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Insulin–eukaryotic model membrane interaction: Mechanistic insight of insulin fibrillation and membrane disruption

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ABSTRACT

Injection of exogenous insulin in the subcutaneous mass has been a proven therapy for type II diabetes. However, chronic administration of insulin often develops local amyloidosis at the injection site, pathologically known as "Insulin Ball". This reduces the insulin bioavailability and exacerbates the disease pathology. Thus, the molecular interaction between insulin and the recipient's membrane surface plays a co-operative role in accelerating the amyloidosis. This interaction, however, is different from the molecular interaction of insulin with the native membranous environment of the pancreatic β-cells. The differential membrane mediated interaction that directly affects the aggregation kinetics of insulin remains elusive yet intriguing to understand the mechanism of pathological development. In this study we have characterized the interactions of insulin at different states with model eukaryotic membranes using high and low-resolution spectroscopic techniques in combination with microscopic investigation. Our results show that insulin amyloid intermediates are capable of interacting with model membranes with variable functional affinity towards the different compositions. Fluorescence correlation spectroscopy confirms the aggregation states of insulin in presence of the eukaryotic model membranes while solid-state NMR spectroscopy in conjugation with differential scanning calorimetry elucidates the molecular interaction of insulin intermediates with the lipid head groups along with the acyl chains. Additionally, dye leakage assays support the eukaryotic model membrane disruption by insulin intermediates, similar to hIAPP and Aβ40, as previously reported. Thus, the present study establishes the distinct mode of interactions of insulin amyloid with pancreatic β-cell and general mammalian cell mimicking membranes.

1. Introduction

Protein amyloidosis is a result of protein misfolding, which gives rise to fibrillar products with a characteristic cross β sheet structure. There are more than twenty identified diseases associated with protein amyloidosis such as Alzheimer's disease, Parkinson's disease, type II diabetes and Huntington's disease [1–3]. However, at present, the understanding of amyloid diseases and the associated pathogenesis is obscure [4–6]. The research to understand the precise mechanism of protein amyloidosis and the disease pathogenesis is on high thrust since the last few decades. Amyloidogenic proteins such as Amyloid beta (Aβ), α-synuclein, and human islet amyloid polypeptide (hIAPP) have been extensively studied for exploring the amyloidosis mechanism and mode of action that results in cytotoxicity. There are hypotheses and supportive in vitro [7–9] and in vivo [10] results, which indicate that the interaction of the amyloid intermediates [11] or fibril [9] with the cell membrane causes cell death. Hence, deciphering the amyloid toxicity mechanism is a potential challenge to the scientific community. Insulin is a well-known protein of amyloidosis model. It is widely used because of its ease of availability from animal sources, unlike other amyloid proteins, which are either synthetic or produced with recombinant DNA technology. Insulin is a peptide hormone comprising of two chains, namely, chain A and chain B constituted by 21 and 30 amino acids respectively; disulfide linkages connect the two chains with each other [12,13]. The Zn bound insulin is hexamer and adopts helical conformation [14].

Abbreviations: ThT, thioflavin T; ANS, 8-anilinonaphthalene-1-sulfonic acid; TMR, tetramethylrhodaminesuccinimidyl ester; 6-CF, 6-carboxyfluorescein; Aβ, amyloid beta; hIAPP, human islet amyloid polypeptide; FCS, fluorescence correlation spectroscopy; AFM, atomic force microscopy; CD, circular dichroism; TEM, transmission electron microscopy; NMR, nuclear magnetic resonance; ssNMR, solid state NMR; TDDS, transdermal drug delivery systems

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Insulin is a protein of commercial importance because of the ever-growing global diabetic population. According to diabetes fact sheet released by World Health Organization (WHO) in November 2016, the consumption of insulin by global diabetic population has increased almost four times (108 million to 422 million) in 24 years (from 1980 to 2014). A large proportion of the diabetic populations depend on insulin-based therapeutics; it necessitates the requirement of safe and efficient insulin formulations for the consumers. To date, insulin is administered as a subcutaneous injection, however, recently researchers have focused on the development of oral dosage forms (reviewed in detail: [15]), biodegradable polymer [16], nanoparticle based delivery [17], and transdermal drug delivery systems (TDDS) [18] for insulin drug delivery. Moreover, researchers are trying to improve the bioavailability of insulin with the use of liposomes [19].

The recombinant form of insulin demonstrates the formation of local amyloid fibrils at the site of administration [20–25]. The localized insulin amyloidosis also creates challenge in the diagnosis of systemic amyloidosis [26]. Existing reports on the fibrillation of hIAPP, Aβ and α-synuclein indicated potential role of the biological membranes [27–34] and hydrophobic surfaces in modulating the fibrillation kinetics of the amyloidogenic proteins [35]. However, there are few reports on insulin amyloid interaction with membrane models; amphiphilic surfactant DHPC inhibits insulin fibrillation [36], while presence of salt accelerates the insulin aggregation for DOPC in the presence of DOPS or DOPE [37]. In spite of all these efforts, the absence of a clear picture on the effect of membrane on insulin amyloidosis hinders safer and high efficacy drug delivery options of liposome or lipid-based drugs in comparison to the traditional subcutaneous route of insulin administration.

