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Copper Enhances Amyloid-β Peptide Neurotoxicity and non β-Aggregation: A Series of Experiments Conducted upon Copper-Bound and Copper-Free Amyloid-β Peptide

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Abstract Alzheimer's disease is characterized by the abnormal aggregation of amyloid-β peptide (Aβ) in extracellular deposits known as senile plaques. However, the nature of the toxic Aβ species and its precise mechanism of action remain unclear. Previous reports suggest that the histidine residues are involved in copper–Aβ interaction, by which resulting in the neurotoxicity of Aβ and free radical damage. Here, we employed a mutant Aβ (Aβ H13R) in which a histidine residue was replaced by arginine. Copper facilitated the precipitation of both wild-type and mutant Aβ in the spectrophotometric absorbance assay but suppressed β-structure aggregates according to Thioflavine-T assay. Wild-type Aβ alone is more cytotoxic but produced less amount of H_2O_2 than AβH13R–copper complexes, suggesting that Aβ–membrane interaction may also implicated in the pathologic progress. Aβ toxicity is in positive correlation to its competence to aggregate despite the aggregation is mainly composed of non-β fibril substances. In short, these findings may provide further evidence on the role of copper in the pathogenesis of Alzheimer's disease.

Keywords Alzheimer's disease · Amyloid-β peptide · β aggregation · Neurotoxicity

Abbreviations

AD	Alzheimer's disease
Aβ	amyloid-β peptide
APP	amyloid precursor protein
SPs	senile plaques
NFTs	neurofibrillary tangles
CD	circular dichroism
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
ThT	Thioflavine-T
DMEM	Dulbecco's modified Eagle's medium
OD	optical density
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
DMSO	dimethyl sulfoxide
LDH	lactate dehydrogenase
$^{13}A\beta_{40}$	Aβ(1–40)H13R
WT $A\beta_{40}$	wild-type Aβ (1–40)

Growing evidence highlights that oxidative stress-mediated cytotoxicity triggers progressive neurodegeneration leading eventually to Alzheimer's disease (AD) and Parkinson's disease (PD). AD is characterized by senile plaques (SPs), neurofibrillary tangles (NFTs), and the loss of neurons in the brain (Selkoe 2002). The predominant component of extracellular SPs is amyloid-β peptide (Aβ), which is believed to facilitate the neurodegeneration that occurs in AD (Wisniewski and Konietzko 2008). Central to this disease is altered proteolytic processing of the amyloid precursor protein (APP) resulting in the production and aggregation of neurotoxic forms of Aβ (Mattson 2004).

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Since Aβ contains part of the transmembrane domain of APP, it is not surprising that the peptide interacts with cell membranes and lipoproteins (Kourie 2001).

The interaction between copper and Aβ1–40 was first observed by the stabilization of an apparent Aβ1–40 dimer by Cu^{2+} on gel chromatography (Bush et al. 1994). Aβ binds Zn^{2+}, Cu^{2+} in vitro, and these metal ions are markedly elevated in the neocortex and especially enriched in amyloid plaque deposits of AD patients (Cuajungco et al. 2000). Aβ coordinates and reduces Cu^{2+} leading to the peptide being oxidized, verified by mass spectrometry showing that copper oxygenates Aβ to Aβ radicals (Nishino and Nishida 2001). Aβ radicals may in turn attack cell membranes and initiate lipoperoxidation resulting in neurodegeneration (Dikalov et al. 2004).

Due to the potential importance of Aβ–Cu^{2+} interactions to the pathophysiology of AD, alternative strategies have been sought to characterize more precisely the multiple binding sites on Aβ (Smith et al. 2006). A study using Raman spectroscopy has provided direct evidence that copper is bound to isolated senile plaques via the histidine imidazole rings (Dong et al. 2003). Rats do not develop amyloid, even in mice transgenic for familial AD-linked mutant presenilin that overexpresses $Aβ_{1–42}$, probably due to the three residues substitution in their homolog of Aβ (Arg5→Gly, Tyr10→Phe, and His13→Arg). Provided histidine residues are related to Aβ-induced pathology, we employed Aβ (1–40) H13R to determine its neurotoxicity and competence to bind copper with histidine missing. Further, the correlation between the toxicity of Aβ and its competence to form aggregation was also explored.

