Abstract

The 37-amino acid residue peptide human amylin, also known as human islet amyloid polypeptide (hIAPP), forms amyloid protein deposits in the pancreas which are regarded to play a key role in the pathogenesis of type two diabetes mellitus. The factors promoting hIAPP aggregation are still not completely clear, however rising evidence indicates that oligomeric aggregates are cytotoxic to β-cells, probably by interaction with and subsequent disruption of β-cell membranes. Consequently, increasing research is aimed at developing inhibitors of cytotoxic amyloid aggregation of hIAPP. In the last times great attention has been paid to the multiple health benefits coming from a diet rich in antioxidants. Cytotoxicity of amyloid aggregates is frequently associated to early modifications of the intracellular redox status, and this has raised interest on the possible protection provided by natural antioxidants. In this respect, extra virgin olive oil is attracting great attention as an important source of polyphenols.

In this study we have shown that oligomers of hIAPP are highly toxic to cultured mammalian cells and that oleuropein aglycon, the main phenolic component found in Mediterrean extra virgin olive oil, can reduce the cytotoxicity of human amylin.

We tested the cytotoxicity of hIAPP aggregates obtained in the presence and in the absence of oleuropein aglycon on RIN-5F rat pancreatic β-cells by the MTT reduction inhibition assay. Human amylin treatment resulted in a highly significant impairment of cells viability with respect to controls, while the viability of cells treated with hIAPP incubated with oleuropein aglycon, was not significantly different from that of control cells.

Then we have shown by immunofluorescence analysis that this protective action may come from the ability of oleuropein aglycon to inhibit the interaction between aggregates and the cell surface, and this had led us to hypothesise that hIAPP aggregates grown in the absence or in the presence of oleuropein are somehow structurally different.

This interaction leads to membrane destabilization with consequent membrane permeabilization, oxidative stress, and eventually cell death by apoptosis. In fact we have verified the apoptotic response, in terms of caspase-3 activation, in cells treated with hIAPP aggregates, while there was no increase of caspase-3 activity in cells treated with hIAPP aggregates grown with oleuropein. These results confirm the protective action of oleuropein aglycon showed by the MTT data and the necessity of aggregates-membrane interaction to induce toxicity.

To further confirm the latter hypothesis, we have carried out an in vitro experiment, incubating synthetic phospholipid unilamellar vesicles (DOPS:DOPC = 3:7) with hIAPP aggregates aged in the presence or in the absence of oleuropein aglycon. We have shown that the toxic hIAPP assemblies induce a significant calcein release from negatively charged phosphatidylserine vesicles, whereas no significant permeabilization was induced by the aggregates grown in the presence of oleuropein aglycon.

Finally, structural studies performed by ThT assay, Circular Dichroism and Electron microscopy have indicated that oleuropein aglycon interferes with the first phases of hIAPP aggregation,

during which cytotoxic hIAPP aggregates are formed, resulting in a different path of amyloid aggregation.

Overall, our results suggest both a possible beneficial effect coming from extra virgin olive oil consumption in the prevention of type two diabetes and a pharmacological use of oleuropein in the treatment of this disease.

INTRODUCTION

1. Protein aggregation and amyloidoses

1.1. Amyloid diseases

Many human degenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), transmissible spongiform encephalopathies (TSEs) and non-insulin-dependent type II diabetes (NIDDM), are associated with an abnormal deposition of proteinaceous fibrillar aggregates (amyloid fibrils) in various tissues and organs [**1**; **2**]. In medicine these pathologies are called ***"Amyloidoses"***. Approximately 25 different proteins and peptides are known to be able to form amyloid fibrils in various diseases. The polypeptides involved include full-length proteins (e.g. lysozime), biological peptides (e.g. human amylin) and fragments of larger proteins produced by specific processing or by more general degradation (e.g the Alzheimer β-peptide or AβP). The peptides and proteins associated with the main amyloid diseases are listed in Table 1.1.

Amyloidoses are also known as *misfolding* or *conformational diseases,* because protein aggregation into amyloid fibrils results from the presence of "misfolded" forms of a specific protein/peptide that lose their functional, native conformation and are often devoid of their normal biological activity. Such proteins, failing to reach or maintain their correct native three-dimensional structure may aggregate and/or interact inappropriately with other cellular components, leading to impairment of cell viability. These events can be due to mutations, changes in the environmental conditions, such as pH or temperature, misprocessing or proteolysis. The possible fates of a newly synthesized polypeptide chain are described in Figure 1.1. Perturbations of the conformational properties of the polypeptide may affect equilibrium 1 in Fig. 1.2, increasing the population of partially unfolded or misfolded species which are more prone to aggregation than the native state.

These diseases can be grouped into three different categories [**3**]:

- **neurodegenerative diseases** in which aggregation occurs in the brain (such as AD, PD, TSEs and Huntington's disease).
- **nonneuropathic localized amyloidoses** in which aggregation occurs in a single kind of tissue other than the brain (such as NIDDM and medullary carcinoma of the thyroid).
- **nonneuropathic systemic amyloidoses** in which aggregation occurs in multiple tissues (such as lysozyme amyloidosis and fibrinogen amyloidosis).

It has also been demonstrated that many unpathological peptides and proteins can aggregate *in vitro*, under appropriate conditions, into fibrils which are indistinguishable from those associated with amyloid diseases [**4**; **5**; **6**]. The amyloidogenic proteins, either related or unrelated to disease, are very different about their sequence, function, size and tertiary structure, but all of them are able to form fibrils that show very similar morphological, structural and tinctorial features. All these evidences led to the idea that the propensity to form amyloid fibrils is not an unusual feature of the small number of proteins associated with diseases, but is instead a generic property of polypeptide chains [reviewed in **1**]. A modification in the three-dimensional

structure can be therefore sufficient to enable the production of aggregation-prone species by many, if not all, proteins or peptides.

Disease	Main aggregate component
Alzheimer's disease	Aβ peptides (plaques); tau protein (tangles)
Spongiform encephalopathies	Prion (whole or fragments)
Parkinson's disease	α-synuclein (wt or mutant)
Primary systemic amyloidosis	Ig light chains (whole or fragments)
Secondary systemic amyloidosis	Serum amyloid A (whole or 76-residue fragment)
Fronto-temporal dementias	Tau (wt or mutant)
Senile systemic amyloidosis	Transthyretin (whole or fragments)
Familial amyloid polyneuropathy I	Transthyretin (over 45 mutants)
Hereditary cerebral amyloid angiopathy	Cystatin C (minus a 10-residue fragment)
Haemodialysis-related amyloidosis	β_2-microglobulin
Familial amyloid polyneuropathy III	Apolipoprotein AI (fragments)
Finnish hereditary systemic amyloidosis	Gelsolin (71 amino acid fragment)
Type II diabetes	Amylin (fragment)
Medullary carcinoma of the thyroid	Calcitonin (fragment)
Atrial amyloidosis	Atrial natriuretic factor
Hereditary non-neuropathic systemic amyloidosis	Lysozyme (whole or fragments)
Injection-localised amyloidosis	Insulin
Hereditary renal amyloidosis	Fibrinogen α-A chain, transthyretin, apolipoprotein AI, apolipoprotein AII, lysozyme, gelsolin, cystatin C
Amyotrophic lateral sclerosis	Superoxide dismutase 1 (wt or mutant)
Huntington's disease	Huntingtin
Spinal and bulbar muscular atrophy	Androgen receptor [whole or poly(Q) fragments]
Spinocerebellar ataxias	Ataxins [whole or poly(Q) fragments]
Spinocerebellar ataxia 17	TATA box-binding protein [whole or poly(Q) fragments]

Table 1.1. A summary of the main amyloidoses and proteins or peptides involved [**1**].

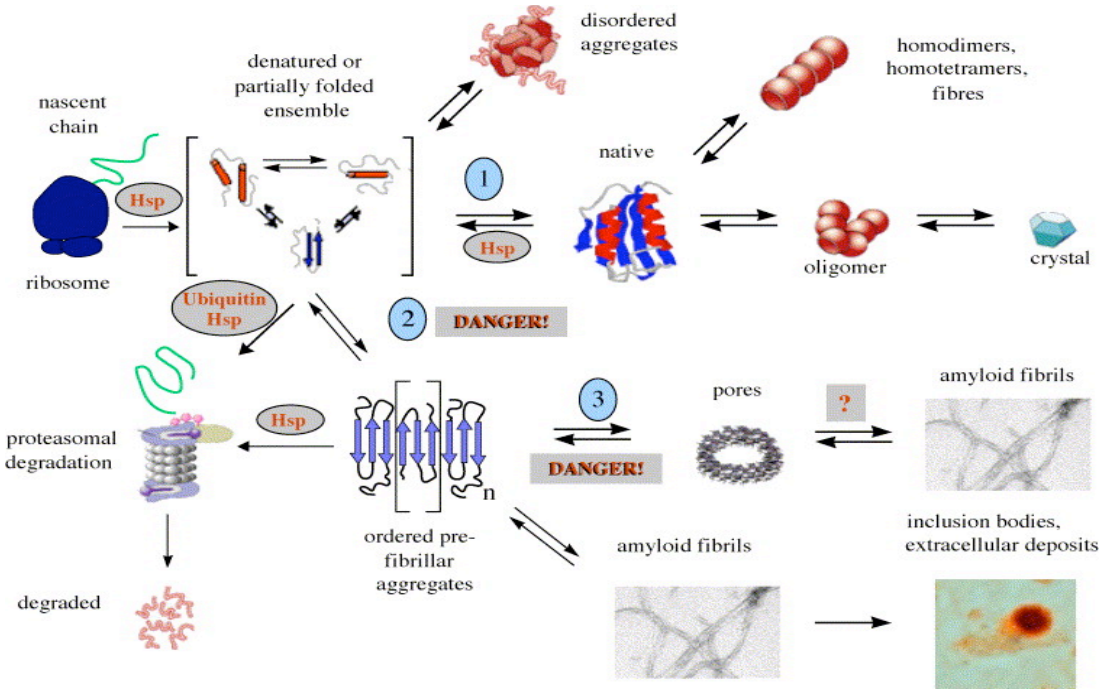

Fig. 1.1. The possible fates of newly synthesized polypeptide chains. The equilibrium 1 between partially folded and native molecules is usually in favour of the latter, except as a result of mutations, chemical modifications or destabilizing solution conditions. Under normal conditions, the increased partially or completely unfolded populations are refolded by molecular chaperones (Hsp) or cleared by the ubiquitin–proteasome machinery. When these clearance machineries are impaired, disordered aggregates arise or the aggregation path (equilibrium 2) is undertaken, towards the nucleation of prefibrillar assemblies that may grow into mature amyloid fibrils (equilibrium 3). The formation of prefibrillar assemblies as amyloid pores could be directly related to the cytotoxic effects of amyloids. The question mark indicates that it is not known if amyloid pores are on path or dead end intermediates of fibril formation. DANGER! indicates the processes producing prefibrillar assemblies, presently considered mostly associated with cell impairment. Molecular chaperones may suppress the appearance of prefibrillar aggregates by reducing the population of misfolded proteins assisting their correct folding or favouring their complete misfolding for proteasome degradation [**2**].

1.2. Structure of amyloid fibrils

A protein or a peptide is amyloid if, due to an alteration in its native state, it converts from its soluble functional state to a particular insoluble form, called the β-pleated-sheet, that produces highly organized fibrillar aggregates. These structures are defined as "**amyloid fbrils** or **plaques**" when they accumulate extracellularly and as "**intracellular inclusions**" when formed inside the cell [**3**]. The presence of amyloid fibrils (*ex vivo* or *in vitro*) is defined by three criteria [**7**]:

1) they bind the dye Congo Red giving a green birefringence under cross-polarized light.

2) they have a fibrillar morphology, that is they appear as long, straight, unbranching fibers, with a diameter of 70-120 Å and a variable length [**8**], when investigated by electron microscopy (EM) and atomic force microscopy (AFM) (Fig. 1.2).

3) they have β-sheet secondary structure.

The fibrils are also stained with Thioflavin T (ThT) giving a shift in the fluorescence of the dye [**9**; **10**]. Both circular dichroism (CD) and Fourier transform infra red (FTIR) spectroscopy support a high β-sheet content, even when the precursor monomeric peptide or protein is disordered or

rich in α-helical structure. Finally, amyloid fibrils reveal a typical X-ray diffraction pattern indicating the presence of the characteristic cross-β structure in the fiber [**11**].

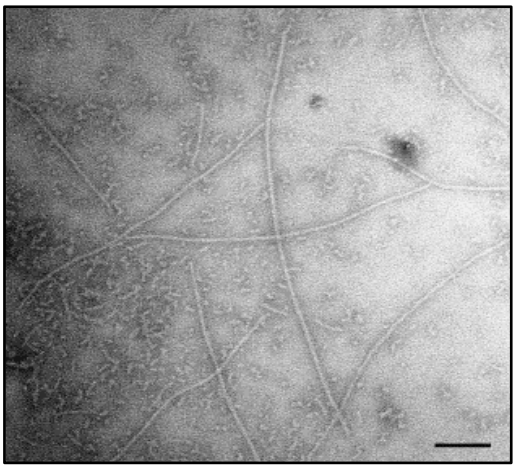

Fig. 1.2. Electron micrograph of amyloid fibrils formed in water from Aβ1–42, stained with 0.1% phosphotungstic acid showing long straight fibrils of 70–80 Å diameter as well as a fibrillar aggregate background. Bar=1000 Å [**8**].

The cross-β structure is a robust, stable structure where the protein chains are held together by repetitive hydrogen-bonding that extends the length of the fibrils. An amyloid fibril is usually composed of two to six 20-35 Å wide "protofilaments", which are often twisted around each other to form supercoiled rope-like structures arranged around a hollow centre [**12**]. These protofilaments have a highly ordered inner core, consisting of polypeptide chains arranged in the characteristic cross-β structure. In this organization, the β-strands run perpendicularly to the protofilament axis, giving a series of β-sheets that propagate along the fibril axis (Fig. 1.3). So the cross-β structure of the amyloid aggregates is their main structural peculiarity.

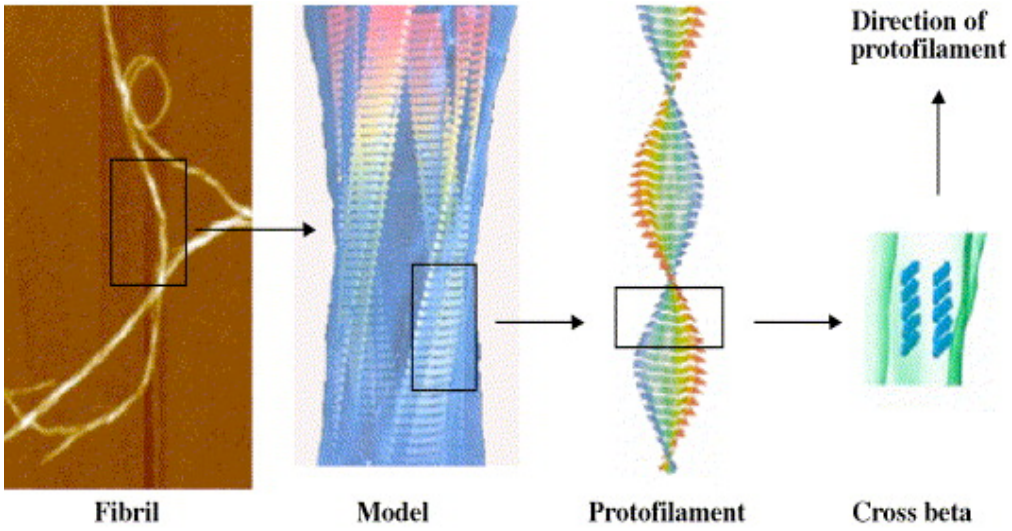

Fig. 1.3. The structural organization of an amyloid fibril. Four protofilaments are wound around each other; their core structure is a row of β-sheets where each strand is perpendicular to the fibril axis [**2**].

The X-ray diffraction pattern characteristic of the cross-β structure consists of a sharp 4.7 Å meridional reflection arising from the spacing between hydrogen-bonded β-strands within a β-sheet. A broad reflection centred at ~10 Å on the equator is also observed that arises from the intersheet spacing (Fig. 1.4, 1.5). The spacing between the β-sheets depends on the size of the side-chain groups [reviewed in **13**].

So far little is known about the detailed arrangement of the polypeptide chains into the amyloid fibrils, either those parts which formed the core β-strands or the regions that connect the several β-strands. Recent studies indicate that the sheets are relatively untwisted and may exist, in some cases at least, in specific supersecondary structures such as β-helices [**14**] or the μ-helix [**15**]. There may be important differences in the way the strands are assembled depending on the characteristics of the polypeptide chain involved, including length, sequence [**14**; **16**] and presence of intramolecular disulfide bonds that are able to stabilize the proteins [**10**].

1.3. Amyloid formation

At present, the physicochemical basis of amyloid formation remains poorly understood. It is largely believed that most amyloidogenic proteins aggregate *via* a nucleation dependent pathway from an ensemble of partially unfolded conformations. This may occur under solution conditions (such as low pH, lack of specific ligands, high temperature, moderate concentrations of salts or cosolvents), such that the native structure is partially or completely disrupted but under which interactions such as hydrogen-bonding are not completely inhibited. However, native-like mechanisms of aggregation have also been described. For example “lithostathine” maintains its native content of secondary structure upon aggregation into fibrils [**17**]. Association of protein molecules in their native-like states can therefore be the first event in the aggregation process, with the structural conversion into an amyloid conformation occurring subsequently.

