Coffee components inhibit the amyloid formation of human islet amyloid polypeptide in vitro: a possible link between coffee consumption and diabetes mellitus.

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J. Agric. Food Chem., Just Accepted Manuscript • DOI: 10.1021/jf201702h • Publication Date (Web): 07 Nov 2011

Downloaded from http://pubs.acs.org on November 10, 2011
Coffee components inhibit the amyloid formation of human islet amyloid polypeptide in vitro: a possible link between coffee consumption and diabetes mellitus.

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Abstract

Global epidemic studies suggested that coffee consumption is reversely correlated with the incidence of type 2 diabetes mellitus (T2DM), a metabolic disease. The misfolding of human islet amyloid polypeptide (hIAPP) is regarded as one of the causative factors of T2DM. Coffee extracts have three major active components: caffeine, caffeic acid (CA) and chlorogenic acid (CGA). In this study, the effects of these major coffee components, as well as dihydrocaffeic acid (DHCA) (a major metabolite of CGA and CA) on the amyloidogenicity of hIAPP were investigated by thioflavin-T based fluorescence emission, transmission electronic microscopy, circular dichroism, light-induced cross-linking, dynamic light scattering and MTT-based cell viability assays. Our results suggest that all components show varied inhibitory effects on the formation of toxic hIAPP amyloids, in which CA shows the highest potency in delaying the conformational transition of the hIAPP molecule with the most prolonged lag time, whereas caffeine shows the lowest potency. At a 5-fold excess molar ratio of compound to hIAPP, all coffee-derived compounds affect the secondary structures of incubated hIAPP as suggested by the circular dichroism spectra and CDPro deconvolution analysis. Further photo induced cross-linking based oligomerization and dynamic light scattering studies suggested CA and CGA significantly suppressed the formation of hIAPP oligomers, while caffeine showed no significant effect on oligomerization. Cell protection effects were also observed for all three compounds, with the protection efficiency being greatest for CA, and least for CGA. Our findings suggest that the beneficial effects of coffee consumption on T2DM may be partly due to the ability of the major coffee components and metabolites to inhibit the toxic aggregation of hIAPP.
Key words:

amyloid; caffeine; caffeic acid; chlorogenic acid; dihydrocaffeic acids; islet amyloidogenic polypeptide; type 2 diabetes

Abbreviations:

CA  caffeic acid;
CD  circular dichroism;
CGA  chlorogenic acid;
CQA  caffeoylquinic acid;
DHCA  dihydrocaffeic acids;
diCQA  dicafeoylquinic acid;
DLS  dynamic light scattering;
EGCG  (-)-epigallocatechin 3-gallate;
FCQA  feruloylquinic acid;
HFIP  hexafluoroisopropanol;
hIAPP  human islet amyloid polypeptide;
MTT  3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;
*p-CoQA  *p-coumaroylquinic acid;
T2DM  type 2 diabetes mellitus;
TEM  transmission electron-microscopy;
ThT  thioflavin-T.
Introduction

Diabetes mellitus is one of the most common metabolic diseases. Type 2 diabetes mellitus (T2DM) comprises 90-95% of diabetic patients worldwide (1). The presence of amyloid fibrils in the pancreas, arising from the polymerization of human islet amyloid polypeptide (hIAPP, also known as amylin), is a key indicator of T2DM (2). hIAPP is a 37-residue peptide hormone synthesized by the pancreatic β-cells (3; Figure 1) with a physiological circulating concentration ranging from 1.6 to 20 pM in non-diabetic people (4). The formation of amyloid by hIAPP is regarded to be a causative factor of T2DM (2). Increasing evidence suggests that the formation of hIAPP amyloids can result in β-cell apoptosis (5). It is also accepted that the formation of hIAPP oligomers is more toxic than the mature fibrils (6). Thus preventing the formation of toxic hIAPP amyloid fibrils, especially the most toxic oligomers, has been considered as a novel therapeutic approach for T2DM (7).

