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Inhibiting Islet Amyloid Polypeptide Fibril Formation by the Red Wine Compound Resveratrol

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Cellular systems keep a balance between protein synthesis and degradation with the help of molecular chaperones and the proteasome, and the quality-control mechanisms in the cell prevent deposition of partially folded, misfolded, or degraded protein.[1] When the balance between protein synthesis and protein degradation is disturbed, pathological conditions can appear in the form of amyloids. In pathological conditions, such amyloids are found in different organs, such as the brain, liver, spleen and pancreas. One of the characteristics of the amyloids is that, in spite of their precursor proteins, they form cross β-sheet-rich structures and a largely similar fibril morphology. A number of diseases like Alzheimer's, Parkinson's, type II diabetes mellitus, and prion-related diseases are characterized by the presence of such amyloids.[2]

In type II diabetes, amyloid deposition occurs near pancreatic β-cells. Biochemical analysis of the amyloid plaques led to the identification of the 37-residue islet amyloid polypeptide (IAPP) as a major constituent.[3] IAPP is synthesized and secreted from pancreatic β-cells along with insulin, and during the post-translational modifications it acquires a disulfide bond between cysteine residues 2 and 7 and an amidation at the C terminus of the 37th residue tyrosine.[4] In its native state, IAPP controls hyperglycemia by controlling the blood glucose level.[5] It is still not known what triggers IAPP to convert from a soluble monomer into insoluble amyloid fibrils. Recent studies have demonstrated that the interaction with membranes might play an important role in the IAPP amyloid fibril formation.[6,7] Despite inherent problems like nonhomogeneity, polymorphism and insolubility, our understanding of IAPP amyloid fibril formation is gradually increasing. Efforts have been made towards a biophysical and structural characterization of the monomeric, oligomeric and fibrillar states of IAPP,[8–10] and the amino acid sequence and environmental factors, such as the presence of membranes and cosecreted proteins, have been under scrutiny concerning their role in IAPP fibril formation.[11–15] Results from recent studies indicate that it might not be the fibrils but precursors of the fibrils, such as particular oligomers, that are the most toxic species.[16]

Preventing IAPP amyloid fibril formation is a rational approach in the direction of drug discovery for type II diabetes. It has been difficult to rationally design drugs due to the lack of structural information about the prefibrillar and fibrillar states of IAPP and of amyloidogenic peptides in general. Despite the limited knowledge about the structure of the amyloid fibrils, screening of inhibitors, in particular, small-molecule inhibitors, might prove promising. Many small molecules have the advantage of crossing the blood–brain barrier, they are stable in biological fluid and can avoid immunological response. Amyloid fibrils generally share the overall basic features at a molecular level, like cross β-sheet-rich hydrogen-bonded fibrils. Hence, corresponding studies on other amyloid, like Aβ or τ, the Alzheimer peptides, might help us explore strategies for inhibition of IAPP amyloid formation. Based on this assumption we have recently shown that rhodanine-based small-molecule inhibitors, which are active against τ-fibril formation,[17] are also effective against IAPP amyloid fibril formation.[18] Heparin-induced τ-filament assembly has also been inhibited by different classes of compounds like phenothiazines, porphyrins and polyphenols, and, interestingly, these compounds also inhibit Aβ-(1–40) fibril formation.[19] In another approach, which is based on peptide inhibitors, small fragments of the peptide have been methylated to prevent IAPP fibril formation, but crossing of the lipid membrane by these peptides remains a challenge.[20]

A group of compounds, called polyphenols, with more than one aromatic phenolic rings has emerged as inhibitors of Aβ, α-synuclein, and prion amyloids.[21] In a recent study, a polyphenol, (−)-epigallochatechin gallate (EGCG), has been shown to divert aggregation-prone proteins like Aβ and α-synuclein into an off-pathway, and they are able to prevent fibril formation.[22] Another phenolic compound from grapes, resveratrol, has been shown to be effective against Aβ(25–35) amyloid fibril formation.[23] It has been shown to reduce the secreted and intracellular Aβ level.[24] In fact, IAPP shares amino acid sequence similarity with Aβ in the presumably ordered region and shows a similar secondary structure in the fibrillar state.[25] Hence, it may be speculated that small molecules that are effective inhibitors of Aβ amyloid formation could also be effective against IAPP fibrillation.

