A novel assay *in vitro* of human islet amyloid polypeptide amyloidogenesis and effects of insulin secretory vesicle peptides on amyloid formation

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Human islet amyloid polypeptide (IAPP) is a 37-residue peptide that is co-secreted with insulin by the β -cell and might be involved in the pathogenesis of non-insulin-dependent diabetes mellitus. We developed an improved assay *in vitro* based on the fluorescence of bound thioflavin T to study factors affecting amyloidogenesis. Monomeric IAPP formed amyloid fibrils, as detected by increased fluorescence and by electron microscopy. Fluorimetric analysis revealed that the initial rate of amyloid formation was: (1) proportional to the peptide monomer concentration, (2) maximal at pH 9.5, (3) maximal at 200 mM KCl, and (4) proportional to temperature from 4 to 37 °C. We found that 5-fold and 10-fold molar excesses of proinsulin inhibited

fibril formation by 39 % and 59 % respectively. Insulin was somewhat more potent with 5-fold and 10-fold molar excesses inhibiting fibril formation by 69 % and 73 % respectively, whereas C-peptide had no effect at these concentrations. Thus at physiological ratios of IAPP to insulin, insulin and proinsulin, but not C-peptide, can retard amyloidogenesis. Because insulin resistance or hyperglycaemia increase the IAPP-to-insulin ratio, increased intracellular IAPP compared with insulin expression in genetically predisposed individuals might contribute to intracellular amyloid formation, β -cell death and the genesis of non-insulin-dependent diabetes mellitus.

INTRODUCTION

In humans non-insulin-dependent diabetes mellitus (NIDDM) is characterized by islet amyloid [1], which is principally composed of islet amyloid polypeptide (IAPP, also referred to as amylin) [2,3] a 37-residue peptide normally expressed in β -cells, where it is co-secreted with insulin [4]. Although the importance of the central amino acids (20–29) of this peptide in amyloidogenesis has been well recognized [5], little is known about the factors that lead to the aggregation of IAPP into amyloid in patients with NIDDM. Because recent reports suggest that both intracellular and extracellular human IAPP (hIAPP) amyloid might be cytotoxic and contribute to the loss of β -cell mass in NIDDM [6,7], a greater understanding of the kinetics of IAPP amyloidogenesis and factors that influence its formation is important.

In the present studies we developed a highly specific and sensitive assay *in vitro* for measurement of the kinetics of IAPP aggregation on the basis of the binding of the fluorescent dye thioflavin T (ThT) to amyloid [8–13] and characterized the amyloid formation with respect to IAPP concentration, pH, salt concentration and temperature. This assay provides a quantitative assessment of amyloid formation and overcomes many of the problems associated with previous methodologies, because the binding of ThT and subsequent fluorescence are dependent on the crossed- β -sheet type structure present in a variety of amyloids. Consequently this assay can distinguish amyloid formation from non-specific precipitation, whereas turbidimetric and light scattering assays cannot. In addition, the assay avoids a concentration step that is employed in all light and electron microscopic observations as well as amyloid binding assays. Using this sensitive assay we assessed the effects of proinsulin,

insulin and C-peptide on the rates of IAPP amyloidogenesis *in vitro*.

MATERIALS AND METHODS

Synthesis and purification of IAPP

Human IAPP (hIAPP_{1–37}) peptide was synthesized on an ABI 431A Peptide Synthesizer (Perkin Elmer Corporation/Applied Biosystems Division, Foster City, CA, U.S.A.) by using the fluoren-9-ylmethoxycarbonyl/diphenylcarbamoyl chloride protocol and reagents. The peptide was purified by reverse-phase HPLC with 0.1 % (v/v) trifluoroacetic acid/acetonitrile buffers and a Vydac C₈ column (Separations Group, Hesperia, CA, U.S.A.). The peptide integrity and molecular mass were confirmed by amino acid analysis and plasma desorption time-of-flight MS (results not shown).

