Research Article

Hsp70 binds reversibly to proteasome inhibitor-induced protein aggregates and evades autophagic clearance in ARPE-19 cells

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Abstract: Age-related macular degeneration (AMD) is characterized primarily by degeneration of the macular retinal pigment epithelium (RPE) that secondarily leads to cell death of photoreceptors and impaired central vision. Hallmarks of AMD are accumulation of lysosomal lipofuscin and extracellular drusen, which indicate impaired proteolysis in RPE cells. Cellular proteostasis is strongly regulated by molecular chaperones such as Hsp70 and proteasomal and autophagic clearance systems. We have recently shown that autophagy receptor SQSTM1/p62 binds irreversibly to proteasome inhibitor–induced perinuclear protein aggregates and undergoes autophagic clearance in RPE cell cultures. Revealing decreased autophagy, SQSTM1/p62 accumulates in macular area of donor AMD patient samples. In this study, we show that Hsp70 binds reversibly to proteasome inhibitor–induced perinuclear protein aggregates and does not become degraded by autophagy in ARPE-19 cells. Our observation reveals new opportunities to use a cytoprotective Hsp70 as a therapy target in the prevention of RPE cell degeneration and development of AMD.

Keywords: aging, autophagy, degeneration, heat shock protein, proteasome, macula, retina

Introduction

Age-related macular degeneration (AMD), the leading cause of irreversible blindness in the Western world, is characterized primarily by degeneration of the macular retinal pigment epithelium (RPE) [1]. RPE damage leads secondarily to cell death of photoreceptors (rods and cones) and impaired vision. The cells of RPE are exposed to chronic oxidative stress originated mainly from three sources: (i) high levels of oxygen consumption, (ii) exposure to lipid peroxidation products derived from the ingestion of photoreceptor outer segments, and (iii) exposure to constant light stimuli. Chronic oxidative stress may evoke misfolding and aggregation of proteins especially during aging. Heat shock proteins (HSPs) attempt to repair the misfolding damage. If this fails, soluble proteins become ubiquitinated and targeted into proteasomes for degradation [2, 3]. Increased levels of HSPs have been observed in RPE cells with accumulated lipofuscin and in retina samples of AMD patients [4, 5]. Moreover, proteasomal activity declines during aging, which leads to aggregation of oxidized and ubiquitinated proteins, as has been documented to happen in

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RPE [6, 7]. Impaired proteasomal clearance in RPE cells induces selective macroautophagy (referred to as autophagy) [7, 8], which is the most prevalent form of autophagy. It involves formation of a double-membrane structure (autophagosome) and engulfment of cytoplasmic proteins, lipids and damaged organelles [9]. Thereafter, autophagosomes fuse with primary lysosomes, and their contents become degraded by lysosomal enzymes [10].

