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Extracellular chaperones prevent Aβ42-induced toxicity in rat brains

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ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterised by cognitive decline, formation of the extracellular amyloid β (Aβ42) plaques, neuronal and synapse loss, and activated microglia and astrocytes. Extracellular chaperones, which are known to inhibit amyloid fibril formation and promote clearance of misfolded aggregates, have recently been shown to reduce efficiently the toxicity of HypF-N misfolded oligomers to immortalised cell lines, by binding and clustering them into large species. However, the role of extracellular chaperones on Aβ oligomer toxicity remains unclear, with reports often appearing contradictory. In this study we microinjected into the hippocampus of rat brains Aβ42 oligomers pre-incubated for 1 h with two extracellular chaperones, namely clusterin and α2-macroglobulin. The chaperones were found to prevent Aβ42-induced learning and memory impairments, as assessed by the Morris Water Maze test, and reduce Aβ42-induced glia inflammation and neuronal degeneration in rat brains, as probed by fluorescent immunohistochemical analyses. Moreover, the chaperones were able to prevent Aβ42 colocalisation with PSD-95 at post-synaptic terminals of rat primary neurons, suppressing oligomer cytotoxicity. All such effects were not effective by adding pre-formed oligomers and chaperones without preincubation. Molecular chaperones have therefore the potential to prevent the early symptoms of AD, not just by inhibiting Aβ42 aggregation, as previously demonstrated, but also by suppressing the toxicity of Aβ42 oligomers after they are formed. These findings elect them as novel neuroprotectors against amyloid-induced injury and excellent candidates for the design of therapeutic strategies against AD.

1. Introduction

Alzheimer's disease (AD) is a progressive human neurodegenerative disorder characterised by cognitive decline, neuronal and synapse loss, and the formation of two pathological lesions, extracellular amyloid plaques composed largely of the amyloid-β peptide (Aβ), and intracellular neurofibrillary tangles formed by the hyperphosphorylated tau protein [1]. Impairment in synaptic function and plasticity might be the most significant early event in the pathogenesis of AD [2,3], with such events being particularly relevant in the hippocampal region [4].

Clinicopathological hallmarks of AD correlate with the presence of soluble Aβ oligomers as the principal neurotoxic agent [2,5,6]. It has been demonstrated that synaptic plasticity is rapidly disrupted by Aβ42 oligomers [7–12]. Moreover, Aβ oligomers have been found to colocalise with postsynaptic density protein 95 (PSD-95) in hippocampal and cortical primary neurons [13–15] and impair cognitive functions of animal models when microinjected in their brains [5,16]. PSD-95 is a critical scaffolding component of postsynaptic terminals found in excitatory Central Nervous System (CNS) signaling pathways [17] and clusters of PSD-95 have been established previously as definitive markers for postsynaptic terminals in mature hippocampal and cortical cell cultures [18–20]. In addition, the concentration of PSD-95 in the brain of AD patients is significantly lower than that of normal brains [21]. Aβ also plays a central role in the pathogenesis of AD as a mediator of oxidative stress and neuronal cell apoptosis [22–29].

Abbreviations: α2M, α2-macroglobulin; AD, Alzheimer's disease; Aβ, amyloid-beta peptide; BSA, bovine serum albumin; CNS, Central Nervous System; Clu, clusterin; CM-H2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; DMSO, dimethylsulfoxide; DTT, dithiothreitol; D-PBS, Dulbecco's phosphate-buffered saline; ED, embryonic day; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; Hp, haptoglobin; Iba-1, ionised calcium binding adaptor molecule 1; MWM, Morris water maze test; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NBM, neurobasal medium; Neu-N, neuronal nuclei; NGS, normal goat serum; HypF-N, N-terminal domain of the HypF protein from Escherichia coli; PMSF, phenylmethylsulfonyl fluoride; PSD-95, postsynaptic density protein 95

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Neurons are not the only cell type in the brain that is affected in AD; vulnerable brain regions exhibit activated microglial cells and astrocytes, which often associate with amyloid deposits suggesting a central role of these non-neuronal cells in AD pathology [30–33]. It has also been suggested that atrophy of astroglia, which occurs at the early stages of AD and is likely to accompany early stages of other neurodegenerative diseases, determines synaptic malfunction, synaptic loss, and cognitive deficits [34].

