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Proteasome regulates turnover of toxic human amylin in pancreatic cells

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Abstract

Toxic human amylin (hA) oligomers and aggregates are implicated in the pathogenesis of type 2 diabetes mellitus (T2DM). Although recent studies demonstrated a causal connection between hA uptake and toxicity in pancreatic cells, the mechanism of amylin's clearance following its internalization and its relationship to toxicity is yet to be determined, and hence was investigated here. Using pancreatic rat insulinoma β -cells and human islets as model systems, we show that hA, following its internalization, first accumulates in the cytosol followed by its translocation into nucleus, and to a lesser extent lysosomes, keeping the net cytosolic amylin content low. An increase in hA accumulation in the nucleus of pancreatic cells correlated with its cytotoxicity, suggesting that its excessive accumulation in the nucleus is detrimental. hA interacted with 20S core and 19S lid subunits of the β -cell proteasomal complex, as suggested by immunoprecipitation and confocal microscopy studies, which subsequently resulted in a decrease in the proteasome's proteolytic activity in these cells. *In vitro* binding and activity assays confirmed an intrinsic and potent ability of amylin to interact with the 20S core complex thereby modulating its proteolytic activity. Interestingly, less toxic and aggregation incapable rat amylin (rA) showed a comparable inhibitory effect on proteasome activity and protein ubiquitination, decoupling amylin aggregation/toxicity and amylin-induced protein stress. In agreement with these studies, inhibition of proteasomal proteolytic activity significantly increased intracellular amylin content and toxicity. Taken together, our results suggest a pivotal role of proteasomes in amylin's turnover and detoxification in pancreatic cells.

Keywords

human amylin; lysosome; proteasome; protein aggregation; proteotoxicity

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AUTHOR CONTRIBUTION

Sanghamitra Singh, Saurabh Trikha and Anjali Sarkar performed the experiments. Sanghamitra Singh and Aleksandar Jeremic designed the experiments. Sanghamitra Singh and Aleksandar Jeremic wrote the paper.

INTRODUCTION

Islet amyloid polypeptide (IAPP) also known as amylin, is a pancreatic hormone produced and co-secreted with insulin from islet β -cells. Amylin is initially produced and synthesized as an immature 67 amino acid long precursor peptide that is subsequently cleaved by proconvertases to yield a mature biologically active 37 amino acid peptide [1]. Although the exact hormonal function of amylin is still unclear, it has been proposed that amylin controls food intake and energy homeostasis [2–4]. Endogenous amylin plays a role in modulating insulin secretion from islet β -cells via amylin receptor-dependent mechanism [5–7]. In addition to its physiological roles, in humans amylin can also be pathogenic. Human amylin (hA), under conditions associated with impaired glucose homeostasis, such as in type 2 diabetes mellitus (T2DM) readily aggregates, which is cytotoxic to pancreatic β -cells [1,8,9].

T2DM is a complex metabolic disease characterized by peripheral insulin resistance, by the decline in the production and secretion of insulin, loss of β -cell mass and formation of islet amyloid composed mostly of aggregated hA [8–11]. The role of hA and islet amyloidosis in the pathogenesis of T2DM is supported by clinical and pathological studies showing the presence of amylin-derived amyloid plaques in over 90% of diabetic patients [12]. In addition to humans, diabetes associated amyloid plaques have been detected in monkeys and cats, species known to develop T2DM, but not in rats and mice, species that do not spontaneously develop T2DM [8,9]. This is strong yet indirect evidence correlating T2DM and islet amyloidosis. However, expression of hA in mice and rat islets leads to the onset of T2DM in transgenic rodents further supporting the detrimental role of hA in the aetiology of the disease [13–15].

In vitro studies show that hA, but not rat amylin (rA), undergoes rapid aggregation in physiological buffers and that insulin, but not pro-insulin, inhibits hA aggregation by forming heteromolecular complexes [16]. Therefore, faulty insulin processing could account for amylin aggregation in diabetic people. Defective processing of pro-hA into mature hA is another candidate for amylin aggregation in T2DM, as partially processed hA has been identified in islet β -cells of diabetic subjects, and accumulation of partially cleaved hA was linked to its toxicity in hA rodent models [17,18]. Increased accumulation of hA derived oligomers and aggregates inside and outside islet β -cells accounts for downstream pathological events such as calcium overload, cell membrane disruption, ER (endoplasmic reticulum) stress, mitochondrial dysfunction, defects in autophagy, oxidative stress and activation of JNK and caspase-3 death signalling pathways [19–29]. These studies are consistent with the idea that both intracellular and extracellular accumulation of hA is toxic to the cells. These findings, together with the fact that hA aggregation, internalization and toxicity are strongly concentration and time dependent [7,28,29], suggest that the dynamics of its turnover inside β -cells could be an important factor in hA aggregation and hA-induced β -cell toxicity. This could be true for intracellularly produced and internalized hA alike [28,30].

Indeed, recent studies using the intracellular model of islet amyloidosis demonstrated that hA is degraded by autophagosomes and lysosomes, thereby preventing its intracellular

accumulation, oligomerization and toxicity [23,31]. In addition to lysosomes, extracellular and intracellular proteases neprilysin and insulin degrading enzyme (IDE) respectively participated in the clearance of hA in pancreatic β -cells thereby protecting cells from amylin insult [32–34]. Whether only lysosomes, IDE and PM (plasma membrane)-bound neprilysin are involved in degradation of hA, and if intracellular proteolysis of internalized hA monomers and oligomers is relevant for hA toxicity in the pancreas are yet to be fully clarified. To answer the aforementioned unresolved questions, we investigated the pathways of hA internalization and the cellular compartments involved in its uptake and degradation, focusing on the major proteolytic complex in cells, the proteasome. Previously, involvement of proteasomes in the clearance and detoxification of other amyloid proteins, such as tau protein and α -synuclein was demonstrated [35,36]. However, the role of proteasomes in hA clearance is yet to be elucidated. Hence, we hypothesized that proteasomes mediate degradation of hA in pancreatic β -cells and by doing so prevent its toxicity.

Using biochemical methods and confocal microscopy, we show that following internalization, hA selectively localizes in the nucleus and lysosomes and to a lesser extent mitochondria of pancreatic rat and human islet cells. In accordance with our hypothesis, results of the present study demonstrate a physical interaction between internalized hA and proteasome's catalytic and regulatory subunits 20S α 4 and 19Srp8 in the pancreatic cells, a decrease in the cell's proteasome activity in response to both hA and rA treatments, and a substantial increase in nuclear, and much less cytosolic accumulation of hA and its cytotoxicity upon inhibition of the enzyme's proteolytic activity. Taken together, these findings suggest a mutual cause and effect relationship between hA clearance and the proteasome proteolytic activity in pancreatic cells.