To address the above problem, we have used model membrane mimics related to pancreatic β-cell membrane and/or mammalian cell membrane [38] and their effect in the context of insulin aggregation. High and low resolution spectroscopic techniques in conjugation with microscopic analysis were used to explore the interaction of insulin fibrillation intermediates with the biological membrane models. The results corroborate well with the fact that the insulin doesn't undergo amyloidosis inside the pancreatic β-cell, despite it is stored at a very high (~40 mM) concentration in the pancreatic β-cell [39], whereas the chronic administration of therapeutic insulin, forms local amyloids at the site of injection.

2. Materials and methods

2.1. Reagents

Bovine Pancreatic insulin (Catalogue no. I5550) was procured from Sigma Aldrich Co. (St. Louis, USA). 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-ditetradecanoyl-sn-glycero-3-phosphocholine (DMPC), 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3- phosphor (1′-rac-glycerol) sodium salt (POPG) and Cholesterol were obtained from Avanti Polar Lipids Inc. (AL, USA). All other reagents used in the experiments were of the highest grade either from Sigma Aldrich Co. (St. Louis, USA), Merck & Co. (NJ, USA). Tetramethylrhodamine (TMR) succinimidyl ester was purchased from Thermo Fisher Scientific Co. (IL, USA).

2.2. Tetramethylrhodamine (TMR) labeling of insulin

Zn free Bovine insulin was prepared as described elsewhere [40]. A solution of Zn free bovine insulin (2 mg/ml) in 10 mM phosphate buffer pH 7.4 was prepared by degassing to avoid interference of dissolved oxygen on subsequent reaction. To the resultant solution, tetramethylrhodamine (TMR) succinimidyl ester was gradually added under continuous stirring at room temperature. The complex solution was kept overnight at 277 K. The resultant solution was passed through the superdex peptide 10/300 GL column (GE Global research, India) to get rid of the unreacted dye. The labeled protein was stored at 193 K freezer.

2.3. Preparation of large unilamellar vesicles (LUVs)

The desired volume of lipids in chloroform was dried under Nitrogen gas flow, followed by overnight lyophilization to get rid of remaining solvent. Appropriate quantity of Tris buffer (10 mM Tris, pH 7.4, 100 mM NaCl) was added to the dried lipid film and vortexed for few minutes. This was followed by 3 freeze-thaw cycles to make homogeneous MLV. Then the mixture was passed through a mini-extruder using a 100 nm filter membrane. The extruder setup was maintained above the transition temperature of the corresponding lipid mixtures to prepare the LUVs. The lipid mixture was passed for 21 times, before collection of the LUVs into a fresh tube.

2.4. ThT and ANS fluorescence assay

Bovine pancreatic insulin was solubilized in aqueous HCl solution (pH = 1.9) at a final concentration of 2 mg/ml. Concentration was determined by UV absorbance using an extinction coefficient of 0.91 mg/ml for 1 OD at 276 nm. The molar extinction coefficient of 36,000 M^{-1} cm^{-1} was used to determine the concentration of thioflavin T (ThT) solution. 2 mg/ml insulin solution was incubated at 335 K in a water bath; aliquots were drawn at regular time intervals. The aliquots were diluted in 10 mM phosphate buffer (pH 7.4) with 100 mM NaCl [41,42]. A quartz cuvette with path length of 0.1 cm was used to measure ThT Fluorescence in the spectrofluorometer (PTI QuantaMaster/Hitachi F-7000 FL) where the excitation was set at 440 nm and an emission maximum was observed around 482 nm. In a similar fashion 8-Anilinonaphthalene-1-sulfonic acid (ANS) was added to the solution at a final concentration of 10 μM and fluorescence was observed with excitation at 350 nm and emission was recorded from 420 nm to 520 nm. The ThT and ANS kinetics experiments were repeated for 3 times. In another set of experiment, LUVs were added at a molar ratio of 1:7 to that of insulin and pH was adjusted to 1.9. As a control, equivalent amount (volume of LUVs added) of 10 mM Tris buffer was added to the insulin sample and pH was re-adjusted to 1.9. The ThT data was recorded as mentioned earlier. Observed fluorescence intensity at 482 nm for different time points was plotted against time, and the curve was fitted by Boltzmann equation (Eq. (1)) as follows:

$$Y = A_2 + \left[\frac{A_2 - A_1}{1 + e^{(t0-t)/\tau}}\right] \tag{1}$$

Here, A_1 is the initial fluorescence, A_2 is the maximum fluorescence, t_0 stands for the time where the fluorescence has reached to half of the maximum value, and $1/\tau$ is the apparent rate constant of fibril growth and lag time approximated to t_0-2τ.

2.5. Circular dichroism (CD) spectroscopy

Alterations in the secondary structure of insulin fibrillation intermediates in the presence and absence of large unilamellar vesicles (LUVs) consisting either 7:3 DOPC:DOPG or 6:4 POPC:Cholesterol at 1:1 (insulin:membrane) molar ratio were monitored with the help of CD spectroscopy. Aliquots of incubated insulin were drawn at indicated time point and were diluted to 25 μM in 10 mM Tris buffer (pH 7.4 with 100 mM NaCl). The sample was taken in a 0.2 cm quartz cuvette and the spectra was recorded in Jasco J-815 spectrophotometer at 298 K. Scanning speed was set to 100 nm/min and the data was collected over a range of 200–260 nm. Here, each CD spectrum denotes an accumulation of four subsequent scans. A control spectrum for the similar concentration of only the LUVs was recorded to eliminate the contribution of LUVs in CD spectrum. The raw data in millidegrees were subtracted from the blank buffer and transformed to molar ellipticity using the following equation (Eq. (2)):

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