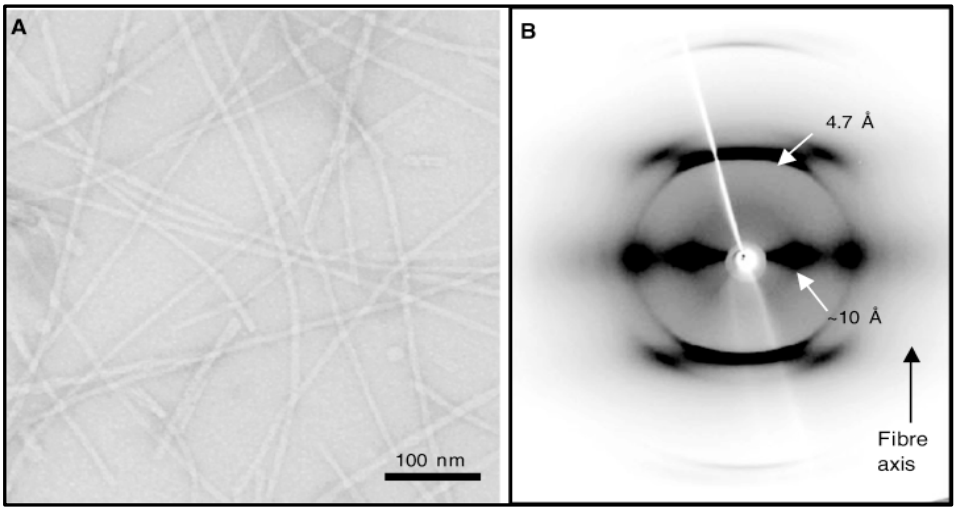

Fig. 1.4. The characteristics of amyloid fibrils include their appearance in the electron microscope and the cross-β diffraction pattern. (**A**) EM of negatively stained amyloid fibrils formed by islet amyloid polypeptide (IAPP), showing long, unbranching fibrils of ≈ 100 Å in diameter. (**B**) X-ray fibre diffraction pattern from aligned IAPP amyloid fibrils, showing the positions of the 4.7 Å meridional and ≈ 10 Å equatorial reflections in a cross-β pattern [**13**].

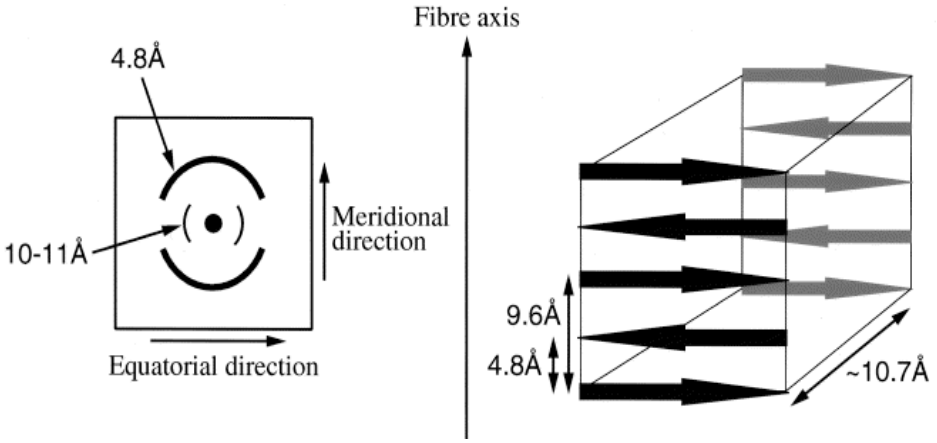

Fig. 1.5. The characteristic cross-β spacings from X-ray fibre diffraction from amyloid fibrils. A strong 4.8 Å reflection on the meridian corresponds to the hydrogen bonding distance between β-strands (shown right), and a more diffuse 10–11 Å reflection on the equator shows the intersheet distance of about 10.7 Å. A spacing of 9.6 Å would correspond to the repeat distance for an anti-parallel arrangement of β-strands [**8**].

The time course to convert a peptide or a protein into its amyloid fibrils typically includes a lag phase which is followed by a rapid exponential growth phase [reviewed in **3**]. The lag phase is assumed to be the time required for “nuclei” to form, where a nucleus is an ordered oligomeric amyloid specie that can serve as a template for the amyloid fibrillation. When a nucleus is formed, fibril growth proceeds rapidly by further association of either monomers or oligomers with the nucleus. This process eventually gives rise to short fibrillar structures referred to as protofibrils, which appear to be precursors of mature fibrils and are generally shorter than the latter. Moreover, to form amyloid fibrils proteins adopt at least in most cases, β-sheet-rich conformations. For example, in the case of the Aβ peptides, the main constituent of amyloid plaques in AD, small ordered, β-sheet-rich aggregates or “protofibrils” formed at early stages of amyloid formation have been described [**18**]. Protofibrils have been observed in heterogeneous populations of small, roughly spherical or tubular assemblies, 2.5-5.0 nm in diameter [**19**; **20**]. These species are often associated into bead-like chains or annular “doughnut” shaped rings and result, in many cases, as precursors of longer protofilaments and mature fibrils that appear only at later stages of assembly (Fig. 1.6).

These “early aggregates” formed from different peptides and proteins may be very important to understand the nature and origins of the pathological properties of amyloid structures associated with neurodegenerative diseases. The soluble prefibrillar aggregates of different peptides and proteins have been shown to be equally recognised by polyclonal antibodies raised against prefibrillar assemblies made from Aβ peptides [**21**]. The same antibodies, however, are unable to recognise the corresponding monomers and fibrillar aggregates, thus

confirming that prefibrillar assemblies from very different peptides and proteins share common structural features which are different form those exhibited by the monomers or mature fibrils.

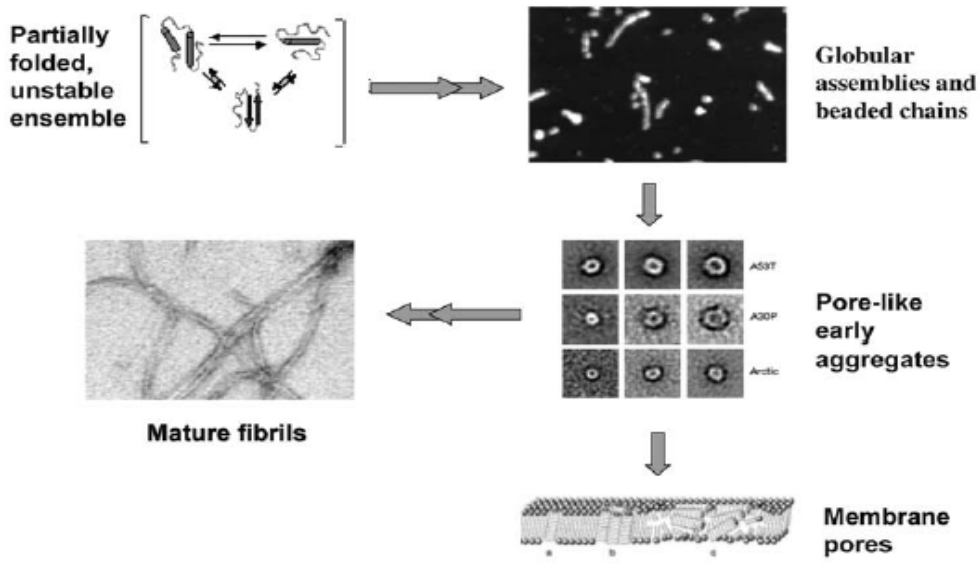

Fig. 1.6. Some amyloid-related peptides/proteins form early aggregates of globular appearance that further organise into beaded chains, globular annular 'doughnut' shaped assemblies eventually giving mature protofilaments and fibrils. Pre-fibrillar aggregates may interact with reconstituted phospholipid membranes and with cell membranes where they form aspecific channels (pores) disrupting cellular homeostasis [**1**].

1.4. Cytotoxicity of amyloid aggregates

The presence of amyloid fibrils in post-mortem brains of demented patients led to the first description of AD and resulted in the hypothesis that mature fibrils themselves could be the primary pathogenic species. However, the absence of any correlation between the amount of fibrillar Aβ deposits at autopsy and the clinical severity of AD, the appearance of clinical symptoms of the disease before amyloid plaques can be detected, and a several other pieces of evidence, led to the failure of this initial hypothesis.

At present, it is widely believed that early soluble, oligomeric precursors, rather than mature fibrils, are the main pathogenic species of amyloidoses [reviewed in **22**; **1**]. Indeed, many studies carried out on Aβ peptides and other amyloidogenic proteins showed that spherical and/or chain-like oligomers are highly neurotoxic [reviewed in **23**]. Moreover, the soluble Aβ content of human brain is better correlated with the severity of AD than are plaques [**24**].

The mature fibrils could be therefore viewed as inert material substantially harmless to cells, although great controversy still exists on the biological role of fibrils.

Indeed, it has been suggested that the large insoluble amyloid deposits may serve as reservoirs that release toxic soluble oligomers [**25**] and recent findings support the existence of a dynamic equilibrium between fibrils and their constituent monomers [**26**]. Recently, mature amyloid fibrils produced from full-length recombinant mammalian prion protein (PrP) have been shown to be

highly toxic to cultured cells and primary hippocampal and cerebellar neurons, in a manner comparable to the soluble small β-oligomers generated from the same protein [**27**].

Similarly, both hen lysozime oligomers and amyloid fibrils exert toxicity on cultured cells, even if with different mechanisms and time-scales [**28**].

Finally, mature fibrils from Aβ1-40 can exhibit different morphologies when they are produced under different conditions. Such different morphologies also exhibit significantly different cytotoxicity [**29**].

In conclusion, it remains to be established which aggregation state is the main responsible for the neurotoxicity, and difficulties in preparing highly homogeneous and stable populations of monomers, oligomeric intermediates and fibrils may account for observations of highly variable experimental results [**30**].

1.5. Structural basis and molecular features of amyloid toxicity

Many amyloid proteins have been shown to induce cellular toxicity by common mechanisms; such a toxicity is likely to arise from the "misfolded' nature of the aggregated species and their precursors and from the exposition in such species of hydrophobic residues or regions that are normally buried in the native state. As many of these regions are likely to be aggregation-prone (or "sticky") they may be able to interact with, and damage, membranes and other cellular components [**31**]. Indeed, the intrinsic instability of prefibrillar species that enables them to assemble further and organize into more ordered structures itself reflects the presence of accessible regions of the structures. In accord with these conclusions, prefibrillar assemblies have been shown to interact with synthetic phospholipid bilayers [**32**; **33**] and cell membranes [**34**; **35**], possibly destabilizing them and impairing the function of specific membrane-bound proteins [**36**; **37**]. Pre-fibrillar amyloid aggregates may interact with cell membranes in a way similar to the action of many prokaryotic or eukaryotic peptides or proteins (e.g. some bacterial toxins) that oligomerize into the membranes of the target cells forming pore-like assemblies that destabilize cell membranes and impair ion balance across these structures. So membrane damage may be a common molecular basis for impairment of viability in cells exposed to misfolded proteins or amyloid aggregates.

Because of the initial membrane perturbation, changes in the intracellular redox status and free Ca^{2+} levels in cells exposed to toxic aggregates have been described as crucial events in the impairment of cell function by the aggregates [**34**; **36**; **38**].

A modification of the intracellular redox status in cells exposed to amyloid aggregates is associated with a sharp increase in the quantity of reactive oxygen species (ROS), resulting in a high oxidizing activity towards several molecular substrates. In addition, changes have been observed in reactive nitrogen species, lipid peroxidation, deregulation of NO metabolism, protein nitrosylation and upregulation of heme oxygenase-1, a specific marker of oxidative stress [reviewed in **1**]. Moreover, it has been evidenced that cells can be protected against amyloid toxicity by treatment with antioxidants [**39**].

Even if it is not clear why protein aggregation induces production of ROS, however in general, oxidative stress could be related to some form of destabilization of cell membranes by toxic

species leading to an upregulation of the activity of hydrogen peroxidase-producing membrane enzymes, such as NADPH- oxidase and a failure in regulation of other plasma membrane proteins, such as receptors and ion pumps [**40**] and/or to impairment of mitochondrial function. Mitochondria play an important role in oxidative stress and apoptosis; in this regard, a key factor in Aβ peptide neurotoxicity could be the opening of mitochondrial permeability transition pores by Ca^{2+} entry in neuronal mitochondria followed by release of cytochrome *c*, a strong inducer of apoptosis.

It has been suggested that intracellular ROS elevation following exposure to amyloid aggregates is a consequence of Ca^{2+} entry into cells followed by stimulation of oxidative metabolism aimed at providing the ATP needed to support the activity of membrane ion pumps involved in clearing excess Ca^{2+} [**41**]. ROS elevation would in turn oxidize not only the proteins involved in ion transfer but also proteins such as calmodulin, that when oxidized is unable to activate the Ca^{2+}-ATPase. The down-regulation of the Ca^{2+}-ATPase activity would then reduce the need for ATP, and hence ROS production by oxidative metabolism, leading to a further increase in intracellular Ca^{2+} concentration [**41**]. This hypothesis can explain the relationship between ROS, apoptosis, mitochondrial damage and intracellular free Ca^{2+} increase shown by cells exposed to toxic amyloid aggregates. Calcium dysregulation and oxidative stress have been observed in AD, PD, NIDDM and prion diseases, as well as in cultured cells exposed to prefibrillar aggregates of disease-unrelated proteins. The increase in intracellular free Ca^{2+} levels is probably consequent to the impairment of membrane permeability and may come from the presence into the membrane of aspecific amyloid pores or may follow oxidative stress, membrane lipid peroxidation producing reactive alkenals such as 4-hydroxynonenal, and chemical modification of membrane proteins acting as ion pumps [**42**; **43**].

1.6. Protein-membrane interaction and cytotoxicity

It is accepted that the toxicity of protein amyloid aggregates is mainly induced by their interaction with the cell membranes. The toxic aggregates are known to interact with cellular membranes and to compromise their integrity, by forming a range of ion channels through which they lead to imbalance of ion homeostasis, oxidative stress and eventually cell death.

Such a mechanism is reminiscent of the action of pore-forming proteins such as peptides found in venoms and antimicrobial secretions, bacterial toxins, perforin [**1**]. The protein-membrane interaction is enabled by the presence, at the surface of the misfolded protein/peptide, of abundant exposed hydrophobic residues and clusters, which prefer the hydrophobic environment of lipid membranes [**44**]. In addition to the hydrophobic interactions, also electrostatic interactions can play a role in this association: the amyloidogenic peptides often possess distinct positively charged regions allowing them to interact with negatively charged membranes.

The extent and the mechanism of membrane destabilization depends upon the balance of the electrostatic and hydrophobic properties and of the amphipaticity of the protein.

The membrane alterations induced by proteins can vary depending on the nature of proteins and lipids. Some peptides disrupt the membrane by binding of their charged residues or regions

to the charged headgroups of the phospholipids through electrostatic interaction. This disturbance, likely to be reversible by itself, may be followed by insertion of some hydrophobic regions of the protein into the bilayer. Following protein insertion, the cell membrane can be affected in several ways (general electrostatic disturbance, fusion of vesicles, loss of membrane integrity, alteration in membrane thickness around the protein and/or pore formation).

The similarity between the mechanism of toxicity of pore-forming toxins [**37; 44**] and the cytotoxicity of amyloid aggregates led to propose, since 1993, the "channel hypothesis" [**45**]; a number of experimental data, coming from both artificial lipid bilayers and cell membranes, indicate that the ability of misfolded proteins and amyloid aggregates to interact with lipid membranes is of crucial importance.

The protein aggregates can exert their toxic effect by:

1) inserting themselves into the membrane lipid bilayer to form ion channels [**46**]

2) modifying the membrane viscosity and lipid packing [**47**] and therefore interfering with membrane proteins

3) entering themselves into the cell and interacting with intracellular components [**44**].

Many proteins have been shown to undergo changes in secondary structure upon their toxic interaction with membranes. Both α-helix into β-sheet and β-sheet into α-helix structure transformations, as well as an increase in secondary structure from a random coil conformation, have been described. For example, for what concerns some natively unfolded polypeptides like Aβ peptides, calcitonin and human amylin, the literature strongly suggests that these peptides populate an early oligomeric helical intermediate during amyloid aggregation *in vitro.* So unfolding of the α-helix of a protein followed by refolding to β-sheet may be an important step in inducing a membrane-active structure for cytotoxic peptides, such as prion and Aβ peptides or hIAPP. It is important to note, however, that both β-sheet and α-helix-based protein structural change could confer cytotoxicity on refolded proteins.

While it appears that no particular secondary structure confers an inherent advantage in membrane interaction, for any particular peptide it may be that one structure may allow better access for the hydrophobic residues to the membrane. This fact means that a change in secondary structure may generate the ability to interact with the membrane. Anyway it is important to underline that the changes in secondary structure often arise as a consequence of a contact with lipid environment: the hydrophobic lipids allow for lower energy exposure of hydrophobic residues, which lead to a rearrangement of the whole protein.

The importance of secondary structure in the exposure of hydrophobic residues was demonstrated by Gasset *et al*., who analyzed the structural requirements of α-sarcin to destabilize lipid bilayers. It was hard to explain the hydrophobic interactions of α-sarcin with a membrane, because this protein is highly polar in its native conformation. Gasset *et al*. demonstrated that, upon interaction with lipid vesicles, α-sarcin undergoes a structural change, with an increase in α-helix and decrease in β-sheet content from the mostly unfolded variant [**48**].

The fragment 106-126 of PrP, which is the main part of the small amount of β-sheet in the normal cellular prion (PrP^{C}), contains a hydrophobic core sequence (residues 113-120), hypothesized to be involved in prion toxic properties *via* membrane interaction and

destabilization [**49**], thus playing a key role in the pathological effect of the protein. It has been demonstrated that β-sheet content of PrP[106-126] increases in the presence of lipids [reviewed in **44**], and this structural change contributes to membrane destabilization and the toxicity of PrP[106-126].

