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To assay different concentrations of arginine and glycine as two osmolytes on human islet amyloid polypeptide conformation under experimental setting

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Aggregation of amylin peptide that cosecretes with insulin has an important role in the pathogenesis of type 2 diabetes. Hence, inhibition of the formation of β-amyloid fibrils would be an ideal goal for management of diabetes. Here, we investigated the inhibitory effect of glycine and arginine on the amylin aggregation in experimental conditions. Using fluorescence spectrographic analysis with thioflavin T and visualization of amyloid fibers by atomic force microscopy, different concentrations of arginine and glycine were evaluated on amylin conformation under near-physiological circumstances. The results obtained from the *in vitro* study showed that 240 h incubation by shaker incubator at 37°C, arginine with concentration of 50, 100 and 150 µmol/L inhibited fibril formation significantly (P <0.001). However, at 10 µmol/L, it had insignificant effect on human isle amyloid peptide conformation. The obtained data also demonstrated that glycine with concentrations of 50, 100 and 150 µmol/L had inhibitory effects on formation of beta-amyloid sheet significantly (P <0.001). It may be concluded that isle amyloid toxicity to β-cells may be reduced by the two amino acids so that these compounds are suggested for development of new therapeutics for diabetes.

Keywords: Amylin fibrils, β-Amyloid sheet, Chaperone, Diabetes mellitus

Diabetes mellitus, as one of the four priority noncommunicable diseases (NCDs), is now a target disease for world community for prevalence and the increasing number of cases over the past few decades. Type 2 diabetes mellitus has become a significant global health care problem, accounts for about 90-95% of all the cases of diabetes and estimated to rise to 439 million by 2030[1-3]. Obesity, unhealthy diet and physical inactivity are the main risk factors. WHO's voluntary global targets for prevention and control of NCDs include halting the rise in diabetes and to reduce its mortality by 25% relatively by the year 2025[1].

Technically, type 2 diabetes is described by a deminiuation in β-cell mass as a consequence of misturned human islet amyloid polypeptide (hIAPP) which forms toxic aggregates that destroy pancreatic β-cells[4-6]. Human islet amyloid polypeptide or amylin is a small hormone secreted with insulin from β-cells of pancreas. The polypeptide function is the management of gastric emptying, glucose homeostasis, and other metabolic actions[7]. In type II diabetic patients, amylin is abnormally increased, self-assembled into amyloid sheet, and finally results into the apoptotic death of β-cells by mechanisms that are not completely understood[8,9]. Osmolytes are small molecules synthesized by all organism to balance osmotic pressure, stabilize protein structure in the native form and reestablish enzymatic activity[10-12]. They share the ability to discriminate and attach to nonnative proteins thus prevent indefinite aggregation[13-15].

Increasing occurrence of diabetes worldwide demands multidisciplinary approach for effective prevention and treatment. The presence of amyloid deposits within the islets of Langerhans is the prominent feature of type 2 diabetic persons[16]. Inhibition of the formation of β-amyloid fibrils would be enticing therapeutic targets for the treatment of diabetes. Taking this clue, here in this study we tried to evaluate latent osmolytic functions of arginine (Arg) and glycine (Gly) on human isle amyloid peptide folding in near to physiological setting for possible application in managing diabetes.

Material and Methods

Synthetic peptide of human amylin and additional applied materials were bought from Sigma-Aldrich Corporation. Human amylin used in this project had the following characteristics: (1-37) (Lys-Cys-Asn-Thr-Ala-Thr-Cys-Ala-Thr-Gln-Arg-Leu-Ala-Asn-Phe-Leu-Val-His-Ser-Ser-Asn-Asn-Phe-Gly-Ala-Ile-Leu-Ser-Ser-Thr-Asn-Val-Gly-Ser-Asn-Thr-Tyr-NH2, intra-molecular disulfide bridge: between Cys2 and Cys7). Its purity was 97% and the lyophilized salt included

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70% peptide by weight. Amylin stock solution was prepared by adding 1.0 mL dimethylsulfoxide (DMSO) to dry purified peptide, sonicating at room temperature for 15 min[15].