Coffee is one of the most common and popular beverages worldwide. Caffeine, caffeic acid (CA) and chlorogenic acid (CGA) are the three most abundant components in coffee (8-10). The major coffee CGAs include 3-, 4-, and 5-caffeoylquinic acids (3-, 4-, and 5-CQA), 3,4-, 3,5-, and 4,5-dicaffeoylquinic acids (3,4-, 3,5-, and 4,5-diCQA), 3-, 4-, and 5-feruloylquinic acids (3-, 4-, and 5-FQA), and 3-, 4-, and 5-p-coumaroylquinic acids (3-, 4-, and 5-p-CoQA) (11). The 3-CQA, which is tested in the present study, accounts for 12% of total CGA (12). It has been estimated that for a regular coffee drinker (3 cups per day), the approximate daily intake of caffeine, CA and CGA from coffee are 500 mg, 500 mg and 1000 mg, respectively (13). Studies have shown that the plasma concentrations of caffeine, CA and CGA after coffee consumption fall in the range of nanomolar to micromolar (11, 14, 15); for example, the plasma concentration of CA reported in different studies range from 81 nM to 1.56
µM after coffee in-take (11, 12). It is also well recognized that these compounds all undergo extensive metabolic modification in vivo after coffee consumption. For example, CGAs have at least more than thirty common metabolites (11, 16), including dihydrocaffeic acid (DHCA) which accounts for greater than 90% of CGA metabolites; moreover, DHCA is also an immediate caffeic acid metabolite (16).

Epidemiological studies indicate that coffee consumption is reversely correlated with the risk of T2DM (17, 18). It has been reported that consumption of four or more cups of coffee per day is generally associated with a substantially 50% lower risk of T2DM (19), and every additional cup of coffee consumed daily was associated with a 7% reduction in the excess risk of T2DM (20). In cross-sectional studies conducted in Japan, Northern and Southern Europe, similar inverse associations between coffee consumption and T2DM or impaired glucose tolerance (an early signal of T2DM), have been observed (21).

Despite all these beneficial effects of coffee consumption on T2DM, the exact underlying molecular mechanisms remain unclear. It has been published that certain polyphenols can inhibit hIAPP amyloid formation and protect β-cells from apoptosis (22). For example, it has been reported that a polyphenol derived from tea, (-)-epigallocatechin 3-gallate (EGCG), can inhibit the toxic amyloid formation of Aβ in Alzheimer’s disease and α-synuclein in Parkinson’s disease (23, 24). Recent studies have found that EGCG can inhibit the fibril formation of hIAPP and also exhibit protective effects on β-cells (25). CA, and CGA, as well as their metabolite DHCA, also have typical polyphenolic structures (Figure 1), raising the possibility that these major coffee components and metabolite may exert their beneficial effects on T2DM by inhibiting the formation of toxic hIAPP amyloids. To test this hypothesis, we examined the effects of these compounds on the formation of hIAPP amyloid with a series of assays.
The fluorescence dye thioflavin-T (ThT) (which specifically binds the β-sheet structure of protein fibrils and gives a strong emission) was used to probe the kinetic process of amyloids formation, circular dichroism (CD) spectroscopy was applied to monitor the secondary structure transition of hIAPP, and the fibril morphology was detected with transmission electron-microscopy (TEM). To explore how coffee components may affect the oligomerization status of hIAPP, Photo-induced Cross-Linking of Unmodified Proteins (PICUP) assay and dynamic light scattering (DLS) measurements were used.
Materials and Methods

Materials

Synthetic hIAPP (1-37) was obtained from Genscript Inc. (Piscataway, NJ, USA). Caffeine, caffeic acid and chlorogenic acid (3-caffeoylquinic acid) were obtained from Aladdin-reagent (Shanghai, China). Dihydrocaffeic acids (DHCA), tris(2,2’-bipyridyl)dichlororuthenium(II) (Ru(bpy)), hexafluoroisopropanol (HFIP) and thioflavin-T (ThT) were purchased from Sigma-Aldrich (St. Louis, USA). INS-1 cells were obtained from the China Center for Type Culture Collection (CCTCC). All other chemicals were of the highest grade available.

