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Full Length Research Paper

The role of two natural flavonoids on human amylin aggregation

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Misfolded amylin, the human polypeptide hormone, forms amyloid deposits in pancreatic islets. These amyloid deposits contribute to the dysfunction of beta-cells and the loss of beta-cell mass in type 2 diabetes mellitus (T2DM). Inhibition of amylin fibrillization has been regarded as a potential therapeutic approach for T2DM. Using fluorescence spectroscopic analysis with thioflavin T, the role of two naturally occurring flavonoids named myricetin and epigallocatechin gallate on human amylin hormone fibrillization and destabilization of fibrillar aggregates were examined under near physiological conditions. The results showed that after 168 h incubation by shaker incubator in 37°C. Myricetin at 10 and 40 µM repressed amylin amyloid formation by 25.3 and 22.4%, respectively ($p<0.05$), and the similar values of epigallocatechin gallate inhibited the formation of β-sheet structure by 18.1 and 16.7%, respectively ($p<0.05$). The obtained data also confirmed that amyloidal sheet opening was induced by myricetin and epigallocatechin gallate significantly ($p<0.05$). Therefore, it was concluded that islet amyloid cytotoxicity to β-cells may be reduced by these two flavonoids, and these compounds should be key molecules for the development of the therapeutics for diabetes mellitus.

Key words: Human islet amyloid polypeptide, hyperglycemia, myricetin, epigallocatechin gallate, flavonoids.

INTRODUCTION

Protein misfolding plays an important role in more than twenty destructive human diseases, including Alzheimer's disease, Parkinson's disease and type 2 diabetes mellitus. A key factor in the development of type 2 diabetes is the loss of insulin producing beta-cells. Amylin, a peptide hormone co-secreted with insulin in the pancreatic beta-cells, is suggested to play a crucial role in this process since amyloid deposits of this peptide are found in the islets of Langerhans in the vast majority of type 2 diabetes patients (Young, 2005; Reddy Nanga et al., 2011; Cheng et al., 2012). Owing to the increasing prevalence of diabetes, multidisciplinary study aimed at prevention and treatment is one of the world-wide research priorities. Considering the high cost of medication and side effects of synthetic medicine, as well as lack of full recovery of diabetic patients treated with chemical agents, has encouraged the researchers to use herbal medications (Akbarzadeh et al., 2012).