Groups designing

In order to assay the effects of different concentrations of Arg and Gly on amylin aggregation and amyloidogenesis, control and treated groups were considered. The peptide stock solution was diluted by modified Krebs-Hensleit (KH) buffer (Nacl 123.5 mmol/L, Glucose 11.0 mmol/L, $CaCl_2$ 1.4 mmol/L and NaN_3 0.05%W/V)at pH 7.4 to the final concentration of 10 µmol/L. Different concentrations of Arg (10, 50, 100 and 150 µmol/L) and Gly (10, 50, 100 and 150 µmol/L) were prepared in KH buffer containing 10 µmol/L amylin as treated groups, separately. The samples without Arg and Gly were selected as the control group. All the studied groups were incubated at 37°C for 240 h with shaking by a shaker incubator (GFL 3031, Germany)[15].

Conformational and folding monitoring

The likely antiamyloidogenic role of Arg and Gly on human islet amyloid polypeptide folding assessed through fluorescence spectrographic analysis with Thioflavin T (ThT), The intrinsic fluorescence (IF) of the peptide tyrosine residue and so visualization of amyloid fibers using atomic force microscopy (AFM).

ThT assay & IF assay

Thioflavin T assay was performed by adding 40 µL of each incubated solution to 700 µL of 10 µmol/L ThT solutions. Fluorescence measurements were recorded in a Perkin-Elmer LS55 fluorescence spectrometer (Perkin-Elmer LS55, USA) at 25°C using a 1-cm path length quartz cell. The ThT signal was quantified by averaging the fluorescence emission at 485 nm (slit width = 10 nm) when excited at 440 nm (slit width = 5 nm)[15]. The intrinsic fluorescence (IF) of the peptide tyrosine residue was measured for the studied groups after 168 h averaging the fluorescence emission at 304 nm when excited at 270 nm[15].

Atomic force microscopy (AFM)

Samples were prepared for AFM imaging by drying a 5µL sample from the reaction mixture on freshly cleaved mica plates. Mica plated containing the samples were dried for about 2 min at ambient temperature. Buffer and salt components were washed from the surface of the mica with deionized water, and the mica was dried again. The samples were imaged with a Veeco AFM. The images were taken in the noncontact AFM imaging mode. Recorded images were analyzed using WSxM software[17].

Statistical analysis

Descriptive statistics was accomplished to obtain means and standard deviations. Statistic significance level was established at $P < 0.05$. Analysis of data was performed using SPSS statistical software package.

Results & Discussion

Fluorometric assay indicated that amylin itself readily aggregated and produced a ThT positive material in control group. Data indicated that before incubation at zero time, ThT fluorescence mean value for control group was 39.16 which at 240 h had increased to mean value of 58.30 ($P < 0.001$). In Arg treated groups, ThT fluorescence assay indicated that 10 µmol/L of Arg had no significant effect on amylin conformation ($P > 0.05$) whereas 50, 100 and 150 µmol/L concentrations of Arg significantly ($P < 0.001$) inhibited amylin misfolding by 14.9, 22.8 and 23.7%, respectively after 240 h incubation at 37°C. It was interesting that by increasing of Arg concentration more than 100 µmol/L, inhibitory level of this amino acid was constant so that 100 and 150 µmol/L of Arg had no significant difference in the ThT fluorescence at the end of incubation time ($P > 0.05$) (Fig. 1). Effects of different concentrations of Gly (10, 50, 100 and 150 µmol/L) were evaluated on amylin folding. These data indicated that compared to control group, 10 µmol/L of Gly had no significant effects on ThT fluorescence of amylin ($P > 0.05$), whereas by increasing of Gly concentration to 50, 100 and 150 µmol/L, ThT fluorescence was decreased significantly ($P < 0.001$). Glycine at 10, 50, 100 and 150 µmol/L reduced amylin aggregation and misfolding by 3.8 ($P > 0.05$) 10.9, 15.6 and 16.1%, respectively compared to the control group ($P < 0.001$) (Fig. 1). It should be noted that the inhibitory strength of Arg in amyloid formation was more than Gly. Addition of Arg and Gly significantly ($P < 0.001$) reduced the intrinsic fluorescence (IF) of amylin compared to the control (Fig. 2). Morphological changes of amyloid fibers in the control and treated groups were monitored using atomic force microscopy (AFM). The figure showed in control group after incubation time, human islet amyloid polypeptide had self aggregation and made long and thick fibrils (Fig. 3A). In treated groups, arginine and glycine reduced the amylin aggregation and fibrils diameters significantly so that in comparison to

controls, almost no fibril was observed in the presence of Arg and much less fibrils were detected in the presence of Gly (Fig. 3 B & C).

