectrophotometer (Varian, USA). To study Aβ fibril inhibition, experiments were carried out by using a reaction mixture containing 80 μL phosphate buffer (10 mM final concentration), 10 μL Aβ (25-35) (100 μM final concentration) or 10 μL full-length peptide (50 μM final concentration) and 10 μL polyphenol (5 and 10 μM final concentration), pH 7.2.

3.3. Cell culture and MTT assay

PC12 cells were obtained from the American Type Culture Collection (ATCC, Manassas, USA). PC12 cells were maintained routinely in DMEM-Glutamax supplemented with 15% heat-inactivated horse serum, 2.5% fetal bovine serum, and 1% penicillin/streptomycin antibiotics at 37 °C in humidified atmosphere of 10% CO_2/90% air. All cells were cultured in poly-D-lysine-coated culture dishes.

Cells were harvested from flasks and plated at a density of 10,000 cells per well in 96-well plates and incubated at 37 °C for 24 h. Aβ (25-35) Aβ (1-40) or Aβ (1-42) preincubated with or without VG at 37 °C for 48 h was diluted with fresh DMEM-Glutamax and added to individual wells. The final concentration of Aβ was 10 μM. After 24 h of incubation, cell viability was determined by the conventional MTT reduction assay. Cells were treated with MTT solution (final concentration, 0.5 mg/ml DMEM-Glutamax) for 3 h at 37 °C. The dark blue formazan crystals formed in intact cells were solubilized with dimethylsulfoxide for 0.5 h. The absorbance was measured at 595 nm with a microplate reader (Dynex, USA). Results were expressed as the percentage of MTT reduction in relation to the absorbance of control cells at 100%.

3.4. Mass spectrometry

A direct infusion-electrospray ionization-mass spectrometry (ESI–MS) Esquire 3000^{plus} Ion Trap mass spectrometer (Bruker Daltonics, Germany) was used to study the non-covalent interactions between VG and Aβ (1-40). Sample solutions were infused directly into ESI source with a syringe pump (74900 Series, Cole-Parmer Instrument) at a constant flow-rate of 180 μL/h. Mass spectra were recorded from m/z = 150–2000 in a positive and negative ionization mode. Normal scan resolution (13,000 m/z s^{-1}) was selected. The source parameters were: +4000 V for negative ion mode and −4000 V for positive ion mode; nebulizer gas (N_2), 10 psi; drying gas (N_2), 7 L/min; dry temperature, 300 °C.

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