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Effect of aldehydes derived from oxidative deamination and oxidative stress on β-amyloid aggregation; pathological implications to Alzheimer's disease

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Summary Formaldehyde and methylglyoxal are generated *via* deamination from methylamine and aminoacetone respectively catalyzed by semicarbazide-sensitive amine oxidase (SSAO). Malondialdehyde (MDA) and 4-hydroxynonenal (HNE) are end products of lipid peroxidation due to oxidative stress. These aldehydes are capable of inducing protein cross-linkage. Elevated levels of aldehydes were found in Alzheimer's disease (AD). These reactive metabolites may potentially play important roles in β-amyloid (Aβ) aggregation related to the pathology of AD. In the present study thioflavin-T (ThT) fluorometry, an immuno-dot-blot assay and atomic force microscopy (AFM) were employed to reveal the effect of aldehydes on Aβ aggregation *in vitro*. The target on Aβ for interaction with formaldehyde was identified. The results support the involvement of endogenous aldehydes in amyloid deposition related to AD.

Keywords: Alzheimer's disease, β-amyloid, aldehydes, SSAO, AFM, fibrillogenesis

Introduction

Extracellular β-amyloid (Aβ) deposition is a major pathological hallmark of Alzheimer's disease (AD) (Selkoe, 2002). Aβ peptides readily form β-sheets structure, oligomers, protofibrils, fibrils, and subsequently co-aggregate with other proteins to produce senile plaques (Stanyer et al., 2004; Ma et al., 1994). Aβ is cytotoxic and capable of triggering oxidative stress and neurodegeneration (Loo et al., 1993). Its oligomers (i.e. 5–10 Aβ monomers) are the most toxic forms (Klein et al., 2004; Kayed et al., 2003). AD shares common pathological features and risk factors with other vascular disorders, such as atherosclerosis and diabetes mellitus (Messier, 2003). A large body of literature suggests that vascular disorders are involved in the pathogenesis of AD (Jellinger, 2002). Substantial Aβ deposits were found to be associated with cerebral vasculature degeneration (Coria et al., 1988; Kawai et al., 1993). Aβ isolated from the vascular tissues contains significantly less isomerized and racemized aspartyl residues than does the neuritic Aβ plaques suggesting that the vascular amyloid is "younger" (Roher et al., 1993).

Semicarbazide-sensitive amine oxidase (SSAO), an enzyme located on the plasma membrane surface of vascular smooth muscle and endothelial cells, may contribute to the pathogenesis of vascular disorders (Yu and Zuo, 1993; Yu and Deng, 1998). Increased SSAO activity is considered a potential risk factor for vascular disorders (Boomsma et al., 2000). SSAO catalyzes the deamination of endogenous substrates methylamine and aminoacetone producing toxic formaldehyde and methylglyoxal respectively, as well as hydrogen peroxide and ammonia. Formaldehyde derived from SSAO-mediated deamination of methylamine cross-links with proteins *in vivo* (Gubisne-Haberle et al., 2004). The enzyme is co-localized with cerebral vascular Aβ deposits in Alzheimer brains (Ferrer et al., 2002). Serum SSAO activity is elevated in different conditions of vascular disorders (see review by Yu et al., 2003) including AD (del Mar Hernandez et al., 2006). Methylglyoxal can also be synthesized *via* other metabolic pathways and is well known to contribute to advanced protein glycation in diabetes (Thornalley, 2002). Diabetes mellitus is a well-known risk factor for AD (Luchsinger et al., 2001). Interestingly, CSF samples from the AD patients exhibit significantly increased levels of methylglyoxal than that of the control

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subjects and this has been implicated in amyloidosis (Kuhla et al., 2005).

Oxidative stress, which leads to lipid peroxidation, are enhanced in aging and AD (Subbarao et al., 1990). Malondialdehyde (MDA), 4-hydroxynonenal (HNE), acrolein, *etc.* are end products of lipid peroxidation. These aldehydes are also very reactive and induce protein cross-linkage. Increased levels of MDA and HNE were detected in the plasma of AD patients (McGrath et al., 2001; Dib et al., 2002) as well as carbonyl protein adducts have been detected in the senile plaques (Dei et al., 2002; Sayre et al., 1997).

