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Oleuropein aglycon prevents cytotoxic amyloid aggregation of human amylin☆

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Abstract

Pancreatic amyloid deposits of amylin are a hallmark of Type II diabetes and considerable evidence indicates that amylin oligomers are cytotoxic to β-cells. Many efforts are presently spent to find out naturally occurring molecules, or to design synthetic ones, able to hinder amylin aggregation or to protect cells against aggregate cytotoxicity. In this context, a protective effect of some polyphenols against amyloid cytotoxicity was reported. Actually dietary polyphenols are endowed with multiple health benefits, and extra virgin olive oil is attracting increasing interest as a source of these substances. Here, we investigated the effects on amylin aggregation and cytotoxicity of the secoiridoid oleuropein aglycon, the main phenolic component of extra virgin olive oil. We found that oleuropein, when present during the aggregation of amylin, consistently prevented its cytotoxicity to RIN-5F pancreatic β-cells, as determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide test and caspase-3 activity assay. A lack of interaction with the cell membrane of amylin aggregates grown in the presence of oleuropein was shown by fluorescence microscopy and synthetic lipid vesicle permeabilization. Moreover, our ThT assay, circular dichroism analysis and electron microscopy images suggested that oleuropein interferes with amylin aggregation, resulting in a different path skipping the formation of toxic pre-fibrillar aggregates. These results provide a molecular basis for some of the benefits potentially coming from extra virgin olive oil consumption and pave the way to further studies on the possible pharmacological use of oleuropein to prevent or to slow down the progression of type II diabetes.

Keywords: Oleuropein; Amylin; hIAPP; Amyloid cytotoxicity; Type two diabetes; Extra virgin olive oil

1. Introduction

The presence in tissues of amyloid plaques consisting mainly of amyloid fibrils arising from the polymerization of specific peptides/proteins is a key hallmark of several degenerative conditions including Alzheimer's, Parkinson's and prion diseases; severe systemic amyloidosis and non-insulin-dependent Type II diabetes (NIDDM) [1]. Amylin, or human islet amyloid polypeptide (hIAPP), was first discovered in the amyloid substance found in human insulinomas as well as in pancreatic islets in people affected by NIDDM. hIAPP is a 37-residue peptide cosecreted with insulin by the β-cells in pancreatic islets in response to the elevation of plasma glucose levels. Although full knowledge of the exact function of amylin is still lacking, there is evidence suggesting a role of this peptide in blood glucose regulation [2].

hIAPP is characterised by low solubility and high fibrillogenic propensity leading it to easily aggregate into amyloid fibrils via a multistep nucleation-dependent process [3]. Pancreatic amyloid deposits of hIAPP occur in up to 90% of people affected by NIDDM [4]. Furthermore, transgenic rats expressing hIAPP develop a diabetic phenotype associated with the deposition of hIAPP amyloid fibrils [5]. Accordingly, hIAPP aggregation is considered to play an important role in NIDDM pathogenesis, since cytotoxic hIAPP oligomers trigger apoptosis in replicating β cells [6,7].

Presently, prefibrillar aggregates of different proteins and peptides are considered the most toxic amyloid species, whereas mature fibrils are substantially devoid of cytotoxicity [8]. Accordingly, intracellular or extracellular prefibrillar aggregates are considered the main responsible for cell impairment and tissue degeneration in amyloid diseases [1] and, more specifically, in NIDDM [9]. In most cases, the toxic effects of amyloid aggregates to exposed cells involve shared early biochemical modifications, including nonspecific membrane permeabilization resulting in intracellular free Ca^{2+} increase, oxidative stress, mitochondria impairment and eventually apoptosis [10,11]. For these reasons, many efforts are presently spent to find out naturally occurring molecules, including polyphenols, or to design synthetic ones, able to protect cells against oxidative stress or to inhibit amyloid aggregation mainly at its earliest stages.

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Many pieces of evidence support the widely recognized assumption that the Mediterranean diet is greatly beneficial for the prevention of several diseases ranging from atherosclerosis to cancer. In this respect, the consideration that the cytotoxicity of amyloid aggregates is frequently associated to early modifications of the intracellular redox status has recently raised interest on the possible protection by natural antioxidants occurring in fruits, vegetables and their products.

In this respect, attention has also been paid to the role of polyphenols, particularly those occurring in wine and in extra virgin olive oil. Oleuropein, a secoiridoid glucoside characteristic of Oleaceae, is the main phenolic component of Mediterranean extra virgin olive oil [12]. Oleuropein has been shown to possess several pharmacological properties, including antioxidant [13], anti-inflammatory [14], anti-atherogenic [15] and anti-cancer [16] activities, and for these reasons, it is commercially available as food integrator in many countries. In addition, oleuropein has been shown to be cardioprotective against acute adriamycin cardiotoxicity [17] and to exhibit anti-ischemic and hypolipidemic activities [18].

Oleuropein hydrolysis, carried out by an endogenous β-glycosidase during olive ripening and in the technological process of olive oil production, releases the aglycon moiety of the molecule in the olive oil [12]. Recently, oleuropein has been shown to form a noncovalent complex with the Aβ peptide [19,20]. Here, we report on the oleuropein aglycon interference with hIAPP aggregation and on its protection against aggregate cytotoxicity using a RIN-5F rat insulinoma cell model. Our data provide the rationale to support further research aimed at assessing the beneficial effect of the Mediterranean diet and, particularly, of the use of extra virgin olive oil as a main diet lipid, against the appearance of Type II diabetes mellitus.

2. Methods and materials

2.1. Materials and cell culture

Oleuropein was from Extrasynthese (Lyon, France). Almond β-glycosidase (EC 3.2.1.21) was from Fluka (Sigma-Aldrich, Steinheim, Germany). hIAPP (Calbiochem, La Jolla, CA, USA) was dissolved in 80% hexafluoroisopropanol (HFIP) (Sigma-Aldrich) to a concentration of 512 μM and stored at −20°C until use. Caspase-3 substrate (Ac-DEVD-AFC) and inhibitor (DEVD-CHO) were from Biomol (Exeter, UK). Anti-amylin rabbit polyclonal antibody (H-50) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Alexafluor 488-labeled chicken anti-rabbit IgG antibody was from Molecular Probes (Eugene, OR, USA); propidium iodide was from Fluka (Sigma-Aldrich). 1,2-Dioleoyl-*sn*-glycero-3-[phospho-L-serine] (DOPS) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were from Avanti Polar Lipids (Alabaster, AL, USA). Thioflavine-T (ThT), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), calcein and all other chemicals and supplements, unless otherwise stated, were from Sigma-Aldrich.

