Clustering and Internalization of Toxic Amylin Oligomers in Pancreatic Cells Requires Plasma Membrane Cholesterol

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*Running Title: Cholesterol mediates clearance of toxic amylin oligomers

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Background: Amylin oligomers are implicated in the pathology of diabetes.

Results: Plasma membrane (PM) cholesterol stimulates clustering and uptake of toxic amylin oligomers in pancreatic cells.

Conclusion: Cholesterol prevents accumulation of toxic amylin oligomers on the cell PM through a lipid-raft-like uptake endocytotic mechanism.

Significance: Impaired amylin oligomer clearance in islet cells with disturbed cholesterol homeostasis may contribute to β-cell mass loss in diabetics.

SUMMARY

Confocal microscopy reveals an increased nucleation of amylin oligomers across the plasma membrane in cholesterol-depleted cells, with a 2-fold increase in cell surface coverage and a 3-fold increase in their number on the PM. Biochemical studies confirm accumulation of amylin oligomers in the medium following depletion of PM cholesterol. Replenishment of PM cholesterol from intracellular cholesterol stores, or by addition of water soluble cholesterol restores amylin oligomer clustering at the PM and internalization, which consequently diminishes cell surface coverage and toxicity of amylin oligomers. In contrast to oligomers, amylin monomers followed clathrin-dependent endocytosis, which is not sensitive to cholesterol depletion. Our studies identify an actin-mediated and cholesterol-dependent mechanism for selective uptake and clearance of amylin oligomers, impairment of which greatly potentiates amylin toxicity.

Human islet amyloid polypeptide (hIAPP) or amylin is a 37-aa peptide hormone produced and co-secreted with insulin by pancreatic β-cells. Amylin has a broad range of both physiological and pathological effects on cells, ranging from the regulation of insulin release to β-cell apoptosis (1-3). The presence of amylin-derived amyloid deposits within the islet of Langerhans is a pathological hallmark of type-2 Diabetes Mellitus (TTDM) (4-6). The important role of amylin in the progression of the TTDM is underscored by the positive relationship between amylin extracellular deposition and the clinical severity of diabetes revealed both in humans and in rodent models (7-
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11) Interestingly, reports from several laboratories suggest that soluble amylin oligomers rather than mature insoluble fibrils are the main cytotoxic species (6, 12-14). However, studies show that amylin aggregation may also contribute to the pathogenesis of TTDM (2, 15, 16-18). Pathological mechanisms may involve amylin oligomers and small aggregates formed intracellularly, extracellularly or both (9, 13, 16). Although studies using islets and clonal β-cell lines suggest that amylin induces apoptotic rather than necrotic cell death (2, 3), currently little is known about the cellular mechanisms that protect β-cells from amylin toxicity. Direct contact of amylin aggregates with β-cell membranes is required to elicit apoptosis (2, 3, 19). Amylin-evoked membrane destabilization and cation channel formation in cell membranes are proposed as two cytotoxic mechanisms (17, 20-25). Other suggested mechanisms include an endoplasmic reticulum stress response (26), activations of stress-activated kinases (3, 27) and induction of reactive oxidative stress species (ROS) or radicals (28, 29).

Several laboratories including ours have used high resolution imaging approaches to investigate amylin oligomerization and fibrilogenesis at the nm-level (30-35). These studies demonstrated two distinct phases in amyloid formation on solid surfaces: oligomeric growth followed by fibril elongation (31, 33). Ultrastructural studies frequently reveal physical associations of amylin aggregates with pancreatic β-cell plasma membranes (PM) and model membranes (36, 37). In spite of this, the role of membrane composition in amylin assembly and aggregation at the membrane interfaces has not been investigated until very recently (33-35, 38, 39). The N-terminal region and His18 play crucial roles in the self-assembly and interactions of human amylin with membranes (24, 40). Concomitant with these structural studies, biochemical studies identify membranes as a potent determinant of amylin aggregation in vitro (33, 38, 41-43). These studies reveal that amylin undergoes facilitated aggregation and conformational change in the presence of membranes composed of anionic lipids such as phosphatidylserine (PS). Cholesterol inhibits phospholipid catalysis of amylin aggregation in solution and on planar membranes (33, 34). Collectively, these studies indicate that cholesterol is a key factor in regulating amylin polymerization and deposition on planar membranes, acting in opposition to PS. Incorporation of cholesterol in anionic and neutral membranes has dual effects on amylin aggregation: it decreases overall amylin accumulation while it facilitates formation of dense 200-500 nm protein clusters on planar membranes (34). However, the question as to whether cholesterol also affects binding and aggregation of human amylin on native β-cell plasma membranes remains open. Based on the intrinsic property of cholesterol to regulate amylin oligomerization and aggregation on model membranes (34), it is reasonable to expect that PM cholesterol may also influence amylin interactions with the native β-cell PM.

To test this idea, in the current study, we investigated amylin binding and internalization pathways in rat insulinoma and human islet cells with depleted, normal and enriched PM cholesterol levels, and explored the extent to which PM cholesterol contributes to amylin uptake and amylin-mediated cell death. Our studies demonstrate that PM cholesterol is required for uptake and clearance of toxic amylin oligomers but not monomers from the cell plasma membrane and that cell susceptibility to amylin insult is inversely related to PM cholesterol levels.

EXPERIMENTAL PROCEDURES

Cell Cultures

Rat insulinoma (RIN-m5F) β-cells (ATCC, Gaithersburg, MD) were cultured in RPMI 1640 medium (ATCC) supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified incubator with 5% CO2. Cells were passage bi-weekly. Passages 10-50 were used for all the experiments. Rat insulinoma cells were plated at density 50,000 cells / well and cultured for 24 h prior to experiments with amylin. Human islets from non-diabetics with >90% purity and viability were obtained through the NIH Pilot Program for Human Islet Research. Upon arrival, islets were further purified by handpicking (≥ 98% based on dithizone staining), gently dissociated in TrypLe cell dissociation medium (Invitrogen) using a 1ml-glass pipette and plated on 96-well glass bottom SensoPlates pre-coated with poly-D-Lysine for confocal studies...
or on plastic 96-well cell culture plates (Greiner Bio-One) for biochemical and toxicity studies at a density ~100 islet equivalents (IEQ) per well. Dissociated islets were cultured in CMRL medium (ATCC) supplemented with 10% (v/v) fetal bovine serum for 24 h prior to experiments.

**Preparation of amylin**

Lyophilized C-terminal amidated synthetic human amylin (American Peptide Co., Sunnyvale, CA) was used for preparations of amylin stock solution using hexafluoride isopropanol (HFIP) as a solvent. The appropriate amount of the peptide was solubilized in HFIP overnight in order to completely dissolve the amylin. This approach efficiently removes any preformed amylin aggregates (33). A 500 μM amylin stock was prepared, and prior to experiments HFIP was evaporated under a stream of dry nitrogen gas to remove the organic solvent, which is toxic to cells. Human amylin was then reconstituted using freshly prepared culture medium or phosphate buffer saline (PBS) at a final conc. of 0.5-10 μM.

