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Amyloidogenic Proteins, in their Fibrillar States, may be Detrimental via Different Mechanisms: Perspective of Potential Curative/Preventive Strategies against Neurodegenerative Disorders

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The aberrant assembly of proteins into fibrillar aggregates is accused to be the primary cause of pathogenesis of neurodegenerative diseases. But the structural determinants of protein fibrils that are responsible for cell dysfunction are not yet clear. In the current study, cell culture, spectroscopic techniques as well as theoretical and structural investigations were used to determine the ability of different fibrillar aggregates to impair cell viability. We evaluated two types of amyloid fibrils that efficiently impair cell viability when added to cell culture. Theoretical and structural investigations indicated differences in the hydrophobic characteristics of protein molecules in the fibrils, so that our findings suggest that surface hydrophobicity may be considered as a main determinant of fibrillar assemblies to cause cellular dysfunction and its consequences such as neurodegeneration. Also, the main objective of the present study was to discuss the potential role of peroxidase activity of "heme-amyloid fibril" complex in neurodegenerative disorders onset/progr-ession using the protein-based experimental models. The results of the present study also suggest that oxidative stress may be involved in neurodegenerative cell toxicity via several independent (mechanistic) routes. The data on origin of amyloid-mediated cell dysfunction may help us to postpone/attenuate the onset/extent of irreversible part of neurodegenerative pathogenesis.

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Introduction

Despite continued efforts, the development of an effective treatment for neurodegenerative diseases (e.g. Alzheimer's disease, AD) remains elusive. Current therapeutic strategies are limited to those that attenuate AD symptomology without deterring the progress of the disease itself and thus only postpone the inevitable deterioration of the disease-affected individual [1,2]. Recent studies have suggested a critical role for abnormal protein aggregation in pathogenesis of neurodegenerative disorders [1-3]. Mature amyloid fibrils are aggregated protein-based thread-like structures and typically consist of two to six unbranched protofilaments, a few nanometers (2-5 nm) in diameter, associated together to form fibrils with 4-13 nm diameter and with polydispersed length of the order of micrometers [4]. Crystallin proteins have been found to be converted readily into amyloid fibrils, under slightly destabilizing conditions [4]. Alpha-crystallin is a mixture of two subunits, αA- and αB-crystallin with ratio of approximately 3:1, respectively [4]. The αB-crystallin is located in many parts of the human body and associated, in significant quantities, with a diversity of neurodegenerative diseases, whereas αA-crystallin is also present to a much lesser extent in the spleen and thymus [4-7]. Bovine α-chymotrypsin, a well-known serine protease, has been also found to be converted readily to amyloid fibrils under certain conditions [8-10]. Due to clinical importance of (*in vitro* and *in vivo*) protein amyloidogenesis, there is now a strong motivation to examine the effects of various conditions to prevent protein aggregation. But, the molecular mechanisms of pathogenesis remain unknown. On the other hand, there is accumulating evidence indicating that intermediate oligomers are the main cytotoxic species. Since the critical role of aggregation intermediates of amyloid fibrils (and their related biological activity) in the neuronal dysfunction and pathogenesis of AD and the other protein conformational diseases (PCDs) is well-known [1,11,12], the inhibition of misfolding and fibril oligomerization, using (structural unrelated) molecular additives, as an attractive therapeutic target for these diseases is extensively studied [13]. Additionally, it has been postulated that there is a generic pathogenic mechanism for amyloid toxicity but this question remains that whether any polypeptide is able to form amyloid (e.g. α-crystallin and α-chymotrypsin) will be toxic to cells or on the contrary, there is any structural-/sequence-/hydrophobicity-dependency in the process [14,15]. However, a variety of morphologically different prefibrillar aggregates has been described as being the pathogenic agents [1,14-16]. In this context, it remains controversial if amyloid-induced toxicity derives from the formation of non-specific membrane pores or is caused by changes in membrane fluidity [17-19].

On the other hand, it has recently been proposed (by several groups [20-23]) that amyloid beta ($A\beta$) [20,21] and even other non-disease-related amyloid fibrils [21,22] can potentially bind heme, *in vitro*, and, therefore, may induce oxidative stress, *in vivo* [20,21,24]. Since considerable amounts of intracellular peroxide species [25-33], free heme [20,21,34] and amyloid fibrils [35,36] are present in intracellular space and appearance of uncontrollable H_2O_2–dependent peroxidase activity of "heme-amyloid" system [20-22], under the pathogenic conditions, there is the possibility that intracellular DNA, neurotransmitters and vital enzymes (as potential substrates) are damaged under the effect of the combined action of peroxides and peroxidase systems. Also, since some anti-oxidant compounds display strong anti-fibrillogenic activity, a specific inter-connection between oxidative stress (especially peroxide formation) and fibrillogenesis/neurodegeneration can be expected [37,38]. Additionally, because oxidative stress is intimately linked to other components of the degenerative process, it is difficult to determine whether oxidative stress leads to, or is a consequence of, these events [21,22,24,25].

