Hsp27 reduces glycation-induced toxicity and aggregation of alpha-synuclein

Hugo Vicente Miranda¹ | Ana Chegão¹ | Márcia S. Oliveira¹ | Bárbara Fernandes Gomes¹ | Francisco J. Enguita² | Tiago Fleming Outeiro¹,³,⁴,⁵

¹CEDOC, Chronic Diseases Research Center, NOVA Medical School, Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Lisboa, Portugal
²Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal
³Department of Experimental Neurodegeneration, Center for Biostuctural Imaging of Neurodegeneration, University Medical Center Göttingen, Göttingen, Germany
⁴Max Planck Institute for Experimental Medicine, Göttingen, Germany
⁵Translational and Clinical Research Institute, Faculty of Medical Sciences, Newcastle University, Newcastle Upon Tyne, UK

Correspondence
Hugo Vicente Miranda, CEDOC, Chronic Diseases Research Center, NOVA Medical School, Rua Câmara Pestana nº 6, Edificio CEDOC II, Lisboa 1150-082, Portugal.
Email: hmvmiranda@nms.unl.pt

Tiago Fleming Outeiro, Experimental Neurodegeneration, Universitätsmedizin Göttingen, Waldweg 33, 37073 Göttingen, Germany.
Email: touteir@gwdg.de

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Abstract
α-synuclein (aSyn) is a major player in Parkinson's disease and a group of other disorders collectively known as synucleinopathies, but the precise molecular mechanisms involved are still unclear. aSyn, as virtually all proteins, undergoes a series of posttranslational modifications during its lifetime, which can affect its biology and pathobiology. We recently showed that glycation of aSyn by methylglyoxal (MGO) potentiates its oligomerization and toxicity, induces dopaminergic neuronal cell loss in mice, and affects motor performance in flies. Small heat-shock proteins (sHsps) are molecular chaperones that facilitate the folding of proteins or target misfolded proteins for clearance. Importantly, sHsps were shown to prevent aSyn aggregation and cytotoxicity. Upon treating cells with increasing amounts of methylglyoxal, we found that the levels of Hsp27 decreased in a dose-dependent manner. Therefore, we hypothesized that restoring the levels of Hsp27 in glycatng environments could alleviate the pathogenicity of aSyn. Consistently, we found that Hsp27 reduced MGO-induced aSyn aggregation in cells, leading to the formation of nontoxic aSyn species. Remarkably, increasing the levels of Hsp27 suppressed the deleterious effects induced by MGO. Our findings suggest that in glycatng environments, the levels of Hsp27 are important for modulating the glycation-associated cellular pathologies in synucleinopathies.

KEYWORDS
alpha-synuclein, glycation, Hsp27, neurodegeneration, Parkinson's disease

Abbreviations: aSyn, alpha-synuclein A; AGE, Advanced glycation end-product; CEL, carboxymethyl-lysine; FBS, fetal bovine serum; HMW, high molecular weight; Hsp, heat-shock protein; LDH, lactate dehydrogenase; MGO, methylglyoxal; MSA, multiple system atrophy; PD, Parkinson's disease; PTMs, posttranslational modifications; sHsps, small heat-shock proteins; ThT, Thioflavin T.

Hugo Vicente Miranda, Ana Chegão, and Márcia S. Oliveira contributed equally to this study.

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INTRODUCTION

Alpha-synuclein (aSyn) is a small protein of 140 amino acid residues abundant in the brain and in various other tissues. In the brain, it is abundant in the presynaptic compartment, but exists also in the nucleus of neurons.\(^1\) aSyn is the major component of pathognomonic protein inclusions, known as Lewy bodies and Lewy neurites, present in the brains of patients affected by Parkinson’s disease (PD) or dementia with Lewy bodies.\(^6\) aSyn undergoes several posttranslational modifications (PTMs) that can modulate its aggregation and cytotoxicity.\(^15\) aSyn is a long-lived protein that while it does not contain any arginine residues, it is lysine-rich, making it a preferably target for glycation in the lysine residues.\(^16,17\) Several studies suggest that glycation might be an important contributor to the pathobiology of aSyn.\(^17,18\) In a previous study, we found that aSyn is glycated in the brains of PD patients and that this modification modulates aSyn pathogenicity, contributing to the neurodegenerative process.\(^19\) Furthermore, methylglyoxal (MGO), the strongest glycating agent in living cells, exacerbates aSyn aggregation and toxicity in several models of synucleinopathies, such as yeast, human cells, and animal models of PD.\(^19\) In addition to inducing aSyn oligomerization, MGO also affects the correct clearance of aSyn by impairing the activities of the proteasome and autophagy clearance systems.\(^19\)

The small heat-shock proteins (sHsps) are a family of small molecular weight proteins that are characterized by a highly conserved domain, known as alpha-crystallin domain.\(^20,21\) sHsps play an important role in cellular proteostasis,\(^22-25\) in particular by preventing protein aggregation. They act as molecular chaperones, promoting protein folding/refolding, stabilization, and by targeting damaged proteins for degradation.\(^24-26\) Furthermore, sHsps are stress response proteins, upregulated under unfavorable conditions such as heat-shock, metabolic, oxidative, and chemical stress.\(^27,28\) However, sHsps are not only involved in stress responses, but also in stress tolerance, cell death, differentiation, cell cycle, and signal transduction.\(^29\) sHsps have also been reported to play a role in aging and protein aggregation disorders, such as neurodegenerative diseases like Alzheimer's disease and PD.\(^30-34\) Remarkably, Hsp27, one of the members of the sHsp family, is present in Lewy bodies.\(^35,36\)

