Biochimica et Biophysica Acta 1832 (2013) 2302–2314

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Transthyretin suppresses the toxicity of oligomers formed by misfolded proteins in vitro

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ARTICLE INFO

Article history:
Received 14 May 2013
Received in revised form 16 September 2013
Accepted 18 September 2013
Available online 25 September 2013

Keywords:
Molecular chaperone
TTR protective effect
TTR-mediated oligomer clustering

ABSTRACT

Although human transthyretin (TTR) is associated with systemic amyloidoses, an anti-amyloidogenic effect that prevents Aβ fibril formation in vitro and in animal models has been observed. Here we studied the ability of three different types of TTR, namely human tetramers (hTTR), mouse tetramers (muTTR) and an engineered monomer of the human protein (M-TTR), to suppress the toxicity of oligomers formed by two different amyloidogenic peptides/proteins (HypF-N and $A\beta_{42}$). muTTR is the most stable homotetramer, hTTR can dissociate into partially unfolded monomers, whereas M-TTR maintains a monomeric state. Preformed toxic HypF-N and $A\beta_{42}$ oligomers were incubated in the presence of each TTR then added to cell culture media. hTTR, and to a greater extent M-TTR, were found to protect human neuroblastoma cells and rat primary neurons against oligomer-induced toxicity, whereas muTTR had no protective effect. The thioflavin T assay and site-directed labeling experiments using pyrene ruled out disaggregation and structural reorganization within the discrete oligomers following incubation with TTRs, while confocal microscopy, SDS-PAGE, and intrinsic fluorescence measurements indicated tight binding between oligomers and hTTR, particularly M-TTR. Moreover, atomic force microscopy (AFM), light scattering and turbidimetry analyses indicated that larger assemblies of oligomers are formed in the presence of M-TTR and, to a lesser extent, with hTTR. Overall, the data suggest a generic capacity of TTR to efficiently neutralize the toxicity of oligomers formed by misfolded proteins and reveal that such neutralization occurs through a mechanism of TTR-mediated assembly of protein oligomers into larger species, with an efficiency that correlates inversely with TTR tetramer stability.

1. Introduction

Transthyretin (TTR) is a homotetrameric protein with a total molecular mass of 55 kDa that was originally thought to be synthesized only in the liver, choroid plexus of the brain, and retina [1,2]. Further studies have demonstrated significant and perhaps physiologically important synthesis in the pancreas, kidneys, Schwann cells and neurons [3–7]. In the plasma TTR transports thyroxine (T_4) and the retinol binding protein charged with retinol (RBP), whereas in the cerebrospinal fluid (CSF) TTR is the primary transporter of T4 [8,9]. TTR is also one of 30 human proteins associated with systemic amyloidoses, a group of disorders originally defined pathologically by the formation and aggregation of misfolded proteins which result in extracellular congophilic deposits that impair organ function [10]. Amyloidotic deposition of wild-type TTR occurs in the heart of 10–25% of humans older than 80 years, resulting in senile systemic amyloidosis (SSA), often leading to congestive heart failure [11,12]. TTR fibrillogenesis is accelerated by the presence of any of the approximately 100 different amyloidogenic mutations responsible for early-onset TTR amyloidoses (http://www.amyloidosismutations.com/mut-attr.php). These mutations are responsible for the reduced thermodynamic and/or kinetic stability of native tetrameric TTR [13], leading to the autosomal dominant disorders familial amyloid neuropathy (FAP), familial amyloid cardiomyopathy (FAC) and the rare leptomeningeal amyloidosis [14,15].

