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IAPP aggregation and cellular toxicity are inhibited by 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose

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Abstract

The polyphenol, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (PGG) has been found to exhibit a host of positive pharmacologic activities, including anti-cancer and anti-diabetic. Little is known about the mode of action of PGG in yielding these positive activities. We show here that PGG is a potent inhibitor of IAPP (islet amyloid polypeptide, amylin) aggregation. Preventing the initial aggregation event of IAPP is one strategy for slowing, and possibly preventing, the toxic effects of IAPP oligomeric intermediates. Equal molar ratios of PGG to IAPP substantially reduced the ability of IAPP to bind thioflavin T. Atomic force microscopy revealed that PGG prevented amyloid-based fiber formation under rigorous conditions conducive to forming IAPP aggregates. PGG was also found to protect PC12 rat cells from toxic IAPP. PGG was compared to the known amyloid inhibitors (and structural relatives); tannic acid and gallic acid. In every test, PGG was far superior to tannic and gallic acids at inhibiting amyloid aggregation. These results indicate that PGG is a potent inhibitor of IAPP amyloid aggregation and a potential lead molecule for development of an amyloid inhibiting therapeutic.

Keywords

Amyloid inhibition; diabetes; islet amyloid polypeptide

Introduction

The amyloid diseases represent a serious challenge to the overall health of our society. While the death rates of most major diseases continue to drop, those of the amyloid-based diseases (such as Alzheimer's disease and type 2 diabetes) continue to rise. While the ultimate cause of these diseases remains uncertain, it is evident that the misfolding, and concomitant aggregation, of proteins is a factor in their progression. In type 2 diabetes, the amyloidogenic protein, islet amyloid polypeptide (IAPP), appears to be linked to the loss of the pancreatic β cells necessary for insulin secretion. A growing body of evidence suggests that the toxic form of IAPP responsible for cell death is not necessarily the amyloid fibers, but is instead the small soluble oligomers which may be "on the pathway" to fiber formation. While the exact structure of the toxic oligomeric species has not been directly

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determined, several structures that may be closely related to the toxic oligomers have been determined. Ramamoorthy and co-workers have solved the NMR structures of several human and rat IAPP peptides under a variety of conditions, including within micelles [1-5]. Recent work by Eisenberg [6] and coworkers on the amyloidogenic protein αB crystallin suggests these soluble and toxic oligomers adopt a β-barrel fold termed “cylindrins”. While it remains to be seen if the soluble oligomers of IAPP adopt a cylindrin fold, it is clear that the aggregation of IAPP leads to cellular toxicity and the loss of pancreatic β-cells.

A potential therapeutic strategy for preventing the progression of amyloid diseases, such as type 2 diabetes, is to prevent the formation of toxic oligomers. This strategy requires a substance to inhibit the aggregation of IAPP long enough to allow the body to remove the monomeric/soluble IAPP before self-assembly into toxic oligomers can occur. Several polyphenol compounds have been shown to act as inhibitors of peptide aggregation for the amyloidogenic proteins Aβ and tau (involved in Alzheimer’s disease) and α-synuclein (in Parkinson’s disease) [7-9]. The polyphenol, epigallocatechin 3-gallate, inhibits the aggregation of several peptides and its interactions with the amyloidogenic peptides human calcitonin and SEVI have been determined at the molecular level [10,11]. Recently, several polyphenols have been shown to inhibit IAPP aggregation [12-14]. Many of these polyphenols, such as resveratrol, appear to interact with the prefibrillar structures of the amyloid proteins, rather than with the monomeric amyloid proteins [15]. It remains to be seen as to whether inhibition of aggregation at the monomeric level or the prefibrillar level is the more desirable for production of a therapeutic compound. Here, we describe the ability of the polyphenol, 1,2,3,4,6-penta-O-galloyl-β-d-glucose (PGG; Figure 1) to inhibit the aggregation of IAPP in a concentration-dependent manner. More importantly, we describe the ability of PGG to rescue mammalian PC12 cells from the toxic effects of amyloidogenic IAPP. The amyloid inhibiting ability of PGG was directly compared to that of the structurally related compounds; tannic acid and gallic acid (both of which have been reported to inhibit amyloid aggregation [16-19]). In all cases, it was found that PGG was a superior inhibitor of IAPP-based amyloid formation compared to tannic and gallic acids.