Materials

All cell culture reagents were obtained from Invitrogen (Carlsbad, USA). Hydrogen Peroxide Assay Kit was purchased from Beyotime Biotech (Haimen, China). Lactate dehydrogenase (LDH) Detection Kit was obtained from Jiancheng Bioengineering Institute (Nanjing, China). 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), poly-L-lysine, dimethyl sulfoxide (DMSO), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetra-zolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemical reagents were commercial products of analytical grade.

Methods

Peptide Preparation

Wild-type Aβ1–40 (WT $Aβ_{40}$) and Aβ (1–40) H13R ($^{13}Aβ_{40}$) were purchased from Sigma–Aldrich (Table 1), and the purity of peptides used has achieved 90% at least. The lyophilized peptide was initially dissolved in HFIP to a final concentration of 1 mg/ml, then the stock solution was aliquoted into siliconized tubes and stored at −20°C. Upon experiment, the stock solution was spin-vacuumed using Integrated Speed-Vac System (Thermo Savant) then further diluted in culture medium or Tris-buffered solutions.

Cu^{2+} was prepared using 0.1 M copper–glycine ($CuGly_2$) solution by dissolving 1 mol equivalent $CuCl_2$ and 2 mol equivalent glycine in deionized water. $CuGly_2$ solution was mixed with Aβ samples in appropriate ratios when copper was needed (Lau et al. 2006).

Circular Dichroism Spectroscopy

Metal-induced changes in peptide secondary structures were detected using CD spectroscopy (Yoshiike et al. 2001). First, the peptide dissolved in HFIP was spin-vacuumed then dissolved in 20 mM Tris–HCl (pH 7.4), and the final concentration of Aβ in each sample was ~50 μM. Each test sample was added into a 1-mm pathlength quartz cuvette and scanned with a J-810 CD spectropolarimeter (Jasco, Japan). To investigate copper-induced Aβ secondary structure transition, copper ions were titrated into Aβ solution at 0.5–3.0 peptide mol equivalent. All CD measurements were carried out between 190 and 240 nm using the following parameters: 2-nm bandwidth, 20 nm/min run speed, 0.5-nm step size, and 2-s response time. Background values for each test were subtracted from the corresponding CDs of each sample.

ThT Fluorometric Assay

The fibril formation of the peptides was measured by a Thioflavine-T (ThT) fluorometric assay according to published reports with some modifications (LeVine 1993). ThT can specially bind to fibrillar structures, and this binding produces a shift in its emission spectrum and fluorescent signals proportion to the amount of fibrils formed. A solution of 10 μM Aβ with or without 10 μM copper ions, respectively, was incubated at 37°C for 24 h in 20 mM Tris–HCl (pH 7.4). After incubation, ThT was added to each test sample to a final concentration of 5.0 μM. Fluorescence was monitored at an excitation of 450 nm and an emission of 485 nm using Varioskan multimode microplate spectrophotometer (Thermo, USA) under kinetic fluorometric mode. To account for background fluorescence, the fluorescence intensity of ThT measured from control solution without Aβ peptides was subtracted from that containing Aβ.

UV Spectroscopy

The effect of copper ion on total aggregation of Aβ was assessed using an optical density (OD) assay (Kourie 2001;

Table 1 Peptides

Designation	Sequence
Wild-type Aβ (1–40)	DAEFR HDSGY EVHHQ KLVFF AEDVG SNKGA IIGLM VGGVV
Aβ (1–40)H13R	DAEFR HDSGY EV**R**HQ KLVFF AEDVG SNKGA IIGLM VGGVV

Yoshiike et al. 2001). Peptides diluted by 20 mM Tris–HCl (pH 7.4) with or without 10 μM copper was incubated at 37°C for 30 min, centrifuged at 10,000×*g* for 10 min at 4°C, and the supernatant was collected for following experiments. The final concentration of Aβ in each test sample was ~10 μM.