Since the basic and clinical aspects of structural features, peroxidase activity/catalase inhibitory of amyloid system (which is documented in the present study, also see [20-22]) have yet to be identified, clearly, these characteristics might be considered as an (partially) uncharacterized mechanisms which may link amyloidogenesis process to cell toxicity and neurodegeneration. Therefore, the present study was also designed (or has tried) to address that amyloid fibril-induced cell toxicity may be modulated by various factors though additional data on this field are still needed. Using the α-chymotrypsin-/α-crystallin-based experimental systems, this paper also highlights a possible role of peroxidase-mediated oxidative stress in Parkinson's disease (PD) or AD where there is evidence for a primary (not secondary or by-stander) contribution of oxidative stress in

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neuronal death. The resulting data may be useful in providing mechanistic insights to develop potential curative and/or preventive strategies *in vivo* against amyloid-related neurodegenerative disorders.

Materials and Methods

Materials and equipments

Commercial hemin (which is referred to as heme), 3,3′,5,5′–tetramethylbenzidine (TMB), 30% H_2O_2, Congo red (CR), 3,4-dihydroxyphenylalanine (DOPA) and Thioflavin T (ThT) were obtained from Sigma chemical company (St. Louis, MO, USA). Gel reagents and all other chemicals were the highest analytical grade of purity available and were used without further purification. Unless otherwise stated, all solutions were prepared using double distilled water and as 100 mM sodium phosphate buffer, pH 7.6, were used. A stock solution of heme (1 mM) was freshly prepared in dimethyl sulphoxide (DMSO) and stored in dark. The stock solution of heme was gradually diluted with buffer to the desired concentration. A Shimadzu UV-Vis spectroph-otometer (model UV-2450) was used for protein determination, CR binding analysis, peroxidase assay and turbidimetric measurements [10,22]. Standard deviations were approximately within 5% of the experimental values of two or three independent experiments and values of P less than 0.05 (versus control experiment) were considered significant.

Protein purification/determination

Alpha-crystallin was purified from bovine lenses, obtained from cows of less than two years old, as described previously [22,39,40]. Additionally, SDS-polyacrylamide gel electrophoresis was used to confirm the purity (less than or equal 95%) of protein [40]. The protein samples were loaded on a 12% slab gel under nonreducing conditions according to the method of Laemmli [41]. Protein concentrations of α-crystallin were determined according to Lowry's method [42] and standard curve was generated using bovine serum albumin (BSA).

Formation of amyloid fibrils by bovine α-crystallin and α-chymotrypsin turbidimetric measurements

Amyloid fibril preparation and turbidimetric analyses were done by using the methods described elsewhere [4,22,10].

ThT fluorescence analyses and CR binding assays

To investigate whether α-crystallin and α-chymotry-psin were converted to amyloid fibrils, ThT-based fluorimetric method as well as CR binding assay were performed, as reported in our previous works [22]. A red shift accompanied with increased intensity in the CR absorption band and increase in the intensity of the ThT emission were together taken to be indicative of the formation of amyloid structures.

Sequence and hydropathy profile analyses

Similarity searches were carried out using BLAST P and FASTA services through the NCBI and EBI (www.ebi.ac.uk) servers, respectively. Amino acid sequences (FASTA format) of αA-crystallin, αB-crystallin, amyloid beta and α-chymotrypsin were derived from swissprot (or PDB) databases. The multiple sequence alignment was performed with the CLUSTAL W_2 service on EBI server (http://www.ebi.ac.uk/Tools/clustalw2/index.html) [43]. The calculation and analyses of hydropathy profiles/hydrophobicity scales of αA-crystallin, αB-crystallin and α-chymotrypsin, were performed by the method of Roseman [1,44] with window size 9.

Heme binding and peroxidase activity

Heme binding property of amyloid structures and peroxidase activity of "amyloid-heme" complexes, in the presence of substrates (Serotonin or DOPA) were evaluated, according to previously described methods [20-22].

Cell culture and cytotoxicity assay

SK-N-MC cells were obtained from the National Cell Bank, Pasteur Institute of Iran, started from frozen stock and cultured on tissue culture-treated plastic in basal medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and maintained at 37 °C with 5% CO_2 until 90% confluent. To determine maximum non-toxic (and cytotoxic) concentrations of protein aggregates, several aliquots of suspensions containing aggregated proteins were added to medium containing confluent SK-N-MC cells. After usual incubation of 48 h, the effect of aggregates on cell viability was evaluated by using lactate dehydrogenase (LDH) assay [45]. The absorbance of converted dye in LDH assay was measured at wavelength of 490 nm with background

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subtraction at 630 nm. The percentage of viable cultured cells exposed to differing concentrations of α-chymotrypsin and α-crystallin are reported. Each concentration was tested in three independent experiments.

