Administration of Minor Polar Compound-Enriched Extra Virgin Olive Oil Decreases Platelet Aggregation and the Plasma Concentration of Reduced Homocysteine in Rats

Raffaella Priora, Domenico Summa, Simona Frosali, Antonios Margaritis, Danila Di Giuseppe, Chiara Lapucci, Francesca Ieri, Fabio M. Pulcinelli, Annalisa Romani, Flavia Franconi, and Paolo Di Simplicio

Abstract

We investigated the effect of extra virgin olive oil (EVOO) on platelet aggregation and plasma concentrations of homocysteine (Hcy) redox forms in rats in relation to the minor polar compound (MPC) concentration of EVOO. We used 3 olive oil samples with similar fatty acid but different MPC concentrations: refined olive oil (RF) with traces of MPC (control oil), native EVOO with low MPC concentration (LC), and EVOO with high MPC concentration (HC) enriching LC with its own MPC. Oil samples were administered to rats by gavage (1.25 mL/kg body weight) using 2 experimental designs: acute (24-h food deprivation and killed 1 h after EVOO administration) and subacute (12-d treatment, a daily dose of oil for 12 d, and killed after 24 h of food deprivation). Platelet aggregation was induced by ADP (ex vivo tests) and a reduction in platelet reactivity occurred in cells from rats given LC in both the acute and subacute studies, whereas LC had this effect only in the subacute experiment. Moreover, in rats administered HC in both experiments, the plasma concentration of free reduced Hcy (rHcy) was lower and Hcy bound to protein by disulfide bonds (bHcy) was greater than in RF-treated rats. bHcy was also greater in rats given LC than in RF-treated rats in the subacute experiment. Plasma free-oxidized Hcy was greater in rats given LC and HC than in those administered RF only in the subacute experiment. In conclusion, these results show that MPC in EVOO inhibit platelet aggregation and reduce the plasma rHcy concentration, effects that may be associated with cardiovascular protection.

Introduction

Epidemiological studies suggest that Mediterranean diets are associated with a reduced risk of cardiovascular diseases (CD). Compared with a saturated fat diet, the Mediterranean diet, rich in oleic acid, has been correlated with lower blood pressure and lower serum lipids (3–5). Whether the beneficial effects of extra virgin olive oil (EVOO) on the cardiovascular system are exclusively due to monounsaturated fatty acids such as oleic acid remains to be elucidated (6).

Besides monounsaturated fatty acids (mainly oleic acid) and PUFA (mainly linoleic acid), which may have healthy properties (7–10), EVOO contains sizeable amounts of minor polar compounds (MPC; prevalently phenols) and tocopherols (11), which may act as antioxidants (6,12,13) and contribute to the benefit derived from the Mediterranean diet. Epidemiological studies have shown that regular consumption of phenol-rich foods is inversely associated with CD (14); in addition, certain MPC, such as 2-(3,4-di-hydroxyphenyl)-ethanol (hydroxytyrosol), luteolin, and oleuropein, seem to inhibit platelet aggregation in vitro to different degrees (15). However, this finding is not univocal; e.g., Turner et al. (13) found that oleuropein,
tyrosol, and hydroxytyrosol did not have antiaggregating effects in whole blood when collagen was the agonist.

Several studies of the biological properties of individual MPC (6,12,15) and of the effects of EVOO itself have been performed (16). On the other hand, data concerning effects of EVOO, in relation to its MPC content, on variables linked associated with platelet function are relatively scarce. Visioli et al. (17) showed that administration of EVOO naturally rich in phenols decreased thromboxane B\textsubscript{2} (TXB\textsubscript{2}) concentrations in serum of mildly dyslipidemic patients with respect to refined olive oil (RF) with low phenol concentrations. Similar beneficial effects (TXB\textsubscript{2} decrease) were obtained after consumption of EVOO with high phenol concentrations in healthy subjects (18). Qualitative and quantitative differences in MPC (19,20) amount may therefore be responsible for different biological effects of EVOO.

There is evidence that a diet rich in polyphenols (found in coffee and black tea) can increase total homocysteine (Hcy) concentrations in plasma (21). Total Hcy represents the sum of various plasma redox forms of Hcy: reduced (rHcy), free oxidized (oxHcy), and bound to protein by disulfide bonds (bHcy) (22). High Hcy plasma concentrations may favor onset of CD (23–25), but it is unclear to what extent the putative Hcy toxicity is related to specific redox forms. In more specific studies, rHcy has been considered an appropriate marker of CD risk (26,27).

