RESULTS

6.1 Results

6.1. The oleuropein aglycon reduces hIAPP cytotoxicity

6.1.1. MTT assay

The effect of the oleuropein aglycon on hIAPP aggregate cytotoxicity was evaluated using rat RIN-5F pancreatic β-cells, as model cell line, which have been extensively used by other groups for similar aims [**214**; **216**; **217**].

Human amylin was incubated in aqueous buffer at 25°C for growing times, up to 72 h, in order to obtain increasingly structured aggregates, either alone [**221**] or in the presence of oleuropein aglycon (nine fold molar concentration excess). Then the cells were treated for 24 h with the aggregates diluted 110 times in the culture medium, and cell viability was determined by the MTT assay. During the incubation period, the cells were cultured without FCS since, under these conditions, the basal level of apoptosis was reduced (data not shown). As a control, the cells were treated with mixtures containing oleuropein alone incubated at 25°C for increasing time periods and similarly diluted in the cell culture medium to verify the absence of any its eventual cytotoxic effect.

At the end of the treatment, the viability of cells treated with oleuropein alone did not significantly differ from that of control cells (treated with the culture medium alone). Furthermore, no significant differences were detected in cells treated with differently aged oleuropein mixtures (t0 and 30 min to 72 h) (data not shown). These data were cumulated and a single average “oleuropein control” was reported in Fig. 6.1 A. The “t0” treatment, that is cell treatment with hIAPP dissolved in the aggregation buffer and immediately diluted 110 times in the culture medium, did not produce any significant cytotoxicity with respect to the control treatment. This result suggests that hIAPP did not produce toxic aggregates in the culture medium during the time of cell treatment (24 h), possibly due to its low concentration (30 nM). On the contrary, the administration of hIAPP incubated under aggregation conditions for time periods ranging from 30 min to 8 h resulted in a decrease in cell viability with respect to controls; the latter was highly significant for hIAPP aggregates aged in the 30 min–3 h time period and significant for aggregates aged in the 5–8 h time period (Fig. 6.1 A). These data confirm the generally accepted idea that, like other amyloidogenic peptides, hIAPP results toxic during the first steps of aggregation, corresponding to the presence of oligomeric species [**1**; **120**].

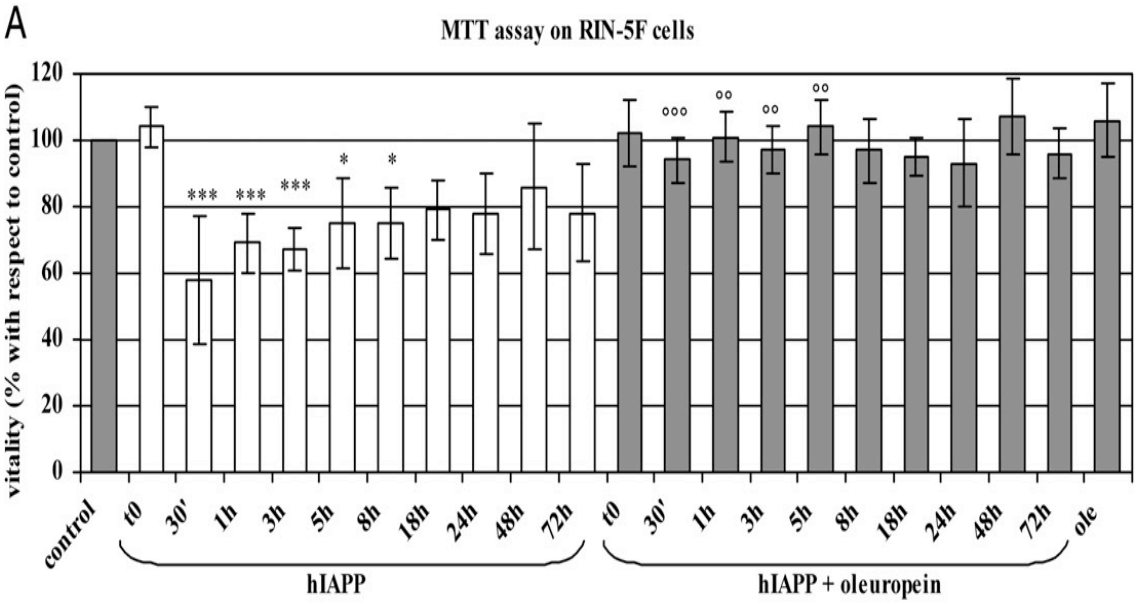

Fig. 6.1 A. 3.25 μM hIAPP solution was incubated for time periods ranging from 0 min to 72 h with or without 30 μM oleuropein aglycon. Then the reaction mixtures were diluted 1:110 in the culture medium and delivered to RIN-5F cells for 24 h; at the end of the incubation, the MTT assay was performed. Control cells received vehicle only; the data coming from cells treated with the incubation buffer containing oleuropein, incubated at 25°C for various time periods, were averaged. In this panel the circles (°) above columns indicate significant statistical differences among cells treated with hIAPP, equally aged in the presence or in the absence of oleuropein; here and in all the other panels, the asterisks (*) indicate significant statistical differences among each treatment and the relative control, treated with vehicle. Statistical analysis was performed with the Tukey–Kramer multiple comparisons test.
* or °Pb.5; ** or °°Pb.1; *** or °°°Pb.01 [Rigacci S., Guidotti V. et al., 2009 **224**].

At variance with the above results, the cells treated with hIAPP previously incubated in the aggregation buffer in the presence of the oleuropein aglycon showed similar viability with respect to the controls. Moreover, the cells exposed to hIAPP aggregates, aged from 30 min to 5 h, displayed a significantly lower viability than those exposed to hIAPP incubated in the presence of oleuropein for the same time periods (Fig. 6.1 A).