The cytotoxic effect of amyloid β protein has been shown to involve the formation of ion channels within cell membranes, so altering cell function and regulation [4**5**; **34**; **36**]. The Aβ peptide fragments (between 39 and 42 residues in length) are involved in forming these channels through a hydrophobic region at the C-terminus [**44**]. The incorporation of Aβ peptides into membranes to form ion channels is thought to be determined preferentially by the presence of negatively charged, rather than by neutral, phospholipids [4**5**; **34**; **50**].

α-synuclein protofibrils, that are thought to be the main responsible of cell death in PD and form annular structures, reminiscent of the known structures of toxins pores [**19**; **35**], have also been found to bind and permeabilize acidic phospholipid vesicles in a pore-like fashion, with a strong size selectivity in allowing molecules to cross cell membrane [**51**; **33**].

Calcitonin, another amyloid-forming peptide of 32 residues, is known to be able to interact either directly with membranes and/or at receptor sites [reviewed in **44**]. Recent data showed that annular oligomers from salmon calcitonin form Ca^{2+}-permeable pores when inserted into liposomes, and that such an interaction is accompanied by a higher β-sheet content of the protein [**52**].

Figures 1.7 and 1.8 show pore-like/channel structures formed by different amyloidogenic proteins in the presence of membranes, imaged by AFM and EM, respectively.

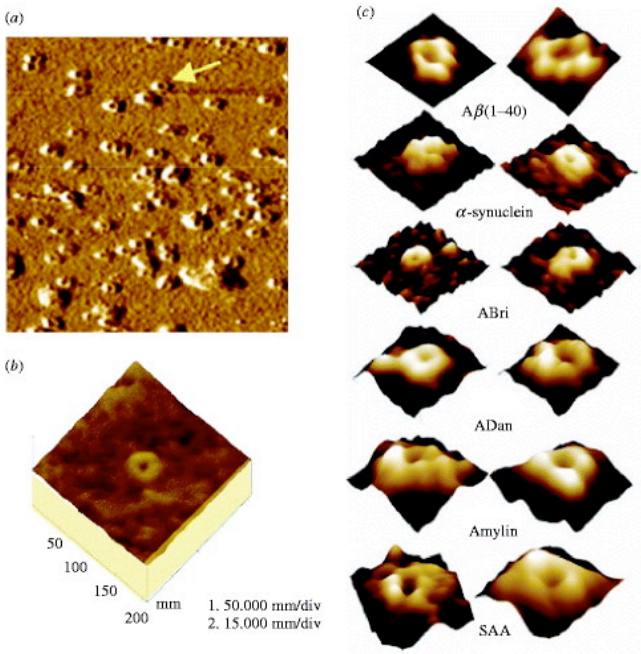

Fig. 1.7. Amyloid forming proteins form pore-like/channel structures on artificial as well as biological membranes. (a) AFM image of pore-like structures formed during the reconstitution of Aβ in lipid bilayers [**34**]. (b) Pore-like structure obtained by incubating α-synuclein protofibrils (WT and A53T) with brain-derived vesicles [**35**]. (c) Pore/channel-like structures formed from various amyloid-forming proteins/peptides in lipid bilayers [**23**].

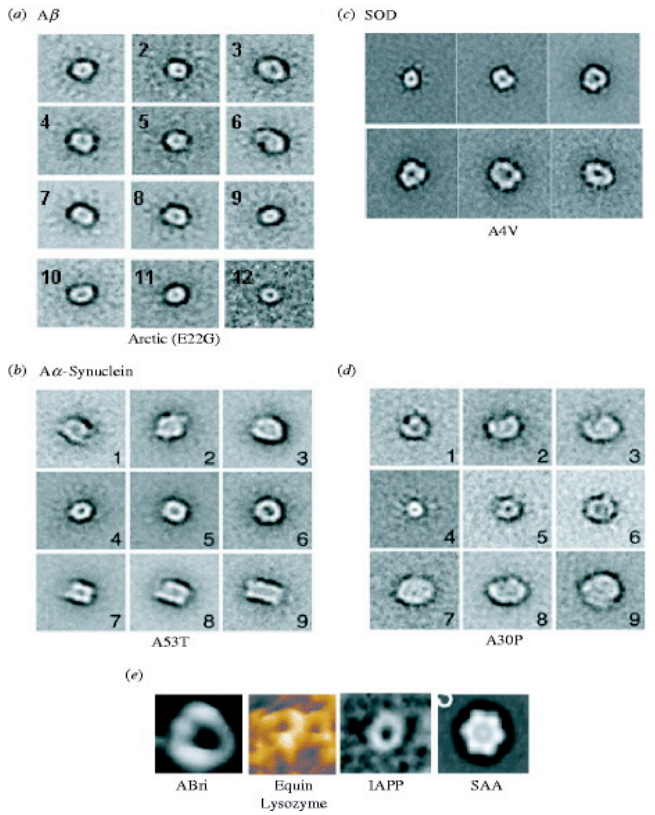

Fig. 1.8. Annular pore-like structures with variable diameters form during the in vitro fibrillogenesis of disease associated mutants of Aβ [Arctic variant (E22G) of Aβ40] (*a*, *e*), α-synuclein (A53T & A30P) (*b*, *d*), SOD1 (A4V) (*c*), and other amyloid-forming proteins (*e*) [**23**].

Mechanisms of membrane disruption others than the formation of pores have also been described: for example, according to some studies human islet amyloid polypeptide (hIAPP), which is associated with death of insulin-producing pancreatic β-cells in type 2 diabetes mellitus, and permeabilizes a variety of model membranes, could destroy the barrier properties of cell membrane by extracting lipids from the latter and taking them up in the forming amyloid fibrils [**53**]. It is important to note that the exact mechanism of membrane disruption by hIAPP aggregates is not known, however such a lipid uptake has been proposed as a generic mechanism underlying amyloid toxicity [**53**] and it has been shown to occur for a variety of proteins [**54**].

The interaction between protein aggregates and membranes may be modulated by changes in the lipid composition of membranes. For example, it can be influenced by the cholesterol content. Cholesterol is the predominant sterol in the plasma membrane and is required for numerous cellular functions. Changes of its content in the membrane can modulate lipid fluidity, affecting various cellular functions related to the cell membrane, including endocytosis, enzyme activities and receptor functions. It has been reported that in AD brains, the cholesterol/ phospholipid ratio is reduced by as much as 30% whereas the phospholipid/protein ratio remains unchanged [**55**]. It has previously been reported that PC12 cells become resistant to

the toxic effect of Aβ peptides when incubated in a medium that enriches cholesterol levels in the plasma membrane [**56**].

Moreover, increasing cholesterol content of phosphatidylserine liposomes suppresses the liposome aggregation induced by these peptides [**56**]. The presence of cholesterol in artificial lipid bilayers also inhibits the permeabilizing activity of human amylin [**57**]. This protective role played by cholesterol could be explained by its ability to modulate the fluidity of membranes, so hindering the incorporation and pore formation of amyloidogenic peptides/proteins into cell membranes.

1.7. Phospholipid membranes as catalysts for protein aggregation

As outlined above, the ability of protein-lipid interactions may play an important role in amyloid toxicity. Membranes may be implicated not only as targets of aggregate toxicity, via disruption of membrane integrity, but also as catalyst that facilitate protein conformational changes and oligomer formation [reviewed in **58**]. Indeed, it is well established that lipid membranes can promote the aggregation of many different proteins/peptides. In particular, lipid composition, especially the presence of negatively charged lipids in the membrane, appears as one of the primary factors that determine the extent of membrane-mediated aggregation.

Many studies have been carried out to investigate the potential of acidic phospholipid-containing membranes in providing an environment able to enhance amyloid formation. For example, the formation of fibrous aggregates by several proteins in the presence of acidic, negatively-charged phospholipids, such as phosphatidylglycerol (PG), cardiolipin, or phosphatidiylserine (PS), has been described in recent studies. Membranes containing PS, an acidic phospholipid normally expressed in the outer surface of the plasma membrane of cancer cells and vascular endothelial cells in tumours [**59**], have been suggested to create surfaces with a high local concentration of protons, required for aggregation [**54**].

All aggregation-prone proteins share the presence of cationic residues or cationic amino acid clusters able to interact with the negatively charged lipids, with subsequent fusion. The binding to acidic phospholipids neutralizes the positive charges in these proteins; accordingly, protein-protein interactions would not been counteracted by repulsion due to cationic residues, protein aggregation being facilitated. The fibrils formed under these conditions have also been shown to incorporate lipid molecules, so suggesting a lipid extraction by protein on the membrane [**53**; **54**]. The liposome-induced fiber formation has been described as very rapid, with macroscopic structures clearly visible almost immediately after the addition of the proteins to a solution of PS-containing liposomes. Enhanced fiber formation has been verified to occur also on negatively charged mica, thus underlining the important role of surfaces for all amyloidogenic proteins [**60**].

2. Diabetes mellitus and human amylin

2.1. The two kinds of diabetes mellitus

Diabetes mellitus is a group of metabolic diseases, widely diffused and characterized by hyperglycaemia resulting from some defects in insulin secretion by pancreatic islet β-cells, insulin action or both [**61**]. There are two main kinds of diabetes [**61**]. Type 1 diabetes (insulin dependent) develops in individuals mostly under the age of 30 years and is due to autoimmune-mediated destruction of pancreatic islet β-cells which determins a dramatic insulin deficiency. Its frequency is low with respect to Type 2 diabetes mellitus (T2DM) or NIDDM, which represents over 90% cases. T2DM occurs with increasing frequency in aged individuals and is characterized by fasting hyperglycaemia that worsens as the disease progresses. In fact it presents an abnormal insulin secretion, associated with varying degrees of chronic insulin resistance and progressive decline in pancreatic β-cells function [**62**]. Despite improved therapeutic strategies, the burden of diabetes remains dramatic. Mortality is up to 5 times higher in diabetic than in non-diabetic people and a chronic hyperglycaemia is often associated with damage of several organs, such as heart, blood vessels, eyes, kidney and nerves [**61**].

NIDDM results from a combination of genetic and acquired factors that impair β-cell function but also tissue insulin sensitivity [**63**]. Several gene polymorphisms have been associated with this form of diabetes. Among acquired factors, glucotoxicity, lipotoxicity and altered processing of hIAPP, a neuroendocrine hormone co-expressed with insulin by pancreas, play an important role. However growing evidence is showing there is no hyperglycaemia without β-cell dysfunction: in fact most people who develop insulin resistance (because of obesity, for example) can increase their insulin secretion appropriately and maintain glucose homeostasis for years, avoiding diabetes [**63]**. Instead in people developing diabetes, insulin levels become progressively too low to support insulin requirements of peripheral tissues [**63**], so blood glucose levels rise, with a progression from normal to impaired glucose tolerance first and to overt diabetes eventually. Notably, deterioration of diabetes control and insulin secretion occurs with years in NIDDM patients, despite insulin resistance remains stable [**64]**. Therefore insulin resistance induces T2DM in people genetically predisposed and β-cell dysfunction is central to the development of diabetes, probably due to a combination of decreased β-cell mass and insulin secretion defects.

2.2. β-cell mass

In a normal pancreas there are over million islets of Langerhans which contain several different kinds of endocrine cells: the insulin secreting β-cells represent the majority (60-80%), followed by glucagon containing cells (α-cells, 20-30%), somatostatin cells (δ-cells, 5-15%) and pancreatic polypeptide cells (PP-cells). β-cell mass is regulated by four different mechanisms, that is apoptosis, size modification (hypo- and hyperplasia), replication (mitotic division of

differentiated β-cells) and neogenesis (development from precursor cells) [**65; 66**]. The contribution made by each of these mechanisms is variable and may change with species as well as different states of life or metabolic demand.

In healthy humans after birth there is a transient burst of β-cells replication, followed by a transitory rise in neogenesis; since in this phase the rate of apoptosis is low there is a marked increase in β-cells growth early in life. Then, during childhood and adolescence the shares of β-cells replication, neogenesis and apoptosis adjust to reach a balance that guarantees the adequate β-cell mass through adulthood. In the senior age, β-cell mass may decrease as apoptosis slightly outweighs replication and neogenesis [reviewed in **67**].

Normally, β-cell mass can adapt to changes in metabolic homeostasis. Obesity and the inherent insulin resistance mainly cause β-cells increase through enhanced replication, neogenesis and size. However the progression from a condition of insulin resistance to a state of diabetes is associated to a decrease in β-cell mass [**63**; **68**]. This loss is due to a marked increase in β-cells apoptosis, which clearly outweighs replication and neogenesis. This condition can proceed further and a severe condition of T2DM may develop which needs insulin replacement therapy [**65**; **66]**.

The role of reduced β-cell mass in human NIDDM, the primary importance of β-cell apoptosis and the insufficiency of replication/neogenesis have been studied [**69; 70**; **71]** and decreased islet mass, reduced β-cell mass and diminished β-cell insulin secretory granules have been reported [reviewed in **67]**. Recently, pancreatic autopsy samples from obese patients with diabetes or with impaired fasting glucose (IFG) or without diabetes as well as from lean patients were examined [**71**]. The authors found that obesity in non-diabetic people is accompanied by a 50% increase in relative β-cell mass as compared with lean non-diabetic humans. However, obese humans with IFG and T2DM had a 40% and 63% deficit in β-cell volume, respectively, while lean subjects with T2DM had a 41% deficit in relative β-cell volume compared with lean non-diabetic patients. These differences were due to a reduced number of β-cells rather than a a smaller volume of individual cells. Neogenesis, while increased in obesity, was comparable in all groups. Moreover β-cell replication was found not significantly decreased in people affected by T2DM or IFG. However an important increase of apoptotic events was detected in type 2 diabetic *vs* non-diabetic cases.

2.3. Insulin secretion function

Even a 50% β-cell mass reduction might not be sufficient to cause hyperglycaemia, since in experimental partial pancreatectomy in rodents normoglycaemia can be maintained. So alterations of insulin secretion, intrinsic or acquired, have to play a role.

The β-cells produce and secrete the hormone insulin (derived from the precursor proinsulin) to maintain circulating glucose concentrations within a narrow, physiological range [**72**]. Glucose is the main physiological insulin secretagogue, even if several other molecules (nutrients, hormones, neurotrasmitters and drugs) can induce and/or amplify its release. According to the most widely accepted hypothesis, glucose induces insulin release as described in Fig. 2.1: glucose equilibrates across the cell membrane by specific glucotransporters (GLUT1 and

GLUT2 especially) and it is phosphorylated by glucokinase which determines metabolic flux through glycolysis. Reducing equivalents are then produced in the mitochondria by the tricarboxylic acid cycle from carbohydrates and from fats and they are transferred to the electron transport chain (ETC). The energy released by the ETC is used to pump protons out of the mitochondrial inner membrane, creating the transmembrane electrochemical gradient, which is used to make ATP from ADP and phosphate. These events result in an enhanced ratio of ATP to ADP in the cytoplasm which causes the closure of the ATP-sensitive K^+ channels. So there is a depolarization of the plasma membrane, an influx of extracellular Ca^{2+} and an activation of insulin exocytosis [**67**]. Classically, glucose-stimulated insulin secretion presents an early phase, which ends after few minutes, and a more prolonged second phase. Moreover it has been demonstrated that the release of insulin is an oscillatory event [**73**].

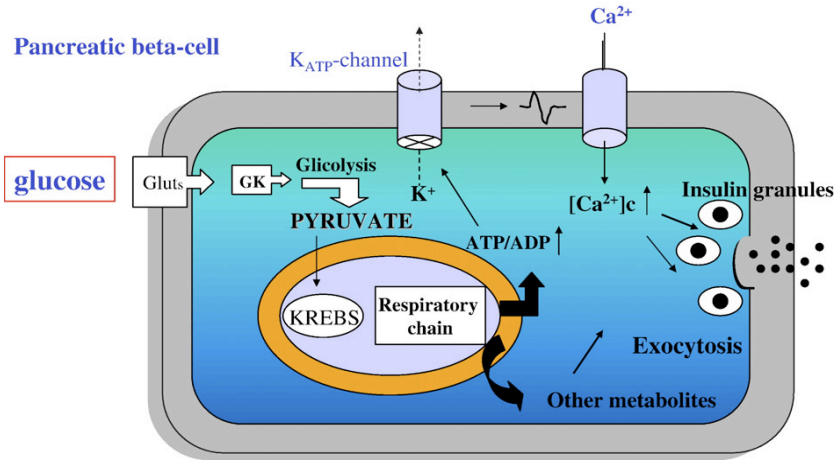

Fig. 2.1. Mechanism of glucose-induced insulin secretion [**67**].

In T2DM there are quantitative and qualitative alterations of insulin release [**63**; **74**; **75**]. Commonly found abnormalities include reduced or absent first phase insulin secretion in response to intravenous glucose, delayed responses to mixed meal ingestion and, with time, reduced second phase release and diminished response to non-glucose stimuli. Qualitative defects are mainly represented by alterations of oscillatory patterns and increased pro-insulin release. As reported before, insulin is released in pulses [**73; 76**] which take place every 8 to 10 minutes in normal subjects and are superimposed on much longer oscillations. The higher-frequency pulses are intrinsically controlled by the islets themselves while the lower-frequency oscillations may be regulated by signalling from outside the islets. In T2DM this pattern is disrupted [**77**; **78**].

Increased pro-insulin/insulin ratio has been also reported in T2DM [**75; 79**]: in fact within β-cell secretory granules, under normal conditions, pro-insulin is cleaved into insulin and C peptide. An amount of intact or incompletely cleaved pro-insulin precursor molecules remains in the granules (around 2%) which are released along with insulin. In patients affected by NIDDM the

presence of pro-insulin molecules is increased 4-5 folds when compared with that of healthy individuals, indicating less efficient conversion of pro-insulin to insulin and this effect seems to be proportional to the level of hyperglycaemia [**75**]. Therefore the increased ratio of pro-insulin to insulin in circulation is a good marker of qualitative β-cell dysfunction, even if it cannot account for the quantitative defect in insulin secretion which occurs in people with impaired glucose tolerance and diabetes.