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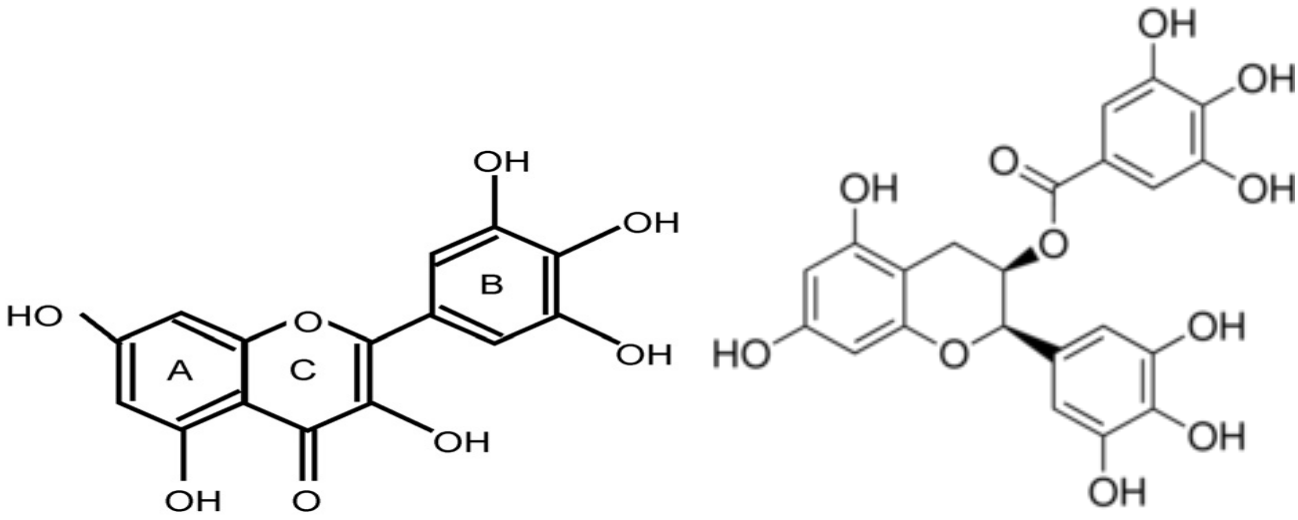
Flavonoids are ubiquitous group of polyphenolic substances present in a variety of plants, such as onion (Nasri et al., 2012). Myricetin (Figure 1) is a natural flavonoid that is commonly found in tea, berries, fruits, vegetables and the medicinal herb *Abelmoschus moschatus* (Harnly et al., 2006). Myricetin is reported to have many therapeutic applications, such as anticarcinogenic action (Ko et al., 2005), antioxidative and cytoprotective properties, and ability to lower plasma glucose in diabetic rats (Liu et al., 2006, 2007). Epigallocatechin gallate (Figure 1), the major component of polyphenols in green tea, has also attracted considerable attention for its antioxidative, anti-inflammatory, anti-mutagenic, anti-thrombotic and neuroprotective properties (Higdon and Frei, 2003; Sheng et al., 2011; Mandel et al., 2008; Koh et al., 2006: Sachdeva et al., 2011).

The improvement of efficient inhibitors against the toxic formation of amylin amyloids has been enormously challenging and would be attractive therapeutic targets for the treatment of diabetes mellitus. Hence, this present study was carried out to assess the potential effects of



Myricetin **Epigallocatechin gallate**

Figure 1. Structures of myricetin and epigallocatechin gallate.

two flavonoids, myricetin and epigallocatechin gallate, on amylin aggregation and destabilization of preformed amyloid under near physiologic circumstances.

MATERIALS AND METHODS

Preparation of amylin stock solution

Human amylin full length peptide and other materials were purchased from Sigma-Aldrich Company.The human amylin used in this study 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 70% peptide by weight. Amylin stock solution was prepared by adding 1.0 ml dimethylsulfoxide (DMSO) to dry purified peptide, and then sonicating at room temperature for 15 min. The experiments were performed in the two different phases as follows:

The first series of experiment: In order to assay the effects of different concentrations of myricetin and epigallocatechin gallate on amylin aggregation and amyloidogenesis, control and treated groups were considered.

The peptide stock solution was diluted by PBS 50 mM at pH: 7.5, to the final concentration of 10 µM. Different concentrations of myricetin (10 and 40 µM) and epigallocatechin gallate (10 and 40 µM) were prepared in PBS buffer containing 10 µM amylin as treated groups, separately. The samples without myricetin and epigallocatechin gallate were selected as the control group. All studied groups were incubated at 37°C for 168 h with shaking by a shaker incubator (GFL 3031, Germany).

The second series of experiments: These series of experiments were carried out to elucidate the destabilizing effect of the two flavonoids on preformed amyloid sheet of amylin. For this purpose, the amyloid prepared from the previous step was used. Amyloid was incubated with different concentrations of each of the agents for 6 h in 37°C.

Amyloid formation and destabilization assay

To determine the level of amyloid beta-pleated sheets at the end of the two series of experiments, thioflavin T (ThT) fluorescent assay was used. Thioflavin T assay was performed by adding 40 µL of each incubated solution to 700 µL of 10 µM ThT solution. Fluorescence measurements were recorded in a Perkin-Elmer LS55 fluorescence spectrometer (Perkin-Elmer LS55, USA) at room temperature 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).