Rat RIN-5F insulinoma cells were from ATCC (American Type Culture Collection). This cell line was extensively used to assess the toxicity of amyloid hIAPP aggregates [7,21,22]. The cells were cultured in RPMI medium supplemented with 10% foetal calf serum (FCS) (Sigma-Aldrich, Steinheim, Germany), 2.25 g/L glucose, 10 mM HEPES, 1.0 mM sodium piruvate, glutamine and antibiotics and maintained in a 5.0% CO_2 humidified atmosphere at 37°C.

2.2. Oleuropein deglycosilation

Oleuropein deglycosilation was performed according to Konno et al. [23] with minor modifications. Briefly, a 10 mM solution of oleuropein in 310 μl of 0.1 M sodium phosphate buffer, pH 7.0, was incubated with 8.90 IU of β-glycosidase overnight at room temperature. The reaction mixture was centrifuged at 18,000 rpm for 10 min to precipitate the oleuropein aglycon, which was then dissolved in DMSO (dimethyl sulfoxide). The complete oleuropein deglycosylation was confirmed by assaying the glucose released in the supernatant with the Glucose (HK) Assay Kit (Sigma). GC-MS (gas cromatography-mass spectrometry) analysis showed the absence of any glycated oleuropein in the precipitate and the substantially total recover of the aglycon in the same precipitate. The 100 mM stock solution was protected from light and used by the day it was prepared. Dilutions in aqueous buffers were made immediately before use.

2.3. Cytotoxicity assay

hIAPP aggregate cytotoxicity was assessed on RIN-5F cells by the MTT reduction assay, as previously reported [24]. The cells were seeded into 96-well plates at a density of 20,000 cells/well in fresh complete medium and grown for 72 h. hIAPP aggregates were prepared by diluting stock solution to 3.25 μM in 10 mM phosphate buffer, pH 7.4, containing 1.0% HFIP [25], in the presence or in the absence of 30 μM oleuropein (oleuropein molar concentration was 9× with respect to hIAPP molar concentration), and aged for different lengths of time at 25°C. Then, the aggregate mixtures were diluted 110 times in the culture medium without FCS and phenol red and given to cells (final concentrations: 30 nM hIAPP, 270 nM oleuropein). The medium without phenol red was preferred since an inhibitory activity of phenol red on hIAPP amyloid aggregation was previously demonstrated [26]. In the dose dependence experiments, 3.25 μM hIAPP was also incubated with a 3× concentration of oleuropein (9.8 μM). As additional controls, the cells were also treated with oleuropein alone, or with hIAPP aggregates (obtained in the absence of oleuropein) together with 270 nM or 2.70 μM oleuropein aglycon; the secoiridoid was previously incubated at the same conditions and for the same length of time as hIAPP aggregates, before being given to cells. At the end of the exposure (24 h), the culture medium was removed and the cells were incubated for 2 h in a 0.5 mg/ml MTT solution. Finally, cell lysis solution [20 % (w/v) sodium dodecyl sulfate, 50 % (v/v) *N*, *N*-dimethylformamide] was added, and the multiwell plate was kept overnight at 37°C in a humidified incubator. Blue formazan absorbance was measured at 570 nm using an automatic plate reader (BioRad).

2.4. Caspase-3 activity assay

The cells were seeded, cultured and treated with hIAPP exactly as indicated for the MTT assay. In this case, the cells were exposed to hIAPP aggregates aged for 30 min at 25°C in the presence or in the absence of oleuropein and then diluted in the culture medium. After 3 h of incubation, the cells were lysed and caspase-3 activity assay was performed as previously described [27], using the Ac-DEVD-AFC substrate (fluorometric, $400_{Ex}/505_{Em}$) and 50 μg of proteins from total lysates for each determination.

2.5. Immunofluorescence analysis

The cells were seeded on glass cover slips and treated with hIAPP aggregates aged for 30 min at 25°C (3.25 μM in 10 mM phosphate buffer, pH 7.4, containing 1.0% HFIP, with or without 30 μM oleuropein) prior to dilution in the culture medium in the presence or in the absence of the oleuropein aglycon (final concentrations: 200 nM hIAPP, 1.8 μM oleuropein). In this case it was necessary to treat cells with a higher concentration of hIAPP (and, consequently, of oleuropein) to subsequently obtain a clear immunodetection. After 5 h, the cells were washed with phosphate-buffered saline, fixed with 2.0% *p*-formaldheyde and incubated overnight at 4°C with rabbit anti-amylin antibodies (1:300). Then the cells were stained with Alexafluor 488-labeled anti-rabbit antibodies (1:400), and propidium iodide was used to image nuclei. Control cells, not treated with hIAPP, were incubated with primary and secondary antibodies as well. Images were obtained using a Leica SP5 laser scanning confocal microscope.

2.6. Preparation of phospholipid unilamellar vesicles

DOPS and DOPC in chloroform solutions were mixed (DOPS:DOPC=3:7 [28]) and dried in glass tubes (8 mm in diameter) under a gentle nitrogen stream in a fume hood. For the calcein release assay, the dry lipid film was resuspended in 1.0 M Tris–HCl, pH 8.0, containing 60 mM calcein, at a final lipid concentration of 1.0 mg/ml. The resuspended samples were incubated for 1 h at room temperature to allow lipid vesicles formation, vortexing occasionally. The resulting suspension was then subjected to five freeze-thaw cycles of 2.0 min each, using liquid nitrogen and a 37°C water bath, and sonicated for 20 min at 20 kHz in ice to obtain a suspension of small unilamellar vesicles (SUV). To remove large lipid aggregates the preparation was centrifuged for 10 min at 10,000×g. The nonencapsulated calcein was removed by gel-filtration using a Sephadex G-50 column (1.5×7.0 cm); the vesicles were eluted in 10 mM phosphate buffer, pH 7.4, and used within 48 h. Before each experiment, the size distribution of the vesicle population was checked using a Zetasizer Nano S dynamic light scattering device from Malvern Instruments (Malvern, Worcestershire, UK) and the nonhomogeneous lipid populations were discarded.

2.7. Calcein release assay

The assay was performed according to Engel et al. [28] with some modifications. 3.25 μM hIAPP was induced to aggregate at 25°C in the presence or in the absence of 30 μM oleuropein in 10 mM phosphate buffer, pH 7.4, containing 1.0% HFIP in a multiwell microplate for increasing time periods. Control mixtures without hIAPP were similarly prepared. At the end of the aggregation periods, the sample volumes were adjusted to 200 μl and the SUV suspension (1:40) was added. Calcein is self-quenched into the vesicle aqueous core; therefore, calcein leakage to the external medium upon hIAPP aggregates-vesicles interaction can be monitored as an increase in calcein fluorescence, due to its dilution and the consequent reduction of self-quenching. At regular time intervals, measurements were performed using the fluorescence microplate reader

Fluoroskan Ascent FL (Thermo Electron). The excitation and emission wavelengths were 485 nm and 538 nm, respectively. The percentage of calcein release was calculated according to the formula:

$$\text{Calcein release } (\%) = (F - F_0) \times 100/(F_{max} - F_0)$$

where F is the fluorescence measured at the different time intervals during the experiment, F_0 is the fluorescence measured at the beginning of the experiment and F_{max} is the maximum fluorescence, as determined by disrupting the vesicles at the end of each experiment by adding 0.1 % Triton X-100. Each curve was obtained after a point-by-point subtraction of the data coming from its control mixture.