IAPP aggregation

The fibril formation experiments *in vitro* were performed in sterile 1.5 ml cryogenic screwcap vials (polypropylene; Labcraft). All plasticware used in this and the fluorescence assay (below) was treated with Sigmacote® (Sigma, St. Louis, MO, U.S.A.) [14]. Each experiment was performed at least three times. Reactions were performed in 10 mM Tris/HCl/100 mM NaCl/0.3 % BSA/0.1 % (w/v) Triton X-100 in a final volume of 300 μ l with constant stirring (7 mm $\times$ 2 mm magnetic spin bar). IAPP_{1–37} was dissolved in DMSO, which allowed solubilization at concentrations of 2–3 mM without aggregation, and diluted into the final buffer, yielding final IAPP and DMSO concentrations of

Abbreviations used: NIDDM, non-insulin-dependent diabetes mellitus; ThT, thioflavin T; IAPP, amylin or islet amyloid polypeptide; h, human.

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50–150 μM and 5% (v/v) respectively. The reaction was carried out for 7 h in a rack attached to a magnetic stir plate at a temperature of 22 °C unless otherwise stated. The zinc-stripped proinsulin, insulin and C-peptide were obtained from Eli Lilly (Indianapolis, IN, U.S.A.).

Fluorescence assay

Fluorescence was measured with a ratio two-system fluorimeter (Optical Technology Devices, Elmsford, NY, U.S.A.) in the ratio mode at 22 °C essentially as described by Naiki et al. [8–10]. The excitation wavelength was 450 nm and the emission wavelength was 482 nm; excitation and emission slits were maintained at 5 and 10 nm respectively. The hIAPP reaction mixture (5 μl) at the indicated time point was transferred to a separate vial containing 1.5 ml of 1 μM ThT and 50 mM glycine/NaOH, pH 9.0. All measurements were made in triplicate within 10 min of addition of the sample to the assay mixture. Fluorescence of ThT binding to amyloid was proportional to its concentration, stable for at least 10 min and maximal at pH 9.0 (results not shown). Fluorescence was measured at 30 min intervals for 2 h, and hourly thereafter for a further 5 h. Initial rates of amyloid formation were obtained by linear regression analysis of the data through the first 5 h of the reaction. Initial rates from at least three separate experiments were averaged. All results were analysed by multivariate analysis of variance followed by *post hoc* Bonferroni *t* tests to assess individual differences.

Electron microscopy

hIAPP aggregate suspensions tested for fluorescence as described above were spread on carbon-coated Formvar grids and stained with 1% (w/v) phosphotungstic acid for 15 min. The grids were then washed with distilled water over dental wax. These specimens were examined with a JEM-1200EXII transmission electron microscope (Jeol) with an acceleration voltage of 60 kV. Photographs of amyloid fibrils detected were taken and the widths of all amyloid fibrils were calculated from a calibration bar photographed automatically on each film.

RESULTS

Inclusion of BSA and Triton X-100 are required to obtain amyloid formation *in vitro*

In our initial efforts to establish the amyloidogenesis assay *in vitro*, we were puzzled by the finding that in the absence of BSA and Triton X-100 the Alzheimer $\text{A}\beta_{1-42}$ peptide readily formed amyloid fibrils [11] in 10 mM Tris/HCl (pH 7.4)/100 mM NaCl/5% DMSO, whereas under the same conditions hIAPP₁₋₃₇ produced no time- and ThT-dependent fluorescence signal (Figure 1). Indeed, electron microscopic evaluation of reaction mixtures containing 50–150 μM hIAPP₁₋₃₇ failed to reveal the presence of amyloid fibrils, whereas these were abundant in identical incubations containing the $\text{A}\beta_{1-42}$ peptide (results not shown). This led us to suspect either that hIAPP₁₋₃₇ would not form amyloid under these conditions or that the hIAPP monomer might be prevented from aggregating, possibly through interactions with the reaction vessel wall. Because BSA and Triton X-100 are often used to prevent 'sticky' peptides from binding to reaction vessel walls, we chose to evaluate their influence on amyloid formation by hIAPP₁₋₃₇. As shown in Figure 1, in the absence of BSA or Triton X-100 no time-dependent increase in the ThT fluorescence signal occurred. In addition, when the fluorescence signal was monitored after 24 h of incubation, there was still no increase in the ThT signal, indicating that the lack of signal generation was not due to a kinetic lag in amyloid

Figure 1 BSA and Triton X-100 are required to generate hIAPP amyloid that can interact with ThT (arbitrary fluorescence units/h)

The results represent the initial rate of amyloid formation in 100 mM Tris/HCl (pH 7.4)/100 mM NaCl/5% (v/v) DMSO as described in the Materials and methods section and in the legend to Figure 2.