**Dot-Blot and Western Blot Assays**

For the dot-blot assay, samples (4 μl) of 10 μM amylin were spotted at the regular time intervals on the nitrocellulose membranes and the extent of oligomers formation in the medium determined by probing blots with conformation-specific, anti-oligomeric A11 antibody from Invitrogen Co. (AHB0052) or Millipore Co. (AB9234) (1:1000 dilution). Blots were developed using the enhanced chemiluminescence (ECL) kit (Thermo Fischer Scientific, Rockford, IL) and the intensity of spots determined using Kodak Gel Logic 1500 Imaging system and Kodak Molecular Imaging Software ver. 4.0 (Kodak Co., Rochester, NY). Both antibodies revealed similar time-courses of amylin oligomerization, and therefore we used the Millipore antibody in all experiments for consistency.

Amylin oligomerization and accumulation in solution and in the culture medium were quantified by Western blot analysis. Cells were exposed to amylin in the presence or absence of cholesterol-modulating agents or inhibitors as described for the toxicity studies. Samples (20μl of cell culture medium) were collected and separated by a 10-20 % Tris-Tricine SDS-PAGE (to resolve monomers and dimers) or by a 4-20% Tris-Glycine SDS-PAGE (to resolve oligomers). Resolved proteins were blotted on nitrocellulose membranes and incubated with human amylin antibody from Santa Cruz (sc-20936, 1:500) or A11 anti-oligomer antibody (Millipore, 1:1000) followed by horseradish peroxidase conjugated secondary antibody (Fischer Scientific). Blots were developed using ECL and documented using the Kodak imaging station and software. Data are expressed as % change from controls.

**Cell Toxicity Assays**

Actual in vivo concentrations of amylin required for formation of toxic oligomers and aggregates in the islet of Langerhans are yet to be determined. Hence, we used low μM amylin concentrations commonly adopted across the field to generate amylin oligomers and aggregates required to study their toxicity in cells (3, 12, 14, 17-19, 25, 27, 28, 35, 44). Rat insulinoma and human islet cells were incubated with human amylin (0.5-10 μM) for 24 hour, in the presence and absence of cholesterol-modulating agents lovastatin, methyl-β-cyclodextrin, filipin and/or water soluble cholesterol (Sigma, St. Louis, MO). Amylin was also incubated with the specific oligomer inhibitor methylene blue (MB). The 3-(4,5-Dimethylthiazol-2-)-2,5-Diphenyl tetrazolium Bromide (MTT) reduction assay, lactate dehydrogenase (LDH) release assay and caspase-3/ Annexin V apoptotic assays were used to determine effect of amylin and treatments on cell’s viability (see supplementary information).

**Quantification of Total and Plasma Membrane Cholesterol Content**

Changes in relative PM cholesterol levels were measured by staining the PM with the specific, fluorescent cholesterol binding drug filipin according to a published protocol (45, 46). Following treatments, the cultured rat insulinoma and human islet cells were washed twice in ice cold PBS and fixed in 4% paraformaldehyde (PFA) for 30 min. on ice. The cells were then washed with ice cold PBS (2x) and stained with 100 μg/ml of fillipin (Sigma) in PBS for 1 hr at RT in the dark. The cells were washed to remove non-bound fillipin, and solubilized in DMSO:PBS (1:4 vol/vol). Samples were collected and transferred to black 96 well fluorescence microplates. The
fluorescence intensities from each well, indicative of the PM cholesterol levels, were read in a Spectramax M5 microplate reader set at 338/480nm (ex/em).

Total cholesterol was measured by the Amplex Red cholesterol quantification kit (Invitrogen Life Science Tech., Carlsbad, CA). Cholesterol was extracted from the cells by addition of chloroform: methanol (2:1 vol/vol) mixture, followed by ice cold PBS. The solution was transferred to microcentrifuge tubes and centrifuged for 5 min at 10,000 g. The top water phase was removed and the lower cholesterol-containing organic solvent phase was evaporated under a stream of nitrogen gas. To this, PBS / 5% Triton x-100 (TX-100) was added and then diluted with 1X Amplex Red reaction buffer. Samples containing the Amplex Red reaction mixture were distributed to the corresponding wells in black fluorescence plates and were allowed to incubate for 1hr at 37° C in dark. The fluorescence was measured at 530 nm / 590 nm (ex/em). Background fluorescence was corrected by subtracting the blanks. Data were expressed as % change from controls (PM cholesterol) or normalized to protein content in the sample and expressed as ng cholesterol per μg of protein (total cholesterol).

Confocal Microscopy
We adopted approach by Saavedra and colleagues to generate and study internalization pathways of toxic amyloid oligomers (47). Human and rat insulinoma cells were first exposed to freshly prepared human amylin (2-10 μM) for the indicated periods of time, washed repeatedly with ice-cold PBS to remove non-bound monomers and oligomers and then incubated with endocytotic markers, cholera toxin (CTX-Alexa555) or transferrin (Trf-Alexa633), for 30 min. on ice or at 37° C. CTX and Trf were purchased from Invitrogen (Carlsbad, CA) and were used at 20 μg/ml and 50 μg/ml final concentrations, respectively. Following incubations with amylin and endocytotic markers, cross-linking of CTX-labeled lipid rafts with anti-CTX antibody (1:200 dilution; Invitrogen) was performed for 15 min. at 4° C. Cells were then washed, fixed with 4% PFA, and incubated in Image iT signal enhancer (Invitrogen) for 30 min. at RT to reduce background. Cells were then incubated in blocking/permeabilization solution (3% BSA, 0.5% normal goat serum, 0.1 % Triton X-100 in PBS) for 30 min. at RT. In some experiments, the permeabilizing agent TX-100 was omitted from the labeling procedure. Membrane-bound and internalized soluble amylin monomers and oligomers were detected by incubating cells with rabbit anti-human amylin antibody (1:200, Santa Cruz) or rabbit anti-oligomer A11 antibody (1:200, Millipore), respectively. Cells were then exposed to goat anti-rabbit Alexa 488-conjugated secondary antibody (1:300, Invitrogen) for 60 min. at RT. Three random fields in each well (treatment) were imaged, with each treatment performed in three to six separate experiments. 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, Thornwood, NY) equipped with oil 63x and 100x (1.4 N.A.) objectives. 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 Pearson colocalization coefficient (R) was determined using the Zeiss colocalization software (release 4.0). The intensity thresholds of the channels were set using the Zeiss crosshair function to avoid an arbitrary background threshold being set. In that way, the background pixels that may contribute to false colocalization were excluded from analysis. Images were also analyzed using 1.37c software version of Image J (NIH, Bethesda, MD). Image J confirmed R values obtained by the Zeiss colocalization software, and therefore all data are reported using the Zeiss software for consistency. Images were assembled using Adobe Photoshop software program (Adobe Systems Co., San Jose, CA). The extent of amylin binding and internalization in rat insulinoma and human islet cells was quantified using Zeiss and NIH Image J single particle analysis software as explained in the supplementary section (image / section analysis).