Oleuropein was also found to inhibit hIAPP cytotoxicity in a dose-dependent way. We report, as an example, the data obtained using aggregates aged for 5 h in the presence of a 3X or 9X molar concentration of oleuropein (Fig. 6.1 B).

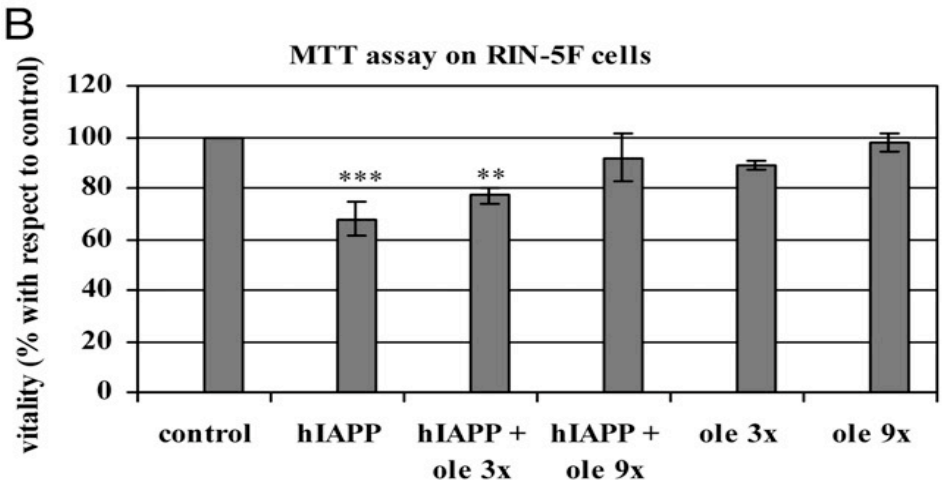

Fig. 6.1 B. The cells were cultured for 24h in the presence of hIAPP aggregates, aged for 5 h in the absence or in the presence of a 3X (9.75 μM) or 9X(30 μM) molar concentration of oleuropein, diluted 1:110 in the culture medium. At the end of the incubation, the MTT assay was performed [Rigacci S., Guidotti V. et al., 2009 **224**].

Finally, we wondered whether oleuropein aglycon should be present during hIAPP aggregation to reduce the cytotoxicity of the aggregates.

To this purpose, the cells were treated with hIAPP aggregates grown for 30 min or 1 h in the absence of oleuropein, and administered together with the same concentration of oleuropein experienced by the cells treated with hIAPP aggregates grown in the presence of different concentrations of oleuropein (9X or 90X) (Fig. 6.1 C).

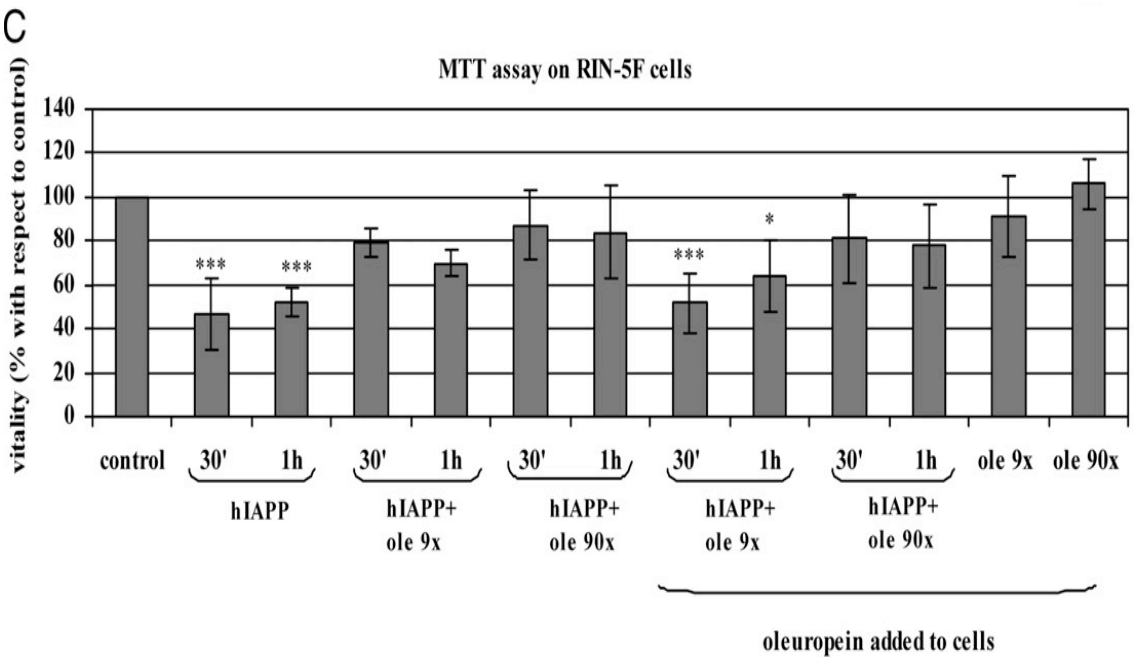

Fig. 6.1 C. The cells were cultured for 24 h in the presence of hIAPP aggregates aged for 30 min or 1h in the absence or in the presence of a 9X or 90X excess (molar concentration) of oleuropein aglycon. The cells were also treated with hIAPP aggregates grown for 30 min or 1 h in the absence of oleuropein, but delivered to the cells together with 30 or 300 μM oleuropein aglycon. At the end of the incubation, the MTT reduction assay was performed [Rigacci S., Guidotti V. et al., 2009 **224**].