2.8. ThT assay

hIAPP 3.25 μM was incubated at 25°C in 10 mM phosphate buffer, pH 7.4, containing 1.0% (v/v) HFIP in the presence or in the absence of increasing concentrations of the oleuropein aglycon, for 24 h; 50 μl of this solution was withdrawn at different time intervals and mixed with 350 μl of 20 μM ThT dissolved in 0.1 M Gly/NaOH buffer, pH 8.5. Blanks were prepared similarly but without hIAPP. As a control, oleuropein was also added to every hIAPP sample aggregated in the absence of oleuropein, before or after ThT addition, and just prior to fluorescence reading. Fluorescence emission intensity was measured ($440_{Ex}/485_{Em}$) [29] using a Perkin Elmer LS 55 spectrofluorimeter equipped with a thermostated cell-holder attached to a Haake F8 water-bath. Dose-dependence analysis was performed by assaying hIAPP samples incubated with a 1×, 3× or 9× molar concentration of oleuropein for 24 h. In experiments parallel to circular dichroism (CD) analysis 6.5 μM hIAPP was used and oleuropein concentration correspondingly adjusted.

2.9. Circular dichroism

hIAPP (6.5 μM) was diluted in 10 mM phosphate buffer, pH 7.4, containing 1.0% (v/v) HFIP, in the presence or in the absence of 50 μM oleuropein aglycon. The spectra of the mixtures were recorded at 25°C (1.0 nm data pitch, 50 nm/min speed, and 1 s response) with subsequent accumulations, using a Jasco J-810 spectropolarimeter equipped with a thermostated cell holder attached to a Thermo Haake C25P water-bath. Background spectra were acquired for buffer with and without 50 μM oleuropein and subtracted to the sample spectra.

2.10. Electron microscopy

hIAPP was incubated with or without oleuropein aglycon as for CD analysis. A 3.0 μl sample was loaded onto a formvar- and carbon-coated copper grid and negatively stained with 30 μl of 1.0% (w/v) uranyl acetate. The grid was air-dried and examined using a JEM 1010 transmission electron microscope at 80 kV excitation voltage.

3. Results

3.1. The oleuropein aglycon reduces hIAPP cytotoxicity

3.1.1. MTT assay

The effect of the oleuropein aglycon on hIAPP aggregate cytotoxicity was evaluated using rat RIN-5F pancreatic β-cells, which have been extensively used for similar purposes [7,21,22]. hIAPP was incubated in aqueous buffer at 25°C for varying lengths of time, up to 72 h, to obtain increasingly structured aggregates, either alone [25] or in the presence of oleuropein (ninefold 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 deprived of 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.

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 (t_0 and 30 min to 72 h) (data not shown). These data were cumulated and a single average "oleuropein control" was reported in Fig. 1A. The "t_0" treatment (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 finding suggests that hIAPP did not produce toxic aggregates in the culture medium during the time period 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. 1A). These data confirm the generally accepted idea that, similarly to other amyloidogenic peptides, hIAPP is toxic in the first steps of aggregation, corresponding to the presence of oligomeric species [1,9].

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 for a time period ranging 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. 1A). 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 3× or 9× molar concentration of oleuropein (Fig. 1B).

Finally, we wondered whether oleuropein 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 (9× or 90×) (Fig. 1C). 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 (9×) to cells concomitantly treated with hIAPP aggregates (30 nM) did not attenuate aggregate cytotoxicity, which remained highly significant (Fig. 1C). The cells partially recovered from the cytotoxic insult given by the aggregates only in the presence of a tenfold molar excess (2.7 μM, 90×) 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.

3.1.2. Caspase-3 activity 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 3 h to 30 min-aged hIAPP aggregates (grown as in the MTT cytotoxicity assay). 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 9× oleuropein, confirming the MTT data (Fig. 1D). These findings indicate that oleuropein suppresses the cytotoxic potential of the hIAPP aggregates.

3.2. hIAPP aggregates grown in the presence of the oleuropein aglycon do not interact with the cell membrane

3.2.1. Immunofluorescence analysis of cells after incubation with hIAPP aggregates

Many pieces of evidence indicate that hIAPP oligomers cytotoxicity is closely related to their ability to interact with, and to destabilize, cell membranes [30]. 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 spatial