Because EVOO from different geographical areas have different phenol concentrations (11), it may be interesting to evaluate the extent to which the health benefits of EVOO consumption are related to its MPC (prevalently phenols) concentration. This study was designed to analyze the biological effect of EVOO intake, acutely or subacutely administered, on parameters linked to cardiovascular function such as platelet aggregation (ex vivo tests) and plasma concentration of Hcy redox forms (rHcy, oxHcy, and bHcy). The relation between biological activity and MPC concentration was analyzed using 3 olive oils with similar fatty acids (FA) but different MPC amounts: an RF containing traces of MPC, an EVOO with low MPC concentration (LC), and an EVOO with high MPC concentration (HC). The HC was LC artificially enriched with its own MPC.

### Materials and Methods

#### Olive oil samples

RF containing traces of MPC and native EVOO (LC) naturally rich in MPC were kindly supplied by Olivicoltori Toscani Associati (O.T.A.). One part of LC oil was conserved in dark glass bottles at room temperature and the other part (4800 mL) was used to extract MPC and prepare the MPC-enriched olive oil (HC). Briefly, LC was extracted with ethanol and water acidified with formic acid (70:30, v:v) and defatted with n-hexane. The ethanol extract was concentrated to 200 mL and 50 mL was dried under vacuum and dissolved with 1 mL of ethanol and water (50:50, v:v) and used to enrich 1 L of native oil. This HC artificially enriched in MPC was created to amplify the biological effects of MPC and to compare them with those of LC.

#### MPC, FA, and tocoferol composition of olive oil samples

RF, LC, and HC were analyzed by HPLC using an HP-1100 liquid chromatograph equipped with a DAD detector and an HP-1100 MSD API-electrospray (Agilent Technologies) according to Romani et al. (28). MPC identification was carried out on the basis of retention times and spectroscopic and spectrometric data, using hydroxytyrosol (5-OH tyrosol) (from Cayman Chemical, SPI-BIO-Europe), tyrosol, lutelenin, and oleuropein (from Extrasynthese) as reference compounds. Identification and analysis of lignan were performed as previously described (29). Quantification of the single minor compounds was performed directly by HPLC-DAD using a 4-point regression curve constructed with the standards. Calibration curves with an $r^2 \geq 0.9998$ were considered. Methyl esters of the FA were prepared according to the EC Official Gazette and GC analysis was performed according to Pinelli et al. (30). RF, LC, and HC were characterized by similar FA (Table 1) but different MPC concentrations (Table 2).

The usual range of MPC fraction in EVOO is 44–704 mg/L (50–800 mg/kg) (31). Thus, native EVOO (LC) had medium-low MPC concentration (Table 2). In our study, we used LC as an EVOO with low MPC concentration and HC as an EVOO with higher MPC concentration (Table 2). Moreover, because we wanted to analyze the effect of EVOO in relation to its MPC concentration, RF was used as control, because it only contained traces of MPC (Table 2) and had a similar FA concentration to LC (Table 1).

Total tocopherol concentrations in RF and LC olive oil assayed according to Rossi et al. (32) were: RF, 123 mg/L (corresponding to 140 mg/kg) and LC, ~209 mg/L (corresponding to ~237 mg/kg).

#### Animals and study design

Experiments were carried out in accordance with the guidelines of the Council of European Communities 86/609/EEC and the Bioethical Committee of the University of Siena approved the protocols. Adult male Sprague-Dawley rats (Charles River), weighing ~450 g, were housed under controlled light (12 h on and 12 h off), temperature (20 ± 2°C), and humidity (55 ± 5%) conditions. Rats consumed food and water ad libitum. Rats were randomly assigned to 6 groups for different treatments (10 rats per group). We used 2 experimental designs: acute and subacute.

In the acute experiment, rats that were food deprived for 24 h received 1 administration of olive oil (RF, LC, or HC; 1.25 mL/kg body weight corresponding to ~1.1 g/kg) by gavage and were killed 1 h later (anaesthetization: 50 mg/kg sodium pentobarbital and 0.4 mg/kg acepromazine). In the subacute experiment, rats that were food deprived for 24 h received 1 administration of olive oil (RF, LC, or HC; 1.25 mL/kg body weight corresponding to ~1.1 g/kg) by gavage and were killed 1 h later (anaesthetization: 50 mg/kg sodium pentobarbital and 0.4 mg/kg acepromazine).
scopolamine, intraperitoneally). We chose the time of 1 h because pilot experiments had indicated that the antiaggregating effect reached the peak within 1 h (data not shown). The oil dose protocol was deliberately chosen even though it was nearly double the normal daily olive oil intake of Mediterranean populations (33), because rats have high basal excretion of certain MPC (34) and we wanted to amplify any effects of MPC.

