Inhibition of platelet aggregation by olive oil phenols via cAMP-phosphodiesterase

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The aim of the present study was to confirm that olive oil phenols reduce human platelet aggregability and to verify the hypothesis that cAMP- and cGMP phosphodiesterases (PDE) could be one of the targets of the biological effect. Four extracts from oils characterized by a high phenol content (HPE), and low phenol levels (LPE) were prepared and analyzed qualitatively and quantitatively by HPLC-UV and electrospray ionization–MS/MS. Human washed platelets stimulated with thrombin were used for the aggregation assay. Human platelet cAMP-PDE and recombinant PDE5A1 were used as enzyme source. Platelet aggregation and enzyme activity were assayed in the presence of HPE, LPE and individual phenols. The phenol content of HPE ranged between 250 and 500 mg/kg, whereas the LPE content was 46 mg/kg. The compounds identified were hydroxytyrosol (HT), tyrosol (TY), oleuropein aglycone (OleA) and the flavonoids quercetin (QU), luteolin (LU) and apigenin (AP). OleA was the most abundant phenol (range 23.3 to 37.7 %) and LU was the most abundant flavonoid in the extracts. Oil extracts inhibited platelet aggregation with an 50% inhibitory concentration interval of 1.23–11.2 μg/ml. The inhibitory effect of individual compounds (10 μM) including homovanillyl alcohol (HVA) followed this order: OleA > LU > HT = TY = QU = HVA, while AP was inactive. All the extracts inhibited cAMP-PDE, while no significant inhibition of PDE5A1 (50 μg/ml) was observed. All the flavonoids and OleA inhibited cAMP-PDE, whereas HT, TY, HVA (100 μM) were inactive. Olive oil extracts and part of its phenolic constituents inhibit platelet aggregation; cAMP-PDE inhibition is one mechanism through which olive oil phenols inhibit platelet aggregation.

Olive oil extracts: Platelet aggregation: Flavonoids: Phosphodiesterases: Phenols

A Mediterranean-style diet, where olive oil is the main source of fat, has been associated with a reduced risk of CVD. Health benefits of olive oil have been in part attributed to minor phenol components, whose composition varies qualitatively and quantitatively depending on several factors such as the cultivar, stage of fruit ripeness and region of cultivation. In addition, the agronomic conditions and the process of oil extraction strongly affect the quality of olive oil and its content of phenols.

The type of phenols in olive oil include flavonoids, in particular luteolin (LU) and apigenin (AP), and secoiridoids oleuropein aglycone (OleA), ligstroside aglycone and their hydrolysis products hydroxytyrosol (HT), and tyrosol (TY), respectively. Olive oil phenols possess antioxidant properties and influence many biological activities that may, at least partially, account for the observed effects of olive oil on the cardiovascular system. Some of these include: (1) inhibition of LDL oxidation, (2) production of nitric oxide, (3) the down regulation of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression in endothelial cells in the presence of olive oil phenols. Platelet aggregation, which often accompanies and aggravates CVD, was inhibited by olive oil phenols, although this remains somewhat controversial.

Abbreviations: AP, apigenin; ESI, electrospray ionisation; HPE, high phenol extract; HT, hydroxytyrosol; HVA, homovanillyl alcohol; IC50, concentration that reduces the effect by 50%; LPE, low phenol extract; LU, luteolin; OleA, oleuropein aglycone; PDE, phosphodiesterase; QU, quercetin; TY, tyrosol.

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involved in the modulation of the platelet activity remains unclear. PDE inhibition has been speculated as one factor in this regard, but there are no supporting data thus far.

In this paper we describe the effects of phenolic extracts obtained from five commercially available olive oils on aggregation of human platelets and on cAMP- and cGMP-PDE activities. The olive oils were chosen on the basis of their phenol content: four extracts (extracts A–D) were obtained from oils characterized by high phenol content (high phenol extract, HPE), while the fifth extract (extract E) was obtained from an olive oil with low phenol levels (low phenol extract, LPE). Each extract was analyzed qualitatively and quantitatively by HPLC-UV and electrospray ionization (ESI)–MS/MS in order to verify whether compositional differences affected biological activity.