Immunofluorescence (IF) assay

The intrinsic fluorescence of the peptide tyrosine residue was measured for the studied groups after 96 h by averaging the fluorescence emission at 304 nm when excited at 270 nm.

Statistical analysis

Descriptive statistics was accomplished to obtain means and standard error of mean. Between groups, comparisons were performed with independent sample t-tests. Statistic significance level was established at $p<0.05$. Analysis of data was performed using SPSS statistical software package.

RESULTS AND DISCUSSION

The first run of experiments showed that amylin itself readily aggregated and formed a ThT-Positive material in control group. The results indicated that at zero time, ThT-fluorescence mean value for control group was 30.35, which at 168 h had increased to mean value of 49.1 ($p<0.05$). In myricetin treated groups, ThT fluorescence assay indicated that 10 and 40 µM of myricetin inhibited amyloid formation by 25.3 and 22.4%,

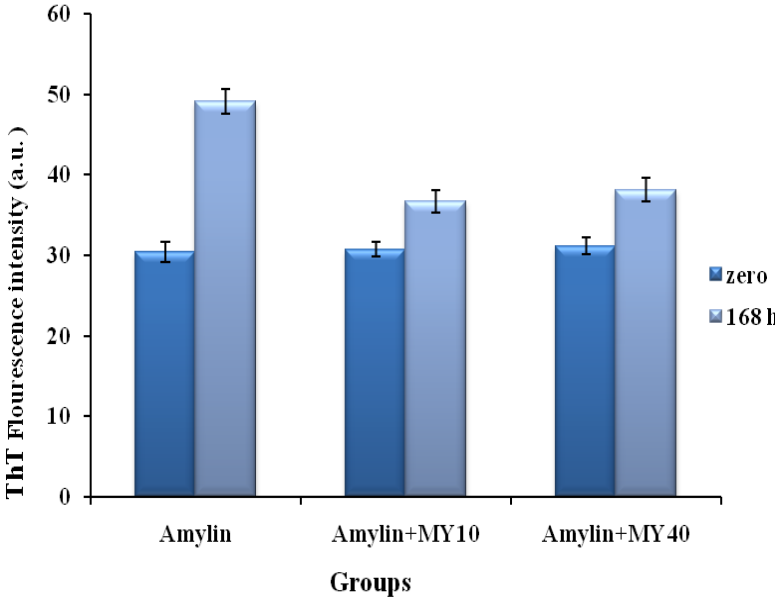


Figure 2. Thioflavin T fluorescence assay of inhibitory effect of Myricetin on amylin aggregation. All groups were incubated at 37°C for 168 h with shaking by a shaker incubator. At zero time (before incubation), there were no significant differences between the three groups: amylin, amylin+MY10 and amylin+MY40 ($p>0.05$). However, myricetin (MY) inhibited amylin aggregation significantly ($p<0.05$) at the end of incubation time. Data are shown as mean ± SEM, n = 5.

