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Pro-oxidative effects of aggregated transthyretin in human Schwannoma cells

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ABSTRACT

Neurotoxicity mechanisms of amyloidogenic polypeptides such as transthyretin (TTR) are not well understood. Misfolded and aggregated TTRs (agTTR) lead to age-related diseases such as senile systemic amyloidosis and familial amyloid polyneuropathy (FAP). Among other clinical manifestations in TTR amyloidic disease, peripheral nerve tissue, including Schwann cell, degeneration has been observed. In this study, we examined potential toxic effects of agTTR in human Schwannoma cells (sNF94.3 peripheral nerve sheath line). Cells were treated with agTTR (2.4 μM pre-aggregation concentration) or, as controls, normal, soluble TTR (2.4 μM) or no-TTR treatment, and then analyzed for different pro-oxidant and anti-oxidant markers: hydrogen peroxide (H_2O_2), catalase (CAT), glutathione (GSH), and more generalized cellular antioxidant capacity. In the latter case, cytosolic fractions were prepared after agTTR (or control) treatments and analyzed in oxidation assays. Relative to treatment with normal soluble TTR, cells treated with agTTR increase their release of H_2O_2. Residual CAT activity is decreased after agTTR treatment. The Schwannoma cells also exhibit significantly lower levels of GSH after agTTR treatment ($p < 0.05$, relative to controls). More generally, cytosols from agTTR-treated cells exhibited a lower capacity to prevent oxidation relative to those from control cells (TTR-treated, or non-TTR-treated). These results suggest that agTTR (a) stimulates production of reactive oxygen species, (b) leads to lower levels of endogenous antioxidants, and (c) decreases overall cellular antioxidant capacity, in Schwannoma cells.

1. Introduction

Amyloidogenic polypeptides contribute to various age-related diseases including neurodegenerative disorders such as Alzheimer's, Parkinson's, and familial amyloid polyneuropathy (FAP). Molecular and cellular mechanisms of toxicity, however, remain poorly understood for most amyloidogenic polypeptides. For amyloid-beta (Alzheimer's) and alpha-synuclein (Parkinson's) there is evidence that pathological mechanisms include production of reactive species and oxidative damage (Atamna and Boyle, 2006; She et al., 2011).

Transthyretin (TTR) is another polypeptide with amyloidogenic potential. Misfolded and aggregated wild-type and mutant TTRs (agTTR) can contribute to age-related amyloidogenic diseases such as senile systemic amyloidosis (SSA) and FAP (e.g., Sousa and Saraiva, 2003; Westermark et al., 1990). Different TTR mutations have been found to be associated with FAP (Sousa and Saraiva, 2003; Westermark et al., 1990; Said and Plante-Bordeneuve, 2009; Suhr et al., 2009). Interestingly, in contrast to its pathological role, TTR has also been reported to be a protective factor against Alzheimer's disease through interaction with amyloid-beta aggregates (Choi et al., 2007; Buxbaum et al., 2008; Costa et al., 2008).

As is the case with other amyloidogenic polypeptides, the toxicity mechanisms of agTTR are not well understood; and a major objective of our current work in this area is to examine the potential of agTTR to promote cellular oxidative stress. Schwann cell degeneration has been reported in cases of FAP (e.g., Ando et al., 2005); and we have selected a Schwannoma model to examine the oxidative stress parameters.

To date, there are few reports of pro-oxidative actions of agTTR. Oxidative modifications of lipids and proteins have been reported in colon tissues, and protein nitration in nerves, of FAP patients (Ando et al., 1997; Sousa et al., 2001). In the current study, we provide novel evidence for the involvement of reactive oxygen species (ROS) in agTTR-mediated cellular toxicity in this cell type; agTTR increases cellular hydrogen peroxide production. Evidence is also provided for agTTR-mediated depletion of antioxidant factors such as glutathione, and more generally, for agTTR-mediated depletion of cellular antioxidant capacity. In the latter case, various antioxidant factors such as apocynin

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(1-(4-hydroxy-3-methoxyphenyl)ethanone) and L-NMMA (N-monomethyl-L-arginine) were tested to further examine the effects of agTTR and to try to gain further insight into the pro-oxidative reactions promoted by this amyloidogenic factor.

2. Materials and methods

2.1. Reagents and cells

Misfolded-aggregated TTR (agTTR) was prepared by a standard procedure (Hammarström et al., 2002) using low pH treatment of purified human TTR. We have recently reported use of this method as well the characterization (turbidity and thioflavin binding, cf. Saito et al., 2005) of the agTTR (Fong and Vieira, 2012). In accordance with other studies (see also Section 4), we express agTTR concentration in terms of pre-aggregate TTR concentration (tetrameric) because of the large possible numbers of molecular species with different molecular weights formed upon agTTR preparation. All the reagents used were of analytical grade and purchased from Sigma-Aldrich, unless noted otherwise. The human Schwann cell-like line from human malignant peripheral nerve sheath tumor (sNF94.3) was obtained from American Type Culture Collection (ATCC, USA). Cells were grown in DMEM containing 1 mM sodium pyruvate; the medium was supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium). Cells were maintained in humidified 5% CO_2 incubator at 37 °C. For the hydrogen peroxide assays (below), 1×10^5 cells were plated per well on 96-well plates; for all other assays, the number was ca. 1.5×10^6 cells per well on 6-well plates.

