Intake of phenol-rich virgin olive oil improves the postprandial prothrombotic profile in hypercholesterolemic patients

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ABSTRACT
Background: Oxidative stress associated with postprandial lipemia contributes to endothelial dysfunction, which shifts hemostasis to a more thrombogenic state.
Objective: We investigated whether a high concentration of phenols in olive oil can partly reverse this phenomenon.
Design: Twenty-one hypercholesterolemic volunteers received 2 breakfasts rich in olive oils with different phenolic contents (80 or 400 ppm) according to a randomized, sequential crossover design. Plasma concentrations of lipid fractions, factor VII antigen (FVIIa), activated factor VII (FVIIa), and plasminogen activator inhibitor-1 (PAI-1) activity were measured at baseline and postprandially.
Results: Concentrations of FVIIa increased less (P = 0.018) and plasma PAI-1 activity decreased more (P = 0.021) 2 h after the high-phenol meal than after the low-phenol meal. FVIIa concentrations 120 min after intake of the olive oil with a high phenol content correlated positively with fasting plasma triacylglycerols (P = 0.001), area under the curve (AUC) of triacylglycerols (P = 0.001), and AUC of nonesterified fatty acids (P = 0.024) and negatively with hydroxytyrosol plasma concentrations at 60 min (P = 0.039) and fasting HDL-cholesterol concentrations (P = 0.005). PAI-1 positively correlated with homeostasis model assessment of insulin resistance (P = 0.005) and fasting triacylglycerols (P = 0.025) and inversely with adiponectin (P = 0.026). In a multivariate analysis, the AUCs of nonesterified fatty acids (R² = 0.467; β: 0.787; SE: 0.02; P < 0.001) and adiponectin (R² = 0.232; β: -1.594; SE: 0.629; P < 0.05) were the strongest predictors of plasma FVIIa and PAI-1, respectively.
Conclusions: A virgin olive oil with a high content of phenolic compounds changes the postprandial hemostatic profile to a less thrombogenic state.

KEY WORDS Olive oil, polyphenols, postprandial lipemia, plasminogen activator inhibitor-1, PAI-1, activated factor VII, FVIIa

INTRODUCTION
Endothelial dysfunction is one of the first steps in the development of arteriosclerosis, and it is characterized by a thrombogenic state caused by an imbalance between procoagulant and profibrinolytic activity (1). Among the procoagulant factors, plasminogen activator inhibitor-1 (PAI-1) and factor VII (FVII) concentrations have been linked to coronary heart disease (CHD) (2), and both can be regulated, at least partly, by alimentary lipemia (3). Attention is currently focusing on investigating whether different components of the diet can regulate acute postprandial changes in coagulation and fibrinolysis.
Factor VII coagulant (FVIIc) has been linked to postprandial plasma triacylglycerol concentrations, which suggests an acute effect of triacylglycerol-rich lipoproteins (TRLs) on the activity of FVII (FVIIa) (4). We previously reported that a Mediterranean diet reduces fasting plasma concentrations of FVIIa in healthy men (5), a fact that might be related to the presence of olive oil in this diet (6, 7). Williams (8) observed a reduction in postprandial FVIIa and factor VII antigen (FVIIa) plasma concentrations after the acute intake of an olive oil–based meal.
On the other hand, it has been suggested that PAI-1 activity declines after the intake of meals rich in oleic acid as part of a Mediterranean-type diet, in both the postprandial (9) and fasting states (10, 11). Virgin olive oil, which is the principal fat in this dietary pattern (12), contains both oleic acid and a wide range of micronutrients, among which phenolic compounds have displayed anti-thrombotic effects in cell culture and in vitro studies (13, 14). Studies in humans, however, are scarce, and more evidence on these biological activities is needed (15). To further investigate whether postprandial phenols from virgin olive oil affect hemostasis, we tested whether 2 breakfasts rich in this oil,
but with different contents of phenolic compounds, had different effects on hemostasis postprandially.