2.4. Physiological functions of human amylin

Under normal conditions human amylin is a low soluble 37-residue peptide, product of a gene located on chromosome 12 [**80**].

It is a naturally occurring neuroendocrine hormone that is co-expressed with insulin by pancreatic β-cells [**81**] and co-secreted in the same secretory vesicles with insulin from pancreatic β-cells (molar ratio hIAPP:insulin of approximately 1:100). Consequently the plasma concentrations of the two hormones display a similar diurnal pattern of low fasting levels and rapid and robust increases in response to meals: in fact human amylin and insulin are cosecreted in response to the elevation of plasma glucose levels [**82**]. In healthy people, plasma hIAPP concentrations generally range between 4 ρmol/L (fasting) and 25 ρmol/L (postprandial). Instead in obese people both human amylin and insulin secretion are increased, while in T2DM and normal aging both tend to decline. Small amounts of hIAPP have also been detected in pancreatic α- and δ-cells [**83]**, in the stomach [**84**] and in various neurons of the nervous system.

The peptide is highly conserved between species, implying a functional significance, and it belongs to a family of peptides (amylin, calcitonin, calcitonin-gene-related-peptide and adrenomedullin), sharing to varying extents metabolic functions in the control of nutrient assimilation, storage and disposal and bone resorption [**85**; **86]**. In fact, even if its physiological functions are not completely clear, there are indications that human amylin binds to specific membrane-located receptors and experimental studies have shown that hIAPP complements the effects of insulin in regulating postprandial glucose homeostasis through several centrally mediated effects [**87**]. This function of human amylin takes on special significance in NIDDM since, as we have just said, the patients affected by this disease have a deficient secretion of insulin and so glucagon secretion is inappropriately upregulated following a meal. The cumulative effect of the ingestion of glucose and the inappropriate upregulation of glucagon causes total blood glucose to rapidly rise. They have been identified at least four effects by which hIAPP complements insulin, reported below with their respective mechanisms of action (Fig. 2.2):

1) **Reduction of postprandial glucagon secretion** and therefore the reduction of endogenous glucagon-stimulated hepatic glucose output [**88**]. The mechanism by which human amylin suppresses glucagon secretion has not been directly determined. However possibilities include a central effect, a direct effect on the α-cells and a reduced rate of exposure to protein nutrients that stimulate glucagon secretion because of a reduced rate of gastric emptying. Results from preclinical studies indicate that this action is mediated centrally.

2) **Regulation of gastric emptying** (reduction) and therefore the rate of nutrient delivery (exogenous glucose) to the small intestine [**89**]**.** Amylin-sensitive neurons in the area postrema, acting via a vagal pathway, are implicated in the regulation of gastric emptying. Results also support the presence of a regulatory feedback mechanism via glucose-sensitive amylinergic neurons present in the area postrema whereby hypoglycaemia can override the regulation of gastric emptying by hIAPP.

3) **Reduction in food intake** and therefore the reduction of exogenous glucose entering the circulation [**90]**. As with the effect of human amylin on gastric emptying, the effect on food intake appears to be mediated via amylin receptors, located in the area postrema. However, this effect does not appear to involve vagal transmission. In one recent trial, patients with type 2 diabetes who were administered “pramlintide” (a soluble, nonaggregating, synthetic peptide analogue of human amylin) had their nutrient intake reduced by 23% from baseline [**91**].

4) **Reduction in body weight**, which is at least partially due to a reduction in food intake.

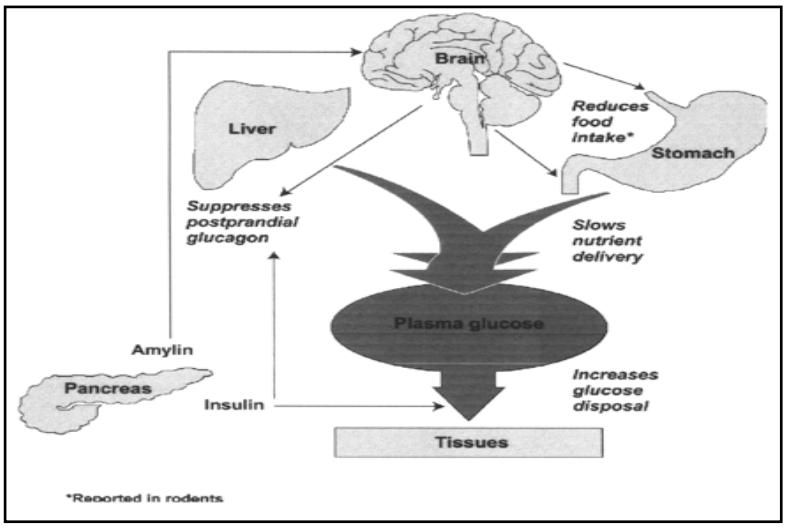

Fig. 2.2. Proposed model of human amylin and insulin action in postprandial glucose homeostasis. Insulin is the major hormonal regulator of glucose disposal. Preclinical and clinical studies indicate that human amylin complements the effects of insulin by regulating the rate of glucose inflow to the bloodstream [**92**].

So these mechanisms collectively reduce the total insulin demand.

It is important to report that application of hIAPP to rat soleus muscle strips was shown to inhibit insulin mediated glucose uptake [**93**]. It was assumed that large amyloid pancreatic deposits in T2DM would be associated with high circulating levels of hIAPP which in turn contributed to the insulin resistance of this disease. Instead the circulating concentrations of the peptide (4-20ρM) were found to be far below that required to inhibit insulin action (nM) and furthermore plasma hIAPP levels in NIDDM were not increased compared with non diabetic controls [**81**]. Subsequent clinical studies indicated that the initial preclinical observations were not due to a direct effect of human amylin to oppose insulin action in muscle but to a direct insulin-independent activation of glycogen phosphorylase in skeletal muscle.

2.5. Factors of β-cell damage in NIDDM: genetic factors, glucotoxicity, lipotoxicity and islet amyloid deposits of human amylin

From few studies focused on some primary alterations in insulin secretion and islet cell survival in NIDDM isolated islets, it has been found that the functional defects of such islets were accompanied by reduced mRNA expression of insulin, GLUT1, GLUT2 and glucokinase and diminished glucose oxidation [**70**; **94**]. Moreover isolated diabetic islets were charcaterized by increased apoptosis with enhanced capsase-3 and -8 activity [**70**]. All these alterations were associated with increased oxidative stress, as shown by higher concentrations of oxidative stress markers (nitrotyrosine for example), increased expression of ROS producing enzymes (PKC β2 and NADPH-oxidase), changes in the expression of free radical scavenging enzymes and alterations of mitochondria [**70**; **95**]. In particular mitochondria in T2DM look round-shaped, with undefined membranes and cristae, and increased density volume [**96**]. Moreover several mitochondrial proteins, such as complex-1 and -5 of the respiratory chain, as well as uncoupling prptein-2 (UCP-2), have higher expression in diabetic islet cells [**95**]. However the ATP/ADP ratio in the diabetic cells is lower than in the non diabetic cells [**95],** suggesting than in the former the respiration is possibly trying to cope with the increased substrate availability and that, however, the energy produced is likely to be wasted, at least in part, through the UCP-2 pathway.

All these molecular events are due to β-cell damage in NIDDM, which is the result of a combination of genetic and acquired factors.

Several genes have been associated with islet cell dysfunction in T2DM, including some encoding for transcription factors, glucose metabolism proteins, molecules of the insulin signalling pathways and others [**97**; **98**; **99**]. For example islet cells carrying the Arg972 polymorphism of the insulin receptor substrate-1 (IRS-1) have increased serum deprivation induced β-cells apoptosis and reduced insulin secretion [**100**]. In addition β-cells with the polymorphism have a lower number of mature insulin granules, suggesting some defects in the process of formation of the granules themselves.

Among acquired factors, glucose toxicity and lipotoxicity have been deeply investigated.

Glucotoxicity, which associates β-cell desensitization to glucose and increased apoptosis with increased glucose concentrations, has been demonstrated with human islets *in vitro* by some studies [**101**; **102**]. These researches support the clinical evidence that lowering serum glucose levels in people affected by NIDDM or with impaired glucose tolerance, can increase their acute insulin response to glucose. A number of mechanisms have been proposed to explain the deleterious effects of prolonged exposure to high levels of glucose on islet cells, including increased oxidative phosphorylation, over activation of the hexosamine pathway, enhanced activity of PKC and others (**101**; **103; 104**).

Notably, all these pathways lead to higher production of ROS and the consequent oxidative stress could explain most of the observed β-cell defects.

Lipotoxicity is another acquired factor of β-cell damage and it is the deleterious effect on β-cells of accumulated fatty acids and their metabolic products which may be observed in subjects with insulin resistance, glucose intolerance and T2DM. Fluctuations in free fatty acids levels are necessary for normal β-cell function but prolonged increases in these levels induce a negative

effect on human β-cell function. The main possible causes of lipotoxicity are inhibition of insulin gene expression and increased ceramide production [**104**]. It is important to note that different fatty acids have different effects on β-cells and in addition it has been proposed that whereas glucose toxicity occurs independently of lipotoxicity, this latter requires the presence of increased glucose levels to be fully manifest [**104**]. Both glucotoxicity and lipotoxicity can also have an impact on β-cell death. A prolonged exposure of cultured human islets to high glucose levels increases β-cell apoptosis in a dose dependent manner [1**01**], such as increased fatty acid levels induce apoptosis [**102**].

Finally the presence of **amyloid deposition** in the islets is another acquired factor which contributes to β-cell dysfunction and death [**105**; **106**]. Pancreatic amyloid deposits are a common pathophysiological feature of NIDDM, found in over 90% of all cases, mainly as extracellular deposits near the insulin-producing β-cells [**107**; **108**] and the degree of amyloid deposition correlates with severity of the disease in humans (Fig. 2.3). The groups of Westermark and Cooper independently identified the peptide called human amylin or hIAPP as the major component of these amyloid aggregates [**109**; **110**], even if these deposits contain a variety of other products including serum amyloid P component (SAP), apolipoprotein (APO) E and the heparan sulfate proteoglycan perlecan [**108**], whose role is not completely understood, however they are also present in other types of amyloid deposits (for example in AD). hIAPP-containing amyloid deposits are also found in insulinomas [**110**]**.** Many piece of evidence demonstrate that hIAPP aggregation generates species that are toxic, when added exogenously, to cultured human islets and β-cells since they induce β-cell apoptosis [**111**]. In addition, overexpression of human amylin in COS-1 cells results in accumulation of hIAPP aggregates and cell death [**112**]. Furthermore, while IAPP in humans, cats and monkeys is amyloidogenic and these species develop T2DM with reduced β-cell mass, in contrast rats and mice do not develop the disease since their IAPP is not amyloidogenic [**113].** However transgenic rats and mice expressing hIAPP develop a diabetic phenotype associated with the deposition of human amylin amyloid fibrils [**114**] and they show increased risk of becoming diabetic in a background of obesity (a major risk factor for T2DM) [**115**]. Further support for the role of human amylin in T2DM comes from the discovery that the S20G mutation in hIAPP has been linked to a familial form for early onset NIDDM in some Asian countries [**116]**. The mutant is resulted more cytotoxic than the wild-type peptide and it aggregates more rapidly**.** Although this mutation is restricted to some ethnic groups only, its existence suggests that other mutations might contribute to the amyloidogenicity of hIAPP.

Accordingly, taken together, these studies suggest that human amylin misfolding and amyloid aggregation play a key role in NIDDM pathogenesis.

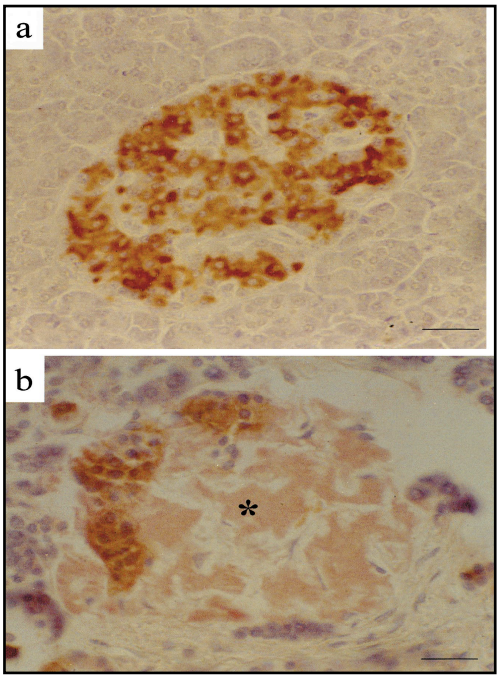

Fig. 2.3. Pancreatic islet structure in non-diabetic and T2DM subjects. (a) Islet of a non-diabetic subject immunolabelled for insulin (brown): insulin-containing β-cells occupy over 80% of the islet space. (b) Islet of a diabetic subject labelled for insulin (brown) and stained with Congo red for amyloid (pink): over 50% of the islet space is filled with Congo Red stained, amorphous amyloid deposit (asterisk). The remaining β-cells are at the edges of the deposits. (a, b) Scale bar =20 μm [**117**].

2.6. Molecular characterization, structural properties and fibril formation of human amylin

Human amylin is synthesized as the prohormone precursor prohIAPP (89-aminoacids) along with proinsulin and it is characterized by the presence of a signal sequence at the N-terminus, which is proteolytically removed. The resultant 67-residue prohormone (prohIAPP) and proinsulin, prior to cosecretion, undergo disulfide bond formation in the rough endoplasmic reticulum and then they are processed by the same subtilisin protein family, known as prohormone convertase (PC)2 and PC1/3 or furin within β-cell secretory granules. The action of this enzyme is followed by the activity of carboxypeptidase E to remove the basic PC1/3 recognition motif (Lys-Arg) and then by the activity of the peptidil amidating monooxygenase complex to remove Gly38 and to amidate the exposed carboxy-terminus of Tyr37 in hIAPP [**118**]. Thus, normal processing of the 67-residue prohIAPP to yield the 37-residue hIAPP requires the formation of a disulfide bridge between residues Cys13 and Cys18 of prohIAPP, followed by cleavage of 11 residues at the N-terminus, 19 residues at the C-terminus, 2 basic residues from the resultant C-terminus and finally amidation of the carboxy end of tyrosine at the C-terminus of hIAPP [**80**] (Fig. 2.4).

Under normal conditions, upon secretagogue stimulation, mature human amylin is secreted into the extracellular matrix along with mature insulin and excreted via the kidney [**119**].

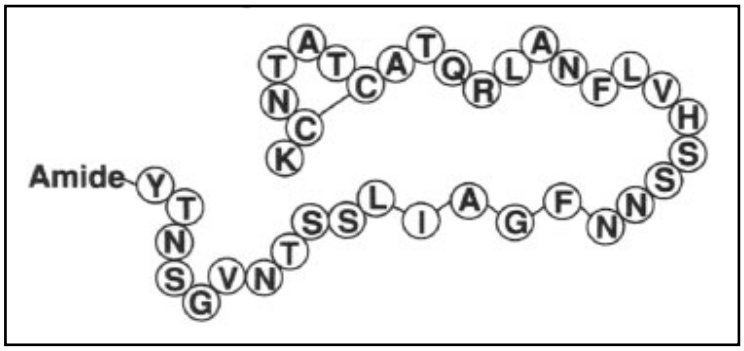

Fig. 2.4. **Amino acid sequence of hIAPP**. Note disulfide bond (C2 and C7) and amidated C-terminus.

The primary sequence of hIAPP is closely conserved between species but the physco-chemical properties of IAPP obtained from different sources are quite dissimilar [**7]**, due to some important differences in $IAPP_{20-29}$ region. It is interesting to note that non-human primates, humans and cats share close homology in this region, the synthetic forms of these peptides form amyloid aggregates in aqueous environment and these are the only members of the animal kingdom that develop spontaneous T2DM [reviewed in **120**]. Instead rats and mice have an identical $IAPP_{20-29}$ region but it is not amyloidogenic, due to three proline substitutions at position 25, 28 and 29 that render rat and mouse IAPP soluble in water and they are not known to be affected by NIDDM [**120**], except for transgenic mice expressing human amylin [**114**]. Accordingly, these data suggest that the amino acid sequence of hIAPP plays a relevant role in the pathogenesis of T2DM.

The structural information about the hIAPP monomer is very limited. The native structure of hIAPP in unknown, although CD spectra show that the peptide, solubilised in 1,1,1,3,3,3-hexafuoro-2-isopropanol (HFIP), filtered and lyophilised, remained in stable random-coil structure for up to 7 days in water, indicating that it may be a natively unfolded protein [**121**]. Recent NMR experiments also suggest that the human amylin peptide chain is unfolded, even if part of the chain, approximately residues 8-19, can dynamically adopt α-helical structure [**122**] since addition of SDS or binding to a membrane results in a stabilezed, mostly α-helical, monomeric hIAPP states [**122**]. The interaction of monomeric hIAPP with insulin is also suggested to increase the helical tendency of human amylin [**122**], although this interaction seems to be very dependent on the physical forms of hIAPP and insulin, i.e. soluble or fibrillar hIAPP and soluble or crystalline insulin [**123**]. Both insulin and lipids thus seem to induce helical structure, but surprisingly their effect on hIAPP aggregation is opposite. Engel reviewed that lipids have a tendency to promote hIAPP aggregation, whereas insulin is well known as an inhibitor of hIAPP fibril formation [**124**]. The effect of lipids and insulin on the secondary structure of hIAPP monomers *in vitro* could suggest that hIAPP might be structured under *in vivo* conditions through interaction with physiological binding partners (insulin and lipids are both present in secretory vesicles, the cellular storage of hIAPP and insulin). The lack of data on the structure of monomeric hIAPP in solution is due to the fact that human amylin has high

fibrillogenic propensity *in vitro* (in aqueous solutions), leading it to quickly aggregate monomers into insoluble amyloid fibrils (within few hours) [**125**].

