Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 6, 2014

Original Article

PROTECTIVE EFFECTS OF A - CRYSTALLIN ON B - AMYLOID (Aβ) INDUCED TOXICITY

K. GOMATHI GOPINATHAN[1,*] AND D. GAYATHRI[2]

[1]Department of Biotechnology, Dr MGR Educational Research Institute and University, Chennai 95 India. [2]Centre of Advanced Study in Crystallography and Biophysics, University of Madras, Guindy Campus, Chennai 25 India.
Email: drgomathigopinathan@gmail.com

Received: 05 Mar 2014 Revised and Accepted: 09 Apr 2014

ABSTRACT

Objectives: To investigate the protective role of α-crystallin against β-amyloid aggregation.

Methods: In vitro spectroscopic methods and cell culture studies were done to validate our objective.

Results: The molecular basis of alzhemiers disease has been proposed to be accumulation and aggregation of β-amyloid (Aβ). However, prevention of β-amyloid aggregation is still a promising means to reduce its neurotoxicity. In this work, we show that α-crystallin was able to inhibit cellular toxicity of Aβ on astrocytes and lymphocytes. Theα–crystallin (αA and αB): the two vertebrate eye lens proteins that are related to the small heat shock protein family, was able to reverse the oxidative stress induced by $A\beta_{1\text{-}42}$. Treatment of α-crystallin enhances the activity of proteasome and it also induces the expression of Hsp70 which is known to inhibit the intramolecular misfolding. We also demonstrate that $A\beta_{1\text{-}42}$ suppresses the expression of TriC chaperonin subunits TCPβ and TCPε, which are known to play a role in folding of misfolded proteins.α-crystallin reverses this effect and enhances the expression of TCPβ and TCPε.

Conclusions: Research findings in this study provide the basis for the development of novel pharmacotherapy for Alzhemier's disease.

Keywords: Alzhemiers disease, α-crystallin, Proteasome, HSP70, TCPβ and TCPε..

INTRODUCTION

Accumulation of beta amyloid peptide (Aβ) in the brain has been believed to be a pathological cascade that causes Alzheimer disease (AD) [1]. Aβ peptides emerged to be the prominent form that accumulates outside the extra cellular space of neurons in AD brains and in transgenic mice expressing familial AD mutations [2]. Fibrillar aggregates are known to act as physical barriers to transport and other essential neuronal functions [3]. So far, the reasons for the neuronal degeneration have not been identified. It is speculated that Aβs form pore in the membrane that leads to an unregulated flux of Ca^{2+} [4]. Furthermore, free radical formation [5], mitochondrial dysfunction [6] and caspase activation [7] have also been assumed as reasons for neurotoxicity. However, the causes of neurotoxicity and the initial events in the cascade of neuronal cell death are still unclear. It has been reported that Aβ promotes the toxicity by suppressing the molecular chaperones by inhibiting the AKT signaling as chaperones play a very important role in preventing aggregation of proteins [8].

Research findings suggest that heat shock proteins can suppress neurotoxicity of Parkinson's and polyglutamine diseases [9, 10]. Hsp40, Hsp90 have been implicated in maintaining tau solubility and suppressing tau aggregate and are reported to inhibit self assembly of polyglutamine proteins in to amyloid like fibrils [11]. *In vivo* treatment of molecular chaperones in amyloid affected cells may improve the cells to overcome the toxicity. α- Crystallin is one such molecular chaperone that inhibits the Aβ aggregation *in vitro* [12]. Apart from this, α-Crystallin has been known to inhibit the free radicals and suppress the inflammatory reactions [13, 14]. In the work described here, we use α-Crystallin to study its role on Aβ toxicity induced in astrocytes and human lymphocytes.

MATERIALS AND METHODS

Peptide Synthesis and Purification

The peptide Aβ 1-42 was synthesized by manual solid phase chemistry, using Fmoc as the protective group for N-terminal ends, and 1-Hydroxy benzotriazole (HOBt), and N,N-dicyclohexyl-carbodiimide as activators of carboxylic ends. The peptides were cleaved from Wang resin with trifluoromethane sulphonic acid/thioanisole/ethanedithiol/trifluoroacetic acid (1:1:1:7) [15] and precipitated with cold ether. The composition of peptides was determined by amino acid analysis using Phenyl isothiocyanate (PITC) method. All reagents used in peptide synthesis were of the purest analytical grade; t-Butyl carbazide, HOBt, N,N-dicyclohexyl-carbazide and trifluoroacetic acid were purchased from ALDRICH, GERMANY. Fmoc-amino acids were prepared using standard procedures [16] and were characterized by thin layer chromatography and FTIR spectroscopic studies.

Preparation of amyloid fibrils

All the following procedures were done inside the laminar hood to avoid contamination. $A\beta_{1\text{-}42}$ was dissolved and disaggregated as given in the procedure [17] by using a 1:1 mixture of trifluoroacetic acid (TFA): 1,1,1,3,3,3-hexafluoro-2-isopropyl alcohol (HFIP) and then aggregated by incubation of 0.5mg/ml solution in sterilized PBS at 37°C for 2 weeks. The aggregation is followed by Thioflavin T assay [18].

Isolation of α-crystallin

Lenses obtained from freshly slaughtered young bulls were washed in cold saline and homogenized with cold 50mM Tris HCl buffer (pH 7.4) containing 0.2M KCl, 0.01% NaN3, 1mM EDTA, and 0.2mM DTT [19]. The homogenized solution was centrifuged and the supernatant was loaded in to Sephacryl S_{200} column material. Isolated crystallin was purified using RP-HPLC. Molecular weight was determined by GPC.

Fluorescence Resonance Energy Transmission

Labeling of Pyrene to Aβ was performed by following a method used by Liang [20]. To label mainly the terminal amine, a buffer close to neutral pH was used. In brief, 2mg of Aβ was dissolved in 1ml of 0.1M sodium phosphate buffer pH 7.4. 100µl of Pyrene solution (0.5mg in 0.5 ml DMSO) was added and incubated at room temperature for 1hr. The reaction was stopped by adding 100µl of fresh 1.5M hydroxylamine. The conjugated Aβ was separated from unconjugated Aβ in a column of Sephadex G-10. The presence of pyrene does not interfere the formation of fibril. Upon excitation at 340nm, an emission spectrum with three emission peaks at 370, 390

and 420nm were observed for pyrene. In the fluorescence resonance energy transfer experiment, the tryptophan in the unlabeled α-crystallin served as a donor and Pyrene in the labeled Aβ act as an acceptor. Both the excitation and emission slits were set at 10nm.

