Islet amyloid polypeptide-induced membrane leakage involves uptake of lipids by forming amyloid fibers

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Abstract Fibril formation of islet amyloid polypeptide (IAPP) is associated with cell death of the insulin-producing pancreatic β-cells in patients with Type 2 Diabetes Mellitus. A likely cause for the cytotoxicity of human IAPP is that it destroys the barrier properties of the cell membrane. Here, we show by fluorescence confocal microscopy on lipid vesicles that the process of hIAPP amyloid formation is accompanied by a loss of barrier function, whereby lipids are extracted from the membrane and taken up in the forming amyloid deposits. No membrane interaction was observed when preformed fibrils were used. It is proposed that lipid uptake from the cell membrane is responsible for amyloid-induced membrane damage and that this represents a general mechanism underlying the cytotoxicity of amyloid forming proteins.

Keywords: Amylin; Type II diabetes mellitus; Protein–membrane interaction; Amyloid; Model membrane

1. Introduction

Amyloid fibril proteins are associated with a number of seemingly unrelated diseases. Examples are the (human) islet amyloid polypeptide (hIAPP) in type 2 diabetes mellitus and the neuronal Aβ in Alzheimer's disease. During the pathogenesis of these diseases, amyloid formation is associated with death of insulin-producing pancreatic islet β-cells [1] and of neuronal cells [2], respectively. Amyloidogenic proteins share the ability to form oligomeric and fibrillar aggregates with a common structure [3,4]. Since the cytotoxic mode of action of the various amyloid-forming proteins seems to be similar, independent of whether their aggregates are formed inside or outside of the cell, it was recently suggested that these protein aggregates also share a common target and that this target is the cell membrane [3]. Indeed, there is increasing evidence that amyloid-forming proteins can interact strongly with membranes [5–12]. However, it is not clear what the precise consequences are of these interactions for the membrane, or how they are related to fibril formation. Yet the process of fibril formation is critical for cytotoxicity: small oligomeric aggregates that form early in the aggregation process appear to be the most detrimental to cells, while mature fibrils do not show any cytotoxicity [3,12,13].

Recent studies on hIAPP have shown that binding of this protein to model membranes increases the rate of fibril formation and it was proposed that the membrane surface acts as catalyst for this process [8]. Other studies have shown that hIAPP can induce aspecific membrane leakage in a variety of model membranes [9,10,14]. Here, we studied simultaneously the process of hIAPP-induced membrane leakage and amyloid-fiber formation in the presence of lipid membranes. For this, we used model systems of giant unilamellar lipid vesicles (GUVs) and living rat insulinoma tumor (RIN) cells, to which monomeric IAPP was added. The aggregation process was investigated by means of laser confocal fluorescence microscopy. This assay allows us to simultaneously monitor the localization of lipids and peptides, alterations in the membrane structure and vesicle leakage by using different fluorescent dyes. We show that during amyloid formation, the membrane barrier becomes disrupted and that this process is accompanied by extraction of lipids from the membrane. These lipids are taken up in the forming amyloid aggregates. We propose that it is the extraction of the lipids from the cell membrane that is responsible for the membrane leakage and, based on our own results combined with recent literature data, we suggest that this represents a general mechanism that leads to amyloid-induced cell death.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) were obtained from Avanti

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Polar Lipids (Alabaster, AL). The fluorescent lipids were purchased from Molecular Probes (the Netherlands). C-terminally amidated human and mouse IAPP1-37 were custom synthesized and HPLC-purified (>98%) (from Bachem (Switzerland) and Dutch Cancer Institute (the Netherlands)). Aβ(1–40) was synthesized and purified by Dr. D.T.S. Rijkers (Faculty of Pharmacy, Utrecht University).

2.2. Vesicle preparation

GUVs were prepared by electroformation [15,16] in a flow chamber with Pt electrodes. Lipids in chloroform/methanol (9/1) (5 mM) were deposited at the Pt electrodes and the solvent was evaporated at 50 °C. After adding ~300 μl of 50 mM HEPES buffer at pH 7.0 into the chamber, a voltage of ~4 V at 10 Hz was applied for at least 1 h. GUVs were prepared of DOPC and of mixtures of DOPC with 30 molar% DOPS, and the lipid fluorescent probes were added at a concentration of 0.05% of the total amount of lipids.