As reviewed by Khemtemourian, the *in vitro* aggregation and fibril formation of human amylin have been studied extensively in the last years and in most of these studies this aggregation is initiated by dilution of, usually synthetic, monomeric hIAPP into a physiological buffer [**126**]. This results in the "spontaneous" aggregation of human amylin monomers into amyloid fibrils, significantly faster than the aggregation of most other amyloidogenic peptides. Fibril formation of hIAPP, as well as of some other amyloidogenic peptides, generally occurs via a nucleation dependent aggregation process and next to the monomeric and fibrillar states of human amylin, several intermediate (oligomeric) states have been observed (see paragraph 1.3). The kinetics of the peptide fibril growth can be monitored in time by the commonly used method of specific binding of the fluorescent molecule ThT to amyloid fibrils (see paragraph 1.3). A kinetic trace of hIAPP fibril growth shows a lag phase and a sigmoidal transition that are typical of fibril growth of amyloidogenic proteins and peptides (Fig. 2.5).

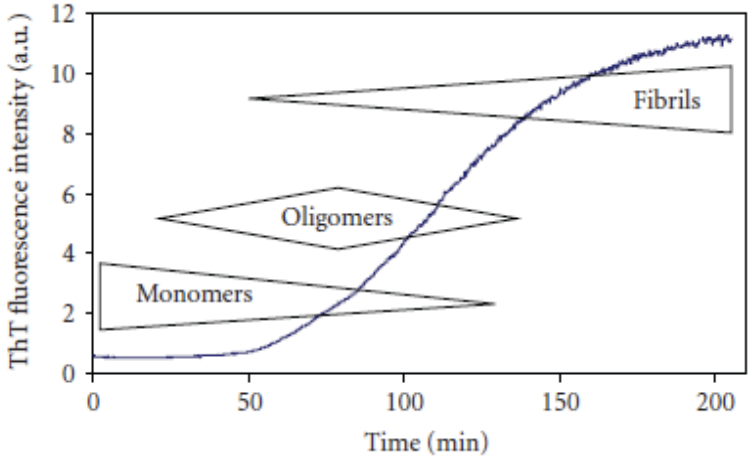

Fig. 2.5. Typical kinetic of hIAPP fibril formation, characterized by a lag phase and a sigmoidal transition. The approximate aggregation state of hIAPP is indicated at the various time points. Fibril formation was induced by adding, at time 0, a monomeric stock solution of human amylin in dimethyl sulfoxide (DMSO) to buffer containing ThT [**126**].

Khemtemourian reviewed that this fibrillogenic propensity is dependent on specific residues, placed in different regions [**126**]. Structural studies have shown that the amino acid residues 20-29 are crucial for amyloid formation [**113**]: a proline scan of this decamer has demonstrated that substitution of a single proline at either position 22, 24 or at postitions 26-28 leads to a drastic reduction of hIAPP amyloid aggregation [**127**]. Note that, as we have previously said, three differences between hIAPP and the nonamyloidogenic mIAPP involve a proline, a residue predicted to disrupt ordered structures, such as β-sheet structure in amyloid fibrils. Moreover it has been shown that $hIAPP_{8-20}$ and $hIAPP_{30-37}$ fragments also can form amyloid-like fibrils, like the hIAPP fragment consisting of residues 14-20 [reviewed in **126**]. These observations suggest

that there are several potential β-strand-containing regions in human amylin, in addition to the well known residues 20-29.

It has been hypothesized that aromatic-aromatic interactions are also important in hIAPP fibril formation [**128**]. Human amylin contains three aromatic residues at positions 15, 23 and 37 and their aromatic-aromatic and aromatic-hydrophobic interactions in amyloid formation were studied using a hIAPP triple mutant [**129**]. The triple mutant F15L/F23L/Y37L, lacking aromatic residues, still forms amyloid fibrils in vitro, indicating that the aromatic residues are not essential in human amylin amyloid aggregation. However the substitutions decrease the rate of fibril formation and alter the tendency of fibril to aggregate.

hIAPP contains a single histidine at position 18 which is the only residue that has a charge that depends on pH in a physiological pH range. Consequently fibril formation of human amylin could depend on pH. A recent study showed that hIAPP fibril formation is faster at a lower pH (4.0) than at a higher pH (8.8) [**130**]. This could be important in a physiological context, since in the β-cell granules of the pancreas where human amylin is stored, the pH is 5.5 but when it is released into the extracellular compartment, it experiences a pH of 7.4 [**131**].

Another characteristic of hIAPP is the intramolecular disulfide bond between cystein residues 2 and 7. This bond does not contribute to the amyloid fiber core structure, however it must play a central role in the assembly mechanism, since the loss of the disulfide bond significantly reduces fibril formation [**132**].

Human amylin oligomers and fibrils are not single, morphologically homogeneous species, but each of them represents a subset of species of different sizes, shapes (polymorph) and with variations in secondary, tertiary and quaternary structure. The information about the structure of hIAPP oligomers is sparse. As reviewed in Engel [**124],** all our knowledge of the hIAPP oligomer structure comes from *in vitro* produced oligomers, which present large variations in shape and size, ranging from a cluster of a 10-20 hIAPP monomers to more than 500. Some studies evidenced spherical hIAPP oligomers, others have detected ring-shaped hIAPP oligomers [**124**]. About their secondary structure, time-dependent studies of the *in vitro* aggregation of human amylin suggest a transient increase in helicitly, as judged by CD, during its assembly to form amyloid although the authors did not explicitly consider helical intermediates [**133].** However it is important to underline that, as already said (see paragraphs 1.5-1.6-1.7), like other amyloidogenic peptides and proteins, human amylin is able to interact with cell membranes which have been implicated both as the targets of oligomer toxicity and as the catalysts that facilitates oligomer formation. Even if many aspects of the structure and formation of these oligomers are unknown, there are recent indications that hIAPP oligomers, in the presence of membranes, exhibit α-helical structure before the formation of the final cross-β structure [**134**] (see below paragraph 2.8.). A significant finding that has helped in detecting and characterizing human amylin oligomers has been the realization of an Aβ oligomer-specific antibody, named A-11, which also binds specifically to oligomers of other amyloid-related peptides and proteins (see paragraph 1.3.). It recognizes hIAPP oligomers but not hIAPP monomers or hIAPP fibrils, accordingly this suggests that such oligomers are structurally unique, and different from hIAPP fibrils. Two other antibody/antisera have been produced against human amylin oligomers: the I-11 antibody reacts to the same hIAPP oligomers as A-11, whereas the antiserum APF

specifically recognizes the bigger, annular hIAPP oligomers that are rich in β-sheet [reviewed in **124**]. Thus, both α-helix rich hIAPP oligomers and β-sheet rich hIAPP oligomers have been observed but it is possible that helix formation could play an important role in fibrillogenesis. The use of aggregation-state specific antibodies is expected to reveal more structural characteristics of these species on the aggregation pathway in future studies.

Instead, the three-dimensional structure of human amylin fibrils is best known, despite they being the most insoluble species, because of their stable nature. These fibrils have been studied by various high-resolution techniques, EM, X-ray diffraction, electron diffraction, nuclear magnetic resonance spectroscopy (NMR) and electron paramagnetic resonance (EPR) [reviewed in **126**]. These studies clearly reveal that human amylin fibrils contain a relevant amount of well-ordered cross-β structure, typical of amyloid fibrils (see Fig. 1.4). During fibril formation, the peptide undergoes a conformational change from random coil to a mixture of β-sheet and α-helical structure. hIAPP fibrils are polymorphic, ranging from thin protofilaments with a diameter of about 5 nm to thicker fibrils, with diameter of up to 15 nm that appear to be rope-like bundles of protofilaments. The predominant type of fibril contains three parallel protofilaments in a left-handed coil with a pitch of 25-50 nm [**135**]. The most recent and most detailed model for the structure of an hIAPP fibril suggests that it is composed of stacked layers of two symmetric human amylin molecules that form a parallel β-sheet structure, running perpendicular to the length axis of the fibril (Fig. 2.6) [**136**]. Residues 8-17 and 28-37 form the β-structure, whereas residues 1-7 are largely unstructured. Importantly, this model is based on experimental data of a morphologically homogeneous sample of hIAPP fibrils, so called striated ribbons.

Instead Kajava [**137**] proposed “the parallel superpleated β-sheet structure” as a model for amyloid fibrils of human amylin. According to this model, consistent with the data of the fibril structures of hIAPP and their assembly just reported, individual polypeptides from residues 9 to 37 have a planar S-shaped fold with three β-strands. These serpentines are stacked in register, with a 0.47 nm axial rise and a small rotational twist per step, generating an array of three parallel β-sheets in cross-β conformation (Fig. 2.7 A). The interior, the two “bays” sandwiched between adjacent sheets, are occupied by non polar and by polar/uncharged residues that are predicted to form H-bonded ladders, similar to those found in β-helical proteins. The N-terminal peptide containing a disulfide bound occupies an extraneous peripheral position in the protofilament, implying a loop conformation that would be incompatible with extending the serpentine on this side. Accordingly, the first eight residues are not considered to be part of the serpentine core and since hIAPP 8-37 forms protofilaments with the same characteristics of hIAPP 1-37, it follows that the N-terminal part does not have a major role in fibrillogenesis. Finally, Kajava and coworkers identified left-handed twist of the β-sheets to underlie left-handed coiling of human amylin protofilaments in fibrils (Fig. 2.7 B).

It is important to note that this model also explains mutational data, that is why rodent amylin does not form fibrils. As already said, diabetes-associated amylin amyloid occurs in primates and cats but not in rodents by proline substitutions in positions 25, 28 and 29 in rodents. In this model, Pro in position 25 is located in the middle of the central strand and at this site it would be expected to inhibit protofilament formation by preventing formation of β-structural H-bonds.

Instead residue 28 is also predicted to be involved in inter-strand H-bonding and Pro at this site may also decrease fibrillogenesis.

However most proposed models, combined with aggregation studies on hIAPP fragments, place residues 9-37 in the amyloidogenic core [reviewed in **126**] which, as said above, adjusts the historic view that only residues 20-29 would be important for amyloid formation [**126**]. Different models are likely required to account for the various hIAPP fibrils morphologies commonly observed. Studies of human amylin fibrils in pancreatic islets show that their morphological character is grossly the same as that of *in vitro* produced fibrils but even large-scale features of *in vivo* fibrils, like the number of protofibrils, are unknown.

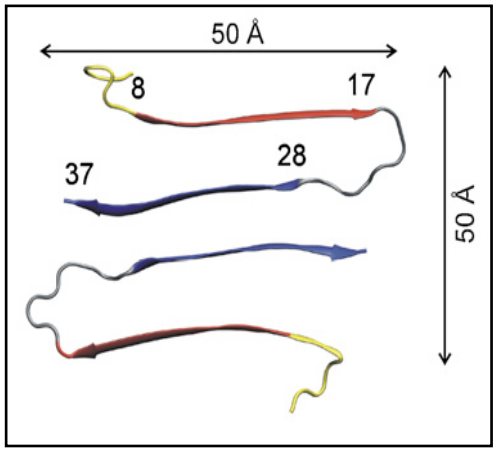

Fig. 2.6. Model of a hIAPP fibril based on NMR data of a morphologically homogeneous fibril sample. The picture shows a cross-sectional view of the fibril, with 2 hIAPP monomers back to back. The long-axis of the fibril is perpendicular to the plane of the paper [**136**].

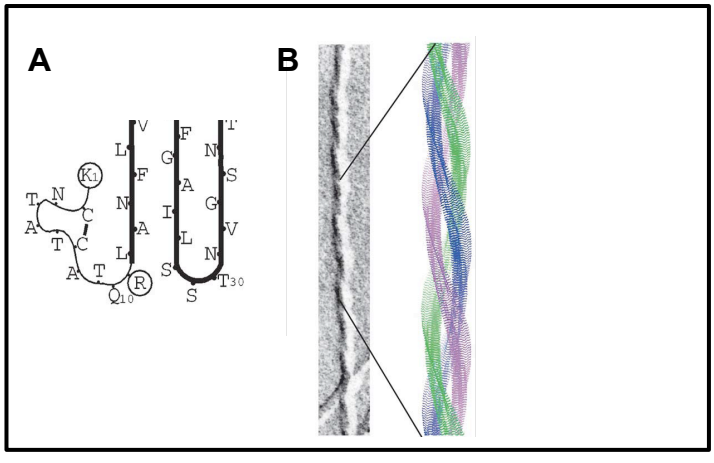

Fig. 2.7. Diagram of the proposed β-serpentine fold for human amylin and Electron micrograph of an hA fibril. (A) The model has three β-strands.Charged residues are circled. The first 11 residues, which contain a disulfide-bonded loop that would be incompatible with extending the serpentine on this side, are shown by a thin line. Accordingly, the first 11 residues are not considered to be part of the serpentine core. **(B)** It reveals that the coiling is left-handed as well as the crossover spacing of 37 nm alongside a fibril model formed by three protofilaments having the superpleated β-structure. The individual protofilaments are coded in different colors [adapteted from **137**].

2.7. Possible causes of hIAPP aggregation and islet amyloid formation

The factors promoting hIAPP aggregation are still not completely known but it is clear that it is consequent to the failure of cellular mechanisms to prevent oligomerization and amyloid deposition of amyloidogenic proteins. It is known that proteins must properly fold into three dimensional structures in order to carry out their proper functions. The endoplasmic reticulum (ER) is responsible for the synthesis, folding and appropriate targeting of all client secretory proteins before their export to the Golgi (most prominently insulin and hIAPP in β-cells).

ER has several important properties to facilitate protein folding, which include a Ca^{2+} concentration of approximately 300 μM (versus 0.1 μM in the cytosol), a relatively oxidative state favoring disulfide bond formation and a protein quality control system [reviewed in **120**]. When precise folding goes awry, unfolded proteins are exported from the ER by retrograde translocation to the cytosol where most are tagged with ubiquitin-conjugating enzymes for degradation by the 26S proteasome, as well as the lysosome [**120**].

Moreover the ER contains abundant chaperone proteins that shield hydrophobic regions of unfolded proteins from surrounding proteins. Given that the ER protein concentration is approximately 100g/liter, these properties are remarkably successful at preventing ER protein aggregation.

This is particularly the case for proteins like hIAPP that are highly prone to form self-aggregates at much lower concentrations in an aqueous environment [**138**].

As reviewed by Haataja, besides these abilities of the ER, in secretory cells the unfolded protein response (UPR) balances ER protein delivery with the capacity of the ER to fold and traffic these proteins to the Golgi and secretory vesicles [**120**]. So the UPR defends the ER from being overwhelmed by misfolded and aggregated proteins that may induce ER stress and apoptosis (see below paragraph 2.9).

Three independent proteins, PERK (protein kinase-like ER kinase), IRE1 (inositol requiring 1), and ATF6 (activating transcription factor 6) detect increased abundance of unfolded proteins in ER and activate a sequence of events that globally decreases translation of major ER client proteins, increases transcription and translation of ER chaperone proteins, such as binding Ig protein (BiP), and increases expression of proteins involved in clearance of unfolded ER proteins [**120**] (Fig.2.8).

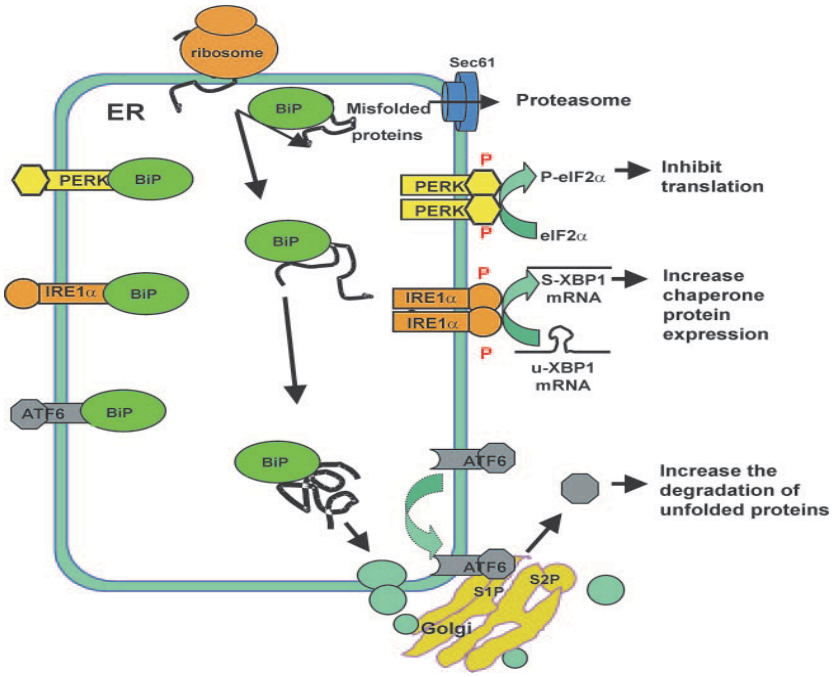

Fig. 2.8. The schematic illustration of the UPR in protein secretory cells. Increased demand for BiP leads to detachment of BiP from PERK, IRE1 and ATF6, which get activated. In this form PERK phosphorylates α-subunit of eukaryotic translation initiation factor 2 (eIF2α), which subsequently suppresses ER protein translation and leads to more ATF4. Activated IRE1α has RNA editing function and removes the hairpin structure on inactive X-box-binding protein-1 (XBP1) mRNA (unspliced, u- XBP1), which later becomes active transcription factor (spliced, s-XBP1). Activated ATF6 translocate into the Golgi and undergoes partial intramembrane proteolysis by site-1 protease (S1P) and site-2protease (S2P), then migrates to the nucleus. Then these activated transcriptional factors induce a series of responses to increase chaperone proteins, limit new protein translation and increase the degradation of unfolded proteins [**120**].