Experimental methods

Reagents
Culture medium Dulbecco’s modified Eagle’s medium, trypsin, protease inhibitors, and all chemical reagents for cell culture were purchased from Sigma Aldrich (Milan, Italy). Penicillin, streptomycin, and L-glutamine were from Gibco (Grand Island, NY, USA); foetal calf serum was provided by Mscia Brunelli S.p.A. (Milan, Italy). COS-7 cell line was purchased from ATCC (Manassas, VA, USA). Superfect reagent for transient transfections was obtained from Qiagen GmbH (Hilden, Germany). The expression plasmid pcDNA3 containing the full-length cDNA of PDE5A1 was a kind gift of Prof. C. S. Lin (Department of Urology, University of California, San Francisco, CA). [3H]cGMP and [3H]cAMP were from Amersham Pharmacia Biotech (Amer- sham Place, Little Chalfont, Buckinghamshire, UK). DEAE-Sephadex A25 was from Pharmacia (Uppsala, Sweden), cGMP, cAMP, AMP, Crotaulus adamanteus snake venom, quercetin (QU), aminophylline, phloretin, homovanillyl alcohol (HVA) and TY were purchased from Sigma Aldrich. Sildenafil was provided by Sequoia Research Products (Oxford, UK). AP and LU were purchased from Extrasythése (Lyon, France). OleA was obtained from oleuropein glucoside (Extrasythése, Lyon, France) by enzymatic digestion, and the purity (99 %) was confirmed by both TLC and ESI–MS analysis. HT was from Cayman Chemical Company (Tallinn, Estonia). All compounds used for the analytical determinations and for the biological assays were of HPLC purity grade.

Preparation and quantification of HPE and LPE total phenols
Extracts A–E, obtained from olive oils available from various drugstores, were prepared according to the method of Montedoro et al. with minor modifications. Briefly, 100 g olive oil was delipidized with hexane (100 ml) and extracted twice with methanol–water (80:20 v/v; 100 ml) for 20 min on a mechanical shaker. The collected methanolic phases were then taken to dryness under N2 and the extracts stored at –20 °C until analysis. Spectrophotometric analyses of total phenols reactive to Folin-Ciocalteu, expressed as oleuropein equivalents, were carried out as described (Fig. 1).

Evaluation of HPE and LPE by HPLC-UV, GC-MS and ESI–MS/MS
HPLC-UV analyses were performed on a JASCO instrument PU 980 using a LiChroCart® 4.0 × 250 mm (5 µm) HPLC-Cartridge Lichrosorb® RP-18 column (Merck, Darmstadt, Germany) at flow rate 1 ml/min. The UV-VIS detector (Jasco mod 875-UV; Jasco Europe, Italy) was set at 278 nm. A gradient elution was performed using water acidified with 2 % acetic acid (A) and methanol (B). The gradient program was: 0–5 min, 90 % (A); 5–14 min, from 90 % to 80 % (A); 14–15 min, 80 % (A); 15–20 min, from 80 % to 60 % (A); 20–30 min, from 60 % to 50 % (A); 30–50 min, from 50 % to 0 % (A); 50–60 min, 0 % (A); 60–65 min, from 0 % to 90 % (A). Aliquots of HPE and LPE were dissolved in ethanol. OleA, HT, AP, QU, and LU were identified by comparison of the retention times and mass spectra with those of authentic standards. In the case of unresolved peaks, identification was obtained by co-injection of the extract with the authentic standards.

Quantitative determination of the single phenols was carried out by ESI–MS/MS using a linear ion-trap mass spectrometer (LTQ; Thermo Finnigan, USA) equipped with an ESI source operating in the negative mode. Hydroxytyrosol (HT), and tyrosol (TY), were quantified by GC–MS using deuterium-labelled compounds as internal standards. Results represent the mean and SD of at least three injections. Results were determined as phenols reactive to Folin-Ciocalteu, expressed as oleuropein equivalents. * Not detectable.
Calibration curves were prepared with phloretin (100 ng/sample), as internal standard, and increasing amounts of the authentic phenols (0–200 ng for AP and QU, 0–500 ng for LU); for the determination of OleA, samples for the calibration curve contained 0–100 µg OleA and 100 µg phloretin. Each sample was then extracted and analysed, as already described. Quantification was performed by monitoring ions derived from the collision of the [M-H]⁺ ion of each compound. One or more specific product ions were selected for each compound and used for quantitative analysis (Table 1).

HT and TY were quantified by GC–MS (GCQ, Thermoquest) using HT deuterium-labelled as internal standard 21.

Table 1. Precursor and product ions selected for ESI–MS/MS analysis. Quantification was performed by monitoring ions derived from the collision of the [M-H]⁺ ion of each compound. One or more specific product ions were selected for each compound and used for quantitative analysis.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor ion (m/z) [M-H]⁺</th>
<th>Product ion (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OleA</td>
<td>377</td>
<td>307</td>
</tr>
<tr>
<td>AP</td>
<td>269</td>
<td>148–150, 182, 201, 225</td>
</tr>
<tr>
<td>LU</td>
<td>286</td>
<td>151, 175, 199, 241, 285</td>
</tr>
<tr>
<td>QU</td>
<td>301</td>
<td>179</td>
</tr>
<tr>
<td>Phloretin</td>
<td>273</td>
<td>167</td>
</tr>
</tbody>
</table>

OleA, oleuropein aglycone; AP, apigenin; LU, luteolin; QU, quercetin.

