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Article

The Concentration of Hydrogen Peroxide Generated during Aggregation of α-Synuclein *in vitro* Is Lower than 5 nmol/L

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Using a fluorometric method with a detection limit of 5 nmol/L, here it is reported that albeit positive results were got from bovine serum albumin (BSA) and chicken ovalbumin (OVA) as published in literature, no detectable amount of hydrogen peroxide (H_2O_2) was generated during α-synuclein (α-Syn) aggregation *in vitro* even in the presence of transition metal ions Cu(II) or Fe(III). The results suggest that the concentration of H_2O_2 generated during aggregation of α-Syn *in vitro* be lower than 5 nmol/L beyond the detection limit of the adopted method and it is far too poor to be responsible for the cytotoxicity of α-Syn aggregates, thus allowing people to extensively elucidate the mechanism underlying neurotoxicities of the aggregates formed by some amyloidogenic proteins.

Keywords　α-synuclein, hydrogen peroxide, aggregation, cytotoxicity

Introduction

α-Syn is a major component of Lewy bodies and Lewy neurites in the brains of patients with neurodegenerative diseases, such as Parkinson's disease, the Lewy body variant of Alzheimer's disease, dementia with Lewy bodies and multiple system atrophy.[1,2] α-Syn is prone to aggregate *in vitro* and the aggregates appear to be toxic to neurons, though the reason for the cytotoxicity remains unclear.[3-6] Previous studies by electron spin resonance (ESR) showed that α-Syn could liberate hydroxyl radicals upon incubation *in vitro* followed by the addition of Fe(II).[7,8] The given explanation was that α-Syn and necessary metal ions established a local condition required for H_2O_2 formation, which was detected by ESR via the Fenton reaction and responsible for the death of vulnerable cells.

In order to get more information in this new field, we decided to directly detect and quantify H_2O_2 generated during aggregation of α-Syn *in vitro*. As we know, four analytical methods are usually adopted for H_2O_2 detection. One is a fluorometric method based on the oxidation of non-fluorescent reagents into fluorescent products by H_2O_2, the second is founded on chemiluminescence produced by chemical reaction caused by H_2O_2, the third employs optical absorbance of products generated by reaction of H_2O_2 with reagents, and the last one is an electrochemical method using selective electrodes.[9-14] Of the four, the fluorometric method gives maximum sensitivity and specificity to H_2O_2 detection besides its convenience in practice. As a result, we adopted Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) in the presence of horseradish peroxidase (HRP) and H_2O_2 to form fluorescent resorufin in our experiments.[15,16] The experimental results demonstrate that although α-Syn could readily aggregate into fibrils upon incubation, no detectable amount of H_2O_2 was generated during the whole aggregation process. Even after the addition of transition metal ions, Cu(II) or Fe(III), no H_2O_2 was detected in α-Syn solutions. On the contrary, the positive controls of BSA and OVA both generated remarkable H_2O_2-specific fluorescence signals which can be blocked by catalase. Possible explanations for the results were considered.

Results and discussion

By Thioflavine T (ThT) binding assay,[17] Figure 1 shows the time course for the aggregation of α-Syn and four control proteins. α-Syn readily aggregated into fibrils as revealed by atomic force microscopy in concomitance with secondary structural change from random coil to β-sheet.[6] However, no such characteristic changes were found in others.

As shown in Figure 2a, the calibration curve of fluorescence intensity vs. the concentration of H_2O_2 was linear in the 10—400 nmol/L range with a detection limit of 5 nmol/L and the correlation coefficient was

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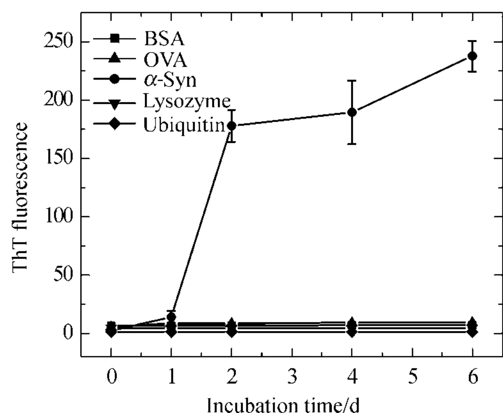

Figure 1 Comparison of BSA (squares), OVA (up triangles), α-Syn (circles), lysozyme (down triangles) and ubiquitin (diamonds) for their abilities to aggregate. Data are presented as the means ± SE, next as the same.