2.3. Rat insulinoma tumor (RIN) cells

The RIN cells were cultured in RPMI 1640 (Gibco) containing 10% fetal calf serum, 11 mM glucose, 300 mg/l L-glutamine, 2000 mg/l $NaHCO_3$, 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified incubator with 5% CO_2 at 37 °C. The cells were incubated with the membrane lipid probes in the serum-free medium for 20 min and excess dye was washed away prior to the addition of the protein. The experiments were performed in PBS buffer, pH 7.2, supplemented with 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 5 mM glucose.

2.4. Fluorescence labeling

The GUVs and the RIN cells were labeled with different membrane probes; the headgroup labeled Rhodamine-PE (*N*-(6-tetramethylrhodaminethiocarbamoyl)-1,2-di-palmitoyl-*sn*-glycero-3-phosphoethanolamine) and the acyl chains labeled NBD-PC (1-palmitoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphocholine), β-Bodipy® (581/591)-PC (1-palmitoyl-2-(4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)-*sn*-glycero-3-phosphocholine) and β-Bodipy® (FL)-PC (1-palmitoyl-2-(4,4-difluoro-5,7-dimethyl-4-bora-3a, 4a-diaza-*s*-indacene-3-pentanoyl)-*sn*-glycero-3- phosphocholine).

2.5. Confocal fluorescence microscopy

Images were taken with a Nikon Eclipse TE2000-U inverted microscope, equipped with C1 confocal laser scanning unit and CFI Plan Fluor 40× objective, N.A 1,30. The green and red fluorescence signals were acquired using double excitation (488 nm line from an Argon–Ion laser and 543 nm line from a He–Ne laser) and detection (emission bandpass filters 515/30 and 585/30). The GUVs and the RIN cells were inspected by microscopy before the addition of the protein. The proteins were added from a freshly prepared DMSO stock solution to a final protein concentration of 3–5 μM.

3. Results and discussion

Confocal fluorescence microscopy allows monitoring of the effects of amyloid forming proteins on membrane integrity and morphology by using a combination of water-soluble fluorescent dyes and lipid-specific probes, which are incorporated in the membrane. In addition, it allows monitoring the formation of amyloid fibers by using an amyloid fiber-specific reagent. Therefore, with this technique it is possible to analyze several processes involving hIAPP–membrane interactions in one sample. As illustrated in Fig. 1A, the membrane of giant unilamellar vesicles (GUVs) of DOPC can be visualized by including the fluorescent lipid dye rhodamine-PE (red) in the membrane. Simultaneously, the intactness of the barrier function is demonstrated by the inability of the water-soluble dye carboxyfluorescein (green), added on the outside, to penetrate the vesicles. The addition of monomeric hIAPP to GUVs results in a loss of barrier function, as shown by the appearance of the green water-soluble dye inside the vesicles (Fig. 1B). It also results in the appearance of small irregular

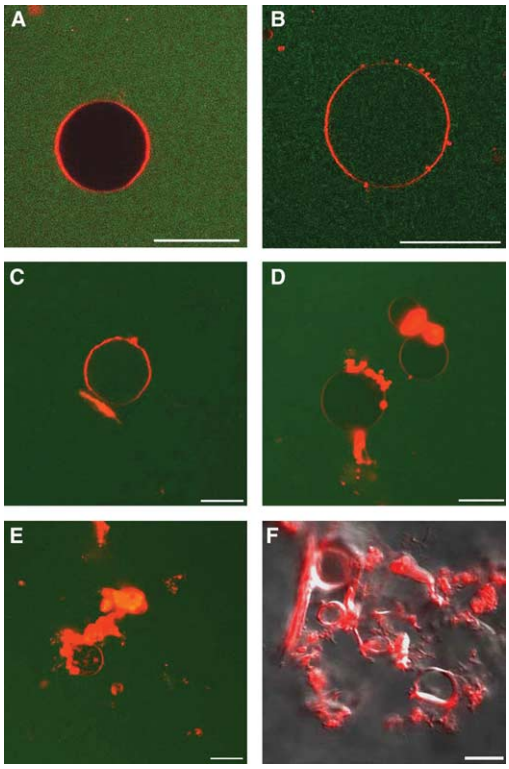

Fig. 1. hIAPP–membrane interactions. Confocal fluorescence images showing equatorial sections of GUVs before (A) and after (B–F) the addition of monomeric hIAPP; B: 1 min, C: 2–3 min, D: 4–5 min, E: 5–6 min and F: ca 10 min. The GUVs are composed of DOPC and rhodamine-PE (red) (0.05% of the total amount of lipids). The aqueous phase contains carboxyfluorescein (green), which is added after the preparation of the GUVs. Membrane disruption, leakage and protein–lipid aggregate formation is observed after the addition of hIAPP. Images (A–E) superimpose the green and the red channels, and image (F) superimposes the red channel and the simultaneously obtained Nomarski optical microscopy image. Scale bars: 25 μm.