In the subacute experiment, rats received 1 administration of olive oil (RF, LC, or HC; 1.25 mL/kg body weight) every day for 12 d (12 doses) and were then were food deprived for 24 h and killed.

Administration by gavage rather than addition to food was preferred to prevent any oxidation of phenol compounds on contact with air; to avoid handling stress, known to alter platelet aggregation (35), a single dose of oil was preferred to division into 2 doses.

We conducted acute and subacute pilot experiments and found that the effects of RF olive oil did not differ from those of saline administered by gavage (untreated rats, data not shown).

Blood drawn from the abdominal aorta was collected into 2 tubes containing different anticoagulants [3.8% sodium citrate at a ratio of 1:9 (v:v; citrate:blood) and EDTA] for platelet aggregation study and measurement of plasma concentrations of Hcy redox forms, respectively.

**Platelet aggregation**

All assays were carried out within 2–3 h of blood collection. Sodium citrate-treated blood was centrifuged at 170 × g; 15 min at 25°C to obtain platelet-rich plasma. Platelet-poor plasma (PPP) was prepared by further centrifugation at 2000 × g; 10 min at 25°C. Platelet count was performed in a Burker chamber and platelet concentration was adjusted to 1.5 × 10^11 platelets/L with PPP. Platelet aggregation was carried out in a Chrono-Log 540 aggregometer (ChronoLog) at constant stirring (900 rpm) at 37°C using PPP as reference system for platelet-rich plasma and monitoring for 4 min after addition of ADP. This agonist was selected because certain MPC were more active in inhibiting aggregation induced by ADP than aggregation induced by collagen (15). Aggregation was measured as percent transmittance (T%) at maximum amplitude.

To analyze the effect of olive oil intake on platelet reactivity, we evaluated the agonist half maximal effective concentration (EC50) using T% obtained with different doses of ADP (0.2–20 μmol/L range). The EC50 of ADP was calculated using the program SigmaPlot 8.0 (SPSS); it represents the dose of agonist that induces 50% of maximum aggregation. A higher EC50 denotes less platelet reactivity.

We also analyzed the effect of olive oil treatment on reversible and irreversible platelet aggregation induced by low (final concentrations: 1, 1.5, and 2 μmol/L) and high (10 μmol/L) doses of ADP, respectively, because these modes of aggregation occur by different mechanisms of platelet activation (36–38).

**Determination of plasma concentrations of Hcy redox forms**

EDTA-treated blood was centrifuged at 10,000 × g; 15 s at 25°C and the plasma was collected. We measured rHcy, oxHcy, and bHcy using monobromobimane (mBrB) according to Mansoor et al. (39) with slight modifications (see below) using an HPLC apparatus (Hewlett-Packard 1100 Series) equipped with fluorescence detection and an Omnispher C-18 reversed-phase column (Varian).

**rHcy determination.** We deproteinized 150 μL of plasma with 6% (w/v) trichloracetic acid (TCA, final concentration), and centrifuged at 10,000 × g; 2 min at 25°C. After neutralization with saturating solid NaHCO₃, the supernatant was incubated with 1 mmol/L mBrB (final concentration) in the dark at room temperature for 15 min and then centrifuged (10,000 × g; 2 min at 25°C). We acidified 90 μL of the supernatant with 10 μL 37% HCl (pH 3–4) and injected it into the HPLC column (40 μL).

**oxHcy determination.** We mixed 100 μL plasma with 2 mmol/L N-ethylmaleimide (NEM, final concentration) for 3 min and then deproteinized and centrifuged as above. The NEM excess in the supernatant was removed by extraction with dichloromethane (0.2 mL of sample plus 2.5 mL dichloromethane) and 100 μL of sample was brought to alkaline pH by addition of solid NaHCO₃ and treated with 1 mmol/L dithiothreitol (final concentration) at room temperature for 20 min. After centrifugation (10,000 × g; 2 min at 25°C), the supernatant was treated with an excess of mBrB (3 mmol/L final concentration) and processed as described above.