Acrylamide Quenching

Fluorescence quenching experiments [21] were performed using pyrene labeled Aβ with/without crystallin. Concentration of pyrene Aβ and crystallin used was 10μM . Samples were excited at 335nm and emission observed from 375-500nm.

Induced Circular dichroism

Induced circular dichroism spectra was recorded using a Jasco J-175 circular dichroism spectropolarimeter (Japan). Spectra were recorded with 1nm bandwidth and 0.2nm step resolution at a scan speed of 50/min over the range of 300-450nm. Concentration of pyrene Aβ used was 100μM . Concentration of crystallin used was 5-15 μM (5, 7.5, 10, 15 μM)

□hioflavin T binding assay

The rates of fibril formation in $A\beta_{1-42}$ in presence and absence of α-crystallin was analysed using the Thioflavin T binding assay [18]. Concentration of $A\beta_{1-42}$ and α-crystallin used in this experiment was 10μM.

Light scattering

Light scattering measurements were conducted on $A\beta_{1-42}$ aggregates in the presence and absence of α-crystallin. The aliquots of aggregation reactions were transferred to a fluorescence cuvette and read as apparent fluorescence in a Perkin Elmer Luminescence spectrometer with excitation and emission wavelengths both set at 600nm. The concentration of $A\beta_{1-42}$ and α-crystallin used in this experiment was 10μM.

Preparation of primary astrocytes cultures

Cultured astrocytes were prepared from the cerebral cortex of 1-2 days old Sprague–Dawley rats) [22]. Briefly, brain was aseptically removed, then placed on ice in sterile culture dishes containing phosphate-buffered saline (HBSS; Gibco-BRL, Invitrogen, Paisley, UK). The midbrain, meninges and blood vessels were removed by dissection. The remaining cerebral cortices were treated in 1% trypsin-EDTA. Every ten minutes (for 40 minutes), solution was removed and centrifuged to pellet the released cells. Pelleted cells were washed thoroughly in HBSS and resuspended in DMEM-F12 (Dulbecco's modified Eagle medium, Gibco-BRL, Invitrogen, Paisley, UK) containing 4 ml/litre of a mixture of penicillin/streptomycin 10000 U/10000 lg/ml (Gibco-BRL). Then, the cells were plated in culture flask (25 cm^2, TPP, Techno Plastic Products, Trasadingen, Switzerland) and maintained in DMEM-F12 supplemented with 10% fetal bovine serum (FBS; Gibco-BRL) at 37ºC in an humidified atmosphere of 5% CO2 and 95% air. The culture medium was changed three times a week with the incubation medium supplemented with 10% FBS. After one week, the flasks were shaken at 200 rpm for 2 h to dislodge microglia. The medium was immediately removed and replaced with a fresh medium containing 10% FBS. Then, up to confluence, the culture medium was used with 10% FBS.

Isolation of peripheral Blood lymphocytes (PBL)

Peripheral blood lymphocytes were isolated from the Blood.[23]. Briefly heparinised blood obtained from normal healthy volunteers was layered over lymphoprep gradient (Sigma chemicals) centrifuged at 1880 rpm for 40 min, the top two thirds of the supernatant were removed. PBL was aspirated and washed twice with DMEM-F12 medium (Sigma. USA)

MTT reduction

Cells treated with 3.5 μM 7 μM 10.5 μM of $A\beta_{1-42}$ and 4.5 μM 6.75 μM , 9 μM of α-crystallin. Cell viability was measured 24 hr after peptide addition to cells using the 3, (4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT) reduction assay [24]. 10 μl of 12 mM MTT was added to 200 μl of cells plus medium in a 96-well plate. The cells were incubated with MTT for 4hr in a CO_2 incubator. Then, 100 μl of a 5:2:3 N, N-dimethylforamide (DMF): sodium dodecyl sulfate (SDS): water solution (pH 4.7) was added to dissolve the formed formazan crystals. After 18hr incubation in a humidified CO_2 incubator, the results were recorded by using a Microplate reader at 585 nm.

Assessment of free radicals in Astrocytes and Lymphocytes

The level of radical generated in Astrocytes and Lymphocytes treated with 7 μM of $A\beta_{1-42}$ and 6.75 μM of α-crystallin was measured with DCFH-DA (Sigma, St Louis, USA) as a fluorescence probe [23]. Cells were incubated for 4hr with DCFH-DA and recorded spectrofluorimetrically with excitation at 488nm and emitted at 530 nm.

Proteasome assay in Astrocyte and lymphocytes [25]

$A\beta_{1-42}$ (7 μM) and α-crystallin (6.75 μM) treated astrocytes and lymphocytes were washed thrice in PBS and lysed using lysis buffer without proteasomal inhibitor. The crude extract was pelleted. The 50 μl of the supernatant was assayed for the chymotrypsin like activity of the proteasome using 20 μM of suc-LLVF as substrate.

Determination of chaperone expression using ELISA

The ELISA was used to determine the expression of Hsp70, Tcp□□□ cp□in the Astrocytes and lymphocytes. Briefly cells were lysed using the lysis buffer. The lysed cells were centrifuged and pelleted. The 50 μl fractions from the supernatant were added followed by subsequent washing and treated with Polyclonal antibody in microplate and incubated for 24hr at 4°C. After washing with phosphate buffered saline, the microplate were incubated with ALP conjugated secondary-goat anti rabbit IgG. Proteins bound to antibodies were assayed, by color development using the *p*-nitro phenyl phosphate in carbonate buffer system at 405nm. (Biotek instruments EL310 Micro plate Autoreader, Winooski, Vermont).