Protein conformational disorders similar to type-II diabetes results from misfolding and aggregation of related proteins or peptides into amyloid fibrils[18]. Normal solution of hIAPP tolerates misfolding giving rise to beta-amyloid fibrils in the pancreas of diabetic individuals that impair the functionality and viability of beta-cells and may lead to apoptosis[18-20]. It has been documented that certain small organic molecules, known as osmolytes have the capacity to fixate origin conformation of proteins and prevent misfolding and aggregation[21]. Only little data is available in literature relating to the effect of Arg and

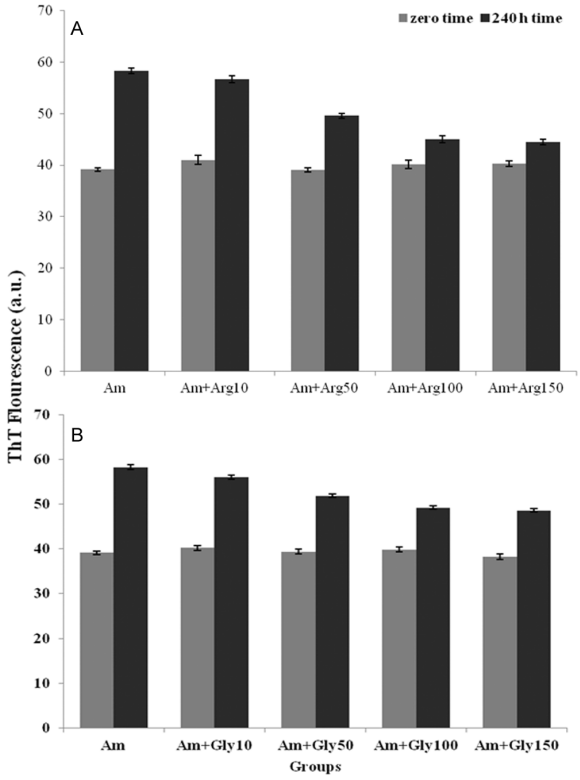

Fig. 1— Thioflavin T fluorescence assay of different concentration of (A) Arginine; and (B) Glycine on human islet amyloid polypeptide conformation. [All groups were incubated at 37°C for 240 h with shaking by a shaker incubator. At zero time (before incubation) there were no significant differences between groups (P >0.05). At the end of incubation time, compared to control, Arg and Gly inhibited amylin aggregation and misfolding significantly (P <0.001). Data have been shown as Mean±SEM, n=5. Am, Amylin]

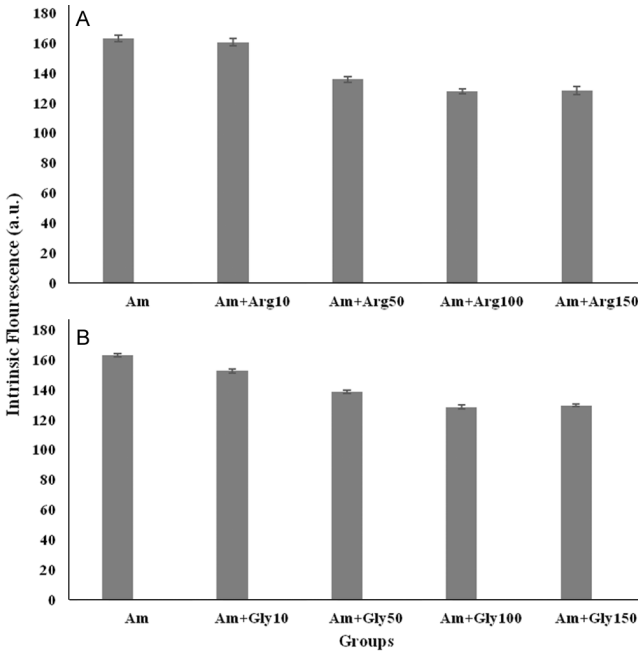

Fig. 2— Intrinsic fluorescence of the Amylin, Arginine and Glycine treated groups. [Tyrosine intrinsic fluorescence of amylin solutions in the absence and presence of the (A) arginine; and (B) glycine were measured after 168 h incubation in 37°C. Data have been shown as Mean±SEM, n=5. Am, Amylin]