Viability staining assay using Ethidium Bromide and Acridine Orange

1X Working solution of Ethidium Bromide/Acridine Orange was prepared by thawing a 1 ml aliquot of the 100X stock solution (50 mg Ethidium Bromide and 15 mg Acridine Orange, dissolved in 1 ml of 95% ethanol and 49 ml distilled water) and its 1/100 diluting/mixing in PBS. The working solution then was stored in an amber bottle at 4 °C for up to one month. Aliquots of the SKNMC cell suspensions were adjusted to an estimated 1-5 × 10^6 cells/ml in phosphate-buffered Minimal Essential Medium or other isotonic medium. Then, equal 25 µl volumes of cell suspension and ethidium bromide/acridine orange solution were added to a tube, mixed gently and a small aliquot (approximately 25 µl) was placed underneath the coverslip on a hemocytometer slide. The cells were initially observed under the microscope using visible light, 100-400×, by adjusting the diaphragm to reduce light, and then keeping the visible light on, the viability of cells was evaluated by switching to fluorescence mode (using 495 nm primary filter and a 515 nm secondary filter). Live cells were green (with acridine orange) and dead cells were orange (with ethidium bromide) [46].

ANS fluorescence analyses

To gain insights into the structural differences of toxic amyloid aggregates, both types of α-crystallin and α-chymotrypsin aggregates formed at identical concentrations (0.4 mg/ml) were centrifuged (10 min) and the pellets were resuspended in phosphate buffer. Aliquots of ANS from stock solution (1 mM) were subsequently added to the aggregates. The final concentrations of ANS and proteins were 0-20 µM and 0.4 mg/ml, respectively. ANS fluorescence spectra were recorded over a range that included the wavelength of 470 nm using excitation wavelength of 380 nm. The excitation and emission slits were both at 5 nm. All fluorescence spectra were corrected by subtraction of the apparent fluorescence of the respective blanks (that measured with protein alone in the absence of ANS), under the identical conditions [1].

Dopachrome determination and assay of dopaquinone formation

Formation of dopachrome was measured by monitoring the increase in absorbance at 475 nm ($\varepsilon = 3.313 \times 10^3$ M^{-1} cm^{-1}) over an initial 2 min period. Peroxidase-catalysed conversion of L-DOPA to dopaquinone was also measured using Besthorn's hydrazone (3-methyl-2-benzothiazolinone hydrazone, MBTH). A pink product with an absorbance maximum at 505 nm is formed in this reaction [47].

Results

Kinetics of protein aggregation

To compare the fibrillar aggregation behavior of α-crystallin and α-chymotrypsin, the kinetics of aggregation were registered by monitoring the absorbance increment at 400 nm. First, aggregation reaction was studied under the effect of different protein concentrations ($(P)_o$). Additional characterization of amyloid formation was performed using CR binding assay (Fig. 1), according to our previous works [10,22].

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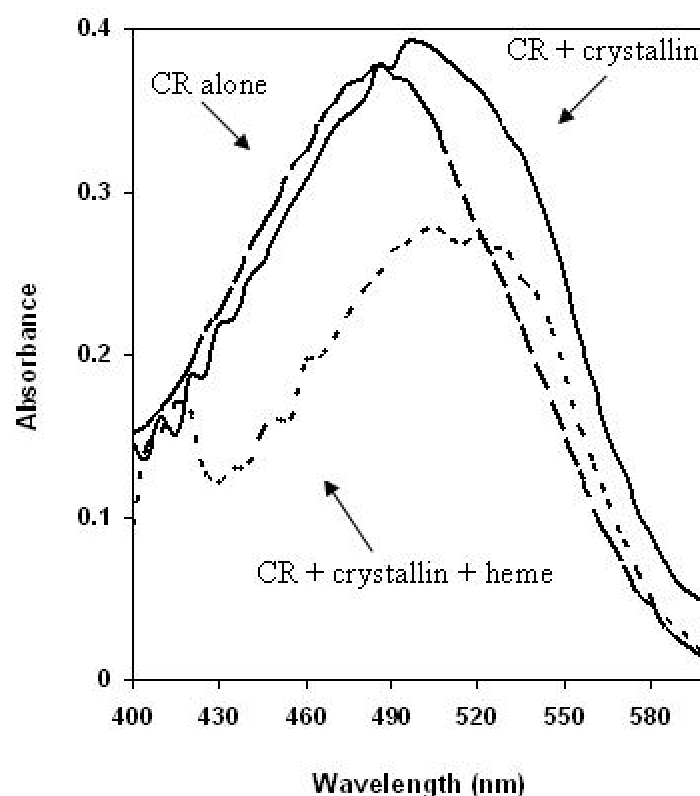

Fig. 1. Effect of heme on crystallin amyloid fibrillation, as measured by monitoring changes in CR absorbance spectra. The spectrum of CR alone (dashed line) was compared with that of CR solutions in the presence of 0.5 mg/ml Crystallin (bold line), Crystallin plus 10 μM heme (dotted line). Difference spectra are shown, which have been corrected for the contribution from buffer. Further details are given in experimental procedures.

Cell toxicity of amyloid fibrils

To assess if non-disease-related amyloid fibrils are able to reproduce amyloid-induced cytotoxicity produced by natural pathogenic proteins, we analyzed the effect of the α-crystallin and α-chymotrypsin on viability of SK-N-MC cells. It has repeatedly been reported that amyloid fibrils (and oligomers) interact with cell membrane and permeabilize it [12,17-19].