RESULTS

Interaction between Aβ and α-Crystallin

The interaction of α-Crystallin with $A\beta_{1-42}$ results in FRET from excited tryptophan of α-Crystallin to pyrene moiety of Aβ1-42. Figure 1 shows the tryptophan fluorescence spectrum of α-Crystallin (λ_{ex} = 295 nm). When $A\beta_{1-42}$ was added to the excited α-Crystallin, fluorescence of tryptophan gets decreased. The emission spectrum of α-Crystallin was excited at 295 nm at which tyrosine and phenylalanine have no absorption and tryptophan is the only stimulated fluorophore. The emission maximum was centered at 330 nm and this was accompanied by the appearance of two sharp fluorescence peaks at 380 nm and 400 nm. Decrease in fluorescence intensity of tryptophan on addition of pyrenated $A\beta_{1-42}$ with increase in pyrene intensity was observed in 24hr. On 48, 72, 96 hrs there was regular decrease in tryptophan absorbtion with concominant decrease in pyrene absorbtion.

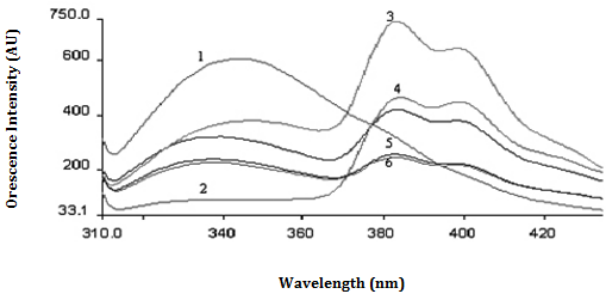

Fig. 1

FRET was observed between tryptophan moiety of α-Crystallin and pyrene moiety of $A\beta_{1-42}$. Concentration of α-Crystallin (donor) used in this experiment was 3 μM and the concentration of $A\beta_{1-42}$ (acceptor) was 7μM. Figure inset number 1 - donor alone, 2 -

acceptor without donor, 3 to 6 - donor in the presence of the acceptor on 1st, 2nd, 3rd & 4th day, respectively.

Acrylamide quenching

On addition of acrylamide to pyrenated Aβ in the presence and absence of α-Crystallin results in regular quenching of the pyrene emission. The quenching constant was found to be 0.2103 M^{-1} for pyrene Aβ without α-Crystallin and it was 0.044 M^{-1} with α-Crystallin (figure 2).

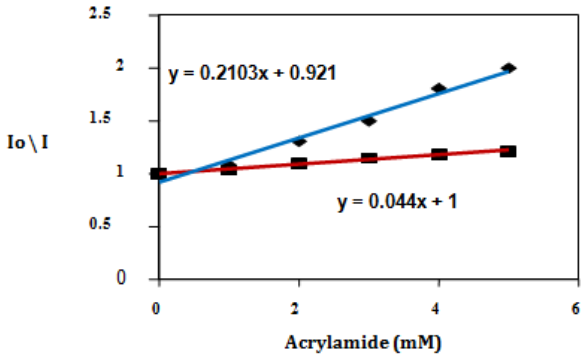

Fig. 2: Solvent accessibility of the pyrene of Aβ1-42 peptide.

Emission spectra of Aβ1-42 in PBS were collected at 298 K at a series of quencher concentrations. Samples were excited at 335 nm and emission observed from 345-500 nm. Stern-Volmer plots were constructed by plotting the peak intensity in the presence of quencher divided by the peak intensity in the absence of quencher (I0/I) as a function of quencher concentration. Stern-Volmer plot derived from acrylamide quenching of 10 µM Aβ1-42 (♦)and 10 µM Aβ1-42 + α-Crystallin (■)The continuous lines represent the best fit of the experimental data to the general Stern-Volmer equation. The Stern-Volmer constants (KSV) obtained from this analysis are presented inside the figure.

Induced CD

From the induced CD, it was observed that Pyrene Aβ produced a weak negative CD band at 350nm. Addition of different concentrations of α-Crystallin (figure 3) changes the asymmetric environment on pyrenated Aβ and produces an intense strong negative band at 350 nm.

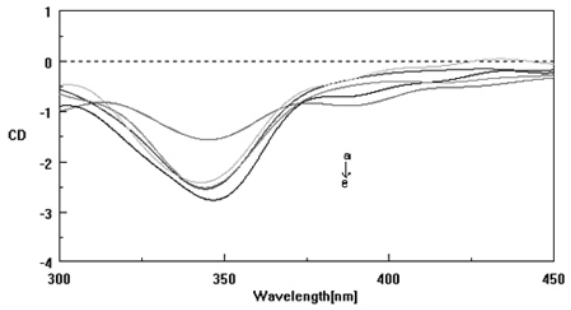

Fig. 3: Induced circular dichroism of pyrene Aβ (100 µM) in PBS (spectra a) represents 100 µM of pyrene Aβ. Spectras b to e depicts pyrene Aβ in the presence of increasing concentrations of α-Crystallin (5, 7.5, 10 & 15 µM).

Thioflavin T binding analysis

Binding of $Aβ_{1-42}$ to Thioflavin T was used to monitor fibrillar kinetics in the presence and absence of α-Crystallin. $Aβ_{1-42}$ shows very short lag phase. Aggregation phase is very steep and it resulted in stationary phase at 48hr. α-Crystallin alone at a concentration of 10 µM showed no change in Thioflavin T intensity. α-Crystallin incubated with $Aβ_{1-42}$ is characterized by the decrease in binding of Thioflavin T which shows an initial lag phase after 24hr with steady log phase followed by a stationary phase at 72hr (figure 4).

Light Scattering

Time dependence aggregation of $Aβ_{1-42}$ in the presence and absence of α-Crystallin was monitored. Kinetic profiles of $Aβ_{1-42}$ aggregation shown in figure5 was characterized by an initial lag phase, a rapid increase in fibrillization and a plateau phase. $Aβ_{1-42}$ exhibits a short lag time and proceeds more aggressively once log phase gets initiated and reaches the plateau phase at 48hr. In presence of α-Crystallin the light scattering of $Aβ_{1-42}$ decreases and reached the plateau at 72hr.