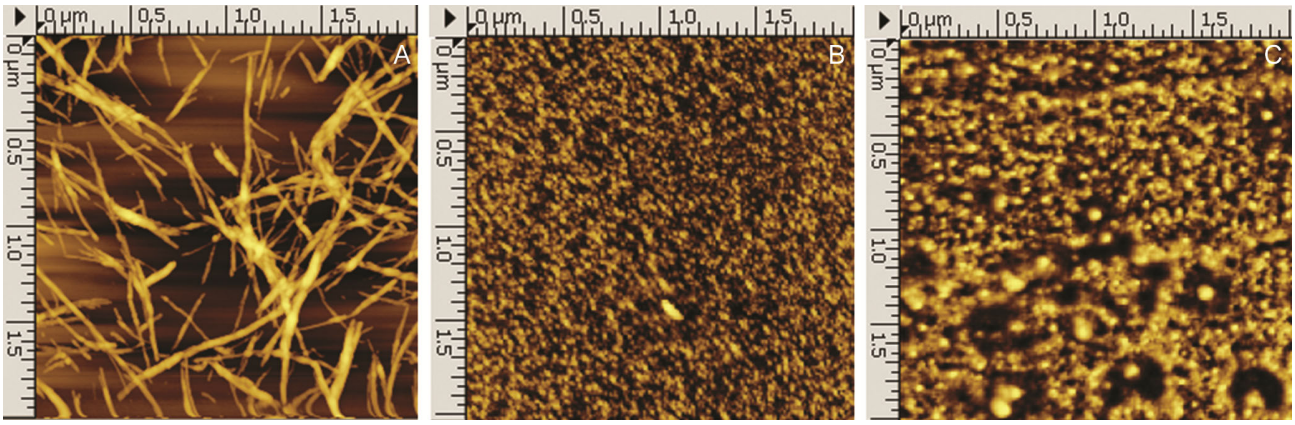

Fig. 3—AFM imaging for human islet amyloid polypeptide (hIAP) in the absence and presence of Arginine and Glycine. [It shows human islet amyloid polypeptide had self aggregation and made long and thick fibrils (A), but argentine and glycine significantly inhibited amylin misfolding and aggregation so as to prevent fibril formation (B and C)]

Gly on amylin amyloidogenesis, thus the present study was designed. This study showed that Arg and Gly inhibited amylin aggregation significantly (P <0.05). The formation of amyloid fibrils via self-assembly of the peptide is assumed to be a crucial stage in the etiology of many amyloid disorders, including type-2 diabetes mellitus[22-24]. Preceding documents have revealed that fibrillation of several polypeptides such as amylin is occurred with formation and extension of oxidative stress[8]. In turn, reactive oxygen species (ROS), accelerate fibril creation, probably by oxidation reactions so that the free radicals produced through amyloid fibrillation develop fibrill production[24,25]. ROS may impact disulfide bond formation and next impress the expansion of hIAPP misfolding. Disulfide bonds have significant role in the native folding of secretory and membrane proteins[26,27]. Although the exact mechanism by which Arg and Gly inhibit amyloid formation *in vitro* persists indistinct, it may be stated the repressive power of these amino acids on amyloid fiber formation may be due to their osmolytes or chaperon-like properties. It seems that Arg and Gly exert their effects indirectly by changing the interaction of the peptide with solvent, rather than through any direct interaction with the peptide. Unfavorable contacts among proteins and osmolytes propagate the solvation of the protein with water. This amplified hydration tends toward more condensed polypeptide conformations, in which hydrophobic residues are more tightly separated from polar solvent[28-30]. Thus, Arg and Gly are thought to work by structuring partly folded intermediates and thermo-dynamically fixing folded conformations to a bigger level than unfolded conformations. Understanding the exact mechanism requires further study.

Conclusion

Molecular chaperones are small molecules known to stabilize protein conformation and assist protein folding *in vitro*. They share the ability to recognize and bind nonnative proteins thus preventing unspecific aggregation and toxicity. chaperon-like properties of Arg and Gly were examined on the formation, and destabilization of amylin amyloid fibril, *in vitro*. For the first time in the literature, we expressed that different concentrations of these amino acids inhibited amylin amyloid formation significantly. It may be accomplished that these two compounds should be used as a supplement in the development of effective drugs used in diabetes management.

Conflict of interest

There is no conflict of interests.

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