Molecular chaperones are proteins that play a central role in the avoidance of protein misfolding and aggregation [35,36]. Chaperones are known to have a range of different functions, such as assisting protein folding [35], inhibiting protein aggregation [37], causing the disaggregation of aberrant protein oligomers [38], and facilitating the degradation of misfolded proteins [39]. In particular, extracellular chaperones, such as clusterin (Clu), haptoglobin (Hp) and α_2-macroglobulin (α_2M), have been shown to colocalise with amyloid plaques in AD [40–42], inhibit Aβ fibril formation *in vitro* [43–47] and promote the clearance of protein aggregates via endocytosis [48–50]. It is unclear, however, whether extracellular chaperones inhibit directly the toxicity of Aβ oligomers, as reports have often been contradictory, with a few of them supporting a toxicity-enhancing role [7,51,52], others claiming a protective action [43,53,54] and one report describing a dose-dependent effect [46].

Our recent data showed that a representative set of chaperones can inhibit efficiently the toxicity of extracellularly added protein oligomers formed by three different peptides and proteins in SH-SY5Y neuroblastoma cells, provided the chaperones are incubated with the preformed oligomers before the resulting mixtures are added to the extracellular medium of the cells [54]. Such an inhibition is very effective as it occurs even at low chaperone concentration [54]. Using preformed HypF-N oligomers as a representative toxic species, the protective effect of the chaperones was shown to result from the ability of these proteins to bind to the oligomers and promote their further assembly into larger species, in the absence of any significant reorganisation of their internal molecular structure [54].

Following this encouraging result obtained with HypF-N oligomers and cultured neuronal cells, the purpose of this study was to determine whether pre-incubation of $A\beta_{42}$ oligomers with two types of extracellular chaperones can prevent $A\beta_{42}$-induced hippocampal injury in rats following hippocampal microinjection and to determine how it correlates with reduced brain pathology and behavioural injuries. In these experiments, toxic oligomers from $A\beta_{42}$ were formed, incubated with Clu or α_2M, and then injected into rat brains or added to the extracellular medium of rat primary neurons. Thus, the oligomers were formed before adding the chaperones, with the aim of assessing whether the protective action of the latter also includes neutralisation of toxic oligomers after they have formed. We demonstrate that the chaperones ameliorate the $A\beta_{42}$-mediated learning and memory impairment in injected rats, reduce $A\beta_{42}$-induced glia inflammation and neuronal degeneration in injected rat brains and protect against the oxidative-stress conditions and apoptotic cell death associated with $A\beta_{42}$ exposure. In addition, the extracellular chaperones were found to prevent $A\beta_{42}$ binding to PSD-95 in dendritic spines, neutralising oligomer toxicity in cultured rat primary neurons.

2. Materials and methods

2.1. Formation of $A\beta_{42}$ oligomers

$A\beta_{42}$ were obtained from Sigma-Aldrich (St. Louis, MO). Human Clu and α_2M were purified as previously reported [55,56]. $A\beta_{42}$ oligomers were prepared as previously described [57] and resuspended in F12-medium to 12 μM. Native proteins were diluted to 12 μM into the same medium. Oligomers were incubated in the medium for 1 h at 37 °C while shaking, without or with chaperones, and then injected into rats or added to cultured cells. The oligomer:chaperone molar ratio was 10:1 for Clu and 100:1 for α_2M ($A\beta_{42}$ is considered as monomers, Clu as αβ dimers and α_2M as a tetramer, according to the functional oligomeric state).