Cellular homeostasis is largely dependent on the quality control of proteins, which is called proteostasis [11]. Hsp70 is one of the key chaperone proteins in the regulation of proteostasis together with proteasomal and autophagic clearance [11]. SQSTM1/p62 (Sequestosome 1) is the best-characterized and most ubiquitously expressed autophagy adaptor that connects proteasomal clearance with autophagy [7, 8, 12-15]. We have previously shown that SQSTM1/p62 binds irreversibly to perinuclear protein aggregates and undergoes autophagic clearance [7]. On the other hand, alleviation of autophagy is usually accompanied by accumulation of SQSTM1/p62, especially on areas that are rich of ubiquitin-stained protein aggregates, as recently reported in AMD patients [7, 8].
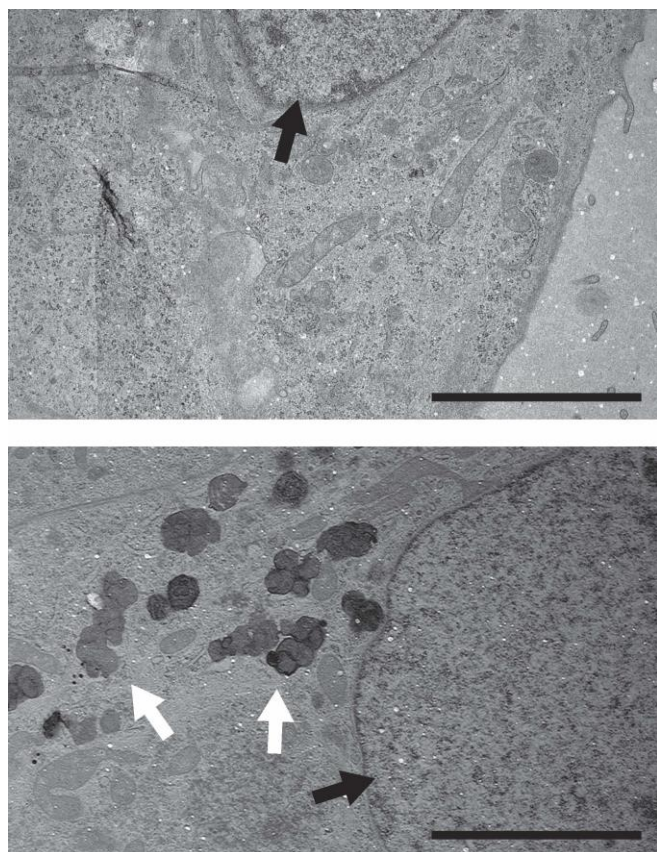

Figure 1. Representative transmission electron micrographs on untreated control ARPE-19 cells (upper) and cells exposed to 5 µM MG-132 for 24 h. Aggregates are indicated by white and nuclei by black arrows. Scale bar = 3 µm.

When proteasomal clearance becomes inhibited in RPE cells, Hsp70 accumulates in same protein aggregates with SQSTM1/p62 [7, 16]. SQSTM1/p62 rather than Hsp70 is considered to be a marker for autophagic activity [7, 8]. In this study, we show that Hsp70 binds in a reversible manner to proteasome inhibitor–induced perinuclear protein aggregates and does not become degraded by autophagy in ARPE-19 cells.

Materials and methods

ARPE-19 cells originated from human retinal pigment epithelium were obtained from the American Type Culture Collection (ATCC). The cells were grown to confluence in a humidified 10% CO_2 atmosphere at 37°C in Dulbecco's MEM/Nut MIX F-12 (1:1) medium (Life Technologies, 21331) containing 10% inactivated fetal bovine serum (Hyclone, SV30160-03), 100 units/ml penicillin, 100 µg/ml streptomycin (Lonza, DE17-602E), and 2 mM L-glutamine (Lonza, BE17-605E). In order to induce protein aggregates, the cells were exposed to 5 µM of MG-132 proteasome inhibitor (Calbiochem, 474790). Lysosomal function was disturbed by 50 nM of bafilomycin A1 (Sigma, B1793). Autophagy was activated with the AMPK (AMP-activated protein kinase) activator AICAR (5-Aminoimidazole-4-carboxyamide ribonucleoside, Toronto Research Chemical, A611700) at 2 mM concentration or induced by starving the cells in serum-free medium. All exposures lasted for 24 hours.

Transmission electron microscopy

Cell culture samples were prefixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hours at room temperature. After 15 minutes washing with 0.1 M phosphate buffer, the cells were post-fixed in 1% osmium tetraoxide and 0.1 M phosphate buffer for 1 hour, and washed with phosphate buffer for 15 minutes prior to standard ethanol dehydration. Subsequently, the samples were infiltrated and embedded in LX-112 resin. Polymerization was carried out at 37°C for 24 h and at 60°C for 48 hours. The sections were examined with a JEM-2100F transmission electron microscope (Jeol) at 200 kV.