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-beta peptide; BSA, bovine serum albumin; CSF, cerebrospinal fluid; CM-H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; DMSO, dimethylsulfoxide; DTT, dithiothreitol; D-PBS, Dulbecco's phosphate-buffered saline; ED, embryonic day; FAC, familial amyloid cardiomyopathy; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NBM, neurobasal medium; HypF-N, N-terminal domain of the HypF protein from *Escherichia coli*; PMSF, phenylmethylsulfonyl fluoride; PSD-95, postsynaptic density protein 95; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; RBP, retinol binding protein charged with retinol; SSA, senile systemic amyloidosis; ThT, thioflavin T; T4, thyroxine; TTR, transthyretin

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http://dx.doi.org/10.1016/j.bbadis.2013.09.011

In spite of its link to human pathology, an anti-amyloidogenic effect that prevents fibril formation of the amyloid β (Aβ) peptide associated with Alzheimer's disease (AD) has been proposed for TTR. In 1993 it was found that in vitro amyloid fibril formation by $Aβ_{40}$ was inhibited by human CSF [16]. Although clusterin and apolipoprotein E were also present in the CSF, the responsible protein was later found to be TTR, through the formation of stable complexes between $Aβ_{40}$ and TTR [17]. In the same study it was also found that purified human TTR inhibited $Aβ_{28}$ fibril formation in vitro. Overexpression of human TTR in transgenic *Caenorhabditis elegans* expressing human $Aβ_{42}$ led to the reduction of ThS-positive Aβ deposits in the muscle cells of the nematodes [18]. Similarly, overexpression of WT human TTR in transgenic AD model mice carrying a mutant of the human Aβ gene led to a reduction in the Aβ deposits in the brains of these mice, while silencing the endogenous murine TTR genes accelerated disease progression in some mouse models [19,20]. Importantly, the reduction of amyloid deposition by overexpression of human TTR prevented the loss of spatial memory and learning usually seen in the AD mice [20].

Analyses of the in vitro interaction between human TTR and Aβ, using predominantly solid phase assay systems, have shown that TTR binds to all forms of Aβ: monomers, oligomers and fibrils [20–23]. The binding is highly dependent on the quaternary structure of TTR, with monomeric TTR binding Aβ with higher affinity than tetrameric TTR [23]. Moreover, the binding occurs with higher affinity for Aβ oligomers, aggregates and fibrils with respect to Aβ monomers [20,21,23,24]. In addition to inhibiting Aβ fibril formation, TTR was also shown to bind to preformed Aβ oligomers and fibrils and reduce their toxicity to murine primary neurons and human neuroblastoma SH-SY5Y cells [25].

In the light of the results obtained so far, one can hypothesize that TTR may act as an endogenous detoxifier of protein oligomers with potential pathological effects, in addition to inhibiting amyloid fibril formation [9]. However, it is not clear if such an effect is specific for Aβ oligomers or more generic, interacting with a variety of misfolded protein oligomers. In addition, previous data do not offer any insight into the mechanism by which TTR inhibits oligomer toxicity and on the TTR form responsible for such an effect. We have previously shown that the in vitro cytotoxicity of protein oligomers formed by Aβ, IAPP and HypF-N can be suppressed by molecular chaperones, with three of the five tested chaperones being extracellular [26]. In particular, it was shown that the chaperones inhibit the toxicity of the oligomers by binding to them and promoting their clustering into large aggregates, in the absence of any disaggregation or structural reorganization within the individual oligomers.

In this study we have examined the ability of three types of TTR having different tetramer stability, i.e., human TTR (hTTR), mouse TTR (muTTR) and an engineered monomer of human TTR carrying the F87M and L110M mutations (making it unable to form tetramers under our experimental conditions) (M-TTR), to suppress the in vitro toxicity of oligomers formed by two different peptides/proteins, i.e., $Aβ_{42}$ and HypF-N. muTTR is the most stable homotetramer that does not dissociate into partially unfolded monomers under physiologic conditions on any timescale [8]; hTTR forms stable tetramers but dissociates to monomers at a finite rate depending on environmental conditions [27], whereas M-TTR exists as a stable monomer [23,28,29]. As recently shown in experiments using these three conformers as well as a series of mutant human TTR tetramers of varying stability, the capacity of TTR to inhibit Aβ fibril formation is generally inversely related to tetramer stability [Li et al., submitted]. We now show that the three types of TTR display different protective effects against oligomer-induced cytotoxicity. We have gained molecular insight into the underlying mechanism by which suppression of oligomer cytotoxicity occurs in vitro, showing that the degree of TTR-mediated protection is greatest for the monomeric M-TTR and least for the most stable murine tetramer. Binding to the HypF-N and $Aβ_{42}$ oligomers appears to promote their clustering into larger aggregates in the absence of any structural reorganization.