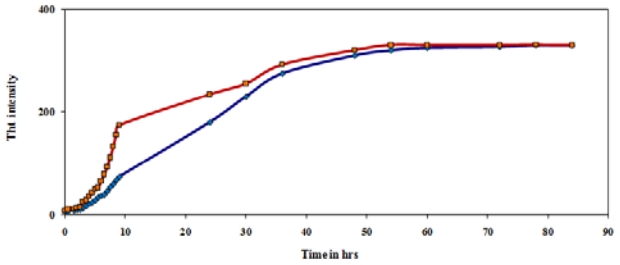

Fig. 4: Fibrilisation of 30 µM of $Aβ_{1-42}$ in 10 mM phosphate (pH 7) at 37 C depicted as the normalized intensity of Thioflavin T fluorescence as a function of incubation time in the absence and presence of 30 µM α-Crystallin.

Red curve represents $Aβ_{1-42}$ and blue for $Aβ_{1-42}$ with α-Crystallin. Excitation wavelength was set at 445nm with slit width of 10nm.

Cell viability in Astrocytes assessed by MTT assay

The assay of the conversion of MTT to colored formazan by mitochondrial reductase serves as an indirect measurement of cell proliferation and viability. Figure 6 shows the dose dependent toxicity of $Aβ_{1-42}$. In $Aβ_{1-42}$, at a concentration of 3.5µM, the viability of cells was about 56% where as 48% and 49% of viable cells were observed at a concentration of 7 µM and 10.5 µM, respectively. Treatment of α-Crystallin in various dose concentrations to $Aβ_{1-42}$ was able to reduce the MTT effectively. At a concentration of 4.5 µM of α-Crystallin, 60% of $Aβ_{1-42}$ treated cells were able to survive (figure 7). Effective dose concentration of α-Crystallin was found to be 6.75 µM in which 77% of viability was found in $Aβ_{1-42}$ treated cells.

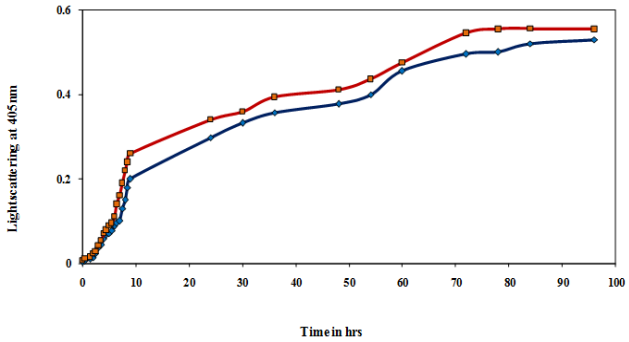

Fig. 5: $Aβ_{1-42}$ aggregates in the presence and absence of α-Crystallin.

Aggregation was monitored by light scattering using fluorescence spectrofluorimeter (excitation and emission wavelength were set at 405nm with slit width of 10nm). Concentration of $Aβ_{1-42}$ and α-Crystallin used in this experiment was 5 µM. Red curve represents $Aβ_{1-42}$ and blue for $Aβ_{1-42}$ with α-Crystallin.

Cell viability in lymphocytes assessed by MTT assay

$Aβ_{1-42}$ at a concentration of 3.5 µM decreases the viability of lymphocytes cells to 60%. At concentrations of 7 µM and 10.5 µM only 48% and 46% of cells, respectively, were able to survive (figure 8). Incubation of α-Crystallin to $Aβ_{1-42}$ (figure 9) treated cells were able to reduce the MTT more effectively than 73% where cells were able to survive a concentration of 6.75 µM α-Crystallin.

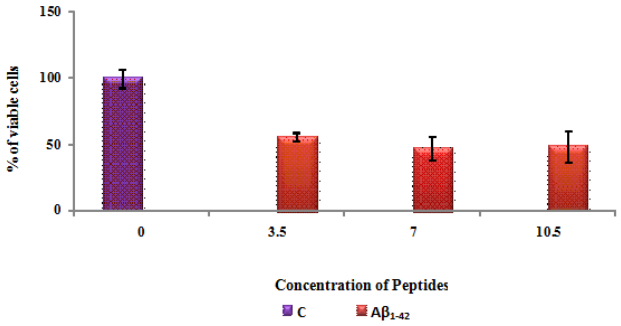

Fig. 6: Cytotoxic effect of Aß$_{1-42}$ on Astrocytes. Concentration of Aß$_{1-42}$ used was 3.5, 7, & 10.5 µM.

Data are mean ± SEM triplicates and are representatives of three separate experiments.

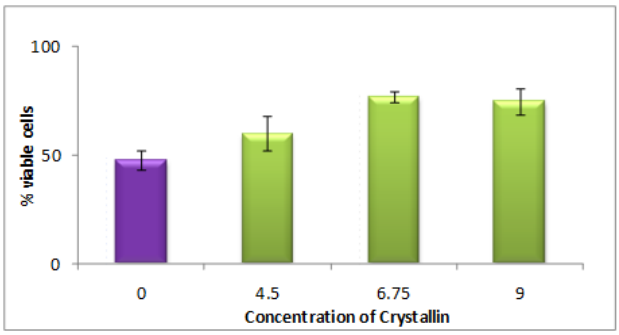

Fig. 7: Cytotoxic effect of Aß$_{1-42}$ on Astrocytes in the presence of α-Crystallin. Concentration of Aß$_{1-42}$ used was 7µM.Concentration α-Crystallin used was 4.5, 6.75 & 9µM.

Data are mean ± SEM triplicates and are representatives of three separate experiments.

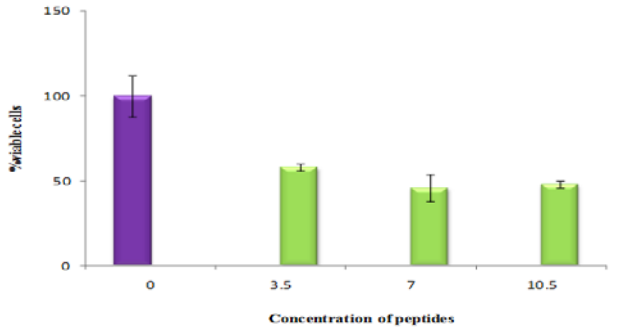

Fig. 8: Cytotoxic effect of Aß$_{1-42}$ on lymphocytes. Concentration of Aß$_{1-42}$ used was 3.5, 7 & 10.5 µM.