Preparation of washed platelets for aggregation
Venous blood from healthy volunteers was collected with sodium citrate 3·8 % (9:1 v/v) and centrifuged at 160 g for 10 min. The supernatant was centrifuged at 600 g for 6 min and the pellet solubilized in a buffer containing Tris-HCl 15·4 mM, EDTA 2·2 mM, glucose 5·5 mM, NaCl 154 mM, pH 7·4. After centrifugation at 600 g for 20 min, pellet was solubilized in Tyrode-HEPES buffer (NaCl 137 mM, KCl 2·68 mM, NaHCO₃ 11·9 mM, NaH₂PO₄ 0·416 mM, MgCl₂ 1 mM, dextrose 0·55 mM, HEPES 5 mM, pH 7·35). Platelet number was adjusted to 1×10⁸ platelets/ml with buffer. All the procedure was performed at room temperature.

Aggregation was carried out in an Elvi 840 aggregometer (Elvi Logos, Milano, Italy). Washed platelets (250 µl) were pre-incubated at 37°C, stirring at 1000 rpm for 10 min in the presence or in the absence of the compounds to be tested, and stimulated with thrombin 10U/ml (Sigma, Milano, Italy). The reaction lasted 5 min. Oil extracts were tested in the concentration range of 0·1–20 µg/ml, and single compounds were tested at 10 µM. The concentration of the vehicle in the control and treated samples was 0·1 %.

Platelet homogenate preparation and assay for cAMP-PDE activity
The blood fraction enriched in platelets, obtained from blood of healthy volunteers, was submitted to two centrifugations at 160 g for 10 min at room temperature. The pellet was removed and platelet-rich plasma was centrifuged at 1000 g for 15 min. The resulting pellet was resuspended in 10 mM Tris-HCl, pH 7·4 (2/5 of the initial volume). The suspension was centrifuged at 1000 g for 15 min and the pellet suspended in the Tris-HCl buffer, pH 7·4 (1/12 of the initial volume). All these steps were performed at 4°C. Cells were disrupted by freezing and thawing three times obtaining the homogenate 22, and cell lysates were stored at –80°C. Total protein concentration was measured according to Bradford 22.

cAMP-PDE activity was determined according to the method of Kincaid & Manganiello 23 with minor modifications. Platelet lysate (64 µg protein/ml) was incubated with 0·5 µM-cAMP and 63 nCi [³H]cAMP suspended in 30 mM Tris-HCl, pH 7·4, 4 mM MgCl₂; final reaction volume was 250 µl. After 5 min incubation at 30°C, the reaction was stopped with 0·1 M HCl. Samples were then incubated for a further 4 min at 70°C with AMP 5 mM and cAMP 5 mM, and the pH adjusted to 7 on ice with 0·1 M NaOH. Samples were then incubated for 20 min at 37°C with 50 µl nucleosidase from Crotalus adamanteus snake venom (1 mg/ml in Tris-HCl 0·1 M, pH 8·0) to cleave AMP to the corresponding nucleoside. The reaction was stopped with 50 µl 200 mM NaEDTA, containing 5 mM adenosine. The nucleoside formed during the incubation was separated from the unreacted substrate by dietylamino ethanol–Sephadex A25 column chromatography. The eluted [³H]adenosine was counted in a β-scintillation counter. cAMP-PDE activity was expressed as picomoles of product formed per minute per milligram of protein. HPE and LPE were tested at 1–100 µg/ml, while concentration–response curves of the single compounds ranged between 0·1 and 25 µM. Concentrations reducing the effect by 50 % (IC₅₀) were calculated from the sigmoidal curves using Graph Pad Prism 4. Inhibition (%) by aminophylline 100 µM used as reference compound was 74·5 (SD 1·30; n 11). Each result is the mean and standard deviation of two experiments in triplicate.

Expression and enzyme assay of human recombinant PDE5A1
Human recombinant PDE5A1 was prepared by expression of the full-length cDNA of PDE5A1 into COS-7 cells as previously described 25. PDE5A1 activity was determined according to the method of Kincaid & Manganiello 24 with minor modifications 26. The extracts were assayed at 50 µg/ml, expressed as total phenol content, whereas the pure compounds were tested at 50 µM. The PDE-5 inhibitor sildenafil used as reference compound showed 70 % inhibition at 100 nm.