aggregates containing fluorescent lipids on the outside of the vesicles (Fig. 1B). In time, the irregular aggregates grow in size on the outside of the vesicles (Fig. 1C–E) and, finally, the vesicles collapse (Fig. 1F). In the final state of the experiment, no intact vesicles are observed in the vicinity of the formed aggregates. The visibility of the formed aggregates by Nomarski optics (Fig. 1F) indicates that they contain protein aggregates. However, the aggregates must also include substantial amounts of lipids as implied from the presence of the fluorescent lipids and from the observation that the membrane lipids from the GUVs are consumed by the aggregates during the association process.

That lipids are taken up in the forming hIAPP aggregates is further confirmed by the co-localization of the amyloid-specific dye Congo-Red (red) with lipid-specific probes (green) at various stages of the process (Fig. 2A and B). Formation of lipid-containing amyloid aggregates is also observed when hIAPP is added to intact living cells, where the cell membrane is visualized after incubation with a fluorescent lipid dye (green). Fig. 2C and D show that the addition of monomeric hIAPP to rat insulinoma tumor (RIN) cells leads to extraction of lipids from the cell membrane and co-localization of protein- and lipid-specific probes in the fibrillar aggregates. The presence of fluorescent lipid in the aggregates was observed

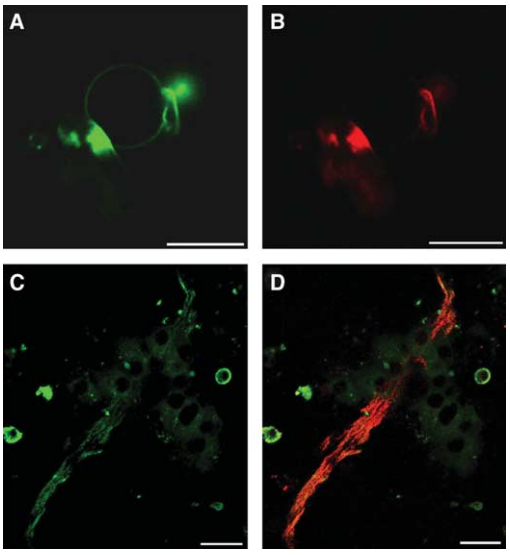

Fig. 2. Co-localization of hIAPP and membrane lipids. Confocal fluorescence images showing the equatorial sections of GUVs 4–5 min after the addition of monomeric hIAPP (A,B) and an amyloid aggregate at the surface of rat insulinoma tumor (RIN) cells (C,D). The GUVs are composed of DOPC and NBD-PC (green) (0.05% of the total amount of lipids). The cells were incubated with Bodipy(FL)-PC (green). In both cases, hIAPP amyloid aggregates are localized by staining with the amyloid-specific dye Congo Red (red). The images show co-localization of the amyloid protein (B,D) and lipids (A,C). Scale bars: 25 μm.

(not shown) for a range of fluorescent lipids with labels either in the headgroups or in the acyl chains (e.g., Rhodamin-PE, NBD-PC, Bodipy(581/591)-PC and Bodipy(FL)-PC).

We also observed that hIAPP amyloid formation is sensitive to the lipid composition. Incorporation of anionic lipids, such as phosphatidylserine, in the zwitterionic phosphatidylcholine vesicles resulted in significant acceleration of both the formation of the protein–lipid aggregates and the loss of barrier function (not shown). This is consistent with the recent literature data, which show that hIAPP interacts more strongly with membranes containing anionic lipids [8] and suggests that electrostatic interactions between the positively charged proteins and the negatively charged membrane surface enhance the protein–membrane interactions that are responsible for membrane damage and formation of lipid-containing hIAPP aggregates.