Hsp27 is ubiquitously expressed, at highest levels in the skeletal, smooth, and cardiac muscles.\(^37\) Like other sHsps, it can assemble in smaller oligomers, such as dimers or tetramers, or in large oligomeric complexes.\(^38-42\) Hsp27 is a target of PTMs, such as phosphorylation, which induces the shift in the oligomeric status of Hsp27.\(^43,44\) Hsp27 has different functions, depending on its oligomerization state: in large oligomers, it acts mainly as a molecular chaperone, also exhibiting anti-apoptotic properties\(^42,43\), in small oligomers, Hsp27 has reduced chaperone activity and acts in the dynamics of microfilaments, contributing to the stabilization of F-actin filaments.\(^44,45\) The role of Hsp27 in protein aggregation is well documented. The levels of Hsp27 are increased in PD brain (cortex),\(^46,47\) and Hsp27 is also present in Lewy bodies and co-localizes with aSyn.\(^35,36\) In vitro, Hsp27 binds to aSyn preventing aggregation and fibril elongation, and also reducing aSyn cytotoxicity in a concentration-dependent manner.\(^23,33,48-50\)

Like aSyn, Hsp27 can also be glycated by MGO.\(^51-55\) Interestingly, this modification promotes the formation of large oligomeric complexes of Hsp27, enhancing its chaperone activity and anti-apoptotic properties.\(^51,55\) Moreover, high levels of glucose increase Hsp27 glycation and reduce its levels in a dose-dependent manner.\(^56\)

Here, we report that MGO reduces the levels of Hsp27 in a dose-dependent manner. Therefore, we hypothesized that restoring the levels of Hsp27 could prevent glycation-associated aSyn pathobiology. Consistently, Hsp27 overexpression reduced the deleterious effects of MGO in a cellular model of synucleinopathies. Our findings suggest that Hsp27 might constitute an important therapeutic target for modulating glycation-induced pathogenesis of aSyn.

MATERIALS AND METHODS

2.1 Cell culture

Human H4 neuroglioma cells were maintained at 37°C in OPTI-MEM (Gibco, Invitrogen, CA, USA) supplemented with 10% of fetal bovine serum (FBS) (Gibco, Invitrogen, CA, USA). H4 neuroglioma cells were seeded in 35 mm imaging dishes (Ibidi, 170,000 cells/dish) for immunocytochemistry assays, 12-well plate (TPP, 90,000 cells/well) for protein chemistry assays, 6-well plate (TPP, 190,000 cells/well) for 6-well plate (TPP, 190,000 cells/well) for cytotoxicity assays, 6-well plate (TPP, 190,000 cells/well) for protein analysis, or 6 cm plates (TPP, 300,000 cells/dish) for triton-X 100 solubility assay.

2.2 Analysis of protein glycation and Hsp27 profile

H4 neuroglioma naïve cells were plated and treated 24 hours later with increasing amounts of MGO (0.2; 0.5; 0.75;
1 mM), prepared as previously. After 24 hours of treatment, cells were collected and protein extracts prepared and quantified as previously. Protein glycation profile was assessed by western blotting, probing for argpyrimidine (a kind gift from K. Uchida, Laboratory of Food and Biodynamics, Nagoya University Graduate School of Bioagricultural Sciences, Japan) as previously. Hsp27 levels were probed with anti-Hsp27 (F-4 1:2000; Santa Cruz Biotechnology, Dallas, TX, USA). Detection procedures were performed according to ECL system (GE Healthcare, Life Sciences; Little Chalfont, UK), and the signal was detected using a ChemiDoc Imaging Systems (Bio-Rad, Hercules, CA, USA). Densitometry was performed using ImageJ - Image Processing and Analysis in Java. When required, membranes were incubated with stripping solution (250 mM Glycine, 0.1% of 10% SDS, pH 2.0) for 45 minutes at room temperature with agitation, followed by four washing steps, twice with 1x TBS and twice with 1x TBS supplemented with 10% Tween 20 solution. Membranes were then incubated with blocking solution for 30 minutes, before reprobing.

2.3 | Mass spectrometry

H4 neuroglioma cells protein extracts were separated by SDS-PAGE and the correspondent 25 kDa fragment was excised from the gel. Peptide mass fingerprinting analysis was performed as previously, in an Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF ion optics. A double miscleavage was allowed, and oxidation of methionyl residues, acetylation of the N-terminal region, and carboxymethylation (CEL) of lysine residues, as well as argpyrimidine or hidroimidazolones formation at arginine residues were assumed as variable modifications. All peaks with S/N 4-5 were included, with a taxonomic restriction to Homo sapiens. The criterion used to accept the identification was significant homology scores achieved in Mascot (P < .05).

2.4 | Expression and purification of recombinant aSyn and Hsp27

Human aSyn was expressed and purified as we previously described. SDS-PAGE, followed by western blotting analysis (using standard procedures), confirmed the monomeric purification of aSyn (anti-aSyn dilution 1:1000; BD Transduction Laboratories, San Jose, CA, USA).