Results
Phenol content of HPE and LPE
The total phenol content of HPE ranged between 250 and 500 mg/kg as expected, whereas the LPE content was 46 mg/kg (Fig. 1). The compounds identified were the phenolic alcohols HT and TY, OleA and the flavonoids QU, LU, and AP. LPE showed a qualitatively, but not quantitatively, similar profile. The results of the quantitative determination of the identified compounds are shown in Fig. 1. OleA was the most abundant phenol present in the extracts (range 23·3 to 37·7 %) and LU was the most abundant flavonoid in all the extracts analyzed. LPE showed the lowest amount of OleA and LU, while AP and QU were undetectable.
Effect of oil extracts and single phenols on platelet aggregation and PDE activities

In order to determine whether oil phenols inhibit platelet aggregation, human washed platelets were first stimulated with thrombin in the presence of oil extracts. Under these conditions the oil extracts inhibited platelet aggregation in a concentration-dependent fashion (Fig. 2), with an IC₅₀ interval of 1.23–11.2 µg/ml, (Table 2). When the individual compounds were tested similarly, the inhibitory effect obtained at 10 µM followed this order: OleA > LU > HT = TY = QU = HVA, while AP was inactive.

Since it is known that reduced platelet aggregation is associated with elevated levels of cAMP, the next step was to verify the ability of oil phenols to inhibit cAMP-PDE and cGMP-PDE-5. All the extracts inhibited cAMP-PDE in human platelet lysates, again in a concentration-dependent manner (Fig. 3). The observed IC₅₀ in this instance ranged from 14.2 to 33.8 µg/ml for HPE, and was 28.2 (SD 2.5) µg/ml for LPE (Table 2). On the other hand, no significant inhibition of PDE5A1 occurred at concentration as high as 50 µg/ml (data not shown).

All individual compounds were tested on cAMP-PDE, as well. HVA, although absent in oil extracts, was included in these studies since it is the main HT metabolite in human subjects. All the flavonoids showed a concentration-dependent inhibition of cAMP-PDE (Fig. 4). The IC₅₀ are reported in Table 2. Among the non-flavonoid constituents tested, only OleA showed a concentration-dependent inhibition, although at higher concentrations (Fig. 4, Table 2), whereas HT, TY, and HVA were inactive up to 100 µM.

In order to verify whether the lack of inhibition of PDE5A1 by oil extracts could be due to the inability of the identified constituents to inhibit the enzyme, PDE5A1 was assayed in the presence of AP, LU, QU, OleA, HT and TY at 50 µM. The inhibition by AP, LU and QU of PDE5A1 was 21.5 (SD 5.7), 50.9 (SD 2.2) and 44.1 (SD 8.1) %, respectively lower in comparison with the inhibition of cAMP-PDE. OleA had a rather moderate effect: 9.3 (SD 2.7) % inhibition at 50 µM and TY was inactive. While HT inhibited PDE5A1 activity in a concentration-dependent fashion (IC₅₀ 40.6 (SD 7.3) µM), it was without effect on cAMP-PDE.

Discussion

In the present study our objective was to confirm that various olive oil phenols reduce human platelet aggregability and to verify the hypothesis that the cAMP and cGMP dependent PDE could be one of the targets of the biological effect. For this purpose five commercially available olive oils were processed to obtain extracts with differing phenol contents, both qualitatively and quantitatively. The phenol content in four oils (HPE) ranged between 250 and 500 mg/kg in agreement with the previously reported data. In LPE, phenol content was from 5- to 10-fold lower than that of HPE; most likely due to the chemical treatment required to reduce the exceedingly high acidity of this olive oil. HPLC–UV analysis of the extracts showed a complex profile and some peaks could be clearly identified as HT, TY, OleA, AP, QU, and LU, and quantified. Cultivar, ripening stage, and production technologies (used to obtain olive oils) could explain the observed levels and compositional differences.

This report shows that olive oil extracts and part of their phenolic constituents inhibit platelet aggregation. Our results disagree with those obtained by Turner et al., which reported no effect by olive oil phenols on collagen-challenged platelet in blood, thus in assay conditions differing from ours. The concentration of the identified compounds in oil extracts, and the extent to which they inhibit platelet aggregation are not consistent with the inhibitory activity shown by the whole extracts. It is thus likely that HPE contain unidentified constituents with anti-aggregating activity. These could include isochromans, a new class of compounds recently described to occur in olive oil which were shown to inhibit human platelet reactivity.