The viability state of the cells was monitored by performing LDH assay and microscopic (and dye staining) analysis. The amyloid aggregates formed by α-chymotrypsin were found to induce cell toxicity, substantially and in a concentration-dependent manner, relative to that of the aggregates formed by α-crystallin (Fig. 2A). Both protein aggregates were fully cytotoxic at protein concentration of 0.04 mg/ml. Fluorescence microscopy images (Fig. 2B) indicate that only the nucleus of (dead) cells treated with amyloid aggregates were prominently stained with EB dye. By contrast, the control cells treated with native α-crystallin/α-chymotrypsin proteins (or control untreated cells) exhibited a high degree of AO staining.

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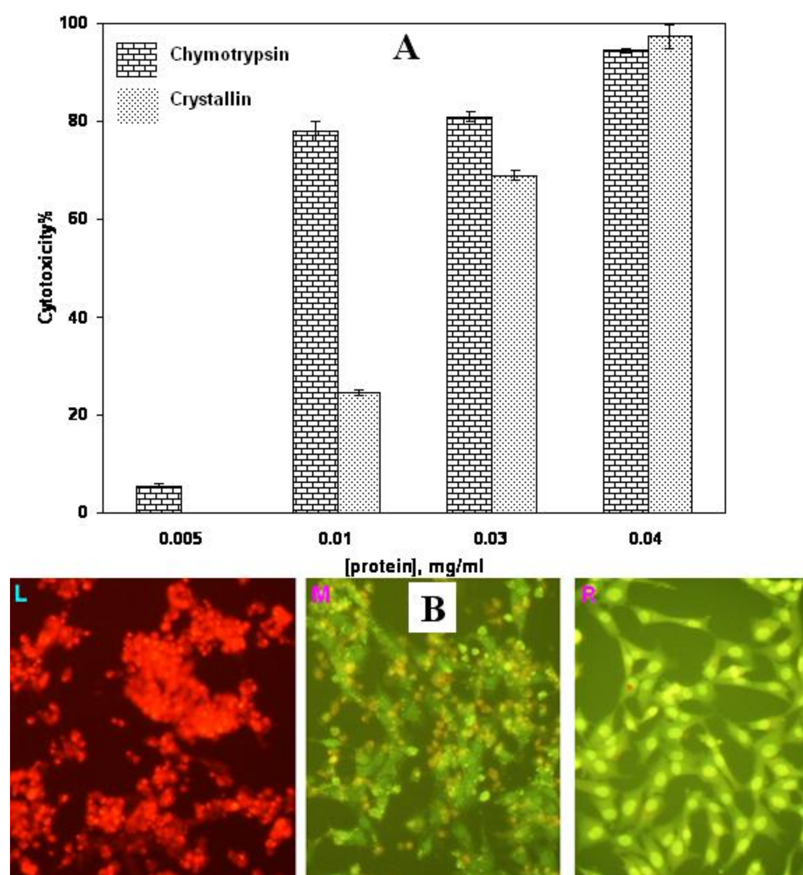

Fig. 2. (**A**) Dose response effects of protein aggregates on SK-N-MC cell viability upon incubation with cytotoxic amyloid fibrils of α-crystallin and α-chymotrypsin. The cells were seeded (5×10^4 cells per well, in 24-well plates) and incubated at the various concentrations of proteins. Cell viability was measured by the LDH assay. Each column represents mean ± SEM of three independent experiments. Further details are given in experimental procedures. (**B**) Fluorescence microscopy images of SK-N-MC cells untreated (Right, R) or treated with various concentrations of aggregated α-crystallin and α-chymotrypsin (Middle image, M represents the effect of moderate concentration and Left image, L represents cell mortality at highly toxic concentration). In case of viable cells (R), a diffuse intracellular green fluorescence of AO is evident. Reciprocally, red nuclei show dead cells under the effect of counterstaining with EB. Photomicrographs (×250 Magnification) shown are representative example of three independent experiments. Further details are given in experimental procedures.

Theoretical analyses of hydropathy profiles

As we know, the structural determinants of the formation of amyloid fibrils and the relationship between their structure and their ability to cause cell dysfunction are not clear. We, therefore, tried not only to investigate hydropathy profile in α-crystallin and α-chymotrypsin but also to find possible inter-connection between protein hydropathy and cell-lethality of related protein aggregates. Based on hydropathy profiles (Fig. 3), in contrast with α-crystallins (and similar to Aβ peptide, with average hydropathy score of -0.194), the amino acid residues of α-chymotrypsin revealed a greater average score (-0.262 versus -0.53), although, there is a gross discontinuity (with no uniformity) among hydropathy values of consecutive residues. Reciprocally, apolar residues of crystallins consecutively have been distributed mainly through the aggregation-prone segments of polypeptide sequences [4,22]

(Fig.3).