In the present study we report the interactions of reactive aldehydes with Aβ *in vitro*. Four aldehydes, which are generated from SSAO-mediated deamination and lipid peroxidation, were assessed. Different methodologies were employed to assess the effects of these aldehydes on Aβ β-sheet formation, oligomerization and fibrillogenesis *in vitro*.

Experimental procedures

Preparation of seed-free $A\beta_{1\text{-}40}$

$A\beta_{1\text{-}40}$ was dissolved in 100% hexafluoroisopropanol (HFIP) (1 mg/ml) and sonicated in a water bath sonicator at 4°C for 2 h. The HFIP was removed under a gentle stream of nitrogen. The seed-free $A\beta_{1\text{-}40}$ crystals were dissolved in nanopure water and used immediately. The purity of $A\beta_{1\text{-}40}$ monomers free of oligomers was ensured using A11 antibody from BioSource (Camarillo, CA, USA).

Interactions of $A\beta_{1\text{-}40}$ with aldehydes

Freshly prepared seed-free $A\beta_{1\text{-}40}$ (200 μM) was incubated in the presence or absence of various concentrations (ranging from 1 μM to 10 mM) of aldehydes in PBS (pH 7.4, 20 mM) in 0.2 ml Eppendorf tubes at 37°C without shaking or pipetting. For the AFM imaging experiment volatile buffer (ammonia/formic acid, 20 mM, pH 7.4) was used to avoid salt crystallization after drying of the samples.

ThT fluorometry

A ThT (2 μM, in 50 mM glycine-NaOH buffer, pH 9.0) reaction solution of 200 μl reacted with $A\beta_{1\text{-}40}$ (2 μM) and was transferred to the black microfluor plates at different time intervals for fluorescence readings. Fluorescence was monitored at λex 450 nm and λem 482 nm using a Spectra Max Gemini XS fluorescence reader (Molecular Devices, Sunnyvale, CA, USA).

FMOC-HPLC

The bond between formaldehyde and $A\beta_{1\text{-}40}$ was stabilized by $NaBH_4$ (10 mM) and then hydrolyzed by HCl (6N) at 110°C for 24 h. FMOC derivatization-HPLC was carried out as described by Kazachkov and Yu (2005).

Dot-blot assay

Two μl aliquot of each sample was spotted onto the nitrocellulose membrane and air dried. The membrane was blocked in 10% non-fat dry milk TBST overnight and washed. A11 antibody (1 μg/ml in 5% non-fat dry milk TBST) was added to cover the membrane and incubated for 1 h and washed. Then the membrane was incubated with anti-rabbit secondary antibody and revealed with peroxidase catalyzed reaction.

AFM imaging

Under ambient conditions 1 μl aliquot of formaldehyde and methylglyoxal samples as described was placed on freshly cleaved mica (Structure Probe Inc., West Chester PA, USA) until dry. MDA and HNE samples were imaged using wet method, in which 10 μl aliquot was placed onto freshly cleaved mica in a sample well for 2 min, and subsequently diluted and imaged in 250 μl PBS (20 mM, pH 7.4). The scanning was carried out on a Pico-SPM (Molecular Imaging Inc., Tempe, AZ, USA) with an AFM M-scanner in MAC mode. The cantilever force constant was approximately 1.2–5.5 N/m, and had a resonant frequency of approximately 60–90 kHz under ambient conditions. All measurements were taken at the ratio of the set-point oscillation amplitude to free air oscillation amplitude of 0.80 with the instrument mounted in a vibration isolation system. The scan rate was 1–2 lines/s (256 pixel per line) for all images. Each image was conducted in 2 opposite directions simultaneously and the final image was averaged.