Methods

Thioflavin T binding assays

Synthetic IAPP (GenScript Corporation, Picataway, NJ, >95% pure) was first disaggregated in hexafluoroisopropanol (HFIP; Sigma-Aldrich, St. Louis, MO) in a sonicating water bath. HFIP was removed under speed-vacuum. The resulting peptide sample was dissolved in 20 mM of Tris buffer, pH 7.40, containing various concentrations of PGG, tannic acid or gallic acid. The final in-solution concentration of IAPP was 106 μM. Aggregation of IAPP was promoted by incubating samples at 37 °C with shaking (200 rpm). A 17 μL aliquot of each sample was removed at indicated time points and mixed with 663 μL of 50.0 μM thioflavin T (ThT) in 20 mM of Tris buffer, pH 7.40. The ThT mixture was incubated at room temperature in the dark for five minutes before recording the ThT fluorescence emission spectrum ($Ex_{450\ nm}$) using an F-7000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan).

Atomic force microscopy

Synthetic IAPP was prepared as described above and incubated with shaking in the presence or absence of PGG, tannic acid or gallic acid. After 30 min of incubation, 15 μL of each sample was deposited onto a freshly cleaved mica surface. The sample was incubated for five minutes at room temperature. The mica surface was washed with 200 μL sterile water and allowed to air dry. The samples were scanned using an MFP-3D atomic force microscope (Asylum Research, Goleta, CA) set on A/C mode and a 240 μm silicon

cantilever (Olympus, Tokyo, Japan). Images shown are the raw data with no flattening. Images were recorded at scan speeds between 0.40 and 0.60 Hz. The data are shown as height images with a range of 15 nm and an offset of 0 nm.

PC12 cell rescue

PC12 cells were incubated with 10 μM of synthetic IAPP prepared as described above. Equal amounts of PC12 cells were plated in triplicate and incubated overnight in a 96-well plate. After 24 h, F-12K media with phenol red was replaced by DMEM/F-12 (1:1) without phenol red. HFIP was evaporated from IAPP using a centrivap concentrator (LabOnco, Kansas City, MO). IAPP was re-suspended in DMEM/F12K media and added to the appropriate wells at a final concentration of 10 μM. Stock solutions of gallic acid, tannic acid and PGG were diluted with DMEM/F12 media and added to the appropriate wells to a final concentration of 10 μM. The treated cells were incubated for 22 h at 37 °C. Thiazolyl blue tetrazolium bromide (MTT) was added into each well and incubated at 37 °C for two hours. Formazan crystals, formed on the bottom of the wells, were re-suspended in a solubilization buffer (20% SDS and 50% dimethylformamide). The absorbance in each well was taken at 562 nm using a BioRad 550 microplate reader (Hercules, CA). PC12 cells and media were obtained from ATCC (Manassas, VA). All incubations took place in a water jacketed incubator in 5% carbon dioxide at 37 °C (Shell Lab, Cornelius, OR). The MTT assay was done in triplicate, with each well containing the same number of cells. The average percentage of cell viability was calculated for each of the conditions. The averages of three separate experiments are shown.

Results

Gallic and tannic acids are known inhibitors of amyloid aggregation. The goal of this work was to investigate the inhibitory potential of these compounds, and the structurally related compound PGG, on the aggregation of IAPP. ThT-binding experiments are a common method for quantitating amyloid formation over time. When dissolved in an aqueous solution, ThT has negligible fluorescence emission when irradiated with 450 nm light. However, ThT fluorescence increases in a concentration-dependent manner upon binding to amyloid [20]. Under the aggregation-promoting conditions described above, IAPP began to bind ThT in less than 30 min of incubation (Figure 2). PGG, tannic acid and gallic acid were added separately to test their ability to prevent amyloid-based ThT binding to IAPP. The inhibitors were added to an in-solution concentration of 500 μM, nearly a 5× molar excess compared to IAPP. In the presence of PGG, ThT binding was greatly reduced. Tannic and gallic acids showed a moderate decrease in ThT binding. The decrease in ThT fluorescence after reaching its maximum is commonly witnessed in these assays and is consistent with previously reported amyloid aggregation reactions [21-23]. Tannic acid, gallic acid and PGG had no noticeable effects on ThT fluorescence in the absence of IAPP.

ThT binding is a facile method for estimating the capacity of a given substance to inhibit IAPP aggregation. However, it is possible for the inhibitor compound to interfere with ThT binding, rather than preventing amyloid-based aggregation [24]. To test the ability of PGG to disrupt amyloid aggregation, atomic force microscopy (AFM) was used to visualize the amount of IAPP fiber formation (Figure 3). IAPP incubated under the aggregation-promoting conditions described above produced a substantial amount of fibers (Figure 3) under all conditions tested. The longest fibers were found to have measurable heights of approximately 2 nm and lengths over 3 μm, suggesting the formation of protofibrils. Smaller fibers are also visible with heights of approximately 1.1 nm, suggesting the formation of protofilaments. When IAPP was incubated with a 106 μM solution of PGG (1:1 molar ratio), virtually no insoluble aggregates were detected (Figure 3). Under no conditions tested were fibers detected when IAPP was incubated with an equal concentration of PGG.