To eradicate the possibility of signals omitted to be detected due to peptide precipitation, automatic 30 s shake mode was selected for the plate reader to evenly suspend any aggregates in the wells before reading. Absorbance (OD) was measured at a wavelength of 214 nm. The percentage of total aggregate increase after 24 h incubation was calculated as the ratio of difference between OD_{after} and OD_{before} as follows:

$$(OD_{after} - OD_{before})/OD_{before} \times 100\%$$

Primary Neuronal Cultures

Cortical neuronal cultures were performed as described previously (Dai et al. 2007). This study was performed with the approval of the local ethical committee, and all animals received humane care in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. The dissociated cells were plated onto poly-L-lysine-coated 48-well culture plates at a density of 125,000 cells/cm^2. After 2 h, the plating medium was replaced with fresh neurobasal medium plus 2% B27 supplements. Cells were allowed to mature for 5 days before commencing treatment. Mixtures were freshly prepared at peptide: Cu^{2+} molar ratios of 0:1 and 1:1, respectively, then the mixtures were added to neurons for 72 h. For all the treatment in neuronal cultures, Aβ stock solution should be further diluted to a concentration of 5 μM in the neurobasal medium.

Cell Viability Assay

Cell viability was quantitatively determined using the MTT assay as described previously. Briefly, a final concentration of 0.5 mg/ml MTT was added to each well and then incubated for 4 h at 37°C. Upon removal of the medium, blue formazan formed was solubilized in DMSO, and the absorbance values at 570 nm were recorded. Background readings of MTT incubated in cell-free medium were subtracted from each value before calculations. The data were normalized and calculated as a percentage of untreated control values prior to analysis.

LDH is a stable cytoplasmic enzyme present in cells, and it is rapidly released into the cell culture supernatant when the cell is damaged (Saad et al. 2006). In the present study, we determine the LDH activity in the medium to enzymatically convert the lactate and NAD^+ to pyruvate and NADH. The dinitrophenylhydrazine salt produced in the enzymatic reaction was then reduced to red formazan in the presence of pyruvate, thus, allowing optical detection of neuronal membrane integrity.

Hydrogen Peroxide Assay

Analyses were carried out using hydrogen peroxide assay kit (Beyotime Biotech, China). Hydrogen peroxide could oxidize Fe^{2+} to Fe^{3+}, and then Fe^{3+} reacted with xylenol orange leading to colorimetric reaction that could be further detected by a spectrometer. Briefly, test tubes containing 50 μl supernatants and 100 μl test solutions were placed at room temperature for 20 min and measured immediately with a spectrometer at a wavelength of 560 nm. The concentration of H_2O_2 released was calculated from standard concentration curve with triplicate experiments.

Statistical Analysis

We diluted and measured each sample in triplicate and used the averaged value for analysis. Error bars represent SEM of each group analyzed. Data were evaluated statistically using SPSS 13.0 Software (Chicago, USA). Comparisons were conducted using one-way ANOVA followed by post hoc Student–Newman–Keuls methods and/or Student's *t* test. *P* values of <0.05 were considered significant.

Results

Analyses of Structural Changes of Aβ Peptides with Copper Addition

AD is characterized by changes in the secondary structure of Aβ from predominantly random coil to β-sheet forming amyloid fibrils (Syme and Viles 2006). The change in the main-chain conformation of Aβ induced by Cu^{2+} binding is, therefore, of great interest.

The spectral curve of WT $A\beta_{40}$ in the absence of Cu^{2+} showed a negative ellipticity at around 200 nm (Fig. 1a), suggesting that the secondary structure of WT $A\beta_{40}$ is mainly random coil (Perczel and Hollosi 1996). As Cu^{2+} was titrated in, the spectral curve slightly shifted to 198 nm, and the negative minimum had a decrease in intensity, suggesting that either the peptide is becoming more ordered or alternatively the change observed is due to peptide precipitation. To determine if the effect observed was due to precipitation of Aβ and therefore loss of CD signal, a range of concentrations of Aβ was used. The loss of signal at 200 nm was dependent on the concentration of Aβ (data not shown) and was evident at higher Aβ concentrations, indicating the effect is due to precipitation upon Cu^{2+} addition. Figure 1b showed $^{13}A\beta_{40}$ spectra with copper titration. The spectrum of $^{13}A\beta_{40}$ alone is similar to that of the WT $A\beta_{40}$ though a slight increase on β-sheet structure