Figure 2 Concentration dependence of hIAPP on amyloid formation

(A) Kinetic analysis of ThT fluorescence produced by 100 μM hIAPP (arbitrary units, means $\pm$ S.E.M.). The results were analysed by linear regression and represent the initial rate of amyloid formation. (B) Analysis of initial rates of amyloid formation for 50–150 μM hIAPP (arbitrary fluorescence units/h, means $\pm$ S.E.M.). In both (A) and (B) the incubations were performed in 100 mM Tris/HCl (pH 7.4)/100 mM NaCl/5% (v/v) DMSO.

formation (results not shown). Inclusion of 0.1% Triton X-100 by itself, but not BSA, did result in a time-dependent increase in ThT fluorescence (10.1 arbitrary units/h; $P < 0.05$). The

Figure 3 Characterization of dependence on temperature (A), pH (B) and salt (C) of hIAPP amyloid formation (arbitrary fluorescence units/h, means $\pm$ S.E.M.)

Incubations were performed in 100 mM Tris/HCl (pH 7.4)/100 mM NaCl/5% (v/v) DMSO, except for those in (B) and (C), where the pH and salt concentrations respectively were varied as indicated.

'negative' fluorescence in the presence or absence of BSA and the absence of Triton X-100 was due to a spurious fluorescent signal that was generated immediately and then decayed throughout the course of the incubation. BSA and Triton X-100 together acted synergistically, resulting in a much larger time-dependent increase in ThT fluorescence signal (28.6 arbitrary units/h; $P < 0.05$). Because the fluorescent signal generated by ThT is highly dependent on the presence of a specific amyloid structure [8–10], these results indicate that in the absence of BSA and Triton X-100, aggregation might be limited to non-specific, amorphous forms, including aggregation on the reaction vessel wall.

Dependence of hIAPP_{1–37} amyloid formation on concentration, pH and temperature

We characterized the effect of hIAPP_{1–37} concentration on ThT fluorescence. Figure 2(A) shows the linear time-dependent increase in ThT fluorescence for a reaction with 100 μ M hIAPP_{1–37} during the early time course of the reaction. Maximal fluorescence

Figure 4 Electron micrograph of amyloid fibrils formed with 100 μ M hIAPP in 100 mM Tris/HCl (pH 7.4)/100 mM NaCl/5% (v/v) DMSO as described in the Materials and methods section

Scale bar, 200 μ m.

signal was observed between 24 and 48 h and was stable for 72–96 h (results not shown). When the initial rate was calculated by linear regression analysis of the early time points (as in Figure 2A) for reactions containing 50, 100 or 150 μ M hIAPP_{1–37}, there was a direct relationship between the concentration and the initial rate of amyloid formation (Figure 2B). Thus the kinetics is well behaved over the concentrations of hIAPP_{1–37} used in these studies. The initial rate of amyloid formation was directly related to the temperature (Figure 3A), suggesting that amyloid formation is a hydrophobically driven reaction. ThT fluorescence increased with increasing pH (3.4–9.5) and was maximal at pH 9.5 (Figure 3B). At pH 3.4, ThT fluorescence was minimal and fibril formation as assessed by electron microscopy was absent. Whereas fluorescence increased in the pH range 5.5–8.5 ($P < 0.05$ in comparison with pH 3.4), the increase at pH 9.5 was significantly greater ($P < 0.05$ in comparison with pH 5.5–8.5). The initial rate of increase of ThT fluorescence was enhanced by increasing concentrations (50–200 mM) of NaCl and KCl (Figure 3C). The initial rate of amyloid formation was minimal in the absence of salt ($P < 0.05$ compared with all salt concentrations) and maximal in the presence of 200 mM KCl; however, this difference was not significant.

Figure 4 shows a typical electron micrograph of hIAPP amyloid formed at 100 μ M monomeric hIAPP under the standard aggregation conditions [100 mM NaCl/5% DMSO/0.3% BSA/0.1% Triton X-100 (pH 7.4)], demonstrating the presence of amyloid fibrils with an average diameter of 10 nm. In addition, as a negative control we have examined the related non-amyloidogenic rat (r)IAPP with the fluorescence assay and electron microscopy. Under identical conditions rIAPP_{1–37} fails to generate a fluorescent signal and amyloid fibrils by electron microscopic examination (results not shown). Taken together these results confirm that the ThT fluorescence assay is a highly specific and quantitative method for measuring hIAPP amyloid formation.