2. Materials and methods

2.1. Materials

All reagents were of analytical grade or of the highest purity available. $Aβ_{42}$, bovine serum albumin (BSA), fetal bovine serum (FBS), hen egg white lysozyme (HEWL), pluronic acid F-127, staurosporine and other chemicals were from Sigma-Aldrich (Milan, Italy), unless otherwise stated. Fluo3-AM, calcein-AM and CM-H_2DCFDA (Life Technologies, CA, USA) were prepared as stock solutions in dimethylsulfoxide (DMSO), dried under nitrogen and stored in light-protected vessels at −20 °C until use. Neurobasal medium and B-27 were from Gibco (Life Technologies, CA, USA).

2.2. Cell cultures

Human SH-SY5Y neuroblastoma cells (ATCC Microbiology, Manassas, VA) were cultured in DMEM, F-12 Ham with 25 mM HEPES and $NaHCO_3$ (1:1) and supplemented with 10% FBS, 1.0 mM glutamine and antibiotics. Cell cultures were maintained in a 5.0% CO_2 humidified atmosphere at 37 °C and grown until they reached 80% confluence for a maximum of 20 passages.

2.3. Formation of protein oligomers

HypF-N was expressed, purified, and converted into toxic aggregates as previously reported [30]. $Aβ_{42}$ oligomers were produced as reported [31] and resuspended in the cell culture medium at a concentration of 12 μM. HypF-N oligomers were centrifuged at 16,100 rcf for 10 min, dried under N_2 and resuspended in cell culture media in the absence of cells (for cell biology tests) or in 20 mM potassium phosphate buffer at pH 7.0 (for biophysical/biochemical analysis). As reported, no significant dissolution of the HypF-N oligomers or change in morphology/structure could be detected after this procedure [30]. Native proteins were diluted to a final concentration of 12 μM into the same media. Oligomers were then incubated in the appropriate media for 1 h at 37 °C while shaking, in the absence or presence of each TTR, and then added to cultured cells or subjected to biophysical/biochemical analysis. The protein:TTR molar ratio was 10:1, unless stated otherwise (hTTR, muTTR and M-TTR are considered as tetramers).

2.4. Preparation of TTRs

hTTR, muTTR and M-TTR were prepared and purified in an *Escherichia coli* expression system as described elsewhere [28,32,33]. The three protein variants were purified by gel filtration on a Superdex 75 column (Amersham Biosciences, Inc.) in 10 mM phosphate buffer, 100 mM KCl, 1 mM EDTA pH 7.6 before each experiment to ensure that no aggregates were present in the starting material. Liquid chromatography–electrospray ionization mass spectrometry was used to confirm the molecular weight of the proteins.

2.5. MTT reduction assay

The effect of protein oligomers on cell viability was assessed using SH-SY5Y cells and primary neurons from rat brains seeded in 96-well plates, and using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described [34]. Preformed oligomers of HypF-N and $Aβ_{42}$ (12 μM monomer concentration) were incubated for 1 h in the absence or presence of hTTR, muTTR, M-TTR, haptoglobin, $α_2$-macroglobulin, HEWL or BSA (protein:TTR molar ratio was 10:1 unless otherwise stated; protein:haptoglobin, protein:HEWL and protein:BSA molar ratio was 5:1; protein:$α_2$-macroglobulin was 100:1), and then added to the cells. Each TTR (1.2 μM tetramer concentration) or 12 μM native HypF-N and $Aβ_{42}$ were also used as controls. In additional experiments, 1 μM staurosporine