Far-UV circular dichroism (CD) and data analysis

CD spectra were recorded at 25 °C under a constant flow of N₂ by using a JASCO-810 spectropolarimeter. Data were recorded from 260 to 190 nm with a 1 mm pathlength. hIAPP was dissolved in 25 mM PBS (pH 7.4) containing 50 mM NaCl and 1% HFIP to a final concentration of 15 µM. The spectra were recorded at time intervals indicated with a scanning speed of 50 nm/min, a response time of 1 s and a bandwidth of 2 nm. Each result is given as the average of three measurements. The data were converted to mean residue ellipticity [θ] and were further analyzed using the software package CDPro as previously described (26).

Amyloid formation and Thioflavin-T (ThT) fluorescence assays

For amyloid formation, hIAPP was dissolved in HFIP and sonicated for 2 min to homogenize the sample. hIAPP was diluted and dissolved in 25 mM PBS (pH 7.4) containing 50 mM NaCl, 1% HFIP to
a final concentration of 15 µM. The solution was incubated at 25 °C with different molar ratios of freshly prepared caffeine, CA, CGA, DHCA and an equimolar mixture of caffeine, CA and CGA for amyloid formation. Solutions were aliquoted at designated time intervals and thioflavin-T based fluorescence assays were used to detect the formation of amyloid; experiments were performed on a Hitachi FL-2700 fluorometer. The excitation and emission wavelengths were set at 450 nm and 482 nm, respectively. The assay solution contains 25 mM PBS (pH 7.4) containing 50 mM NaCl, and 20 µM thioflavin-T. All experiments were repeated at least three times. The following formula was used to fit the kinetic curves as described (27, 28), where Y is defined as the fluorescence intensity, t_{50} is defined as the time when the fluorescence intensity is half of the Y_{max}, and k is the curve constant; the lag time is defined as t_{50}-2/k:

\[ Y = Y_0 + (Y_{max} - Y_0)\left[1 + \exp\left(-\frac{t - t_{50}}{k}\right)\right] \]

**Transmission electronic microscopy (TEM)**

The TEM was performed as we previously described (28); briefly, 5 µl of sample was applied onto a 300-mesh Formvar-carbon coated copper grid (Shanghai, China) followed by staining with 1% fresh prepared uranyl formate. Samples were air dried and observed under a transmission microscope (Hitachi, Tokyo, Japan) operating at an accelerating voltage of 100 kV.

**Photo-induced Cross-Linking of Unmodified Proteins (PICUP) assay**

hIAPP was first dissolved in HFIP and sonicated for 2 min to homogenize the solution. Then hIAPP was diluted in 10 mM phosphate buffer (pH 7.4) to a final concentration of 82 µM peptide and...
1% HFIP. Caffeine, CA and CGA were also freshly dissolved in 10 mM phosphate buffer (pH 7.4). Samples were photo-cross-linked using the PICUP method as described (29). The reaction buffer consists of 150 µM Ru(bpy), 3 mM ammonium persulfate, 41 µM IAPP and different molar ratios of caffeine, CA, CGA, DHCA and an equimolar mixture of caffeine, CA and CGA. The mixture was photoinduced cross-linked by irradiating for 5 s with a 150 W incandescent lamp installed in a house-made dark-box. After irradiation, 7.5 µl lammeli loading buffer was immediately added, followed by denaturation at 97 °C for 10 min. The cross-linked samples were separated on a 20% Tricine-Urea gel as previously described (30) and visualized by silver staining (Beyotime Fast Silver Stain Kit).

Dynamic light scattering

Dynamic light scattering was performed by using a zeta pals potential analyzer (Brookhaven Instruments Corporation, New York, USA). Solutions containing 32 µM hIAPP, 25 mM PBS, 50 mM NaCl in the presence or absence of 160 µM different compounds (1:5 molar ratio) were incubated in the cuvette at 37 °C in the analyzer (Footnote 1), the mean particle size was recorded every 12 min at a 90° angle.