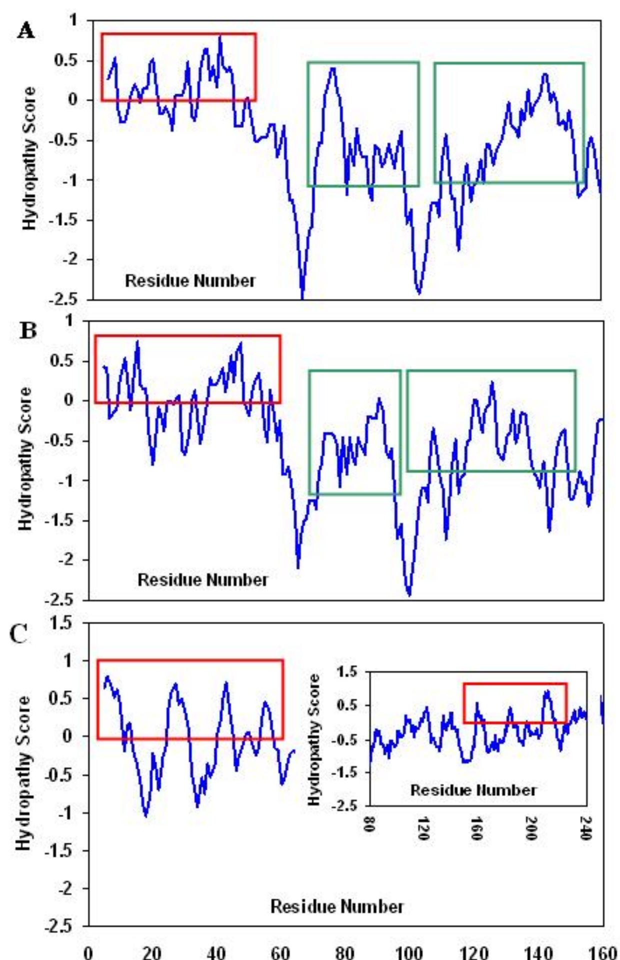

Fig. 3. Hydropathy profiles of αA-crystallin (A), αB-crystallin (B) and α-chymotrypsin (C), calculated using the Roseman hydrophobicity scale [1,44] with window size 9. Note and compare the continuity (in A and B) and discontinuity (in C and its inset) in positive hydropathy scores, as indicated by red rectangulars. Also, note the homogeneity among hydropathy values in aggregation-prone segments [4] of crystallin sequences (A and B), as indicated by green rectangulars. See text for further details.

ANS fluorescence analyses

When ANS binds to solvent-exposed hydrophobic clusters, it generates a remarkable increase in its fluorescence intensity and a blue shift of its maximum emission wavelength. As indicated in Fig. 4, at equivalent protein/ANS concentrations, the fluorescence intensity measured in the presence of the α-chymotrypsin aggregates was higher than that obtained with α-crystallin, indicating that the dye interacts with more nonpolar environment on the accessible surfaces of more toxic species.

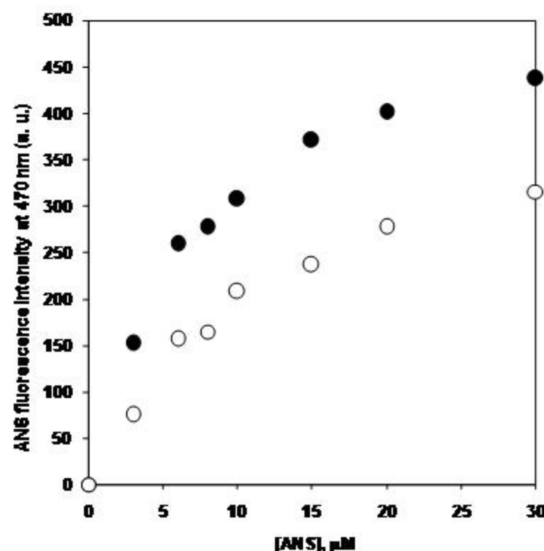

Fig. 4. ANS binding to α-crystallin (empty circles) and α-chymotrypsin (filled circles) aggregates. The ANS fluorescence intensity measured at 470 nm is reported as a function of the ANS concentration [1]. Both the excitation and emission slits were set at 5 nm. Protein concentration was 0.4 mg/ml in all cases. Data values shown are the averages of three independent experiments and standard deviations were approximately within 5% of the experimental values. Further details are given in experimental procedures.

The "amyloid aggregate-heme" complexes exhibit peroxidase activity, in vitro: Peroxidase-mediated formation of dopaquinone, as a neurotoxin

It has been proposed that *in vivo* formation of (intracellular) floating "amyloid-heme" complexes with outstanding peroxidase activity is the main cause of mitochondrial heme deficiency (and its subsequent up-regulation). As depicted in Fig. 5, DOPA, which is a classic substrate for tyrosinases, was also rapidly oxidized to dopaquinone (and red-colored dopachrome) by peroxidase activity of "heme-amyloid" system. Fig. 5 also shows that the neurotransmitter serotonin is suitable substrate for non-specific peroxidase system and may explain the decline in specific neurotransmitters in some

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degenerative diseases due to probable uncontrollable peroxidase activity of “amyloid fibril-heme” complex.