MATERIALS AND METHODS

Reagents

Pepstatin A (PepA) and Methylene Blue (MB) were purchased from Sigma. Lactacystin (Lac) was obtained from Calbiochem. Mitotracker (MT), nuclear dyes (DRAQ5) and DAPI, Golgi marker (GM130) and Alexa fluor secondary antibodies were from Invitrogen. hIAPP, PARP, actin, Tim rabbit polyclonal primary antibodies were purchased from Santa Cruz Biotechnology. HSP90, 20S proteasome (α -4), Ub (ubiquitin) (P4D1), γ -tubulin mouse monoclonal primary antibodies were also purchased from Santa Cruz Biotechnology. Histone rabbit polyclonal antibody was purchased from Abcam. Giantin and Lamp1 rabbit polyclonal primary antibodies were a generous gift from Dr Julie Donaldson, NIH.

Preparation of human and rat amylin

Lyophilized C-terminal amidated synthetic 37 amino acid hA and rA peptides (American Peptide or Anaspec) were used for preparation of amylin stock solutions (500 μ M) using hexafluoro isopropyl alcohol (HFIP) as a solvent. The appropriate amount of peptide was solubilized in HFIP overnight to completely dissolve amylin. This approach efficiently removes any preformed amylin oligomers and aggregates [37,38]. Before experiments, HFIP was evaporated under a stream of dry nitrogen gas to remove the organic solvent, which is toxic to cells. For fresh amylin preparation, hA was reconstituted using freshly prepared

culture medium to final concentration of 10 μM (confocal microscopy) or 30 μM (toxicity and biochemical studies) and immediately applied to cultures. Lower hA concentration was used for microscopy studies to prevent detachment of cells. Pre-aggregated hA was prepared in the following way: after evaporating HFIP, hA was reconstituted in the same volume of PBS in order to return to the stock concentration (500 μM) and incubated further for 10 min. Calculated volumes of human or rA were then added directly to each well to yield the peptides' desired final concentrations.

Cell cultures and treatments

Rat insulinoma (RIN-m5F) β -cells (A.T.C.C.) were cultured in RPMI 1640 medium (A.T.C.C.) supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified incubator with 5% CO₂. Cells were passaged biweekly. Passages 10–50 were used for all the experiments. Rat insulinoma β -cells were plated at density 50000 cells/well and cultured for 12 h prior to the experiments. Human or rA was then added to cultures for additional 0–24 h depending on experiments. Human islets from non-diabetics with >90% purity and viability were obtained through the Integrated Islet Distribution Program (IIDP) for Human Islet Research. Upon arrival, islets were handpicked under a dissecting microscope to yield purity of 95% (based on dithiazone staining). Intact islets were plated on 24-well tissue culture treated plates (50 islets per well) and suspended in CMRL media (A.T.C.C.) for biochemical studies. For confocal studies, 15–20 islets per well were gently dissociated in TrypLe cell dissociation medium (Invitrogen) using a 1 ml-glass pipette and plated on 96-well glass bottom SensiPlates (Greiner) precoated with poly-D-lysine. Pancreatic cells were treated with 10–30 μM hA in the absence or presence of pharmacological inhibitors, PepA (1 μM), Lac (1 μM), MB (100 μM), for a total of 12 h. H₂O₂ (100 μM) treatment for 4 h was used as a positive control for PARP and caspase-3 cleavage and MTT toxicity assays respectively.

Immunofluorescence microscopy

Following treatments, cultured RIN-m5F or human islet pancreatic cells were further incubated with specific organelle markers, MT (90 nM) for additional 45 min at 37°C or 1 μM of nuclear specific dye, DRAQ5 for 10 min at RT (room temperature) or 1 μM DAPI for 5 min at RT. Cells were washed and fixed with 4% paraformaldehyde. hA was detected by incubating the cells with rabbit anti-hA antibody (1:200) at 4°C overnight. No measurable fluorescence signal was detected in control (hA-lacking) RINm5F cells as reported previously [29]. In addition to organelle probes, cells were also co-incubated overnight with hA antibody and one of the primary mouse, goat or rabbit antibodies (1:200) raised against a respective protein of interest at 4°C. Cells were then exposed to goat anti-rabbit Alexa 488, goat anti-mouse Alexa 647 or donkey anti-goat Alexa 555-conjugated secondary antibodies (1:300) for 60 min at room temperature. Three random fields in each well were imaged by confocal microscopy, with each treatment performed in duplicates. Single optical sections (1 μm z axis) through the middle of the cells were acquired for each field using an LSCM-510 Meta confocal microscope (Carl Zeiss) equipped with 63 $\times$ (1.4 N.A.) oil objective. The pinhole was adjusted to keep the same size of z-optical sections for all channels and objectives used. Multitrack imaging was performed to ensure that there was no cross-talk between the channels. The intensity thresholds of the channels were set using the Zeiss

cross-hair function to avoid an arbitrary background threshold being set. In that way, the background pixels that might contribute to false co-localization were excluded from analysis. The Pearson co-localization coefficient (R) was determined using Zeiss co-localization and Image J software (release 4.0). Nine to twelve cells per image with three images per sample (treatment), each performed in duplicates, were analysed. Obtained R -values were averaged and grouped in three categories: low colocalization $R < 0.3$, partial colocalization $R = 0.3$ – 0.6 and high colocalization $R > 0.6$. Images were assembled using Adobe Photoshop software (Adobe Systems).

Cell fractionation and quantification of human and rA intracellular content

Pancreatic cells were incubated overnight with hA or rA in the absence or presence of proteolytic inhibitors. Following incubations, cytosolic, organelle and nuclear fractions were sequentially isolated using a cell fractionation kit (Cell Signaling, catalogue number 9038S) according to the manufacturer's protocol, with minor modifications. Purity of fractions was determined by Western blot analysis using specific nuclear, cytosolic and organelle markers. Pure mitochondrial fraction was isolated from control and hA treated cells using cell fractionation kit (Abcam, catalogue number 109719) according to the manufacturer's protocol. Purity of mitochondrial fraction was confirmed using mitochondrial marker, cytochrome *c*.

Following addition of hA to cells, real time changes in hA content in pancreatic cell extracts or fractions, reflecting its internalization and accumulation, were quantified by a hA ELISA kit (Millipore catalogue number EZHA-52K) according to manufacturer's guidelines. Prior to analysis, samples were collected and diluted 100 $\times$ for their concentrations to be in the linear range, established by running the standards provided in the kit. The concentrations obtained were normalized to the protein content measured in each sample. Because this kit also recognizes rodent amylin isoforms, controls (cells not exposed to hA) were always run in parallel with hA treated cells to distinguish between rA and hA accumulation in these cells.

Western blot analysis

PARP/caspase 3 cleavage, apoptotic markers, and the extent of protein ubiquitination in pancreatic cells in response to treatments, reflecting protein stress, were estimated by Western blot analysis. Pancreatic cells were incubated with 10 or 30 μ M hA or rA in the absence or presence of proteasomal inhibitor Lac (1 or 10 μ M) for 12 h. Following the above treatments, cells were either subjected to cell fractionation to obtain nuclear and cytosolic fractions or lysed with 1 $\times$ RIPA buffer and samples obtained separated by 4–15% Tris/glycine SDS/PAGE. The proteins were blotted on to nitrocellulose membranes for detection with Ub specific P4D1 mouse monoclonal antibody or PARP rabbit polyclonal antibody (Santa Cruz) at 1:200 dilutions respectively for 1 h. This was followed by addition of horseradish peroxidase-conjugated goat anti-rabbit secondary antibody at 1:2000 dilutions (Thermo Fisher). Blots were developed using ECL substrate (Pierce) and quantified using the Kodak 1500 imaging station.

