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Small Heat Shock Proteins Protect Against α -Synuclein-Induced Toxicity and Aggregation

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Abstract

Protein misfolding and inclusion formation are common events in neurodegenerative diseases, such as Parkinson's disease (PD), Alzheimer's disease (AD) or Huntington's disease (HD). Alpha-synuclein (aSyn) is the main protein component of inclusions called Lewy bodies (LB) which are pathognomic of PD, Dementia with Lewy bodies (DLB) and other diseases collectively known as LB diseases. Heat shock proteins (HSPs) are one class of the cellular quality control system that mediate protein folding, remodeling and even disaggregation. Here, we investigated the role of the small heat shock proteins Hsp27 and α B-crystallin, in LB diseases. We demonstrate, via quantitative PCR, that Hsp27 messenger RNA levels are ~2–3-fold higher in DLB cases compared to control. We also show a corresponding increase in Hsp27 protein levels. Furthermore, we found that Hsp27 reduces aSyn-induced toxicity by ~80% in a culture model while α B-crystallin reduces toxicity by ~20%. In addition, intracellular inclusions were immunopositive for endogenous Hsp27, and overexpression of this protein reduced aSyn aggregation in a cell culture model.

Keywords

α -synuclein; Parkinson's disease; Lewy body; protein misfolding; heat shock proteins; neurodegenerative disease

Introduction

Alpha-synuclein (aSyn), an abundant presynaptic protein of unknown function, is implicated in a variety of neurodegenerative disorders collectively known as 'synucleinopathies' [1]. PD, the second most common neurodegenerative disease, has a complex etiology with three mutations in aSyn (A30P, E46K, and A53T) [2–4], and a triplication of the aSyn gene [5] linked to familial PD. In both familial and sporadic PD, aSyn is found in intracellular fibrillar inclusions called LBs and also in Lewy neurites (LNs) [6].

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aSyn-immunoreactive LBs are also the characteristic pathological hallmark of another neurodegenerative synucleinopathy, DLB, where LBs can be found throughout the brain [7]. In DLB, LBs appear scattered throughout several brain regions, whereas in PD they are more restricted to the substantia nigra [8,9].

Overexpression of aSyn in a variety of animal models, yeast, and mammalian cells, results in cytotoxicity and inclusion body formation [10–12], modeling some of the key aspects of synucleinopathies. It has been suggested that sub-microscopic oligomeric species of aSyn, found in vitro and also in PD, may represent a toxic species [13]. A common aspect of many neurodegenerative diseases is the presence of proteinaceous inclusions, which seem to result from the misfolding of otherwise normal endogenous proteins [14]. Pre-aggregated, oligomeric species of these proteins have been implicated in these disorders, ranging from AD to HD, suggesting the oligomeric species may indeed constitute a toxic conformation [14,15].

Heat shock proteins (HSPs) are a ubiquitous class of proteins that represent part of the quality control machinery of the cell and are upregulated in response to various types of stress [16]. HSPs exert chaperone activity by stabilizing partially unfolded proteins, disaggregating protein inclusions, or directing misfolded proteins for degradation [17]. Overexpression of Hsp70 reduces the toxicity of aSyn in a variety of animal models and also in cellular systems [18, 19]. Importantly, Hsp70 activity was also shown to be protective against polyglutamine-induced toxicity in HD models [20], suggesting this chaperone may have a broad protective effect over the toxicity associated with protein misfolding, oligomerization, and aggregation.

Small HSPs share a number of features, including low monomeric molecular mass, formation of large oligomeric complexes, and the presence of an α B-crystallin domain that occurs near the C-terminal region [21]. Along with Hsp70, Hsp27 is one of the main inducible HSPs in the nervous system and it has been shown to be neuroprotective against a variety of stresses and stimuli, including heat stress or oxidative conditions [22,23,24]. It has also been shown to interfere with apoptosis by preventing caspase activation [25,26]. Notably, Hsp27 prevents polyglutamine-induced toxicity in a cellular model for HD, where it acts upon the oxidative stress response caused by overexpression of mutant huntingtin [27]. In a separate study, Hsp27 was found to be protective against a variety of stresses applied to aSyn-expressing cells [28].