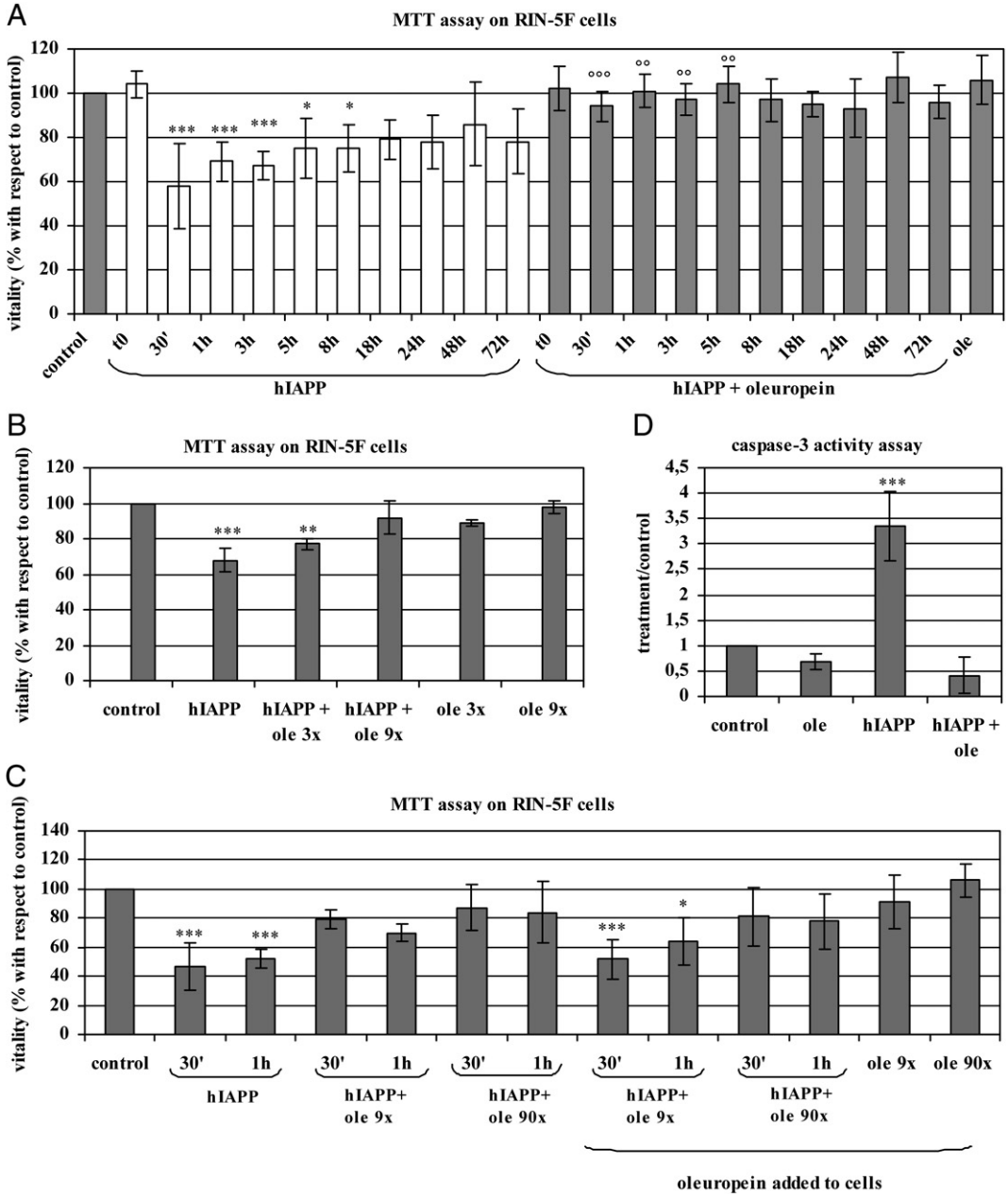

Fig. 1. hIAPP cytotoxicity in the presence or in the absence of the oleuropein aglycon. (A) A 3.25 μM hIAPP solution was incubated for time periods ranging from 0 to 72 h with or without 30 μM oleuropein. Then the reaction mixtures were diluted 1:110 in the culture medium and delivered to RIN-5F cells 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 °$P<.5$; ** or °°$P<.1$; *** or °°°$P<.01$. (B) The cells were cultured for 24 h in the presence of hIAPP aggregates, aged for 5 h in the absence or in the presence of a 3× (9.75 μM) or 9× (30 μM) molar concentration of oleuropein, diluted 1:110 in the culture medium. At the end of the incubation, the MTT assay was performed. (C) The cells were cultured for 24 h in the presence of hIAPP aggregates aged for 30 min or 1.0 h in the absence or in the presence of a 9× or 90× excess (molar concentration) of oleuropein aglycon. The cells were also treated with hIAPP aggregates grown for 30 min or 1.0 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. (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 (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.

distribution of such aggregates with respect to the cells was detected by immunofluorescence using anti-hIAPP antibodies (Fig. 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. 2A); the different types of aggregates imaged could possibly arise from a non-synchronous 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

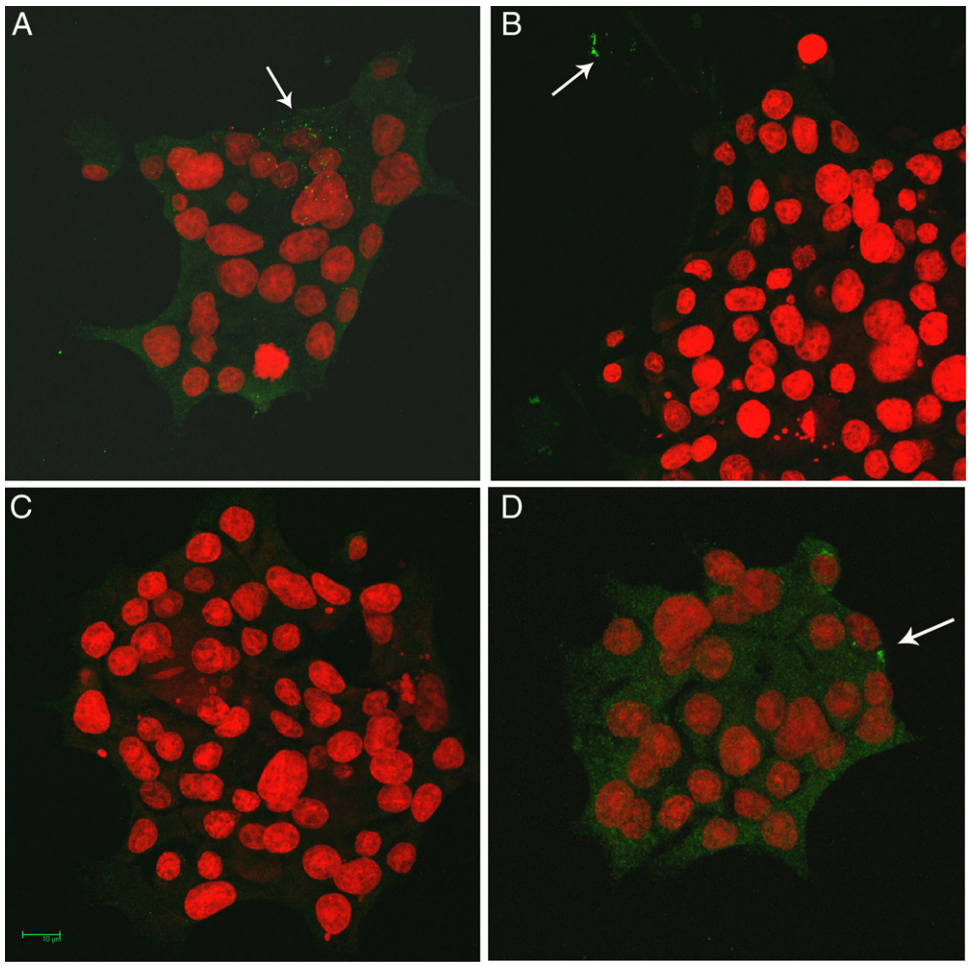

Fig. 2. Immunofluorescence analysis of RIN-5F cells treated with hIAPP aggregates. The cells were treated with 30 min-aged hIAPP aggregates (final concentrations: 200 nM hIAPP, 1.8 μM oleuropein). After 5 h, the cells were fixed and stained with rabbit anti-amylin and Alexafluor 488-labeled anti-rabbit 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.

indicated by the arrow in Fig. 2B). No staining was observed when control cells, not exposed to amylin, were incubated in the presence of both primary and secondary antibodies (Fig. 2C), 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 9× concentration of oleuropein (the same condition used in the MTT assay depicted in Fig. 1C) (Fig. 2D). These data indicate that oleuropein does not prevent the association of pre-formed amyloid aggregates with the cell membrane; rather, oleuropein must be present during the aggregation process to drive the formation of aggregates unable to interact with the cells. These results suggest that hIAPP aggregates grown in the absence or in the presence of oleuropein are somehow structurally different (see later); 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, the main responsible of cell sufferance [30].