Native PAGE analysis for cationic proteins

Monomeric and oligomeric amylin species were visualized using Biorad's cathodic, discontinuous native PAGE protocol (technical note 2376), with minor modifications. L-Lactate dehydrogenase (146 kDa), lectin (49 kDa) and trypsinogen (24 kDa) were used as basic standard proteins. Freshly prepared hA (30 μ M) was incubated for 4 h at 4°C alone or in the presence/absence of MB (500 μ M) at RT. Samples were then run on 9% native polyacrylamide gel and resolved protein bands detected using Coomassie Brilliant Blue staining.

20S proteasome activity assay

Proteasome activity was assessed using a 20S proteasome activity assay kit (Cayman Chemicals, catalogue number 10008041), according to the manufacturer's protocol. Detection is based on a specific 20S substrate, SUC-LLVY-AMC that, upon cleavage by the active enzyme, generates a highly fluorescent product. Increase in fluorescence intensity, reflecting proteasome activity in sample, was measured using M5 Spectromax fluorometer (Molecular Devices) with excitation/emission set at 360/480 nm. Data (relative fluorescent unit, RFU) were collected and plotted using GraphPad Prism 5 graphic program. For *in vitro* experiments, instead of cellular extracts, purified 20S complex (1 μ g/ml, Enzo Biosciences) was used in the absence or presence of increasing concentrations of peptides or Lac. Log (inhibitor) dose–response curves were then constructed using the same graphic program. The half maximal inhibitory concentration (IC₅₀) was determined from the slope following curve fitting and non-linear regression analysis. A standard slope model (Hill factor of – 1.0) was used for fitting.

MTT cell toxicity assay

We used 10–30 μ M amylin concentrations, commonly adopted across the field, to generate amylin oligomers and aggregates to study amylin toxicity in cells [24,28,29,39,40]. RIN-m5F cells and human islet cells were plated on to 96-well plates for 24 h followed by incubation with various reagents in the absence or presence of hA for additional 24 h. Cells were then incubated with 1 mg/ml MTT (Sigma) solution for 3 h and precipitated formazan crystals inside cells, a product of mitochondrial metabolism, dissolved in HCl–propan-2-ol solution and absorbance determined at 570 with subtraction at 690 nm. A decrease in cell viability results in a decrease in the amount of MTT formazan formed and a decrease in absorbance. Raw data (absorbance) were recorded and expressed as percentage change from control (taken as 100%).

Immunoprecipitation from cell fractions

Nuclear and cytosolic fractions of hA treated or non-treated (control) RIN-m5F cells were prepared using cell fractionation kit (Cell Signaling). The samples were precleared with magnetic dynabeads (Invitrogen), at 4°C, under rotation, for 2 h. Ten micrograms of anti-hA rabbit polyclonal (Santa Cruz) was coincubated with 50 μ l proteinG Dynabeads (Invitrogen) at 4°C, under rotation, for 24 h. The Dynabeads–IgG complex was then cross-linked with 1 ml 20 mM DMP (dimethylpimelimidate dihydrochloride) solution in 0.2 M triethanolamine pH 8.2 and reaction terminated with Tris/HCl pH 8 buffer after 30 min. The precleared

nuclear and cytosolic fractions and cross-linked dynabeads were then co-incubated at 4°C, under rotation, for 24 h. After three washes in PBS, the immunoprecipitated material was detached from the magnetic beads with 5× Laemmli buffer and resolved by SDS/PAGE on 4–15% Tris/glycine gels. Western blot analysis using anti-20S α 4 or anti-19S Rpn8 primary antibody (1:200) was then performed. Blots were washed three times with PBS-Tween buffer, incubated with goat anti rabbit HRP linked secondary antibodies (1:2000) for 1 h and developed using a chemiluminescence method.

***In vitro* immunoprecipitation**

The intrinsic property of hA to interact with 20S catalytic particle was examined *in vitro*, under cell free conditions. hA (30 μ M) was incubated with purified 20S peptide (1 μ g/ml) from human erythrocytes (Enzo Biosciences) for 1 h with constant and even rotation at + 4°C. Thereafter, the hA/20S proteasome complex was immunoprecipitated using standard protocol described above, with polyclonal anti hA antibody (Santa Cruz) cross-linked to Dynabeads as baits. After washing (3×), immunoprecipitated material was collected in sample prep buffer separated on 4–15% Tris/glycine SDS/PAGE followed by Western blot analysis using anti-20S β 1 mouse monoclonal antibody (Santa Cruz, CA).

Amylin fibrilization assay

The Thioflavin-T (Th-T) fluorescence assay was used to determine the lag phase, kinetics and the extent of amylin aggregation as explained previously [37,38]. Freshly prepared stock solutions (500 μ M) of hA in the organic solvent HFIP were used to ensure the absence of proto-fibrils in incubation medium. Final concentration of 30 μ M of preaggregated or monomeric amylin was prepared in PBS and 3 μ M Th-T was added to each well in the microplate. Real-time changes (5 min interval) in Th-T fluorescence intensities, expressed as arbitrary units (au), were continuously recorded using Spectramax M5 spectrofluorometer, Molecular Devices, with the excitation wavelength set at 450 nm and the emission recorded at 482 nm. In order to avoid any possible secondary effect of Th-T on amylin aggregation and to test the effect of temperature on amylin aggregation, we used the end-point approach: following incubations at + 4°C or RT, aliquots from 30 μ M samples were withdrawn and then mixed with Th-T reagent to detect the aggregated species using the settings described above. Data were collected, plotted and compared using the GraphPad Prism 5 graphic program.

Statistical analysis

The Graph Pad Prism 5 Program was used for plotting and statistical analysis. The unpaired Student's *t* test or one-way ANOVA followed by the Tukey post-hoc test were used for pair wise comparisons among groups when appropriate with significance established at $P < 0.05$.

RESULTS

Internalized hA accumulates in the nucleus and to a lesser extent cytosol of RIN-m5F cells and human islets

Previous studies established that small cationic peptides such as hA cross the cell's plasma membrane (PM) using both endocytotic and non-endocytotic pathways in a concentration

and time dependent manner [7,28,29,41]. In the present study, we focus on the fate of hA post internalization, and the cellular compartments sequestering it. Typically, RIN-m5F cells and human islets were treated with preaggregated hA for 24 h, and intracellular hA redistribution was determined by microscopy and biochemical methods (Figure 1, Supplementary Figure S1). At this (μ M) concentration and prolonged time exposures (0–24 h), hA readily oligomerizes and aggregates (Supplementary Figure S2) and is cytotoxic to cells [7,27,29], thus allowing us to examine how cells defend themselves against high molecular weight toxic hA species. Following hA's internalization into the cells, nuclear, cytosolic and organelle fractions were isolated using cell fractionation approach (see Materials and methods). Fraction purity and redistribution of organelles and other cellular components within fractions were determined by Western blot analysis using antibodies raised against the following markers: histone and PARP for the nucleus, HSP90 and actin for the cytosol, TIM for mitochondria, Giantin for Golgi apparatus, LAMP1 for lysosomes and VAMP-1/2/3 for secretory vesicles (Supplementary Figure S1A). Western blot analysis revealed that the nuclear (N) and cytosolic (C) preparation were highly enriched, and the cytosolic fraction was devoid of larger organelles such as Golgi complex, mitochondria and secretory vesicles but contained smaller organelles such as lysosomes, ascertained by the absence or presence of their respective markers: Giantin, TIM, VAMP and LAMP, in these two fractions (Supplementary Figure S1A). Heavier organelles, mitochondria and Golgi were co-purified as a third separate large organelle-enriched (O) fraction (Supplementary Figure S1A).