MTT cell toxicity assay

MTT-based cell toxicity assays were performed as described (28). Briefly, the INS-1 cells were plated at a density of 5×10^5 cells/ml (80 µl/well). Following 24 h incubation at 37 °C in 5% CO₂ atmosphere, the medium was aspirated and replaced with fresh medium containing 15 µM hIAPP and various amounts of caffeine, CA and CGA. Cells were further incubated for 24 h. Wells treated with
PBS or EGCG were used as controls. After incubating for 24 h, 20 µl MTT (5 mg/ml) was added to each well and further incubated for 4 h. The absorbance was measured at 570 nm.

*Statistical analysis*

Data were presented as mean ± SD. Data were analyzed by the nonparametric Kruskal-Wallis test followed by the Mann-Whitney test, where $p < 0.05$ was considered significant.
Results

Secondary structures determination by Far-UV circular dichroism (CD) and deconvolution.

To gain insight into the effects of coffee components on the secondary structures of hIAPP aggregation, far-UV CD spectroscopy was applied to monitor conformational changes over time. Consistent with a previous report (31), the CD spectrum of hIAPP was characteristic of predominant random coil structure at the beginning of incubation (Figure 2), which is also supported by CDPro deconvolution analysis (Figure S1). The spectra started to change after incubating for 2 h, with the intensity of the negative band at 225 nm gradually increasing as the incubation time increased, reaching a maximum after 4 h of incubation. This indicates a gradual conformational conversion from an unstructured random coil structure, to a β-sheet-rich structure. Further CDPro deconvolution analysis after incubating for 24 h showed the percentage of unordered structure decreased to 22%, whereas that of the β-structure increased from the initial 29% to 71% (Figure S1).

EGCG has been reported to bind to the oligomers and mature fibrils of hIAPP, and inhibit its amyloid formation (25). Our results showed that equimolar amounts of EGCG slightly altered the secondary structure of hIAPP (Figure 2B). Following time course studies suggested that although EGCG delayed the onset of secondary structural change of hIAPP by about an hour, the extent of β-structure conformational conversion is still comparable to that of hIAPP alone, as suggested by CDPro analysis (Figure S1). In comparison, the presence of equimolar amounts of caffeine, CA and CGA also showed a tendency of delaying the onset of hIAPP conformational change, although less effective compared with EGCG (Figure 2 and Figure S1). In contrast, in the presence of a 5-fold molar excess of caffeine, CA and CGA, the conformational changes of hIAPP were further suppressed, as
suggested by the time course results, in which the β-sheet-rich content started to increase only after incubating for 5 h (which is two hours later than the IAPP alone); the inhibitory effects of 5-fold molar excess of EGCG was not tested by CD due to a very noisy baseline caused by the high concentrations of EGCG. Furthermore, the shapes of spectra obtained with 5-fold molar excesses of caffeine, CA and CGA, were also notably different from those obtained without the compounds or with equimolar amounts of the compounds. A broader negative peak at 220 nm was observed, and the positive peak at 200 nm was absent. It is interesting to note that from the CDPro analysis, the addition of caffeine, CA and CGA all changed the percentages of final secondary structure components after incubation for 24 h, with notably higher percentages of residual random coil structures and lower percentages of converted β-structures (Figure 2 and Figure S1).

Amyloid formation

The kinetics of amyloid formation of hIAPP in the presence of different molar ratios of freshly prepared caffeine, CA and CGA were monitored by ThT-fluorescence based assays. The ThT-binding assay and the following TEM data suggested that hIAPP readily formed typical long linear fibrils and gave strong ThT emissions with a lag time of 3.6 ± 0.3 h (Figures 3 & 4, Table 1). The addition of equimolar amounts of EGCG exhibited strong inhibition on the ThT emission (Figure 3), which agreed with a previous report (25).