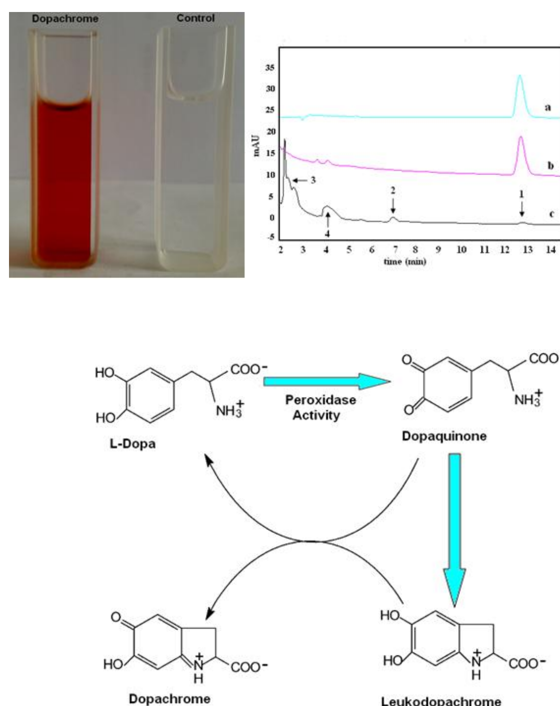

Fig. 5. (Leftt, Top) Non-specific peroxidase activity of the “aggregate–heme” system in the presence of DOPA produces dopachrome (red). (Right, Top) Oxidation of serotonin by peroxidase activity of “crystallin aggregate-heme” complex. The retention times of serotonin (denoted by 1), heme (denoted by 4 on **c**) and oxidation products (denoted by 2 and 3 on **c**) are indicated in **a** (serotonin alone), **b** (serotonin + H_2O_2) and **c** (serotonin + peroxidase system plus H_2O_2) panels. For further details, see experimental procedures. (Bottom) Proposed enzyme-mediated L-DOPA to dopachrome converting pathway under the effect of “heme-amyloid” complex peroxidase activity (inset: see the red cuvette). Peroxidase-catalyzed DOPA oxidation was also efficiently inhibited by synthetic radical scavengers (data not shown) confirming that radicals are involved in this enzymatic process. At *in vivo* conditions, as a abnormal process in the substantia nigra, excess amounts of free dopamine quinone as well as dopaquinone exist in the cytosol and conjugate with cysteine residues of glutathione and functional proteins.

Discussion

The objective of the present study was to address any inter-connection between cell toxicity and amyloidogenesis using *in vitro* protein models. We first determined whether the cell viability was affected in the presence of fibril, alone. To assess the disrupting activity of the α-crystallin and α-chymotrypsin fibrils, we added different amounts of protein aggregates to the cell culture media of SK-N-MC neuroblastoma cells. Incubation of 0.005 mg/ml of α-chymotrypsin with SK-N-MC cells was enough to trigger the cascade of events that lead to cell death (Fig. 2A), while incubation with α-crystallin aggregate present a cell death rate equal to untreated cells. However, higher concentrations increased the percentage of cell death (Fig. 2A,B). For example, incubation of SK-N-MC cells with α-chymotrypsin amyloid fibrils (0.03 mg/ml) decreased cell viability to 80%, slightly higher than the toxicity exerted by the α-crystallin fibrils. Moreover, no detrimental effect was observed when the cells were treated with native forms of α-crystallin and PMSF-inhibited α-chymotrypsin [8-10]. We also stained SK-N-MC cells with acridine orange/ethidium bromide, AO/EB, giving rise to a strong fluorescence signal.

We observed that samples containing mature fibrils affected cell viability, confirming that mature fibrils may have not an innocuous nature. But, there is this possibility that fibril pathogenicity of α-crystallin and α-chymotrypsin is due to fibril depolymerization to