Western blotting

For Western blotting, whole cell extracts containing 20 µg of protein were run in 10% SDS-PAGE gels and then transferred to nitrocellulose membranes (Amersham Biosciences) using a semi-dry transfer apparatus (Bio-Rad Laboratories). Successful transfer of proteins from gels to membranes was monitored by Ponceau S staining (Sigma). The membranes were blocked in 0.3% Tween 20/PBS (phosphate-buffered saline) containing 3% fat-free dry milk for 1.5 hours at room temperature (RT). Subsequently, the membranes were incubated overnight at 4°C with a mouse

monoclonal Hsp70 antibody (StressGen) or with a mouse monoclonal anti-α-tubulin (Sigma). Primary antibodies were diluted 1:5000 and 1:8000, respectively, in 0.3% Tween 20/PBS containing 0.5% bovine serum albumin. After three 10-minute washes with 0.3% Tween 20/PBS, the membranes were incubated for 2 hours at RT with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences). Secondary antibodies were diluted 1:20 000 for Hsp70 and 1:10 000 for α-tubulin in 0.3% Tween 20/PBS containing 3% fat-free dry milk. Before detection, all membranes were washed as mentioned above. Protein-antibody complexes were detected with an enhanced chemiluminescence method (Millipore).

pDendra2-Hsp70 fusion plasmid construction

The functional open reading frame (ORF) of human Hsp70 A1A gene (NCBI Nucleotide accession no. NM_005345) was amplified from DNase-treated (DNase I, Roche) total RNA, which was extracted using the Eurozol reagent (Euroclone) from human ARPE-19 cells. RNA was reverse-transcribed by MultiScribe reverse transcriptase (Applied Biosystems), and Hsp70 cDNA was amplified with Phusion Hot start DNA polymerase (Finnzymes). Primers were 5'-ATA *CTC GAG* at**A TG**G CCA AAG CCG C (forward) and 5'-AAT *AAG CTT* g**CT A**AT CTA CCT CCT CAA TGG TG (reverse), containing target sites for restriction endonucleases *Xho*I and *Hin*dIII (italics) and stuffers (minuscules) for in-frame ligation. Sites for the initiation and termination of translation are in boldface.

Sticky ends for the amplified Hsp70 ORF as well as for the multiple cloning site of the vector pDendra2-C (Evrogen) [17] were produced with above-mentioned

restriction endonucleases (MBI Fermentas). Ligated (T4 DNA Ligase, Roche) DNA containing a fusion gene of Dendra2 and Hsp70 was transfected into competent DH5α *E. coli* cells, which were prepared using the protocol described by Inoue et al. [18] Following the bacterial growth, plasmids were purified as previously described [19]. Integrity of the construct, named pDendra2-hHsp70, was determined initially by the restriction endonuclease digestion analysis and finally by sequencing the junction sites and the entire inserted Hsp70 ORF.

The plasmid pDendra2-hp62 producing a fusion protein containing Dendra2 and human SQSTM1/p62, analogous to pDendra2-hHsp70 described above, was prepared as previously described [7]. Preliminarily, the structure and functionality of the fusion proteins were initially monitored using RT-PCR amplification of a 365-bp fragment flanking one junction and by Western hybridization with a Hsp70 antibody (StressGen) detecting the fusion protein (not shown).