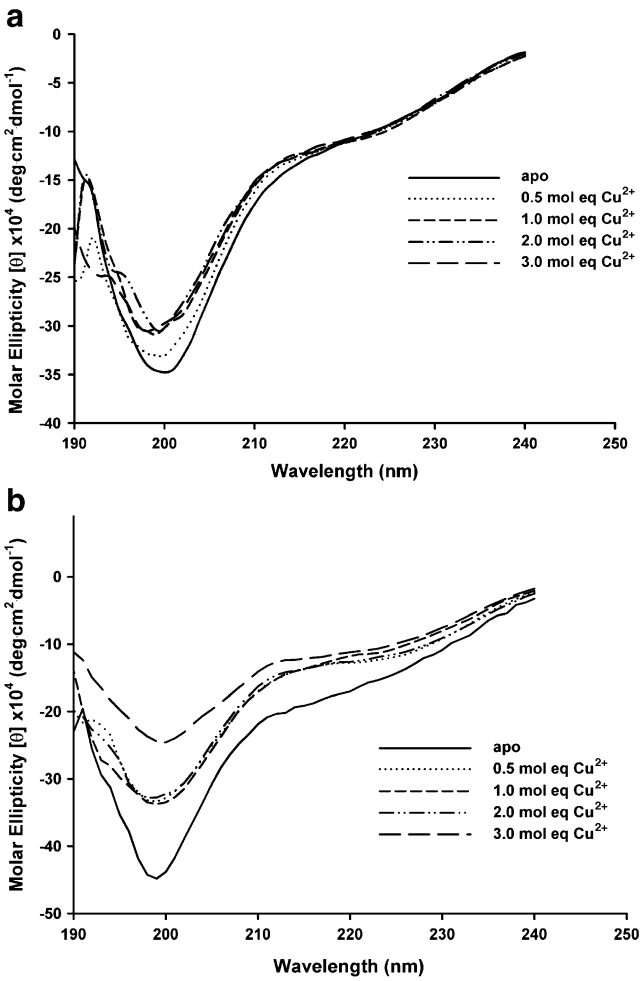

Figure 1 UV region CD spectra of Aβ peptides titrated with copper. Molar ellipticity of WT $A\beta_{40}$ (**a**) and $^{13}A\beta_{40}$ (**b**) was recorded in the UV region (190–240 nm) with addition of increased mol equivalent of Cu^{2+}. All peptides were diluted using 20 mM Tris–HCl (pH 7.4) to a final concentration of 50 μM in each sample. The data represent the average of six runs

according to Yang's formula (Yang et al. 1986). The spectra of $^{13}A\beta_{40}$ with addition of copper dramatically decreased in negative minimum, suggesting copper readily precipitate the peptide and a decrease on β-sheet secondary structures (Yang et al. 1986).

To quantify the fibrils formed after 24 h incubation, ThT fluorometric assay was employed. In accordance with CD spectroscopic data, the ThT assay showed that fibril formation of $^{13}A\beta_{40}$ alone was higher than that of WT $A\beta_{40}$, while a decrease in fluorescent signal was observed either in WT $A\beta_{40}$ or $^{13}A\beta_{40}$ in the presence of copper (Fig. 2a). However, these results were in discordance with former reports that favored copper as a fibrillar promoting factor especially under slightly acidic conditions (Atwood et al. 1998, 2004). Thus, we further resorted to absorbance assay that assess the total aggregation formed in the solution to find out the exact reason for this discrepancy. Either WT or mutant $A\beta_{40}$ had similar OD values before incubation whether in the presence of copper or not (data not shown). As shown in Fig. 2b, both WT and mutant $A\beta_{40}$ exhibited strong aggregate properties after 24 h incubation, and these peptides were more prone to precipitate upon copper and peptide co-incubation. The aggregates of WT $A\beta_{40}$ in the presence of copper increased by 81% compared to that before incubation, while $^{13}A\beta_{40}$ was also dramatically precipitated by copper (in accordance to the results obtained from circular dichroism) though exhibited much lower aggregate capacity or Cu^{2+}-induced aggregation than that of WT $A\beta_{40}$. In conclusion, copper induces Aβ aggregation and precipitation (absorbance assay) but suppresses fibril formation (ThT fluorometric assay), suggesting a smaller fraction of the precipitated aggregates is fibril.