**bHcy determination.** The protein pellet from NEM-treated plasma samples (oxHcy) was washed 3 times with 1 mL 1.5% (w/v) TCA to remove traces of NEM and other compounds. The pellet was resuspended in 400 μL of 1 mmol/L K₂EDTA and saturated with solid NaHCO₃. Then 18 μL 50 mmol/L dithiothreitol was added to the resuspension and the sample maintained under continuous agitation at room temperature for 20 min. After centrifugation for 2 min, 100 μL supernatant was deproteinized with 40 μL 60% (w/v) TCA and the supernatant was diluted in water (1:3, v/v), neutralized with saturating solid NaHCO₃, reacted with 3 mmol/L mBrB, and processed as described above.

**Chromatographic conditions.** Mobile phases were 0.25% (w/v) acetic acid, adjusted to pH 3.09 with 1 mol/L NaOH (A) and methanol (B). Elution profile was as follows: 0–8 min, 20% B; 8–15 min, 20–40% B; 15–25 min, 40–100% B (1.0 mL/min flow rate; fluorimetric detector: excitation, 380 nm; emission, 480 nm; 10.1 min Hcy retention time).

**Statistical analysis**

Data are means ± SEM, n = 10 rats per group. Statistical analysis was performed using commercially available software (GraphPad Prism 4.0, GraphPad Software). Data in each experiment (acute and subacute) were tested by 1-way ANOVA. When the F-test was significant, Tukey’s post hoc test was used to identify the means that differed. In case of nonhomogeneous variances, as verified by Bartlett’s test, the nonparametric Kruskal-Wallis 1-way ANOVA was used. Dunn post hoc test was used to identify the means that differed. The designated level of significance was P < 0.05.

**Results**

**Acute treatment: platelet aggregation and plasma concentrations of Hcy redox forms.** Platelets from rats administered RF, LC, and HC olive oils differed in reactivity, as evidenced by a greater ADP EC50 value in the HC group than in RF (156%) or LC (101%) groups (P < 0.05) (Table 3).

Platelet aggregation was inhibited in rats given HC compared with those administered RF and LC when platelets were stimulated by low ADP doses (reversible aggregation) (Fig. 1A). Groups did not differ when aggregation was induced by a high dose of ADP (irreversible aggregation).

In rats administered HC, the plasma concentration of rHcy was 40% lower and bHcy was 51% greater than in RF-treated rats (Fig. 2A). Those of the LC group were intermediate and not different from the RF or HC groups.

**Subacute treatment: platelet aggregation and plasma concentrations of Hcy redox forms.** Platelets from rats administered RF, LC, or HC olive oils for 12 d differed in reactivity, but in contrast to the acute experiment, the ADP EC50 values were not different from those administered RF and LC when platelets were stimulated by low ADP doses (reversible aggregation) (Fig. 1A). Groups did not differ when aggregation was induced by a high dose of ADP (irreversible aggregation).

**TABLE 3** Effect of administration of RF, LC, or HC olive oils in rats on EC50 of ADP in acute and subacute experiments 1

<table>
<thead>
<tr>
<th>Olive oils</th>
<th>Acute</th>
<th>Subacute</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF</td>
<td>0.812 ± 0.064b</td>
<td>0.887 ± 0.046b</td>
</tr>
<tr>
<td>LC</td>
<td>1.032 ± 0.091b</td>
<td>1.626 ± 0.106b</td>
</tr>
<tr>
<td>HC</td>
<td>2.067 ± 0.189b</td>
<td>1.816 ± 0.340b</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 10. Means in a column without a common letter differ, P < 0.05.
value was greater in rats administered both LC (83%) and HC (104%) than in the RF group ($P < 0.05$) (Table 3).

Compared with the RF group, aggregation of platelets from rats in the LC and HC groups was inhibited only when ADP was present at low concentrations (Fig. 1B).

In the subacute experiment, the plasma concentrations of the Hcy redox forms differed among the groups (Fig. 2B). As in the acute experiment, the rHcy plasma concentration was lower (38%) than in the RF group only in rats administered HC (Fig. 2B). The plasma oxHcy concentration was greater than in the RF group in rats given LC (+69%) and HC (+80%). Similarly, the concentrations of bHcy in plasma of LC (+91%) and HC (+129%) treated rats (Fig. 2B) were greater than in the RF group.