For human Hsp27, the E.coli strain BL-21 was transformed by heat-shock with HSP27 PET16b construct (a kind gift from Dr Paul Muchowski) and expression induced for 3 hours with IPTG (0.3 mM). Cells were pelleted and re-suspended in lysis buffer, supplemented with 10 mg/mL of lysozyme (Sigma-Aldrich, MO, USA). The cell suspension was then incubated on ice with constant stirring for 20 minutes, followed by the addition of 0.33 mL/L benzonase (Sigma-Aldrich, MO, USA), and subsequently incubated at room temperature for 20 minutes with constant stirring. Insoluble cellular debris were removed by centrifugation. About 53 mL of a 200 mM dithiothreitol (Sigma-Aldrich, MO, USA) solution was added to the soluble supernatant, followed by incubation at room temperature with constant stirring for 10 minutes. Insoluble contaminants were removed by centrifugation and the supernatant was filtered with a 0.22 μm filter. The sample was loaded into an ion-exchange chromatography Q Sepharose TM fast flow column equilibrated with 20 mM Tris-HCl, pH 8.0. Proteins were eluted with a linear NaCl gradient (0-1.0 M), at a flow rate of 1.5 mL/min and the elution monitored at 280 nm. Protein-containing fractions were collected and probed by SDS-PAGE analysis using Coomassie staining. Fractions containing the Hsp27 were collected, concentrated by centrifugation using Amicon filters and applied to a gel filtration Superdex 75 column, equilibrated with 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl. Proteins were eluted with the same buffer at a flow rate of 1 mL/min. Fractions containing Hsp27 were collected and concentrated by centrifugation using Amicon filters. SDS-PAGE, followed by western blotting analysis, confirmed the monomeric purification of Hsp27 (anti-Hsp27 1:2000; Santa Cruz Biotechnology, Dallas, TX, USA).

2.5 | In vitro aSyn aggregation

Recombinant monomeric aSyn was diluted at 140 μM in 30 mM Tris-HCl pH 7.4. Aggregation was induced as previously described. aSyn aggregation was evaluated alone or in the presence of Hsp27 (0.45 or 3 μM), and or in the presence of MGO (0.5 mM) or corresponding vehicle.

2.6 | Thioflavin T binding assay

The formation of amyloid fibrils was monitored by Thioflavin T (ThT) binding assay as previously described. Emission wavelength scan at 490 nm was performed with an excitation wavelength of 450 nm using a plate reader (Tecan Infinite 200, Männedorf, Switzerland).

2.7 | Native-PAGE electrophoresis

About 15 μg of total protein extract from purified proteins were separated by native electrophoresis using a Tetra cell (Bio-Rad; Hercules, CA, USA), in 12% of polyacrylamide separation gel, and a 4% of polyacrylamide stacking gel in non-denaturing conditions, applying a constant voltage of 120 V.
2.8 | aSyn cytotoxicity assays

To assess exogenous aSyn toxicity, cells were plated and treated 24 hours later with recombinant aSyn species, either glycated or not, alone or in the presence of Hsp27 (0.45 or 3 μM). Cells were collected 24 hours later, and cytotoxicity was determined by lactate dehydrogenase (LDH) cytotoxicity assay (see below).

2.9 | Hsp27 overexpression

24 h post-seeding, H4 neuroglioma cells were transfected with pcDNA3.1 vectors containing aSyn WT,36 SynT,36 or Hsp2735 alone, or co-transfected with aSyn WT and Hsp27, or SynT and Hsp27 pcDNA3.1 vectors, using FuGENE 6 (Roche, Basel, Switzerland), using standard procedures. 20 hours post-transfection, cells were treated with MGO (0.2 mM). After 24 hours, a second MGO treatment (0.2 mM) was performed. Cells were collected for analysis 48 hours post-transfection.

2.10 | aSyn, Hsp27 and glycation levels

H4 protein extracts were analyzed by western blotting probing for aSyn (anti-aSyn dilution 1:1000; BD Transduction Laboratories, San Jose, CA, USA), Hsp27 (anti-Hsp27 F-4:2000; Santa Cruz Biotechnology, Dallas, TX, USA), anti-MGO-derived Advanced Glycation and Products (AGEs) (KAL-KH001 Cosmo-Bio, USA, 1:500 dilution), and normalized to β-actin levels (anti-b-actin 1:5000; Thermo Fisher Scientific; Waltham, MA, USA).

2.11 | LDH cytotoxicity assay

Cytotoxicity was measured using lactate dehydrogenase (LDH) kit (Clontech; Mountain View, CA, USA), according to manufacturer's instructions.

2.12 | Triton-X 100 solubility assay

The solubility assays were performed as previously described.19

2.13 | Immunocytochemistry

Immunocytochemistry was performed as previously described.19 Microscopy images were acquired in a Widefield fluorescent microscope Zeiss Axiovert 40 CFL (Carl Zeiss MicroImaging).

3 | RESULTS

3.1 | MGO induces the formation of argpyrimidine modifications and reduces the levels of Hsp27

First, we determined the profile of argpyrimidine-modified proteins in human H4 cells induced by increasing concentrations of MGO (0.2; 0.5; 0.75 mM) after 24 hours of treatment. Argpyrimidine is a specific MGO-derived AGE at arginine residues. We identified a protein migrating with an apparent molecular weight of ~25 kDa (Figure 1A). Surprisingly, that signal decreased in an MGO concentration-dependent manner, in contrast to the effect on other proteins (Figure 1A).

**FIGURE 1** The levels of Hsp27 decrease in an MGO-dependent manner. H4 cells were treated with increasing concentrations of MGO for 24 h. A, Protein extracts were separated by SDS-PAGE and were immunoblotted with anti-Argpyrimidine and anti-GAPDH antibodies (loading control). Two exposure times are shown (30 or 180 s of signal acquisition in ChemiDoc™ Touch Imaging System, Bio-Rad). B, A gel fragment at ~25 kDa was cut and processed for mass spectrometry analysis, leading to the identification of Hsp27. Hsp27 schematics show the different glycated residues (yellow), with a 55% of sequence coverage (dark green). C, Protein extracts were probed for Hsp27 and β-actin (loading control). Data presented as fold ratio to vehicle-treated cells (n = 3). ****P < .0001, one-way ANOVA, followed by Tukey's multiple comparisons test.
To identify this protein, cell extracts were separated by SDS-PAGE and the correspondent ~25 kDa fragment was excised from the gel and processed for mass spectrometry analysis, resulting in the identification of Hsp27 (55% of sequence coverage). We identified several glycated residues in Hsp27, mainly in arginine residues (Figure 1B and Table S1), and immunoblot analysis confirmed that the ~25 kDa protein was recognized by an antibody specific for Hsp27. Interestingly, we confirmed that the levels of Hsp27 decreased in cells treated with increasing concentrations of MGO (Figure 1C).