Oleuropein was incubated at the same conditions as in the hIAPP aggregation experiments (30 min or 1 h in phosphate buffer, pH 7.4, at 25°C), prior to cell supplementation. At these conditions, supplementation of 270 nM oleuropein (9x) to cells concomitantly treated with hIAPP aggregates (30 nM) did not reduce aggregate cytotoxicity, which remained highly significant (Fig. 6.1 C). The cells partially recovered from the cytotoxic insult given by the aggregates only in the presence of a tenfold molar excess (2.7 μM, 90X) of similarly incubated oleuropein, possibly as a consequence of its generic anti-oxidant power.

These data indicate that, at our conditions, the protection provided to the cells by oleuropein arises during the process of hIAPP aggregation, not simply from its anti-oxidant activity.

6.1.2. Caspase-3 activity assay and LDH relaese assay

The protective effects of oleuropein was confirmed by assaying the apoptotic response, in terms of caspase-3 activation, in cells treated with hIAPP aggregates.

At our conditions, a peak of caspase-3 activity was present in cells exposed for 3h to 30 min-aged hIAPP aggregates (grown in the same conditions used in the MTT cytotoxicity assay). Instead, we did not detect any increase of caspase-3 activity in cells treated with hIAPP, aged under the same conditions but in the presence of 9X oleuropein, thus confirming the MTT data (Fig. 6.1 D).

These findings indicate that oleuropein is able to suppress the cytotoxic potential of the hIAPP aggregates.

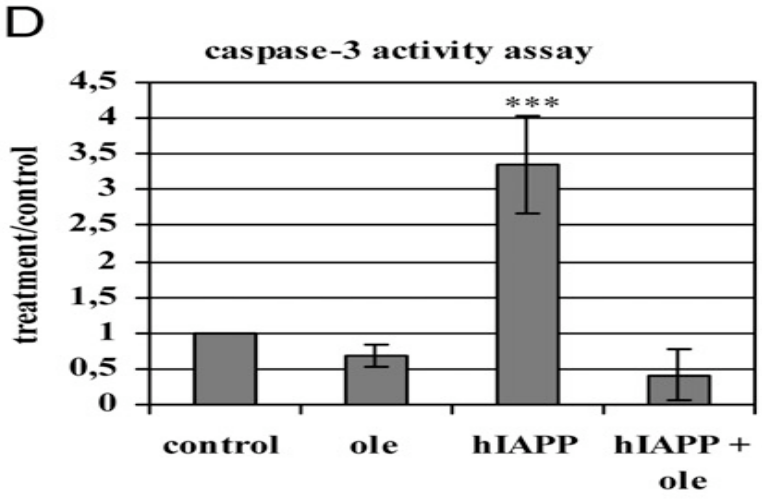

Fig. 6.1 D. Caspase-3 activity was determined in cells cultured for 3.0 h in the presence of 30 min-aged hIAPP aggregates grown in the presence or in the absence of 30 μM oleuropein and diluted in the culture medium, as described for 6.1 A. The data refer to caspase-3 activity increase (fold) with respect to controls that were incubated with vehicle. All experiments were carried out at least five times and S.D. is reported [Rigacci S., Guidotti V. et al., 2009 **224**].

Moreover to confirm the apoptotic nature of cell death induced by the hIAPP aggregates, we also measured LDH release in cells exposed for 24 h to 30 min aged hIAPP aggregates (grown as those used in the MTT cytotoxicity assay).

No significant differences in lactate dehydrogenase release were detected in treated and untreated cells (data not shown), indicating that the biochemical modifications occurring in cells exposed to the human amylin toxic forms trigger the apoptotic pathway, leading to cell death by apoptosis rather than by necrosis.

6.2. hIAPP aggregates grown with oleuropein aglycon do not interact with the cell membrane

6.2.1. Immunofluorescence analysis

Many pieces of evidence indicate that hIAPP oligomers cytotoxicity is closely related to their ability to interact with, and to destabilize, cell membranes [**126**]. Accordingly, we checked whether oleuropein interfered with the ability of hIAPP aggregates to interact with the cell membrane. For this reason, the cells were exposed to 30 min-aged hIAPP aggregates obtained as in the MTT assay, and the distribution of such aggregates with respect to the cells was detected by immunofluorescence using anti-hIAPP antibodies (Fig. 6.2).

Confocal image analysis showed that small hIAPP aggregates were specifically present on the cell membrane, both as a diffuse staining and as more discrete structures (indicated by the arrow in Fig. 6.2 A); the different types of aggregates imaged could possibly arise from a nonsynchronous aggregation, with different species co-existing at the same aggregation time. On the contrary, we did not find any significant interaction of the aggregates with the plasma membranes of cells exposed to hIAPP aggregates grown in the presence of oleuropein. Under these conditions, hIAPP could be retrieved as bulky aggregates dispersed among the cells (as exemplified by the material indicated by the arrow in Fig. 6.2 B). No staining was observed when control cells, not exposed to amylin, were incubated in the presence of both primary and secondary antibodies (Fig. 6.2 C), confirming the specificity of the hIAPP immunodetection. The same interaction of hIAPP aggregates with the cells was observed when the treatment was performed with aggregates obtained in the absence of oleuropein and concomitantly supplemented with a 9X concentration of oleuropein (the same condition used in the MTT assay depicted in Fig.6.1C) (Fig. 6.2 D).

These data indicate that oleuropein aglycon does not prevent the association of pre-formed amyloid aggregates with the cell membrane; rather, oleuropein aglycon must be present during the aggregation process to drive the formation of aggregates unable to interact with the cells.