Figure 5 Effect of proinsulin (A), insulin (B) and C-peptide (C) on hIAPP amyloid formation (arbitrary fluorescence units/h, means $\pm$ S.E.M.)

All incubations were performed with 100 μ M hIAPP in 100 mM Tris/HCl (pH 7.4)/100 mM NaCl/5% (v/v) DMSO as described in the Materials and methods section.

Proinsulin and insulin, but not C-peptide, inhibit hIAPP₁₋₃₇ amyloid formation *in vitro*

Having established a sensitive assay for hIAPP amyloidogenesis, we examined the effects of proinsulin, insulin and C-peptide, which are co-secreted with hIAPP from the β -cell secretory vesicle (Figure 5). Proinsulin had no effect on amyloidogenesis at an equimolar concentration, but inhibited amyloid formation by 39% and 59% respectively at 5-fold and 10-fold molar excesses ($P < 0.05$) (Figure 5A). Similarly, insulin had no effect on amyloid formation at an equimolar ratio; however, it was somewhat more potent, inhibiting amyloid formation by 69% and 73% respectively ($P < 0.05$) at 5-fold and 10-fold molar excesses (Figure 5B). C-peptide had no effect on hIAPP amyloidogenesis at any concentration tested (Figure 5C).

DISCUSSION

We have developed a highly specific fluorescence assay based on the binding of ThT to measure the rate of hIAPP amyloid formation *in vitro*. With this assay we evaluated the effect of physical factors such as pH, temperature, salt and hIAPP concentration on amyloid formation. Amyloidogenesis was

maximal at pH 9.5, increased with increasing NaCl and KCl concentration, increased with increasing temperature, and was directly proportional to the hIAPP concentration. Amyloid formation as ascertained by increased ThT fluorescence was directly correlated with the presence of amyloid fibrils by electron microscopy. At physiologically relevant molar ratios (insulin and proinsulin to hIAPP), insulin and proinsulin inhibited hIAPP amyloid formation, whereas C-peptide had no effect on the process.

Previous studies evaluating the influence of insulin, proinsulin and C-peptide on hIAPP amyloid formation *in vitro* arrived at opposite conclusions by different methods and under different conditions [15,16]. Amyloid formation was measured by Chargé et al. [15] by electron microscopy and binding of radio-labelled hIAPP to preformed amyloid at very low pH, which favours hIAPP solubility. Westermark et al. [16] used direct light microscopic observation of the characteristic green birefringence of amyloid in the presence of Congo Red and electron microscopy to observe the characteristic fibrils. These assays have several disadvantages that limit their utility in evaluating amyloidogenesis. Both electron microscopy and light microscopic methods use samples that have been dried, introducing large increases in substrate concentrations during this process. Consequently sample preparation can contribute to the amount of observed amyloid, compromising the quantitative evaluation. The radio-labelled binding experiments require a low pH, which favours solubilization of the monomeric peptide. This pH range might not be representative of that present at the site of amyloidogenesis *in vivo*. Indeed, with these methods Chargé et al. [15] found that insulin enhances the binding of hIAPP to preformed amyloid, whereas Westermark et al. [16] found a marked inhibitory effect of insulin on hIAPP amyloidogenesis. Consequently we sought to develop a method that overcomes these problems and allowed a quantitative measurement of IAPP amyloidogenesis *in vitro*.

To achieve this we adapted the method originally developed by Naiki et al. [8–10] to evaluate amyloid formation by serum amyloid A protein and apoAII by using ThT. ThT is an azo-free dye that by an unknown mechanism specifically interacts with the crossed- β -pleated sheet structure common to a variety of amyloid fibrils. This property allows the ThT assay to distinguish highly ordered amyloid structures from amorphous precipitates, which light scattering, a commonly employed method to evaluate amyloid formation, cannot. ThT binds to amyloids derived from the Alzheimer A β_{1-40} [12] and A β_{1-42} peptides [11] as well as amyloids generated from diverse peptides and proteins [13]. Interestingly, under conditions in which most amyloidogenic precursors form amyloid, hIAPP₁₋₃₇ (Figure 1) and hIAPP₂₀₋₂₉ [13] do not seem to interact with ThT to produce measurable fluorescence emission at 482 nm. This observation led us to consider whether hIAPP precipitation occurred in non-amyloidogenic forms or whether adsorption of hIAPP on the reaction vessel wall prevented amyloidogenesis. We demonstrated that BSA and Triton X-100, agents commonly used to prevent self-aggregation and adsorption of 'sticky' peptides on glass and plastic [14], were absolutely required to observe a ThT fluorescence signal or amyloid fibril by electron microscopy (Figures 1 and 4). BSA/detergent might prevent self-aggregation and adsorption on reaction vessel walls, thus maintaining the substrate in a form that is capable of forming amyloid, although more complex mechanisms might also be involved. Because BSA alone lacks the ability to promote amyloid formation (Figure 1), it does not seem to be acting as a nucleation promoter. We established that concentrations of Triton X-100 that were above the critical micelle concentration were required to produce this effect (results not shown), suggesting that micelle formation *per*