SUBJECTS AND METHODS

Subjects

Twenty-one hypercholesterolemic subjects (5 men and 16 women) aged 53 to 68 y and with a mean body mass index (in kg/m²) of 25.4 ± 4.1 (range: 23.5–27.1) participated in the study. All were patients who were being followed up in the Lipids and Atherosclerosis Unit at the Reina Sofia University Hospital in Cordoba. Plasma total cholesterol concentrations were between 200 and 350 mg/dL and plasma triacylglycerol concentrations were <200 mg/dL. The women were postmenopausal (but were not undergoing hormone replacement therapy). None of the participants showed evidence of chronic diseases, high alcohol consumption, or family history of early-onset cardiovascular disease. None of the subjects were active smokers. The study was approved by the Human Investigation Review Committee at the Reina Sofia University Hospital. All the participants gave their informed consent before joining the study.

Experimental design

The participants were instructed to not take vitamins, soya supplements, or any drug treatments, including hormone treatment, for the 6 wk preceding the study. Three patients were taking atorvastatin, 10 mg/d, which was discontinued 6 wk before the randomization step. The subjects were shown how to follow a low-fat, carbohydrate-rich diet during that period to eliminate potential differences in their usual dietary habits. Compliance with the stabilization diet was assessed after 2 and 4 wk by means of a 3-d record and a food-frequency dietary questionnaire. The participants were instructed to avoid consuming polyphenol-rich foods (such as fruit or juices, wine, grape juice, chocolate, coffee, tea, olive oil, or soya) or performing intense physical exercise in the 24 h before the experimental breakfast. The following morning they came to the hospital after fasting for 12 h. By use of a randomized, sequential crossover design, the participants were given 1 of 2 breakfasts consisting of 60 g white bread, 40 mL virgin olive oil (Carapelli Firenze SpA, Florence, Italy) with either a high (A, 400 ppm) or a low (B, 80 ppm) content of phenolic compounds and 60 000 IU vitamin A/m² body surface area. Patients starting with the A type breakfast consumed the B type after 1 wk, and conversely. Olive oil B was obtained by the extraction of most of the phenolic compounds in olive oil A, so that both oils had similar contents of their remaining macro- and micronutrients. The procedure involved washing olive oil A in a separation funnel with an equal quantity of double-distilled water. The mixture was shaken for 3 min and left to settle to facilitate the separation process. The aqueous phase was then discarded and the procedure repeated. The concentration of phenolic compounds was measured until it fell to trace levels.

Throughout the 4-h duration of the study, the subjects neither performed physical activity nor consumed anything but water. Venous blood was drawn at 0, 30, 60, 120, and 240 min after consumption of the breakfast.

Composition of the olive oils

Tocopherols were measured by separating the different tocopherol isomers by means of HPLC (Beckman, Palo Alto, CA) and were subsequently analyzed in a Jasco FP-920 spectrophotometer (Jasco, Tokyo, Japan) at an excitation wavelength of 294 nm and an emission wavelength of 340 nm. A spectrophotometer (UNICAM 5625, Cambridge, UK) was used to measure total carotenoid (670 nm) and chlorophyll (472 nm) contents. No significant differences in any of the micronutrient concentrations were found, except for the polyphenol fraction.

Plasma samples

Samples from the fasting and postprandial states were collected in tubes containing 1 g EDTA/mL or 3.8% citrate and were stored in containers with ice and kept in the dark. Special care was taken to avoid exposure to air, light, and ambient temperature. Plasma was separated from whole blood by low-speed centrifugation at 1500 × g for 15 min at 4 °C within 1 h of extraction.

Plasma polyphenol concentrations

Concentrations of tyrosol, hydroxytyrosol, and 3-O-methylhydroxytyrosol (MHT), a biological metabolite of hydroxytyrosol, were measured by gas chromatography–mass spectrometry in plasma samples at 0 and 60 min (16, 17).