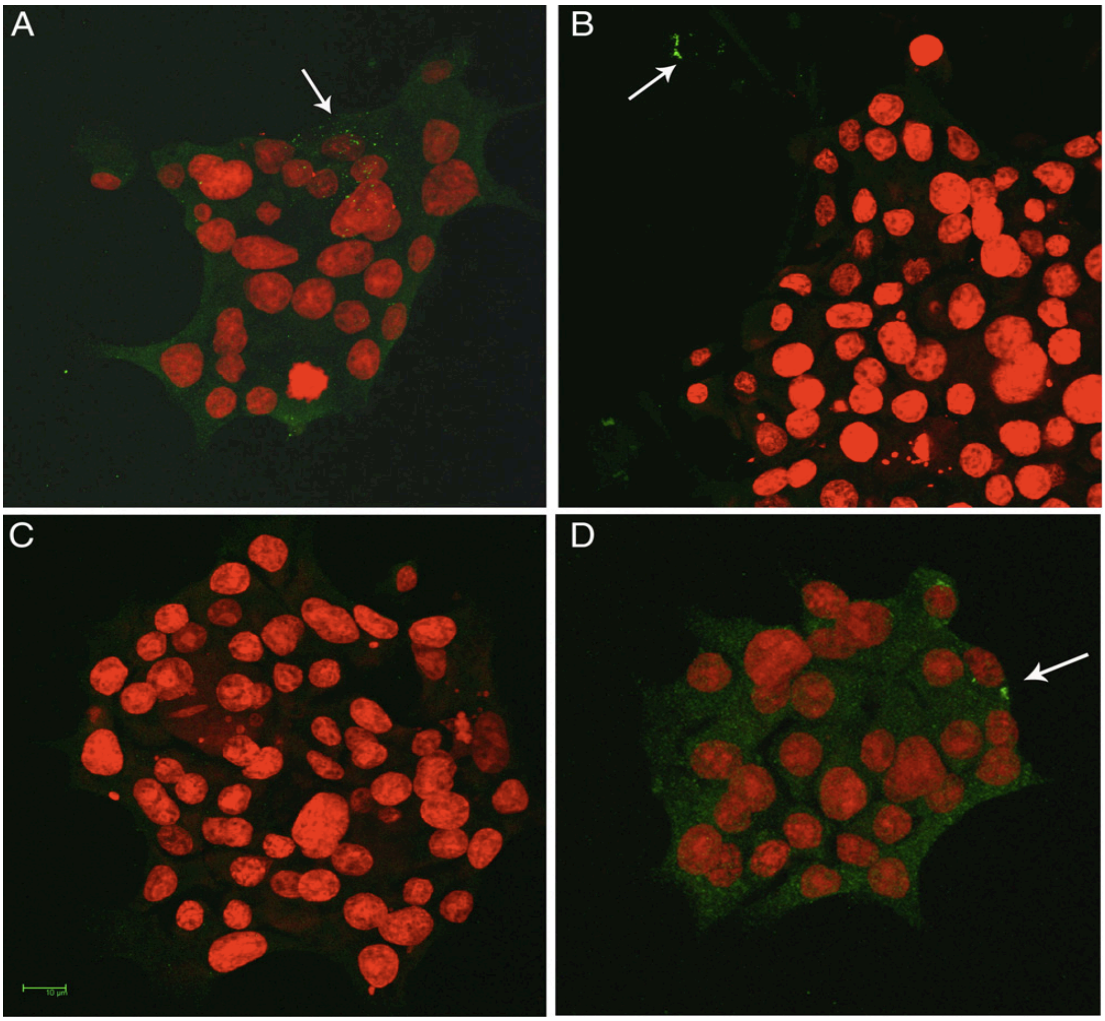

Fig. 6.2. Immunofluorescence analysis of RIN-5F cells treated with hIAPP aggregates. The cells were treated with 30 min-aged hIAPP aggregates (final concentrations: 200 nM hIAPP, 1.8 μM oleuropein). After 5 h, the cells were fixed and stained with rabbit anti-amylin and Alexafluor 488-labeled anti-rabbit antibodies. Nuclei were stained with propidium iodide. (A) Cells treated with hIAPP. (B) Cells treated with hIAPP incubated with oleuropein. (C) Control, untreated cells. (D) Cells treated with hIAPP that was aged without oleuropein and given to cells together with oleuropein [Rigacci S., Guidotti V. et al., 2009 **224**].

These results suggest that hIAPP aggregates grown in the absence or in the presence of oleuropein are somehow structurally different (see later paragraph 6.3); they also show that the decreased cytotoxicity of the aggregates grown in the presence of oleuropein correlates with a significant reduction of their interaction with the cell membrane and, possibly, of membrane damage, regarded as the main responsible of cell sufferance [**126**].

6.2.2. Permeabilization of synthetic phospholipid vesicles by hIAPP aggregates

To further confirm the latter hypothesis, we performed an *in vitro* experiment, incubating synthetic phospholipid vesicles (DOPS: DOPC = 3:7) with hIAPP aggregates aged for different lengths of time (10, 30, 60 min) in the presence or in the absence of oleuropein.

Membrane damage (resulting in vesicle permeabilization) was monitored in calcein-loaded vesicles in terms of increase of fluorescence following calcein release.

We found that the permeabilizing activity of hIAPP aggregates was significantly reduced (from around 80% of total calcein release to around 40%) when the aggregates were grown in the presence of oleuropein for all the assayed times (Fig. 6.3 A–C).