In this work, we describe the inhibitory effect of resveratrol (*trans*-3,5,4′-trihydroxystilbene, Figure 1), a polyphenol that is found in significant amounts (130–220 μM) in red wine, on IAPP fibril formation. We have used different biophysical methods like ThT fluorescence, atomic force microscopy (AFM), attenuated total reflection (ATR) FTIR spectroscopy, and a cytotoxicity assay on a pancreatic cell line to show the inhibitory effect of resveratrol on IAPP amyloid formation. Moreover, the effect of resveratrol is also investigated in the presence of aggregation-fostering lipid membranes.

IAPP fibril formation was started by dissolving the peptide in amyloid-forming buffer (10 mM sodium phosphate, pH 7.5) to a final IAPP concentration of 10 μM at 25 °C for 96 h. To reveal the effect of resveratrol on the IAPP fibrillation reaction, 0.01 to 100 μM of the compound was added to the fibril-forming

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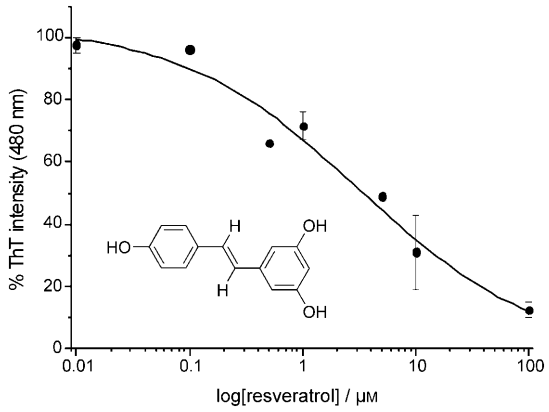

Figure 1. Effect of different concentrations of resveratrol on 10 μM IAPP amyloid fibril formation. Fibril formation was carried out in 10 mM phosphate buffer, pH 7.5, 25 °C for 96 h. The chemical structure of resveratrol is shown inside the plot.

buffer. ThT, an extrinsic fluorescence dye, binds to amyloid fibrils, and upon binding its fluorescence intensity increases. The fibril formation was quantified by measuring the fluorescence intensity of ThT at 480 nm after excitation at 440 nm. The fluorescence intensity of IAPP without resveratrol was taken as 100%. The effect of resveratrol was monitored by measuring the ThT fluorescence intensity of samples containing different resveratrol concentrations. As is evident from Figure 1, at low concentrations (0.01 and 0.1 μM) almost no inhibitory effect of resveratrol was observed. Upon increasing the resveratrol concentration, the ThT fluorescence started to decrease drastically, with an IC_{50} for amyloid fibril inhibition of 3.3 μM.

As the ThT assay might be prone to error because of an interaction between ThT and resveratrol, additional techniques were employed. We used AFM to study the fibril morphology. A 10 μM IAPP solution was incubated with and without resveratrol in amyloid-forming buffer (10 mM sodium phosphate, pH 7.5) at 25 °C for 96 h. IAPP forms long unbranched fibrils with heights of 3–5 nm, which is typical of IAPP amyloid fibrils (Figure 2A). Conversely, in the presence of 10 μM resveratrol, spherical structures with heights of typically only 3–4 nm are formed (Figure 2B). These results thus support the ThT data and indicate that resveratrol inhibits IAPP amyloid fibril formation.