Statistical analysis
The GraphPad Prism 5 Program was used for plotting and statistical analysis. The unpaired Student’s t test or One-Way ANOVA followed by Newman-Keul post hoc test were used for pairwise comparisons among groups when appropriate with significance established at P<0.05.
RESULTS

Depletion of PM cholesterol enhances amylin toxicity in rat insulinoma cells. We investigated the extent to which variations in PM cholesterol levels affect amylin toxicity in rat insulinoma cells. Cells were incubated with 10 μM human amylin (hA) for 24 h in the presence or absence of the specific cholesterol-depleting drugs methyl-ß-cyclodextrin (BCD), lovostatin (Lov) or filipin (48). Following the treatments, changes in the cell cholesterol content and cell viability were assessed (Fig. 1, Table 1). Pretreatments of cells with the BCD (15 min) and then with the inhibitor of cholesterol biosynthesis, Lov, for 24 h depleted PM and total cholesterol to 63 ± 4 % and 65 ± 9 % of control values, respectively (Table 1). This protocol was used because prolonged (>60 min.) applications of BCD were toxic to rat insulinoma and human islet cells (data not shown), and cells were also able to replenish PM cholesterol in the absence of lovastatin (97 ± 6 % of control values, Table 1). While this combination of BCD/Lov was not toxic to cells during 24 h, it significantly increased amylin toxicity by 21 ± 4% respective to controls (hA) (Fig. 1A). In the absence of Lov, acute depletion of PM cholesterol by BCD (15 min) had little effect (≤ 5%) on the cellular cholesterol levels and amylin toxicity measured at 24 h (Fig. 1A-C, Table 1). However, the total and plasma membrane cholesterol levels following 15 min treatments with BCD were 74 ± 6 and 73 ± 5 % of control values, respectively (Table 1). Thus, without Lov, cells were able to replenish PM and cellular cholesterol levels and consequently to reduce amylin toxicity. Next, we tested the effects of endogenous cholesterol depletion on amylin toxicity by inhibiting cholesterol biosynthesis with lovastatin (Lov). Lov is an inhibitor of 3-hydroxyl-3-methylglutaryl coenzyme A reductase, a crucial enzyme in cholesterol biosynthesis. Cells incubated with Lov had reduced PM (82 ± 8%) and total (78 ± 9%) cholesterol levels as compared to controls (Table 1). These moderate decreases in PM and total cholesterol levels had no significant effect (P=0.6-0.9) on amylin-evoked cell death in our MTT, LDH-release and caspase-3 apoptotic studies (Fig. 1A-C). We further tested effect of cholesterol reloading on amylin toxicity by incubating cells with soluble cholesterol, which readily incorporates into the cell plasma membranes (45, 46). Following exposure to BCD for 15 min., rat insulinoma cells were further incubated with water soluble cholesterol and lovastatin (BCD/Lov/Chol) for 24 h. This treatment replenished PM and total cholesterol levels (Table 1), which in turn reversed the stimulatory effect of BCD/Lov on amylin toxicity in the MTT assay (Fig. 1A). Finally, external loading of PM cholesterol using soluble cholesterol (Chol.) raised PM cholesterol levels by 17 ± 6 % compared to controls (Table 1), which further attenuated amylin toxicity in insulinoma cells (Fig. 1A). These results indicate that PM and to lesser extent endogenous cholesterol protect cells from the deleterious effect of amylin.

It is well established that the MTT reduction assay reflects both reversible and irreversible cellular dysfunction that may or may not lead to cell death. Hence, the modulatory effect of PM cholesterol on amylin toxicity was verified using the LDH release assay (Fig. 1B). The amount of LDH released into the medium was used to quantify the extent of membrane damage evoked by amylin. In cells in which PM cholesterol levels were reduced by BCD/Lov treatments, amylin evoked a significant 56 ± 9% increase in LDH release when compared to control (hA-treated) cells (Fig. 1B). Cell viability was restored by addition of soluble cholesterol that replenished PM and total cholesterol content (Table 1) and consequently attenuated amylin-evoked LDH release into the medium (Fig. 1B). As with MTT assay enrichment of the PM cholesterol pool with soluble cholesterol abolished amylin-evoked LDH leakage in the medium (Fig. 1B). The protective effect of PM cholesterol on amylin toxicity was confirmed with the caspase-3 apoptotic assay (Fig. 1C). In cells co-incubated with BCD/Lov and human amylin there was a significant 58 ± 12 % increase in cytosolic caspase-3 activity as compared to control (hA-treated) cells (Fig. 1C). As demonstrated with the other two toxicity assays (Fig. 1A, B), the stimulatory effect of BCD/Lov on amylin toxicity was reversed by reloading PM cholesterol using water soluble cholesterol (Fig. 1C). Enrichment of the PM cholesterol pool using soluble cholesterol (Table 1) further reduced amylin-induced caspase-3 activation in rat insulinoma cells (Fig. 1C). Analogous to BCD/Lov,
incubation of rat insulinoma cells with the sterol binding drug, fillipin, potentiated amylin-evoked cell death, further demonstrating the preventative effect of PM cholesterol on amylin toxicity (data not shown). The significance of these results is supported by clinical studies showing a comparable 41 % loss in β-cell mass in type-2 diabetics (49), and by the fact that a 20 % decrease in islet β-cell mass is enough to produce insulin deficiency and diabetes in humans (50).

Amylin oligomers are toxic to cells. Accumulated data suggest that small soluble oligomers play an important role in the toxicity of amylin and other amyloid proteins (12-14, 44, 51-53). To determine the identity of toxic molecular species in our experiments, the dot-blot oligomeric assay and dynamic light scattering (DLS) were performed in parallel with the cell viability experiments described above. Human amylin (10 μM) was added to PBS, and the kinetics and the extent of amylin oligomerization in solution were monitored by the dot-blot assay (Fig. 2A). To detect amylin oligomers we used a conformation specific anti-amyloid oligomer A11 antibody that does not react with either monomers or fibrils (44). The half-time for amylin oligomerization was $t_{1/2}=37±9$ min. (Fig. 2A). Amylin oligomerization reached a maximum at 1 h and remained steady thereafter (Fig. 2 A). The specific oligomer inhibitor methylene blue (MB) (51) blocked amylin oligomerization and amylin-induced cell death suggesting a causal link between amylin oligomerization and toxicity in cells (Fig. 2B, C). These results are in agreement with the previously published findings on amylin oligomer toxicity in vitro and in vivo (12-14). Dynamic light scattering (DLS) studies confirmed formation of distinct populations of small and intermediate-sized (IS) oligomers with a particle hydrodynamic radius ($R_H$) of $R_H=2-8$ nm, and larger oligomers of $R_H=60-90$ nm (Suppl. Fig. S1). The peak of amylin oligomer formation revealed by the immuno-detection method (60 min, Fig. 2A) correlated with the formation of these amylin oligomer species in our DLS experiments (60 min, Supp. Fig. S1A). Amylin oligomers were detected by DLS as early as 10 min following amylin addition to buffer (data not shown). DLS confirmed that the same populations of amylin oligomers were still present in solution after 24 h with concomitant appearance of large $R_H > 200$ nm particles, presumably fibrils (Supp. Fig. S1B).