Aβ/AβCu^{2+} Toxicity to Primary Cortical Neurons

To examine the toxicity of Aβ peptides to neuronal cultures, cortical neurons were exposed to 5 μM Aβ with or without 5 μM Cu^{2+} for 72 h. As determined by MTT assay, copper aggravated the toxicity of both WT and mutant $A\beta_{40}$. Samples containing WT $A\beta_{40}$ exhibited much stronger neurotoxicity, with the Cu^{2+}: peptide molar ratio of 1:1 the most toxic that the cell viability reduced to ~49.4% (Fig. 3). Treatment with 5 μM $^{13}A\beta_{40}$ alone or $^{13}A\beta_{40}Cu^{2+}$ complexes was less deleterious than WT $A\beta_{40}$ or its peptide–copper complexes but still markedly decreased to less than 80% in cell activity.

Increased LDH released from cells into culture medium also reflects the damage of neurons. Treatment of either WT or mutant $A\beta_{40}$ in the presence or absence of copper led to a significant increase in extracellular LDH concentration versus control after 72 h exposure. Groups treated with Aβ–copper complexes gave rise to more LDH release

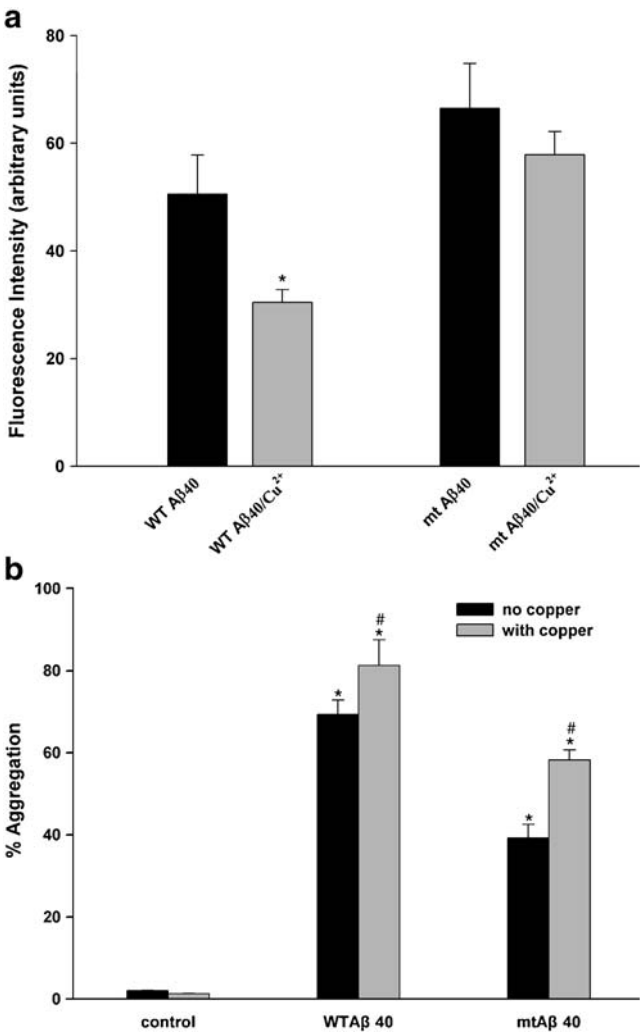

Figure 2 Aggregative state of Aβ in the presence or absence of copper measured by fluorescence or OD assay. **a** β-fibril aggregation of Aβ peptide assessed by ThT fluorescence assay. Solutions of 10 μM peptides in the presence or absence of 10 μM copper were incubated at 37°C for 24 h. ThT fluorescence was monitored at an excitation of 450 nm and emission of 485 nm. Data are the mean values ± SEM (n=3). *P<0.05, comparison between peptides with vs without copper. **b** The percentage of total aggregation increase was shown as a ratio of difference between OD values after and before 24 h incubation (see details in "UV spectroscopy"). Data points are means ± SEM, n=3. *Asterisk* denotes significant difference between samples and control, and #(P<0.05) denotes significant difference between samples with or without copper

compared to those with peptide alone (Fig. 4). LDH concentration in the supernatant of cells exposed to $^{13}A\beta_{40}$ alone or $^{13}A\beta_{40}Cu^{2+}$ complexes was significantly increased though still lower than that in WT $A\beta_{40}$.

