学 位 論 文 の 要 旨

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学　位　論　文　名　A Carboxylated Zn-Phthalocyanine Inhibits Fibril Formation of Alzheimer's Amyloid β Peptide

発　表　雑　誌　名　FEBS Journal
（巻，初頁～終頁，年）　(**282**: 463-476, 2015)

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論 文 内 容 の 要 旨

INTRODUCTION

Alzheimer's disease (AD) is a common dementia disease of the elderly. Evidences suggest that Aβ peptide has a critical role in AD pathogenesis. For example, gene mutations causing Aβ burden in brains are found to be related to AD. Increased Aβ leads to aggregation, affording oligomers or polymeric fibrils. Aggregated species, such as oligomers, are more neurotoxic than monomers, suggesting that Aβ aggregation process in brains also have key roles in the disease pathogenesis by increasing neurodegeneration. Hence, Aβ accumulation and aggregation processes might be good targets for diagnosis and therapy of AD. Several compounds having anti-amyloid activity *in vitro* or in animal models are already in clinical trials. However, an effective disease-modifying therapy remains elusive.

Diagnosis is also an important issue. Aβ in CSF or deposited in brain parenchyma could be a diagnostic marker. Deposited Aβ can be visualized by PET imaging with high-affinity compounds, but the equipment is expensive and not widely available. Conversely, near-infrared (NIR) spectroscopy is technically favorable for in vivo imaging due to its optical window from approximately 600 to 1000 nm, where the absorption coefficient of tissue is at a minimum, resulting in a low background. Recently, it was reported that phthalocyanines (Pcs), which are metal-containing, aromatic, macrocyclic NIR fluorophores, interact with α-synuclein and affect fibril formation. Further, Fe-containing Pc interacts with toxic $A\beta_{1\text{-}40}$ oligomers and converts them to an amyloid fibril meshwork. Thus, we hypothesized that Pcs might bind to Aβ and serve as amyloid fibril-modifying agents. For such property, they might be used for AD therapy, also as amyloid-specific NIR imaging probes to visualize deposited Aβ.

Most Pc species are hydrophobic, and tend to aggregate in aqueous medium, resulting a

self-quenching effect on their excited state. Therefore, we prepared water-soluble Zn-containing Pcs (ZnPcs) bearing sodium carboxylate groups as candidate for amyloid fibril-modifying agents or *in vivo* NIR probes, and investigated their effects on Aβ during fibril formation process.

MATERIALS AND METHODS

In this study, 4 types of Pcs were used. $ZnPc(COONa)_8$ and $ZnPc(COONa)_{16}$ were dissolved in H_2O. $ZnPc(COOC_5H_{11})_8$ and PdPc dimer were dissolved in chloroform. These Pcs were prepared following described protocols. To check their effects on Aβ fibril formation, Aβ monomer was added to a fibril-forming buffer containing various concentrations of Pcs, and incubated for the indicated times at 37°C. The amount of fibrils in a sample was determined by ThT fluorescence assay using a spectrofluorimeter. To determine the effects of Pcs on the stability of Aβ fibrils, first fibrils were prepared, then $ZnPc(COONa)_8$ was added to Aβ fibrils, and further incubated for 24 h. Then fibril levels were determined by ThT assay. Morphological analysis of Aβ fibrils was done by electron microscopy.

To understand the underlying mechanisms, we checked the binding of $ZnPc(COONa)_8$ with Aβ. $ZnPc(COONa)_8$ was incubated with Aβ. Then Aβ was immunoprecipitated with Aβ-specific monoclonal antibody, and $ZnPc(COONa)_8$ in the immunoprecipitate was detected by NIR scanning. The changes in hydrophobicity during Aβ fibril formation were determined using 8-anilino-1-naphthalenesulfonic acid (ANS). ANS binds to exposed hydrophobic amino acids causing an increase in fluorescence with a blue shift. After incubation, ANS was added to the samples, and fluorescence intensity was measured with excitation at 360 nm, and emission was scanned from 380 to 600 nm. To check different Aβ aggregated species in a sample, the sample was separated by SDS PAGE using 4–20% gradient tris-glycine gel in a non-reducing condition, and stained with Coomassie Blue. Moreover, high molecular weight aggregated oligomeric species of Aβ was analyzed by dot blot immunoassay. After fibril formation, the samples were spotted on a nitrocellulose membrane, and the oligomers on the membrane were detected by oligomer-specific antibody.