Plasmid transfection and confocal microscopy

The pDendra2 fusion plasmids were transfected into subconfluent cultures of ARPE-19 cells using the ExGen500 *in vitro* transfection reagent (MBI Fermentas) following manufacturer's instructions. In all transfection experiments for confocal microscopy, ExGen 500 *in vitro* Transfection Reagent (MBI Fermentas) was used according to the manufacturer's protocol. ARPE-19 cells were cultured in 8-well plates (μ-Slide, ibiTreat, tissue culture treated, Ibidi) to subconfluent density. Thereafter, the pDendra2 fusion plasmids containing 1 μg DNA per well were added, and the cells were incubated for 24 hours. Subsequently, the culture
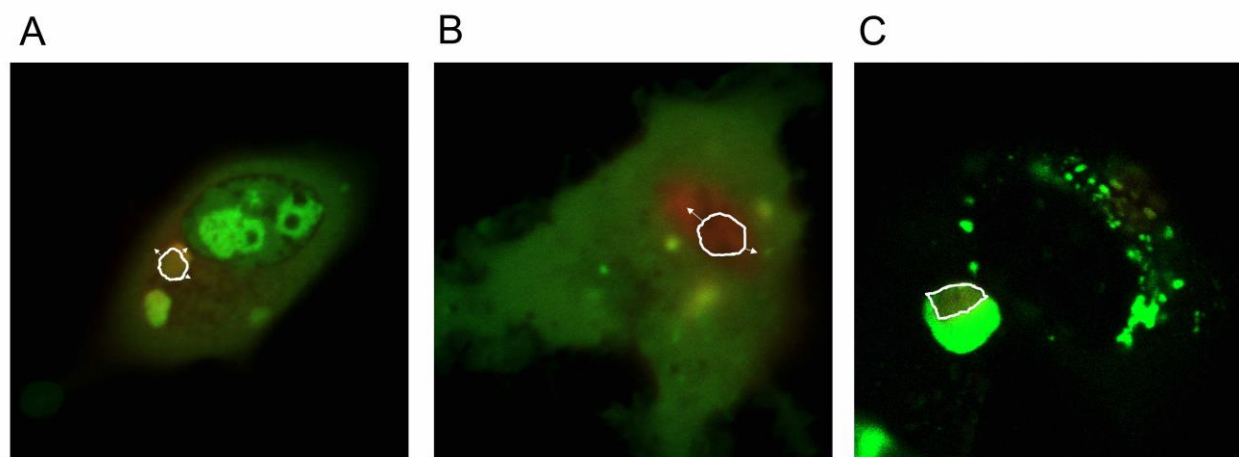

Figure 2. Transfections of ARPE-19 cells with pDendra2-hHsp70 (A and B) and with pDendra2-hp62 (C) constructs. After 24-hour transfections, cells were treated for 24 hours with bafilomycin (50 nM) and MG-132 (5 μM) (panel A) or with MG-132 alone (B and C). The pictures were taken at the moment of UV exposure within the zones drawn with a white line. White arrows indicate the spreading of red colour outside of the exposed spot. Zone diameter = 1 μm.

medium was changed and MG-132 (5 μM) and bafilomycin (50 nM) were added for 24 hours. Transfected cells were exposed to UV radiation (405 nm), which changed the photo-switchable colour of Dendra2 from green to red. Fluorescent images were obtained with a Zeiss Axio Observer inverted microscope (40 × NA 1.3 or 63 × NA 1.4 oil objectives) equipped with the Zeiss LSM 700 confocal module (Carl Zeiss). For live cell imaging, Zeiss XL-LSM S1 incubator with temperature and CO_2 controls was utilized. ZEN 2009 software (Carl Zeiss) was used for image processing.

Results

We recently documented that SQSTM1/p62, a shuttle protein between proteasomal and autophagic clearance, binds permanently in perinuclear aggregates and subsequently undergoes autophagic clearance [7]. Since Hsp70 binds to the same aggregates [16], we wanted to evaluate whether the binding of Hsp70 is a reversible or an irreversible process. First, ARPE-19 cells were exposed to 5 μM concentration of MG-132 for 24 hours, which evoked

strong formation of perinuclear protein aggregates (Fig. 1) [16]. Then pDendra2-hHsp70 fusion plasmid construct was transfected to the cells and confocal microscopy was used for live cell imaging. The movement of pDendra2-hHsp70 fusion protein was evaluated after photo conversion (Fig. 2). We detected that Hsp70 protein moved from the converted area to other parts of the cell, which suggests that the binding of Hsp70 to protein aggregates is reversible. As a comparison, pDendra2-hp62 did not leave the exposed area (Fig. 2, panel C) [7].

