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Effect of *N*-homocysteinylation on physicochemical and cytotoxic properties of amyloid β-peptide

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ABSTRACT

Hyperhomocysteinemia has recently been identified as an important risk factor for Alzheimer's disease (AD). One of the potential mechanisms underlying harmful effects of homocysteine (Hcy) is site-specific acylation of proteins at lysine residues by homocysteine thiolactone (HCTL). The accumulation of amyloid β-peptide (Aβ) in the brain is a neuropathological hallmark of AD. In the present study we were interested to investigate the effects of *N*-homocysteinylation on the aggregation propensity and neurotoxicity of $A\beta_{1-42}$. By coupling several techniques, we demonstrated that the homocysteinylation of lysine residues increase the neurotoxicity of the Aβ peptide by stabilizing soluble oligomeric intermediates.

Structured summary of protein interactions:
A Beta 1-42 and **A Beta 1-42** bind by fluorescence technology (View interaction)
A Beta 1-42 and **A Beta 1-42** bind by electron microscopy (View interaction)

1. Introduction

Elevated plasma level of homocysteine (hyperhomocysteinemia) has recently been identified as an independent risk factor for the development of cardiovascular diseases and neurodegenerative disorders (Dementia, Alzheimer's disease, Schizophrenia) [1–4]. Several studies have shown that one of the likely mechanisms underlying harmful effects of homocysteine (Hcy) is a chemical modification of protein by homocysteine thiolactone (HCTL), a highly reactive cyclic thioester of Hcy [5–8], which is formed by methionyl-tRNA synthetase [5,6,9] in the human body [10,11] and is greatly elevated in CBS and MTHFR deficiencies [12].

It has been demonstrated that HCTL preferentially forms amide bonds with ε-amino group of protein lysine residues in a non-enzymatic mechanism; a process referred to as "protein *N*-homocysteinylation" [13]. This modification neutralizes the positive charge of the ε-amino group of protein lysine residue and introduces a thiol group. This causes large changes in structure and biochemical properties of the protein [7,8,14].

Alzheimer's disease (AD), the most common age-related neurodegenerative disorder, is a progressive disease that results in the death of specific neuronal populations and a devastating loss of cognitive function. It is well known that aggregation of amyloid β-peptide (Aβ) plays a central role in mediating neurotoxicity in AD [15–17].

Aβ is a normal product of cellular metabolism which has been implicated in numerous physiological activities including cell survival and synaptic activity [18]. It is a 39–43 amino acid peptide generated by sequential proteolytic cleavage of the transmembrane amyloid precursor protein (APP) catalyzed by β- and γ-secretases [19]. The concentration and physicochemical properties of Aβ species and the relative abundance of $A\beta_{1-42}$ are the likely determinants that switch its function from physiological to pathological [20].

The aggregation process involves formation of intermediate oligomers that elongate to form fibrils. In the first "amyloid cascade hypothesis", Aβ fibrils were supposed to be responsible for the initiation of AD [19]. However, subsequent studies have shown that neurological dysfunction and degeneration can be linked to much smaller, oligomeric intermediates of Aβ, which have now been incorporated into a revised version of the amyloid cascade hypothesis [21–23].

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β-peptide; Hcy, homocysteine; HCTL, homocysteine thiolactone; ThT, thioflavin T; CD, circular dichroism; TEM, transmission electron microscopy; LDH, lactate dehydrogenase; MTT, 3-[4,5-dimethylthiazol-2-y]-2,5-diphenyl tetrazolium bromide

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There are two regions in Aβ that play an important role in the aggregation process and eventually in pathogenesis of AD. Previous studies have demonstrated that the key aggregation motif of Aβ (16KLVFF20), in the central region, is an essential sequence but not sufficient for the self-assembly of Aβ. $A\beta_{16-20}$ binds to the homologous regions $A\beta_{17-21}$ or $A\beta_{18-22}$ and forms an antiparallel β-sheet structure [24–27]. The 21–30 region of Aβ ($A\beta_{21-30}$) is another important sequence involved in the aggregation process. The formation of β-turn structure in this region nucleates the folding process of Aβ monomer, and initiates the molecular association to form oligomeric intermediates [28–31]. The intra- or intermolecular salt bridge between Lys28 and Glu22/Asp23 in this region is one of the important factors that contribute to the stability of the turn [30,31].

Despite the proven increment of plasma homocysteine level and its metabolite (HCTL) in AD, their effects on the Aβ structure and its neurotoxicity are not well understood [2]. The Aβ is generally considered to be fully unfolded in the monomeric state in aqueous solution [2,32] and therefore, its lysine residues in regions 16–20 and 21–30 can be *N*-homocysteinylated by HCTL.

According to these facts, we were interested to investigate the effects of *N*-homocysteinylation in the mentioned regions on the aggregation propensity and neurotoxicity of $A\beta_{1-42}$. In the present study, we demonstrated that the homocysteinylation of lysine residues increase the neurotoxicity of the Aβ peptide by stabilizing soluble oligomeric intermediates.