Data are mean ± SEM triplicates and are representatives of three separate experiments.

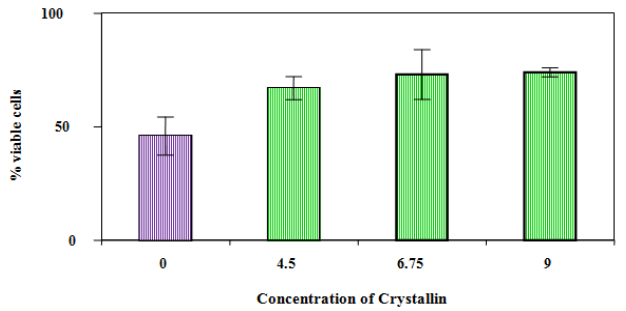

Fig. 9: Cytotoxic effect of Aß$_{1-42}$ on Lymphocytes in the presence of α-Crystallin. Concentration of Aß$_{1-42}$ used was 7 µM. Concentration of α-Crystallin used were 4.5, 6.75 & 9 µM.

Data are mean ± SEM triplicates and are representatives of three separate experiments.

Oxidative stress in Astrocytes

Intracellular ROS concentration in Aβ$_{1-42}$ (Figure 10) treated cells were estimated by the conversion of DCFH$_2$ – DA to DCF. The ROS production was expressed as a percentage compared with positive control cells, which were treated with H_2O_2. Aβ$_{1-42}$ treated cells showed 65% ROS production. When the α-Crystallin was added to Aβ$_{1-42}$ about 66% of radicals were significantly scavenged in the α-Crystallin treated cells.

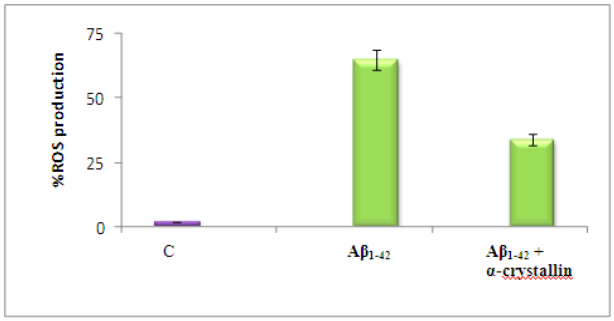

Fig. 10: Inhibition of reactive oxygen species by α-crystallin in Aß1-42 treated Astrocytes. Concentration of Aß$_{1-42}$ and α-Crystallin used in this experiment were 7µM and 6.75 µM.

Data are mean ± SEM of triplicates and are representative of three separate experiments.

Oxidative stress in Lymphocytes

In lymphocytes Aβ$_{1-42}$ treated cells showed 75% ROS production. When the α-Crystallin was added to Aβ$_{1-42}$ treated cells the percentage of ROS production was reduced (figure11) to about 78%.

Fig. 11: Inhibition of reactive oxygen species by α-crystallin in Aß1-42 treated Lymphocytes. Concentration of Aß$_{1-42}$ and α-Crystallin used in this experiment was 7µM and 6.75µM

Data are mean ± SEM of triplicates and are representative of three separate experiments

Effect of α-Crystallin on proteasomal activity of Astrocytes

To investigate the perturbation of Aβ$_{1-42}$ on proteasome and the role played by α-Crystallin was investigated using the fluorescent substrate suc-LLVF. Aβ$_{1-42}$ inhibited about 40% proteasomal activity of Astrocytes. α-Crystallin reversed the proteasome inhibition. α-Crystallin enhances its enzyme activity to 80% in Aβ$_{1-42}$ treated cells (figure12).

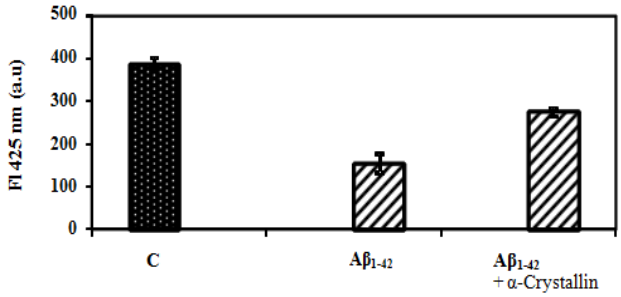

Fig. 12: Proteasome inhibition assay in astrocytes. Concentration of Aß$_{1-42}$ and α-Crystallin used in this experiment were 7µM and 6.75 µM respectively.

Data are mean ± SEM of triplicates and are representative of three separate experiments.

Effect of α-Crystallin on proteasomal activity of Lymphocytes

In lymphocytes (figure 13) Aβ$_{1-42}$ inhibited the proteasomal activity by 50%. α-Crystallin treatment reversed the proteasomal inhibition

brought by $A\beta_{1-42}$ and the treated cells maintain their activity as that of control cells. More than 80% of enzyme activity was restored in α-Crystallin treated cells.

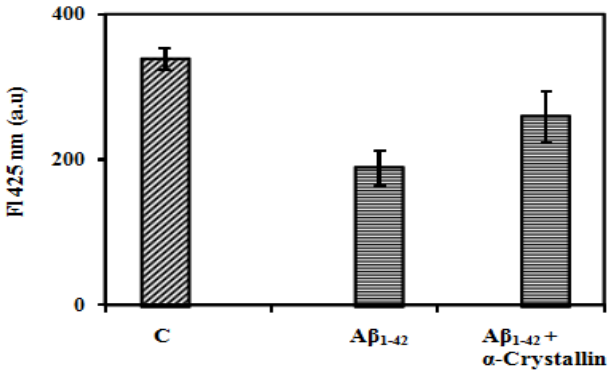

Fig. 13: Proteasome inhibition assay in Lymphoctes. Concentration of Aß$_{1-42}$ and α-Crystallin used in this experiment were 7µM and 6.75µM.