Clustering of amylin oligomers on the cell PM requires cholesterol. The ability of PM cholesterol to inhibit amylin-mediated membrane perturbation and toxicity (Fig. 1) can be in part due to cholesterol’s intrinsic property to diminish amylin oligomerization and aggregation on the membranes as demonstrated for synthetic planar membranes (34). We explored amylin oligomer binding and uptake mechanisms in rat insulinoma cells by confocal microscopy using the oligomer-specific A11 antibody (Fig. 2A, B; Suppl. Fig. S2A) (44, 51). To detect human amylin monomers, we used a human-specific amylin antibody that does not cross-react with the rat isoform (Suppl. Fig. S2B). In addition to human amylin, we used the lipid raft marker, cholera toxin (CTX), and the clathrin endocytotic marker, transferrin (Trf), to determine the specificity of amylin monomer and oligomer binding to the cell PM (Fig. 3-4, Suppl. Fig. S3). Amylin and CTX were sequentially (Fig. 3, 4) or concurrently (Suppl. Fig. S3) incubated with cells for the indicated periods of time, fixed and processed for immunochemical analysis. In experiments in which amylin and CTX were concurrently incubated for 30 min, immuno-confocal microscopy revealed a punctuated staining pattern of CTX and amylin oligomers on the cell PM, exhibiting high spectral overlap (yellow) and a high co-localization coefficient, (R=0.74±0.09) in discrete membrane regions (Suppl. Fig. S3). Changes in the cells’ morphology characterized by the appearances of elongated protrusions were frequently observed in cultures treated with human amylin (Suppl. Fig. S3). Amylin oligomer binding and uptake were particularly noticeable in protrusions. However, amylin oligomers also co-patched with the lipid raft marker CTX in other plasma membrane regions (Suppl. Fig. S3). This suggests that amylin oligomers accumulate at specific microdomains, possibly lipid rafts, on the cell PM prior to their uptake. In order to exclude a possible modulatory effect of CTX on amylin binding, sequential incubations of rat insulinoma cells with amylin and CTX were performed. Following the incubation with amylin, cells were further incubated with CTX for 30 min at 4° C. Amylin oligomers and CTX once more co-localized on the cell PM (Fig. 3A, B). Prolonging...
the incubation period from 30 min. to 24 h allowed amylin oligomers in the microdomains to internalize, as demonstrated by a significant ~50% drop in amylin oligomer/CTX colocalization values (Fig. 3 A, B).

Upon depletion of PM cholesterol with BCD/Lov, a significant decrease in colocalization of amylin oligomers with cholera toxin on the cell PM was observed indicating amylin/CTX particle de-clustering and their dispersions across the cell surface (Fig. 3A, B). In line with this finding, image and single particle analysis revealed that the mean particle area of PM-bound amylin oligomers (Fig. 3A, right boxed panel) in cholesterol-depleted cells (BCD/Lov) decreased significantly as compared to control cells (Fig. 3D). Conversely, the number of amylin oligomer clusters or puncta on the PM of cholesterol-depleted cells increased by 3-fold relative to control cells (Fig. 3C). Consequently, amylin oligomer cell surface coverage increased by ~2-fold in cells with reduced PM cholesterol content as compared to control cells (Fig. 3A, right boxed panel). This inhibitory effect of BCD/Lov on clustering of amylin oligomers on the PM was also observed at earlier time points, 30 min and 3 h (data not shown). These results demonstrate that the seeding (nucleation) capacity of amylin oligomers and their ability to form a dense network of amyloid aggregates on the PM were augmented in cells with impaired cholesterol homeostasis. Clustering of amylin oligomers on the PM was fully restored following replenishment of PM cholesterol (Fig. 3 A-D), indicating that amylin oligomer deposition on the PM is modulated by cholesterol and is reversible.

Binding and clustering of amylin monomers on the cell PM is largely independent of PM cholesterol. In contrast to oligomers (Fig. 3 B), fewer amylin monomers co-localized with the lipid raft marker CTX on the PM during the first 30 min of incubation with cells (Fig. 4 B). However, analogous to the binding of oligomers (control 30 min, Fig. 3A right boxed panel), amylin monomers exhibited a discrete, punctuated staining pattern on the cell PM during that period (control 30 min, Fig. 4A right boxed panel). Interestingly, the degree of colocalization between amylin monomers and CTX also decreased significantly upon cholesterol depletion with BCD/Lov (Fig. 4 B), albeit to much lesser extent than for amylin oligomers (Fig. 3 B). In contrast to oligomers (Fig. 3C, D), the number and the mean particle area of amylin monomer puncta at the cell PM remained almost the same in control and BCD/Lov-treated cells (Fig. 4C, D), indicating that clustering of amylin monomers into microdomains on the cell PM is not affected by PM cholesterol.

Specificity of amylin and CTX binding is further demonstrated in experiments in which transferrin, a marker of clathrin-dependent endocytosis, was used (Fig. 5). Colocalization analysis revealed that neither amylin monomers (bottom panel, Fig. 5) nor oligomers (middle panel, Fig. 5) colocalize with transferrin on the cell PM. Similarly, CTX and Trf were found to bind to distinct regions on the cell PM (top panel, Fig. 5). Collectively, our studies (Fig. 3-5) imply that PM cholesterol reversibly and specifically determines binding and distribution of amylin oligomers but not monomers on the cell PM.

PM cholesterol stimulates amylin oligomer internalization and clearance in rat insulinoma cells. The inhibitory effect of BCD/Lov on clustering and internalization, but not on binding of amylin oligomers to PM (Fig. 3), was frequently observed in cultures (>90% cells) as shown in lower magnification confocal images (Suppl. Fig. S4 B, F). To infer more about the relationship between extracellular accumulation and cholesterol-mediated internalization of amylin oligomers, we studied accumulation of amylin oligomers on the PM in cells with normal and depleted PM cholesterol levels (Fig. 6). In cells incubated with BCD/Lov amylin oligomers preferentially accumulated on the PM as 83±7% of total cellular amylin immunoreactivity was detected at the cell PM after 24 h (Fig. 6 B, D). A significant, concurrent decrease in amylin internalization was observed in cells treated with BCD/Lov but not with Lov alone (Fig. 6 D). In control cells, internalized amylin oligomers accounted for almost half of total immunoreactivity: 51±8% internalized versus 49±7% PM-associated oligomers (Fig. 6 A, D). In accordance with toxicity (Fig. 1) and binding studies (Fig. 3), the replenishment of PM cholesterol fully restored internalization of amylin oligomers in the cells, and that consequently reduced the total accumulation of amylin oligomers on the cell PM (Fig. 6C, D). The section
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analysis confirmed increased deposition of amylin oligomers on the PM in cells treated with BCD/Lov (Fig. 6 A-C, right panels). Further, there was a marked decrease in the number of internalized amylin oligomers upon cholesterol depletion with BCD/Lov (section analysis, Fig. 6B). Endocytic inhibitors, cytochalasin D and colchicine (54), were also effective in preventing internalization of soluble amylin oligomers (Supp. Fig. S4 C, D). Like BCD/Lov, cytochalasin D and colchicine increased accumulation of amylin oligomers on the cell PM (Suppl. Fig. S4F) and enhanced amylin toxicity (data not shown). Labeling of amylin oligomers in the absence of the permeabilizing agent Triton X-100 resulted in less than 3% of the fluorescent signal being detected from the cell interior (Supp. Fig. S4E, F), thus validating our approach to detect PM-associated and internalized amylin oligomers in the same cells.