3.2 Hsp27 reduces aSyn aggregation and cytotoxicity

Hsp27 was previously shown to reduce the oligomerization of aSyn. However, the effect of Hsp27 on the oligomerization of glycated aSyn has not been investigated. To address this, we tested the effect of sub-stoichiometric levels of Hsp27 (300:1 or 300:7, aSyn:Hsp27) on aSyn aggregation (140 μM), in the presence or absence of glycation conditions. To study the kinetics of aggregation, aSyn was incubated under controlled temperature and shaking conditions, and samples were collected at specific timepoints. Using native-PAGE electrophoresis followed by immunoblot analysis, we observed that, with time, aSyn species appeared at the top of the gel (Figure 2A), indicating the formation of high molecular weight (HMW) species. In the presence of Hsp27, the formation of these HMW species was reduced (Figure 2A).

We and others previously reported that while suppressing fibril formation, glycation of aSyn by MGO increases its oligomerization. Here, we evaluated if Hsp27 protects from glycation effects on aSyn oligomerization and found that it reduced the formation of HMW species of aSyn.

**FIGURE 2** Hsp27 decreases aSyn aggregation and toxicity in a dose-dependent manner. aSyn aggregation kinetics (140 μM) in the absence or presence of Hsp27 (300:1 or 300:7, aSyn:Hsp27) was evaluated by (A) native-PAGE followed by immunoblotting with an anti-aSyn antibody. B, SDS-PAGE followed by immunoblotting with an anti-aSyn antibody, in control or MGO-glycation conditions. C, ThT aggregation assays. D, The cytotoxicity of the resulting species was evaluated in H4 cells using LDH release as a readout. Data normalized to control conditions without Hsp27 or MGO (n = 3). *P < .05, **P < .01, one-way ANOVA, followed by Tukey’s multiple comparisons test.
(Figure 2B). In particular, at a 300:7 (aSyn:Hsp27) molar ratio, aSyn remains mainly monomeric or as lower molecular weight species, suppressing the formation of HMW species, even at 80 hours of incubation. We also confirmed a reduction in the formation of aSyn amyloid structures in the presence of Hsp27, using ThT as a readout (Figure 2C).

Next, to determine whether Hsp27 affected the toxicity of glycated aSyn species, we exposed H4 cells to those species, for 24 hours, and assessed the overall membrane integrity (LDH assay) as a readout of cytotoxicity. While aSyn exposed to MGO was toxic, as we have previously shown, the species incubated with Hsp27 were not (Figure 2D).

### 3.3 Hsp27 reduces MGO-associated aSyn pathobiology in cellular models

Next, we investigated the effects of Hsp27 on MGO-associated aSyn cellular pathologies in cells. Briefly, cells expressing aSyn alone or together with Hsp27 were treated with MGO 20 hours post-transfection, and then, received a second treatment 24 hours later, in order to ensure glycation conditions were maintained. Protein extracts were then prepared and analyzed by immunoblot analyses. In contrast to the effect of MGO observed on the endogenous levels of Hsp27 (Figure 1), we found that in cells overexpressing Hsp27 the levels did not decrease (Figure 3A-D), while the general glycation increased in an MGO-dependent manner (Figure 3E-H). In cells expressing aSyn WT, we observed that Hsp27 prevented aSyn cytotoxicity by ~10%. Impressively, Hsp27 overexpression also prevented the MGO-associated increase of aSyn levels (~56%) (Figure 4A) and cytotoxicity (~28%) (Figure 4B).

In order to assess whether Hsp27 modulated the MGO-induced aggregation of aSyn, we used an established cell model using an aggregation-prone variant of aSyn (known as SynT). This model consists of a modified form of aSyn where the C-terminus is fused to a truncated, expressed aSyn alone or together with Hsp27 were treated with MGO 20 hours post-transfection, and then, received a second treatment 24 hours later, in order to ensure glycation conditions were maintained. Protein extracts were then prepared and analyzed by immunoblot analyses. In contrast to the effect of MGO observed on the endogenous levels of Hsp27 (Figure 1), we found that in cells overexpressing Hsp27 the levels did not decrease (Figure 3A-D), while the general glycation increased in an MGO-dependent manner (Figure 3E-H). In cells expressing aSyn WT, we observed that Hsp27 prevented aSyn cytotoxicity by ~10%. Impressively, Hsp27 overexpression also prevented the MGO-associated increase of aSyn levels (~56%) (Figure 4A) and cytotoxicity (~28%) (Figure 4B).

In order to assess whether Hsp27 modulated the MGO-induced aggregation of aSyn, we used an established cell model using an aggregation-prone variant of aSyn (known as SynT). This model consists of a modified form of aSyn where the C-terminus is fused to a truncated,
nonfluorescent fragment of GFP (known as SynT). Using this model, we have previously shown that MGO treatment of cells expressing SynT increases the percentage of cells with aSyn inclusions (51% to 85%). The aggregation status of SynT was assessed using a Triton X-100 solubility assay. Briefly, H4 cells overexpressing (i) SynT; or (ii) SynT together with Hsp27, were treated with MGO as previously described. Protein extraction was performed in nondenaturing conditions, and protein extracts solubilized in 1% of Triton X-100. Soluble and insoluble fractions were separated by centrifugation and analyzed by western blot. We observed that MGO treatment induced an increase of SynT insolubility (~25%) (Figure 4D). In cells overexpressing SynT, we observed that Hsp27 was also able to prevent the MGO-associated increase of SynT levels (~59%) (Figure 4E) and cytotoxicity (~16%) (Figure 4F).