Aβ/AβCu^{2+}-Induced Neurotoxicity Involves Hydrogen Peroxide Generation

Aβ has been shown to induce cell death in cultured neurons. Previous studies demonstrated that human $A\beta_{40}$-

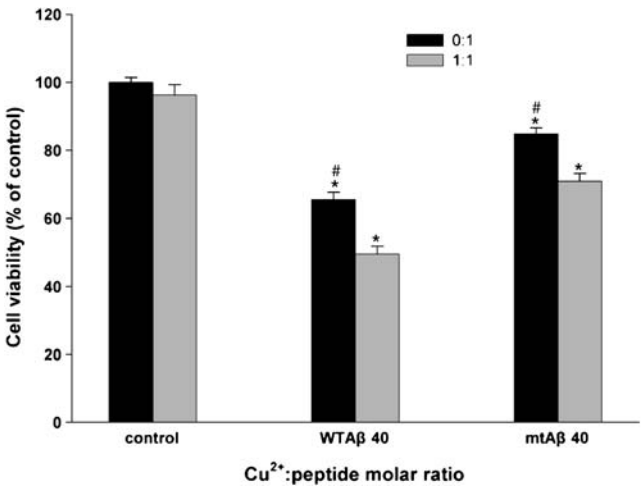

Figure 3 Neurotoxic activity of WT $A\beta_{40}$ and $^{13}A\beta_{40}$. Cell viability following different treatments was determined using the MTT reduction assay. Neurons were treated with 5 μM WT $A\beta_{40}$ or Aβ variant at Cu^{2+}: peptide molar ratio of 0:1 (*black bars*) and 1:1 (*gray bars*) for 72 h in serum free medium. *P<0.05 versus control and #(P<0.05) denotes significant difference between samples with or without copper, n=3

bound copper ion resulting in metal reduction, H_2O_2 generation, and resultant increase in Aβ-mediated neuronal cell death in vitro (Huang et al. 1999). As shown in Fig. 5, WT $A\beta_{40}$ released maximum amount of H_2O_2 (~86 μM) when co-incubated with 1 mol equivalent Cu^{2+} for 72 h. Neurons exposed to $^{13}A\beta_{40}$ or $^{13}A\beta_{40}Cu^{2+}$ also resulted in significantly higher release of H_2O_2 than control (P<0.05). Surprisingly, treatment with $^{13}A\beta_{40}Cu^{2+}$ released more H_2O_2 than that released by WT $A\beta_{40}$. These results together with cell viability assay suggested that $A\beta Cu^{2+}$-induced H_2O_2 generation were correlated with cytotoxicity,

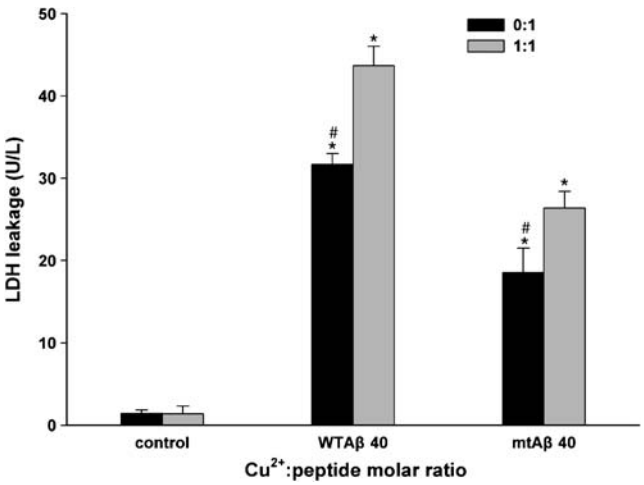

Figure 4 Cell death determined using LDH assay. Primary cortical neurons were exposed to 5 μM WT $A\beta_{40}$ or $^{13}A\beta_{40}$ at Cu^{2+}: peptide molar ratio of 0:1 (*black bars*) and 1:1 (*gray bars*) for 72 h. *P<0.05 symbolizes versus control and #(P<0.05) represents significant difference between samples with or without copper, n=3