The importance of PERK in the protection of β-cells was illustrated by the development of diabetes due to increased β-cell apoptosis in the PERK -/- mouse [**139**]. In common with the ER, the insulin secretory vesicles presumably sustain hIAPP concentrations that far exceed the solubility of hIAPP in a typical aqueous environment. An important property of insulin secretory vesicles to prevent hIAPP oligomer formation is the acid pH (5.5) of the vesicle lumen: in fact hIAPP is maintained in monomers at this value of pH (99). In addition, insulin interacts with hIAPP to reduce oligomer formation [**138**].

It is likely that there are other factors (chaperone proteins, ions) that restrain oligomer formation in insulin vesicles. For several reasons all these protective mechanisms fail in T2DM, making possible human amylin aggregation and consequently islet amyloid formation with deposition of misfolded hIAPP (Fig. 2.9)

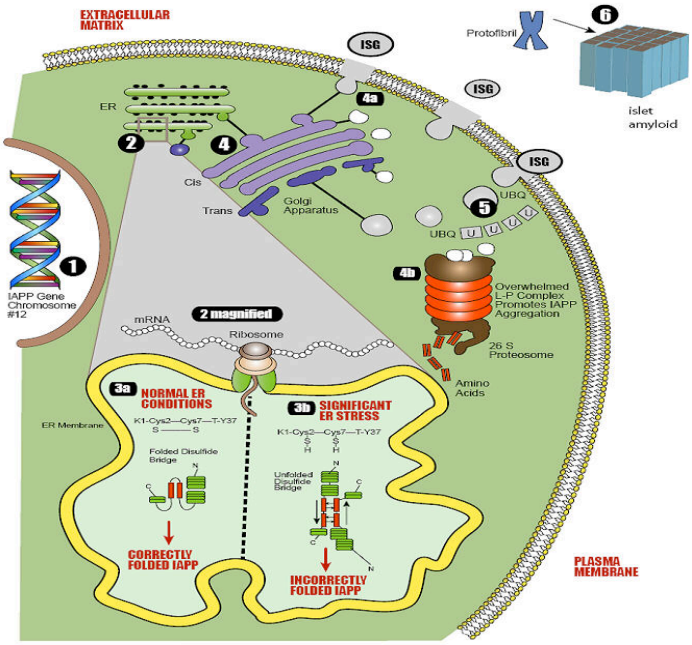

Fig. 2.9. hIAPP misfolding leads to protein aggregates. This cartoon depicts ER, Golgi apparatus, and the lysosome-proteasome complex with respect to the misfolding of hIAPP. The peptide is transcribed from chromosome 12 (1). Translation of hIAPP gene occurs (2). Without significant ER stress, chaperones properly fold hIAPP. Post-translational modifications of hIAPP include the formation of a disulfide bond at positions C2 and C7 and amide formation at the C-terminal tyrosine. The vulnerability of the disulfide bond may play an important role in the unfolding of hIAPP (3a) or in the presence of significant ER stress, hIAPP may become misfolded (3b). hIAPP oligomers may form. hIAPP is transported to the Golgi apparatus (4). Normal processing without significant ER stress results in cosecretion of normal insulin and hIAPP in insulin secretory granules (4a). Once misfolded, aberrant hIAPP is processed initially in the Golgi apparatus, and there is an additional attempt to refold the misfolded protein. If this is unsuccessful, the misfolded protein goes to the lysosome-proteasome complex for degradation to its constituent amino acids (4b). Ubiquitination pathways are also employed to facilitate trafficking to the lysosome-proteasome complex (5). When these organelles are overwhelmed, like in early T2DM before β-cell failure, the result will be apoptosis of the β-cells and the accumulation and aggregation of protofibrils into β-pleated sheets. Finally islet amyloid is formed (6) [**140**].

One widely accepted mechanism to explain this aggregation is that increased production and secretion of hIAPP, associated with increased demand for insulin during the prediabetic stage of T2DM might result in accumulation and aggregation of the peptide [reviewed in **141**]. The fact that non-diabetic obese and/or insulin resistant subjects with elevated human amylin production rarely develop islet amyloid [**108**] speaks against the idea that a simple increase in the level of this peptide secretion is sufficient for amyloid formation in NIDDM. Moreover, as just said (see paragraph 2.4), several groups have reported that transgenic mice with β-cell overexpression of hIAPP develop islet amyloid deposits associated with β-cell death and development of hyperglycaemia but in each of these strains islet amyloid only develops if a predisposing genetic or environmental factor (administration of glucocorticoid, high fat diet, or genetic obesity and hyperlipidemia) is also present in addition to human amylin overexpression [**142**; **115**]. As we have said, each of these factors is known to impact β-cell function. Taken together, these findings suggest that overexpression of hIAPP is important but not sufficient for amyloid formation in T2DM [**141**].

As reviewed by Haataja et al., the increased risk of hIAPP oligomerization with increasing its expression implies that the protective mechanisms against human amylin aggregation and toxicity are saturable [**120**]. This is consistent with the observation that circumstances that increase hIAPP expression by β-cell in humans increase risk for developing NIDDM. Thus insulin resistance (which disproportionately increases hIAPP compared with insulin expression) is a major risk factor for T2DM. Also, a 50% decrease in β-cell mass (which doubles the secretory demand per β-cell) often leads to diabetes in cats and humans (see paragraph 2.4), both of which express an amyloidogenic form of IAPP, but not rats that secrete a soluble form of IAPP [**120**]. Recent genome-wide linkage studies show linkage between risk for T2DM and several cell cycle transcriptional regulatory proteins [**97**]. This together with the wide range of β-cell mass observed in non diabetic humans raises the possibility that a relatively low adult β-cell mass might serve as a risk factor for T2DM. Under these circumstances, insulin resistance would place a substantially increased demand per β-cell in adult humans and, presumably, a greater risk for hIAPP expression rates that exceed the threshold for trafficking soluble human amylin.

Another possible mechanism of islet amyloid formation is that mutations in the prohIAPP gene might facilitate the aggregation process by undesired alterations in the structure of hIAPP [**141**]. This hypothesis is supported by the fact that a serine-glycine substitution at position 20 (S20G) in the hIAPP molecule in a subpopulation of Japanese people with T2DM (frequency 4.1%) is associated with an earlier onset and more severe form of the disease (see paragraph 2.4). However other studies have failed to demonstrate any linkage between abnormality of the hIAPP gene and NIDDM, so it appears that mutations in the hIAPP gene cannot explain amyloid formation in most type 2 diabetic patients, but it may be a contributor in some patients [**141**].

Haataja et al. reviewed other potential mechanisms to increase the risk for hIAPP oligomer formation. One of these would be a decrease in the capacity of β-cells to neutralize toxic oligomers as they form. Insulin degrading enzyme (IDE) has been reported to have this property, and therefore it is of note that the IDE gene shows linkage to both T2DM and AD [**120**]. IDE has been shown *in vitro* to inhibit hIAPP and AβP aggregate formation and cytotoxicity [**143**].

Polymorphisms in chaperone proteins important in trafficking hIAPP are obvious candidates for increased propensity to form hIAPP oligomers. In this regard it is of interest that hIAPP and AβP share close structural properties and that the prevalence of Alzheimer's disease is increased in people with T2DM [**120**].

Also, any factors, inherited or acquired, that disturb the function of the ER might reasonably be expected to increase risk for hIAPP oligomerization. Compromised ER function leads to mitochondrial dysfunction [**120**]. Because ER function requires high energy, it is reasonable to expect that compromised mitochondrial function might lead to ER dysfunction. Therefore mitochondrial dysfunction in β-cells in NIDDM [**95**] might be expected to lead to increased risk of hIAPP aggregation.

Finally factors in the secretory pathway and vesicle environment might also contribute to risk for oligomerization. In cystic fibrosis, acidification of intracellular vesicles is impaired and it is therefore of interest that a high proportion of patients with cystic fibrosis develop T2DM with islet

amyloid [**120**]. Moreover, as we have just said (see paragraph 2.3), there is a higher secretion of incompletely processed insulin in NIDDM, suggesting that the same could be true for hIAPP, since they are in the same vescicles. It has been proposed that defects in the processing of human amylin could play a critical role in triggering islet amyloid formation and deposition [**144]**. In fact incompletely processed hIAPP is also amyloidogenic, even if less than hIAPP [**145; 146**] and immunohistochemical studies of islet amyloid have indicated the presence of the N-terminal region of prohIAPP but not the C-terminal region, thus demonstrating the presence of a processing intermediate designated as prohIAPP$_{1-48}$ [**147**]. One hypothesis is that incorrectly processed hIAPP interacts with heparan sulfate proteoglycans (HSPGs) of the basement membrane and this event may lead to seed formation for amyloid deposits *in vivo* [**148**], as already shown *in vitro* where it was been demonstrated that the process occurs *via* formation of a helical intermediate [**149**]. In fact HSPGs are found in islet amyloid deposits and appear to be a general feature of amyloid plaques [**149**]. The proteoglycan perlecan has been implicated in virtually all human amyloid diseases [**149**]. HSPGs are ubiquitously expressed and have been proposed to serve as scaffolds for amyloid, stabilizing and possibly inducing amyloid formation. Peptide fragments derived from the N-terminal region of prohIAPP$_{1-48}$ have been shown to bind glycosaminoglycans (GAGs) [**144; 148**], suggesting that interactions between HSPGs and prohIAPP$_{1-48}$ might play a role in amyloid deposition in T2DM (Fig. 2.10). Interestingly, studies have demonstrated that prolonged exposure of human β-cells to high glucose concentrations (20mM) increases the relative proportions of prohIAPP and its partially processed N-terminally extended form. Furthermore, *in vitro* studies have shown that prolonged exposure of β-cells to free fatty acids results in impaired processing of proinsulin due to decreased activation of PC2 and PC3 [reviewed in **141**]. It is therefore plausible that hyperglycaemia and/or hyperlipidemia associated with T2DM may contribute to islet amyloid formation indirectly by impairing prohIAPP processing, possibly *via* alterations in the activity of PC2 and PC3 and increased secretion of N-terminally extended prohIAPP.

Disproportionate secretion of incompletely processed prohIAPP and subsequent binding to basement membrane HSPGs may induce conformational changes in prohIAPP that favour β-sheet formation and islet amyloid deposition.

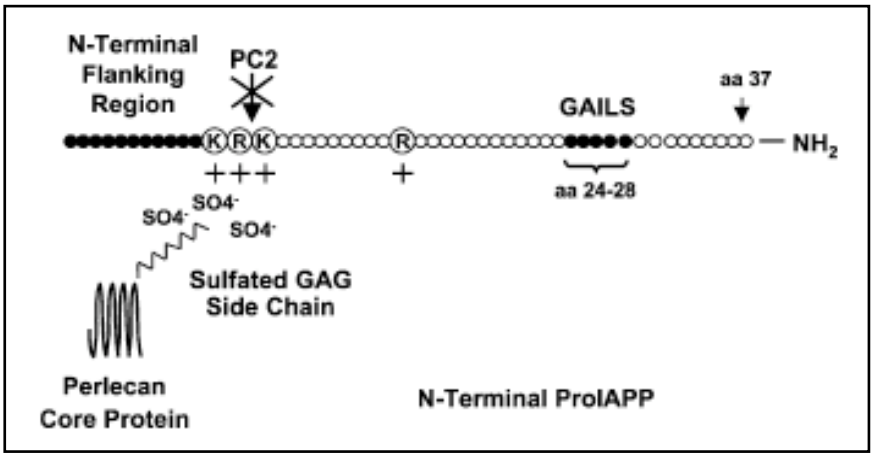

Fig. 2.10. Proposed pathway for islet amyloid formation in T2DM. Impaired processing of proIAPP in NIDDM may result in an increase in the secretion of N-terminally unprocessed prohIAPP which has affinity for GAG side chains of the heparan sulfate proteoglycan perlecan. Binding of prohIAPP to perlecan in the basement membrane of islet β-cells may create a nidus for amyloid formation [**141**].

2.8. Mechanisms of hIAPP cytotoxicity: from the amyloid hypothesis to the oligomer hypothesis and the interaction with the membrane

Just after the discovery of hIAPP as the major component of islet amyloid in 1987 [**109; 110**], it was generally thought that hIAPP fibrils were cytotoxic to β-cells, thereby contributing to T2DM [**150**; **151**]. Interestingly, already in the early seventies a peculiar interaction between extracellular islet amyloid fibrils and β-cell membranes had been noticed [**152**]. It was observed then, and confirmed in later studies, that the islet amyloid fibrils were often orientated perpendicular to the membrane, and co-localized with distinct changes in the morphology of the β-cell membrane [**152**; **117**]. In contrast, in the vicinity of other types of Islet cells (like α-cells), this characteristic orientation of fibrils near membranes was rarely seen, and they were mostly randomly orientated. Whereas these studies involved endogenous hIAPP, also externally synthetic hIAPP added to cultured β-cells induced typical membrane deformations, for example membrane invaginations, budding and vesicle formation [**151**]. These observations made researchers hypothesize that the membrane might be the target of cytotoxic hIAPP and that this could cause death of the insulin producing β-cells, similar to Aβ neurotoxicity in Alzheimer's disease. However it appeared that the amount of amyloid in the Islets of Langerhans did not correlate well with the decrease in the number of healthy β-cells. Thus, the concept that extracellular human amylin amyloid causes β-cell damage (the amyloid hypothesis) is implausible. Currently, the prevailing and well-documented view, based on in vitro evidence, is that soluble hIAPP oligomers are the toxic species and that hIAPP fibrils are biologically inert [reviewed in **120**].

The mechanism of human amylin oligomer cytotoxicity is thought to involve permeabilization of cellular membranes, possibly through formation of membrane pores. As previously said (see paragraph 1.6), the first observation that soluble amyloid oligomers could affect the integrity of a lipid bilayer by forming an ion-channel, was made in 1993 [**45**]. It was shown that Aβ could form cation selective channels in planar lipid bilayers. Soon after that, the group of Kagan showed that also hIAPP could form cation-selective channels [**57**]. In contrast, the non-amyloidogenic rIAPP did not form channels.

As reviewed by Engel, at the moment a substantial amount of experimental data suggests that human amylin, as well as many other amyloid-related peptides and proteins, can form cation-selective channels [**124**]. Visualization by AFM suggests that a hIAPP ion-channel, assembled in a bilayer, is composed of approximately 5 subunits in a circular arrangement [**153**]. Various sizes of the hIAPP-induced membrane pores or openings have been suggested, ranging from Ca^{2+}-permeable to permeable for fluorescent dyes with a size larger than 1 kDa [reviewed in **124**]. Soluble hIAPP oligomers, and amyloid oligomers in general, could have characteristics of pore-forming protein toxins, like α-hemolysin, and might have a similar mechanism of action [**19**; **23**; **154**].

Several groups have reported that membrane permeabilization is caused by hIAPP oligomer-induced distortions of the phospholipid bilayer packing and membrane instability, in contrast to the formation of discrete pores [reviewed in **124**].

Another possibility of oligomer-induced membrane permeabilization is the interaction of amyloid oligomers with specific membrane receptors, which has been shown for HypF-N aggregates [**155**], but not (yet) for hIAPP.

Contradicting reports have appeared suggesting that human amylin oligomer assembly occurs either at the membrane [**134**; **153**] or in solution, after which the preformed oligomers interact with the membrane [**156**].

A recent study suggests that it is not oligomeric hIAPP species, but the process of human amylin fibril growth at the membrane to cause membrane permeabilization [**157**]. It was found that the assembly of hIAPP fibrils at the membrane causes membrane disruption, possibly by forcing the curvature of the bilayer to unfavorable angles or by the uptake of lipids by these fibrils during fibril elongation at the membrane. Importantly, this study shows that pre-formed human amylin fibrils and the non-amyloidogenic rIAPP do not permeabilize membranes, whereas allowing hIAPP to aggregate at the membrane, starting from a monomeric population, leads to fibril growth at the membrane and concomitant membrane permeabilization. The uptake of lipids into forming amyloid has been observed before under *in vitro* conditions [**53**; **158**], and also in various types of amyloid, isolated from patients [**159**]. The tendency of amyloidogenic peptides to fibrillate on the surface of lipid vesicles, and simultaneously damage the lipid bilayer, has also been observed using molecular dynamics simulations [**160**]. Remarkably, this simulation showed that bilayer permeabilization is caused by growing aggregates, but not by mature fibrils, in agreement with the hypothesis that hIAPP fibril growth at the membrane causes membrane damage [**157**]. These effects on membrane morphology were also observed in model membrane studies [**158**] and are straightforward to explain using the hypothesis of fibril growth at the membrane [**157**]. The possibility that amyloid fibrils are able to physically “break” cell membranes was recently suggested in a study that showed that fibrillar polyglutamine can be taken up by cells [**161**].

Fibril growth at the membrane as a membrane-permeabilizing action has not only been suggested for hIAPP, but also for other amyloidogenic proteins, such as the Aβ peptides [reviewed in **124**]. Interestingly, the suggestion that not a human amylin oligomer, but the membrane-located conversion of small spherical oligomers into annular oligomers might be responsible for membrane permeabilization [**154**] also supports the notion that a process occurring at the membrane could lead to membrane permeabilization. Moreover most pore-forming toxins, which have been hypothesized to have a similar mechanism of action as hIAPP oligomers [**23**], are formed from their monomers at the membrane interface and not in solution [**162**]. This includes the bacterial pore-forming toxin α-hemolysin which also reacts with the annular oligomer antiserum [**154**]. Thus, toxicity might be related to a process or a conversion occurring at the membrane and not to a certain species. These new ideas might lead to a focus on mechanisms of membrane permeabilization that are governed by conversion of species along the fibril formation pathway.