The modulatory effect of BCD/Lov on extracellular amylin accumulation was further tested in experiments in which TX-100 was omitted from the immunochemical protocol. A permeabilization step is required for the labeling of intracellular but not extracellular amylin oligomers (Suppl. Fig. S4). In experiments in which TX-100 was omitted from the labeling procedure, depletion of PM cholesterol by BCD/Lov stimulated a 34±6% increase in accumulation of amylin oligomers on the PM relative to controls. This result is comparable to the BCD/Lov-induced 31±4% increase in amylin oligomer accumulation on the PM obtained in the presence of TX-100 (Fig. 6D).

The effect of PM cholesterol on amylin clearance was also studied using biochemical methods. Cells were incubated with human amylin for 24 h in the presence or absence of cholesterol-modulating agents. Following incubations, 20 µl aliquots were collected and the oligomer content quantified by Western blot and densitometry (Fig. 7). In agreement with the confocal microscopy data (Fig. 3-6), Western blot analysis demonstrates the accumulation of intermediate- and low-MW oligomers-dimers in the culture medium of cholesterol-depleted cells, and less oligomers upon replenishment of the PM cholesterol with soluble cholesterol (Fig. 7). Formations of toxic intermediate-sized oligomers and dimers have been demonstrated during amylin and β-amyloid aggregation (12, 55, 56), also observed in our biochemical and DLS studies (Fig. 7; Supp. Fig. S1). Interestingly, monomers were not detected in these samples (Fig. 7), demonstrating their efficient removal by cells.

Amylin monomers and oligomers internalize in cells by distinct endocytic mechanisms. Depletion of plasma membrane cholesterol by BCD/Lov did not prevent internalization of amylin monomers (Fig. 8 B, D), and had no significant effect on their binding to the cell PM (Fig. 4 C, D). Thus, in stark contrast to amylin oligomer internalization (Fig. 3, 6), the uptake of amylin monomers, Trf and to large extent CTX were not inhibited by PM cholesterol depletion. Upon cholesterol depletion with BCD/Lov, CTX switched internalization pathways and entered cells apparently by a clathrin-dependent mechanism as demonstrated by the appearance of CTX/Trf-positive endosomes (Suppl. Fig. S5). Amylin monomers and oligomers also exhibit different sensitivities to chlorpromazine, a selective inhibitor of clathrin-dependent endocytosis (57), and to BCD/Lov: internalization of amylin monomers and transferrin was blocked by chlorpromazine (Suppl. Fig. S6) but their uptake was not inhibited by PM cholesterol depletion (Fig. 4, 8). On the other hand, amylin oligomers were not internalized in cholesterol-depleted cells (Fig. 3, 6), whereas chlorpromazine did not block their cellular uptake (Supp. Fig. S6). These results suggest that, after binding and sorting on the PM, amylin monomers and oligomers internalize by clathrin-dependent and clathrin-independent mechanisms, respectively. The latter pathway is sensitive to depletion of PM cholesterol.

PM cholesterol is required for clearance of toxic amylin oligomers in human islet cells. To determine if the cholesterol effect on amylin oligomer uptake is cell specific, we investigated amylin internalization and toxicity in cultured human islet cells in the absence or presence of the cholesterol-modulating agents. As expected, amylin is also toxic to cultured human islet cells albeit with notably higher potency (LD₅₀=2.5 µM) as compared to its toxic effect in rat insulinoma cells (LD₅₀=15 µM). Human amylin (hA, 2 µM) evokes apoptosis in 43 ± 5 % of cultured human islet cells. The combination of BCD (5 mM, 10 min) and lovastatin (0.5 µM 24
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h), reduces PM cholesterol to 67 ± 4% relative to controls (Table 2), which in turn increases amylin toxicity by a 34 ± 5% as compared to cells with normal membrane cholesterol content (Fig. 9A, C). Addition of soluble cholesterol together with BCD/Lov (BCD/Lov/Chol), replenishes cholesterol levels (94±5% relative to controls), which reverses the stimulatory effect of BCD/Lov on amylin toxicity. As demonstrated in rat insulinoma cells, Lov alone had a small (< 10%) stimulatory effect on amylin toxicity in human islet cells that did not reach significance (data not shown). Finally, incubation of human islet cells with soluble cholesterol (50 µg/ml) increases PM cholesterol levels by 14 ± 6% relative to control cells (Table 2), and further attenuates amylin toxicity (Fig. 9A, C). A strong inverse relationship between PM cholesterol levels and amylin toxicity in human islets is obtained (Fig. 9B). The inhibitory effect of PM cholesterol on amylin toxicity in human islets was confirmed with the LDH-release assay (Suppl. Fig. S7). Similarly, Western blot analysis demonstrates extracellular accumulation of low-MW amylin monomers and dimers (left panel, Fig. 9B) and intermediate-sized oligomers (right panel, Fig. 9B) upon PM-cholesterol depletion with BCD/Lov, and their efficient clearance by islet cells upon PM cholesterol reloading with soluble cholesterol (BCD/Lov/Chol). In line with these findings, confocal microscopy studies further reveal the existence of a cholesterol-sensitive clearance mechanism in rat insulinoma and human islet cells that prevents extracellular accumulation of toxic amylin oligomers, impairment of which may contribute to the β-cell loss and islet dysfunction (4). In support of this notion, a recent study shows an impaired clearance but not production of β-amyloid in patients with Alzheimer’s disease (59).