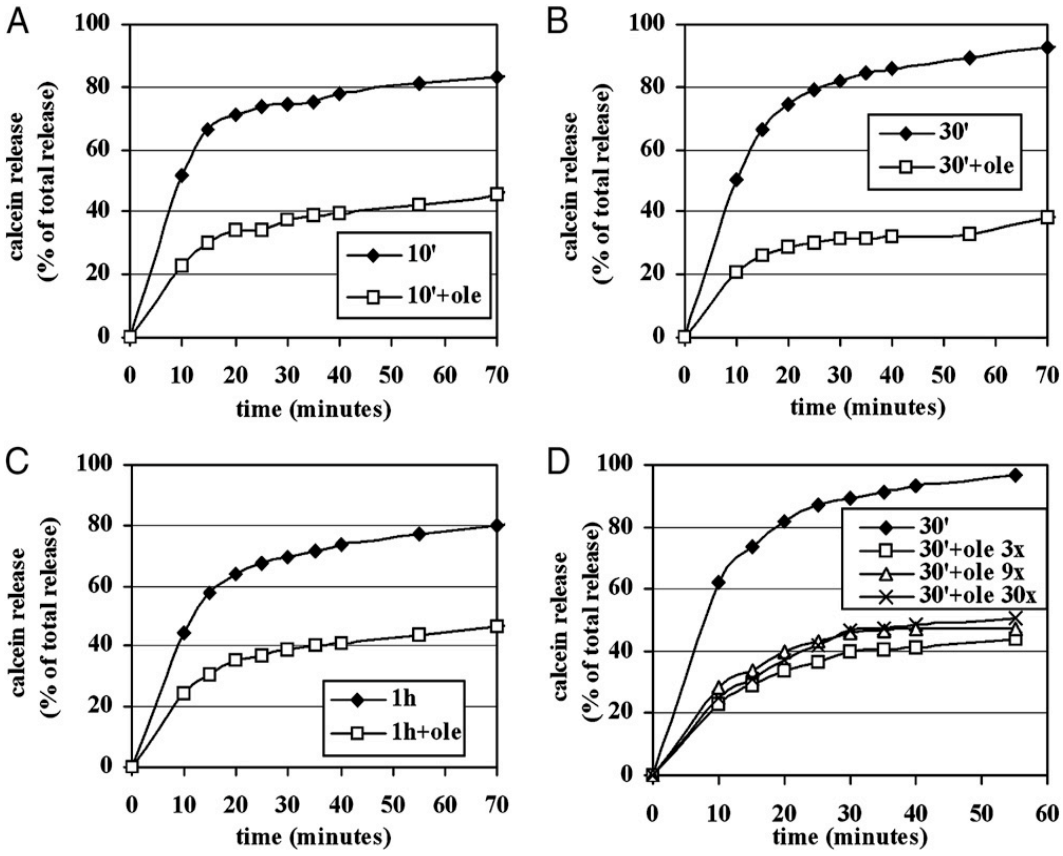

Fig. 6.3. Vesicles permeabilization assay: 3.25 µM hIAPP was aggregated for 10, 30 and 60 min (Panels A–C), in the presence or in the absence of 30 µM oleuropein into a multiwell microplate. The aggregates were then diluted 1:110 and calcein-loaded SUVs were added. At regular time intervals, calcein release was monitored as fluorescence emission (485Ex/538Em), and the values of percentage increase relative to the total calcein release were calculated. The dose-dependence analysis (Panel D) was performed by incubating hIAPP for 30 min with increasing molar ratios of oleuropein, prior to using it in the permeabilization assay. These are representative experiments out of three giving comparable results [Rigacci S., Guidotti V. et al., 2009 **224**].

This result suggests a reduced interaction between the vesicles and the aggregates grown in the presence of oleuropein, confirming the results obtained with cultured cells. No significant dose dependence was apparent from the analysis carried out in the presence of a 3X, 9X or 30X oleuropein aglycon/hIAPP molar ratio (Fig. 6.3 D), thus showing that a 3X molar ratio was sufficient to achieve the maximal inhibition of vesicle permeabilization.

A complete suppression of the permeabilization was not expected at conditions designed to strongly favor hIAPP interaction with phospholipid vesicles.

6.3. In the presence of oleuropein, hIAPP aggregates through a different pathway

6.3.1. ThT assay

The above results suggested that oleuropein aglycon interferes with hIAPP aggregation. Then we tried to investigate the kinetics of hIAPP aggregation in the presence or in the absence of the oleuropein aglycon by the ThT assay.

hIAPP 3.25 μM was incubated at 25°C in 10 mM phosphate buffer, pH 7.4, containing 1.0% HFIP, in the presence or in the absence of 30 μM oleuropein aglycon, and aliquots of the mixture were periodically withdrawn and added to a ThT solution (Fig. 6.4 A). The same final concentrations of the oleuropein aglycon were also added to aliquots of hIAPP aggregates grown in the absence of oleuropein prior to, or after, mixing with the ThT solution (hIAPP/ole/ ThT and hIAPP/ThT/ole in Fig.6. 4 A, respectively).

These controls were necessary to ascertain any inhibitory effect of oleuropein on ThT binding to the aggregates.

Actually, we found some inhibition by oleuropein of ThT binding to the aggregates; however, the reduction of ThT fluorescence was by far more significant when oleuropein was present during hIAPP aggregation (Fig. 6.4 A and Table 6.1). Moreover, the ThT fluorescence was significantly lower when hIAPP was incubated in the presence of oleuropein than when it was incubated in the absence of oleuropein and assayed in the presence of oleuropein (hIAPP+ole vs. hIAPP/ ole/ThT in Table 6.1 and Fig. 6.4 A). Thus, although the reduction of ThT fluorescence can, at least in part, arise from some impairment of ThT binding to hIAPP aggregates in the presence of oleuropein, nonetheless, our results suggest that oleuropein interferes significantly with the formation of ThT-positive (amyloid) hIAPP aggregates.

When ThT was added to hIAPP aggregates prior to oleuropein (hIAPP/ThT/ole in Table 6.1 and Fig. 6.4 A), there was no significant reduction of ThT fluorescence with respect to that recorded in the presence of hIAPP alone, suggesting that ThT successfully competes with oleuropein for binding to hIAPP aggregates. However, when a mixture containing ThT and hIAPP aged for 24 h in the presence of oleuropein was monitored for 24 h, no increase in ThT fluorescence was observed, indicating an intimate interaction between hIAPP and oleuropein that cannot be displaced by ThT competition (data not shown).