After successfully demonstrating the role of resveratrol in IAPP fibril inhibition, we wanted to know whether this compound is also effective against membrane-induced IAPP fibril formation. To this end, a surface-sensitive technique, ATR-FTIR spectroscopy, was used. Lipid bilayers formed from large unilamellar vesicles of dioleoyl-phosphatidylcholine (DOPC) and the anionic dioleoyl-phosphatidylglycerine (DOPG) in a 7:3 ratio were spread out on the ATR crystal. Then, 10 μM IAPP solutions with and without resveratrol were injected into the ATR cell, and the evolution of the IR bands was followed for 20 h. In ATR-FTIR spectroscopy, accumulation of fibrils and oligomers adsorbed at the lipid interface can be detected, while protein molecules distant from the membrane surface (evanescent wave depth < 1 μm), that is, in the bulk solution, do not contribute significantly to the ATR-FTIR signal. IAPP forms amyloid fibrils in the presence of DOPC/DOPG lipid bilayers. This is clearly visible by the strong amyloid-specific amide I band at around 1620 cm^{-1}, which is characteristic of formation of intermolecular parallel β-sheets. In the presence of resveratrol, however, no specific bands are observed; this clearly demonstrates that the IAPP molecules are not in contact with the lipid bilayer (Figure 3). We also analyzed the effect of resveratrol on IAPP fibril formation in the absence of membrane. As shown in Figure 3 as well, no amyloid-specific bands could be seen.

It is evident from the in vitro studies shown above that resveratrol is a strong inhibitor of IAPP amyloid fibril formation. To reveal whether this compound is also an effective inhibitor of IAPP fibril formation in vivo, a cytotoxicity assay was carried out. The effect of resveratrol on IAPP fibril inhibition was studied by using the pancreatic cell line INS-1E. In a control experiment, only 60% cells survived in the presence of 10 μM IAPP (Figure 4). In the presence of 10 μM resveratrol, the survival of the cells increased to about 90%. As depicted in Figure 4, resveratrol itself is nontoxic to the cells at 10 μM concentrations. This shows that resveratrol is not only an effective in vitro inhibitor, it can be considered to be a potent inhibitor in the cellular model system as well.

Inhibiting the formation of oligomeric and fibrillar species during amyloid formation is a promising approach to prevent amyloid-related diseases. One of the synthetic compounds,

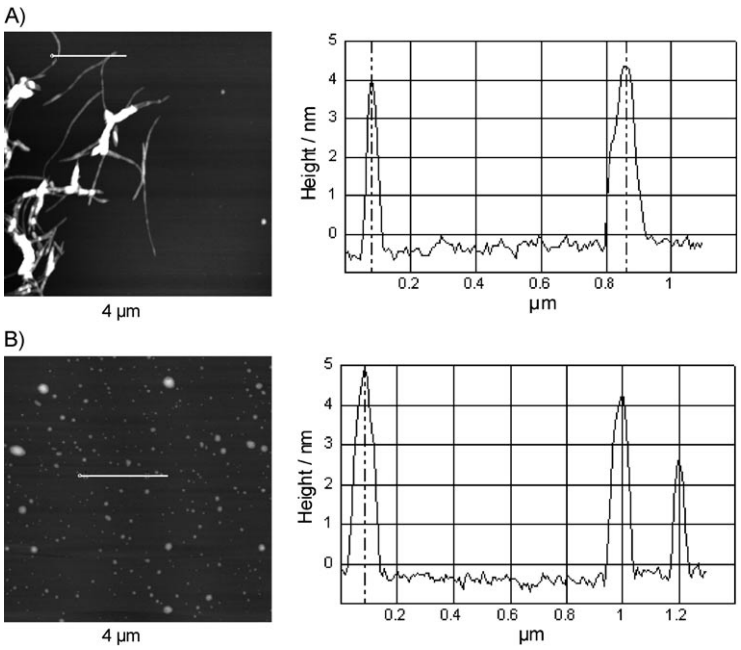

Figure 2. Effect of resveratrol on IAPP amyloid fibril morphology as revealed by atomic force microscopy. A) IAPP without resveratrol and B) IAPP in the presence of 10 μM resveratrol. Right: height profiles corresponding to the lines on the left.