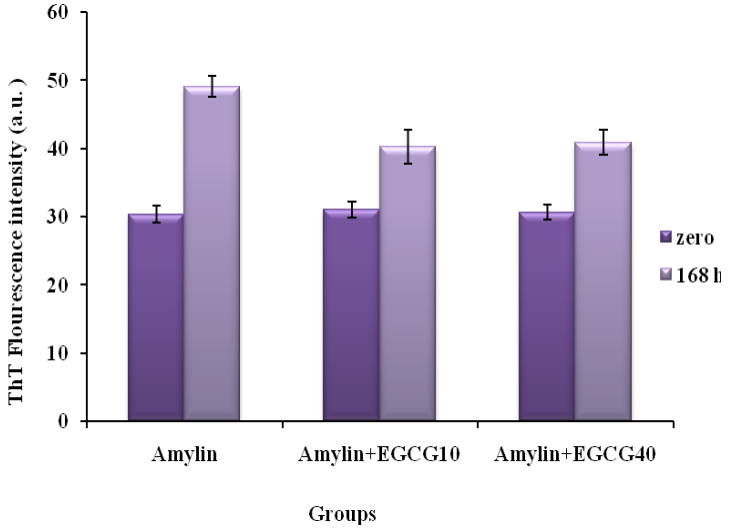


Figure 3. Thioflavin T fluorescence assay of inhibitory effect of epigallocatechin gallate on amylin aggregation. All groups were incubated at 37°C for 168 h with shaking by a shaker incubator. At zero time (before incubation), there were no significant differences between the three groups: amylin, amylin+EGCG10 and amylin+EGCG40 ($p>0.05$). However, epigallocatechin gallate (EGCG) inhibited amylin aggregation significantly ($p<0.05$) at the end of incubation time. Data are shown as mean ± SEM, n = 5.

respectively after 168 h incubation at 37°C ($p<0.05$) (Figure 2). Different concentrations effects of epigallocatechin gallate on amylin aggregation were demonstrated in Figure 3. These data indicated that

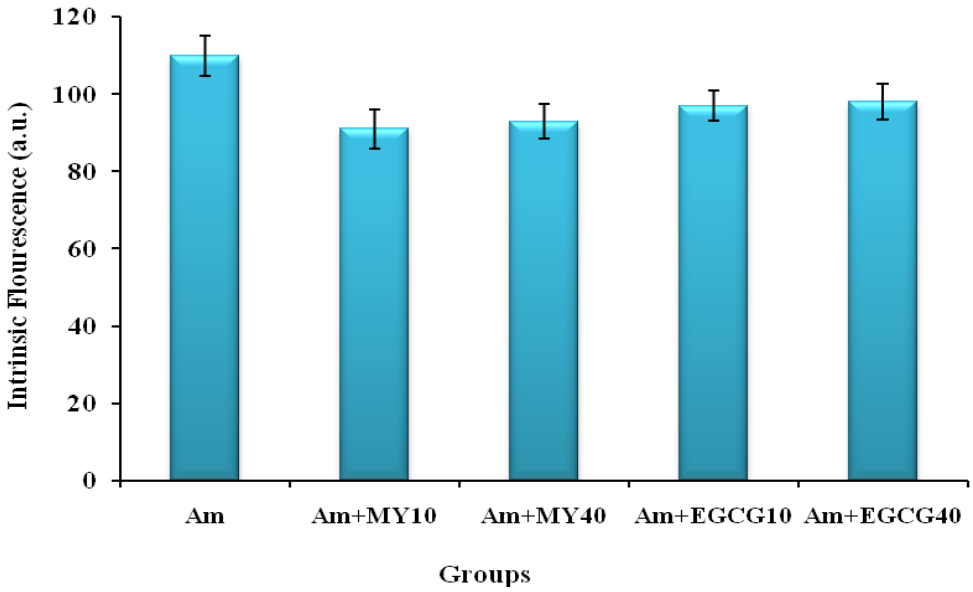


Figure 4. Intrinsic fluorescence of the control and treated groups. Tyrosine intrinsic fluorescence of amylin solutions in the absence and presence of the agents was measured after 96 h incubation at 37°C. Data are shown as mean ± SEM, n = 5.