A dose dependence analysis, performed with two concentrations of hIAPP incubated for 2 h in the presence of different molar ratios of oleuropein, suggests that a 3X concentration of oleuropein is sufficient to almost completely suppress the increase in ThT fluorescence (Fig. 6.4 B). Due to the afore mentioned overestimation of the effect, the 9X molar ratio used in all the other experiments is fully justified.

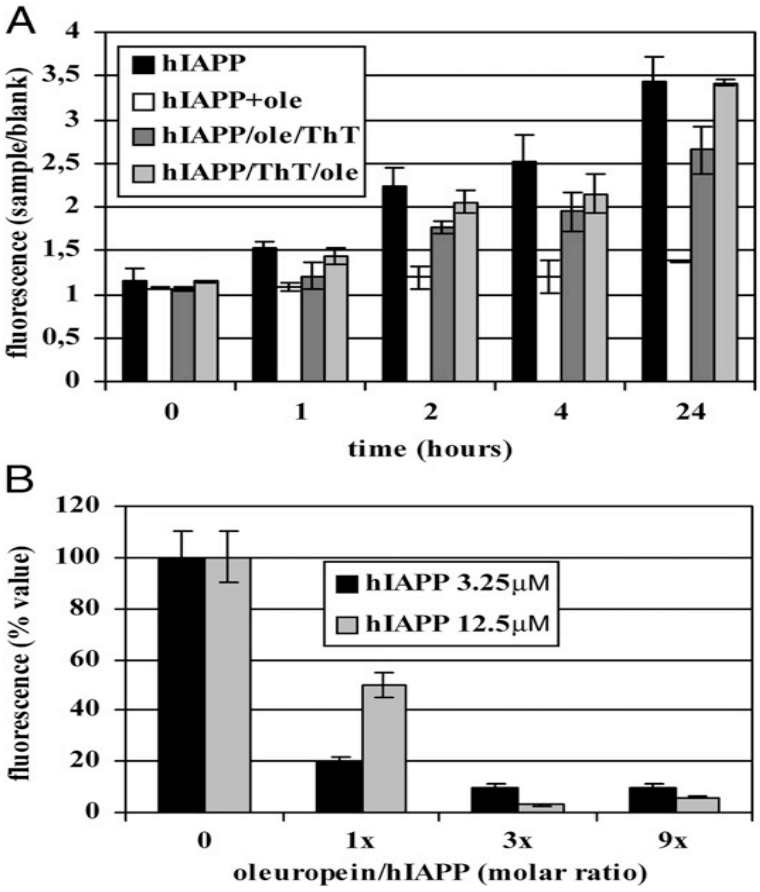

Fig.6.4. ThT assay on 3.25 μM hIAPP. (A) 3.25 μM hIAPP was incubated in 10 mM phosphate buffer, pH 7.4, 1.0% HFIP, in the presence or in the absence of 30 μM oleuropein aglycon, at 25°C. Aliquots were periodically withdrawn from the reaction mixtures and analysed by the ThT binding assay. The interference of oleuropein in ThT binding to hIAPP preformed aggregates was assayed by supplementing oleuropein into aliquots of the hIAPP aggregation mixture either before (hIAPP/ole/ThT) or after (hIAPP/ThT/ole) the addition of ThT. Fluorescence emission values at 485 nm were normalized with respect to the fluorescence of buffer containing all supplements except hIAPP. (B) 3.25 μM hIAPP was incubated for 24 h in the presence or in the absence of a 1X, 3X or 9X molar concentration of oleuropein, then the ThT assay was performed. All experiments were carried out at least five times, and S.D. is reported [Rigacci S., Guidotti V. et al, 2009 **224**].

	0	1h	2h	4h	24h
hIAPP vs. hIAPP+ole	NS	**	***	***	***
hIAPP vs. hIAPP/ole/ThT	NS	* *	NS	**	
hIAPP+ole vs. hIAPP/ole/ThT	NS	NS	**	*	***
hIAPP+ole vs. hIAPP/ThT/ole	NS	*	***	**	***
hIAPP/ole/ThT vs. hIAPP/ThT/ole	NS	NS	NS	NS	**

Table 6.1. Tukey-Kramer analysis of results coming from the ThT assay. Data coming from the ThT assay reported in Fig. 6.4 A were statistically analysed with the Tukey-Kramer multiple comparisons test. The columns refer to the times at which the incubation of hIAPP mixtures were assayed. NS, not significant.

* Pb< 5.

** Pb< 1.

*** Pb< 01.

6.3.2. CD analysis

To gain insight into the structural differences between the hIAPP aggregation intermediates arising in the absence or in the presence of oleuropein, a CD analysis was performed on a 6.5 μM hIAPP solution incubated in 10 mM phosphate buffer, pH 7.4, containing 1.0% HFIP, in the presence or in the absence of 50 μM oleuropein aglycon. hIAPP concentration was doubled (6.5 μM *vs.* 3.25 μM) to obtain acceptable spectra and a ThT assay was also performed at these conditions (Fig. 6.5).