2. Materials and methods

2.1. Materials

Human $A\beta_{1-42}$ was purchased from r-Peptide Company (USA). The cell culture medium (RPMI 1640), penicillin–streptomycin, fetal bovine serum (FBS) and horse serum were purchased from Gibco BRL (Life technology, Paisley, Scotland). The culture plates were obtained from Nunc (Denmark). Ethidium bromide (EtBr) and acridine orange (AO) were purchased from Pharmacia LKB Biotechnology (Sweden). MTT [3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazoliumbromide], thioflavin T (ThT), L-Hcy-thiolactone (HCTL), γ-butyrolactone and 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemicals Co. (Germany).

2.2. Peptide preparation and modification

Peptide solution was prepared according to the manufacturer's instructions. Briefly, $A\beta_{1-42}$ was re-suspended in 1% NH_4OH at a concentration of 1 mg/ml and sonicated for 1 min, then stored at −70 °C until used. $A\beta_{1-42}$ has no free sulfydryl group and possesses only two lysine residues (Lys16 and Lys28) which can be modified by HCTL. In order to *N*-homocysteinylate $A\beta_{1-42}$, peptide solution was diluted with phosphate buffer (50 mM, pH 7.4) containing 400 μM of HCTL to 100 μM and incubated with gentle shaking at 37 °C for 6 h. To investigate the probable effect of lactone ring in HCTL on the aggregation process, γ-butyrolactone (analogue of HCTL) was used in similar concentrations and incubation times. Control experiment was carried out in the absence of lactone compounds. At the end of incubation, the specimens were dialyzed, using cellulose acetate membrane with the molecular weight cut-off of 1-kDa, to remove different types of free lactones. In order to determine the extent of modified peptide, the levels of peptide sulfhydryls were analyzed using the Ellman's test [33]. Briefly, it is based on the reaction of the thiolate anion ($R–S^-$) with Ellman's reagent ($DTNB^{2-}$) whereupon a mixed disulfide ($R–S–TNB^-$) and one equivalent of TNB^{2-} is formed that exhibits intense light absorption at 412 nm. The amount of free sulfhydryl groups per peptide molecule (amount of modified peptide) for the peptide pre-incubated with HCTL was calculated to be 0.94 ± 0.03. No absorption at 412 nm was detected for the control peptide and the peptide pre-incubated with γ-butyrolactone as well. To prepare $A\beta_{1-42}$ fibrils, pre-incubated peptide solutions were incubated at 37 °C for a week. At the indicated times, the extent of fibril formation was assayed by ThT fluorescence and electron microscopy, as described below.

2.3. Thioflavin T assay

Thioflavin T (ThT), a dye that specifically binds to β-sheet structures, is widely used for the identification and quantification of amyloid fibrils [34]. A 100 μM aqueous stock solution of ThT was prepared and filtered through a 0.2 μm filter. The different aged samples of $A\beta_{1-42}$ were diluted in 10 mM phosphate buffer (pH 7.4) to a final concentration of 5 μM. Then, 15 μl of stock ThT was added to 140 μl of each test sample. Fluorescence intensity was monitored immediately using a Varian-spectrofluorometer (Model: Cary Eclipse) with the excitation and emission wavelengths of 450 and 482 nm, respectively.

2.4. Electron microscopy

The ultrastructural characteristics of Aβ-aggregates were investigated by electron microscopy. To prepare the specimens for transmission electron microscopy (TEM), 5 μL aliquot of each sample was spotted onto a formvar/carbon-coated copper grid and incubated for 1 min. The TEM grids were then blotted and washed twice in distilled H_2O prior to negative staining with 2% uranyl acetate for 1 min. Samples were examined using a Hu-12A TEM (Hitachi, Japan), operating at 75 kV.

2.5. Circular dichroism (CD) spectroscopy

Changes in secondary structure of different $A\beta_{1-42}$ samples were analyzed during aggregation by circular dichroism (CD) spectroscopy. CD spectra were recorded on an Aviv model 215 Spectropolarimeter (Lakewood, NJ, USA). Measurements were performed in the spectral range of 195–260 nm using a quartz cuvette (Helma) with a path length of 1 mm and a scan rate of 20 nm/min. Investigation of secondary structure during aggregation was performed at a peptide concentration of 40 μM. A composite buffer containing 10 mM PB at pH 7.4 was prepared and its contribution was subtracted in the CD spectra of $A\beta_{1-42}$ to give a normalized spectrum. Spectra were converted from machine units in millidegrees to delta epsilons.

2.6. Cell culture

PC12 rat pheochromacytoma cells, obtained from Pasteur Institute (Tehran, Iran), were cultured at a density of 1×10^5 cells/ml in RPMI 1640 medium supplemented with 5% horse serum, 10% fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin, and incubated at 37 °C in the presence of 5% CO_2.

2.7. Neurotoxicity assays

Metabolic activity of the assayed cells was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. PC12 rat pheochromacytoma cells were chosen for assessing cytotoxicity because they have been identified as sensitive to the toxic effects of Aβ using MTT [35]. To examine precisely the correlation between inhibition of MTT reduction and cell death, both experiments were performed with the same cultures. Cells were seeded at a density of 7500 cells/well in 96-well plates in 100 μl of fresh medium. After 24 h, the cells were