**FIGURE 4** Hsp27 overexpression reduces cytotoxicity, insolubility, and aggregation of aSyn in glycating conditions. Cells expressing aSyn or SynT, or co-expressing aSyn or SynT together with Hsp27 were treated two times for two consecutive periods of 24 h with vehicle or MGO (0.2mM). A, Protein levels of cells expressing aSyn were determined by immunoblotting with anti-aSyn, -Hsp27 or -β-actin antibodies. B, Cytotoxicity was determined by loss of membrane integrity (LDH release). C, Triton X-100 soluble and insoluble (TS and TI) fractions were probed for aSyn. The ratio between soluble and insoluble fractions is normalized to vehicle-treated ratio and presented as SynT insolubility (n = 4). D, Cells were processed for immunocytochemistry (aSyn, green), and the percentage of cells with inclusions determined. E, Protein levels and (F) cytotoxicity of cells expressing SynT were determined as in (A) and (B). Data presented as fold ratio between MGO and vehicle-treated cells, at least n = 3. *P < .05, **P < .01, ***P < .001 unpaired t test with equal SD

4 | DISCUSSION

Recent reports suggest that type II diabetes is an important risk factor for PD. In particular, protein glycation seems to play an important role in synucleinopathies. We previously showed that aSyn is a target of glycation, which potentiates its oligomerization, impairs its clearance and
membrane binding ability, resulting in its accumulation, aggregation, and cytotoxicity, inducing dopaminergic neuronal loss and motor impairments in animal models.\textsuperscript{19}

However, glycation can affect many other proteins in addition to aSyn, thereby contributing to a variety of cellular pathologies. To investigate this, we evaluated the pattern of glycation in a cellular model of synucleinopathies treated with increasing concentrations of MGO, as a model glycating agent since it is the most significant and highly reactive glycating agent in cells, mainly generated as a by-product of glycolysis.\textsuperscript{70} Surprisingly, although we were expecting an overall increase in the levels of glycated proteins, we observed dominant signal corresponding to a ~25 kDa protein whose levels decreased with increased levels of MGO. Using peptide mass fingerprinting, we identified this protein as Hsp27 and confirmed this using a specific antibody. In fact, we found that the total levels of Hsp27 decreased in an MGO-dependent manner. Hsp27 was previously shown to be protective in models of synucleinopathies. In particular, it reduces aSyn oligomerization\textsuperscript{23,49,71} and prevents the toxicity of exogenous aSyn species in neuronal cells.\textsuperscript{23,50} Hsp27 reduces the aggregation of aSyn\textsuperscript{35} and of other proteins associated with other neurodegenerative disorders.\textsuperscript{33,72-74} Therefore, we hypothesized that under glycatcing conditions, restoring or inducing the levels of Hsp27 might prevent the deleterious effects of cellular glycation in aSyn pathogenesis.

To test our hypothesis, we first compared the kinetics of recombinant aSyn oligomerization alone or in the presence of Hsp27, in control or glycatcing conditions. In contrast to other studies that tested aSyn:Hsp27 ratios of 1:1 or 5:1\textsuperscript{23,49} we performed our analysis in sub-stoichiometric concentrations of 300:1 or 300:7. To assess the effect of much lower amounts of Hsp27, we confirmed our previous findings that glycation potentiates aSyn oligomerization. Moreover, we also validated that Hsp27 is able to reduce non-glycated aSyn aggregation. Remarkably, in the presence of Hsp27, the deleterious effects of glycation over aSyn oligomerization were almost completely abolished, avoiding the formation of stable HMW species. Also, although glycated aSyn species are cytotoxic, the species formed in the presence of Hsp27 were not. These findings provide important evidence that Hsp27 may directly suppress the pathogenicity of aSyn-glycation.

In order to further validate our hypothesis, we evaluated if Hsp27 overexpression would be protective in cellular models of aSyn toxicity and aggregation. Impressively, Hsp27 alleviated the MGO-induced pathogenic phenotypes, reducing cytotoxicity in cells expressing WT aSyn or an aggregation-prone version of aSyn (SynT). Hsp27 significantly reduced the levels of aSyn or SynT and reduced the aggregation of SynT. Notably, the cytotoxic protection provided by Hsp27 in glycation conditions (28\%) is higher than in non-glycation conditions (10\%), suggesting a relevant role of Hsp27 in glycation conditions. Interestingly, MGO was shown to modify the α-crystallin domain of Hsps (namely, Hsp27) probably by exposing hydrophobic sites that would, otherwise, not be available for chaperone function. In addition, MGO might induce the formation of large oligomers of Hsp27, enhancing its activity.\textsuperscript{51,75} This enhanced Hsp27 activity might be responsible for reducing aSyn cytotoxicity, by reducing the formation of aSyn oligomers, or by preventing apoptosis, since Hsp27 glycation potentiates its anti-apoptotic potential, as described in several types of cancers.\textsuperscript{54-55,76} Although in
the present study we could not confirm the formation of large oligomeric species of Hsp27, we hypothesize that the strong reduction of Hsp27 protein levels would prevail over a possible gain-of-function of Hsp27 as a molecular chaperone.