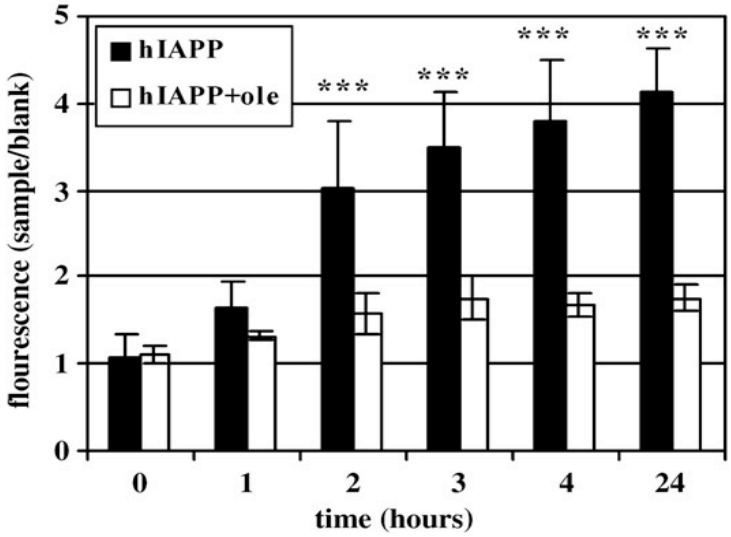

Fig. 6.5 ThT assay on 6.5 μM hIAPP; 6.5 μM hIAPP was incubated in the absence or in the presence of 50 μM oleuropein exactly as for the CD analysis. Fluorescence emission values at 485 nm were normalized with respect to the fluorescence of buffer containing all supplements except hIAPP. In this case, the negative interference coming from the presence of oleuropein during the ThT assay was calculated and added to every point of the "hIAPP + ole" curve. Eight experiments were performed, and S.D. is reported. Statistical analysis was performed with the Tukey–Kramer multiple comparisons test. Asterisks indicate a significant difference between the signals produced at any time point by hIAPP incubated in the absence or in the presence of oleuropein. ***Pb.01 [Rigacci S., Guidotti V. et al., 2009 **224**].

The CD spectra of samples aged for differing lengths of time are shown in Fig. 6.6.

It can be seen that, immediately after dilution in aqueous buffer (Fig. 6.6 A), hIAPP, both in the absence and in the presence of oleuropein, was largely unstructured, in agreement with data previously reported for soluble hIAPP [**215**]. At this stage of the aggregation process, oleuropein

aglycon determines a variation of the intensity of the CD spectrum of hIAPP, although its shape remains substantially unaltered. After 3 h (Fig. 6.6 B), the spectrum of hIAPP alone indicates a conversion to a β-sheet-rich structure (as revealed by the minimum around 218–220 nm).

A different behaviour was seen in the presence of oleuropein: in this case hIAPP transition to a β-sheet-rich structure was somehow inhibited, and the spectrum intensity appeared reduced with respect to the zero time, suggesting the formation of insoluble species (compare panels A and B). At this time point, the ThT assay revealed a highly significant difference between hIAPP incubated in the absence or in the presence of oleuropein (Fig. 6.5).

Both the CD spectra and the ThT assay show that the structural transition of hIAPP is already completed at this time point: in fact, no further changes could be observed in the CD spectrum of hIAPP after 2 h of incubation (Fig. 6.6 B–C), and the statistical analysis of data coming from the ThT assay (Fig. 6.5) revealed no significant difference between the signals produced after 3 and 24 h of incubation.

On the other side, the spectra of hIAPP incubated in the presence or in the absence of oleuropein aglycon were not significantly different after 24 h, although the intensity of the CD spectrum recorded in the presence of oleuropein was further reduced (compare Panels B and C in Fig. 6.6).

This evidence suggests that oleuropein does not modify the fate of hIAPP by inhibiting its amyloid aggregation, but drives the process through an alternative and less cytotoxic pathway.

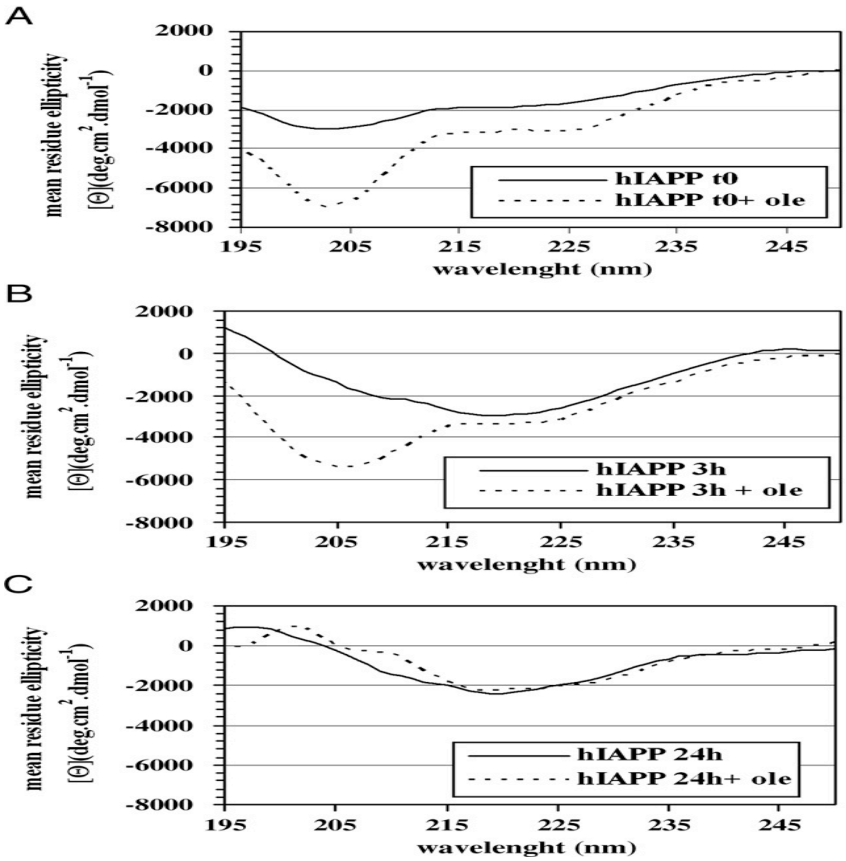

Fig. 6.6. CD analysis. 6.5 μM hIAPP was incubated in 10 mM phosphate buffer, pH 7.4, containing 1.0% HFIP, at 25°C in the absence or in the presence of 50 μM oleuropein aglycon. The CD spectra of the mixtures were acquired in the far UV region immediately (A), after 3 h (B) or after 24 h (C) of incubation. This is a representative experiment out of three giving qualitatively identical results [Rigacci S., Guidotti V. et al., 2009 **224**].