Figure 2.11 shows all various suggested membrane-permeabilizing hIAPP species and processes in relation to cytotoxic human amylin membrane interaction.

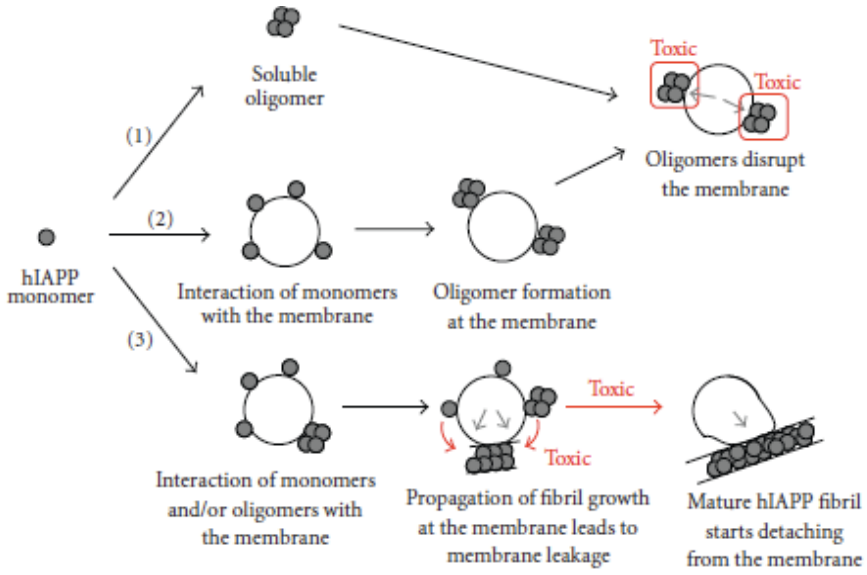

Fig. 2.11. Schematic representation of the different models of hIAPP-membrane interaction in relation to membrane damage and hIAPP cytotoxicity. The red rectangles show the toxic species and the red arrows show the toxic processes according to different hypotheses. The black circle represents a phospholipid membrane (vesicle), the grey circles represent hIAPP monomers, and clusters of 4 or more circles represent hIAPP oligomers and hIAPP fibrils, respectively. Membrane damage is schematically indicated by the grey arrows. Model (1) includes two steps: (i) formation of soluble hIAPP oligomers, (ii) interaction of the toxic oligomers with the membrane leading to membrane damage. Model (2) includes three steps: (i) binding of monomeric, random coil hIAPP to the membrane and folding to α-helix, (ii) oligomer formation of membrane-bound hIAPP, and (iii) interaction of the toxic hIAPP oligomer with the membrane leading to membrane damage. Model (3) includes 3 steps: (i) interaction of monomeric and possibly oligomeric hIAPP to the membrane, (ii) growth of hIAPP fibrils at the membrane (red arrows) leading to a forced change in membrane morphology and concomitant membrane disruption, and (iii) detachment of mature fibrils from distorted membrane [**126**].

In such mechanisms, membranes could have an important function as mediator or accelerator of the conversion of one hIAPP species to the other, possibly representing a cytotoxic event. In fact, as just said (see paragraph 1.7), membranes are able to catalyze human amylin fibril formation: the presence of phospholipid bilayers can reduce the lag phase of hIAPP fibrillation, a most pronounced effect with negatively charged lipids [reviewed in **124**].

Other negatively charged surfaces, like heparin molecules, are also able to catalyze hIAPP aggregation [**163**]. The observation that a dichloromethane/water interface accelerates hIAPP fibril formation seems to indicate that also hydrophobicity at the interface plays an important role in the acceleration of hIAPP aggregation [**164**]. Even human amylin fibrils themselves can accelerate subsequent fibril formation, a process that is known as “secondary nucleation” [**165**]. Moreover it has been suggested that other factors can affect the interaction between hIAPP and membranes, for example calcium ions and crystalline insulin [**123**].

Interfaces can affect the aggregation of peptides/proteins in different ways. They can serve as a template to put molecules in a preferential orientation such that aggregation is favored, as has

been suggested for hIAPP [**168**]. Membrane fluidity is suggested to be an important factor that enables a specific orientation of fibrils on membranes [**166**].

Secondly, adsorption of the peptide at the interface can locally increase the peptide concentration, resulting in bidimensional crowding [**167**]. Consequently, a high local concentration of membrane-bound hIAPP monomers will greatly accelerate aggregation. In addition, interaction or aggregation of membrane-bound hIAPP monomers could result in cooperative binding [**168**].

Thirdly, interfaces are able to change the conformation of a protein [reviewed in **124**] and consequently might also induce structure in a protein or peptide that is unstructured in solution, like hIAPP. Indeed, it has been shown that adsorption of hIAPP at membranes induces helical structure (see beyond).

Interfaces also have a significant effect on the conformation and nucleation of amyloidogenic proteins [**169**]. Possibly, membranes could induce different fibril morphologies, as observed for apolipoprotein fibrils [**170**]. Recent studies address the interaction of amyloidogenic peptides and proteins with various interfaces and in particular membranes [reviewed in **124**].

The effect of interface-mediated catalysis of aggregation might be a very important factor in the mechanism of amyloid-induced cytotoxicity. If membranes act as a “template” for amyloid aggregation, it is not surprising that amyloid species, through their conversion, also affect the barrier properties of the bilayer.

Human amylin has several positively charged residues, all located on the N-terminal region. These residues are suggested to be involved in the initial interaction of hIAPP with lipids, in particular negatively charged lipids [**168**]. Indeed it has been shown that membrane binding of human amylin is most efficient when bilayer lipids are negatively charged [reviewed in **124**]. Hydrophobic interactions also most likely play an important role. Remarkably, the 7 N-terminal residues, including the disulfide, are not required for hIAPP cytotoxicity [**171**]. The residues important for hIAPP–membrane interaction could include the six residues that are different compared to the nonamyloidogenic rIAPP and that are thus important for the fibrillogenesis. The only charged residue of these six , histidine 18, is important for both fibril formation [**130**] and membrane interaction [**172**]. Deprotonation of H18 favors an orientation of the hIAPP1–19 fragment parallel to the membrane, while in the protonated state hIAPP1–19 is suggested to be transmembrane [**172**]. The first 17 N-terminal residues are identical in hIAPP and rIAPP, and seem well conserved in IAPP from several species. The N-terminal region has been suggested to be involved in binding to receptors and binding to insulin [reviewed in **124**].

Recent reports suggest that human amylin fiber formation and hIAPP induced membrane disruption are separate processes localized in two distinct regions of the peptide [reviewed in **124**]. It was suggested that membrane disruption is caused by the N-terminal section of the peptide (residues 1–19) and that amyloidogenicity is not required for this. This is unexpected since many studies have shown a link between amyloidogenicity and membrane permeabilization for many amyloidogenic proteins. In particular, it has been shown that the cytotoxicity of hIAPP is linked to its amyloidogenicity, in contrast to non-amyloidogenic rIAPP that is not cytotoxic and not linked to T2DM [**151**].

A possible explanation for the membrane disrupting ability of non-amyloidogenic hIAPP fragments, as observed in recent works, as well as of rIAPP, is the “carpet mechanism”, in which α-helical peptides disrupt membranes without aggregating into β-sheet-rich structures [reviewed in **124**]. Indeed, hIAPP and rIAPP both insert in lipid monolayers as monomers [**173**], and their similar membrane interaction has been ascribed to the carpet mechanism [reviewed in **124**]. Membrane damage by rIAPP and non-amyloidogenic hIAPP fragments seems to occur only in membranes composed of negatively charged lipids, a condition that is very different from those found in cells (approximately 20–30% negatively charged lipids). Moreover, experimental conditions, for instance different membrane permeability assays, or the presence or absence of an amidated C-terminus, can lead to discrepancies between studies. In conclusion, since membrane damage under certain conditions can be induced by the T2DM-unrelated and non-amyloidogenic rIAPP, it is unlikely that the mechanism for this process relates to a physiologically relevant event that can explain membrane damage and cytotoxicity in T2DM.

Apart from the N-terminus, it has been shown that other residues are in contact with the membrane when human amylin binds to a phospholipid bilayer. Important information comes from a recent residue-level study using site-directed spin labeling and EPR [**174**]. This work shows details of the α-helical structure of monomeric hIAPP bound to large unilamellar vesicles (LUVs) composed of 80% 1-palmitoyl-2-oleoyl-sn-glycero-3-(phospho-l-serine) (POPS) and 20% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC). It was found that residues 9–22 form an α-helix oriented parallel to the membrane surface, embedded in the bilayer at the level of the phospholipid headgroups (Fig. 2.12). Importantly, most of these residues are also thought to be involved in the formation of cross-β structure in the hIAPP fibril. This might explain the success of the study since the high amount of negatively charged lipids trapped this helical state, possibly by preventing key residues from conversion to β-sheet structure. It was also suggested that residues Thr9, Leu12, Leu16 and Ser20 face the hydrophobic core of the membrane, while the charged residues Arg11 and His18 are located at the level of the phospholipid headgroups.

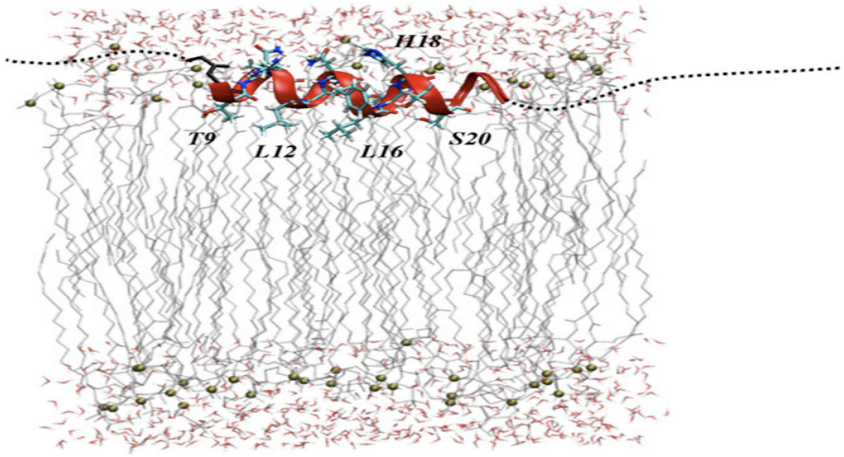

Fig. 2.12. Model for the structure of monomeric membrane-bound hIAPP from EPR data. The red ribbon indicates the α-helical part of the peptide (residues 9–20). The N-terminal and C-terminal part are unstructured. The scheme shows the position of the peptide relative to the bilayer lipids [**174**].

Residues 23–37 of the membrane-bound hIAPP are largely unstructured, and it is implied that this exposure will promote conversion to β-sheet structure [**174**]. It is important to note that the presence of 80% negatively charge lipids is very different from the *in vivo* situation (about 25% negatively charged lipids), consequently this helical state is likely to be less stable in a cellular environment. Still, the observation that physiological levels of negatively charged lipids are sufficient to accelerate human amylin fibril formation [**175**] supports the notion that transient helical structure might play a role in hIAPP fibrillation *in vivo*. In this context it is important to underline that negatively charged lipids are preferentially located at the cytosolic side of the cellular membrane, where they are only available to intracellular hIAPP species. Consequently, membrane permeabilization by the process of fibril growth at the membrane [**157**], which occurs independent of the presence of negatively charged lipids, could affect biological membranes from both sides. The previously mentioned EPR study confirms earlier work, which had shown using CD and FTIR spectroscopy that human amylin adopts helical structure when sufficient negatively charged lipids are present [reviewed in **124**]. Considering that residues 9–22, except for residue 18, are identical for hIAPP and rIAPP, it is not surprising that the nonamyloidogenic rIAPP also forms similar helical structure when bound to membranes [**134**]. The hIAPP and rIAPP1–19 fragments reconstitued in dodecylphosphocholine (DPC) micelles have also been shown to adopt α-helical structure [**172**]. In addition, it was found that in solution, rIAPP sample α-helical structures [**176**]. The acceleration of human amylin fibrillation in the presence of helix-promoting organic solvents like trifluoroethanol (TFE) and HFIP supports the importance of helical structure in the process of fibril formation [**165**]. The observation of α-helical structure in membrane-bound hIAPP seems ordinary, since it is known that many peptides adopt α-helical structure when interacting with a lipid bilayer. However, this helical structure might turn out to be extra-ordinary, in according to the observation that the hIAPP monomer converts from a mostly unstructured peptide in solution to a β-sheet rich fibrillar assembly. Thus, it is interesting the suggestion of a recent spectroscopic study that this α-helical state could be an intermediate promoting human amylin fibrillation by parallel helix associations to bring together regions of hIAPP that could nucleate β-strand structure [**177**].

2.9. hIAPP-induced β-cell apoptosis

As known, two major pathways of apoptosis are the extrinsic and the intrinsic pathway, which includes the ER stress pathway. The extrinsic pathway is mediated by binding of death signals (Fas ligands) to death receptors (Fas) on the cell surface leading to aggregation of Fas receptors, caspase-8 and Fas-associated death domain protein into a death-inducing signaling complex in which caspase-8 is proteolytically activated and then released [**178**]. Activated caspase-8 can then either directly activate the execution phase of apoptosis (via caspase-3) or amplify its signal by proteolytic activation of the proapoptotic member of the Bcl-2 family Bid leading to subsequent release of mitochondrial proapoptotic factors (like cytochrome c). The extrinsic pathway is active in autoimmune-mediated β-cell death and it has also been invoked as a mediator of β-cell glucose toxicity by high glucose concentrations to induce expression of the Fas ligand and IL1-β in β-cells [reviewed in **120**]. The intrinsic pathway is mediated by

several cell stresses. For what concerns degenerative diseases, documented inducers of the intrinsic pathway are ER stress, mitochondrial dysfunction, generation of ROS (mainly free radicals), metabolic toxins, disruption of the actin cytoskeleton and anoxia [**179**].

As just said, islet in T2DM is characterized by increased β-cell apoptosis and hIAPP can form toxic oligomers that may induce apoptosis. Moreover secretory cells, such as β-cells, are particularly vulnerable to the ER stress pathway of apoptosis. The primary defense mechanism against this event (see paragraph 2.7) is the UPR, that is the adaptive effort by secretory cells to prevent ER stress (i.e. the circumstances that induce apoptosis as a consequence of deposits of aggregated proteins) [reviewed in **120**]. When the UPR is unable to clear the ER of unfolded (especially aggregated) proteins, ER stress may develop.

Marchetti showed increased markers of ER stress in isolated islets from patients with T2DM [**180**]. Interestingly, β-cells showed modest signs of ER stress when the islets were cultured at normal glucose, but increased when the islets were cultured at higher glucose. This finding implies a genetic predisposition in islets from individuals with NIDDM to ER stress when β-cells are chronically stimulated, a predisposition absent in islets of nondiabetic individuals. These data are consistent with the notion of a lower capacity to traffic and fold major client secretory proteins, such as hIAPP in T2DM subjects. Moreover ER stress is characteristic of β-cells in humans with NIDDM but not in type 1 diabetes [**181**]. The exact mechanism linking protein oligomer formation and ER stress-induced apoptosis is unknown. Haataja reviewed that one proposed mechanism is that toxic oligomers interact with the ER membrane leading to Ca^{2+} leakage and this might directly lead to mitochondrial membrane permeability, leakage of cytochrome c and activation of executioner caspases (caspase-3), as well as indirectly contributing to apoptosis by increasing the unfolded proteins in the ER due to depletion of ER Ca^{2+} [**120**]. This leakage from the ER can also activate ER-associated calpain, which can then directly induce apoptosis in a caspase-independent manner [**120; 182**]. In rodents, activation of the ER membrane resident caspase-12 is associated with induction of the ER stress pathway of apoptosis, although it is not clear whether caspase-12 activation is a consequence of or a mechanism contributing to the ER stress-induced pathway of apoptosis [**183**]. Caspase-12 expression was detected in hIAPP transgenic mice and rats, but not in rIAPP transgenic mice. Caspase-4 shows the same properties (activated by chronic ER stress) in humans [**184**]. ER stress has been identified as an important mechanism inducing apoptosis in AD, PD and T2DM, so it is a strong candidate for mediating hIAPP oligomer-induced apoptosis [reviewed in **120**]. ER stress has been observed in β-cell lines transduced with hIAPP as well as mice and rats transgenic for hIAPP [**181**]. Nuclear C/EBP homologous protein/GADD153 (CHOP) staining was found in hIAPP, but not rIAPP transgenic mice and also in pancreatic section of a NIDDM subject. The appearance of CHOP preceded execution of apoptosis and furthermore, when CHOP was knocked down by small interfering RNA, apoptosis was decreased. Application of human amylin oligomers extracellularly has also been shown to impair the ubiquitin proteasomal pathway [**181**]. The accumulation of polyubiquitinated proteins was also identified in hIAPP, but not rIAPP transgenic mice. Because human amylin oligomers induce membrane leakage and disruption, application of these oligomers extracellularly or formation of them within the secretory pathway intracellularly might reasonably be expected to permit Ca^{2+} influx into

cytoplasm, a signal known to induce the intrinsic pathway of apoptosis. Moreover, ER stress has been shown to induce expression of death receptor, potentially invoking the extrinsic pathway of apoptosis so that in reality, both classical pathways of apoptosis will likely be active once cell membranes have been disrupted [reviewed in **120**]. Finally, recent studies showed that addition of hIAPP to the cells induces β-cell apoptosis by Fas-associated death receptor and consistent with this, activation of p38 MAPK and JNK1 [reviewed in **120**].