Lipid analysis and biochemical determinations

Concentrations of the different lipid variables were analyzed with a modular autoanalyzer (DDPPII Hitachi; Roche, Basel, Switzerland) with the use of Boehringer-Mannheim reagents. Concentrations of total cholesterol and triacylglycerol were measured by colorimetric enzymatic methods (18, 19). HDL-cholesterol concentrations were measured by colorimetric assay after the lipoproteins containing apolipoprotein (apo) B were precipitated with polyethylene glycol (20). LDL-cholesterol concentrations were estimated by using the Friedewald formula on the basis of total cholesterol, triacylglycerol, and HDL-cholesterol values (21). Apo A-I and apo B concentrations were measured by immunonutribiometry (22). The chylomicron and large VLDL fractions of TRLs were isolated from 4 mL plasma overlaid with 0.15 mol NaCl/L, 1 mmol EDTA/L (pH 7.4; density: 1.006 kg/L) by a single ultracentrifugal spin (20 000 × g, 30 min, 4 °C) in a type 50 rotor (Beckman Instruments, Fullerton, CA). Chylomicrons contained in the top layer were removed by aspiration after the tubes were cut. The infranatant fraction was centrifuged at a density of 1.019 kg/L for 24 h at 115 000 × g in the same rotor. The nonchylomicron fraction of TRL (also referred to as small TRL) was removed from the top of the tube. All operations were performed in subdued light. Large and small TRL fractions were kept at −70 °C until total cholesterol and triacylglycerol concentrations were analyzed. Fasting plasma adiponectin and resistin were measured by enzyme-linked immunosorbent assay with the Quantikine Human Adiponecin/Acrp30 Immunoassay and Quantikine Human Resistin Immunoassay (R&D Systems Inc, Minneapolis, MN). Plasma glucose concentrations were measured with a Hitachi 917 analyzer (Boehringer Mannheim, Mannheim, Germany) by the glucose oxidase method (GOD-PAP). Plasma insulin concentrations were measured by microparticle enzyme immunoassay (Abbott Diagnostics, Matsudo-shi, Japan). Nonesterified fatty acid concentrations were measured by enzymatic colorimetric assay (Roche Diagnostics, Penzberg, Germany). The homeostasis model assessment of insulin resistance (HOMA-IR) was defined by the validated definition: HOMA-IR = [fasting glucose (mmol/L) × fasting insulin (μU/mL)]/22.5 (23).
TABLE 1
Baseline characteristics of the hypercholesterolemic participants in the study

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
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<tbody>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.4 ± 0.05 (4.61–6.22)</td>
</tr>
<tr>
<td>Insulin (µU/mL)</td>
<td>6.22 ± 2.17 (2.80–11.30)</td>
</tr>
<tr>
<td>NEFA (mmol/L)</td>
<td>0.45 ± 0.12 (0.32–0.68)</td>
</tr>
<tr>
<td>HOMA-IR (units)</td>
<td>1.5 ± 0.6 (0.6–2.7)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>6.12 ± 1.22 (5.1–8.62)</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>5.20 ± 1.27 (4.02–6.82)</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.27 ± 0.39 (0.65–2.00)</td>
</tr>
<tr>
<td>Tyrosol (µg/mL)</td>
<td>2.07 ± 2.50 (3.50–11.60)</td>
</tr>
<tr>
<td>Hydroxytyrosol (µg/mL)</td>
<td>2.17 ± 2.80 (2.80–11.30)</td>
</tr>
<tr>
<td>MHT (µg/mL)</td>
<td>2.17 ± 2.80 (2.80–11.30)</td>
</tr>
<tr>
<td>Resistin (ng/mL)</td>
<td>2.07 ± 0.56 (1.15–3.10)</td>
</tr>
</tbody>
</table>

1 All values are x ± SD (range in parentheses); n = 21. NEFA, nonesterified fatty acids; HOMA-IR, homeostasis model assessment of insulin resistance.

Measurement of FVIIag, FVIIa, and PAI-1
We measured concentrations of FVIIag, FVIIa, and PAI-1 in the frozen plasma samples by means of a tissue-type plasminogen activator–based immunoactivity assay (Chromolize PAI-1; Trinity Biotech, County Wicklow, Ireland) and concentrations of FVIIag (Asserachrom VII:Ag; Diagnostica Stago, Asnières sur Seine, France) and FVIIa (Imubind; American Diagnostica Inc, Greenwich, CT) by enzyme-linked immunosorbent assay. All measurements were performed in duplicate.

Statistical analysis
Dichotomous values from each subject were calculated before data analysis. Comparisons among the end values for each treatment period were made after adjustment for baseline values. The percentage change between concentrations at the beginning of each breakfast (basal) and concentrations 60 and 120 min after intervention with the olive oils was calculated. The area under the curve (AUC) of plasma concentration–versus–time curve and a line drawn parallel to the horizontal axis through the 0 h concentration. These areas were calculated by a computer program using the trapezoidal rule. All data presented in the text and tables are expressed as means ± SDs. The normality of variables was assessed by the Kolmogorov-Smirnov test. The data were analyzed by analysis of variance (ANOVA) for repeated measures. Diet, time, and the interaction between diet and time showed a smaller increase in FVIIag, after the phenol-rich olive oil than after the low-phenol breakfast.