To test the potential of PGG to protect living cells from IAPP, the MTT test was performed using PC12 cells. The MTT assay, which quantitates the oxidative capacity of PC12 cells after treatment with IAPP and/or PGG, tannic acid and gallic acid, indicated that PGG had superior protective properties (Figure 4). The MTT levels of PC12 cells alone were set as 100%. The addition of IAPP to the PC12 cells decreased the cell viability to 79% in less than 24 h. The addition of gallic acid or tannic acid to the IAPP kept cell viability at 79–80%, while the addition of PGG rescued the cells (99% viability). Gallic acid, tannic acid and PGG alone (in the absence of IAPP) had no effect on the cells.

Discussion and conclusion

Both tannic and gallic acids have been shown to inhibit amyloid aggregation. Tannic acid has been shown to destabilize Aβ fibrils and inhibit Aβ aggregation in a concentration-dependent manner [16]. Tannic acid was reported to be the most potent polyphenol inhibitor out of the 2000 compounds tested in inhibiting the aggregation of PrP^{sc}, the scrapie form of the prion protein [17]. Tannic acid has also been shown to inhibit fiber formation of α-synuclein, the protein linked to Parkinson's disease [18]. Likewise, gallic acid, found in abundance in green and black teas, appears to inhibit Aβ aggregation [8] and protect living cells from Aβ toxicity [19]. Surprisingly, we are unaware of studies showing tannic acid or gallic acid acting as inhibitors of IAPP aggregation. Our results, here, suggest that both tannic and gallic acids are poor inhibitors of IAPP aggregation and that these two polyphenolic acids are weak protectors of IAPP-based cellular toxicity.

While both tannic and gallic acids appear to be poor inhibitors of IAPP-based aggregation, the structurally related compound PGG appears to be a strong inhibitor of IAPP-based aggregation. We found that PGG functioned in a concentration-dependent manner to inhibit fiber formation and ThT binding under extreme conditions known to promote IAPP aggregation. Additionally, we found that PGG protected mammalian cells from the toxic effects of IAPP. It remains to be determined why PGG functions as a strong inhibitor of IAPP aggregation while its structural relatives, tannic and gallic acids, are not. Studies with inhibitors of Aβ and tau amyloid aggregation indicate that the most potent inhibitors tend to be planar substances [9,25,26]. PGG is likely to interact with IAPP as a planar molecule. Tannic acid, with a second ring of gallate groups compared to PGG, is unlikely to remain constrained to a planar configuration. PGG has been described as a natural diabetes therapeutic [27], yet little is known about how this compound produces these effects. Our results suggest that one possible explanation for PGG's effect as an anti-diabetic comes from its ability to inhibit the formation of IAPP aggregates.

Acknowledgments

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Abbreviations

AFM	atomic force microscope
IAPP	islet amyloid polypeptide
HFIP	hexafluoroisopropanol
MTT	thiazolyl blue tetrazolium bromide
PGG	1,2,3,4,6-penta-*O*-galloyl-β-D-glucose
ThT	thioflavin T

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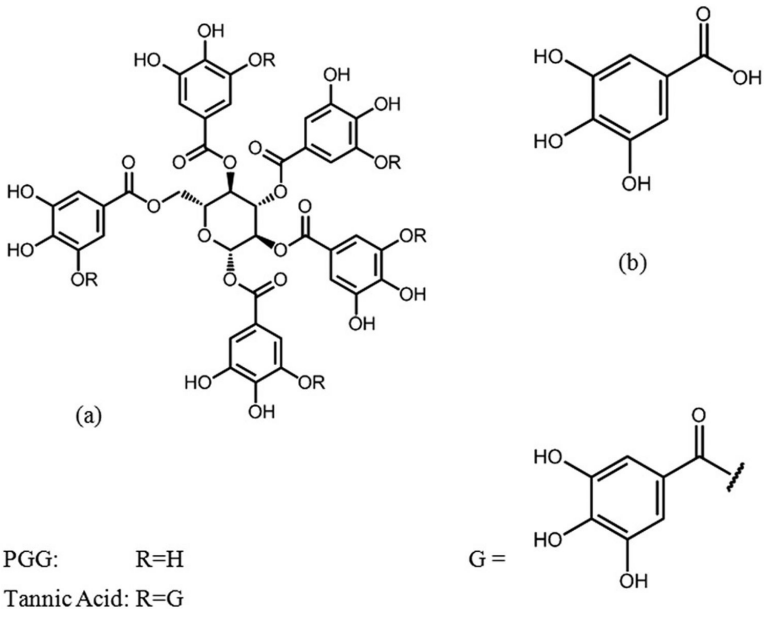

Figure 1.
Chemical structure of: (a) PGG and tannic acid and (b) gallic acid.