The presence of Hsp27 in Lewy bodies suggests it may play a relevant role in PD. Actually, a decrease in the activity and gain-of-function of Hsp27 as a molecular chaperone. Furthermore, the presence of Hsp27 in Lewy bodies suggests it may play a relevant role in PD. Actually, a decrease in the activity of Hsps and of other protein quality control components is associated with aging and neurodegeneration.

Molecular chaperones are crucial for stabilizing protein conformations and for directing misfolded or damaged proteins for degradative pathways. Therefore, our work highlights the importance of Hsp27 in suppressing the pathological conditions induced by glycation (Figure 5) and suggests that Hsp27 may constitute a suitable target for intervention in synucleinopathies and other neurodegenerative disorders.

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AUTHOR CONTRIBUTIONS

H. Vicente Miranda and T.F. Outeiro designed research; H. Vicente Miranda, A. Chegão, M.S. Oliveira, and B.F. Gomes performed research; F.J. Enguita contributed new reagents or analytic tools; H. Vicente Miranda, A. Chegão, M.S. Oliveira, and T.F. Outeiro analyzed data; H. Vicente Miranda, A. Chegão, and T.F. Outeiro wrote the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Maroteaux L, Campanelli JT, Scheller RH. Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal. J Neurosci. 1988;8:2804-2815.
2. Iwai A, Masliah E, Yoshimoto M, et al. The precursor protein of non-A beta component of Alzheimer’s disease amyloid is a presynaptic protein of the central nervous system. Neuron. 1995;14:467-475.
3. McLean PJ, Ribich S, Hyman BT. Subcellular localization of alpha-synuclein in primary neuronal cultures: effect of missense mutations. J Neural Transm Suppl. 2000;53-63.
4. Goncalves S, Outeiro TF. Assessing the subcellular dynamics of alpha-synuclein using photoactivation microscopy. Mol Neurobiol. 2013;47:1081-1092.
5. Pinho R, Paiva I, Jerčić KG, et al. Nuclear localization and phosphorylation modulate pathological effects of alpha-synuclein. Hum Mol Genet. 2019;28:31-50.
6. Spillantini MG, Crowther RA, Jakes R, Cairns NJ, Lantos PL, Goedert M. Filamentous alpha-synuclein inclusions link multiple system atrophy with Parkinson’s disease and dementia with Lewy bodies. Neurosci Lett. 1998;251:205-208.
7. Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M. alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson’s disease and dementia with Lewy bodies. Proc Natl Acad Sci U S A. 1998;95:6469-6473.
8. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. Nature. 1997;388:839-840.
9. Vekrellis K, Xiouri M, Emmanouilidou E, Rideout HJ, Stefanis L. Pathological roles of alpha-synuclein in neurological disorders. Lancet Neurol. 2011;10:1015-1025.
10. Shachar T, Lo Bianco C, Recchia A, Wiessner C, Raas-Rothschild A, Futerman AH. Lysosomal storage disorders and Parkinson’s disease: Gaucher disease and beyond. Mov Disord. 2011;26:1593-1604.
11. Weinreb PH, Zhen W, Poon AW, Conway KA, Lansbury PT Jr. NACP, a protein implicated in Alzheimer’s disease and learning, is natively unfolded. Biochemistry. 1996;35:13709-13715.
12. Soto C. Unfolding the role of protein misfolding in neurodegenerative diseases. Nat Rev Neurosci. 2003;4:49-60.
13. Gadad BS, Britton GB, Rao KS. Targeting oligomers in neurodegenerative disorders: lessons from alpha-synuclein, tau, and amyloid-beta peptide. J Alzheimer’s Dis. 2011;24(Suppl. 2):223-232.
14. Michel PP, Hirsch EC, Hunot S. Understanding dopaminergic cell death pathways in Parkinson disease. Neuron. 2016;90:675-691.
15. Stamel R, Kappe G, Boelens W, Slingsby C. Wrapping the alpha-crystallin domain fold in a chaperone assembly. J Mol Biol. 2005;353:68-79.
16. van Montfort RL, Basha E, Friedrich KL, Slingsby C, Vierling E. Crystal structure and assembly of a eukaryotic small heat shock protein. Nat Struct Biol. 2001;8:1025-1030.
17. Cox D, Ecroyd H. The small heat shock proteins alphaB-crystallin (HSPB5) and Hsp27 (HSPB1) inhibit the intracellular aggregation of alpha-synuclein. Cell Stress Chaperones. 2017;22:589-600.
18. Cox D, Whiten DR, Brown JWP, et al. The small heat shock protein Hsp27 binds alpha-synuclein fibrils, preventing elongation and cytotoxicity. J Biol Chem. 2018;293:4486-4497.
19. Hight J, Bracher A, Hayer-Hartl M. Molecular chaperones in protein folding and proteostasis. Nature. 2011;475:324-332.
24. Treweek TM, Meehan S, Ecroyd H, Carver JA. Small heat-shock proteins: important players in regulating cellular proteostasis. *Cell Mol Life Sci.* 2015;72:429-451.

25. Saibil H. Chaperone machines for protein folding, unfolding and disaggregation. *Nat Rev Mol Cell Biol.* 2013;14:630-642.

26. Bartelt-Kirbach B, Golenhofen N. Reaction of small heat-shock proteins to different kinds of cellular stress in cultured rat hippocampal neurons. *Cell Stress Chaperones.* 2014;19:145-153.

27. Mehlen P, Mehlen A, Godet J, Arrigo AP. hsp27 as a switch between differentiation and apoptosis in murine embryonic stem cells. *J Biol Chem.* 1997;272:31657-31665.

28. Carra S, Alberti S, Arrigo PA, et al. The growing world of small heat shock proteins: from structure to functions. *Cell Stress Chaperones.* 2017;22:601-611.