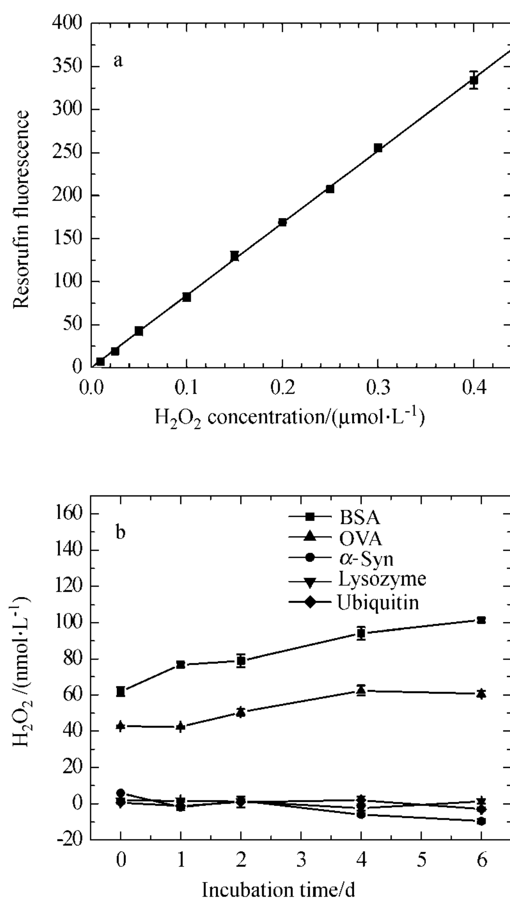

Figure 2 (a) Calibration curve for assaying H_2O_2. Background for the control without adding H_2O_2 was subtracted from each value. (b) Time course of H_2O_2 production by BSA (squares), OVA (up triangles), α-Syn (circles), lysozyme (down triangles) and ubiquitin (diamonds). The fluorescence intensity value of resorufin was converted into H_2O_2 concentration by using the H_2O_2 calibration curve after subtracting the blank (0.1 mol/L phosphate-buffered saline, PBS, pH = 7.0).

0.999 (n = 9). During the incubation, generation of H_2O_2 was detected for BSA and OVA, while for α-Syn, lysozyme and ubiquitin no detectable H_2O_2 was produced (Figure 2b). Furthermore, addition of catalase prior to H_2O_2 assay could block fluorescence change in BSA and OVA samples (Figure 3). This suggests that our adopted H_2O_2 assay system be highly specific to H_2O_2.

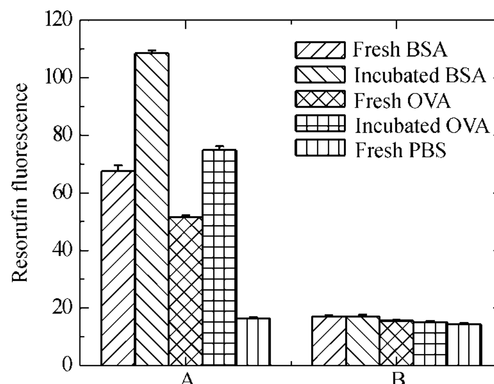

Figure 3 The consumption of H_2O_2 by catalase. (A) Data are shown in the order: freshly prepared BSA (10 μmol/L), 6-day incubated BSA (10 μmol/L), freshly prepared OVA (10 μmol/L), 6-day incubated OVA (10 μmol/L) and 0.1 mol/L PBS. (B) Samples corresponding to the ones in panel A after treatment with catalase.

As it was reported that formation of H_2O_2 by α-Syn was likely to be metal-dependent,[7,8] related experiments were further performed in the presence of $CuCl_2$ or $FeCl_3$. Unexpectedly, the data show that the metal ions had no significant effects on the production of H_2O_2 in the incubated mixtures, though the accelerated aggregation of α-Syn was observed as reported (Figure 4).[18] Repeatedly, H_2O_2 was not found in the α-Syn aggregation system or lysozyme solution upon incubation, while the amount of H_2O_2 generated from BSA remained unchanged.

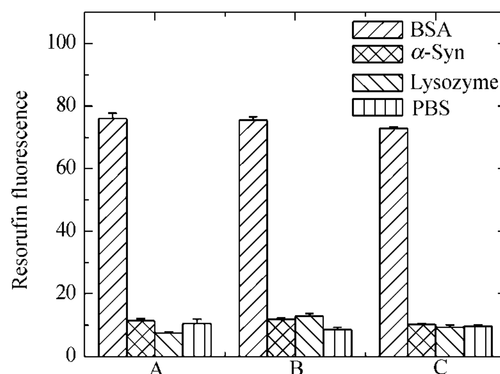

Figure 4 The effect of metal ions on the generation of H_2O_2. (A) Data are shown in the order: 6-day incubated BSA (10 μmol/L), 6-day incubated α-Syn (10 μmol/L), 6-day incubated lysozyme (10 μmol/L) and 0.1 mol/L PBS. (B) Samples corresponding to the ones in panel A after addition of 1 μmol/L Fe(III) before incubation. (C) Samples corresponding to the ones in panel A after addition of 1 μmol/L Cu(II) before incubation.