Several Hsps, including Hsp27 and α B-crystallin, can be found in aSyn-inclusions such as LBs in DLB and glial cytoplasmic inclusions (GCIs) in multiple system atrophy [29,30].

Here, we investigated the role of two members of the small HSP family in DLB, and further explored the neuroprotective role of small HSPs in an in vitro model of synucleinopathies.

Materials and Methods

Human brain tissue

All human brain tissues studied were obtained through the Massachusetts Alzheimer Disease Research Center (ADRC) brain bank, the Harvard Brain Tissue Resource Center or the University of Maryland brain bank and processed as previously described [19,29] (suppl. materials).

RNA Extraction and Quantitative PCR

RNA was isolated using PicoPure RNA Isolation kit (Arcturus, Mountain View, CA, USA) according to manufacturer's guidelines, and subjected to DNase treatment (Qiagen, Valencia, CA, USA). RNA was eluted in 30 μ L of elution buffer, and the column washed with an additional 30 μ L of elution bringing the final volume up to 60 μ L. Eluted RNA was stored at -80°C until further use.

Gene expression of target mRNAs was quantified for each sample using quantitative real-time PCR. The Hsp27 (BC012768) primers used were 5'CAAGTTTCCTCCTCCCTGTC3' and 5'GGCAGTCTCATCGGATTTTG3'. The GAPDH (NM_002046) primers used were 5'GGTCTCCTCTGACTTCAACA3' and 5'GTGAGGGTCTCTCTTCTCCT3'. Real-time quantitative PCR (QPCR) short synthetic PCR primers (18–20 mer) were designed using Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Primer sets were designed to amplify small (200–300 bp) amplicons for the candidate mRNAs. First-strand cDNA synthesis was carried out on mRNA extracted with SuperscriptTM first-strand synthesis kit (Invitrogen Carlsbad, CA) according to the manufacturer's specifications. QPCR was carried out in a 96 well plate using an iCycler (BioRad, Hercules, CA), and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). A concentration curve with known concentrations of plasmid DNA containing the reference sequence was used to calculate the primer set efficiency for each experiment, and to quantitate products. The final concentration of each transcript was calculated with the δC_t method using the software provided by BioRad.

Plasmid construction

The constructs for human wild type aSyn and its C-terminal tagged version (referred to as synT) have been described previously [31]. The Myc-tagged version of Hsp27 was generated by PCR from pcDNA3.1-Hsp27 (a kind gift of Rainer Benndorf, University of Michigan Medical School, Ann Arbor) and sub-cloned into pcDNA3.1/myc-His as a BamHI/HindIII fragment. The construct was verified by DNA sequencing. α B-Crystallin was a kind gift of W. Boelens.

Cell culture, transfections, and immunocytochemistry

Cell culture, transfections and immunocytochemistry were performed as described previously [19]. For more information please see supplementary materials.

RNA interference

For co-transfections of siRNA and plasmid DNA we used HiPerfect transfection reagent from Qiagen (Qiagen, Chatsworth, CA, USA). Cells were seeded into a 24-well plate in 0.5ml of Opti-MEM supplemented with 10% FBS 24 hr prior to transfection, and grown under standard conditions. On the day of transfection, 1 μ g of plasmid and the desired amount of siRNA were transfected according to the manufacturer's instructions. After 4–6 hrs the medium was changed and cells were incubated under standard conditions for 24 hrs or 48 hrs.

Quantitation of cells containing aSyn inclusions

Cells immunostained for aSyn using the syn-1 antibody (BD Biosciences) were imaged using an upright Olympus fluorescence microscope equipped with CAST software (Visiopharm, Denmark). Cells containing at least one aSyn-positive inclusion were counted according to a stereology based unbiased systematic random sampling scheme.

aSyn toxicity assay

Toxicity was analyzed 24 h after transfection using the ToxiLightTM kit (Cambrex, Walkersville, MD) according to the manufacturer's protocol.