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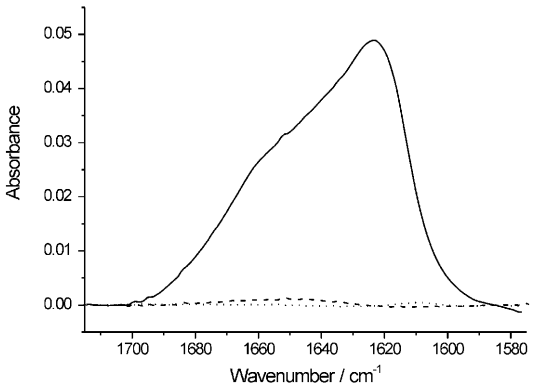

Figure 3. ATR-FTIR-spectra of 10 μM IAPP in the presence of a phospholipid bilayer made up from a mixture of DOPC and DOPG in a 7:3 (*w/w*) ratio (——), 10 μM IAPP mixed with 10 μM resveratrol in the absence of membrane (----), 10 μM hIAPP mixed with 10 μM resveratrol in the presence of a DOPC/DOPG (7:3, *w/w*) bilayer (····). All spectra were collected at 25 °C after 20 h incubation in the ATR-FTIR cell.

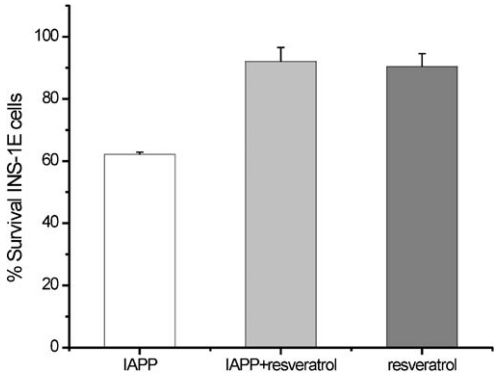

Figure 4. Cell viability of pancreatic β-cells (cell line INS-1E) after exposure to 10 μM IAPP (white), 10 μM of both IAPP and resveratrol (light grey), and 10 μM resveratrol, only (dark gray).

phenolsulfonphthalein, has been shown to inhibit IAPP fibril formation also.[26] Still, with the synthetic compounds, cell toxicity remains an issue for drug development. Naturally occurring polyphenolic compounds have an advantage over synthetic ones because of their nontoxicity as well as their biocompatibility. These characteristics of naturally occurring polyphenolic compounds have been exploited for the discovery of Aβ and other amyloid inhibitors. In an elegant study, Wanker's group has shown that EGCG, a naturally occurring polyphenolic compound, can inhibit amyloid formation of Aβ and α-synuclein.[22] One other important polyphenolic compound, resveratrol, which is found in grapes and red wine, has been shown to inhibit the cytotoxicity of Aβ(25–35) and to prevent its fibril formation.[23,27,28]

We report here the inhibitory activity of resveratrol in IAPP amyloid formation. Although resveratrol is a slightly less-potent inhibitor ($IC_{50} = 3.3$ μM) than the recently described rhodanine-based inhibitors (with IC_{50} values of 0.45 and 1.23 μM), its IC_{50} value is lower for IAPP than value for the Aβ (25–35) amyloid fibrils that were determined recently, which is 5.6 μM.[23] The differences in the IC_{50} of different compounds reflect the fact that these compounds interact at different stages and/or at different interaction sites. It is generally believed that polyphenols interfere by interacting with the hydrophobic pockets in the amyloid fibril formation pathway. Work on the Aβ amyloid inhibitors leads to the assumption that polyphenols do not interact with the monomers of the amyloid-forming proteins, but rather with prefibrillar structures.[19,21] A substoichiometric concentration of resveratrol is required for IAPP fibril inhibition. This indicates that it is interacting and preventing the fibril formation during the early stages of the fibrillogenesis. This is evident from the AFM studies in which only small spherical structures have been observed. However, such inhibition products might not always be desirable. If they are toxic to the cells, then the purpose is defeated. Recently, it has been shown that epigallochatechin promotes the formation of off-pathway oligomers, which are nontoxic to cells.[22]