Data are mean ± SEM of triplicates and are representative of three separate experiments

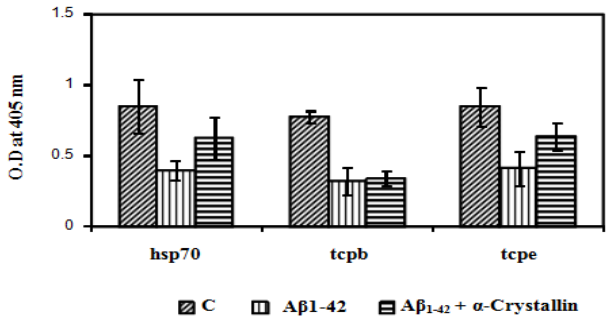

Fig. 14: ELISA analysis to determine the expression of HSP70, TCPβ and Tcpε in astrocytes. Concentration of Aß$_{1-42}$ and α-Crystallin used in this experiment were 7µM and 6.75µM.

Data are mean ±SEM of triplicates and are representative of three separate experiments.

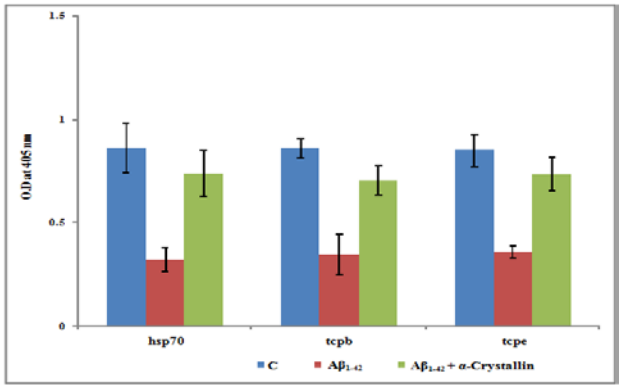

Fig. 15: ELISA analysis to determine the expression of HSP70 TCPβ and Tcpε in Lymphocytes.Concentration of Aß$_{1-42}$ and α-Crystallin used in this experiment were 7µM and 6.75µM respectively.

Data are mean ± SEM of triplicates and are representative of three separate experiments

Expression of Hsp70, TCPβ and Tcpε in Astrocytes

From the ELISA analysis it is evident that the expression Hsp70, TCPβ and ε was reduced to up to 30% in $A\beta_{1-42}$. α-crystallin treatment enhances the expression of protective molecules such as Hsp70, TCPβ and TCPε as that of control whose dysregulation contribute to many toxic response.

Expression of Hsp70, TCPβ and ε in Lymphocytes

ELISA analysis (figure 15) of lymphocytes for the expression Hsp70, TCPβ and TCPε revealed that amyloid down regulates the chaperones and the expression of these chaperones was only about 40%. α- crystallin treatment enhances the expression of Hsp70, TCPβ and TCPε as that of control.

DISCUSSION

There is an overlap between tryptophan emission and pyrene excitation and energy transfer occurs between these two probes if there is interaction between α crystallin with $A\beta_{1-42}$. Decrease in fluorescence intensity of tryptophan on addition of pyrenated $A\beta_{1-42}$ indicates that there was an interaction between α crystallin with $A\beta_{1-42}$. The nature of the interactions may be due to the participation of $A\beta_{1-42}$ in the subunit exchange with the α crystallin. Early work on α B crystallin with Aβ peptide suggests that energy transfer occurs due to subunit exchange [20]. The FRET observed in this study suggested that both the molecules are in close proximity and they interact efficiently providing an evidence of conformational change in $A\beta_{1-42}$ in the presence of α- crystallin. Increase in intense negative bands in induced CD also suggests that on addition of crystallin there was a change in asymmetry of the microenvironment surrounding the pyrenyl chromophore. FRET and CD experiments clearly indicates the intimate association of the pyrene Aβ with crystallin. Two extreme possibilities for binding of these Aβ to crystallin can be considered. Binding may occur on the protein surface such that the pyrenyl chromophore is exposed to the solvent, or pyrene-Aβ might be buried in protein matrix shielded from the aqueous phase. Fluorescence quenching studies clearly suggest that the pyrene-Aβ was buried in the protein surface, as it was not accessible to the quencher. Thioflavin T is a fluoresecent dye used as a diagnostic probe to identify amyloid fibril formation [27]. The emission intensity of the Thioflavin T increases significantly upon binding to the linear array of beta strands in the amyloid [28]. Decreased Thioflavin T fluorescence in α-crystallin treated $A\beta_{1-42}$ clearly indicates that α-crystallin deprive the fast propagation of fibril in $A\beta_{1-42}$. Light scattering measures attenuation in the intensity of light as it passes through a scattering medium. Turbidity is a good indicator of aggregation. Decreased scattering of light was found when $A\beta_{1-42}$ was incubated with α-crystallin suggesting that α-crystallin decreases the fibril formation. From the spectroscopic analysis, it is suggested that α-crystallin prevents the intermediate conformational transition of the so called fibrils of $A\beta_{1-42}$ and stabilizes its structure. Fibril disrupts the leaflet structure of membrane and perturbs to form membrane channels [29]. Aβ fibrils can induce the acute electrophysiological changes and progressive neurotoxicity in cortical neurons. [30]. α-crystallin can effectively bind to the exposed hydrophobic residues and effectively stabilize the intermediately folded states of proteins and it also has the ability to suppress the aggregation completely [31]. α-crystallin binds to the $A\beta_{1-42}$ and prevents its highly toxic intermediate. There was increased degeneration in $A\beta_{1-42}$ on co-incubation with α-crystallin the degeneration greatly reduced and suggested that α- crystallin protects the cells by preventing the cytotoxicity induced by $A\beta_{1-42}$. Toxicity of amyloids mostly correlated with hydrophobicites and ability to form amyloid fibrils and its interaction with membrane [23], and formation of cation selective channels in cellular membranes [32]. The finding of receptor for advanced glycation end products (RAGE) as the specific receptor for Aβ has enhanced understanding of $A\beta_{1-42}$ induced neurotoxicity [33]. Interaction of $A\beta_{1-42}$ with receptors in cell membranes of neurons induces production of ROS [34]. Generation of ROS impair the function of membrane proteins involved in maintenance of ion homeostasis resulting in membrane depolarization and calcium influx through glutamate receptor channels and voltage-dependent calcium channels [35] that leads to dysregulation of cellular signal transduction which may ultimately result in cell death. Increase ROS production in $A\beta_{1-42}$ treated cells suggested that decrease in viability