Results and discussion

ThT fluorometry revealed that formaldehyde, methylglyoxal and malondialdehyde significantly enhance the β-sheet formation of $A\beta_{1\text{-}40}$. The reactions are time- and concentration-dependent manner and saturable. As can be seen in Fig. 1, methylamine, in the presence of SSAO enhances β-sheet formation of $A\beta_{1\text{-}40}$. Methylamine by itself does not affect β-sheet formation of $A\beta_{1\text{-}40}$. The SSAO enzyme preparation quenches the fluorescence. Methylamine in the presence of SSAO enhances β-sheet formation. SSAO inhibitor, MDL-72974A, significantly blocked the formaldehyde effect.

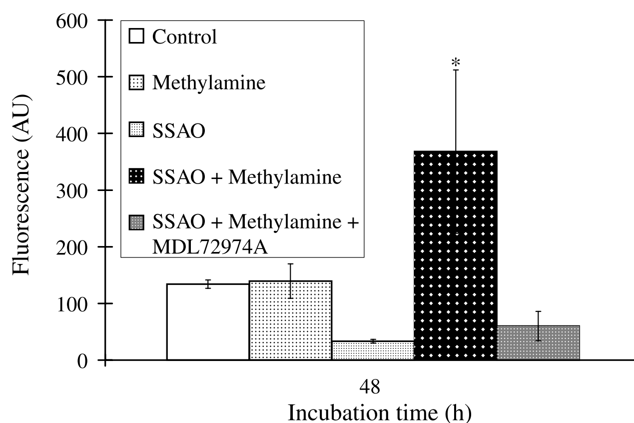

Fig. 1. Effect of formaldehyde, derived from methylamine deamination, on β-sheet formation. $A\beta_{1\text{-}40}$ was incubated with methylamine (1 mM), SSAO (1.3 nmol/min/mg), in the presence or absence of MDL 72974A (1×10^{-5} M) for 48 h. Data represent mean (n = 3) ± SD. $^{*}p < 0.05$, compared to corresponding control values

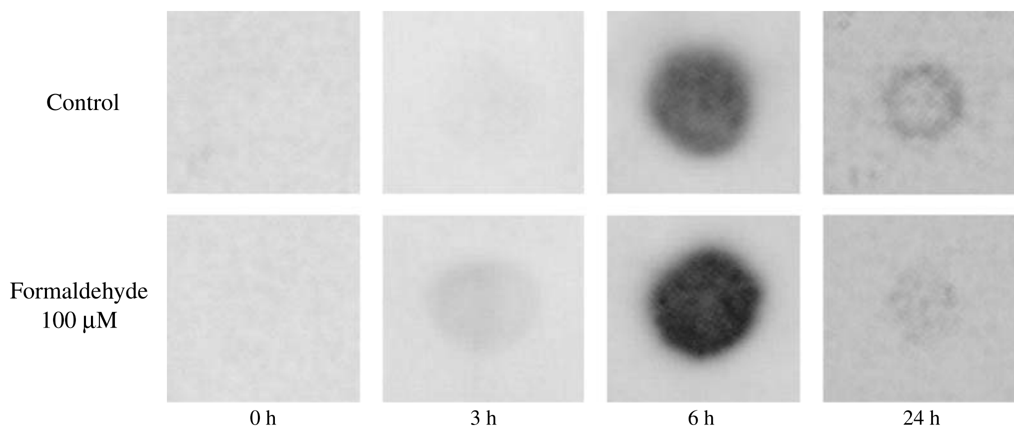

Fig. 2. Effect of formaldehyde on $A\beta_{1\text{-}40}$ oligomerization using a dot-blot assay. Formaldehyde (100 μM) was incubated with seed-free $A\beta_{1\text{-}40}$ (200 μM). Aliquots were applied on a nitrocellulose filter at different time and assessed immunochemically using oligomer specific A11 antibody. Upper row: $A\beta_{1\text{-}40}$ alone; lower row: $A\beta_{1\text{-}40}$ in the presence of formaldehyde

Figure 2 shows the results of a dot-blot assay, where specific A11 antibody for Aβ oligomer (Kayed et al., 2003) was employed for the test. $A\beta_{1\text{-}40}$ was incubated in the presence or absence of aldehydes. Freshly prepared seed-free $A\beta_{1\text{-}40}$ was not stained. After 6 h of incubation more oligomers were formed in the presence of formaldehyde. After 24 h, the positive dots were diminished. Advanced aggregation of Aβ, i.e. protofibrils and fibrils, are not be recognized by the A11 antibody. Methylglyoxal and MDA exhibit very similar effect (data not shown).