One of the important aspects of IAPP fibrillation is the ability of negatively charged membranes to induce IAPP fibril formation.[6,7,13] So far, amyloid inhibition studies have mostly not focused on the role of membranes in amyloid fibril formation. Recently, we have included this aspect as well.[18] We clearly show that resveratrol can inhibit IAPP fibril formation even in the presence of anionic lipid bilayers, such as DOPC–DOPG model membranes. The amyloid-specific IR band (at about 1620 cm^{-1}) band is absent in the sample that contains resveratrol (Figure 3). This clearly demonstrates that resveratrol is able to interact with IAPP and can prevent IAPP fibril formation even in the presence of anionic lipid membranes, which are known to drastically induce and foster fibrillation of IAPP.

Small-molecule inhibitors like resveratrol have the potential to be developed as drug candidate for type II diabetes. Therefore it is necessary to extend these in vitro studies to the cellular system. Resveratrol has already been shown to reduce the Aβ level in cell culture studies.[24] In this study, resveratrol has been shown to have pronounced effects on the survival of the INS-1E cell line treated with IAPP as well (Figure 4). An earlier role of resveratrol in cell survival has been attributed to its antioxidant activity.[28] Our in vitro inhibition results do not depend on the antioxidant activity and are in line with the cell culture studies. The markedly enhanced cell survival in the presence of resveratrol also indicates that the small oligomeric structures that have been observed in the AFM study are not toxic and could very well be off-pathway assembly products. Because resveratrol is nontoxic to the pancreatic cells, this natural polyphenol has the potential to be developed as drug candidate for type II diabetes.

Experimental Section

Materials: Synthetic human IAPP was obtained from Calbiochem (Darmstadt, Germany), hexafluoroisopropanol (HFIP) from Riedel–de Haen (Seelze, Germany), resveratrol from Sigma. Di-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DOPG) and di-oleoyl-*sn*-glycero-3-phosphocholine (DOPC) were from Avanti Polar lipids (Alabaster, AL, USA). INS-1E rat insulinoma cells were a gift from Dr. Pierre Maechler (Department of Cell Physiology and Metabolism, University Medical Centre, Geneva, Switzerland). RPMI 1640

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medium (with 2 mM glutamine) was purchased from Gibco-Invitrogen and fetal calf serum (FCS) from Brunschwig (Basel, Switzerland). The WST-1 was purchased from Roche Diagnostics. HEPES, 2-mercaptoethanol, sodium pyruvate, penicillin, streptomycin and the trypsins were bought from AppliChem (Darmstadt, Germany). All the other chemicals were of highest purity grade available.

Fibril formation and ThT fluorescence spectroscopy: Fibril formation was carried out essentially as described earlier.[18] Briefly, the peptide was dissolved in hexafluoroisopropanol (HFIP) to disaggregate the protein, and the solvent was removed by lyophilization. The lyophilized peptide was dissolved in sodium phosphate buffer (10 mM, pH 7.5) to a final cocentration of 10 μM. A stock of resveratrol (10 mM) was prepared in DMSO, and further substocks were made from this. All the reactions were carried out at room temperature for 96 h. Amyloid fibrils were detected by staining with thioflavin T (ThT; 10 μM) after 96 h. Amyloid fibrils were detected by using an ISS K2 multifrequency phase fluorometer (ISS Inc., Champaign, IL) coupled with a water-circulating bath to keep the chosen temperature constant. The fluorescence signal (excitation at 440 nm) was recorded between 450 and 600 nm by using 1 nm slits both for the emission and excitation measurements. The data shown in Figure 1 are the average (±standard deviation) of at least two independent measurements. To guide the eye, a curve was fitted by using Origin (version 7.0) software, and the IC_{50} value (half maximal (50%) inhibitory concentration) was calculated from the fitted curve.