2.10. Appendix

The β-cells have a highly developed ER, apparently due to an excessive demand for compensatory insulin secretion [**185**]. An increased demand for insulin secretion may result in β-cell overload, ultimately leading to deficient insulin secretion. β-cell mass is reduced in the later phases of T2DM, as a result of apoptosis, especially in rapidly replicating β-cells [**186**]. As known, the toxic hIAPP species induce apoptosis of the β-cells, but for a time, the more primordial ductal cells of the exocrine pancreas (replicative pool) can replace the damaged, apoptotic β-cells and continue the compensatory hyperinsulinemia, causing further β-cell damage. These effects cause a defective diffusion barrier within the islet [**187**].

Table 2.1 shows how deposits of human amylin aggregates may relate to NIDDM pathogenesis.

1- STAGE I (Latent period)

- Increased production of ROS, nitrogen and thiol species
- β-cell ER stress
- Compensatory insulin processing
- Protein misfolding/unfolding
- UPR activation/chaperone challenge
- Impaired first phase insulin secretory response
- Prolific free radical polymerization of hIAPP monomers

2- STAGE II (Transition period)

- Ongoing redox stress
- hIAPP oligomerization/fibril formation
- Impaired insulin secretory response
- Early β-cell apoptosis
- β-cell protein quality control severely challenged

3- STAGE III (Impaired glucose tolerance period)

- Appearance of advanced glycation endproducts (AGEs)
- 50-75% amyloid involvement in islet architecture
- Impaired β-cell function

4- STAGE IV (Impaired fasting glucose period)

- Increasing global insulin resistance
- Increased fasting blood glucose levels
- Excess hepatic and renal gluconeogenesis
- Progressive amyloid deposition

5- STAGE V (Overt T2DM)

- 50% loss of β-cell function
- 75-100% amyloid deposition

Table 2.1. Putative model of phases of T2DM, considered as a conformational disease.

3. Oleuropein aglycon and hIAPP amyloid aggregation

3.1. Structure and beneficial effects of oleuropein aglycon on human health

The oleuropein aglycon is a chemical compound occurring in extra virgin olive oil, a major component of the Mediterranean diet. The latter one is also rich in vegetables, cereals, fruit, fish, milk and wine that, due to the presence of unsaturated fatty acids and antioxidants, have been demonstrated to have beneficial biological effects on health, such as the prevention of several diseases ranging from atherosclerosis, cardiovascular diseases to cancer. Although the composition of olive oil is complex, its chief active components include oleic acid, squalene and phenolic constituents which are responsible for a number of olive oil's salutary biological activities (anti-oxidant, anti-inflammatory and anti-cancer).

As reviewed by Waterman and Lockwood, **oleic acid** represents 70–80% of the fatty acids present in this product, so olive oil is unique for its high oleic acid content since the majority of seed oils are composed primarily of polyunsaturated fatty acids, including the essential omega-6 fatty acid linoleic acid [**188**]. Compared to polyunsaturated fatty acids (PUFA), oleic acid is monounsaturated (MUFA), making it much less susceptible to oxidation and contributing to the antioxidant action, high stability, and long shelf life of olive oil. Data concerning its health benefits are conflicting. It has been reported that oleic acid plays a role in cancer prevention. Whether this is a secondary effect of the fatty acid on oil stability (preventing oxidative stress) or a direct anti-cancer effect remains debatable [**188**]. It reduces plasma levels of low density lipoproteins (LDL)-cholesterol and increases high density lipoprotein (HDL)-cholesterol. Finally, it is known that an increased consumption of MUFA instead of PUFA reduces the risk of atherosclerosis because they make the circulating lipoprotein less sensitive to peroxidation [reviewed in **189**].

Squalene is a triterpene hydrocarbon and a major intermediate in the biosynthesis of cholesterol. Although found in both plants and animals, it is present in different amounts (in olive oil it is approximately 0.7%, other foods and oils typically have levels in the range of 0.002-0.03%) and the majority is transported to the skin [**188**]. Due to its structure, it is more likely to scavenge singlet oxygen species (SOS) than hydroxyl radicals. Exposure to high levels of ultraviolet radiation causes the formation of carcinogenic SOS within the skin, where a high concentration of squalene may provide a chemoprotective effect [**188**]. Since squalene is in high amounts in the Mediterranean diet, it is believed to be responsible for the lower incidence of skin cancer observed in populations consuming this diet. Animal studies have shown topical squalene has an inhibitory action on chemically induced skin carcinomas: in fact when it was added to the diet of rats resulted in an 80% increase in serum squalene levels and inhibition of the hepatic enzyme 3-hydroxy3-methylglutaryl (HMG)-CoA reductase, the rate-limiting enzyme in the biosynthesis of cholesterol. This inhibition may be due to squalene or its metabolites and results in decreased production of cholesterol and the intermediates formed during its

biosynthesis. These intermediates are commonly needed to activate oncogenes. One of them is farnesyl pyrophosphate (FPP), involved in the prenylation of several oncoproteins. Since other dietary substances lowering FPP levels, induce a reduction in tumor growth, squalene is hypothesized to work in the same manner [**190**].

Finally, **phenolic constituents** are the main anti-oxidants of olive oil (carotenoids are also contained but in small concentrations) and they provide some of olive oil health benefits. Many *in vitro* studies have been performed to understand mechanisms by which phenolic compounds may act to confer these positive effects. Several of these studies have shown that they possess strong radical scavenging activity and appear to be at least, as if not more, effective than other important dietary anti-oxidants, such as vitamin C and α-tocopherol. Exogenous antioxidants are useful because they have a twofold function, preventing food oxidation (in particular lipid oxidation) and at the same time increasing the amount of antioxidant agents present in the organism, protecting against degenerative diseases [**189**]. In fact a decreased plasma antioxidant capacity and the uncontrolled production of free radicals have been hypothesized as contributing to the pathogenesis of diseases such as coronary heart disease (CHD), closely related to atherosclerosis, cancer and neurodegenerative diseases [**191**]. As reviewed by Covas, targets for ROS are lipids, deoxyribonucleic acid (DNA) and proteins [**192**]. Oxidation of the lipid part, or directly of apolipoprotein B, of LDL leads to a change in the lipoprotein conformation by which the LDL is better able to enter into the monocyte/macrophage system of the arterial wall and develop the atherosclerotic process, thus promoting cardiovascular disease. In addition, 3-cloro- and 3-nitro-tyrosine generation, *via* myeloperoxidase activity, in HDL, converts the lipoprotein in a pro-inflammatory HDL and reduces its capacity to remove cholesterol from cells and to counteract the LDL oxidation (lipids in HDL are preferentially oxidized before those in LDL) [**192**]. Nucleic acids are also targets of free radicals. Oxidative stress leads to mutagenic DNA lesions in purines, pyrimidines, deoxyribose, and DNA single- and double-strand breaks. Accumulation of mutations from oxidative DNA damage is considered to be a crucial step in human carcinogenesis [**192**].

Moreover ROS production is also related to the modulation of cycloxygenase (COX-2) induced by different factors, which is clearly involved in the inflammatory processes, inflammatory bowel diseases (IBD) and some forms of cancer [reviewed in **193**]. So the ability of polyphenols to scavenge free radicals could be important in explaining how they may play a role in preventing these diseases.

There are at least thirty-six structurally distinct phenolics identified in virgin olive oil but not all of these phenolics are present in every virgin olive oil. Although the reported levels of phenolic compounds vary widely, one consistent conclusion is that extra virgin olive oil has a higher phenolic content than refined virgin olive oil [**194**]. Owen showed that this difference was reflected in the levels of individual phenols as well as the total quantity of phenols in the oil [**195**]. The concentration of phenols depends on several factors, including environmental growth conditions, method of oil production and storage conditions [**196**].

As reviewed by Tripoli, the pulp of olives contains these compounds but they are also found in the oil and they are both lipophilic and hydrophilic [**189**]. The class of phenols includes numerous substances, such as simple phenolic compounds like vanillic, gallic, coumaric and

caffeic acid (acids), tyrosol and hydroxytyrosol (alcohols) and more complex compounds like the secoiridoids (oleuropein and ligstroside), and the lignans (1-acetoxypinoresinol and pinoresinol) [**189**]. Flavonoids like luteolin and apigenin were also reported as phenolic components of olive oil [**197**]. The lipophilic phenols, among which are tocopherols and tocotrienols, can be found in other vegetable oils, while the hydrophilic phenols (flavonoids, phenolic alcohols and acids, secoiridoids and their metabolites) are not generally present in other oils and fats (**189).** The polyphenols include phenolic alcohols and acids, secoiridoids with their metabolites and the lignans, even if, since some of these (tyrosol) have not two hydroxyl groups, it would be incorrect to put them in this class [**196**].

Secoiridoids are the main compounds of olive oil and they are present exclusively in plants of the family of Oleaceae. These substances were found, for the first time, by Montedoro [**198**] and they were identified as aglycone derivatives of secoiridoid glucosides contained in the olive fruit, originating during oil mechanical extraction process, by hydrolysis catalysed by endogenous β-glucosidases [**199**]. The phenolic content of the olive fruit also changes as it grows and develops. After six months of growth, the major phenols are the glucosides of ligstroside aglycon (ligstroside) and oleuropein aglycon (oleuropein) but as the olive matures, they are deglycosylated by glucosidase enzymes to free secoiridoids that, unlike the glucosides, can be detected in olive oil because they are able to cross the oil/water barrier [**188**]. Then, simple phenols hydroxytyrosol and tyrosol are formed from the hydrolysis of the secoiridoid aglycones of oleuropein and ligstroside respectively; In particular, hydrolysis of oleuropein produces hydroxytyrosol, tyrosol, and ethanol [reviewed in **188**]**.**

About the molecular structure, secoiridoids present elenolic acid in its glucosidic or aglyconic form and are produced from the secondary metabolism of terpenes as precursors of several indole alkaloids [reviewed in **189**).

Among them, oleuropein aglycon is their most distinctive and important constituent (Fig. 3.1). This secoiridoid derivative, which is responsible for distinctive sensorial characteristics of extra virgin olive oil (mainly pungency and bitterness), owns several biological properties.

It has an **anti-microbial activity** against a wide spectrum of micro-organisms, known from decades [**200**] but in the last years its other healthy properties have emerged, many of which described as resulting from the high anti-oxidant power of oleuropein aglycon. This ability was demonstrated to be enhanced by increasing the number of hydroxyl group in the phenols and in particular compounds with o-dihydroxyl functionalities, like oleuropein aglycon, the high antioxidant activity is due to the formation of intramolecular hydrogen bonds between the hydroxyl group and the phenoxylic radicals during the reaction with free radicals; also, electron-donating substituents in “ortho” position tend to weaken the O-H bond of the phenol and furnish more stability to the phenoxyl radical [reviewed in **193**]. In particular oleuropein actively scavenges reactive oxygen and nitrogen species.

Among the biological properties coming from its antioxidant nature, we can indicate:

- the **cardioprotective effect** as anti-atherogenic agent. In fact several *in vitro* studies have shown protection of LDL against oxidation [**201**], lower thiobarbituric acid reactive substances which are indicator of lipid peroxidation [**202**], inhibition of platelet aggregation, which often accompanies and aggravates CHD, by cAMP-phosphodiesterases inhibition [**203**] and the down

regulation of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression in endothelial cells (crucial proteins for endothelial activation and the adhesion process, which are the initial events in inflammatory response and atherogenesis, an inflammatory patology) [**204**]. Moreover it was demonstrated the potentiation of the nitric oxide (NO)-mediated macrophagic immune response in endothelial cells, with consequent increase of NO production due to a direct effect on the inducible form of the enzyme NO synthase [**205**]. This increase is useful to avoid LDL oxidation, since it prevents the formation of peroxinitrite (a reactive compound inducing peroxidation in lipids); No can react with superoxide anion (O_2^-), forming peroxinitrite, but if NO levels are higher than O_2^- levels there is an antioxidant effect.

- the **anticancerogenic ability**. In fact it is able to prevent some forms of human cancer as inhibitor of the mutagenic activity caused by oxidative stress. For example it inhibits hydrogen peroxide-induced DNA damage in peripheral blood mononuclear cells and promyelocytic leukemia cells [**206**].

In addition to the antioxidant properties, oleuropein aglycon exhibits a range of indirect actions that may be beneficial to health. For example, it inhibits enzymes involved in the inflammatory process, such as COX-1 and COX-2 [**207**; **208**], important because inflammatory processes, as just said, are also implicated in the development of various chronic diseases, including CVD and certain types of cancer. In particular COX-2 is overexpressed in colorectal cancer cells, and this overexpression has a strong association with colorectal neoplasia. Corona and co-workers showed that the secoiridoid acts as a potent inhibitor of cancer cell proliferation, reducing COX-2 expression [**207**]. So oleuropein aglycon has an **anticarcinogenic** activity also as anti-proliferative agent and in other cases also as pro-apoptic agent. For example the phenol is an anti-breast cancer, since preferentially kills HER2 oncogene-overexpressing breast cancer cells (HER2 plays a pivotal role in oncogenic transformation, tumorigenesis and metastasis) [**209**]. Finally, it has a **hypolipidemic effect** as hypocholesterolemic agent by which oleuropein aglycon may indirectly reduce the incidence of CHD. In fact the administration of the phenol in a series of rats models, fed a cholesterol-rich diet, significantly lowered the serum levels of total cholesterol, triglycerides and LDL-cholesterol and increased the serum level of HDL-cholesterol [**202**]. These results are important since hypercholesterolemia leads to increased cholesterol accumulation in cells that activate oxygen free radicals production and so atherogenesis, while high HDL-cholesterol levels may compete with LDL receptor sites on arterial smooth muscle cells, inhibiting the uptake of LDL ; in addition it could protect the LDL against oxidation *in vivo* since lipids in HDL are preferentially oxidized before those in LDL.

Obviously, information on the absorption and disposition of this compound is essential in determining its potential in exerting healthful affects *in vivo.* Animal and human studies show that olive oil phenols are well absorbed; in particular few studies concerning the absorption and bioavailability of oleuropein aglycon demonstrated that, when administered, it is absorpted for 55-66% in humans, it is stable in the gastric juice and is hydrolysed to hydroxytyrosol before urinary excretion, even if it is not clear whether this happens before or after absorption [**210**].

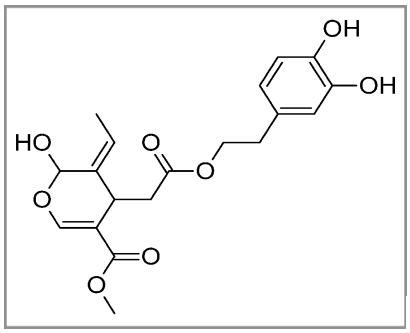

Fig. 3.1. Structure of oleuropein aglycon which shares a catecholic (ortho-diphenolic) structure**.**

3.2. Polyphenols as antiamyloidogenic agents: oleuropein aglycon *vs* hIAPP

So far, several therapeutic approaches have been suggested to treat amyloidogenic diseases. As reviewed by Porat, these include reduction in the production of the amyloidogenic forms of proteins, increase in the clearance rate of misfolded or aggregated proteins, increase of the native state stability in amyloid proteins and direct inhibition of the self-assembly process [**211**].
In the past few years, has been an accumulation of reports describing small molecule inhibitors of amyloid fibril formation. Among them, especially certain natural polyphenolic aromatic-rich compounds dramatically inhibit cell death in cultured cells and several of these were also found to be efficient *in vitro* inhibitors of amyloid fibrils formation. For example catechin, resveratrol and other wine-related polyphenols are able to inhibit AβP aggregation and cytotoxicity [reviewed in **211**]. The interest about the employ of these natural compounds as possible drugs to prevent cytotoxicity of amyloidogenic assemblies comes from the fact that, as just said (see paragraph 1.5), these aggregates increase the formation of reactive species and abnormalities in cell redox systems, so these poyphenols could give a possible protection as natural antioxidant. In this respect, extra virgin olive oil is attracting interest as an important source of polyphenols and since oleuropein aglycon is the main phenolic component of extra virgin olive oil, we have decided to investigate about the effects of Oleuropein aglycon on hIAPP cytotoxicity.

4. Aim of this study

So far, the researchers have already identified inhibitors of human amylin amyloid aggregation. For example, rifampicin prevents hIAPP fibrillization; however, it does not inhibit the formation of toxic oligomers but merely interferes with fibril growth [**214**], while other inhibitors were effective against *in vitr*o aggregation of hIAPP, but they displayed marked cytotoxicity, as it was the case of an octapeptide fragment of hIAPP carrying a Phe to Tyr substitution [**215**]. Thus, presently there was no indication of an inhibitor able to protect the cells against hIAPP toxicity.

Therefore, the first aim of this study was to investigate whether the oleuropein aglycon, such a strong natural antioxidant of the extra virgin olive oil, could prevent human amylin cytotoxicity to cultured mammalian cells, since it is known amyloid assemblies cytotoxicity is frequently associated to early modifications of the intracellular redox status. In particular we produced the toxic hIAPP aggregates and then we checked whether cell viability was differently influenced by the exposure to this kind of assemblies or to hIAPP aggregates grown in the presence of oleuropein aglycon.

In addition, we investigated the ability of these two different aggregates to interact with the cell surface, where they could trigger several toxic events, in order to understand the nature of a possibile different behaviour.

We also tried to determine which type of cell death, namely apoptosis or necrosis, is the final outcome of the cell insult.

Such an investigation was carried out using rat insulinoma RIN-5F cells, previously shown to be highly affected by the exposure to human amylin amyloid aggregates [**216**; **217**].

A better understanding of the molecular features of the interaction between the two kinds of human amylin assemblies and membranes could provide further information on the molecular events that induce or not induce plasma membrane permeabilization and protein aggregate toxicity.

To this aim, we checked whether the two different hIAPP aggregates were able to permeabilize synthetic phospholipid unilamellar vesicles, negatively charged.

Furthermore, we performed structural studies by Circular Dichroism, Electron Microscopy and ThT assay to verify the presence of possible structural differences between hIAPP aggregates formed in the presence or in the absence of oleuropein aglycon, that could explain their possible different effect on cultured mammalian cells.