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 CVD1,2. 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 phenols3.

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 oxidation4–5,(2) production of nitric oxide6 and (3) the down regulation of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression in endothelial cells7,8 in the presence of olive oil phenols. Platelet aggregation, which often accompanies and aggravates CVD, was inhibited by olive oil phenols9,10, although this remains somewhat controversial11. Tight regulation of platelet function and platelet–vessel interaction is an essential requisite for intact vessel physiology. Platelet activation is regulated by a number of physiological activators (thromboxane A2, vasoressin, ADP, thrombin, serotonin) and inhibitors (endothelium-derived relaxing factor, prostaglandin inhibitor-2). Platelet antagonists inhibit platelet function by increasing the intracellular levels of cyclic nucleotides cAMP and cGMP through the activation of the respective cyclases. Cyclic nucleotide levels are down regulated by degradation through phosphodiesterases (PDE). Platelets contain mainly PDE3, which preferentially hydrolyzes cAMP as substrate, and PDE5 which uses preferentially cGMP as substrate12. PDE3 is inhibited by the binding of cGMP; therefore platelet PDE inhibition is reasonably considered a therapeutic tool to treat vascular diseases. Indeed PDE inhibitors are currently used as anti-aggregating agents and in the treatment of arterial occlusive diseases13.

Inhibition of platelet aggregation has been demonstrated for some olive oil phenols14. The exact nature of the mechanisms

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 Mascii 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 Amer sham Pharmacia Biotech (Amer sham Place, Little Chalfont, Buckinghamshire, UK). DEAE-Sephadex A25 was from Pharmacia (Uppsala, Sweden), cGMP, cAMP, AMP, Crotalus adamanteus snake venom, quercetin (QU), aminophylline, phloretin, homovanillyl alcohol (HVA) and TY were purchased from Sigma Aldrich. Sil denafil was provided by Sequoia Research Products (Oxford, UK). AP and LU were purchased from Extrasyntезe (Lyon, France). OleA was obtained from oleuropein glucoside (Extrasyntезe, 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).

**Fig. 1. Levels of phenols in olive oil extracts. A–D are extracts from oils with high phenol content (HPE), E is from oil with low phenol content (LPE).**

**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® 40 × 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: 0–3 min, 90% (A); 3–13 min, from 90% to 80% (A); 13–15 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. 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.

**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 15 min. The supernatant was centrifuged at 160 g for 15 min and the pellet suspended in 10 mM Tris-HCl, pH 7.4. After centrifugation at 600 g for 20 min, pellet was solubilized in a buffer containing Tris-HCl 15.4 mM, EDTA 2 mM, glucose 5.5 mM, NaCl 154 mM, pH 7.4. After centrifugation at 600 g for 20 min, pellet was solubilized in a buffer containing Tris-HCl 15.4 mM, EDTA 2 mM, glucose 5.5 mM, NaCl 154 mM, pH 7.4. After centrifugation at 600 g for 20 min, pellet was solubilized in a buffer containing Tris-HCl 15.4 mM, EDTA 2 mM, glucose 5.5 mM, NaCl 154 mM, pH 7.4.

**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 and the pellet suspended in the 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, and cell lysates were stored at −80°C. Total protein concentration was measured according to Bradford.

cAMP-PDE activity was determined according to the method of Kincaid & Manganelli 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 mM 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 mM NaOH. Samples were then incubated for 20 min at 37°C with 50 μl nuclease 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. PDE5A1 activity was determined according to the method of Kincaid & Manganelli with minor modifications. 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.

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**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.
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-flavonoidic 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 manner (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 ligstroside 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
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 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.

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is shown to be one underlying mechanism in its pathogenesis. 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 platelet aggregation thus potentially reducing the putative risk of vascular diseases. Data presented here show one mechanism, cAMP-PDE inhibition, by which olive oil phenols inhibit platelet aggregation.

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