se was required. This raises the possibility that mixed detergent/hIAPP micelles might be involved in generating amyloid fibrils; however, further work will be required to distinguish between these mechanisms.

Amyloid formation is concentration-dependent (Figure 2), demonstrating that under the conditions of this assay amyloid formation is kinetically well behaved. This observation is consistent with the hypothesis that an increased rate of IAPP synthesis might be one of the causative factors in islet amyloidosis. Insulin resistance is a common risk factor for the development of NIDDM [17]. Induction of insulin resistance results in a marked increase in the expression rate of IAPP [18,19], conditions that would increase the probability for IAPP amyloidogenesis.

Our studies of the effect of pH on hIAPP₁₋₃₇ amyloidogenesis show a maximum rate of amyloid formation between pH 5.5 and 8.5, with a significant increase in amyloid formation at pH 9.5 (Figure 3B). The pH of the insulin secretory vesicle is estimated to be 5–6 *in vivo* [20], a value corresponding to optimal conditions for proinsulin conversion to insulin [21]. Hyperproinsulinaemia and islet amyloid are both characteristic features of NIDDM [22]. Thus an inability to maintain β -cell intravesicular pH could be a common pathogenic mechanism to account for both of these processes. Indeed, we previously demonstrated that islet amyloid is characteristically present in patients with cystic fibrosis complicated by diabetes mellitus [23]. Cystic fibrosis is a disease characterized by failure to achieve intraorganellar acidification [24–26].

Our present results demonstrating that insulin and proinsulin inhibit hIAPP amyloid formation are in general agreement with those of Westermark et al. [16]. However, in our studies somewhat higher concentrations of insulin and proinsulin were required to achieve significant inhibition of amyloidogenesis and we found that C-peptide did not enhance fibril formation. These differences might be related to differences in the pH used in the two studies. Westermark et al. [16] performed their experiments at a final pH of 5–6, a value reflecting the pH of the mature secretory vesicle. In contrast, our studies were performed at pH 7.4, a condition that might be more representative of intravesicular conditions in NIDDM as discussed above. More recently, Janciauskiene et al. [27] presented evidence with electron microscopy and the ThT assay that insulin inhibited IAPP amyloid formation, in agreement with Westermark et al. [16] and the results reported here. However, these authors found that C-peptide inhibited amyloid formation with the ThT assay under pH and salt conditions similar to those reported here with the exception that BSA and Triton X-100 were not included. As these authors do not report the time-dependent behaviour of their ThT fluorescence assay, it is difficult to evaluate their results, as discussed above (Figure 1). Several factors might contribute to the differences observed in these studies, including variations in (1) C-peptide purity, (2) assay conditions and experimental protocol, (3) duration of incubation, or (4) some unique characteristic of the C-peptide that is sensitive to different handling conditions. Although further work will be required to clarify these issues, particularly the

behaviour of the C-peptide, the combined results do indicate that conditions that increase hIAPP expression and the hIAPP-to-insulin ratio could contribute to the formation of amyloid and the genesis of NIDDM.

We thank Jane Kahl and Karen Laakso for technical assistance, Dan McCormick for synthesis and purification of hIAPP₁₋₃₇, and Ruth Kiefer for editorial and secretarial assistance. This work was supported by grants from the National Institutes of Health (AG08031 and AG14522, NLE and DK44341; P.C.B.), a gift from the Quade Amyloidosis Research Fund (N.L.E.), and Mayo Foundation Research Funds.

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