Confocal microscopy studies further reveal that amylin oligomers and CTX bind to the same microdomains on the cell PM, possibly lipid rafts, prior to their internalization. Cholesterol-rich lipid rafts appear to be a major port of entry for amylin oligomers since oligomer internalization is associated with a marked reduction (~50 %) in colocalization between remaining amylin oligomers at the cell PM and the lipid raft marker CTX. This conclusion is further supported by the inhibitory action of the well known lipid raft disrupting agents, BCD and Lov (48), on amylin oligomer internalization. In agreement with the colocalization data, depletion of PM cholesterol in cells by BCD/Lov decreased the size of amylin oligomer clusters, which become more numerous and occupy more of the PM. Specifically, cholesterol reduced amylin oligomer seeding and accumulation on the PM as revealed by a marked ~3-fold drop in the number of amylin oligomers on the PM, and by a comparable ~2-fold decrease in the amylin oligomer surface coverage as compared to cholesterol-depleted membranes. We recently demonstrated the inhibitory effect of cholesterol on seeding and deposition of amylin oligomers and aggregates on synthetic planar membranes (34). Interestingly, following removal of BCD the uptake of amylin oligomers is fully restored within a few hours. This gain of endocytotic function is prevented by addition of choles...
Lov, demonstrating that PM cholesterol levels and amylin endocytosis are restored by newly synthesized cholesterol. In addition to replenishment of the PM cholesterol from an endogenous cholesterol pool, external loading of PM cholesterol using water soluble cholesterol stimulates endocytosis, consistent with the idea of a PM cholesterol-mediated transport process. The inhibitor of cholesterol biosynthesis, Lov, alone had little effect on amylin internalization and toxicity in rat insulinoma and human islet cells. However, its presence is a requirement for stimulatory effect of PM cholesterol depletion by BCD on amylin-mediated cell death. Apparently, cells use the biosynthetic pathway to control PM cholesterol levels, which regulates amylin oligomer internalization and toxicity. Following internalization, amylin and its toxic oligomers are routed to the cell’s acidic compartments, autophagosomes and lysosomes, for degradation (Trikha and Jeremic, unpublished observation). Interestingly, these two proteolytic compartments are also common destinations for intracellularly generated amylin oligomers (60).

When compared to oligomers, amylin monomers and CTX showed lower colocalization at the cell PM. In contrast to oligomers, the binding capacity and uptake of amylin monomers are not significantly affected by cholesterol depletion suggesting less importance of membrane cholesterol in binding and internalization of amylin monomers. Because PM cholesterol is dispensable for clathrin-mediated endocytosis in some cell types (61, 62), and amylin monomers internalize by a clathrin-dependent mechanism, the lack of action of BCD/Lov on binding and internalization of pre-oligomeric amylin in cells is understandable. The results of this study demonstrate that cells can sense and concurrently internalize distinct amylin molecular species, monomers vs. oligomers, using separate endocytic mechanisms as further discussed below. Cargo molecules internalize in cells by clathrin-dependent and clathrin-independent mechanisms. Caveolin-dependent endocytosis is the major clathrin-independent pathway for entry of various cargos in cells (63). It has been previously shown that depletion of PM cholesterol inhibits endocytosis from invaginated caveolae in various cells (63, 64). For example, invaginated caveolae only form when the cholesterol levels are <50% of control values (64). To effectively inhibit internalization of amylin oligomers, PM and total cholesterol levels had to be reduced by 32 % or more in our uptake experiments. By contrast, internalization of amylin monomers and transferrin, a marker of clathrin-dependent endocytosis, is not inhibited by depletion of PM cholesterol. Unlike internalization of amylin monomers and transferrin, amylin oligomer uptake is not sensitive to the clathrin endocytic inhibitor chlorpromazine. However, the inhibitor of actin polymerization, cytochalasin D, blocks internalization of amylin oligomers in uptake experiments. Hence, our results indicate that amylin oligomers internalize via an actin- and cholesterol-mediated, clathrin-independent mechanism(s) such as caveolae, lipid rafts or macropinocytosis. In support of this hypothesis, internalization of β-amyloid peptide by primary neurons is mediated by a clathrin-independent, cholesterol-dependent endocytic mechanism (47).

How does cholesterol regulate binding and deposition of amylin oligomers on the cell’s PM? As amylin is a cationic peptide, it is conceivable that self-assembled amylin oligomers and aggregates bearing positive surface charges are attracted to negatively charged anionic phospholipids such as PS and ganglioside (GM1), which are found to be present in higher concentrations in the lipid rafts (63, 65, 66). We and others have reported that membrane cholesterol mediates clustering of amylin oligomers and aggregates on lipid-raft model membranes, which results in a decrease in the amount of protein deposits (34, 35). Our current studies reveal clustering and accumulation of amylin oligomers together with the lipid raft marker CTX in submicron-sized membrane regions prior to their internalization. The observed heterogeneity in amylin distribution across the PM can be attributed to the well known ability of cholesterol to promote phase separation within the bilayers, crucial for cargo sorting on the PM. An increased binding of amylin fibrils to negatively-charged GM1 and lipid rafts was recently demonstrated in neuroendocrine PC12 cells (67). Other amyloidogenic proteins such as β-amyloid also display higher capacity for binding to GM1 clusters formed both on synthetic and native membranes (66, 68). It is possible that cholesterol, by modulating membrane fluidity and/or membrane curvature (69, 70), stimulates amylin
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Oligomer clustering on the PM and subsequent internalization, as observed in this study. In addition to lipids, protein-protein interactions may also play a role in the binding of amylin to lipid rafts, since direct interactions between lipid raft proteins such as flotillin and β-amyloid in synaptic membranes was demonstrated (71). Another possibility is that amylin directly interacts with cholesterol and/or other lipids in the PM. Amylin, as a monomer, has a strong tendency to insert into phospholipid monolayers (72). This event may set the stage for direct peptide–cholesterol interactions in the membrane core. In any case, amylin segregation into microdomains followed by internalization prevents its aggregation and accumulation on the PM. Other studies show reduced accumulation and toxicity of β-amyloid in cells with intact PM cholesterol (45, 46, 73), suggesting a similar mechanism for detection and clearance of toxic amyloid species. In addition to its stimulatory effect on the clearance of toxic oligomers from the PM, membrane cholesterol may also be required for intracellular oligomer trafficking and/or degradation by membrane-associated amyloid degrading enzymes such as neprylisin and matrix metalloproteinase-9 (16, 74), impairment of which may cause amylin toxicity in cells. This will be investigated in the future studies.

In summary, this study demonstrates that peptide clustering is a prerequisite for amylin oligomer association with cholesterol-regulated microdomains on the PM and subsequent uptake by cells. This in turn prevents amylin oligomer accumulation on the cell PM and cell death. The emerging concept of inhibition of amyloid formation and cellular toxicity by membrane cholesterol may have broader implications for a host of amyloid diseases, such as Alzheimer’s and TTDM, in which cholesterol homeostasis is disturbed.

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FOOTNOTES

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2The abbreviations used in text are: BCD, methylbetacyclodextrin; CTX, cholera toxin; DLS, dynamic light scattering; hA, human amylin; HFIP, hexafluoride isopropanol; LD50, median lethal dose; LDH, lactate dehydrogenase; Lov, lovastatin; MB, methylene blue; PM, plasma membrane; Trf, transferrin; Type-2 Diabetes Mellitus, TTDM.