SDS-PAGE and immunoblotting

Transfections, SDS-PAGE and immunoblotting were performed as previously described [19].

Preparation of lentiviral constructs

The ViraPower Lentivirus Expression System (Invitrogen) was used to generate lentivirus encoding human aSyn (A53T) and Hsp27 with a C-terminal myc tag. A cDNA encoding A53T was amplified by PCR and subcloned into the Kpn I and Xho I sites of the vector pENTR1A to generate pENTR-A53T. A cDNA encoding Hsp27-myc was amplified by PCR and subcloned into the BamH I and Xho I sites of the vector pENTR1A to generate pENTR-Hsp27-myc. The insert from each pENTR1A construct was transferred into the pLENTI6/V5 DEST lentiviral expression vector (Invitrogen) via a recombination reaction. Each lentiviral construct was sequenced using an Applied Biosystems DNA sequencer (University of Wisconsin and Purdue University). Lentiviral constructs were packaged into virus via lipid-mediated transient transfection of the 293FT packaging cell line. A control virus packaged with the pLENTI6/V5-DEST/LacZ DNA (Invitrogen), encoding β -galactosidase fused to the V5 epitope, was also prepared. Recombinant lentivirus was titrated using HEK293 cells.

Preparation of primary mesencephalic cultures

Primary midbrain cultures were prepared using a modified version of a previously described protocol [32,33]. Briefly, whole brains were dissected from day 17 rat embryos, and the mesencephalic region containing the *substantia nigra* and ventral tegmental area were isolated stereoscopically. The mesencephalic neurons and glia were dissociated from neuronal tissue with trypsin (final concentration, 26 μ g/mL in 0.9% [w/v] NaCl) and plated on coverslips previously treated with poly-L-lysine (5 μ g/mL). The media consisted of DMEM, 10% (v/v) FBS, 10% (v/v) HS, penicillin (100 U/ml), and streptomycin (100 μ g/ml). Four days later, the cells were treated for 48 h with AraC (20 μ M) to inhibit the growth of glial cells.

Lentiviral transductions of primary cultures

AraC-treated primary cultures were transduced with lentiviral particles in the presence of polybrene (6 μ g/ml). The cells were incubated with lentiviruses encoding A53T, Hsp27, A53T plus Hsp27, or A53T plus β -galactosidase. Control cells were incubated without lentivirus. After a 72-h transduction period, the cells were treated with fresh media for an additional 48 h and analyzed immunocytochemically.

Measurement of primary neuron viability

MAP2- and TH-immunoreactive primary neurons were counted in at least 10 randomly chosen observation fields for each experimental condition using a Nikon TE2000-U inverted fluorescence microscope. The data were expressed as the percentage of MAP2-positive neurons that were also TH-positive. Each experiment was repeated 3 times using embryonic neurons isolated from independent pregnant rats. Statistical analyses were carried out using the program GraphPad Prism, Version 4.0.

Results

Hsp27 is elevated in DLB cases

To test the hypothesis that cellular stress and protein misfolding are associated with DLB, PD and other synucleinopathies, we investigated the expression levels of several major heat shock proteins in pathologically confirmed cases of DLB as well as matched control cases. SDS-PAGE followed by immunoblot analysis showed that Hsp27 was elevated 2.5-fold in DLB cases compared to control (Fig. 1A). Similar analysis, performed on the same material, revealed that the levels of Hsp40, Hsp70 and Hsp90 were not significantly altered (Fig. 1A). We also found no significant change in the levels of aSyn. To further investigate the type of response leading to elevated levels of Hsp27, we performed quantitative-PCR (qPCR) analysis to measure Hsp27 mRNA. Our results indicate that Hsp27 mRNA is elevated approximately 2–

3-fold in the DLB cases (n=15, p<0.001) (Fig. 1B), consistent with the results from the immunoblot analysis. This suggests that the increase in Hsp27 protein levels is not simply due to an altered half life of the protein, but is possibly a result of a specific stress response that leads to increased production of the protein.