In the presence of coffee-derived compounds, both the lag time and the maximum fluorescence intensity were significantly altered. When a 0.5 molar ratio of CA was added, although the lag time was not significantly changed, the ThT intensity was nevertheless decreased by 40% at 24 h. In the presence
of equimolar amounts of CA, the lag time was prolonged from $3.6 \pm 0.3$ h to $5.5 \pm 0.3$ h ($p < 0.05$; Table 1). When the molar ratio was further increased to 2:1 and 5:1, a stronger inhibition on hIAPP amyloid formation was observed with the lag time increased from $3.6 \pm 0.3$ h to $7.8 \pm 0.1$ h ($p < 0.05$) and $9.2 \pm 0.7$ h ($p < 0.05$), respectively; the intensities of ThT emissions were also greatly decreased by more than 60%. Similar dose-dependent results were found when CGA was added. In the presence of 0.5 molar ratio of CGA, the lag time increased to $7.0 \pm 0.7$ h ($p < 0.05$; Table 1). Unlike CA, in the presence of equimolar and higher ratios of CGA, although the ThT intensities were further decreased, the lag times remained unchanged (Table 1). On the other hand, when hIAPP was incubated in the presence of caffeine at different molar ratios, the lag times were only slightly prolonged (Table 1) compared with those of CA and CGA. We further tested the effects of DHCA and a 1:1:1 mixture of caffeine, CA and CGA on the formation of hIAPP amyloid. The results indicated that DHCA and the mixture have a similar inhibitory effect as CA and CGA at the same molar ratio, however, no obvious synergic or antagonist effects were observed for the combined compounds (Figure S2).

The morphologies of hIAPP aggregates in the presence of freshly prepared caffeine, CA and CGA were examined. Under TEM, hIAPP alone showed extensive long linear amyloid fibrils with a morphology typical of IAPP deposits (Figure 4). No amyloid or amorphous aggregates were observed in an hIAPP sample treated with an equimolar amount of EGCG, which is consistent with a recent report (25). The presence of equimolar or a 5-fold excess of caffeine showed no obvious effects on the morphology of hIAPP amyloids (Figure 4D). In contrast, co-incubation with CA or CGA both inhibited the formation of lineal hIAPP fibrils, with only a small amount of amorphous aggregates being observed under TEM (Figure 4E-4H).
Peptide oligomerization studied by PICUP

The effect of caffeine, CA and CGA on the oligomerization of hIAPP was determined with the PICUP based photo-cross-linking method. Without irradiation, hIAPP migrated mostly as monomer (lane 1, Figure 5); after a short amount of light exposure (5 s), extensive hIAPP oligomerization was detected, consisting of monomer, dimer, trimer, tetramer and higher oligomers (lane 2, Figure 5). The addition of an equimolar amount of EGCG showed a significant inhibitory effect on the oligomerization of hIAPP, with no obvious cross-linked oligomers being identified, except sometimes with a faint dimer band (lane 3, Figure 5). Similar strong oligomerization inhibitory effects were identified for CA and CGA, in which only monomers and small amounts of dimers were observed (lanes 7-12, Figure 5). In contrast, increasing the ratio of caffeine showed little inhibitory effects on oligomerization, only in the presence of a 5-fold excess of caffeine was a weak oligomerization inhibitory effect observed (lanes 4-6, Figure 5). The effects of DHCA and a 1:1:1 mixture of caffeine, CA and CGA on oligomerization were also investigated; the migration patterns clearly indicated that both DHCA and the mixture exhibited an inhibitory effect on oligomerization of hIAPP (Figure S3).

Dynamic light scattering

Dynamic light scattering experiments were performed to determine the particle size distribution. Large particle sizes (about 10 µm, the largest particle size the instrument can record) of hIAPP fibrils were detected after 90 min of incubation without adding any compounds. In contrast, the presence of EGCG, CA, CGA all significantly inhibited this process at a molar ratio of 5 where only smaller particles were found after incubating for 120 min (Figures S4 & S5); on the other hand, caffeine failed
to show an inhibitory effect with large particles being observed at a similar rate to that of hIAPP (Figures S4 & S5), which agrees with the results of ThT fluorescence tests and PICUP assays.