2.2. Hydrogen peroxide production assays

Hydrogen peroxide assays were based on the Amplex red method and performed according to the manufacturer's instructions (Molecular Probes). The conversion of amplex red (N-acetyl-3,7-dihydroxyphenoxazine) to resorufin was quantified. Equal numbers of cells were treated with TTR (2.4 μM), or agTTR (same, pre-aggregate concentration), or no TTR (another control) in Krebs-Ringer phosphate buffer for 30 min at 37 °C. A reaction mixture (100 μl) containing 50 μM Amplex Red reagent and 0.1 U/ml peroxidise (HRP) in the same buffer was added to the above samples, and to hydrogen peroxide standards and blank controls (buffer alone). The reaction was allowed to proceed 30 min at 37 °C. Light absorbance (A) was measured using a microplate reader (StatFax) and results were expressed as $100 \times (A_{\text{sample}} - A_{\text{blank}})/(A_{\text{control}} - A_{\text{blank}})$.

2.3. Catalase assays

Catalase activity was estimated using the Amplex red method according to the manufacturer's instructions (Molecular Probes) through the measurement of unreacted, exogenous hydrogen peroxide. Equal numbers of cells were treated with TTR (2.4 μM), or agTTR (same, pre-aggregate concentration), or no TTR (another control) in 2% FBS-DMEM for 4 h. Cells were then collected by centrifugation ($1000 \times g$, 4 °C, 5 min, Spectrafuge 16 M) after being detached from the plate with trypsin-EDTA. Cell pellets were resuspended in an ice-cold extraction buffer containing 0.1% Triton-X (in 0.1 M potassium phosphate buffer, KPE) and subjected to three freeze–thaw cycles. After centrifugation of the cell suspension ($3000 \times g$, 4 °C, 4 min), 25 μl of supernatants, or catalase standards, were added into wells of a 96-well plate, followed by the addition of 25 μl of 40 μM hydrogen peroxide. After incubation for 30 min at room temperature, 50 μl of a mixture containing 100 μM amplex red reagent and 0.4 U/ml peroxidise (HRP) was added into each well. Light absorbance was measured using a microplate reader (Rainbow). Values of catalase activity were interpolated from the catalase standard curves and expressed by subtracting the sample value from that of the no-TTR control.

2.4. Glutathione (GSH) assays

The quantitative assays for GSH were based on the enzymatic recycling method in which GSH is oxidized by 5,5′-dithio-bis(2-nitrobenzoic acid) and leads to the formation of 5′-thio-2-nitrobenzoic acid (Rahman et al., 2006). Equal numbers of cells were treated with TTR (2.4 μM), or agTTR (same, pre-aggregate concentration), or no TTR (another control) for 4 h, 37 °C. Cells were then collected by centrifugation ($1000 \times g$, 4 °C, 5 min, Spectrafuge 16 M) after being detached from the plate with trypsin-EDTA. Pellets were resuspended using ice-cold extraction buffer containing 0.1% Triton-X and 0.6% sulfosalicyclic acid (in 0.1 M KPE), lysed, and centrifuged as above for the catalase assays. A solution of 120 μl containing 0.34 mg/ml DTNB and 1.65 units/ml glutathione reductase was added to the samples and GSH standards for 30 s in 96-well plate. β-NADPH (60 μl of 1.5 mg/ml) was then added and light absorbance at 412 nm was measured. Values of total GSH were obtained by using the GSH standard curves.

2.5. Hemin-based oxidation assay

The following pre-treatments were performed separately for 30 min, 37 °C, on equal numbers of cells: 200 μM apocynin, 200 μM L-NMMA, or 2 mM sodium azide. Cells subjected to each of these pre-treatments, and cells without any pre-treatment, were then incubated with TTR (2.4 μM), or agTTR (same, pre-aggregate concentration), or no TTR (another control) for 4 h, 37 °C. Cytosol extracts (mitochondrial fraction removed) were obtained as reported by Wieckowski et al. (2009). Briefly, cells were resuspended in buffer containing 225 mM mannitol, 75-mM sucrose, 0.1-mM EGTA and 30-mM Tris–HCl, pH 7.4, and the suspension was homogenized and centrifuged ($600 \times g$) to remove unbroken cells and nuclei. The cytosolic fractions were obtained after a further centrifugation of the supernatant ($7000 \times g$, 10 min). Oxidation assays were performed by incubating hemin with cytosolic fractions for 1 h, 37 °C, followed by the addition of hydrogen peroxide and TMPD (N,N,N′,N′-tetramethyl-p-phenylenediamine). The final concentrations in the assay were as follows: 1 μM hemin, 1 mM hydrogen peroxide, and 100 μM TMPD. Absorbance was measured at different time intervals up to 10 min.

2.6. Statistical analyses

Data are presented as mean ± standard error of the mean (SEM), unless noted otherwise. Statistical analyses were performed using either Student's t-test, or one-way analysis of variance (ANOVA) with SPSS 17.0 software. The alpha level for statistically significant differences was 0.05.

3. Results

3.1. Cellular production of hydrogen peroxide and cellular catalase activity

As shown in Fig. 1, treatment of sNF94.3 Schwannoma cells with agTTR (2.4 μM) resulted in a statistically significant increase of about 125% in the cellular levels of hydrogen peroxide, relative to untreated controls (C). Cells treated with normal, soluble TTR (sTTR) did not exhibit significantly different levels of hydrogen