The uptake of membrane lipids into the amyloid is directly coupled to the process of amyloid formation, as is inferred from the following observations. First, none of the effects described above, i.e., membrane leakage, changes in membrane morphology, or formation of lipid-containing amyloid aggregates, is observed to a significant extent when preformed hIAPP fibrils are added to the membranes (not shown). Second, the structurally related non-amyloidogenic mouse IAPP (mIAPP) (Table 1), which does not form fibrils [17], causes none of these effects when added to GUVs or intact living cells. Moreover, although mIAPP is more positively charged than hIAPP, we did not observe an interaction with negatively charged vesicles under the experimental conditions that we used. Finally, the formation of lipid-hIAPP amyloid aggregates can be inhibited by addition of mIAPP (not shown), which can act as a β-sheet blocker [18]. Thus, aggregation of hIAPP seems directly associated with extraction of lipid from the membrane to the forming protein–lipid aggregates, and with the accompanying alterations in membrane structure and function. This is in line with the recent suggestion that leakage of vesicles upon interaction with hIAPP is due to lipid loss from the membrane rather than to the formation of discrete protein pores [9].

Membrane-disruption and formation of protein–lipid aggregates, albeit at a slower rate, is also observed at the membrane when the amyloidogenic Aβ(1–40) is added to GUVs (not shown). This is consistent with the observations from Michikawa et al, showing that oligomeric Aβ can promote lipid release from neurons and astrocyte cells [19]. Moreover, uptake of lipids in forming fibers has been shown to occur for a variety of water-soluble proteins [20]. Thus, our results suggest

Table 1
Amino acid sequences for hIAPP (top) and mIAPP (bottom)

hIAPP	KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSN-TY-$CONH_2$
mIAPP	KCNTATCATQRLANFLVRSSNNLGPVLPPTNVGSN-TY-$CONH_2$

The peptides differ in the central region comprising amino acids 20–29.

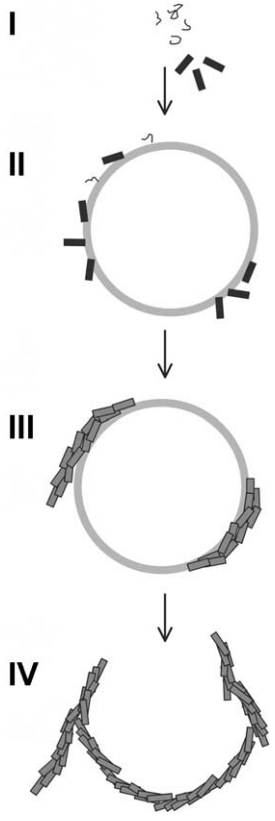

Fig. 3. Schematic representation of the proposed mechanism for the formation of amyloid protein–lipid aggregates in the presence of lipid membranes. I: The aggregation process starts when monomeric proteins are added to the aqueous solution. II: Attractive interaction between the lipid bilayer and the protein oligomers and/or monomers leads to accumulation of proteins at the lipid bilayer. III: Aggregation continues at the lipid membrane, whereby lipids are taken up by the forming aggregates. The extraction of lipids from the membrane also causes local disruption of the membrane barrier. IV: Aggregation continues. Large protein–lipid aggregates are formed for which the membrane may serve as a template. The aggregates include amyloid proteins as well as lipids from the membrane.

that induction of membrane leakage concomittant with the formation of lipid-containing amyloid aggregates is a universal property of amyloid proteins when aggregating in the presence of either natural membranes in living cells or lipid bilayers in model systems.

We propose the following mechanism for the cooperative process of disruption of membrane barrier function and formation of lipid-containing amyloid aggregates (Fig. 3). The oligomeric and/or monomeric amyloid proteins accumulate at the membrane due to strong interactions between the lipid bilayer and the hydrophobic β-sheet-containing amyloid oligomers. Accumulation of amyloid proteins at the membrane then facilitates nucleation and the growth of lipid-containing amyloid aggregates, resulting in membrane disruption. In addition, the lipid membrane can serve as a template for protein aggregation, as suggested by the observation that the hIAPP-lipid amyloid aggregates formed in the presence of GUVs often adopt a nearly circular shape with a diameter of typically 15–40 μm, reflecting the shape and size of the template intact GUVs (Fig. 1F).

In conclusion, we show that aggregating IAPP takes up lipids when added to lipid membranes, and that the extraction of lipids is directly coupled to the process of amyloid formation and to the permeabilization of the lipid membrane. The fact that we observe similar effects for hIAPP and Aβ indicates a general phenomenon that is independent of the way that the amyloid aggregates are formed in nature, lending further support to the recent hypothesis that the membrane is the common target for these proteins [3].

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