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Dual functions of β-amyloid oligomer and fibril in Cu(II)-induced H_2O_2 production

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ABSTRACT

Amyloid-β (Aβ) aggregation and Cu(II)-related oxidative stress are involved in the dysfunction and death of neurons in Alzheimer's disease (AD). However, the relationship between Aβ and Cu(II) is not clear. Furthermore, the pro- or anti-oxidant properties of Aβ are also under great debate. Here the H_2O_2 generating ability of Aβ42 in its monomeric, oligomeric and fibrillar forms was studied in the presence of Cu(II). The results show that Aβ42 in both oligomeric and fibrillar forms can promote H_2O_2 generation at lower concentrations of Cu(II) and Aβ42 oligomer can promote H_2O_2 generation to a higher extent. Nevertheless, the promoting effect of Aβ42 oligomer and fibril may convert to an inhibitory effect when the concentration of Cu(II) is increased. This indicates the dual functions of Aβ42 oligomer and fibril in Cu(II)-induced H_2O_2 production. Hereby we present a new perspective on the roles of Aβ42 in Cu(II)-mediated oxidative stress and add new evidence to the viewpoint that Aβ42 oligomer may be primarily responsible for the pathogenesis of AD.

1. Introduction

Alzheimer's disease (AD) is a highly prevalent neurodegenerative disease that is characterized by the extracellular deposition of a 39-43 amino acid peptide, amyloid-β (Aβ), derived from the cleavage of the amyloid precursor protein (APP) [1]. Monomeric Aβ can assemble into oligomeric and fibrillar forms, and finally become senile plaques in the brain [2]. According to the amyloid hypothesis, the accumulation of Aβ is involved in the pathogenesis of AD and the aggregates of Aβ peptide possess neurotoxicity. In recent years, more and more evidence has shown that oligomeric form of Aβ rather than the fibril is primarily responsible for the neuronal injury and death occurring in AD [3].

The pathogenesis of AD is also believed to be associated with oxidative stress which might be responsible for the dysfunction or death of neurons. Furthermore it is reported that impaired Cu(II) homeostasis is one of the most important factors in the generation of oxidative stress [4]. In several neurodegenerative disorders like AD, Parkinson's disease (PD), Huntington disease (HD) and so on, pathological oxidative stress arises from incorrectly bound Cu(II) to cellular proteins [4]. According to previous studies, Aβ is a representative example where inappropriate binding of Cu(II) may elevate the level of oxidative stress by generating ROS [5,6]. Aβ can serve as a metalloenzyme to catalyze the generation of neurotoxic H_2O_2 from O_2 through binding and reduction of Cu(II) [7]. However, it has also been proposed that Aβ can play a role as an anti-oxidant *in vitro* [8]. As a consequence, there is still a dispute on the pro- or anti-oxidant properties of Aβ.

Although it has been proposed that the interaction between Aβ and Cu(II) is important for the development of AD [6,9], there have been limited reports about the link between Cu(II)-related oxidative stress generation and different aggregated forms of Aβ [10–12]. Meanwhile, current reports mostly focus on the interaction between either Aβ monomer or fibril and Cu(II) [7,13]. However, since impaired Cu(II) homeostasis develops with the pathogenesis of AD, it is important to understand how Cu(II) affects oxidative stress during the Aβ aggregation process which is characterized by different aggregation forms of Aβ. In our study, the abilities of three forms (monomer, oligomer and fibril) of Aβ42 to generate H_2O_2 in the presence of different concentrations of Cu(II) were investigated. It was found that compared to the consistent anti-oxidant property of the Aβ42 monomer, both the oligomer and the fibril can play dual roles in Cu(II)-mediated H_2O_2 generation. Our findings show that the oligomer and the fibril can promote the generation of H_2O_2 when the concentration of co-incubated Cu(II) is below a critical level. When the Cu(II) concentration exceeds that level, both the oligomer and fibril act in a protective manner, similarly to monomer. We have also found that the oligomeric form of Aβ42 is superior to the fibrillar form in the process of H_2O_2 generation, which is concordant with the most prevalent idea that oligomers may be the primary culprit in AD [14].