According to Petroni et al., inhibition of platelet aggregation is thought to be mediated by a decrease in eicosanoid production caused by phenols. In addition to affecting the arachidonic acid cascade, our data show that the mechanism of action could also be through a degradation of cAMP brought about by phenols, since HPE and LPE inhibited cAMP-PDE. Conversely cGMP-PDE5 seems to be less or not affected at all by olive oil phenols when tested as a mixture. AP, LU and QU, OleA and HT, also inhibited PDE5A1, however their concentration in the phenol mixture was insufficient to cause enzyme inhibition. QU, AP, LU and OleA contributed differently to the overall effect of the extract on cAMP-PDE, while HT and TY, the in vivo metabolites of OleA and ligrostide aglycone respectively, were inactive. Taking into consideration the level of each compound (as % of the total phenols) and the IC₅₀ of each phenol, LU would be the major component to inhibit platelet aggregation among the identified compounds. Other components of olive oil, still of unknown nature, could play a role in this process as well. A possible explanation for the lower inhibitory activity of HPE-D is the likely presence of compounds that may counteract the
effects of the active principles. The activity of LPE is lower with respect to HPE A–C, but the degree of inhibition is consistent with the low levels of LU and OleA, and with the absence of AP. Thus the composition and the quantity of phenols in oil extracts affect the degree of inhibition of cAMP-PDE.

The in vivo significance of the results presented here with regard to inhibition of platelet aggregation by olive oil remains to be established. Quite limited data are available on the bioavailability of HT and TY30,32, and only one study33 addressed the question of evaluating the urinary levels of these two compounds after olive oil intake. According to those studies OleA, HT, and TY are adsorbed, extensively modified in the body, and HT and TY are present in plasma only in the conjugated forms28. The maximum concentration of flavonoids in human plasma rarely exceeds 1 μM34 but bioavailability studies on flavonoids were performed after intake of non-oil matrices. When HT was administered as a natural component of olive oil, its urinary excretion in human subjects was higher than after its addition to refined olive oil or yoghurt35. Similarly flavonoids might be more bio-available after olive oil intake.

Finally, since conjugated HT could not be assessed for platelet aggregation due to its current unavailability, the effect of olive oil phenols should be attributed to components of still-unknown structure.

Consumption of olive oil has long been considered to be healthy, particularly with regard to its effects on the cardiovascular system. The role of platelets is increasingly well defined in coronary artery disease and platelet aggregation

| Table 2. Effects of olive oil extracts and single phenols on platelet aggregation and cAMP-PDE activity. Individual compounds were tested on platelet aggregation at 10 μM (Mean values and standard deviations) |
|-----------------------------------------------|------------------|------------------|
| **Platelet aggregation IC₅₀ (μg/ml)**       | **cAMP-PDE IC₅₀ (μg/ml)** |
| **Compound**                               | **Mean** | **SD** | **Mean** | **SD** |
| Olive oil extract                          |          |      |          |      |
| HPE-A                                      | 1·23     | 0·24 | 14·2     | 0·3  |
| HPE-B                                      | 6·54     | 0·23 | 15·4     | 0·4  |
| HPE-C                                      | 6·83     | 0·53 | 15·9     | 0·8  |
| HPE-D                                      | 7·38     | 0·69 | 23·8*    | 2·7  |
| LPE                                        | 11·17    | 0·68 | 28·2*    | 2·5  |
| **Inhibition (%) IC₅₀ (μg/ml)**             |          |      |          |      |
| Phenol                                     |          |      |          |      |
| OleA                                       | 75·4     | 4·5  | 89·0     | 4·3  |
| LU                                         | 23·3     | 0·7  | 1·3      | 0·1  |
| AP                                         | Inactive |      | 4·1      | 0·9  |
| QU                                         | 7·5      | 2·9  | 1·5      | 0·3  |
| HT                                         | 11·4     | 3·4  | Inactive at 100 μM |
| TY                                         | 10·2     | 2·5  | Inactive at 100 μM |
| HVA                                        | 6·8      | 4·4  | Inactive at 100 μM |

PDE, phosphodiesterase; HPE, high phenol extract; LPE, low phenol extract; OleA, oleuropein aglycone; LU, luteolin; AP, apigenin; QU, quercetin; HT, hydroxytyrosol; TY, tyrosol; HVA, homovanillyl alcohol.

*Significantly different v. A–C.
is shown to be one underlying mechanism in its pathogen-
ecis. Phenolic components of commonly consumed foods, in
Mediterranean countries at least, such as red wine and
olive oil rich in these constituents, are shown to inhibit pla-
telet aggregation thus potentially reducing the putative risk
of vascular diseases. Data presented here show one mechan-
ism, cAMP-PDE inhibition, by which olive oil phenols inhib-
itate platelet aggregation.

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