Hsp27 and α B-crystallin are present in LBs

To evaluate the presence of α B-crystallin in LBs, we stained human midbrain sections, from pathologically confirmed cases of DLB, with antibodies against aSyn and α B-crystallin or Hsp27. We confirmed the presence of Hsp27 in LBs and found that α B-crystallin also colocalized with aSyn in LBs as well as Lewy neurites (LNs) (Fig. 2A). Importantly, in accord with our previous findings for Hsp27 and other Hsps, not all aSyn-positive structures (LBs or LNs) were positive for these chaperones [29].

Hsp27 and α B-crystallin are present in aSyn inclusions in vitro

To further investigate the role of small heat shock proteins in aSyn-containing inclusions, we asked whether they were also present in aSyn inclusions in vitro using a cellular model for aSyn aggregation in human H4 neuroglioma cells. We co-stained H4 cells co-transfected with synT and synphilin-1 for aSyn and Hsp27 or α B-crystallin and found that both small heat shock proteins could be detected in synT/synphilin-1 inclusions (Fig. 2B). In agreement with the observations in DLB tissue, co-localization was only partial, as not all aSyn-positive inclusions were positive for either Hsp27 or α B-crystallin.

Hsp27, but not α B-crystallin, reduces aSyn aggregation in vitro

Recruitment of heat shock proteins into inclusion bodies is a widespread phenomenon, possibly constituting a cellular defense mechanism [34,35]. To investigate whether small heat shock proteins can influence inclusion body formation, we asked whether overexpression of Hsp27 or α B-crystallin would interfere with aSyn aggregation. To this end, we co-transfected H4 cells with synT, synphilin-1 and either Hsp27 or α B-crystallin overexpression plasmids and monitored the effect of the small Hsps on aSyn aggregation (Fig. 3A). As a control, we co-transfected cells with Hsp70, which we have previously shown to reduce aSyn aggregation in H4 cells [19]. Here, we found that co-transfection with Hsp27 reduced the percentage of cells with aSyn inclusions by ~20%, a similar effect to that of Hsp70 (Fig. 3B). By contrast, α B-crystallin did not significantly reduce aSyn inclusions (Fig. 3B). SDS-PAGE demonstrated a relatively small increase in the expression of Hsp27 was sufficient to cause the observed reduction in aggregation (Fig. 3C).

Hsp27 reduces aSyn toxicity in H4 cells

It is widely accepted that chaperone-overexpression or pharmacological upregulation of their levels may hold potential for the treatment of several protein misfolding diseases [36]. Work in fly models of synucleinopathies showed that Hsp70 protects against aSyn-induced neurodegeneration [18]. More recently, Hsp27 was found to have a stronger protective effect against aSyn-induced toxicity than Hsp70 [28]. Due to the presence of the small heat shock proteins Hsp27 and α B-crystallin in LBs, we hypothesized they may constitute a cellular response to modulate aggregation and toxicity. Previous work from our lab showed that overexpression of aSyn is toxic in human H4 cells. We used this in vitro paradigm to investigate the effect of Hsp27 and α B-crystallin on aSyn-induced toxicity by co-transfecting H4 cells with aSyn and each of the chaperones. Interestingly, we found that both small heat shock proteins reduced aSyn toxicity, an effect that was similar to that of Hsp70 (Fig. 3D).

To further characterize the effect of Hsp27, we hypothesized that reducing the endogenous levels of this heat shock protein would increase aSyn-induced toxicity. We performed knock

down experiments of Hsp27 using small interfering RNAs (siRNAs). For this purpose, we designed one siRNA according to general rules (see materials and methods section), and utilized a commercially available siRNA, from Qiagen, which was previously validated, to confirm the specificity of the knock down experiments. Both siRNAs knocked down Hsp27 by 20%, as seen by SDS-PAGE and immunoblot analysis (Fig. 4B, C). Although we only obtained a 20% decrease in Hsp27 levels, it was sufficient to result in a ~20% increase in aSyn toxicity (Fig. 4A). We also observed a dose-dependent increase of aSyn toxicity with increasing concentrations of siRNA, although the siRNA treatment by itself was not toxic (Fig. 4A).