of astrocytes and lymphocytes may be due to the ability of $A\beta_{1-42}$ to induce oxidative stress. Increased oxidative stress was greatly reduced in α-crystallin treatment. In our early work, we have showed that in physiological conditions and in slightly perturbed conditions during mild stress, Aβ binds to the antioxidant enzymes causing aggregates and prevents them from retaining their native structure [23]. α- crystalline, a molecular chaperone, might prevent the above deactivation of antioxidant enzymes and inactivated the production of ROS. Masilamani et al has already suggested that α-crystallin has ability to prevent the oxidative stress in mice models [14]. From the western blot and ELISA it was inferred that $A\beta_{1-42}$ suppresses the expression of Hsp70 and Tric subunits. Earlier workers suggested that polyglutamine aggregates down-regulates the signal transduction process and decreases the expression of regulatory proteins such as endogenous chaperones and proteasome [36]. Hsp70 have been implicated in maintaining tau solubility and suppressing tau aggregates. Cell culture experiments have shown that GRP78 an Hsp70 found in endoplasmic reticulum binds to APP and decreases the amyloid $A\beta_{1-42}$. It indicates that Hsp70 shield APP from β/γ secretase cleavage [37]. Apart from this Hsp70 rescues neurons from intracellular $A\beta_{1-42}$ mediated toxicity [38]. Degeneration of cells in this study on treatment of $A\beta_{1-42}$ might be due to the down regulation of Hsp70. In turn, in α-crystallin treated groups, expression of endogenous chaperones have been regulated. Upregulation of chaperones might be due to α-crystallin that activates the expression of the proteins at the level of gene or at the level of protein turn over. This suggests that α-crystallin regulates some critical cellular processes implicated in the production and maintenance of Hsp70 and Tric subunits.

Cross talk between molecular chaperones and the Ubiquitin proteasome might be pivotal in regulating the deposition and toxicity of aggregated proteins. Particularly Hsp70 mediates the misfolded proteasome to the ubiquitin proteasome system or lysosomes result in selective degradation [37]. CHIP is a protein that binds to Hsp70 and acts as an e3 ligase to facilitate the transfer of polyubiquitin chain to misfolded substrates [39]. $A\beta_{1-42}$ diminishes the expression of Hsp70 and Tric and affects the ubiquitin mediated degradation. α-crystallin expresses the Hsp70 and Tric and maintains the cellular function by up regulation of endogenous chaperones and facilitating the CHIP mediated Ubiquinated degradation of misfolded substrates.

In conclusion, this study suggests that α-crystallin accelerates the amyloid formation by preventing the intermediate protofibrils formation and decreases the toxicity by activating various cellular machinery such as high expression of Hsp70 and Tric.

REFERENCES

1. Takuma K, Fang F, Zhang W, Yan S, Fukuzaki E, Du H, et al. RAGE-mediated signaling contributes to intraneuronal transport of amyloid-beta and neuronal dysfunction. Proceedings of the National Academy of Sciences of the United States of America 2009;106(47):20021-6.
2. Zhang Y, Champagne N, Beitel LK, Goodyer CG, Trifiro M, LeBlanc A. Estrogen and androgen protection of human neurons against intracellular amyloid beta1-42 toxicity through heat shock protein 70. The Journal of neuroscience: the official journal of the Society for Neuroscience 2004;24(23):5315-21.
3. Mattson MP. Cellular actions of beta-amyloid precursor protein and its soluble and fibrillogenic derivatives. Physiological reviews 1997;77(4):1081-132.
4. Brown ST, Scragg JL, Boyle JP, Hudasek K, Peers C, Fearon IM. Hypoxic augmentation of Ca2+ channel currents requires a functional electron transport chain. The Journal of biological chemistry 2005;280(23):21706-12.
5. Perluigi M, Joshi G, Sultana R, Calabrese V, De Marco C, Coccia R, et al. In vivo protective effects of ferulic acid ethyl ester against amyloid-beta peptide 1-42-induced oxidative stress. Journal of neuroscience research 2006;84(2):418-26.
6. Abramov AY, Canevari L, Duchen MR. Beta-amyloid peptides induce mitochondrial dysfunction and oxidative stress in astrocytes and death of neurons through activation of NADPH oxidase. The Journal of neuroscience: the official journal of the Society for Neuroscience 2004;24(2):565-75.
7. Fonte V, Kapulkin WJ, Kapulkin V, Taft A, Fluet A, Friedman D, et al. Interaction of intracellular beta amyloid peptide with chaperone proteins. Proceedings of the National Academy of Sciences of the United States of America 2002;99(14):9439-44.
8. Magrane J, Rosen KM, Smith RC, Walsh K, Querfurth HW, J. Gouras Gk, Intraneuronal beta amyloid expression downregulates the Akt survival pathway and blunts the stress response. Biochemical and biophysical research communications 2005;25(47):10960-9.
9. Flower TR, Chesnokova LS, Froelich CA, Dixon C, Witt SN. Heat shock prevents alpha-synuclein-induced apoptosis in a yeast model of Parkinson's disease. Journal of molecular biology 2005;351(5):1081-100.
10. Vacher C, Garcia-Oroz L, Rubinsztein DC. Overexpression of yeast hsp104 reduces polyglutamine aggregation and prolongs survival of a transgenic mouse model of Huntington's disease. Human molecular genetics 2005;14(22):3425-33.
11. Muchowski PJ, Schaffar G, Sittler A, Wanker EE, Hayer-Hartl MK, Hartl FU. Hsp70 and hsp40 chaperones can inhibit self-assembly of polyglutamine proteins into amyloid-like fibrils. Proceedings of the National Academy of Sciences of the United States of America 2000;97(14):7841-6.
12. Franklin TB, Krueger-Naug AM, Clarke DB, Arrigo AP, Currie RW. The role of heat shock proteins Hsp70 and Hsp27 in cellular protection of the central nervous system. International journal of hyperthermia: the official journal of European Society for Hyperthermic Oncology, North American Hyperthermia Group 2005;21(5):379-92.
13. Wang K. alpha-B- and alpha-A-crystallin prevent irreversible acidification-induced protein denaturation. Biochemical and biophysical research communications 2001;287(3):642-7.
14. Masilamoni JG, Jesudason EP, Bharathi SN, Jayakumar R. The protective effect of alpha-crystallin against acute inflammation in mice. Biochimica et biophysica acta 2005;1740(3):411-20.
15. Stewart JM, Young JD. Solid Phase Peptide Synthesis. Pierce Chemical Company Rockford; 1984.
16. Bodanszky M, Bodanszky A. The Practice of Peptide Synthesis. Springer-Verlag Berlin Heidelberg New York Tokyo; 1984.
17. Chen S, Wetzel R. Solubilization and disaggregation of polyglutamine peptides. Protein science: a publication of the Protein Society 2001;10(4):887-91.
18. Rekas A, Adda CG, Andrew Aquilina J, Barnham KJ, Sunde M, Galatis D, et al. Interaction of the molecular chaperone alphaB-crystallin with alpha-synuclein: effects on amyloid fibril formation and chaperone activity. Journal of molecular biology 2004;340(5):1167-83.
19. Luthra M, Balasubramanian D. Nonenzymatic glycation alters protein structure and stability. A study of two eye lens crystallins. The Journal of biological chemistry 1993;268(24):18119-27.
20. Liang JJ. Interaction between beta-amyloid and lens alphaB-crystallin. FEBS letters 2000;484(2):98-101.
21. Rodriguez WG, Vega A, Becerrai MS, Milton S, Johnson DA, Yatsimirsky AK, et al. Change in the Carboxyl Terminus of Alzheimer's Ab Accompanies the Transition from Dimer to Fibril as Revealed by Fluorescence Quenching Analysis. Chem 2264522649 2000;275(30):1-40.
22. Abe K, Abe Y, Saito H. Evaluation of L-glutamate clearance capacity of cultured rat cortical astrocytes. Biological & pharmaceutical bulletin 2000;23(2):204-7.
23. Jayakumar R, Murali J, Koteeswari D, Gomathi K. Cytotoxic and membrane perturbation effects of a novel amyloid forming model peptide poly(leucine-glutamic acid). Journal of biochemistry 2004;136(4):457-62.
24. Liu KZ, Schultz CP, Johnston JB, Lee K, Mantsch HH. Comparison of infrared spectra of CLL cells with their ex vivo sensitivity (MTT assay) to chlorambucil and cladribine. Leukemia research 1997;21(11-12):1125-33.
25. Hanson AJ, Prasad JE, Nahreini P, Andreatta C, Kumar B, Yan XD, Prasad KN. Over expression of amyloid precursor protein is associated with degeneration decreased viability and increased damage caused by neurotoxins (Prostaglandins A1 and E2