At present, only hydroxyl radicals were detected in

the α-Syn aggregation system by ESR and it is significant to directly determine H_2O_2 for clarification of the cytotoxicity caused by α-Syn. Here a H_2O_2-specific and sensitive method was used to determine H_2O_2 possibly generated from α-Syn aggregates in a cell-free manner, but it was found that only the positive controls of BSA and OVA produced H_2O_2. Now the absence of H_2O_2 formation stands in contrast to the previous studies that hypothesized formation of H_2O_2 by aggregated α-Syn based on ESR detection of hydroxyl radicals, so it is necessary to further consider the reliability of our adopted system. Amplex Red is a highly sensitive and chemically stable fluorogenic probe for assaying H_2O_2. It shows very low background fluorescence and its reaction with H_2O_2 follows a 1∶1 stoichiometry to produce fluorescent resorufin in the presence of horseradish peroxidase.[16] Because resorufin has absorption and fluorescence emission at 563 and 583 nm respectively, there is much less interference from intrinsic fluorescence of the proteins under study. Under optimal conditions, the detection limit of the fluorometric method is 5 nmol/L H_2O_2. Meanwhile, the amount of H_2O_2 formed by BSA and OVA is proportional to their concentration separately (data not shown). As for α-Syn, ubiquitin and lysozyme, there is no such correlation. The fact that catalase can block fluorescence enhancement also confirms the reliability of the adopted method. Therefore, the concentration of H_2O_2 generated during α-Syn aggregation *in vitro* is likely to be lower than 5 nmol/L, beyond the detection limit of our adopted method.

On the other hand, previous cytotoxicity assay showed that aggregated α-Syn was significantly toxic to neurons.[5,6] It is generally accepted that high levels of H_2O_2, usually above 50 μmol/L, are toxic to cultured neurons in a dose-dependent manner.[19-22] Thus, the cytotoxicity of α-Syn *in vitro* is in contradiction with the poorly generated H_2O_2. Although in the earlier report the cytotoxicity of α-Syn was linked to the generation of H_2O_2, no experiments have ever been done to eliminate H_2O_2 and protect neurons against toxicity. Furthermore, the circumstances for H_2O_2 generation *in vivo* is much more complicated than *in vitro*. Under continuous oxidative stress associated with aging, the generation of H_2O_2 and lipid peroxidation of polyunsaturated fatty acids probably occur accompanied with the aggregation of α-Syn *in vivo*, though it is hard to convincingly correlate the aggregation of α-Syn and the generation of H_2O_2 *in vivo* based on current research work. Such a complicated situation prompted the authors to extensively elucidate the mechanism underlying neurotoxicities of the aggregates formed by some amyloidogenic proteins. At present, a growing body of evidence supports the hypothesis that the membrane permeabilization by protofibrillar intermediates formed during aggregation might be associated with the cytotoxicities of some neurodegenerative disease-related proteins.[23,24]

Experimental

Materials

Recombinant BL21 human α-Syn was overexpressed in *Escherichia coli* (DE3) and purified as previously described.[25] BSA (Amresco), OVA (Sigma), ubiquitin (Sigma) and lysozyme (Amresco) were used as controls.

Methods

Each 200 μmol/L of the five protein solutions in 0.1 mol/L PBS and the protein-null buffer were incubated at 37 ℃ with continuous agitation. Aliquots of each sample were taken at intervals and stored at −20 ℃ for future use. The aggregation process was monitored by ThT fluorescence assay. The enhancement of ThT fluorescence was used to semiquantitatively estimate the relative rate of fibril formation. The protein aliquots were added to 5 μmol/L ThT (Aldrich) in 50 mmol/L Glycine-NaOH buffer (pH=9.0) to give a final protein concentration of 4 μmol/L. Fluorescence measurements were performed on a Hitachi F-4010 spectrofluorimeter at 482 nm with an excitation at 446 nm.

A sensitive and specific hydrogen peroxide assay kit (A-12212, molecular probes) was used for the following assays, by which H_2O_2 was directly detected by its ability to convert Amplex Red into resorufin.[15,16] Immediately after each stored sample was thawed, 10 μL of working solution (400 μmol/L Amplex Red containing 2 U/mL horseradish peroxidase) and 940 μL of reaction buffer (50 mmol/L phosphate buffer, pH=7.4) was added into the protein sample and its final concentration became 10 μmol/L. The mixed solution was then incubated for 30 min at 37 ℃ in dark before measuring the fluorescence at 583 nm (excitation at 563 nm). Since the samples were stored frozen prior to assay, the production of H_2O_2 was tested with frozen samples and fresh ones and it was confirmed that there was no obvious difference between them. To ensure that the fluorescence enhancement was specifically due to the generation of H_2O_2, fluorescence data were recorded after incubation of the samples with 500 U catalase (Sigma) for 10 min at 25 ℃.

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