Cell viability assays

The effects of caffeine, CA and CGA on the cytotoxicity of hIAPP were evaluated using pancreatic INS-1 cells. Consistent with previous reports (32), the presence of 15 µM hIAPP for 24 h resulted in significant cytotoxicity, with cell viability being only 36% that of the untreated control (p < 0.05). The presence of an equimolar amount of EGCG significantly suppressed the cytotoxicity of hIAPP with the cell viability increasing to 75% (p < 0.05), which is in good agreement with a previous report (25). The three coffee-derived compounds all protected INS-1 cells from hIAPP induced apoptosis to varying degrees, among which CA exhibited the highest protection. An equimolar amount of CA significantly decreased the cytotoxicity of IAPP by increasing the cell viability from 36% to 71% (p < 0.05, Figure 6). When further increasing the molar ratio of CA/hIAPP to 5:1, the cell viability increased to 96% (p < 0.05, Figure 6). Caffeine and CGA, whilst exhibiting some protection, were less effective than CA. The one and five molar ratio of caffeine to hIAPP increased the cell viability to 48% (p < 0.05) and 54% (p < 0.05), respectively (Figure 6); for CGA, the viabilities were 40% (p < 0.05) and 49% (p < 0.05), respectively (Figure 6). In the absence of hIAPP, all compounds had no significant effect on INS-1 cell viability at various concentrations (95-105% viabilities, data not shown).
4. Discussion

Amyloid proteins including hIAPP can aggregate into mature fibrils and induce cell apoptosis, eventually leading to diseases including Alzheimer's disease, Parkinson's disease and T2DM (2, 5, 33-36). Previous studies have demonstrated that certain small molecules, such as multiple polyphenols (including EGCG and curcumin), can be used as chemical probes to affect the formation of toxic protein aggregations, such as Aβ and α-synuclein fibrils (23, 37, 38). In this study, we found two coffee derived polyphenols, CA and CGA all have significant inhibitory effects on hIAPP fibril formation and alleviate the cytotoxicity of hIAPP to pancreatic INS-1 cells; the other major active component of coffee, caffeine, only demonstrated a weak inhibitory effect on amyloid formation and protection of INS-1 cells (Figures 2-4). It is interesting to note that from our CD study data, these compounds not only delayed the onset of secondary structure conversion of hIAPP from random coil to β-structure, but that the addition of higher molar ratios of these compounds even altered the secondary structure of the final products (Figure 2 and Figure S1). This suggests that these compounds may directly bind to the hIAPP molecule and interfere with its secondary structural conversion. To the best of our knowledge, this is the first report that these coffee-derived compounds can affect secondary structure of amyloids.

The process of hIAPP fibril formation can be divided into two steps: an initial oligomerization step that forms toxic soluble oligomers, which can penetrate the cell membranes and lead to cell death; and a further oligomer self-assembly step that forms mature linear fibrils and deposits in the pancreatic islets (39). The inhibition of amyloid fibril formation may take place at either the initial oligomerization step or the self-assembly step. The observations that CA and CGA show significant inhibitory effects on hIAPP oligomerization (Figure 5) and that there is an absence of classic linear fibrils (Figure 4),
suggests that these two polyphenol molecules may suppress the initial oligomerization step and disrupt the formation of fibrils. CA and CGA may thus redirect the amyloidogenic molecules into off-pathway aggregates. In contrast, caffeine seems to show no obvious effects on these steps, since the oligomerization status was unchanged (Figure 5) and typical fibrils were observed in the presence of caffeine. However, the decreased ThT-based fluorescence and altered secondary structures in the presence of caffeine suggest there may be some structural difference between the mature fibrils of hIAPP formed with or without caffeine, which awaits further investigation.