3.2.2. 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. 3A–C). 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 3×, 9× or 30× oleuropein aglycon/hIAPP molar ratio (Fig. 3D), thus showing that a 3× 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.

3.3. In the presence of oleuropein, hIAPP aggregates through a different path

3.3.1. ThT assay

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

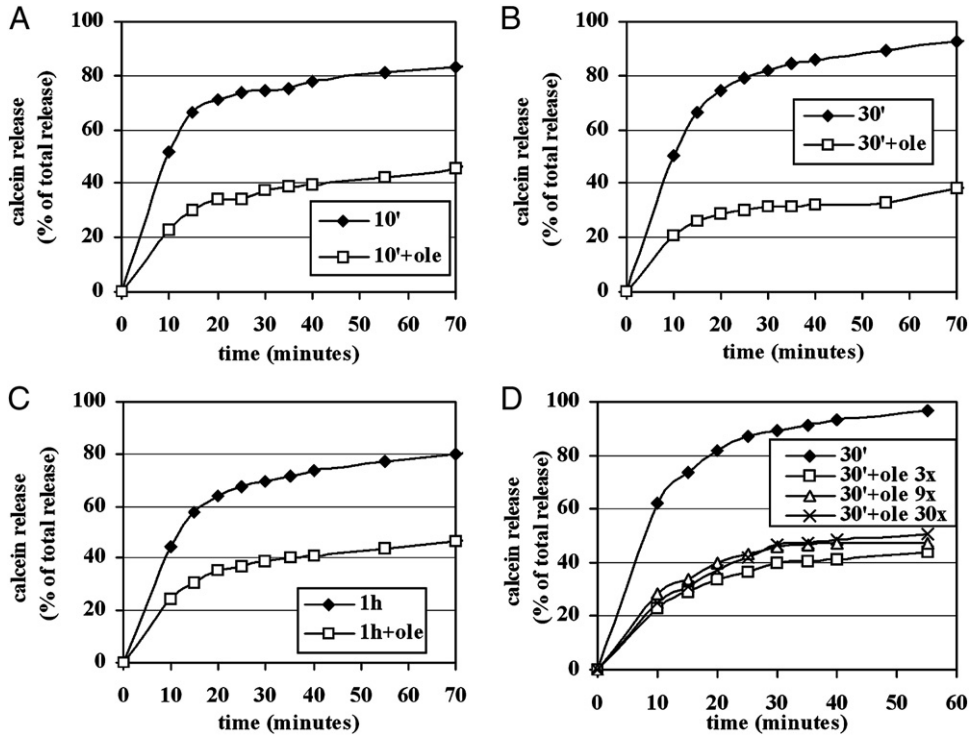

Fig. 3. Vesicles permeabilization assay: 3.25 μM hIAPP was aggregated for 10, 30 and 60 min (Panels A–C), in the presence or in the absence of 30 μM oleuropein into a multiwell microplate. The aggregates were then diluted 1:110 and calcein-loaded SUVs were added. At regular time intervals, calcein release was monitored as fluorescence emission ($485_{Ex}/538_{Em}$), 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.

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. 4A). 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. 4A, 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. 4A and Table 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 1 and Fig. 4A). 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 1 and Fig. 4A), 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 24 h in the presence of different molar ratios of oleuropein, suggests that a 3× concentration of oleuropein is sufficient to almost completely suppress the increase in ThT fluorescence (Fig. 4B). Due to the aforementioned overestimation of the effect, the 9× molar ratio used in all the other experiments is fully justified.

3.3.2. 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).

The CD spectra of samples aged for differing lengths of time are shown in Fig. 5. It can be seen that, immediately after dilution in aqueous buffer (Fig. 5A), hIAPP, both in the absence and in the presence of oleuropein, was largely unstructured, in agreement with data previously reported for soluble hIAPP [26]. At this stage of the aggregation process, oleuropein determines a variation of the intensity of the CD spectrum of hIAPP, although its shape remains substantially unaltered. After 3 h (Fig. 5B), 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

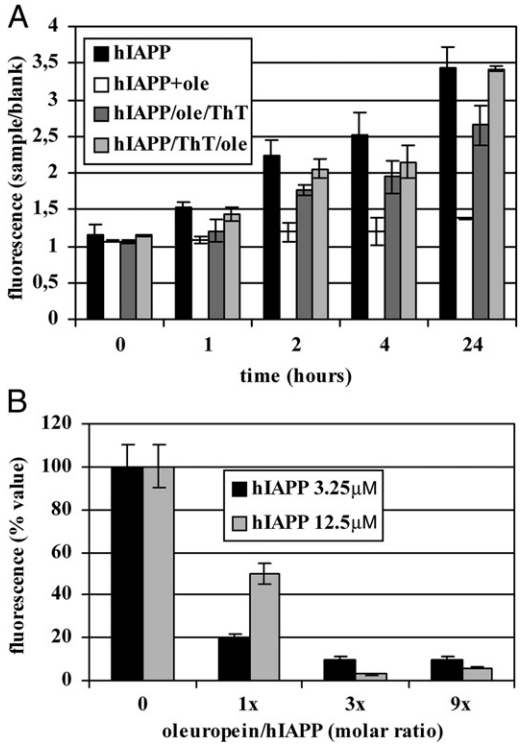

Fig. 4. ThT assay on 3.25 μM hIAPP. (A) 3.25 μM hIAPP was incubated in 10 mM phosphate buffer, pH 7.4, 1.0% HFIP, in the presence or in the absence of 30 μM oleuropein aglycon, at 25°C. Aliquots were periodically withdrawn from the reaction mixtures and analysed by the ThT binding assay. The interference 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 1×, 3× or 9× molar concentration of oleuropein, then the ThT assay was performed. All experiments were carried out at least five times, and S.D. is reported.

between hIAPP incubated in the absence or in the presence of oleuropein (Fig. 6). 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 24 h of incubation (Fig. 5B–C), and the statistical analysis of data coming from the ThT assay (Fig. 6) 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. 5). This evidence suggests that oleuropein does not inhibit the amyloid aggregation of hIAPP, but drives the process through an alternative pathway.

Table 1
Tukey-Kramer analysis of results coming from the ThT assay

	0	1 h	2 h	4 h	24 h
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	**

Data coming from the ThT assay reported in Fig. 4A were statistically analysed with the Tukey-Kramer multiple comparisons test. The columns refer to the times at which the hIAPP incubation mixtures were assayed. NS, not significant.