Bafilomycin is known to inhibit autophagy by preventing the fusion of autophagosomes with lysosomes [9]. To examine how bafilomycin affects the expression of Hsp70 protein in ARPE-19, the cells were treated either with 50 nM of bafilomycin, 5 μM of MG-132 or both. Thereafter, total protein extracts were analysed for the expression of Hsp70 using the western blotting technique. In contrast to our previous observations with SQSTM1/p62 [7], ARPE-19 cells treated with bafilomycin displayed no changes in Hsp70 protein levels (Fig. 3). When the cells were exposed simultaneously to both bafilomycin and MG-132, there was a significant increase in the amount of Hsp70 protein. A
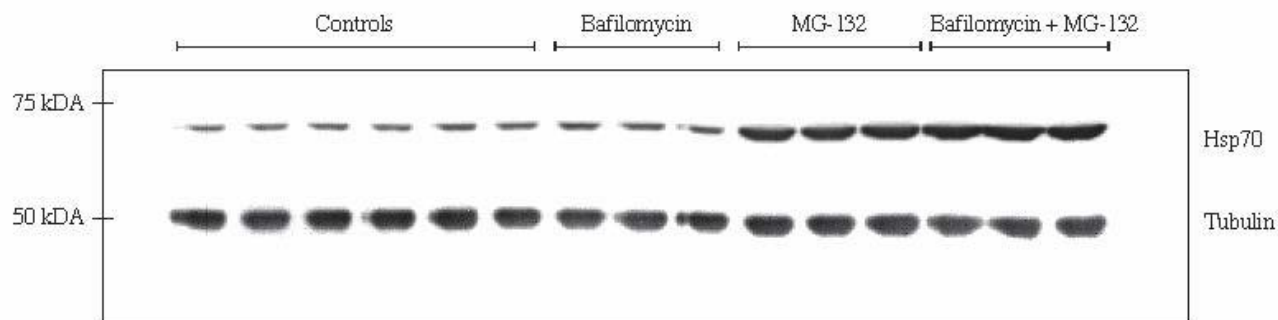

Figure 3. Image from a representative western blot showing the expression of Hsp70 in control ARPE-19 cells and in cells exposed to 5 μM of MG-132 and 50 of nM bafilomycin, together or alone for 24 hours. α-tubulin was used as a loading control.
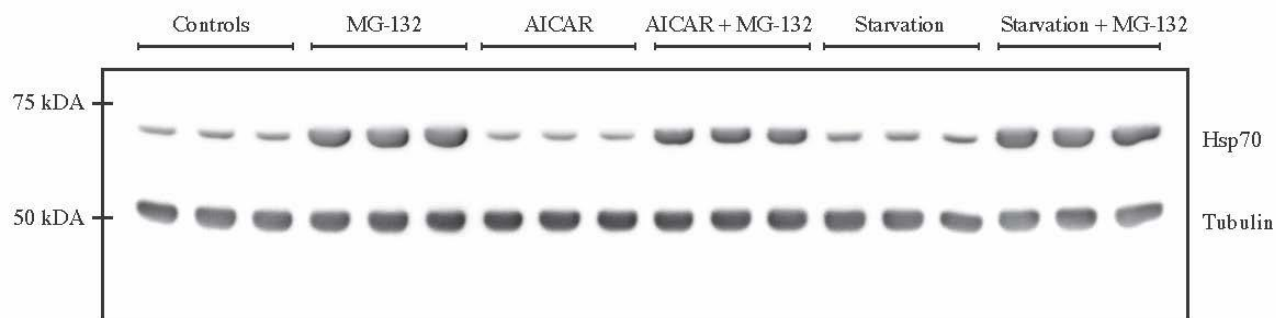

Figure 4. Image from a representative western blot showing the expression of Hsp70 protein in control ARPE-19 cells and in cells exposed to 5 μM of MG-132, 2 mM of AICAR, starvation or simultaneously to 5 μM of MG-132 and 2 mM of AICAR or starvation for 24 hours. α-tubulin was used as a loading control.

similar increase in the levels of Hsp70 was seen in cells treated with MG-132 alone. This suggested that Hsp70 is not degraded by autophagy.

In order to confirm this hypothesis, possible effects of AICAR and serum starvation on the Hsp70 expression under MG-132 stimulation were studied. Induction of autophagy by either way did not change the Hsp70 protein levels (Fig. 4). These results support the idea that Hsp70, in contrast to SQSTM1/p62, does not bind permanently to proteasome inhibitor–induced protein aggregates and does not undergo the autophagic clearance.