29. Schultz C, Dick EJ, Cox AB, Hubbard GB, Braak E, Braak H. Expression of stress proteins alpha B-crystallin, ubiquitin, and hsp27 in pallido-nigral spheroids of aged rhesus monkeys. *Neurobiol Aging.* 2001;22:677-682.

30. Braak H, Del Tredici K, Sandmann-Kiel D, Rub U, Schultz C. Nerve cells expressing heat-shock proteins in Parkinson’s disease. *Acta Neuropathol.* 2001;102:449-454.

31. Bjorkdahl C, Sjogren MJ, Zhou X, et al. Small heat shock proteins Hsp27 or alphaB-crystallin and the protein components of neurofibrillary tangles: tau and neurofilaments. *J Neurosci Res.* 2008;86:1343-1352.

32. Wilhelmus MM, Boelens WC, Otte-Holler I, Kamps B, de Waal RM, Verbeck MM. Small heat shock proteins inhibit amyloid-beta protein aggregation and cerebrovascular amyloid-beta protein toxicity. *Brain Res.* 2006;1089:67-78.

33. Walther DM, Kasturi P, Zheng M, et al. Widespread proteome remodeling and aggregation in aging C. elegans. *Cell.* 2015;161:919-932.

34. Outeiro TF, Klucken J, Strathearn KE, et al. Small heat shock proteins protect against alpha-synuclein-induced toxicity and aggregation. *Biochem Biophys Res Comm.* 2006;351:631-638.

35. McLean PJ, Kawamata H, Hyman BT. Alpha-synuclein-enhanced green fluorescent protein fusion proteins form proteasome sensitive inclusions in primary neurons. *Neuroscience.* 2001;104:901-912.

36. Sugiyama Y, Suzuki A, Kishikawa M, et al. Muscle develops a specific form of small heat shock protein complex composed of MKBP/HSPB2 and HSPB3 during myogenic differentiation. *J Biol Chem.* 2000;275:1095-1104.

37. Mehlen P, Hickey E, Weber LA, Arrigo AP. Large unphosphorylated aggregates as the active form of hsp27 which controls intracellular reactive oxygen species and glutathione levels and generates a protection against TNFalpha in NIH-3T3 cells. *Biochem Biophys Res Comm.* 1997;241:187-192.

38. Mymrikov EV, Bukach OV, Seitz-Nebi AS, Gusev NB. The pivotal role of the beta 7 strand in the interubumin contacts of different human small heat shock proteins. *Cell Stress Chaperones.* 2010;15:365-377.

39. Rogalla T, Ehrnsperger M, Preville X, et al. Regulation of Hsp27 oligomerization, chaperone function, and protective activity against oxidative stress/tumor necrosis factor alpha by phosphorylation. *J Biol Chem.* 1999;274:18947-18956.

40. Arrigo AP, Pauli D. Characterization of HSP27 and three immunologically related polypeptides during *Drosophila* development. *Exp Cell Res.* 1988;175:169-183.

41. Abisambra JF, Blair LJ, Hill SE, et al. Phosphorylation dynamics regulate Hsp27-mediated rescue of neuronal plasticity deficits in tau transgenic mice. *J Neurosci.* 2010;30:15374-15382.

42. Guay J, Lambert H, Gingras-Breton G, Lavoie JN, Huot J, Landry J. Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27. *J Cell Sci.* 1997;110(Pt 3):357-368.

43. Lavoie JN, Hickey E, Weber LA, Landry J. Modulation of actin microfilament dynamics and fluid phase pinocytosis by phosphorylation of heat shock protein 27. *J Biol Chem.* 1993;268:24210-24214.

44. Renkawek K, Bosman GJ, de Jong WW. Expression of small heat-shock protein hsp 27 in reactive gliosis in Alzheimer disease and other types of dementia. *Acta Neuropathol.* 1994;87:511-519.

45. Renkawek K, Stege GJ, Bosman GJ. Dementia, gliosis and expression of the small heat shock proteins hsp27 and alpha B-crystallin in Parkinson’s disease. *NeuroReport.* 1999;10:2273-2276.

46. Cox D, Selig E, Griffin MD, Carver JA, Ecroyd H. Small heat-shock proteins prevent alpha-synuclein aggregation via transient interactions and their efficacy is affected by the rate of aggregation. *J Biol Chem.* 2016;291:22618-22629.

47. Bruinisma IB, Bruggink KA, Kinast K, et al. Inhibition of alpha-synuclein aggregation by small heat shock proteins. *Proteins.* 2011;79:2956-2967.

48. Oya-Ito T, Naito Y, Takagi T, et al. Enhancement of chaperone function of alpha-crystallin by methylglyoxal modification. *Biochemistry.* 2003;42:10746-10755.

49. Sakamoto H, Mashima T, Yamamoto K, Tsuruo T. Modulation of heat-shock protein 27 (Hsp27) expression by methylglyoxal. *Mol Pharmacol.* 2008;73:1391-1399.

50. Nagaraj RH, Oya-Ito T, Padayatti PS, et al. Enhancement of chaperone function of alpha-crystallin by methylglyoxal modification. *Biochemistry.* 2003;42:10746-10755.

51. Schalkwijk CG, van Bezu J, van der Schors RC, Uchida K, Stehouwer CD, van Hinsbergh VW. Heat-shock protein 27 is a major methylglyoxal-modified protein in endothelial cells. *FEBS Lett.* 2006;580:1565-1570.

52. Padival AK, Crabb JW, Nagaraj RH. Methylglyoxal modifies heat shock protein 27 in glomerular mesangial cells. *FEBS Lett.* 2003;551:113-118.