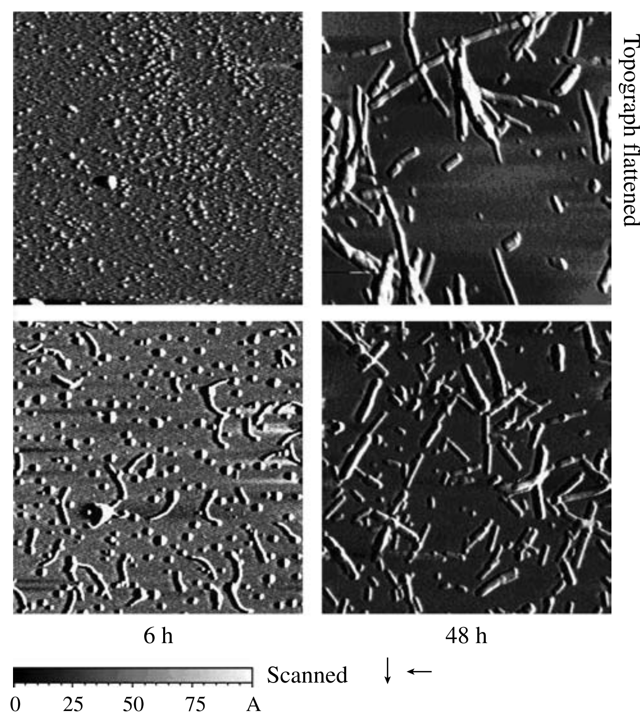

Fig. 3. AFM imaging of the effect of formaldehyde on Aβ aggregation. $A\beta_{1\text{-}40}$ (200 μM) was incubated in the presence or absence of formaldehyde (1 mM). One μl of diluted samples (1:100) was placed on freshly cleaved mica, quickly air-dried and subject of AFM. Upper row, $A\beta_{1\text{-}40}$ alone; lower row: $A\beta_{1\text{-}40}$ aggregated in the presence of formaldehyde. The image size is 5000 × 5000 nm

AFM imaging reveals the morphology of protein aggregation intermediates. As can be seen in Fig. 3, $A\beta_{1\text{-}40}$ alone forms oligomers in 6 h. Formaldehyde significantly increases both the number and the size of $A\beta_{1\text{-}40}$ oligomers. Protofibrils already began to appear. After 48 h, substantial amount of protofibrils aggregates formed. Formaldehyde also increases the formation of more $A\beta_{1\text{-}40}$ protofibrils. Methylglyoxal and MDA also exhibit similar effect as of formaldehyde and HNE has no obvious effect (data not shown).

$A\beta_{1\text{-}40}$ has 2 lysine and 1 arginine residues, which are expected to be the most likely sites for interaction with formaldehyde. Both Aβ alone and formaldehyde-Aβ adducts were incubated with sodium borohydride (to make the Shiff's base covalent) and then hydrolyzed by HCl. The hydrolysates were derivatized with FMOC and the amino acid residues were analyzed by HPLC. As can be seen in Fig. 4, N-methyl-lysine peak was only detected in the hydrolysates of the formaldehyde-$A\beta_{1\text{-}40}$ adducts. This is clearly evident that formaldehyde interacts with the lysine residue of Aβ.