Discussion

In the present study, we have shown that the best-known molecular chaperone Hsp70 binds to perinuclear protein aggregates by a reversible mechanism under proteasome inhibition in human retinal ARPE-19 cells. We have recently shown using pDendra2-hLC3 (MAP1LC3A/LC3) and pDendra2-hp62 (SQSTM1/p62) plasmids, with which localisation of the proteins in a cell can be detected at a desired time point, that both LC3 and SQSTM1/p62 disappeared via autophagic clearance in response to the inhibition of proteasomes and the induction of autophagy. The binding of SQSTM1/p62 protein to perinuclear aggregates was observed to be an irreversible process [7, 9]. In contrast to our previous results, we show here by pDendra2-hHsp70 fusion plasmid construction analyses that Hsp70 moves from protein aggregates during proteasome inhibition, indicating reversible binding of Hsp70 to protein aggregates.

Blocking the expression of SQSTM1/p62 rather than Hsp70 using mRNA silencing results in decreased autophagosome formation in ARPE-19 cells [7]. However, both of them had similar cytoprotective capacity under proteasome inhibition. Cytoprotection was obviously involved in the repairing chaperone function of Hsp70, while SQSTM1/p62 regulated the induction of autophagic clearance. Once autophagy was induced during proteasome inhibition, SQSTM1/p62 degradation also became accelerated. In this study, we have shown that the induction of autophagy by AICAR or by starvation, or the inhibition of autophagy by bafilomycin, did not change Hsp70 levels under proteasome inhibition. This indicates that Hsp70 is protected from autophagic clearance.

Hsp70 consists of two major functional domains; the amino-terminal domain that possesses the ATP/ADP binding site as well as the ATPase activity, and the carboxy-terminal domain, which contains the binding site for substrate peptides [20]. In the ATP-bound state, substrates can easily bind to and dissociate from Hsp70. When ATP is hydrolysed to ADP, the affinity of a substrate to Hsp70 becomes increased [21]. An important feature in the Hsp70's cycle is that chaperone proteins have several co-chaperones that modulate substrate interactions. We found that Hsp70 binds in a reversible manner to proteasome inhibitor–induced

protein aggregates. This process might be controlled by the ATP/ADP regulation cycle. Post-translational modifications of Hsp70 such as SUMOylation, ubiquitination or glycosylation may also control the binding activity of Hsp70 to the client proteins in aggresomes and prevent its autophagic clearance [22-27]. There is evidence that in contrast to SQSTM1/p62, Hsp70 favours proteasomal clearance in certain stress conditions [26].

Damage in cellular macromolecules accumulates during aging and tissue degeneration in postmitotic cells, such as RPE cells [3]. It has recently been proposed that declining heat shock response, reduced levels of HSPs, and the resultant loss of protein quality control may exacerbate protein damage during aging [28-31]. Molecular Hsp70 chaperone therapy might be a new formulation strategy in the treatment of retinal pigment epithelium and AMD [32]. RPE cells are phagocytic and they can internalise extracellular material as occurs e.g. with photoreceptor outer segments during normal visual cycle. The shed photoreceptor outer segment particles are taken up by RPE via endocytic and phagocytic processes and degraded in lysosomes [3]. In aged RPE cells, however, lysosomal enzyme activity is decreased, which leads to harmful lipofuscin accumulation and reflects the severity of AMD [33]. In the Hsp70-based therapy, proteins should be targeted to lysosomes due to phagocytic process [34]. Interestingly, Hsp70 has been found in lysosomal fractions of RPE cells [4, 16]. It might be hypothesized that Hsp70 as a molecular chaperone keeps lysosomal enzymes functionally active, prevents lipofuscin accumulation in RPE cells and thereby suppresses the development of AMD [35]. Hsp70 has also anti-inflammatory capacity [36]. Inflammation is a central hallmark in the pathogenesis of AMD. Therefore, Hsp70 might also prevent inflammatory signalling in RPE cells. The present data importantly show that Hsp70 is protected from lysosomal clearance system and thus provide opportunity to develop its use in protein, drug or laser therapy for the prevention of AMD.

Conflict of interest: None declared

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