Hsp27 protects primary dopaminergic neurons from aSyn-induced neurotoxicity

Our next aim was to determine whether Hsp27 also protected primary dopaminergic neurons from aSyn-induced neurotoxicity. We examined the effect of Hsp27 on A53T-dependent dopaminergic cell death in primary midbrain cultures. We focused on the A53T mutant rather than the wild-type protein because our data (Liu et al, submitted manuscript) and reports from other groups [32,37] indicated that wild-type aSyn is only weakly toxic to primary dopaminergic neurons. To determine whether Hsp27 protected dopaminergic neurons from A53T-induced neurotoxicity, we transduced primary midbrain cultures with lentivirus encoding A53T alone, A53T plus β -galactosidase ('lacZ' virus), or Hsp27 plus A53T. Control cells were cultured in the absence of lentivirus. The relative number of TH-positive neurons was lower in primary cultures infected with A53T lentivirus compared to untransduced control cells (Fig. 3E). A higher percentage of TH-positive neurons was observed in primary cultures that coexpressed A53T and Hsp27 than in cultures expressing only A53T or A53T plus β -galactosidase (Fig. 3E). Transduction of the cells with Hsp27 lentivirus alone had no significant effect on dopaminergic cell viability. These findings suggest that Hsp27 protects dopaminergic neurons from toxicity induced by A53T in a primary cell-culture model.

Discussion

Several neurodegenerative disorders, such as PD, DLB, AD and the polyglutamine diseases, are characterized by the deposition of misfolded proteins inside or outside of neurons, and are commonly referred to as 'protein misfolding diseases'. Proteins misfold due to a variety of reasons, some of which are not fully understood. Molecular chaperones assume a crucial role in neurodegeneration because they can assist in protein folding, refolding, oligomerization or disaggregation [38]. Members of the HSP70 and HSP40 families of proteins are protective against the cellular toxicity associated with protein misfolding and inclusion formation in PD and polyglutamine disease models, both in vitro and in vivo (Drosophila and mouse models) [18,39–43]. However, the formation of protein aggregates is not necessarily associated with cellular toxicity. Whether protein inclusions inside cells are normal, toxic, or a by-product of the cellular life cycle of certain proteins is still unclear and this remains a central question towards the understanding of the molecular mechanisms underlying neurodegenerative disorders [44,45].

The small Hsps play critical roles as a defense against physiological stress, by protecting proteins through an energy-independent process [46–48]. These chaperones have also been implicated in several conformational disorders. Wytenbach and colleagues showed that Hsp27 suppresses poly(Q)-induced cellular toxicity in a cellular model for HD, by reducing reactive oxygen species (ROS) toxicity [27].

Our goal was to investigate the role of small HSPs on aSyn-induced toxicity and aggregation by modeling LB diseases, such as PD and DLB, in a human cell line (H4 cells) where we recapitulate those aSyn effects. Ultimately, we wanted to further understand how aggregation relates to cytotoxicity and to devise novel avenues for therapeutic intervention.

The presence of Hsp27 and α B-crystallin in LBs and LNs in DLB brains, and the fact that Hsp27 levels were 2.5-fold higher in DLB brains when compared to controls, indicated that small HSPs may play a role in the disease process. Initially, we asked whether this represented a response to the presence of misfolded and aggregated aSyn or rather a marker for cell death. We had previously shown that Hsp70 protects against aSyn-induced toxicity in vitro [19] and here we observed that Hsp27 and α B-crystallin were also effective in protecting against aSyn-cytotoxicity. The mechanisms of action of Hsp70 and of the small HSPs are quite different, suggesting that protection against aSyn-toxicity can be achieved through different pathways. Our data suggest that small variations in the levels of this small HSP might have severe consequences in terms of pathology. We next wanted to understand whether the modulation of aSyn toxicity was due to an effect on the aggregation of aSyn. First, we verified that, similar to LBs, aSyn inclusions in H4 cells are also positive for small HSPs. Importantly, we demonstrated a reduction in the percentage of cells with inclusions similar to that we had previously reported for Hsp70 [19]. Our findings suggest that a reduction in aSyn aggregation is related to a reduction in aSyn toxicity. Recently, we reported the identification of a small molecule that reduced toxicity by promoting aggregation [50]. Thus, our work indicates it might be possible to alleviate aSyn toxicity by using different strategies which do not necessarily correlate with a direct effect on aggregation.