FIGURE LEGENDS

FIGURE 1. PM cholesterol protects rat insulinoma cells against amylin toxicity. Cells were incubated with human amylin (10 μM) in the presence or absence of cholesterol-modulating agents, and their effects on cell viability assessed with MTT reduction (A), LDH release (B) and Caspase-3 cleavage (C) cytotoxic assays as described in Material and Methods. Significance established at *P<0.05 and **P<0.01 hA vs. treatments, and #P<0.05 and ##P<0.01 hA+BCD/Lov vs. hA+BCD/Lov/Chol, n=3-6, ANOVA followed by Newman-Keul post hoc test.

FIGURE 2. Amylin oligomerization induces cell death. A. Kinetics of human amylin (hA) oligomerization in solution revealed by a dot-blot oligomeric assay. Raw data were converted into % change from maximum signal and plotted as a function of time (graph, A). B. Western blot analysis demonstrates amylin oligomerization in solution in the absence but not in presence of oligomer inhibitor MB (100 μM). Oligomer formation was monitored using the conformation-specific A11-oligomer antibody (A11 Ab, left panel). In contrast to oligomers, MB does not prevent monomer recognition by human amylin antibody (hA Ab, right panel) demonstrating that the lack of A11 immunoreactivity is due to the MB-mediated oligomer loss (left panel). C. Addition of MB (100 μM) to rat insulinoma cells abolished amylin toxicity measured at 24 h. Significance established at **P<0.01, n=6, Student's t-test.

FIGURE 3. Binding and clustering of amylin oligomers into microdomains on the cell PM requires cholesterol. A. Confocal microscopy analysis of amylin oligomer and CTX distribution on the cell PM. Characteristic binding profiles of amylin oligomers on the cell PM for each treatment (within boxes, right panel) are rendered in gray tones for easier particle comparisons, which are presented side by side with the original fluorescence images (left panels). Note the time-dependent increase in the number of internalized amylin oligomers (control, 30 min vs. 24 h, left panel), which prevents accumulation of amylin oligomers on the cell PM (control, 30 min vs. 24 h, right box). Bar, 5μm. B. Quantitative analysis demonstrates a decrease in hA/CTX colocalization in cells with depleted PM cholesterol levels (BCD/Lov), which was restored after reloading of PM cholesterol with water soluble cholesterol (BCD/Chol/Lov). Single particle analysis demonstrates a 3-fold increase in the number of amylin oligomer clusters or puncta on the PM (C), and their dispersion across the PM in cholesterol-depleted...
cells (D). Significance established at *P<0.05, **P<0.01 control vs. BCD/Lov, and #P<0.05, ##P<0.01 BCD/Lov vs. BCD/Lov/Chol, n=5, ANOVA followed by Newman-Keul post hoc test.

FIGURE 4. Amylin monomer internalization is not blocked by cholesterol depletion. A. Confocal microscopy demonstrates internalization of amylin monomers both in controls (hA) and cholesterol-depleted cells (hA + BCD/Lov). No appreciable change in PM-binding pattern of amylin monomers (right boxes) was noticed upon cholesterol-depletion. Bar, 5 μm. B. Amylin monomers partially co-localize with the lipid raft marker CTX at the cell PM. Following treatment with BCD/Lov there was a little decrease in colocalization of amylin monomers and CTX on PM, which was reversed by replenishment of PM cholesterol levels with soluble cholesterol (hA + BCD/Chol/Lov). C-D. PM cholesterol does not modulate amylin deposition on the PM. The number of amylin puncta on PM (C) and their area (D) did not change significantly (NS, P>0.05, n=4) upon depletion of PM cholesterol by BCD/Lov. Significance established at **P<0.01 control vs. BCD/Lov, and #P<0.01 BCD/Lov vs. BCD/Lov/Chol, n=4, ANOVA followed by Newman-Keul post hoc test.

FIGURE 5. Amylin monomers and oligomers do not colocalize with transferrin at the cell PM. Following 30 min or 24 h incubations with amylin, cells were further incubated with CTX-Alexa555 or Trf-Alexa633 conjugates for 30 min at 4°C to label PM. Amylin monomers and oligomers were detected using specific amylin monomer and oligomer antibodies. Immunochemical analysis reveals distinct binding sites for the lipid raft marker cholera toxin (CTX) and clathrin-dependent endocytotic marker transferrin (Trf) on the cell PM (top panel). Amylin oligomers (middle panels) and monomers (bottom panels) did not co-localize with Trf either during early stage (30 min) or during late stage (24 h) of amylin internalization. Bar, 5 μm.

FIGURE 6. PM cholesterol is required for internalization of amylin oligomers in rat insulinoma cells. Amylin oligomer binding and cellular distribution was determined using oligomer specific A11 antibody and confocal microscopy. Section analysis profiles (shaded bar across each image) and boundaries of the cell are marked by arrows on the panels to the right. A. Internalized amylin oligomers (fluorescence peaks positioned between arrows) and PM-bound oligomers (arrows) are demonstrated in cells with intact PM cholesterol (hA). Bar, 5 μm. B. Depletion of PM cholesterol prevents amylin oligomer internalization (hA + BCD/Lov). Note the increase in the peak widths at the PM (denoted by two arrows) upon cholesterol depletion by BCD/Lov demonstrating accumulation of amylin oligomers on the cell PM (A-B). C. Replenishment of PM cholesterol with soluble cholesterol restored amylin internalization (hA + BCD/Chol/Lov). D. Whole cell analysis confirms that PM cholesterol is required for amylin oligomer internalization in rat insulinoma cells. Significance established at **P<0.01 hA vs. treatments, and ##P<0.01 hA+BCD/Lov vs. hA+BCD/Lov/Chol., n=6, ANOVA followed by Newman-Keul post hoc test.

FIGURE 7. PM cholesterol prevents accumulation of soluble amylin oligomers in the extracellular medium. Cells were incubated with human amylin (hA) for 24 h in the absence or presence of cholesterol-modulating agents BCD/Lov or soluble cholesterol, and extracellular amylin monomer and oligomer content analyzed by Western blot. Blots were probed with human amylin antibody (right panel) or A11-oligomer antibody (left panel). Depletion of PM cholesterol by BCD/Lov stimulates ~30 % increase in intermediate- sized (IS)-oligomer content in the culture medium relative to controls (left panel and graph). Similarly, BCD/Lov increases low-MW oligomer (dimer) content by ~60 % relative to controls (right panel and graph). Reloading of the plasma membrane cholesterol by addition of soluble cholesterol to cells in the presence of lovastatin (BCD/Lov/Chol) reinstates capacity of insulinoma cells to remove low-
MW and IS-amylin oligomers as revealed by a marked drop in their content in the extracellular medium (panels and graph). Significance established at \(^*P<0.05\) and \(^{**}P<0.01\) BCD/Lov vs. BCD/Lov/Chol., \(n=3\), Student’s t-test.