Unexpectedly, amylin specific ELISA revealed a large 20-fold increase in absolute amylin levels in the nucleus and to a much smaller extent (3-fold increase) in the cytosol of hA-treated (for 24 h) RIN-m5F cells as compared with control rA-producing cells (Figure 1A). Because in both these fractions the externally applied hA dwarfs the production of a native (rat) amylin, this allowed us to study turnover of internalized hA in β -cells. hA accumulation in the large-organelle fraction of RIN-m5F cells was comparable to the cytosol, ~3% of the total internalized hA (Supplementary Figure S1B). This finding was reconfirmed by indirect immunocytochemistry (Figure 1C). Immunoconfocal microscopy, using hA specific polyclonal antibody [29] (Supplementary Figure S3), showed a high colocalization value ($R > 0.6$) between nuclear marker DRAQ5 and hA, demonstrating its predominantly nuclear accumulation in RIN-m5F cells (Figure 1C, top panel). In contrast with the nucleus, immunoconfocal data showed relatively lower levels of hA in the cytosol, mostly perinuclear, in agreement with biochemical data (Figure 1C, top panel). To determine if hA trafficking is cell specific, we incubated partially dissociated human islet cells with hA for 24 h and intracellular hA redistribution was again analysed by immunoconfocal and biochemical approaches (Figures 1B and 1C, bottom panel). Similar to RIN-m5F cells, internalized hA predominantly localized in the nucleus of human islet cells (Figure 1C, bottom panel). Biochemical analysis confirmed a predominantly nuclear accumulation of hA in hA-treated human islet cells (Figure 1B). Cytosolic and organelle enriched fractions together accumulated less than 10% of the total internalized hA in both cell types (Supplementary Figure S1B).

Nuclear and cytosolic accumulation of hA takes place in a sequential and time dependent manner

In order to better understand the mechanism and dynamics of hA trafficking in cells, RIN-m5F cells were treated with pre-aggregated hA (Figure 2A) or freshly reconstituted hA (Figure 2B) (see Materials and methods section) for increasing periods of time (0–24 h), cell fractions prepared as described above, and amylin content in both nuclear and cytosolic fractions analysed by ELISA. Biochemical analysis demonstrated that irrespective of the method of hA preparation, hA progressively translocates in the nucleus over a period of 24 h, with a lag phase of ~4 h (Figures 2A and 2B). In contrast, hA content in the cytosolic fractions spiked after 30 min without further significant accumulation after 4 h (Figures 2A and 2B inset). In both preparations, this event (cytosolic influx of hA) preceded hA accumulation in the nucleus. Thus, translocation of hA into the nucleus (Figures 2A and 2B), and to a lesser extent into mitochondria accounts, at least in part, for the small hA accumulation in the cytosol. The time course confocal analysis of amylin internalization (Figure 2C) confirmed ELISA experiments by showing visible amylin accumulation in the perinuclear (arrows) and nuclear (arrowheads) regions of the cell as early as 4 h following the peptide addition, and thereafter. Prolonged and excessive intracellular accumulation of hA (24 h, Figure 2C) often induced nuclear condensation and cell shrinkage indicating apoptosis, as previously reported [29].

The extent of nuclear accumulation of hA and its cytotoxicity depends on its aggregation state

Further evidence of the importance of hA's aggregation state for its clearance and toxicity in cells comes from experiments in which we assessed hA clearance and toxicity using two distinct methods of hA preparation, one that contains high content of pre-aggregated hA and the other (fresh hA) initially featuring mostly soluble but aggregation prone hA (Supplementary Figure S2A). If aggregation state of hA matters for its enhanced accumulation and/or toxicity, we would expect method one (featuring aggregating species) to be more cytotoxic than fresh samples initially lacking larger aggregates (Supplementary Figure S2A). Twenty-four hours after hA treatment, a 5-fold increase in nuclear but not cytosolic hA content is seen when hA is added in its pre-aggregated form (Figure 2A) as compared with fresh amylin (Figure 2B), consistent with the ability of amyloid oligomers to incorporate into and penetrate membranes [42–45]. In accordance with the aggregation (oligomeric) hypothesis [30], MTT metabolic stress assay revealed that pre-aggregated hA was significantly more stressful to cells as compared with fresh hA preparation (Figure 2D), linking nuclear accumulation of hA and its toxicity. Amyloid oligomeric inhibitor and antioxidant MB [29,46] prevented amylin oligomerization (Supplementary Figure S2B) and specifically reversed amylin's toxicity (Figure 2D) but not toxicity due protein stress (Figure 2E) in these cells, further demonstrating the detrimental effect of oligomers, and redox sensitive mechanism of hA evoked β -cell death.

Lysosomes sequester the cytosolic pool of hA

Mitochondria regulate vital cellular functions such as respiration, cell growth, cell division and cell death. Certain extracellular amyloid peptides tend to accumulate in mitochondria

following internalization leading to a collapse of mitochondrial membrane potential, excessive production of reactive oxygen species (ROS) and finally apoptosis in neural cells [47,48]. Several studies suggested mitochondria to be a prime target of hA toxicity [28,47,49]. However, it is unclear if direct or indirect mechanisms account for hA-induced mitochondrial impairment. Direct action is implied by a recent study showing that hA, at its cytotoxic concentration and following its internalization, localizes in the mitochondria in insulin secreting INS-1 cells, resulting in mitotoxicity and β -cell death [28]. However, in our RIN-m5F cells, confocal microscopy revealed no significant co-localization of hA with MT, a selective marker of mitochondria ($R < 0.3$, Figure 3) and in human islets (results not shown). To confirm our microscopy studies, hA accumulation in the mitochondria was examined using biochemical approach. Following exposure of cells to hA for 24 h, non-internalized hA was washed away, cells collected and pure mitochondrial fractions prepared, as described in Materials and methods section. Using a very sensitive method for hA detection (ELISA), we detected a very small pool (<2%) of total internalized hA in isolated mitochondrial fraction. However, this rather low mitochondrial accumulation of hA was not detectable by confocal microscopy due to limitations in the sensitivity of the technique. However, both biochemical and microscopy methods are consistent with a rather minor accumulation of internalized hA in the mitochondria in our model cells, indicating largely indirect effects of hA on mitochondrial dysfunction.