toxic oligomers as a consequence of dilution of aggregates when transferred to cell medium, although ThT assay showed that neither types of amyloids undergo any detectable structural reorganization when added to cell culture medium (data not shown). Despite considerable efforts, the toxicity mechanism derived from amyloid formation still remains unknown. The cell toxicity results emphasize that the α-crystallin aggregates reproduce the comparable cytotoxicity (lethality) caused by the α-chymotrypsin aggregates. This fact that the proteins have no considerable similarity in primary structure, might also suggest that factor(s) other than protein sequence, length and chirality (such as surface hydrophobicity of protein aggregates) may affect amyloid lethality. In agreement with this assumption, it has been previously suggested that amyloid-induced toxicity seems to depend neither on polypeptide length nor on protein sequence, but on the particular morphology (quaternary structure) of the amyloid aggregates [1]. Also, multiple sequence alignments showed no considerable similarity between α-crystallin and α-chymotrypsin sequences, indicating that observed amyloid-induced toxicity (Fig. 2) seems to be protein sequence-independent. On the other hand, many evidences suggest that the plasma membrane is the primary target of amyloid pathogenicity. Although the identification of the precise molecular mechanism by which amyloid fibrils bind to plasma membrane is beyond the scope of this work, the comparative hydropathy analyses point to a lipid interaction rather than to specific receptor-ligand binding [11,17-19]. In addition, it remains controversial if amyloid-induced toxicity derives from the formation of non-specific membrane pores (that leads to leakage of essential ions) or is caused by changes in membrane fluidity [11,17-19]. Furthermore, several data indicate the internalization of amyloid (fibrils/oligomers) into the cytosol of involved cell [35,36]. Irrespective of the mechanism of toxicity, the different sensitivity of SK-N-MC cells to different cytotoxic aggregates (from crystallin and chymotrypsin) might be due to the presence of specific structural features on amyloid fibrils. To evaluate this possibility, we analyzed the ANS fluorescence of protein aggregates and, also, compared the hydropathy profiles of their parent polypeptide sequences Distribution pattern of hydrophobic and hydrophilic amino acids in primary structure of proteins determines their final fold. Additionally, hydrophobicity is a major driving force/determinant of pathological effects in different types of amyloidogenic proteins [22]. It has been recently reported that hydropathy profile of proteins can be used in order to investigate parameters such as evolutionary relationship, signature hydropathy profile of membrane proteins and identification of surface ß-strands in globular proteins [48]. The hydropathy profiles of crystallin sequences (Fig. 3A,B) show that the N-terminal and central regions are hydrophobic and the resulted hydrophobic clusters have possibly a strong tendency to be membrane-interacting segment of (α-crystallin-based) amyloid aggregates (Fig. 4). In this frame, uniformity among hydropathy values of aggregation-prone segment of *Aβ* [49] was also documented. In contrast, profile of α-chymotrypsin shows no (such a) local/processive hydrophobicity in the protein sequence. Furthermore, the profile of hydropathy versus residue number, may suggest that N-terminal and central domains of α-crystallins (the regions spanning approximately residues 1-65 and 66-149) are specific/local regions which have a high tendency to tightly packed within the structural core of the α-crystallin amyloid aggregates [4,22]. Notably, there is an excellent agreement between the amyloid aggregation-prone regions in α-crystallin and the locations of the highest peaks in the hydropathy profile. Reciprocally, some of distributed hydrophobic side chains along the chymotrypsin sequence have this chance to be exposed on the amyloid aggregates (as evident in Fig. 4). By comparing the hydropathy profiles, it is not plausible to conclude that a certain amino acid sequence (including consecutive apolar residues) may be determinant for the effective and noxious amyloid-membrane interaction. But, taken together, our data may highlight this possibility that different (debilitating) abilities (to cause cellular dysfunction) are result of difference in the degree of contribution of the hydrophobic side chains to the structural core of amyloid aggregates. Additionally, human $A\beta_{(1-42)}$ was shown to be more neurotoxic than $A\beta_{(1-40)}$, whereas the reverse sequence was shown to be non-neurotoxic [14]. This indicated that secondary and tertiary structural features played an integral role in toxicity and not just the nature of the amino acid side chains. Also, it has been shown that short peptide $A\beta_{16-21}$ exhibits similar morphology/toxicity as the full length $A\beta_{1-42}$ [11,14], confirming that amyloid

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formation/toxicity are independent of polypeptide length. Furthermore, by comparing hydropathy values of residues along the Aβ sequence [11], it may be proposed that Aβ amyloid-induced cell toxicity is related to a particular hydrophobic motif, but (based on the results of multiple sequence alignment between Aβ and α-crystallin, data not shown) completely independent of a common particular sequence of aggregated peptide/protein. In other word, the fact that different amyloidogenic proteins with different sequences are toxic for different cells could explain that dysfunctionality/lethality is principally side-chain-independent.

Although correlation between hydrophobicity, tendency to form aggregate and aggregate cytotoxicity remained to be established in comparative studies and whereas the ability to form amyloid-like structures is generic to all polypeptide chains [10,22], the key feature in the generation of toxicity is apparently the conversion of a species with extreme buried hydrophobic residues to one in which residues are substantially exposed. It is evidenced that residues 12-24 and 30-40 of $A\beta_{40}$ form two parallel β-strands and hydrophobic side chains form the core structure between sheets [11], highlighting the importance/critical role of hydrophobic interactions in both fibrillogenesis [22] and amyloid-induced cell toxicity. Campioni et al. [1] showed that the aggregate-induced cell toxicity is influenced by the degree of hydrophobic packing. In order to assess whether or not the toxicity differences between α-crystallin and α-chymotrypsin fibrils are associated with structural (or hydrophobic packing) differences, and to obtain information on the polarity of the accessible surfaces/clusters of the (α-crystallin and α-chymotrypsin) aggregates, we tested the ability of aggregate species to interact with ANS and compared ANS fluorescence intensities. When ANS binds to solvent-exposed hydrophobic clusters, it generates a remarkable increase in its fluorescence intensity and a blue shift of its maximum emission wavelength [50]. The intensity values obtained for two types of aggregates indicate that hydrophobic moieties/clusters are more accessible to the solvent in the more toxic α-chymotrypsin aggregates than in the (relatively) less toxic α-crystallin amyloid fibrils. Based on the literature [1,11] and regardless of polypeptide sequence and hydropathy index, it is suggested that amyloid aggregates exert their cytotoxic effects through a common cell death mechanism. Here, we have proposed that the degree of lethality of the toxic species seems to depend on quaternary structure of amyloid aggregates. These observations may also confirm that amyloid-induced neuronal cell death is mediated in part by a mechanism other than simple interaction with aggregates and physical disruption of plasma membrane.