53. Oya-Ito T, Naito Y, Takagi T, et al. Heat-shock protein 27 (Hsp27) as a target of methylglyoxal in gastrointestinal cancer. *Biochem Biophys Acta.* 2011;1812:769-781.

54. Sakamoto H, Mochida T, Yamamoto K, Tsuruo T. Regulation of heat-shock protein 27 (Hsp27) anti-apoptotic activity by methylglyoxal modification. *J Biol Chem.* 2002;277:45770-45775.

55. Gawlowska T, Stratmann B, Stork I, et al. Heat shock protein 27 modification is increased in the human diabetic failing heart. *Horm Metab Res.* 2009;41:594-599.

56. Vicente Miranda H, Xiang W, de Oliveira RM, et al. Heat-mediated enrichment of alpha-synuclein from cells and tissue for assessing post-translational modifications. *J Neurochem.* 2013;126:673-684.

57. Oga T, Naito Y, Takagi T, et al. Heat-shock protein 27 (Hsp27) as a target of methylglyoxal in gastrointestinal cancer. *Biochem Biophys Acta.* 2011;1812:769-781.

58. Winkler M, Mochida T, Yamamoto K, Tsuruo T. Regulation of heat-shock protein 27 (Hsp27) anti-apoptotic activity by methylglyoxal modification. *J Biol Chem.* 2002;277:45770-45775.

59. Gawlowska T, Stratmann B, Stork I, et al. Heat shock protein 27 modification is increased in the human diabetic failing heart. *Horm Metab Res.* 2009;41:594-599.

60. Vicente Miranda H, Xiang W, de Oliveira RM, et al. Heat-mediated enrichment of alpha-synuclein from cells and tissue for assessing post-translational modifications. *J Neurochem.* 2013;126:673-684.

61. Gomes RA, Vicente Miranda H, Silva MS, et al. Yeast protein glycation in vivo by methylglyoxal. Molecular modification of glycolytic enzymes and heat shock proteins. *Fems J.* 2006;273:5273-5287.

62. Klionsky DJ, Abdelmohsen K, Abe A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy.* 2016;12:1-222.

63. Ardash MT, Paleologou KE, Lv G, et al. Structure function relationship of phenolic acid inhibitors of alpha-synuclein fibril formation and toxicity. *Front Aging Neurosci.* 2014;6:197. doi:10.3389/fnagi.2014.00197.

64. Daniele SG, Beraud D, Davenport C, Cheng K, Yin H, Maguire-Zeiss KA. Activation of MyD88-dependent TLR1/2 signaling by
misfolded alpha-synuclein, a protein linked to neurodegenerative disorders. Sci Signal. 2015;8:ra45.

62. Fauvet B, Mbefo MK, Fares MB, et al. Alpha-synuclein in central nervous system and from erythrocytes, mammalian cells, and Escherichia coli exists predominantly as disordered monomer. J Biol Chem. 2012;287:15345-15364.

63. Killinger BA, Moszczynska A. Characterization of alpha-synuclein multimer stoichiometry in complex biological samples by electrophoresis. Anal Chem. 2016;88:4071-4084.

64. Lázaro DF, Rodrigues EF, Langohr R, et al. Systematic comparison of the effects of alpha-synuclein mutations on its oligomerization and aggregation. PLoS Genet. 2014;10:e1004741.

65. Lee D, Park CW, Paik SR, Choi KY. The modification of alpha-synuclein by dicarbonyl compounds inhibits its fibril-forming process. Biochem Biophys Acta. 2009;1794:421-430.

66. de Oliveira RM, Vicente Miranda H, Francelle L, et al. The mechanism of sirtuin 2-mediated exacerbation of alpha-synuclein toxicity in models of Parkinson disease. PLoS Biol. 2017;15:e2000374.

67. Lee S, Carson K, Rice-Ficht A, Good T. Small heat shock proteins differentially affect Abeta aggregation and toxicity. Biochem Biophys Res Comm. 2006;347:527-533.

68. McLean PJ, Kawamata H, Shariff S, et al. TorsinA and heat shock proteins act as molecular chaperones: suppression of alpha-synuclein aggregation. J Neurochem. 2002;83:846-854.

69. Miceli L, Frenk J, Politi K, et al. The small heat shock protein alphaB-crystallin suppresses SOD1 aggregation in vitro. Cell Stress Chaperones. 2013;18:251-257.

70. Oya-Ito T, Liu BF, Nagaraj RH. Effect of methylglyoxal modification and phosphorylation on the chaperone and anti-apoptotic properties of heat shock protein 27. J Cell Biochem. 2006;99:279-291.

71. van Heijst JW, Niessen HW, Musters RJ, van Hinsbergh VW, Hoekman K, Schalkwijk CG. Argpyrimidine-modified Heat shock protein 27 in human non-small cell lung cancer: a possible mechanism for evasion of apoptosis. Cancer Lett. 2006;241:309-319.

72. Auluck PK, Chan HY, Trojanowski JQ, Lee VM, Bonini NM. Chaperone suppression of alpha-synuclein toxicity in a Drosophila model for Parkinson’s disease. Science. 2002;295:865-868.

73. McLean PJ, Kawamata H, Shariff S, et al. TorsinA and heat shock proteins act as molecular chaperones: suppression of alpha-synuclein aggregation. J Neurochem. 2002;83:846-854.

74. Barabási MA, Friguet B. Changes of the proteasomal system during the aging process. Prog Mol Biol Transl Sci. 2012;109:249-275.

75. Chen TS, Richie JP Jr, Lang CA. The effect of aging on glutathione and cysteine levels in different regions of the mouse brain. Proc Soc Exp Biology Med. 1989;190:399-402.

SUPPORTING INFORMATION
Additional Supporting Information may be found online in the Supporting Information section.