DISCUSSION

7. Discussion

7.1. Oleuropein aglycon reduces hIAPP cytotoxicity

In this study, we have demonstrated that oleuropein aglycon, the main polyphenol found in the Mediterranean extra virgin olive oil, is able to substantially reduce the cytotoxicity of hIAPP aggregates to RIN-5F pancreatic β cells (Fig. 6.1 A). In fact, we found a significant improvement of cell viability in cells treated with hIAPP aggregates grown in the presence of the oleuropein aglycon, with respect to that found in cells exposed to aggregates grown in the absence of the latter. This protective effect seems to be related to the inability of hIAPP, aggregated in the presence of the oleuropein aglycon, to interact with the cell membrane and so to induce membrane damage. In fact in most cases, amyloid cytotoxicity requires the binding of the aggregate to the plasma membrane with subsequent membrane destabilization, loss of selective permeability, derangement of ion homeostasis [**225; 226**] and, possibly, alteration of signal transduction pathways [**40**].

The findings we report here are consistent with the above mentioned scenario, since the immunofluorescence analysis showed that human amylin did not interact with cell membranes, only wheter aggregated with oleuropein aglycon (Fig. 6.2) and we found that there was no permeabilization of synthetic negatively charged (PS) phospholipid vesicles only if they were treated with this kind of aggregates (Fig. 6.3). This fact is important since a large body of data suggests that interaction between protein aggregates and cell membranes is favoured by the presence, in these aggregates, of suitable (that is most often hydrophobic) surfaces as well as, in exposed cells, of a suitable membrane lipid composition. Electrostatic interactions are also important. Indeed, it has been repeatedly reported that anionic phospholipids such as phosphatidylserine, that becomes exposed in the outer membrane leaflet in apoptotic and tumoural cells, are putative docking sites for amyloid aggregates or can favour aggregate formation thus initiating membrane impairment [**54**; **227**].

Thus, our results strongly support the idea of a correlation between toxicity and membrane interaction.

Our analysis also revealed that the oleuropein aglycon does not merely protect cells against the cytotoxic insult caused by the aggregates by virtue of its antioxidant properties; in fact, cells treated with toxic hIAPP aggregates are not protected against cytotoxicity by the concomitant presence of the aglycon; the latter hinders the formation of toxic amyloid species only when it is present during human amylin aggregation. Such hypothesis does not imply that cells do not benefit of the oleuropein antioxidant activity in a condition of oxidative stress; rather, it means that, to achieve such effect, a much higher oleuropein concentration (90X, see Fig. 6.1 C) than that resulting in hIAPP aggregate cytotoxicity suppression (9X, see Fig. 6.1 A, B) is needed.

Finally the analysis of apoptotic and necrotic markers indicates that, as it has been shown for most cultured cells exposed to toxic amyloid aggregates, apoptosis, rather than necrosis, is the final outcome of the insult given by the toxic hIAPP forms to RIN-5F cells and confirms the protective effects of oleuropein aglycon (Fig. 6.1 D).

7.2. Structural effects of oleuropein aglycon on hIAPP aggregates

Our studies have shown that the presence of the aglycon of oleuropein during amyloid aggregation of human amylin, induces structural changes that may modify the aggregative pathway of the peptide.

The ThT assay indicated a significant reduction of ThT fluorescence when hIAPP was incubated with oleuropein, suggesting impaired amyloid aggregation since this probe binds specifically amyloid species (Figs. 6.4 and 6.5).

On the other hand, the CD and EM analysis showed that oleuropein aglycon did not suppress the formation of hIAPP amyloid fibrils (Fig. 6.6, 6.7).

Overall, our data show that our compound interferes with the early steps of hIAPP aggregation, as indicated by the differences in the CD spectra that can be observed after 3 h of incubation, the results of the ThT assay and the EM analysis. The early reduction of the CD signal of hIAPP aged in the presence of oleuropein (Fig. 6.6) and the appearance of the latter as bulky deposits (Fig. 6.7) that do not interact with the cells (Fig. 6.2), confirm that oleuropein aglycon anticipates peptide precipitation into macrostructures, devoid of toxic properties, skipping the early steps of amyloid aggregation where toxic oligomers and chains of oligomers are formed. Later on, the amorphous precipitate seems to evolve into amyloid fibrils, as it happens for other amyloidogenic peptides [**228**; **229**], often associated into bundles. These “superstructures” are not ThT positive (see the 24 h point in Fig. 6.5), possibly because their tight structure hinders dye penetration.

Overall, the results coming from the structural analysis are coherent with the cytotoxicity ones, and agree with the widely accepted assumption that most amyloid proteins and peptides, the most toxic species are the oligomeric pre-fibrillar aggregates, preceding the formation of stable, and substantially harmless, amyloid fibrils [**1; 120**].

7.3. The nature of the cytoprotective action of oleuropein aglycon

This protection given by oleuropein against the appearance of early toxic hIAPP assemblies is of particular interest since other inhibitors of hIAPP aggregation were previously identified but, however, they did not protect the cells against human amylin toxicity. For example, rifampicin was found to prevent hIAPP fibrillization; however, it was not able to inhibit the formation of toxic oligomers but merely to interfere with mature fibril growth [**214**]. Other inhibitors were effective against *in vitro* 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**]. On the contrary, oleuropein aglycon resulted to be not toxic to RIN-5F pancreatic cells, not only in the concentration range (90–270 nM) that we used but also in large excess up to 100 μM (data not shown).

As just said, oxidation in biological systems may lead to cellular membrane dysfunction and DNA damage, in particular amyloid aggregates increase the formation of reactive species and abnormalities in cell redox systems. Polyphenols, like oleuropein aglycon, act as free radical scavengers and have shown beneficial health-promoting effects in chronic and degenerative

diseases, so they could give a possible protection from amyloid asemblies as natural antioxidant.