hydrogen peroxide and nitric oxide) in differentiated neuroblastoma cells. J Neuro Sci Res 2003;74148-159.

26. Merienne K, Helmlinger D, Perkin GR, Devys D, Trottier Y. Polyglutamine expansion induces a protein-damaging stress connecting heat shock protein 70 to the JNK pathway. The Journal of biological chemistry 2003;278(19):16957-67.
27. Dzwolak W, Pecul M. Chiral bias of amyloid fibrils revealed by the twisted conformation of Thioflavin T: an induced circular dichroism/DFT study. FEBS letters 2005;579(29):6601-3.
28. Khurana R, Coleman C, Ionescu-Zanetti C, Carter SA, Krishna V, Grover RK, et al. Mechanism of thioflavin T binding to amyloid fibrils. Journal of structural biology 2005;151(3):229-38.
29. Ye CP, Selkoe DJ, Hartley DM. Protofibrils of amyloid beta-protein inhibit specific K+ currents in neocortical cultures. Neurobiology of disease 2003;13(3):177-90.
30. Hartley DM, Ye CP, Diehl T, Vasquez S, Vassilev PM, Teplow DB, et al. WalshDM, Protofibrillar intermediates of amyloid beta-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. Biochemical and biophysical research communications 1999;9(20):8876-84.
31. Lee S, Carson K, Rice-Ficht A, Good T. Hsp20, a novel alpha-crystallin, prevents Abeta fibril formation and toxicity. Protein science: a publication of the Protein Society 2005;14(3):593-601.
32. Aleardi AM, Benard G, Augereau O, Malgat M, Talbot JC, Mazat JP, et al. Gradual alteration of mitochondrial structure and function by beta-amyloids: importance of membrane viscosity changes energy deprivation reactive oxygen species production and cytochrome c release. Int 2005;21(5):403-19.
33. Yan SD, Chen X, Fu J, Chen M, Zhu H, Roher A, et al. RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease. Nature 1996;382(6593):685-91.
34. Lue LF, Yan SD, Stern DM, Walker DG. Preventing activation of receptor for advanced glycation endproducts in Alzheimer's disease. Current drug targets. CNS and neurological disorders 2005;4(3):249-66.
35. Mattson MP, Mark RJ, Furukawa K, Bruce AJ. Disruption of brain cell ion homeostasis in Alzheimer's disease by oxy radicals, and signaling pathways that protect therefrom. Chemical research in toxicology 1997;10(5):507-17.
36. Bonini NC, Sci USA. Chaperoning brain degeneration. Proc Natl Acad 2002;10:16407-11.
37. Muchowski PJ, Wacker JL. Modulation of neurodegeneration by molecular chaperones. Nature reviews. Neuroscience 2005;6(1):11-22.
38. Magrane J, Smith RC, Walsh K, Querfurth HW. Heat shock protein 70 participates in the neuroprotective response to intracellularly expressed beta-amyloid in neurons, J,Neuroscience. Biochemical and biophysical research communications 2004;24(7):1700-6.
39. Esser C, Alberti S, Höhfeld J. Cooperation of molecular chaperones with the ubiquitin/proteasome system. Biochimica et biophysica acta 2004;1695(1-3):171-88.