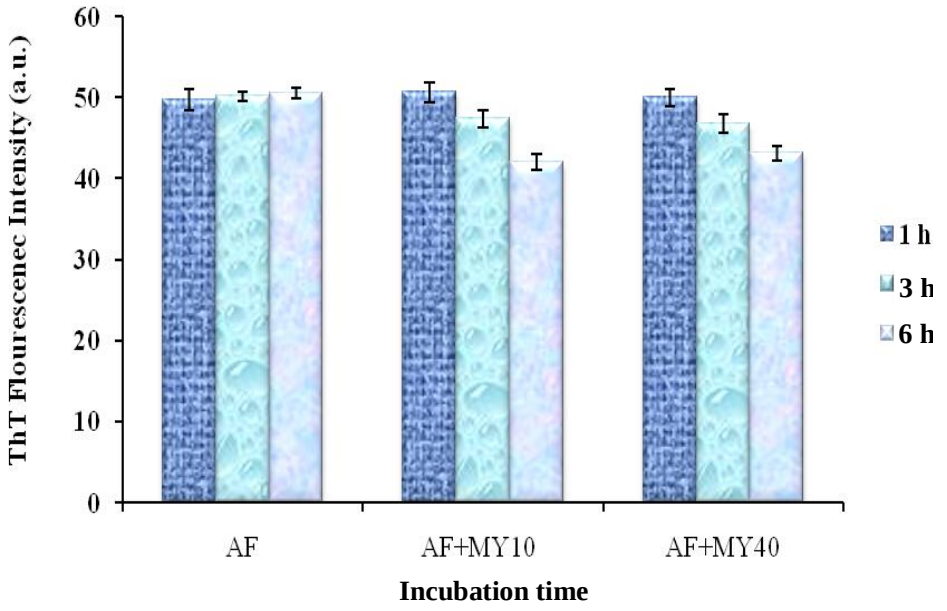


Figure 5. Effect of Myricetin on amylin fibril destabilization. Myricetin (MY) with two different concentrations destabilized amylin fibril (AF) significantly ($p<0.05$) after 6 h incubation.

compared to control group, at the end of incubation time, ThT-fluorescence decreased significantly in the presence of 10 and 40 µM of epigallocatechin gallate by 18.1 and 16.7%, respectively ($p<0.05$). The data also showed that the inhibitory effect of these flavonoids versus amyloid formation were not dose-dependent ($p>0.05$). Figure 4 indicates that the addition of myricetin and epigallocatechin gallate significantly ($P<0.05$) reduced the intrinsic fluorescence (IF) of amylin relative to the control (Figure 4). In addition, amyloid destabilizing effects of these components were shown in Figures 5 and 6. The obtained data from the 2nd run of experiments confirmed that both compounds were able to open the amyloid sheet significantly ($p<0.05$).

Diabetes mellitus is a group of metabolic diseases characterized by abnormally high concentrations of