**FIGURE 8.** PM cholesterol is not required for internalization of amylin monomers in rat insulinoma cells. Cells were incubated with 10 \(\mu\)M human amylin in the presence or absence of BCD/Lov or BCD/Chol/Lov for 24 h. Following incubations, amylin monomers were detected using hA-antibody and their cellular distributions revealed by confocal microscopy. A. Internalized and PM-bound amylin monomers are demonstrated in cells with intact PM cholesterol (hA). Bar, 5 \(\mu\)m. B. Depletion of PM cholesterol did not prevent internalization of amylin monomers (hA + BCD/Lov). C. Similarly, addition of soluble cholesterol had no affect on amylin binding or on internalization (hA + BCD/Chol/Lov). D. Whole cell analysis demonstrates no significant effect of cholesterol depletion or reloading on amylin monomer accumulation in rat insulinoma cells (NS, \(P>0.1\), \(n=4\), ANOVA followed by Newman-Keul post hoc test).

**FIGURE 9.** PM cholesterol prevents extracellular accumulation and toxicity of soluble amylin oligomers in cultured human islet cells. A. Confocal microscopy analysis of phosphatidylserine (PS) externalization and caspase-3 proteolytic activation by amylin in cells with normal, depleted and enriched cholesterol levels. Arrows depict non-apoptotic nuclei (blue) in viable cells, whereas arrowheads depict fluorogenic Caspase-3 substrate found in the nuclei of apoptotic cells, giving these nuclei a green/blue appearance. The majority of PS-positive cells (red) show shrinkage and nuclear condensation (arrowheads) indicative of apoptosis (hA and hA+BCD/Lov). Bar, 10 \(\mu\)m. B. Linear regression analysis demonstrates an inverse relationship between amylin-induced cell death and PM cholesterol levels. The extent of cell death evoked by amylin was plotted as a function of variable PM cholesterol levels in controls and treatments. C. Quantitative analysis of amylin-induced apoptosis in human islets. D. A representative western blot analysis (\(n=3\)) of extracellular amylin monomer and oligomer content in controls and treatments is shown. Arrowheads depict 4-kD and 8-kD bands denoting amylin monomers and dimers, respectively (left). The A11-antibody detects larger-MW (<30kD) oligomeric assemblies (right). Significance established at \(^{**}P<0.01\) hA vs. control, \(^*P<0.01\) hA vs. treatments, and \(^{**}P<0.01\) hA+BCD/lov vs. hA+BCD/Lov/Chol., \(n=6\), ANOVA followed by Newman-Keul post test.
Cholesterol mediates clearance of toxic amylin oligomers

Table 1. Cholesterol content in rat insulinoma cells following different treatments. Cells were treated with various cholesterol-modifying agents before determination of the cellular cholesterol levels as indicated in Material and Methods. Values are normalized to protein content in samples and expressed as ng cholesterol / µg protein. Results are means ± SEM of six independent experiments.

| Treatment     | Total Cholesterol (ng/µg) | Total Cholest. (%) | PM Cholesterol (%) |
|---------------|---------------------------|--------------------|--------------------|
| Control       | 0.817 ± 0.062             | 100                | 100                |
| BCD-15 min    | 0.605 ± 0.041             | 74 ± 6             | 73 ± 5             |
| BCD           | 0.761 ± 0.067             | 95 ± 7             | 97 ± 6             |
| Lov           | 0.632 ± 0.060             | 78 ± 9             | 82 ± 8             |
| BCD/Lov       | 0.538 ± 0.047             | 65 ± 9             | 63 ± 4             |
| BCD/Lov/Chol  | 0.768 ± 0.080             | 94 ± 11            | 89 ± 4             |
| Chol          | 0.935 ± 0.034             | 114 ± 4            | 117 ± 6            |

Table 2. Cholesterol content in cultured human islet cells following different treatments. Dissociated human islet cells were treated with various cholesterol-modifying agents before determination of the cellular cholesterol levels as described in Material and Methods. Values are normalized to protein content in samples and expressed as ng cholesterol / µg protein (total cholesterol). Results are means ± SEM of six independent experiments.

| Treatment     | Total Cholesterol (ng/µg) | Total Cholest. (%) | PM Cholesterol (%) |
|---------------|---------------------------|--------------------|--------------------|
| Control       | 8.58 ± 0.92               | 100                | 100                |
| BCD           | 8.24 ± 0.87               | 96 ± 7             | 94 ± 6             |
| Lov           | 7.35 ± 0.60               | 85 ± 5             | 88 ± 7             |
| BCD/Lov       | 6.04 ± 0.73               | 70 ± 5             | 67 ± 4             |
| BCD/Lov/Chol  | 7.75 ± 0.80               | 90 ± 6             | 94 ± 5             |
| Chol          | 9.63 ± 0.78               | 112 ± 5            | 114 ± 6            |
Figure 1

A

MTT Reduction (%)

B

LDH Release (%)

C

Caspase-3 cleavage (%)

[w/o hA, hA, Ctr, BCD, LOV, BCD Lov, BCD Lov Chol, Chol]
Figure 2

A

minutes

5 10 15

20 25 30

hours

1 6 24

B

kD

114

78

53

35

28

19

6

hA 10 µM + + + + +
MB 10 µM + + + + +
MB 100 µM + + + + +

hA Ab

A11 Ab

C

MTT Reduction (%)

0 25 50 75 100

Ctr hA hA+MB

Oligomerization (%)

0 25 50 75 100

5 10 20 30 1 6 24 minutes hours

---

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Figure 3

A

Amylin Oligomers  CTX  Merged

30 min
Control

24 hours
Control

+BCD/Lov
+Chol

B

hA/CTX

Pearson Coefficient (R)

|          | 30 min                  | 24 hours               |
|----------|-------------------------|------------------------|
| Ctr      | 1.0                     | 0.2                    |
| Ctr      | 0.8                     | 0.4                    |
| BCD/Lov  | 0.2**                   | 0.0**                  |
| BCD/Lov/Chol | 0.4##                  | 0.2##                  |

C

Particle number/ cell

|          | Ctr    | BCD Lov | BCD Lov Chol |
|----------|--------|---------|--------------|
| + hA     | 50**   | 10#     | 50##         |

D

Mean Particle Area (Pixel$^2$/cell)

|          | Ctr    | BCD Lov | BCD Lov Chol |
|----------|--------|---------|--------------|
| + hA     | 40**   | 20*     | 40##         |
Figure 5
Figure 8

(A) hA

(B) hA+ BCD/Lov

(C) hA+ BCD/Lov/Chol

(D) Internalization and PM accumulation

Monomers (%)

| Condition       | Internalization | PM accumulation |
|-----------------|-----------------|-----------------|
| hA              | 75              | 25              |
| BCD/Lov         | 50 NS           | 50 NS           |
| BCD/Lov/Chol    | 50 NS           | 50 NS           |
| + hA            | 25              | 75              |
Clustering and internalization of toxic amylin oligomers in pancreatic cells requires plasma membrane cholesterol
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