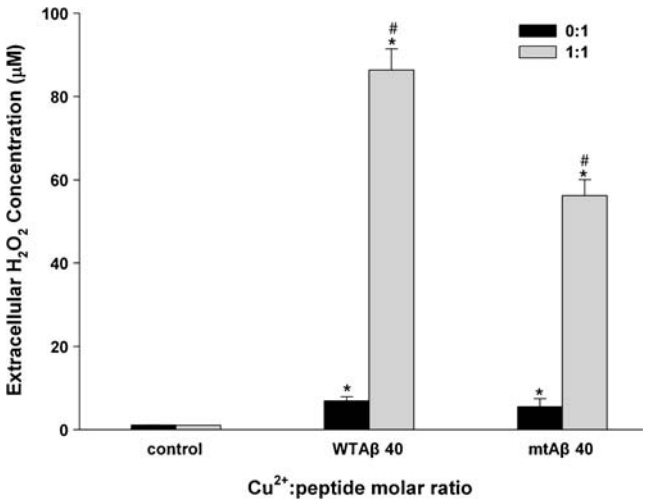

Figure 5 Extracellular H_2O_2 production induced by Aβ or Aβ/ Cu^{2+} complexes. Primary cortical neurons were exposed to 5 μM WT $Aβ_{40}$ or Aβ variant at Cu^{2+}: peptide molar ratio of 0:1 (*black bars*) and 1:1 (*gray bars*) for 72 h. Absorbance readings were taken and compared to control (*P<0.05); with copper versus no copper (#P<0.05), n=3

but H_2O_2 may not be the sole factor responsible for Aβ/ $AβCu^{2+}$-induced toxicity.

Discussion

Aβ peptide forms long, insoluble amyloid fibrils, which accumulate in spherical microscopic deposits known as senile plaques in AD. However, the relevance of these plaques to AD pathogenesis is unknown (reviewed in Haass and Selkoe 2007). Aβ folding and aggregation pattern in the brain has long been considered as an important factor in AD, but whether Aβ toxicity depends on the soluble or aggregated forms of Aβ is still in dispute. Lesne et al. reported a specific Aβ protein assembly in the brain, termed as Aβ*56, impaired memory independently of plaques (Lesne et al. 2006). Thus, disclosing Aβ aggregate state is prerequisite to understand the biological significance of Aβ assembly in AD.

Abnormally high concentration of copper has been found in amyloid plaques, and copper-selective chelators are shown to dissolve Aβ peptide from postmortem brain specimen (Ali et al. 2004). Copper may play a significant role in the formation and neurotoxicity of Aβ fibrils or oligomers, and serum copper measurement is proved to be a peripheral diagnostic marker for AD (Squitti et al. 2002), though whether abnormal copper homeostasis initiates Aβ deposition or the accumulation of Aβ acts as a sink that draws metal ions into its mass is still unclear. Ali et al. (2006) have reported that Cu^{2+} could induce aggregation of the soluble natural fragment $Aβ_{1–16}$. Copper binding will have a profound influence on the electrostatic surface of the Aβ peptide with a reduction in charged groups on Aβ and a change from an overall neutral charge to a highly negatively charged N-terminal tail, and perhaps this change in charged residues is the cause of the aggregation observed upon Cu^{2+} binding (Syme et al. 2004).

In the current study, copper binding and aggregate properties of the neurotoxic WT Aβ and Aβ variant were detected. In line with the findings of former reports (Dai et al. 2007; Gupta et al. 2008), the secondary structure of WT $Aβ_{40}$ is predominately random coil according to circular dichroism. Data from WT $Aβ_{40}$ suggest that copper addition does not induce a profound change on the main-chain conformation, and changes in the spectral curve could be attributed to copper-induced aggregation and loss of CD signal. Aβ (1-40) H13R, resembling Aβ secreted from transgenic mice, displayed similar spectral curve as WT $Aβ_{40}$ but more profound aggregate propensity with copper addition as analyzed by CD. However, the precipitation of the peptide by copper may have lowered the sensitivity of the technique to detect physicochemical changes. Moreover, this analysis of binding capability is limited by both sensitivity and the lack of competitive binding factors that would emulate the physiological situation more closely.