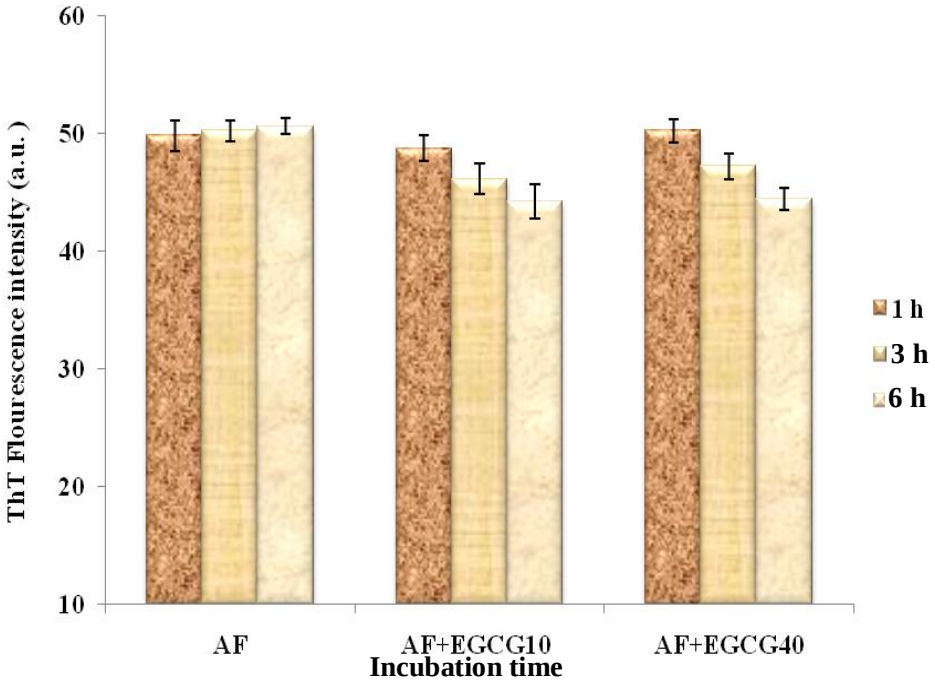


Figure 6. Effect of epigallocatechin gallate on amylin fibril destabilization. Epigallocatechin-gallate (EGCG) with two different concentrations destabilized amylin fibril (AF) significantly ($p<0.05$) after 6 h incubation.

glucose in blood and/or urine. In addition to neurological complications and premature death, consequences of these disorders include vascular complications such as coronary artery disease, cerebrovascular disorders, renal failure, blindness, and limb amputation (Liu et al., 2006). It is implicated that human amylin is a small fibrillogenic protein that is the major constituent of pancreatic islet amyloid, which occurs in most subjects with type 2 diabetes (Konarkowska et al., 2006; Zheng et al., 2010; Wang et al., 2011). Although the amyloid contribution to islet β-cell dysfunction is well clarified in the diabetic patients, the factors affecting this process remain elusive. We previously reported that some herbal compounds influence the aggregation of amylin (Mirhashemi et al., 2012) *in vitro*. Since the existing documents regarding effects of myricetin and epigallocatechin gallate on amylin depositions are very little (Meng et al., 2010; Noor et al., 2012) the present study was designed.

This study showed significant inhibitory role of myricetin and epigallocatechin gallate on amylin amyloidogenesis and thus demonstrated the β-sheet opening ability for these compounds. The formation of amyloid fibrils via self-assembly of peptide is assumed to be a crucial step in the pathogenesis of many amyloid diseases, including type 2 diabetes mellitus (Tabner et al, 2001). Previous investigations have shown that fibrillization of several polypeptides such as amylin is accompanied by formation of free radicals. In turn, reactive oxygen species (ROS), mainly free radicals, accelerate fibril formation, possibly via oxidation reactions so that the free radicals formed during amyloid fibrillization enhance fibrillization (Schoneich, 2005; Shoval et al., 2007). ROS may impact disulfide bond formation (Cummings et al., 2004) and subsequently influence the development of amylin misfolding. Disulfide bonds are important for proper protein structure, biological activity, and stability of many secreted and membrane proteins (Kopito and Ron, 2000; Anelli et al., 2002; Fassio and Sitia, 2002). Although the exact mechanism by which myricetin and epigallocatechin gallate inhibit amyloid formation and destabilize preformed amyloid *in vitro* remains unclear, it may be suggested that the inhibitory power of these compounds in amyloid fiber formation may be due to their potent antioxidant and free radical scavenging properties (Abdel-Raheem et al., 2009; Zhang et al., 2009; Piao et al., 2008). Flavonoids can provide both short and long-term protection against oxidative stress via a variety of mechanisms, including directly neutralizing toxic ROS through the donation of hydrogen ions (Wanga et al., 2010). Further study is required to elucidate the exact mechanism.

Conclusion

The improvement of efficient inhibitors against the toxic formation of amylin amyloids has been enormously challenging, since amylin is one of the most amyloidogenic polypeptides. Flavonoids are structurally heterogeneous polyphenolic compounds that are widely

distributed in plant foods, and which may exert beneficial effects. The antioxidant activity of myricetin and epigallocatechin gallate was examined on the formation and destabilization of amylin amyloid fibril *in vitro*. Our re showed that these two compounds inhibited amylin amyloid formation significantly. In addition, they destabilized preformed amylin fibrils. It may be concluded that these compounds should be key molecules for the development of the therapeutics for diabetic patients.

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