Abbreviations: AD, Alzheimer's disease; Aβ, Amyloid-β; TEM, Transmission electron microscopy; SEC, Size exclusion chromatography; ThT, Thioflavin T; HPLC, High performance liquid chromatography; PBS, Phosphate buffered saline; H_2DCF-DA, 2′,7′-dichlorofluorescein diacetate; HRP, Horseradish peroxidase; BC, Bathocuproinedisulfonic acid; ROS, Reactive oxygen species.

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2. Materials and methods

2.1. Materials

Aβ42 was purchased from Otwobiotech (Guangzhou) Inc. (China). Bathocuproinedisulfonic acid (BC) was purchased from Alfa Aesar (USA). Dopamine was purchased from Sigma (USA). 2′,7′-Dichlorofluorescein diacetate (H_2DCF-DA) was purchased from Molecular probes (USA). Horseradish peroxidase (HRP) and glycine were purchased from Biodee Biotechnology (Beijing) Co. (China). $CuCl_2$ was purchased from Xilong Chemical (Guangzhou) Factory (China). All chemical agents were analytical grade.

2.2. The preparation of Aβ42 monomer, oligomer and fibril

Aβ42 was dissolved to 2 mg/ml in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP, Acros, USA), incubated overnight at RT and stored at −20 °C. For the aggregation protocols [15,16], HFIP was evaporated off by N_2 and then the peptide was redissolved in dimethyl sulfoxide (DMSO, Sigma, USA) to a concentration of 2 mM. To prepare monomer, the 2 mM Aβ42 in DMSO was diluted directly by PBS (phosphate buffered saline, containing NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 2 mM, pH 7.4) to a stock concentration of 100 μM right before using. Aβ oligomer was prepared by adding PBS to bring the peptide to a final concentration of 100 μM and incubating at RT for 24 h. For fibrillar preparation, PBS was added to bring the peptide to a final concentration of 50 μM and the solution was incubated for 72 h at 37 °C. Each sample was centrifuged for 15 min at 10,000×*g* (Biofuge, Heraeus Instruments, Germany). The supernatants of monomer and oligomer were used for succeeding experiments. While the fibril precipitated during the centrifugation according to SEC analysis, the supernatant of fibrillar Aβ42 was discarded and the pellets were redissolved in PBS to a final concentration of 50 μM. Besides, all solution corresponding to the different aggregation forms of Aβ42 was sonicated for at least 10 min to get homogeneous solution before use.

2.3. Size exclusion chromatography (SEC)

The SEC (TSKG3000PWx1; Tosch, Japan) analysis was performed on a Waters 600 HPLC system (Waters, Massachusetts, USA). The column of SEC was equilibrated with elution buffer and then calibrated with six molecular weight standards (catalase (232,000); albumin (62,900); ovalbumin (47,600); chymotrypsinogen A (19,400); ribonuclease A (15,600); and vitamin B12 (1350)) [17]. Samples were prepared as stated above, each sample was centrifuged for 15 min at 10,000×*g* (Biofuge, Heraeus Instruments, Germany). 200 μl of supernatant of each sample was loaded into the HPLC system. The running buffer was PBS and flow rate was 0.60 ml/min. Absorbance was measured by UV detector (Waters 2487, USA) at 280 nm.

2.4. Transmission electron microscopy (TEM)

8 μl of each sample was placed on 200 mesh formvar-coated copper grids for 2 min before removing excess solution. And then the sample was stained with 1% fresh tungstophosphoric acid for another 2 min [18]. JEOL-1200EX electron microscope (JEOL, Japan) was operated at 100 kV.