**Discussion**

In this study, we investigated the biological effect of EVOO with different MPC concentrations but with the same FA composition on platelet aggregation and on plasma concentrations of Hcy redox forms. We considered the influence of treatment duration (acute, subacute) to evaluate the role of MPC, irrespective of FA, which may also influence platelet function (7–10). The importance of MPC in the biological activity of EVOO is evident in the acute experiment where only HC affected the variables studied, whereas in the subacute experiment, LC and HC EVOO had similar effects.

Repeated administration of LC EVOO (1.25 mL/kg body weight) could represent the action of dietary phenols on platelet reactivity, because LC provided an amount of MPC in the normal daily range of phenols (10–20 mg) of Mediterranean diets under these conditions (40). A high dietary intake of MPC, as produced by HC EVOO, could also exert these effects after administration of only 1 dose of oil. The reduction in platelet reactivity to ADP after dietary intake of MPC could help to reduce the risk of CD, because subjects with platelets highly reactive to ADP run a higher risk of developing fatal cardiovascular events (41).

The mechanism of action by which MPC modify platelet function is unclear, but the use of different doses of ADP to induce platelet aggregation showed that only reversible aggregation...
was inhibited. Because reversible and irreversible aggregation express different mechanisms of platelet activation (36–38), our result suggests possible mechanisms of action of MPC. Reversible aggregation is sustained by mechanisms that activate fibrinogen receptors (αIIbβ3) without massive platelet response involving release of granule contents (irreversible aggregation) (36–38). Reactive oxygen species (ROS) are involved in platelet activation (42–44) and regulate activation of fibrinogen receptors (αIIbβ3) without triggering release of granules by platelets (45). Because MPC are free radical scavengers (46), they may quench the effect of ROS on αIIbβ3, inhibiting irreversible platelet aggregation. On the other hand, the effect of antioxidants on platelet function is well known, because several phenol compounds such as hydroxytyrosol, oleuropein, and inhibitors of ROS production in platelets inhibit platelet aggregation (6,15,47,48).

An interesting new result was the modification of plasma concentrations of Hcy redox forms after administration of EVOO. In acute and subacute studies, rHcy concentration was lower in plasma of rats given HC than in those given RF. Instead, oxHcy and bHcy generally increased after LC and HC administration. These effects seem related to MPC, because RF intake did not significantly change Hcy redox forms in untreated rats (data not shown).

In the last 10 y, great interest has been paid to the relationship between increased concentrations of plasma Hcy and the incidence of CD and other diseases (23–25,49). Although a link has been widely documented, the mechanism of putative Hcy toxicity and the contribution of the different Hcy redox forms are still unclear. Despite this, Hcy is considered a major marker of risk of CD (26,27) and its increase has been associated with vascular endothelial dysfunction (27,50), ischemic stroke (51), and kidney disease (26,52). It has also been demonstrated that rHcy is a pro-aggregating agent (53) that increases TXB2 and kidney disease (26,52). It has also been demonstrated that vascular endothelial dysfunction (27,50), ischemic stroke (51), and risk of CD (26,27) and its increase has been associated with toxicity and the contribution of the different Hcy redox forms are still unclear. Despite this, Hcy is considered a major marker of risk of CD (26,27) and its increase has been associated with vascular endothelial dysfunction (27,50), ischemic stroke (51), and kidney disease (26,52).

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The lower dose of MPC provided by LC, which is in the normal range of phenol intake by humans in Mediterranean countries (40), may only raise oxHcy and bHcy concentrations after repeated administration. The biological significance of these increases remains to be clarified.

The analysis of the influence of treatment duration on the effects of EVOO indicated that LC was more effective under subacute than acute conditions, whereas HC was effective either in acute or subacute experiments. It is possible that after longer exposure to EVOO, other minor components (e.g. tocopherols, squalene, etc.) (11,55) could contribute to these results. However, because LC oil showed a weak significant difference in tocopherol concentrations with respect to RF (123 mg/L RF, 209 mg/L LC) and presumably also in other minor components (55), we speculate that the biological contribution of minor components would be less than that of MPC (prevalently phenols).

In conclusion, this study shows that MPC in EVOO reduce platelet function and modify plasma concentrations of Hcy redox forms. These results confirm the antiaggregating properties shown by single MPC and the ability of some phenol compounds to modify Hcy metabolism (15,21). The reduction of platelet reactivity to ADP, a major mediator of hemostasis and thrombosis (38), and the decrease in rHcy plasma concentrations could contribute to the reduced risk of CD found by epidemiological studies in the Mediterranean area (1,2) where EVOO is an important dietary item.

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Literature Cited