Atomic force microscopy: For the atomic force microscopy (AFM) measurements, samples were diluted with deionized H_2O to yield a final concentration of 1 μM. Then the sample (20 μL) was applied onto freshly cleaved muscovite mica and allowed to dry. Data were acquired in the tapping mode on a Multi Mode TM SPM AFM microscope that was equipped with a Nanoscope IIIa controller from Digital Instruments. As AFM probes, Silicon SPM Sensors "NCHR" (force constant, 42 $N m^{-1}$; length, 125 mm; resonance frequency, 300 kHz) from Nanosensors were used.[29]

ATR-FTIR spectroscopy: The peptide was dissolved in hexafluoroisopropanol (HFIP) to disaggregate the protein, followed by the removal of solvent by lyophilization. Reconstitution in buffer (1 mL) and shaking (Vortexer) yielded a 10 μM solution. A stock of resveratrol (10 mM) was added to the reaction mixture to a final concentration of 10 μM. This mixture was shaken for 3 h. Stock solutions of the lipids were prepared by dissolving a mixture DOPC (7 mg) and DOPG (3 mg) in $CHCl_3$ up to a concentration of 10 $mg mL^{-1}$. This solution (50 μL), which contained 0.5 mg of lipids was dried under a stream of N_2. When all $CHCl_3$ was evaporated, the samples were placed in a Speed-Vac for 3 h to remove residual solvent. The DOPC/DOPG (7:3; 0.5 mg) mixture was dissolved in buffer (1 mL) and ultrasonicated for 10 min. After five freeze–thaw cycles, a homogeneous solution of multilamellar vesicles (MLV) was obtained. For obtaining large, unilamellar vesicles (LUV), the solution was passed through an extruder with a membrane filter made of polycarbonate (11×; pore diameter: 0.1 μm). For all FTIR spectroscopic experiments, a phosphate buffer (10 mM, pD 7.4) in D_2O, which contained 100 mM NaCl, was used.

ATR-FTIR spectra were recorded by using a Nicolet 6700 infrared spectrometer at a spectral resolution of 2 cm^{-1}. The ATR out-of-compartment accessory consists of a liquid jacketed Piketech ATR flow-through cell with a trapezoidal Ge-Crystal (Piketech, Madison, WI, USA, 80×10×4 mm, angle of incidence: 45°). The freshly prepared solution of large unilamellar vesicles was injected into the ATR-flow-cell, which was held at 25 °C; this led to the spontaneous formation of supported lipid bilayers. Adsorption of the membrane was controlled by following the increase of the CH_2 lipid band intensities over time. After adsorption overnight, the membrane was washed with buffer over 6 h. To ensure integrity of the membrane, the IAPP and IAPP-inhibitor solutions were injected into the ATR cell by means of a peristaltic pump at 1.4 $mL min^{-1}$. Spectra were processed by using GRAMS software (Thermo Electron).

Cell culture: The INS-1E[30] cells were cultured in RPMI 1640 medium that was supplemented with 5% fetal bovine serum, 10 mM HEPES, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, penicillin (100 $U mL^{-1}$) and streptomycin (0.1 $mg mL^{-1}$), and then incubated at 37 °C under 5% CO_2, pH 7.4. The viability of the cells was determined by a cell proliferation assay by using WST-1 reagent. The cells were seeded into 96-well plates at 10000 cells per well, grown for 24 h prior to exposure to the agent to be tested (IAPP, mixture of IAPP and inhibitor, or inhibitor, respectively), and then exposed for 48 h. The supernatant was replaced with a water-soluble tetrazolium salt, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1, 3-benzene disulfonate sodium salt (WST-1) solution (1:4 diluted with phosphate buffered saline (PBS) and further diluted 1:10 with growth medium) and incubated for 24 h. The absorbance was read at 450 nm (reference at 630 nm). Percentage cell viability was calculated based on the absorbance measured relative to that of cells exposed to culture medium alone.

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