6.3.3. Electron microscopy analysis

To further support the above hypothesis, the aggregates obtained from hIAPP incubated for 30 min, 3 and 24 h in the presence or in the absence of oleuropein were observed with an electron microscope (Fig. 6.7 A-F).

After 30 min, hIAPP formed small spherical aggregates, which join together in short chains or rosettes similarly to the early steps of aggregation of other peptides and proteins (Panel A). At this time, no discrete aggregates but an apparently amorphous precipitate can be seen in the presence of oleuropein (Panel D).

This picture did not significantly change after 3h of incubation (Panel E), except for a further increase in the amount of precipitate, whereas in the absence of oleuropein hIAPP begins to organize into fibrils (Panel B). These morphological differences between aggregates obtained in the absence or in the presence of oleuropein agree with the different CD spectra recorded.

After 24 h, hIAPP formed an extensive network of mature fibrils (Panel C). A large amount of fibrils apparently originating from the previously observed precipitate was also seen in the presence of oleuropein (Panel F); these fibrils seem to further associate in bundles.

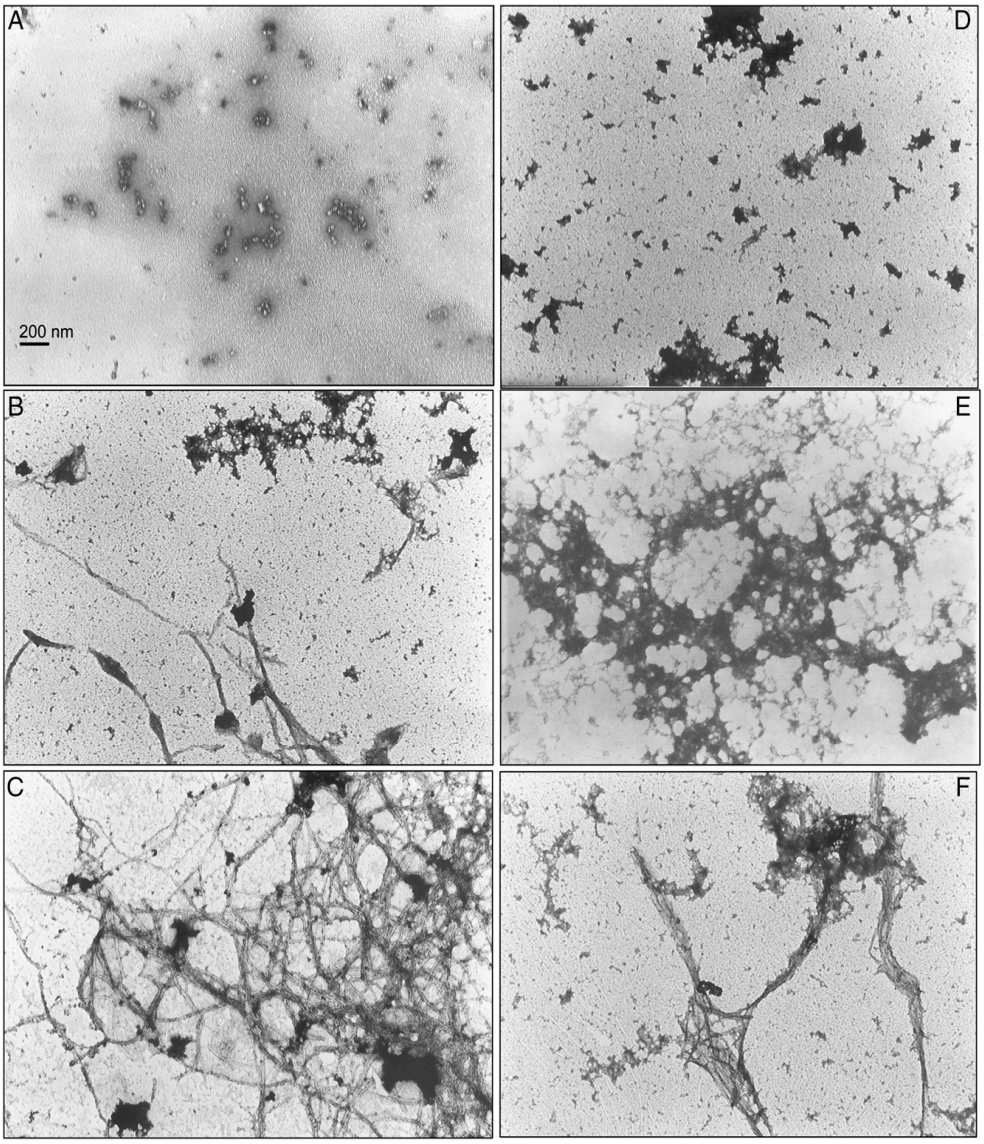

Fig. 6.7 A-F. EM analysis. hIAPP was incubated in the absence (Panels A, B, C) or in the presence (Panels D, E, F) of oleuropein as for CD analysis and analyzed after 30 min (A and D) 3 h (B and E) and 24 h (C and F). Magnification: 25.000X [Rigacci S., Guidotti V. et a., 2009 **224**].