The changes in the secondary structures were evaluated by analyzing circular dichroism (CD) spectra, acquired in the range of 190 – 250 nm, and expressed as mean residue molar ellipticity. The percentage of secondary structures were estimated using a computer program.

Finally, the effects of $ZnPc(COONa)_8$ on Aβ-induced neurotoxicity was evaluated using a neuronal cell (A1) culture. A1 cells were cultured on wells of a 96-well plate for 48 h. The cells were treated with $ZnPc(COONa)_8$ or $A\beta_{1\text{-}42}$, alone or in combination for 48 h. The viability of A1 cells was determined by MTT assay following established assay protocol.

RESULTS AND DISCUSSION

Effects of Pcs on $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ fibril formation. $A\beta_{1\text{-}40}$ (50 μM) or $A\beta_{1\text{-}42}$ (12.5 μM) was each incubated with increasing concentrations of $ZnPc(COONa)_8$, $ZnPc(COONa)_{16}$,

$ZnPc(COOC_5H_{11})_8$ and PdPc dimer for 48 h or 24 h. Measurement of fibrils by ThT fluorescence assay revealed that $ZnPc(COONa)_8$ dose-dependently inhibited fibril formation of both $A\beta_{1-40}$ and $A\beta_{1-42}$. $ZnPc(COONa)_{16}$ had similar but modest inhibitory effects. PdPc dimer inhibited, but $ZnPc(COOC_5H_{11})_8$ increased $A\beta_{1-40}$ fibril formation. Kinetics of Aβ fibril formation showed that $ZnPc(COONa)_8$ extended the lag time of fibril formation. However, it did not affect the overall morphology of the fibrils, as revealed by electron microscopic analysis. Moreover, our fibril stability experiments showed that it increased the breakdown of Aβ fibrils. These results are suggesting that $ZnPc(COONa)_8$ effectively interacted with Aβ and inhibited the fibril formation.

Possible mechanism of $ZnPc(COONa)_8$-mediated inhibition of Aβ fibril formation. Pcs are reported to produce singlet oxygen ($^1O_2$), which might be the cause of $ZnPc(COONa)_8$-induced inhibition. But our experiments with NaN_3 ($^1O_2$ scavenger) showed that $^1O_2$ had no role in the inhibitory effects. Next, we checked whether $ZnPc(COONa)_8$ directly binds to Aβ. After incubation of $A\beta_{1-42}$ monomers or pre-formed fibrils with $ZnPc(COONa)_8$ for 24 h in a fibril-forming environment, $A\beta_{1-42}$ was immunoprecipitated with Aβ-specific antibody, and NIR scanning was done for $ZnPc(COONa)_8$. We found ZnPc in the immunoprecipitate, suggesting its binding with the peptide. Such binding might affect the fibril formation process.

Hydrophobic amino acids in Aβ peptide as well as a hydrophobic microenvironment play a key role in fibril formation. To further explore the mechanism, we checked whether binding of $ZnPc(COONa)_8$ alters hydrophobic microenvironment using a hydrophobic fluorescent probe (ANS). The result showed that $ZnPc(COONa)_8$ decreased hydrophobicity of $A\beta_{1-42}$ during fibril formation process. As hydrophobic interaction is important in secondary structure formation of Aβ, we investigated the effects of $ZnPc(COONa)_8$ on such process by CD spectral analysis. The results showed that $ZnPc(COONa)_8$ increased α helix, and decreased β sheet structures of $A\beta_{1-40}$ during fibril formation process. As a result, low molecular weight species was increased and high molecular weight oligomeric species of Aβ was decreased, as shown by SDS PAGE, and dot blot immunoassay for oligomers, respectively.

$ZnPc(COONa)_8$ on $A\beta_{1-42}$-induced cytotoxicity. Oligomers of Aβ peptides are considered to be more cytotoxic than the monomers. As $ZnPc(COONa)_8$ inhibited oligomer formation, we explored whether it affected $A\beta_{1-42}$-mediated cytotoxicity to a neuronal cell line (A1). Both morphological analysis and MTT cell viability assay showed that $ZnPc(COONa)_8$ had no cytotoxic effect on A1 cells, rather it protected the cells from $A\beta_{1-42}$-mediated cytotoxicity.

CONCLUSION

In conclusion, our results indicate that water-soluble $ZnPc(COONa)_8$ binds to Aβ peptides and inhibits the oligomerization and fibril formation processes. It also destabilizes pre-formed fibrils. We consider that $ZnPc(COONa)_8$ has potential value as a diagnostic probe for near-infrared imaging of fibrils in AD brains, and it may also be a candidate for therapy of AD.