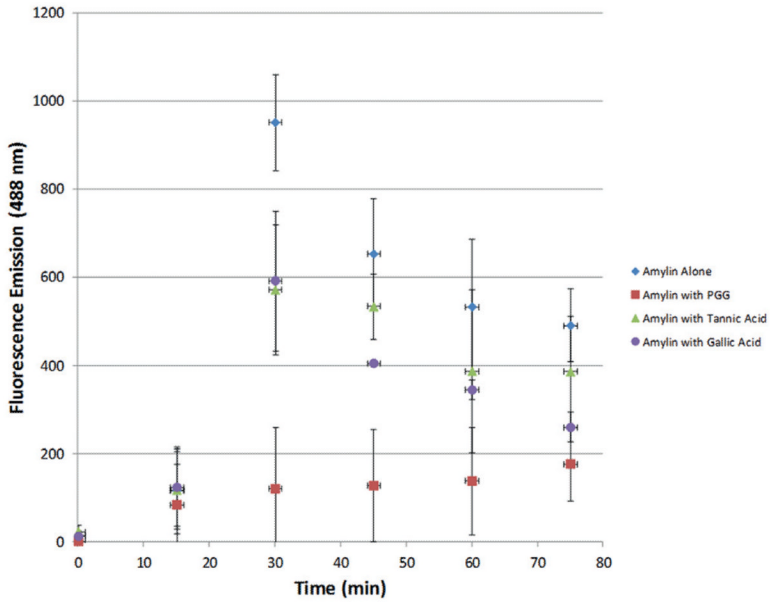

Figure 2.

ThT binding of IAPP alone and in the presence of PGG, tannic acid and gallic acid. IAPP was 106 μM for all samples. Each inhibitor was 500 μM (the highest concentration of tannic acid that remained soluble without the aid of solvents). Data represent the average of four separate runs with error bars indicating the SD.

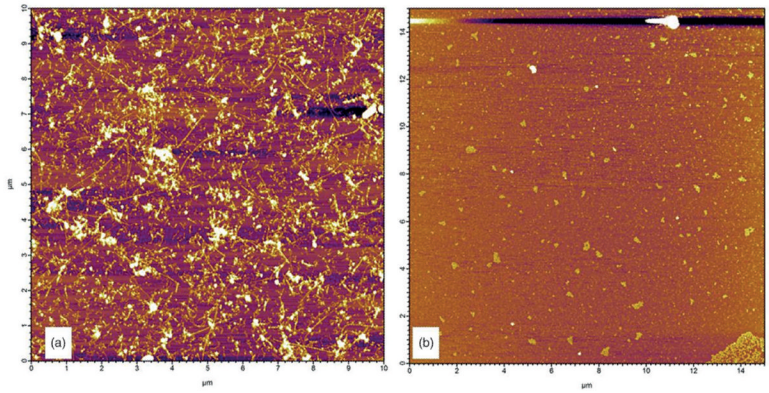

Figure 3.
AFM images of 106 μM IAPP: (a) alone and (b) with a 1:1 molar ratio of PGG.

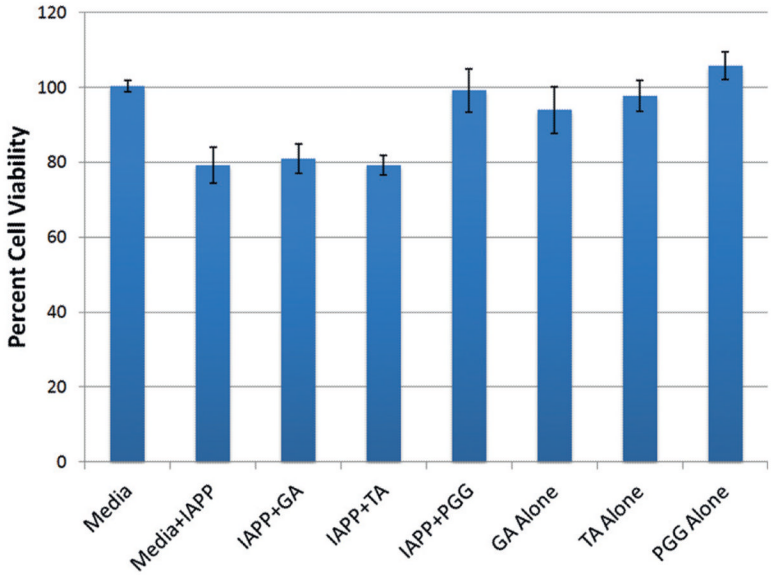

Figure 4.

Viability of PC12 cells after treatment with IAPP and IAPP with PGG, gallic acid and tannic acid. The results shown here are the average of three independent experiments performed in triplicate (total of nine). The error bars represent the SD within the experiment and show a significant effect of PGG on the viability of PC12 cells in the presence of IAPP.