However it is important to underline that most polyphenols are effective as antioxidants in the 5–50 μM concentration range [33], and the EC50 for oleuropein in the 3,3 diphenyl-1-picrylhydrazyl radical scavenging test was shown to be 36.3 μM [**191**], which is over two orders of magnitude greater than the oleuropein effective concentration in our MTT assays (270 nM). This, again, confirms that the protection proided by oleuropein aglycon comes from a different mechanism which does not rely on its antioxidant properties. Its efficacy in inhibiting hIAPP aggregation is comparable to those reported for other polyphenols: Porat et al. [**215**] calculated for phenol red an IC50 of 1.0 μM when used during the aggregation of 5.0 μM hIAPP and Mishra et al. [**230**] estimated an IC50 of 3.3 μM for resveratrol by using 10 μM hIAPP. From our ThT assays, we estimated an IC50 of 1.0 μM for the oleuropein aglycon using 3.25 μM hIAPP.

As recently reviewed by Porat, several mechanisms were suggested to explain polyphenols-induced protection against amyloid assemblies cytotoxicity, besides their known antioxidant properties. In fact various intracellular signaling mechanisms have been indicated to be involved, at least partly, in the protective effects of polyphenols [**213**]. Moreover several authors have shown that *in vitro* inhibition of amyloid formation is not dependent on oxidative conditions [**213**]. Furthermore, it has been reported that structural properties of the polyphenol compounds should be considered as affecting their inhibitory effect. Thus, Porat suggests a new mechanistic approach that implies two assumptions regarding the inhibition mechanism of amyloid fibril formation by small polyphenolic compounds:

1- a specific structural conformation is necessary for β-sheet interaction and stabilization of the inhibitor–protein complex

2- an aromatic interaction between the phenolic compound in the inhibitor molecule and aromatic residues in the amyloidogenic sequence may direct the inhibitor to the amyloidogenic core and facilitate interaction, thus interfering with fibrils assembly [**213**].

Previous research claimed the importance of aromatic interactions in hIAPP fibrillization, possibly related to β-sheet stacking [**215; 221**]. On the other hand, it has also been reported that aromatic-aromatic interactions are less important than hydrophobic clustering and charge-charge interactions in hIAPP aggregation [**231**]. Whatever the case, oleuropein might interfere with either aromatic-aromatic or hydrophobic interactions by masking exposed hydrophobic groups.

We have also shown that oleuropein must be present during hIAPP aggregation to prevent the formation of toxic species. In addition, oleuropein has been reported to form a noncovalent complex with the Aβ peptide [**232**; **233**]. Given the structural similarities among amyloid aggregates grown from different proteins or peptides, these data suggest that oleuropein forms a similar complex even with hIAPP under aggregation conditions.

So, these results are a further confirm that the protective effect of several polyphenols against amyloid cytotoxicity actually comes from their physical association with aggregation-prone proteins, rather than from their antioxidant activity.

7.4. Conclusions

Taken together, our results suggest that the oleuropein aglycon can drive hIAPP aggregation to a path where less cytotoxic or even harmless aggregated species, unable to interact with the cell membrane, are formed. In particular, the oleuropein aglycon appears capable of interfering with the early steps of hIAPP aggregation hindering the proper reorganization of the polypeptide chain and the appearance of the most cytotoxic species, and delaying, but not suppressing, the growth of harmless amyloid fibrils likely structurally different from those grown in the absence of the polyphenol.

Obviously, information on the absorption and disposition of this secoiridoid is essential to evaluate its capacities in exerting healthful effects *in vivo*. Some studies by Visser and coworkers have demonstrated that humans absorb a large amount of the ingested olive oil phenols, mainly in the small intestine; in particular, when administred, the oleuropein aglycon is absorbed for 55-66% in humans, it results stable in the gastric juice and it is hydrolysed to hydroxytyrosol and tyrosol in the gastrointestinal tract, before urinary excretion [**212**]. They also suggested that human body metabolizes this phenol extensively, probably after absorption in the small intestine, although the mechanism of absorption is not clear [**212**].

In contrast, experiments carried out using colonic Caco-2 cells or an isolated rat perfused intestine raised doubts on oleuropein absorption, suggesting that it would reach the large intestine to be rapidly degraded by the colonic microflora to hydroxytyrosol [**234**].

Thus, whatever the case, caution should be used in extending such conclusions to humans, also in view of the fact that the oral intake of oleuropein aglycon together with its natural oily matrix should favour the absorption of this highly nonpolar compound.

We then believe that the new property of oleuropein to modify the path of hIAPP amyloid aggregation and to hinder aggregate toxicity, possibly beneficial against T2DM, further confirms the multiple benefits potentially coming from extra virgin olive oil consumption and paves the way to further studies on the possible pharmacological use of oleuropein first of all to prevent or to slow down the progression of T2DM *in vivo.*

Oleuropein aglycon possible ability to prevent the cytotoxic effects of other pathological amyloid proteins too deserves further investigations.

8. Abbreviations

AD Alzheimer's disease

PD Parkinson's disease

T2DM Type 2 diabetes mellitus

NIDDM Non insulin dependent diabetes mellitus

hIAAP human islet amyloid polypeptide

ROS Reactive oxigen species

PC Phosphatidiylcholine

PS Phosphatidiylserine

DOPS 1,2-Dioleoyl-snglycero-3-[phospho-L-serine]

DOPC 1,2-dioleoyl-sn-glycero-3-phosphocholine

HFIP Hexafluorisopropanol

DMSO Dimethyl sulphoxide

FTIR Fourier transform infra red

ThT Thioflavin T

TEM Trasmission electron miscoscopy