2.5. Thioflavin T (ThT) assay

Concentration of three different forms of Aβ42 was 10 μM in PBS. 20 μL of each sample was added to a black 96-well plate (Costar Ltd., USA), mixing with 180 μL ThT (10 μM). The ThT fluorescence was measured at 485 nm with excitation at 440 nm in a Synergy 4 plate reader (Biotek Company, USA) [19].

2.6. Hydrogen peroxide assay

The generation of H_2O_2 was detected by H_2DCF-DA assay, as described previously [20]. Briefly, H_2DCF-DA (5 mM in DMSO) (Molecular probes, Eugene, OR) is deacetylated in the presence of 0.25 M NaOH for 30 min and neutralized at pH 7.4 to a final concentration of 1 mM. Reactions are carried out in PBS (pH 7.4) in a 96-well microtiter plate (Amresco, USA). Different forms of Aβ42 (10 μM), different concentrations (from 0.2 to 1.4 μM, with 0.2 μM as an interval) of Cu–Gly (molar ratio: 1:6), a reducing agent (dopamine, 5 mM), deacetylated H_2DCF (100 mM), and horseradish peroxidase (1 mM, pH 7.4) were co-incubated for 60 min at 37 °C. Fluorescence is recorded using a Synergy 4 Plate Reader (Bioteck Company, USA) with excitation and emission at 485 nM and 530 nM respectively. The concentration span of Cu(II) was chosen since it is almost the same to *in vivo* environments [4]. The amount of H_2O_2 produced was proportional to the absolute fluorescence intensity difference between a sample and buffer solution-only (PBS) control.

2.7. Cu(II) reduction assay

Cu(II) reduction was analyzed using bathocuproine disulfonate (BC) (Alfa Aesar, USA) as a Cu(I) indicator [21]. The Cu(I)–BC divalent complex has a maximum absorbance at 483 nm when scanned from 400 to 600 nm. The assay was performed in a 96-well microtiter plate. Typically, samples containing 10 μM Aβ42 or ascorbic acid (10 μM), 10 μM $CuCl_2$, and 360 μM BC were co-incubated in PBS buffer (200 μl). The concentration span of Cu(II) in the hydrogen peroxide assay was under the detection limit in this experiment, therefore the Cu(II) concentration of 10 μM was chosen to magnify the differences between different groups. Absorbance readings were monitored at two time intervals, 60 s- or 60 min-incubation, at 37 °C. Ascorbic acid was included as a positive control.

2.8. Statistical analysis

On the *y*-axis of Figs. 3 and 4, we chose the highest value of absolute fluorescence intensity difference between a sample and buffer solution-only (PBS) control and defined it as 100%. Data from three independent experimental groups were presented in mean values ± S.D. Multiple comparisons were performed through Student-*T* test. Differences with $p<0.05$ were considered significant.

3. Results

3.1. Confirmation of the formation of Aβ42 monomer, oligomer and fibril by SEC, TEM and ThT assays.

Aβ42 aggregation generally involves the monomer's self-assembly into the oligomer and further fibril. The three forms of aggregates are thought to have different aggregation properties, cytotoxicities and sizes. In order to investigate the differences among them, it was of great importance to monitor the form into which the Aβ42 had actually aggregated. Here SEC-HPLC [17] was adopted to identify the forms of different Aβ42 aggregates under current preparation conditions. Six molecular weight standard proteins were used to calibrate the column of the SEC. A calibration curve was constructed by regression analysis and used to determine the molecular weights of different forms of Aβ42 (Fig. S1). A solution of monomeric Aβ42 was monitored by SEC-HPLC directly after preparation. A solution of the oligomer or of the fibril was centrifuged and the supernate was analyzed by SEC-HPLC. The SEC-HPLC profile of the Aβ42 monomer showed a main peak at a retention time of 17.5 min corresponding to a molecular weight of about 5.0 kDa (Fig. 1A). The supernate of Aβ42 oligomer solution showed a different peak at a retention time of 11.0 min corresponding to a molecular weight of about 116.0 kDa (Fig. 1B). However, SEC separates molecules