* $P<.5$.

** $P<.1$.

*** $P<.01$.

3.3.3. Electron microscopy analysis

To further support the above hypothesis, the aggregates obtained from hIAPP incubated for 0.5, 3 and 24 h in the presence or in the absence of oleuropein were observed with an electron microscope (Fig. 7). 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 3 h 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

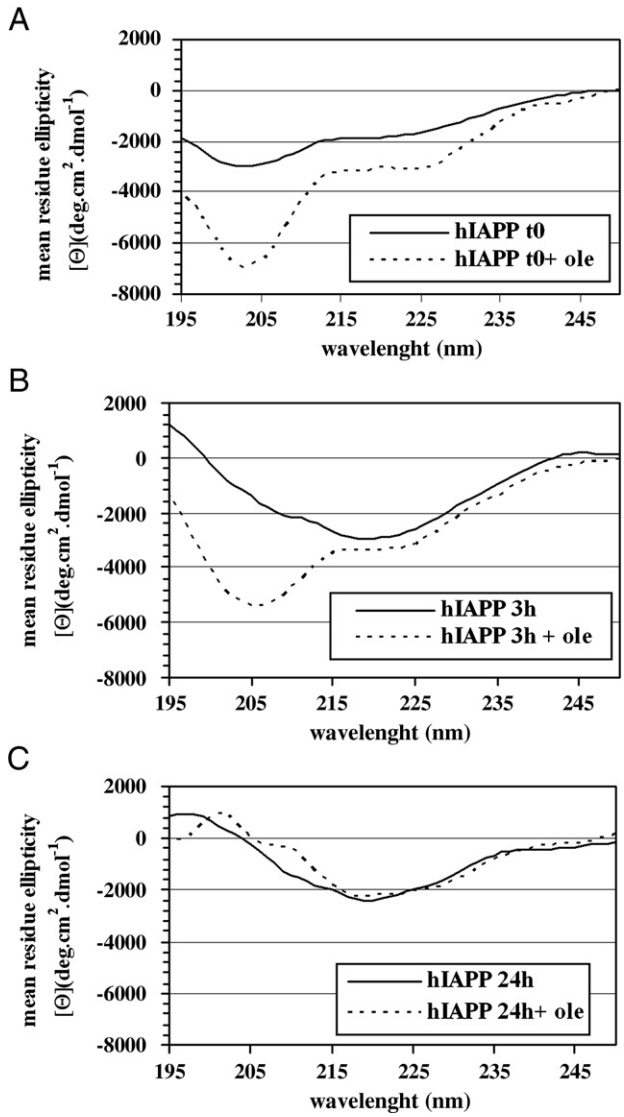

Fig. 5. CD analysis. 6.5 μM hIAPP was incubated in 10 mM phosphate buffer, pH 7.4, containing 1.0% HFIP, at 25°C in the absence or in the presence of 50 μM oleuropein aglycon. The CD spectra of the mixtures were acquired in the far UV region immediately (A), after 3 h (B) or after 24 h (C) of incubation. This is a representative experiment out of three giving qualitatively identical results.

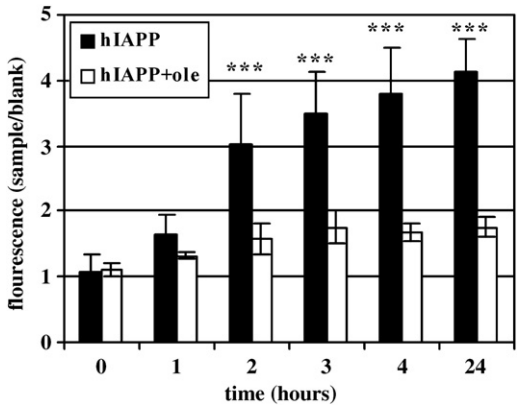

Fig. 6. ThT assay on 6.5 μM hIAPP; 6.5 μM hIAPP was incubated in the absence or in the presence of 50 μM oleuropein exactly as for the CD analysis. Fluorescence emission values at 485 nm were normalized with respect to the fluorescence of buffer containing all supplements except hIAPP. In this case, 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. ***$P<.01$.

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.

4. Discussion

In this article, we demonstrate that the aglycon of oleuropein, the main polyphenol found in the Mediterranean extra virgin olive oil, substantially reduces the cytotoxicity of hIAPP aggregates to RIN-5F pancreatic β cells (Fig. 1). 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 (Fig. 2) and to induce membrane damage (Fig. 3). 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 hIAPP 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 (90×, see Fig. 1C) than that resulting in hIAPP aggregate cytotoxicity suppression (9×, see Fig. 1A, B) is needed.

The ThT assay indicated a significant reduction of ThT fluorescence when hIAPP was incubated with oleuropein, suggesting impaired amyloid aggregation (Figs. 4 and 6). On the other hand, the CD and electron microscopy (EM) analysis showed that oleuropein did not suppress the formation of hIAPP amyloid fibrils (Fig. 5, 7). Overall, our data show that oleuropein 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. 5) and the appearance of the latter as bulky deposits (Fig. 7) that do not interact with the cells (Fig. 2), confirm that oleuropein 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 [31,32], often associated into bundles. These "superstructures" are not ThT positive (see the 24 h point in Fig. 6) 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 the most toxic species are the oligomeric pre-fibrillar aggregates preceding the formation of stable, and substantially harmless, amyloid fibrils [1,9].

The protection given by oleuropein against the appearance of early toxic hIAPP assemblies is of particular interest; in fact, other inhibitors of hIAPP aggregation were previously identified, however, they did not protect the cells against hIAPP toxicity. For example, rifampicin prevents hIAPP fibrillization; however, it does not inhibit the formation of toxic oligomers but merely interferes with mature fibril growth [21]. 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 [26]. On the contrary, oleuropein was not toxic to RIN-5F pancreatic cells not only in the concentration range (90–270 nM) we used but also up to 100 μM (data not shown).

In a recent review [33], it was suggested that the protective effect of several polyphenols against amyloid cytotoxicity comes from their physical association with aggregation-prone proteins, rather than from their antioxidant activity. Most polyphenols are effective as antioxidants in the 5–50 μM concentration range [33], and the EC_{50} for oleuropein in the 3,3-diphenyl-1-picrylhydrazyl radical scavenging test was shown to be 36.3 μM [34], which is over two orders of magnitude greater than the oleuropein effective concentration in our MTT assays (270 nM). Its efficacy in inhibiting hIAPP aggregation is comparable to those reported for other polyphenols: Porat et al. [26] calculated for phenol red an IC_{50} of 1.0 μM when used during the aggregation of 5.0 μM hIAPP and Mishra et al. [35] estimated an IC_{50} of 3.3 μM for resveratrol by using 10 μM hIAPP. From our ThT assays, we estimated an IC_{50} of 1.0 μM for the oleuropein aglycon using 3.25 μM hIAPP. 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 [19,20]. 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. Previous research claimed the importance of aromatic interactions in hIAPP fibrillization, possibly related to β-sheet stacking [25,26]. 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 [36]. Whatever the case, oleuropein might interfere with either aromatic-aromatic or hydrophobic interactions by masking exposed hydrophobic groups.