In addition to the cell damages caused by the formation of membrane-penetrating hIAPP oligomers (39), it has been shown recently that the formation of hIAPP amyloids may also induce oxidative stress, inducing cell apoptosis (40). Since CA has also been reported as an antioxidant (41), the inhibitory effect of CA on hIAPP-induced cytotoxicity may also be partly due to its antioxidative properties. Similarly, polyphenol baicalein has been reported to inhibit the Aβ protein-induced neurotoxicity as an antioxidant (42). On the other hand, our findings that caffeine show higher protection than CGA on cell viability are intriguing, since CGA showed higher potency in inhibiting oligomerization and fibrillization of hIAPP. It has been reported that caffeine can prevent the Aβ-induced neurotoxicity by blocking adenosine A$_2$A receptors (43), suggesting that the protection effects of caffeine on cytotoxicity may come from its impact on various intracellular signaling pathways. However, the exact mechanism of its protection remains unclear.

There are controversial reports about the effects of caffeine on diabetes. Some studies have shown that caffeine in-take can increase thermogenesis and metabolism (44), suggesting that long-term coffee consumption may be associated with weight loss, lowering the risk of T2DM. Compared with
decaffeinated coffee (45), our results demonstrate for the first time that caffeine shows mild beneficial effects, including an inhibitory effect on fibril formation and a protection effect on pancreatic INS-1 cell viability. These results however, do not determine whether decaffeinated or caffeinated coffee has the greater effect on lowering the risk of diabetes.

It should be noted that both the concentrations of hIAPP and coffee-derived compounds used in the present in vitro study are significantly higher than the physiological levels. It has been shown that the physiological circulating concentration of hIAPP ranged from 1.6 to 20 pM in non-diabetic people (4), whereas the concentration of the three coffee derived compounds can reach sub-micromolar or even micromolar level hours after coffee consumption (11, 14, 15), suggesting under physiological conditions, the stoichiometry of coffee-derive compounds to hIAPP may actually be much higher than those used in the present study. Thus even greater misfolding protection effects are predicted. Since T2DM is regarded as a chronic disease, the long process of T2DM development, hIAPP aggregation and deposition may take years to happen in vivo (compared to the hours shown in the present in vitro study), a beneficial effect may thus be expected for a regular coffee drinker.

It is also worth mentioning that the phenolic components tested in the present study, CA and CGA, all undergo extensive metabolism during their passage through the gastrointestinal tract, and vast amounts of the metabolites and catabolites of these parent compounds enter the circulatory system. For example, Stalmach and colleagues have observed that the vast majority (>90%) of circulating compounds after coffee CGA intake are dihydrocaffeic acids, which also have a polyphenol structure (16). It is of great interest to note from this study that DHCA also showed strong amyloid inhibitory effects comparable to those of CGA and CA (Figures S2-S3); moreover, many other CGA metabolites,
such as ferulic acid, isoferulic acid, gallic acid and vanillic acid, also have typical polyphenolic structures. Thus, it is reasonable to expect that similar hIAPP aggregation inhibitory effects may also exist for these coffee metabolites; it will be interesting to study these metabolites in the future.

In summary, the present in vitro study suggests that coffee components may reduce the risk of T2DM through preventing toxic hIAPP assembly, and an in-depth in vivo study will be of future interest. Coffee-derived compounds could be useful candidates for anti-diabetes drug development. In addition to diabetes, it has also been reported recently that coffee consumption is associated with decreased blood Aβ levels and could be an effective therapeutic agent against Alzheimer's disease (AD) (46). It would also be of future interest to extend this study to other amyloidogenic diseases.

Acknowledgements:

This work was supported by the Natural Science Foundation of China (Nos. 30801445, 30970607 and 30870949), the National Basic Research Program of China (2009CB918304), Program for New Century Excellent Talents in University (NECT-10-0623), the Key Project of Chinese Ministry of Education (No.109103), and the Important National Science and Technology Specific Projects (2009ZX09301-014). The authors wish to thank Dr. Mitchell Sullivan (University of Queensland) for proofreading the manuscript, and three anonymous reviewers from the Journal of Agricultural and Food Chemistry for their constructive comments and suggestions on the manuscript.
References:

Table 1. Effects of different compounds on the amyloidogenic properties of hIAPP.  