Given the small but significant cytosolic accumulation of hA (Figures 1A and 1B) we further used confocal microscopy to determine if lytic cytoplasmic compartments such as lysosomes accumulate internalized hA. A partial ($R \sim 0.5$) and mostly perinuclear colocalization of hA and lysosomes was observed at 24 h (Figure 3). This result is in agreement with lysosomal accumulation of endogenous amylin [50], suggesting a similar but not identical recycling mechanism for extracellular and intracellular hA. Under certain conditions, endocytotic vesicles can merge with biosynthetic compartments like Golgi complex, thus routing internalized cargos and recycling vesicles away from lytic compartments, lysosomes and autophagosomes [51]. To explore this possibility, we examined the extent to which internalized hA co-localizes with Golgi complex by confocal microscopy. Pancreatic cells were exposed to unlabelled hA, and then co-stained with Golgi marker, GM130 and hA antibody. However, very low co-localization values between GM130 and hA were detected in both RIN-m5F cells ($R < 0.3$, Figure 3) and human islets (results not shown), refuting a major involvement of Golgi complex in the turnover of internalized hA in these two cell types. This conclusion is consistent with our biochemical results showing a minor pool of total hA (2.6%) in organelle fraction (Supplementary Figure S1B), which was enriched in Golgi and mitochondria (Supplementary Figure S1A).

hA interacts with 20S proteasome complex, but not aggresomes, in the nucleus of pancreatic cells

We extended our studies by investigating whether internalized hA, in addition to mitochondria and lysosomes, interacts with aggresomes in cultured pancreatic RIN-m5F and human islet cells. Aggresomes, a protein complex consisting of proteasome, Ub, heat shock proteins (HSP) and γ -tubulin, serve as an alternative degrading/sequestering centre to lysosomes for many misfolded and potentially toxic cytosolic proteins [52–54]. To

determine if the cytosolic pool of internalized hA is targeted to aggresomes for degradation, immunoconfocal approach was again employed. A low co-localization was observed between hA and HSP70 or HSP90 in RIN-m5F cells ($R < 0.3$, Figure 4) and human islets (results not shown), whereas a high co-localization of the peptide was detected upon co-staining with antibody against Ub (P4D1) in RIN-m5F β -cells ($R > 0.6$, Figure 4) and human islets (results not shown). Aggresomes are usually formed around the microtubule organizing centre (MTOC) in the cells, which requires the presence of polymerized γ -tubulin [55]. Similar to HSP70 and HSP90, very low co-localization values between hA monomers and γ -tubulin were obtained (Figure 4, $R < 0.3$) suggesting that internalized hA is not associated with the MTOC. Immunofluorescence confocal microscopy revealed that despite a significant disruption of the microtubule network by the tubulin depolarization agent colchicine (hA + Colch, Figure 4) hA internalization was not affected, demonstrating that tubulin polymerization is not required for hA monomer uptake and trafficking.

Although the majority of hA did not associate with tubulin or HSP70/90, the internalized peptide showed a significant co-localization with the α -4 subunit of the 20S proteasome complex in the nucleus, and to a lesser extent, in the cytosol of RIN-m5F β -cells and human islets ($R > 0.6$, Figure 5A), suggesting their possible interaction in these cells. The site and nature of interactions between hA and 20S proteasome complex was further analysed by immunoprecipitation approach. Typically, cells were exposed to preaggregated hA for 24 h, washed and cytosolic and nuclear fractions were prepared. The two fractions then were immunoprecipitated using hA specific antibody [29] and immunoblotted with an antibody against the α -4 subunit of the 20S proteasome complex (Figure 5B). In agreement with colocalization studies (Figure 5A), 20S α 4 proteasome subunit was detected in the immunoprecipitated nuclear (Figure 5B) but not cytosolic fraction (results not shown), indicating that hA forms a tight complex with the 20S proteasome catalytic subunit in the nucleus of hA treated RIN-m5F cells (Figure 5B). Interestingly, rpn8 an important regulatory subunit of the proteasome 19S complex co-precipitated with hA (Figure 5C) and 20S α 4 subunit (Figure 5B). Presence of both regulatory (lid) and catalytic (core) subunits in hA-immunoprecipitated samples (Figures 5B and 5C) suggests formation of a hA/26S proteasome complex in pancreatic β -cells.

Proteasome is a multi-subunit proteolytic complex that consists of a 20S α/β catalytic subunits and two 19S lid subunits [56]. Lids regulate binding of cargo to the catalytic 20S core subunit, promoting its unfolding and degradation in an ATP and Ub dependent manner [56]. Proteasomes, besides degrading soluble cytosolic and nuclear proteins, also cleaves misfolded and aggregation prone proteins to prevent their intracellular accumulation and toxicity [35,36,57]. In some cases proteasome mediated degradation of certain proteins does not require a ubiquitination step [58]. Rather, a direct association between 20S and unfolded proteins has been shown [58,59]. To explore whether ubiquitination and aggregation are required for hA interactions with 20S proteasome, *in vitro* pull down studies (Figure 5D) were performed in the absence of the main components of the cellular Ub machinery, Ub and Ub ligases [60,61]. To examine the role of aggregation in hA-proteasome interactions, *in vitro* IP (immunoprecipitation) studies using synthetic hA and 20S purified complex were performed at low temperature ($+ 4^{\circ}\text{C}$), a condition that efficiently abolishes hA oligomerization (Supplementary Figure S2B) and aggregation (Supplementary Figure S2C).

Our *in vitro* immunoprecipitation studies revealed that hA interacts with the integral component of the 20S catalytic core, 20S β 1 (Figure 5D), in confirmation of hA/20S interactions in intact cells (Figure 5B), suggesting that ubiquitination or aggregation of hA are not absolute requirements for its interaction with 20S proteasome. However, this result does not exclude the possibility that a portion of the internalized hA interacts with proteasomes via a Ub dependent step. In accordance with a recent study showing ubiquitination of internally expressed hA [23], our IP studies reveal physical interactions between hA and 19Srp8 subunit in β -cells, suggesting that ubiquitination of hA may indeed play a role in their mutual interactions. Taken together, these results suggest that hA interacts with the catalytic and regulatory subunits of the proteasome complex in the pancreatic cells, and that aggresomes do not serve as major hubs for turnover of internalized hA in pancreatic cells, analogous to aggresome-independent β -amyloid clearance in neuronal cells [62].

Proteasomes, but not lysosomes, mediate turnover of internalized hA in pancreatic cells

Because proteasomes and lysosomes were previously implicated in the degradation of other amyloid proteins [35,36,63], we wanted to examine their role in the turnover (clearance) of internalized hA in pancreatic cells, as suggested from our confocal and immunoprecipitation studies (Figures 3 and 5). If lysosomes and proteasomes are involved in hA degradation, then inhibition of their proteolytic function should enhance amylin accumulation in cells. To test this idea, cells were incubated with hA for 24 h, in the absence (control) or presence of selective lysosomal inhibitor, PepA or proteasomal inhibitor Lac and nuclear and cytosolic hA accumulation in response to these treatments was determined by amylin ELISA (Figure 6). As shown previously (Figures 1A and 1B), addition of hA led to a significant accumulation of hA in the nucleus after 24 h (Figure 6A) and to a much smaller extent in the cytosol (Figure 6B). As suspected and compared with controls, a notable (~70%) increase in nuclear (Figure 6A), and smaller (~20%) but significant cytosolic hA accumulation in response to Lac was detected (Figure 6B). In contrast, no significant change in hA content in response to PepA treatment was observed in either compartment (Figures 6A and 6B). Our results show that RIN-m5F cells express endogenous rA in small quantities (Figure 6B, control) consistent with previous reports [64]. We then analysed whether proteasome also regulates homeostatic balance of rA. As demonstrated for hA, Lac but not PepA significantly augmented endogenous rA levels in cytosol of hA lacking cells as compared with control cells (Figure 6B), further confirming the important and more general role of proteasomes in the clearance of different amylin isoforms. Together, these findings suggest that 20S proteasome complex in the nucleus, and to a lesser extent cytosolic proteasome complex function as major hubs for turnover (degradation) of internalized hA and endogenous rA in pancreatic cells respectively. Interestingly, the major contribution of lysosomes to the degradation of overexpressed pre-pro hA was previously demonstrated [50], suggesting that lysosomes could serve as alternative proteolytic centres to proteasomes for hA degradation in pancreatic cells.