2.2. Rat model and cell cultures

Thirty-six three-month-old (220–250 g) male Wistar rats (Harlan Nossan, Correzzana, Italy) were housed in macrolon cages until surgery and maintained on a 12-hour light/dark cycle at 23 °C. All animal manipulations were performed *in vivo*, according to the European Community guidelines for animal care (DL 116/92).

Primary cortical and hippocampal neurons were obtained from embryonic day (ED)-17 Sprague-Dawley rats (Harlan) as described in Bongers et al. [58]. The experimental procedures were in accordance with the standards set forth in the Guide for the Care and Use of Laboratory Animals (published by the National Academy of Science, National Academy Press, Washington, D.C.). The uteri were removed from the gravid rat under anaesthesia. Cerebral cortices and hippocampi were dissociated in sterile Dulbecco's phosphate-buffered saline (D-PBS; Sigma), and neurons isolated in the same medium containing trypsin (0.5% in D-PBS) for 10 min at 37 ° C. After centrifugation at 2000 rcf for 5 min, dissociated neurons were re-suspended in neurobasal medium (NBM; Gibco, Invitrogen Corporation, Milan, Italy) supplemented with 2% (v/v) B-27 (Gibco) and 0.5 mmol/L glutamine (Gibco), and then plated in poly-L-lysine-coated 96 or 24-well plates at densities of approximately 1.0×10^4/well and 2.0×10^4/well, respectively. Cultures were maintained in NBM at 37 ° C in a 5% CO_2-humidified atmosphere. Neurons were exposed to 12 μM toxic oligomers 14 days after plating.

2.3. Morris water maze test (MWM)

For *in vivo* studies, $A\beta_{42}$ oligomers and chaperones were suspended in F12-medium with 0.1% DMSO at final concentrations of 0.45 mg/ml (100 μM) and 0.7 mg/ml (10 μM for Clu and 1 μM for α_2M), respectively, and incubated for 1 h at 37 °C while shaking. 1.5 μl aliquots of F12-medium, F12-medium with $A\beta_{42}$ oligomers, F12-medium with $A\beta_{42}$ oligomers and Clu and F12-medium with $A\beta_{42}$ oligomer and α_2M, all containing 0.1% DMSO, were injected into the Cornu Ammonis (CA) 1 molecular layer of the right hippocampus of anaesthetised (Zoletyl, 45 mg/kg plus Carprofen, 5 mg/kg) rats for each condition, using a Hamilton microsyringe, at the following stereotaxic coordinates (in mm): AP,-3.7; L,-2.5 from bregma; and H, 3.5 below the dura [59]. The injections lasted 3 min, and the microsyringe was left in the place for 5 min after completing the infusion. Rats were behaviourally tested in the MWM 1 week after the intrahippocampal injection of the different oligomers ($n = 6$/group). This was considered a reasonable time period to allow us to detect long-term effects of the injected aggregates in light of their likely persistence in tissue.

The water maze apparatus consisted of a circular pool (160 cm diameter and 45 cm high) made of plastic. The pool was filled to a depth of 30 cm with water (22 ± 1 °C) that was made dark by the addition of non-toxic dark paint, and virtually divided into four equivalent quadrants. Rats were tested in the reference memory version of MWM with the procedure previously described [60]; briefly, all rats underwent a reference memory training with a hidden platform (13 cm diameter, submerged 0.5 cm under the water level), placed in the centre of one quadrant of the pool (northwest) for 4 days, with 4 trials per day, with the four starting locations varied between trials. Upon release into the water, the rat was allowed to search the platform for 60 s; if the platform was not located within the maximum time of 60 s, the rat was guided to the location. The rat was allowed 20 s on the platform. Extra-maze visual cues around the room remained in fixed positions throughout the experiment. For each trial, latency to find the platform (maximum 60 s) was recorded by a video-tracking/computer-digitising system (HVS Image, Hampton,