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<th>Fibril formation b</th>
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<td>t_{50} (h)</td>
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<td>3.6 ± 0.3</td>
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<td>3.5 ± 0.5</td>
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<td>hIAPP : caffeine</td>
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<td>+</td>
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<tr>
<td>hIAPP : CGA e</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 : 0.5</td>
<td>7.0 ± 0.7</td>
<td>9.5 ± 0.5</td>
<td>66.1 ± 1.3</td>
<td>++</td>
</tr>
<tr>
<td>1 : 1</td>
<td>6.4 ± 0.6</td>
<td>9.8 ± 0.3</td>
<td>36.5 ± 6.9</td>
<td>+</td>
</tr>
<tr>
<td>1 : 2</td>
<td>6.0 ± 0.9</td>
<td>11.6 ± 0.6</td>
<td>33.5 ± 2.8</td>
<td>+</td>
</tr>
<tr>
<td>1 : 5</td>
<td>7.3 ± 1.0</td>
<td>10 ± 0.9</td>
<td>29.1 ± 2.5</td>
<td>+</td>
</tr>
</tbody>
</table>

a All assays were repeated for at least three times.
b The concentration of hIAPP was 15 µm.
c The fluorescence intensity of hIAPP alone was set as 100.
d Semi-quantitative analysis of ThT-fluorescence based amyloid formation: ‘‘+++’’ designates samples with fluorescence intensity change greater than 75 arbitrary units, ‘‘++’’ for values between 40 and 75 arbitrary units, ‘‘+’’ for values between 10 and 40 arbitrary units, and ‘‘- -’’ for values less than 10 arbitrary units.
e CA, caffeic acid; CGA, chlorogenic acid.
Footnotes:

1 To determine the working concentration for DLS, a series of hIAPP concentrations were tested. 32 µM of hIAPP was chosen as the lowest sample concentration with the acceptable signal intensity.

Figure Legends

Figure 1. The primary sequence of hIAPP and compounds. (A) The peptide has an amidated C terminus and a disulfide bridge between Cys-2 and Cys-7; (B) the chemical structures of EGCG, caffeine, caffeic acid, chlorogenic acid and dihydrocaffeic acid.

Figure 2. Far-UV circular dichroism spectra of hIAPP with different compounds.

Figure 3. Relative thioflavin-T fluorescence intensity of hIAPP with different molar ratios of EGCG (A), caffeine (B), caffeic acid (C) and chlorogenic acid (D).

Figure 4. The TEM images of hIAPP co-incubated with different compounds. (A) hIAPP; (B) 1:1 mixture of hIAPP and EGCG; (C), (E), (G), equimolar amounts of caffeine, caffeic acid and chlorogenic acid; (D), (F), (H), five-fold molar excess of caffeine, caffeic acid and chlorogenic acid.

Figure 5. Oligomerization studied by the photo-induced PICUP assays combined with Tricine-UREA-PAGE and silver staining. Lane 1, hIAPP without irradiation; lane 2, hIAPP with irradiation; lane 3, the 1:1 mixture of hIAPP and EGCG (irradiated); lanes 4-6, 0.5, 1, 5 molar
ratio of caffeine/hIAPP (irradiated); lanes 7-9, 0.5, 1, 5 molar ratio of caffeic acid/hIAPP (irradiated); lanes 10-11 0.5, 1, 5 molar ratio of chlorogenic/hIAPP (irradiated). CA, caffeic acid; CGA, chlorogenic acid.

**Figure 6.** INS-1 cell viabilities in the presence of different compounds as determined by the MTT assay. The hIAPP was used as the control. *p* < 0.05.
Figure 1
Figure 2
Figure 3
Figure 5
Figure 6
TOC Figure

Diabetes

Amyloids

Human islet amyloid polypeptide

Lower risk of diabetes

Coffee

In vivo metabolites

Polyphenols and caffeine

Down-regulation