Formaldehyde, methylglyoxal, MDA and HNE are derived from different pathways, such as oxidative deamination, lipid peroxidation and hyperglycemia. Both oxidative stress and vascular disorders, such as diabetes mellitus are risk factors for Alzheimer's disease (Jellinger, 2002). Interestingly, protein-aldehyde adducts have been identified in the senile plaques (Dei et al., 2002; Sayre et al., 1997). Mounting evidence indicates that increased SSAO activity

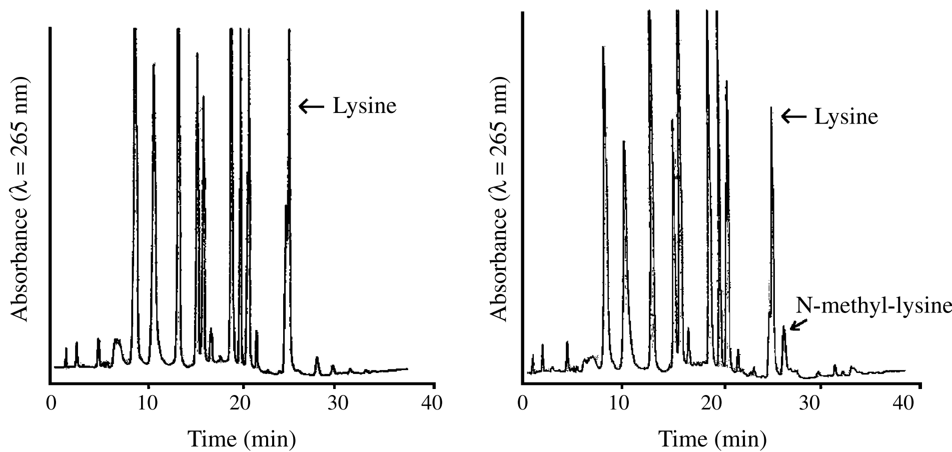

Fig. 4. Detection of N-methyl-lysine in formaldehyde-treated $A\beta_{1\text{-}40}$ by FMOC-HPLC. $A\beta_{1\text{-}40}$ (200 µM) was incubated in the absence or presence of formaldehyde (10 mM) for 48 h. Left: $A\beta_{1\text{-}40}$ alone and arrow indicates the lysine peak; right: formaldehyde treated $A\beta_{1\text{-}40}$. Arrows indicate the lysine and N-methyl-lysine peaks

may contribute to vascular disorders (Yu et al., 2003). Recently, an increase in SSAO has also been found in severe Alzheimer's patients (del Mar Hernandez et al., 2005).

An Aβ molecule composes are 2 lysines and 1 arginine, which are vulnerable for interaction with formaldehyde and other aldehydes (Gubisne-Haberle et al., 2004). We demonstrated that *in vitro*, aldehydes generated from different sources were able to enhance Aβ aggregation at every stage using different techniques. In fact Schiff's base regarding lysine residue has been identified in the present study. We also observed that formaldehyde not only increase the rate of oligomerization, but also the size of the oligomers. The Aβ oligomers (i.e. 5–16 Aβ monomers) are known to be most toxic towards neurons (Klein et al., 2004; Kayed et al., 2003). It is not surprising that aldehydes can also be involved in advanced intra-molecular and intermolecular aggregation of Aβ, such as formation of protofibrils, fibril and senile plaques. However, it remains to be established whether the formaldehyde altered the toxicity of the oligomers.

Since aldehydes are very reactive, soon as they are generated, they would interact with adjacent proteins. The effect on protein misfolding may be insignificant to cause immediate damage, but it may proceed chronically and accumulatively until damage becomes apparent later. This is consistent with the chronic development of amyloidosis in Alzheimer's brain or pancreas associated to diabetic complications. The co-location of Aβ deposits and SSAO on the surface of cerebral blood vessels supports the hypothesis that SSAO-mediated deamination is involved in Aβ deposition and pathogenesis of the disease. Aldehydes generated from lipid peroxidation (a result of oxidative stress) may also enhance Aβ aggregation. However, aldehydes synthesized intra-cellularly would probably be quickly metabolized by dehydrogenase. Neither aldehyde dehydrogenase nor the cofactor are readily available outside the cells. This may explain why Aβ depositions are present primarily extracellularly.

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