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.

A few studies concerning the absorption and bioavailability of the oleuropein aglycon demonstrated that it is stable in the gastric juice

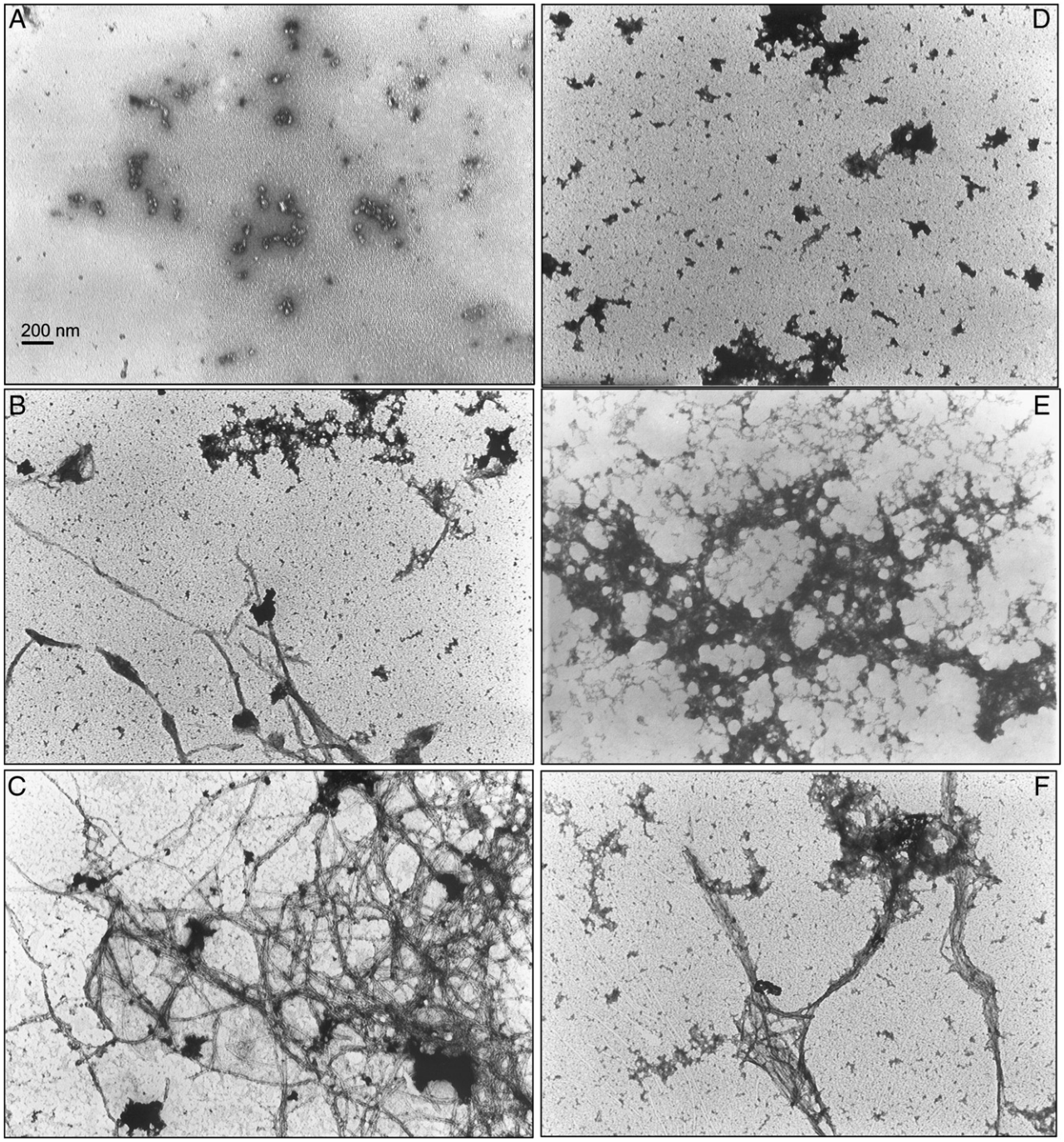

Fig. 7. EM analysis. hIAPP was incubated in the absence (Panels A, B, C) or in the presence (Panels D, E, F) of oleuropein as for CD analysis and analyzed after 30 min (A and D) 3 h (B and E) and 24 h (C and F). Magnification: 25.000×.

and is hydrolysed to hydroxytyrosol before urinary excretion; however, it is not clear whether this happens before or after absorption [37,38]. 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 degraded by the colonic microflora [38]. 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 non-polar 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 Type II diabetes, 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 to prevent or to slow down the progression of Type II diabetes.