Human and rat amylin inhibit proteasome activity *in vitro* and in pancreatic cells

Our immunoprecipitation results confirmed a physical interaction between hA and 20S catalytic subunits both *in vitro* and in pancreatic cells (Figures 5B–5D), which may affect proteasome function. The 20S proteasome proteolytic assay revealed that following 24 h of

the peptide's exposure, both amyloidogenic human and non-amyloidogenic rA significantly diminished the proteolytic activity of 20S proteasome to a similar extent, in RIN-m5F β -cells ($53 \pm 2\%$ hA and $44 \pm 1\%$ rA with respect to the control, Figure 7A). Equivalent inhibitory effect of hA and rA on 20S proteolytic activity was also observed in human islets (Figure 7B). These results are in partial agreement with a previous study in which hA and rA showed a larger and smaller inhibitory effect on proteasome activity respectively [65]. Proteasomal activity was also suppressed by addition of Lac, a highly selective and irreversible proteasome inhibitor [66] in both cell types (Figures 7A and 7B). However, co-incubation of cells with hA and Lac did not produce a further decrease in proteasome activity in pancreatic cells as compared with their solitary applications. This result suggests that hA and Lac inhibit cellular proteasome's peptidase activity (Figure 7) through a common mechanism which may possibly involve direct binding of hA to its 20S catalytic domain (Figure 5), leading to its inactivation, thus mimicking the inhibitory mechanism of action of Lac [66]. This hypothetical mechanism was further verified using reconstituted *in vitro* approach. The proteolytic function of purified 20S complex was measured in real time (Figure 8A) and plotted as a function of increasing concentrations of peptides or Lac (Figures 8B–8D). Synthetic hA efficiently competed with 20S specific fluorogenic substrate for binding to the complex as evident by a marked loss in fluorescent signal over time (Figure 8A), thus confirming the IP studies and formation of hA/20S complex (Figures 5B and 5D). These *in vitro* studies revealed that peptides interact more avidly with 20S proteasome complex ($IC_{50} \sim 100$ nM, Figures 8B and 8C) as compared with Lac ($IC_{50} \sim 1$ μ M, Figure 8D), in agreement with recent complementary study [67]. Thus, amylin directly interacts with the 20S complex and efficiently modulates its proteolytic activity even in the absence of ubiquitination.

Lactacystin but not amylin induces protein stress in pancreatic cells

A previous study has shown that hA, at its cytotoxic (μ M) concentrations, causes a broad increase in ubiquitinated protein levels in pancreatic cells, suggesting general protein stress as a plausible cause for hA-induced cytotoxicity [65]. In order to test whether a similar mechanism accounts for hA toxicity in our model system, we analysed by Western blot the effect of hA, Lac or both on the levels of ubiquitinated proteins, indicative of protein stress (Figures 9A and 9B) and on cell viability (Figures 10A and 10B). In agreement with previous studies, hA is cytotoxic in the μ M range as suggested by MTT toxicity assay (Figure 2D, Figures 10A and 10B). However, at this cytotoxic concentration, hA had a negligible effect on protein ubiquitination as compared with 10 μ M Lac, which alone markedly increased levels of ubiquitinated proteins in RIN-m5F cells (by 8-fold) indicating severe protein stress (Figures 9A and 9B). It is noteworthy that although 1 μ M Lac was of limited or no cytotoxicity (Figures 10A and 10B), it still induced higher Ub levels (~2.5-fold) and thus protein stress as compared with hA or rA (Figures 9A and 9B), demonstrating a threshold for resisting protein stress in these cells.

To further clarify causality between hA-induced protein stress and hA evoked β -cell death, the toxic potential of hA (Figures 10A and 10B) was assessed under conditions of both mild and severe protein stress induced by 1 or 10 μ M Lac respectively (Figures 9A and 9B). The lower (1 μ M) Lac concentration was used to avoid a direct cytotoxic effect of Lac on cell

viability as seen with higher (5 and 10 μM) Lac (Figures 10A and 10B). Lac (1 μM) potentiated hA toxicity as indicated by a significant decrease in mitochondrial metabolic activity (Figure 10A) and concomitant increase in levels of cleaved PARP in the nucleus, a known stress marker [68] as compared with hA alone (Supplementary Figure S4). Western blot analysis confirmed an increase in hA-evoked PARP and caspase-3 cleavage in cells concurrently exposed to 1 μM Lac, as compared with Lac-lacking cells (Figure 10B). A small potentiation by Lac was also observed at higher 5 and 10 μM inhibitor concentrations (Figures 10A and 10B). In line with this gradual stimulatory effect on hA toxicity, Lac also enhanced intracellular hA accumulation in a dose dependent manner (Figure 10C). Although 1 μM Lac significantly elevated hA levels (Figure 10C) and toxicity (Figures 10A and 10B and Supplementary Figure S4), at this concentration, Lac was ineffective in further increasing Ub signal in hA-treated cells (Figures 9A and 9B), thus denoting a major protein stress involvement in these cells. This stimulatory effect of Lac on hA-evoked ubiquitination and vice-versa could be expected if hA toxicity stems from its ability to elicit general protein stress in cells. In contrast, general protein stress induced toxicity in these cells was readily evoked by addition of 5 and 10 μM Lac (Figure 2E, Figures 10A and 10B).

DISCUSSION

Previous studies have demonstrated a causal connection between hA oligomerization/aggregation and toxicity in pancreatic cells and have provided mechanism for its toxicity [1,19–29,31]. Yet, studies detailing the mechanisms and cellular factors protecting these cells from hA insult are sparse. Few studies point to autophagosomes and small proteolytic proteins, insulin degrading enzymes and neprilysin, as gatekeepers of hA turnover and toxicity in pancreatic cells [23,33,34]. However the role of the cells' two main proteolytic centres, proteasomes and lysosomes, in processing and detoxification of exogenous hA was not explored previously, and hence was the main goal of the present study. Our confocal microscopy and biochemical studies independently confirmed the idea that 20S proteasome complex in the nucleus and cytosol, and to a lesser extent, lysosomes participate in the sequestration and degradation of internalized hA, both in RIN-m5F cells and human islets (Figure 10D). Proteasome also degraded native (rat) amylin in RIN-m5F cells. In accordance with these findings, nuclear fraction was enriched in hA and 20S, and IP studies confirmed interactions between hA and 20S $\alpha 4$ subunit in the nucleus. IP studies indicate formation of 20S $\alpha 4/\beta 1$ /hA heterocomplex in these cells which also contained a lid subunit of the fully assembled 26S proteasome complex. Biochemical and viability studies revealed that proteasomal inhibitor Lac significantly increased intracellular hA accumulation and toxicity, further implicating 20S proteasome as a centre for amylin clearance and detoxification in pancreatic cells.