Therefore, we resorted to a fluorescence dye that specially binds to fibrillar structures. Results from ThT and OD assays lead us to hypothesize that in addition to β-aggregate, another type of aggregate is formed. This idea logically origins from higher β-fibril contents in Aβ (1–40) H13R compared to WT $Aβ_{40}$ according to ThT assay but comparatively lower total aggregation than that obtained in WT $Aβ_{40}$ whether in the presence of copper or not. Copper addition diminishes the fluorescent signals both in WT $Aβ_{40}$ and $^{13}Aβ_{40}$ but enhances the overall aggregation in these peptides, suggesting that there must be other forms of aggregate rather than β-aggregate resulting from the copper-peptide chelation. Although CD spectroscopy and ThT assay may suggest that copper induce Aβ to take non β-sheet conformation, more specific method such as electronic microscope would be necessary for the precise identification.

Amyloid plaques are the hallmark of neuropathological lesions in Alzheimer's disease, which consist of abnormally aggregated Aβ protein (Hardy and Selkoe 2002; Lau et al. 2006). Fibrillar Aβ was initially reported to facilitate the neuronal cell death process, while non-amyloidogenic or amorphous aggregates of Aβ are comparatively nontoxic compared with fibrillar Aβ (Lorenzo and Yankner 1994). Interestingly, multiple Aβ-aggregated species have been identified now, and neurotoxicity appears to be correlated with the amount of nonfibrillar oligomers (Finder and Glockshuber 2007). McLean et al. suggest that low molecular weight oligomers are more toxic than the larger β-fibrils (McLean et al. 1999; Roychaudhuri et al. 2009).

The deleterious effect of copper-bound or copper-free WT $A\beta_{40}$ on neurons is remarkable, even at micromolar concentrations according to our results. However, $^{13}A\beta_{40}$ with a residue substitution rendered the peptide less toxic, and this lack of neurotoxicity could be attributed to reduced capability of Aβ variant binds to cell surface membranes, congruent with the notion that Aβ–lipid membrane interactions are central to the cause of neurotoxicity in AD (Butterfield and Boyd-Kimball 2004; Puglielli et al. 2005). We do notice a decrease of copper-induced β aggregation in both wild-type and variant peptides, resulting in increased cytotoxicity as quantitated by MTT and LDH assay compared to peptides alone. One explanation for this finding is that Aβ–copper compound is capable of promoting potentially toxic, pro-oxidative reactions, resulting in the generation of reactive oxygen species (Huang et al. 1999).

Growing evidence supports a pivotal role for oxidative stress in the etiology of AD (Cuajungco et al. 2000; Butterfield et al. 2007). In contrast with the initial hypothesis, demonstrating Aβ spontaneously generates peptide-derived free radicals and even hydroxyl radical (Hensley et al. 1994), our results suggest that little amount of H_2O_2 releases to extracellular fluid by Aβ peptides alone. We suppose it is due to metal ions contamination in the test solution unable to remove, and this conclusion is consistent with some other study (Turnbull et al. 2001). We also note that WT $A\beta_{40}$ alone generates less amount of H_2O_2 but still more toxic than copper-bound $^{13}A\beta_{40}$ that generates more H_2O_2. Whereas the mechanism of Aβ toxicity in AD remains unsolved, a general theme is beginning to emerge whereby the interaction of Aβ with lipid membranes is a necessary step in neurotoxicity (Puglielli et al. 2005; Tickler et al. 2005). These interactions lead to changes in membrane fluidity resulting in depolarization and disorder, pore/channel formation, disrupted calcium homeostasis, and lipid peroxidation via membrane-associated free radical formation (Butterfield and Boyd-Kimball 2004; Puglielli et al. 2005).

Due to the negative correlation between $A\beta Cu^{2+}$-induced neurotoxicity and the contents of β aggregation, it is possible that non-β aggregates are most likely responsible for the neuronal damage and cognitive impairment observed in AD brain. Our findings are in accordance with the lack of correlation between fibrillar aggregates and oxidative stress and neurotoxicity (Drake et al. 2003; Dai et al. 2007). Moreover, the substitution of arginine for histidine could partly decrease the cytotoxicity of Aβ. Studies are still on its way to further elucidate the mechanism by which amyloid plaques are associated with Aβ-induced cytotoxicity. In any case, inhibition of Aβ accumulation in the brain could be a possible therapeutic approach to lower the risk of developing Alzheimer's disease.

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