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References

[1] Stefani M, Dobson CM. Protein aggregation and aggregate toxicity: new insights into protein folding, misfolding diseases and biological evolution. J Mol Med 2003; 81:678–99.
[2] Ludvik B, Kautzky-Willer A, Prager R, Thomaseth K, Pacini G. Amylin: history and overview. Diabet Med 1997;14(Suppl 2):S9–S13.
[3] Padrick SB, Miranker AD. Islet amyloid: phase partitioning and secondary nucleation are central to the mechanism of fibrillogenesis. Biochemistry 2002; 41:4694–703.
[4] Rocken C, Linke RP, Saeger W. Immunohistology of islet amyloid polypeptide in diabetes mellitus: semi-quantitative studies in a post-mortem series. Virchows Arch A Pathol Anat Histopathol 1992;421:339–44.
[5] Matveyenko AV, Butler PC. β-cell deficit due to increased apoptosis in the human Islet Amyloid Polypeptide Transgenic (HIP) rat recapitulates the metabolic defects present in Type 2 Diabetes. Diabetes 2006;55:2106–14.
[6] Höppener JWM, Lips CJ. Role of Islet amyloid in Type 2 diabetes mellitus. Int J Biochem Cell Biol 2006;38:726–36.
[7] Ritzel RA, Butler PC. Replication increases beta-cell vulnerability to human islet amyloid polypeptide-induced apoptosis. Diabetes 2003;52:1701–8.
[8] Chiti F, Dobson CM. Protein misfolding, functional amyloid, and human disease. Annu Rev Biochem 2006;75:333–66.
[9] Haataja L, Gurlo T, Huang J, Butler PC. Islet amyloid in Type 2 diabetes, and the toxic oligomer hypothesis. Endocrin Rev 2008;29:303–16.
[10] Bucciantini M, Calloni G, Chiti F, Formigli L, Nosi D, Dobson CM, et al. Pre-fibrillar amyloid protein aggregates share common features of cytotoxicity. J Biol Chem 2004;279:31374–82.
[11] Butterfield AD. Amyloid β-peptide (1-42)-induced oxidative stress and neurotoxicity: implications for neurodegeneration in Alzheimer's disease brain. A review. Free Radic Res 2002;36:1307–13.
[12] Soler-Rivas C, Espin JC, Wichers HJ. Oleuropein and related compounds. J Sci Food Agric 2000;80:1013–23.
[13] Visioli F, Poli A, Galli C. Antioxidant and other biological activities of phenols from olives and olive oil. Med Res Rev 2002;22:65–75.
[14] Visioli F, Bellosta S, Galli C. Oleuropein, the bitter principles of olives, enhances nitric oxide production by mouse macrophages. Life Sci 1998;62:541–6.
[15] Carluccio MA, Siculella L, Ancora MA, Massaro M, Scoditti E, Storelli C, et al. Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: antiatherogenic properties of mediterranean diet phytochemicals. Arterioscler Thromb Vasc Biol 2003;23:622–9.
[16] Owen RW, Giacosa A, Hull WE, Haubner R, Würtele G, Spiegelhalder B, et al. Olive oil consumption and health: the possible role of antioxidants. Lancet Oncol 2000; 1:107–12.
[17] Andreadou I, Sigala F, Iliodromitis EK, Papaefthimiou M, Sigalas C, Aligiannis N, et al. Acute doxorubicin cardiotoxicity is successfully treated with the phytochemical oleuropein through suppression of oxidative and nitrosative stress. J Mol Cell Cardiol 2007;42:549–58.
[18] Andreadou I, Iliodromitis EK, Mikros E, Constantinou M, Agalias A, Magiatis P, et al. The olive constituent oleuropein exhibits anti-ischemic, antioxidative, and hypolipidemic effects in anesthetized rabbits. J Nutr 2006;136:2213–9.
[19] Bazoti FN, Bergquist J, Markides KE, Tsarbopoulos A. Noncovalent interaction between amyloid-β-peptide (1-40) and oleuropein studied by electrospray ionization mass spectrometry. J Am Soc Mass Spectrom 2006;17:568–75.
[20] Bazoti FN, Bergquist J, Markides K, Tsarbopoulos A. Localization of the noncovalent binding site between amyloid-β-peptide and oleuropein using electrospray ionization FT-ICR mass spectrometry. J Am Soc Mass Spectrom 2008;19:1078–85.
[21] Meier JJ, Kayed R, Lin CY, Gurlo T, Haataja L, Jayasinghe SJ, et al. Inhibition of human IAPP fibril formation does not prevent β-cell death: evidence for distinct actions of oligomers and fibrils of human IAPP. Am J Physiol Endocrinol Metab 2006;291: 1317–24.
[22] Yan LM, Tatarek-Nossol M, Velkova A, Kazantzis A, Kapurniotu A. Design of a mimic of nonamyloidogenic and bioactive human islet amyloid polypeptide (IAPP) as nanomolar affinity inhibitor of IAPP cytotoxic fibrillogenesis. Proc Natl Acad Sci U S A 2006;103:2046–51.
[23] Konno K, Hirayama C, Yasui H, Nakamura M. Enzymatic activation of oleuropein: a protein crosslinker used as chemical defence by privet tree. Proc Natl Acad Sci U S A 1999;96:9159–62.
[24] Bucciantini M, Giannoni E, Chiti F, Baroni F, Formigli L, Zurdo J, et al. Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. Nature 2002;416:507–11.
[25] Kayed R, Bernhagen J, Greenfield N, Sweimeh KM, Brunner H, Voelter W, et al. Conformational transitions of islet amyloid polypeptide (IAPP) in amyloid formation in vitro. J Mol Biol 1999;287:781–96.
[26] Porat Y, Mazor Y, Efrat S, Gazit E. Inhibition of islet amyloid polypeptide fibril formation: a potential role for heteroaromatic interactions. Biochemistry 2004; 43:14454–62.
[27] Bucciantini M, Rigacci S, Berti A, Pieri L, Cecchi C, Nosi D, et al. Patterns of cell death triggered in two different cell lines by HypF-N pre-fibrillar aggregates. FASEB J 2005;19:437–49.
[28] Engel MFM, Khemtémourian L, Kleijer CC, Meeldijk HJD, Jacobs J, Verkleij AJ, et al. Membrane damage by human islet amyloid polypeptide through fibril growth at the membrane. Proc Natl Acad Sci U S A 2008;105:6033–48.
[29] LeVine III H. Quantification of beta-sheet amyloid fibril structures with Thioflavine T. Methods Enzymol 1999;309:274–84.
[30] Khemtémourian L, Killian JA, Höppener JWM, Engel MFM. Recent insight in Islet amyloid polypeptide-induced membrane disruption and its role in β-cell death in Type 2 diabetes mellitus. Exp Diabetes Res 2008 Article ID 421287.
[31] Cerdà-Costa N, Esteras-Chopo A, Aviles FX, Serrano L, Villegas V. Early kinetics of amyloid fibril formation reveals conformational reorganisation of initial aggregates. J Mol Biol 2007;366:1351–3.
[32] Rigacci S, Bucciantini M, Relini A, Pesce A, Gliozzi A, Berti A, et al. The (1-63) region of the p53 transactivation domain aggregates in vitro into cytotoxic amyloid assemblies. Biophys J 2008;94:3635–46.
[33] Porat Y, Abramowitz A, Gazit E. Inhibition of amyloid fibril formation by polyphenols: structural similarity and aromatic interactions as a common inhibition mechanism. Chem Biol Drug Des 2006;67:27–37.
[34] Visioli F, Bellomo G, Galli C. Free radical-scavenging properties of olive oil polyphenols. Biochem Biophys Res Commun 1998;247:60–4.
[35] Mishra R, Sellin D, Radovan D, Gohlke A, Winter R. Inhibiting islet amyloid polypeptide fibril formation by the red wine compound resveratrol. Chembiochem 2009;10:445–9.
[36] Tracz SM, Abedini A, Driscoll M, Raleigh DP. Role of aromatic interactions in amyloid formation by peptides derived from human amylin. Biochemistry 2004; 43:15901–8.
[37] Vissers MN, Zock PL, Roodengburg AJC, Leenen R, Katan MB. Olive oil phenols are adsorbed in humans. J Nutr 2002;132:409–17.
[38] Corona G, Tzounis X, Dessì MA, Deiana M, Debnam ES, Visioli F, et al. The fate of olive oil polyphenols in the gastrointestinal tract: implications of gastric and colonic microflora-dependent biotransformation. Free Radic Res 2006;40:647–58.