In accordance with a previous study by Casas et al. [65], we show a decrease in 20S proteasome activity in RIN-m5F cells in response to hA treatment. However, non-amyloidogenic rA was equally potent to hA in inhibiting proteasomal activity and evoking protein stress, suggesting that hA aggregation is not a requirement for hA-20S proteasome interactions, at least in our model system, as discussed below. CoIP studies performed under experimental conditions that prevent or interfere with proteins' aggregation and ubiquitination, such as low temperature and absence of Ub and ATP, revealed that hA still

interacts with 20S core complex, suggesting that neither ubiquitination of hA nor its aggregation is required for its interactions with catalytic 20S proteolytic subunit. Indeed, several reports, mostly for intrinsically disordered proteins (IDPs) such as p53 demonstrate a direct interaction of non-ubiquitinated proteins with 20S catalytic core, bypassing the 19S mediated recognition step [58,59,69]. Interestingly, hA belongs to the family of IDPs [1,27,38], suggesting a common interaction and degradation mechanism by 20S proteasome for the two proteins. In line with this idea, it is estimated that up to 20% of the total cellular protein pool is directly degraded by 20S catalytic subunit but not 26S complex at their unstructured (disordered) domains [70]. Because ATP-dependent unfolding and ubiquitination steps are dispensable for 20S proteasome mediated degradation of IDPs [58,59,69,70], the intrinsic property of hA to interact with the 20S catalytic particle in a Ub independent manner *in vitro* (Figures 5D and 8) and in cells (Figure 5B) is understandable. As stated above, this finding does not rule out the possibility of hA degradation by proteasome via a Ub dependent mechanism as both mature and pre-pro form of hA have at least one lysine in their protein core. Indeed, intracellular pre-pro hA form was shown to be ubiquitinated [23], and our IP studies revealed complex formation between hA and 19S $\text{rpn}8$ (lid) subunit in β -cells. Because lids regulate binding of cargos to catalytic 20S core subunits in Ub-dependent manner [56], this suggests that ubiquitination of hA may indeed play a role in their mutual interactions.

In addition to proteasomes, other proteolytic centres, such as autophagosomes and lysosomes, were shown to be involved in the uptake and clearance of brain amyloids, β -amyloid [71] and α -synuclein, in neural cells [35,72]. Autolysosomes have also been implicated in degradation of overexpressed hA in pancreatic β -cells [23,31]. We, therefore, tested the hypothesis that lysosomes may also regulate degradation and turnover of extracellular hA following its internalization in pancreatic β -cells. Unexpectedly, lysosomal inhibition did not cause a significant accumulation of internalized hA in either cell type tested. This result is logical given the fact that the majority of hA (90%) in our studies was targeted to the nucleus and not lysosomes. Previous studies have reported that mitochondria are targets of amyloid proteins [47,48,73]. For instance, brain β -amyloid and pancreatic hA share a common toxicity pathway via mitochondrial dysfunction, oxidative stress and apoptosis [47]. Our biochemical and immunoconfocal studies reveal that mitochondria sequester small amounts (<3%) of total internalized hA in both RIN-m5F cells and human islets. In contrast with our cells, in INS-1 cells, mitochondria was the predominant site of hA accumulation [28], reflecting a somewhat divergent pathway of hA trafficking between different cell types. In addition to different cellular backgrounds, different methods of hA preparation, non-labelled (our study) compared with fluorescently labelled hA [28], may also account for observed differences in hA trafficking. Our study and recent report also rule out the involvement of biosynthetic compartments like Golgi complex and ER in turnover of external hA in these cells, as these organelles were essentially free of internalized hA [28].

Our functional and co-localization studies further indicate that hA, following its internalization in cells, forms a protein complex with 20S proteasome in the nucleus, which was devoid of other aggresome components, HSP70, HSP90 and tubulin, thus mirroring the trafficking pathway of β -amyloid peptide in neurons [62]. Aggresomes are the cell's protein depots formed around the microtubule organizing centre consisting of intermediate filament

protein vimentin, tubulin, 20S proteasome subunits, Ub and HSPs. Such aggregates start forming inside the cells, when the rate of synthesis of aggregated/misfolded proteins exceeds the rate of its proteolytic degradation [74]. However, our study and previous reports suggest that aggresomes do not play a significant role in turnover of internalized hA or overexpressed β -amyloid [62]. A previous report showed that hA evoked a decline in proteasome activity and subsequent accumulation of ubiquitinated proteins, which correlated with enhanced hA toxicity [65]. The authors then proposed a causal link between hA toxicity and dysregulation of the Ub/proteasome pathway by hA [65]. However, the role of Ub/proteasome pathway in hA turnover remained undefined. Our study points to a novel mechanism in which proteasome-mediated hA clearance, but not hA-evoked proteasome malfunction and subsequent protein stress, determines hA's toxicity in the pancreatic cells. This idea is supported by four lines of evidence: (i) hA, under conditions that did not induce significant protein stress, was cytotoxic to pancreatic cells; (ii) Lac, at a low concentration (1 μ M) that induced moderate protein stress in pancreatic cells, which was significantly higher than those for hA and rA, was only marginally toxic to these cells; (iii) Lac-mediated proteasome inhibition corroborated with increase in intracellular hA levels and hA-evoked toxicity, the latter of which was alleviated in the presence of amyloid oligomeric inhibitor and antioxidant, MB, whereas MB was ineffective in inhibiting protein stress-mediated cytotoxicity evoked by 10 μ M Lac; and (iv) cytotoxic hA and less-toxic rA failed to induce meaningful ubiquitination under these conditions further decoupling hA from general protein stress induced toxicity in these cells. However, our data do not preclude the possibility that moderate but prolonged general protein stress evoked by hA oligomers and/or aggregates may have a long-term deleterious effect on islet cells as previously proposed [65]. Indeed, ER-evoked protein stress is a common trait in β -cell mass loss in diabetic subjects [75,76], and toxic hA oligomers were found to impair ER function in these cells [26,77].

A quantitative analysis of hA trafficking in cells further revealed that impairment of proteasome function diminished hA degradation in the nucleus, and to a lesser extent in the cytosol, in agreement with hA's selective redistribution in these two compartments. In support of nuclear localization and degradation of hA by the 20S proteasome complex, studies reveal that the nucleus can serve as an alternative storage/proteolytic centre to aggresomes, lysosomes and autophagosomes for other amyloid proteins in cells [62,78–83]. It has long been proposed that lysosomes degrade long-lived membranes and bulky hydrophobic proteins that enter the cells via endocytosis whereas proteasomes primarily degrade smaller, soluble cytosolic and nuclear proteins. Our results are in line with recent evidence pointing towards an important regulatory role of proteasomes in the clearance of soluble misfolded amyloid proteins [35,36,63]. Using aggregated and non-aggregated hA preparations, we showed that the molecular form of hA matters not only for its toxicity but also dictates how much and where hA will accumulate in the cell. In our experiments, a pre-aggregation procedure enhanced hA accumulation in the nucleus and its toxicity, linking these two events.