Despite the resident sequestration mechanisms for intracellular reactive oxygen species (ROS), the number of produced free radicals, in healthy neurons with high metabolic activity, is estimated by some to be 10^{11}/cell/day [51]. Recently, Atamna and Boyle [21] demonstrated that amyloid-induced heme deficiency triggers oxidative stress and the release of ROS (e.g., H_2O_2) from AD-affected mitochondria due to the loss of complex IV so that the vulnerable AD neurons can no longer control the debilitating propagation of oxidants. It has been proposed that *in vivo* [20,21] formation of (intracellular) floating “amyloid-heme” complexes with outstanding peroxidase activity is the main cause of mitochondrial heme deficiency (and its subsequent up-regulation). In this regard, Atamna and Boyle [21] and Khodarahmi et al. [22] have reported the peroxidase activity of “amyloid-heme” complexes using *in vitro* experimental systems. Despite extensive experimental work was carried out on this field, discrepancy on chronological precedence of amyloid aggregation and oxidative reactions as well as the mechanism involved in the peroxidase-induced oxidative stress is still not completely understood. Furthermore, “amyloid-metal” complexes markedly potentiated amyloid neurotoxicity by promoting generation of H_2O_2 and other biological hydroperoxides (as oxidant substrate for non-specific peroxidase activity), at the expense of oxidizing L-DOPA and dopamine [49,65]. Neurotransmitter deficiency, generation of neurotoxins, altered activity/metabolism of key enzymes and cellular DNA damage are possible evidences highlighting importance of the proposed uncontrollable peroxidase activity in AD/PD-involved brain cells [21,51]. Such a scenario may be consistent with this possibility in which mitochondrial abnormalities, peroxidase activities and metal accumulations are synergistically responsible for oxidative stress and neuronal toxicity in AD/PD.

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Peroxidase-mediated formation of dopaquinone, as a neurotoxin

Although melanogenesis is started by the monophenolase and diphenolase activities of tyrosinase enzyme [52], but the pathway, mechanism and involved enzymes of reactions converting tyrosine to neuromelanine is not yet well understood and it is thought to proceed through oxidative conversion to quinone species [52-55]. The quinone consequently produced has an electron-deficient ring, which readily forms covalent bond with available nucleophiles [53]. Naturally, (at physiological concentrations of quinones and) in the absence of competing nucleophiles, the amine side chain of dopamine-/DOPA-derived quinone (Fig. 5) is readily available for 1,4-intramolecular ring closure/oxidation, forming more stable dopa(mino)-chrome, a potential precursor of neuromelanin [53,54]. Dopachrome (dopaminochrome) is a member of the family of red to violet colored indoline-5,6-quinones, known as aminochromes which are readily obtained on oxidation of the corresponding catecholamines under the effect of different enzymes To date, the primary cause of oxidative stress, at pathogenic conditions, has not been clarified but the leading candidate is the oxidation of L-DOPA (or dopamine) itself, which is thought to proceed through formation of various toxic species (such as quinones), is accelerated by a number of different enzyme activities [55]. Also, "heme-amyloid" peroxidase activity has not been suggested as pathological responsible for oxidative reactions in nigral dopaminergic neurons. As discussed earlier [53] and as shown in Fig. 5 (*in vitro* quinone formation by "crystallin aggregate-heme" complex"), in the substantia nigra of AD/PD brain, there is this possibility that excess amounts of quinones are (rapidly) generated in the enzymatic oxidation of DOPA/dopamine neurotransmitters by uncontrollable peroxidizing activity of "amyloid-heme" complexes. Quinone itself has been also found to modify/disrupt dopamine transporter [51,53]. Additionally, peroxidase-mediated catalysis can also lead to formation of 6-hydroxydopamine (a known neurotoxin) from reaction of dopamine quinone with various peroxides. In conclusion, the data of the present study may propose that non-specific quaternary structured-aggregates may interact with plasma membrane, disorganize it and produce cell toxicity. Since disorganized hydrophobic residues within amyloid oligomers are suggested (in part) as origin of the pathogenesis of debilitating neurodegenerative diseases, application of β-sheet breaker peptides (agents), as an (effective!) therapeutic intervention, may lead to the generation of (more) toxic intermediate/oligomer species. Additionally, peroxidase-mediated oxidative stress can play at least partially a causative rather than merely by-stander role in the neurofibrillary pathology in AD/PD, *in vivo*. Based on our data in the present study and the literature [22,53,56], it can be concluded that pathogenic peroxidase activity of "amyloid-heme" complex may affect nucleic acids, enzymes and neurotransmitters. Thus, despite the plethora of studies, more work will be needed if the field is going to reach a consensus on the actual role of peroxidase activity of "amyloid-heme" complex in neurodegenerative diseases. Apparently, ongoing clinical trials should therefore focus on simultaneous application of fibril stabilizers, aggregation inhibitors (no disaggregation inducers) and peroxidase inhibitors as a new generation of amyloid-based therapeutics.

Conflict of interest

Authors certify that no actual or potential conflict of interest in relation to this article exists.

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