Recently, Zourlidou and colleagues used a neuronal cell line model to investigate the effects of several classes of chaperones, including Hsp27, on aSyn-associated toxicity [28]. In their system, cytotoxicity was obtained by challenging cells expressing aSyn with death-inducing stimuli, hence toxicity was not fully derived from aSyn expression [49]. Our work indicates small HSPs may have chaperone activity capable of assisting the proper folding of misfolded aSyn. We cannot exclude the possibility that they may act upon oligomeric species, which are not detectable by fluorescence microscopy, shifting the reaction towards the normal, non-toxic species, therefore reducing aggregation. Most importantly, our findings in H4 cells are confirmed by the effect of Hsp27 in reducing aSyn toxicity in primary dopaminergic neurons.

In summary, we show a direct effect of Hsp27 and α B-crystallin on aSyn toxicity and aggregation, indicating the increased levels of Hsp27 in DLB brains is a protective strategy to cope with misfolded and aggregated aSyn. Ultimately, our work suggests that methods for increasing Hsp27 levels, either by introducing exogenous copies of the Hsp27 gene or by pharmacologically elevating Hsp27 levels, hold great potential as therapeutic strategies in PD and other synucleinopathies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A.

B.

Figure 1.

Levels of Hsp27 in DLB. **A.** DLB tissue was homogenized and western blot analysis was performed with antibodies for the proteins shown. **B.** Q-PCR analysis of Hsp27 levels in DLB and control brains (n=15; p<0.001).

Figure 2.

Small heat shock proteins are present in aSyn lesions in DLB and in aSyn inclusions in cell culture. **A.** DLB tissue was stained with antibodies for aSyn (green) and Hsp27 or αB-crystallin (red). Merged images are shown in yellow. Scale bar, 50 μm. **B.** H4 cells co-transfected with aSyn and synphilin-1 were stained for aSyn (green) and endogenous Hsp27 (red). Merged images are shown in yellow. Scale bar, 20 μm.

Figure 3.

Effect of small HSPs on aSyn aggregation and toxicity. **A.** H4 cells co-transfected with aSyn (green), synphilin-1 and the indicated chaperone (red). **B.** Cells bearing inclusions were counted and percentages calculated. (* $p < 0.005$). **C.** Western blot analysis of Hsp27 levels in H4 cells transfected with Hsp27-Myc. **D.** H4 cells were co-transfected with aSyn and the indicated chaperone and toxicity was assessed using the adenylate kinase release assay. **E.** Primary midbrain cultures were transduced with lentivirus encoding A53T alone, A53T plus β -galactosidase ('lacZ'), A53T plus Hsp27, or Hsp27 alone. Control cells were cultured in the absence of lentivirus. Dopaminergic cell death was evaluated immunocytochemically using an anti-MAP2 monoclonal antibody and an anti-TH polyclonal antibody. Viability is expressed as the percentage of MAP2-positive neurons that were also TH-positive. Data are presented as the mean $\pm$ SEM, $N = 3$; * $p < 0.05$, ANOVA with Newman-Keuls post-Hoc test.

Figure 4.

A. H4 cells were co-transfected with aSyn and increasing concentrations of a siRNA towards Hsp27. Toxicity was assessed by measuring the release of adenylate kinase. **B.** Western blot analysis of H4 cells transfected with siRNA towards Hsp27. **C.** Quantification of western blot analysis (24h vs. 72h). (* $p < 0.005$)