In summary, our results show that, following internalization, the majority of external hA is routed to the nucleus for degradation by the proteasome. The significant increase in hA accumulation and hA-induced β -cell death in proteasome-impaired cells suggests a

cytoprotective role of this proteolytic complex in amylin's clearance and detoxification. Finally, our data indicate that early onset of hA toxicity in pancreatic cells is not mediated by a general protein stress mechanism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

hA	human amylin
HFIP	hexafluoride isopropyl alcohol
HSP	heat shock protein
IAPP	islet amyloid polypeptide
IDE	insulin degrading enzyme
IDP	intrinsically disordered protein
Lac	lactacystin
MB	Methylene Blue
MT	mitotracker
MTOC	microtubule organizing centre
PepA	pepstatin A
rA	rat amylin
T2DM	type 2 diabetes mellitus
Ub	ubiquitin

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Figure 1. Analysis of hA trafficking in pancreatic cells

Cells were treated with hA for 24 h and intracellular redistribution of hA in intact cells and cell fractions determined. **(A)** Analysis of hA accumulation in cytosolic and nuclear fractions revealed by ELISA in RIN-m5F cells. **(B)** Redistribution of internalized hA between the nucleus and cytosol examined by ELISA. Note accumulation of hA in the nucleus and to a lesser extent in cytosol following its uptake in RIN-m5F cells **(A)** and human islets **(B)**. Significance established at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$, $n = 6$, Student's *t* test. **(C)** Confocal microscopy analysis of amylin localization in pancreatic cells. Nuclear marker DRAQ5 colocalizes with hA in RIN-m5F (top panel) and human islets (bottom panel) as indicated by arrows in merged images. Bars, 10 μm .

Figure 2. Temporal analysis of intracellular accumulation and toxicity of hA
Dynamics of hA internalization in RIN-m5F cells was examined by ELISA (A and B) or confocal microscopy (C). Bar, 10 μ m (D) MTT stress assay on cells treated with preaggregated or monomeric hA in the absence or presence of oligomeric inhibitor MB is depicted. (E) Effect of protein stress inducer Lac (10 μ M) on mitochondrial activity in the presence or absence of MB is depicted. Significance was established at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, $n = 6$, ANOVA followed by Tukey's post hoc comparison test.

Figure 3. Confocal microscopy analysis of hA accumulation in cellular organelles in pancreatic cells

hA was incubated with cells for 24 h, cells fixed and its trafficking and association with cellular organelles was analysed by indirect immunocytochemistry. hA interaction or lack of it with lysosome marker LAMP1, mitochondrial marker MT and Golgi marker GM130 are shown. Representative cells in which hA accumulates in LAMP2 positive perinuclear compartments are indicated by arrows. Bars, 10 μ m.

Figure 4. Localization of aggresomes and internalized hA in pancreatic cells by confocal microscopy

hA was incubated with cells for 24 h and its interactions with aggresomal components, HSP70/90, Ub (P4D1) or tubulin was assessed by immunocytochemistry. Representative cells exhibiting high colocalization between hA and P4D1 are depicted by arrows. To assess the effect of tubulin dynamics on hA trafficking, cells were treated with tubulin depolarization agent colchicine (Colch), and hA and tubulin accumulation and location in cells was also analysed by immunoconfocal microscopy. Note disruption of tubulin network in cells treated with colchicine, which however does not prevent hA internalization. Bars, 10 μ m.

Figure 5. hA and proteasomes co-localize and interact *in vitro* and in pancreatic cells hA was incubated with RIN-m5F cells and human islets for 24 h and its interaction with 20S proteasome was assessed by confocal microscopy and immunoprecipitation. **(A)** hA colocalizes (arrows) with 20S proteasome in the nucleus of RIN-m5F cells (top panel) and human islets (bottom panel) as confirmed by indirect immunocytochemistry. Bars, 10 μ m. **(B and C)** hA interacts with the catalytic and lid components of 26S proteasome complex in RINm5F cells. hA was pulled down using hA specific antibody from the nuclear fraction **(B)** or whole cell extract **(C)** of hA-treated RIN-m5F cells and immunoblotted with antibodies against 20S α 4 subunit **(B)** and 19S α 4 subunit **(C)** of the 26S proteasome complex. **(D)** hA interacts with 20S β 1 *in vitro* to form a heterocomplex. Synthetic hA and purified 20S complex were co-incubated and immunoprecipitated using anti-hA antibody as bait. Anti-20S β 1 antibody was used to confirm pull down of hA/20S immunocomplex. Significance was established at * $P < 0.05$, ** $P < 0.01$, $n = 3$, ANOVA followed by Tukey's post hoc comparison test **(B and D, histograms)**.

Figure 6. Nuclear accumulation of hA is upregulated in the presence of proteasome inhibitor Lac hA was incubated with cells in the presence or absence of Lac for 24 h. The extent of amylin accumulation in the presence and absence of Lac (1 μ M) or PepA (1 μ M) in (A) nucleus and (B) cytosol is revealed by ELISA. Significance was established at *** $P < 0.001$, $n = 3-6$, ANOVA followed by Tukey's post hoc comparison test.

Figure 7. Aggregation-independent modulation of 20S proteolytic activity in pancreatic cells by hA

Cells were treated with human or rA for 24 h in the presence or absence of proteasomal inhibitor Lac (1 or 10 μ M) and 20S proteolytic activity was determined in (A) RIN m5F cells and (B) human islets. Significance was established at *** $p < 0.001$, $n = 3-6$, ANOVA followed by Tukey's post hoc comparison test.

Figure 8. hA directly interacts with 20S complex and modulates its function in a Ub independent manner *in vitro*

The proteolytic function of purified 20S complex was measured in real time (A) and plotted as a function of increasing concentrations of (B) hA (C) rA or (D) Lac.

Figure 9. Lac but not amylin induces protein stress in pancreatic cells

RINm5F cells were treated with corresponding concentrations of hA, rA or Lac for 24 h following which cellular Ub levels reflecting levels of protein stress were detected using Western blot analysis (A). Relative protein Ub levels are shown (B).

Figure 10. Analysis of hA cytotoxicity in response to proteasomal inhibition

Dose dependent effect of Lac (1–10 μ M) on hA toxicity was analysed by (A) MTT stress assay and (B) PARP/Caspase 3 cleavage assay. (C) The effect of proteasome inhibition evoked by Lac (1 or 10 μ M) on hA accumulation in RIN-m5F cells as revealed by ELISA. Significance was established at $*P < 0.05$ and $***p < 0.001$, $n = 3-6$, ANOVA followed by Tukey's post hoc test, hA compared with Lac treatments. (D) A schematic representation of 20S proteasome mediated degradation and detoxification of hA in pancreatic cells is depicted.