RESULTS

Basal and postprandial metabolic parameters
The clinical characteristics of the participants at baseline are shown in Table 1. No significant differences were observed in any of the incremental AUCs of the main metabolic variables after the intake of either of the olive oils (Table 2).

Basal and postprandial concentrations of tyrosol, hydroxytyrosol, and MHT
We observed a greater increase in concentrations of plasma tyrosol, hydroxytyrosol, and MHT after the intake of the olive oil with a high phenolic compound content than after the olive oil with a low content of phenols (Figure 1).

FVIIag, FVIIa, and PAI-1
Postprandial plasma concentrations of FVIIag and FVIIa and PAI-1 activity are presented in Figure 2. The intake of both olive oils significantly increased FVIIa activity (P = 0.002) and decreased FVIIag activity (P = 0.050) and PAI-1 activity (P = 0.001) with respect to baseline concentrations, which indicates a change in these variables during postprandial lipemia. Analysis of the interaction between diet and time showed a smaller increase in FVIIag concentration (P = 0.050) and a greater decrease in PAI-1 activity (P = 0.047) after the phenol-rich breakfast than after the low-phenol breakfast.

When we compared percentage changes from baseline values in FVIIag, FVIIa, and PAI-1 values at 120 min, we found a smaller postprandial increase in FVIIa plasma concentrations (39.7 ± 30.8% compared with 20.3 ± 26.5%; P = 0.018) and a greater decrease in PAI-1 plasma activity (−29.1 ± 32.8% compared with −52.8 ± 30.4%; P = 0.021), but no significant differences in FVIIag, after the phenol-rich olive oil than after the low-phenol breakfast.

Regression analysis
To further evaluate predictors of FVIIa and PAI-1, we performed a correlation analysis between these 2 variables and body mass index (BMI), metabolic variables, HOMA-IR, adiponectin, resistin, and plasma phenol concentrations. In a univariate correlation analysis, FVIIa concentrations correlated positively with triacylglycerol (r = 0.503, P = 0.001), the AUC of plasma

TABLE 2
Areas under the plasma postprandial response curves (AUCs) after the intake of the low-phenol and the phenol-rich olive oil (OO)-based breakfasts

<table>
<thead>
<tr>
<th>AUC</th>
<th>Low-phenol OO breakfast</th>
<th>Phenol-rich OO breakfast</th>
<th>P2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg · min/dL)</td>
<td>4510 ± 2094</td>
<td>4530 ± 1976</td>
<td>0.859</td>
</tr>
<tr>
<td>Insulin (µU · min/mL)</td>
<td>5036 ± 990</td>
<td>4962 ± 743</td>
<td>0.827</td>
</tr>
<tr>
<td>NEFAs (mmol · min/L)</td>
<td>−45.2 ± 2.5</td>
<td>−44.3 ± 2.1</td>
<td>0.109</td>
</tr>
<tr>
<td>Plasma TG (mg · min/dL)</td>
<td>8890 ± 6018</td>
<td>9027 ± 6772</td>
<td>0.707</td>
</tr>
<tr>
<td>TG in small TRLs (mg · min/dL)</td>
<td>2427 ± 2063</td>
<td>2300 ± 2181</td>
<td>0.667</td>
</tr>
<tr>
<td>TG in large TRLs (mg · min/dL)</td>
<td>6672 ± 3327</td>
<td>6990 ± 5372</td>
<td>0.616</td>
</tr>
</tbody>
</table>

1 All values are x ± SD; n = 21. NEFAs, nonesterified fatty acids; TG, triacylglycerols; TRLs, triacylglycerol-rich lipoproteins.

2 One-factor ANOVA.
triacylglycerol (0.567, \( P = 0.016 \)), and the AUC of nonesterified fatty acids (\( r = 0.234, P = 0.024 \)) and negatively with concentrations of hydroxytyrosol (\( r = -0.465, P = 0.039 \)) and HDL cholesterol (\( r = -0.593, P = 0.005 \)). Strong direct relations were found between PAI-1 activity and HOMA-IR (\( r = 0.602, P = 0.005 \)) and triacylglycerol (\( r = 0.487, P = 0.025 \)). PAI-1 activity correlated negatively with fasting adiponectin concentrations (\( r = -0.485, P = 0.026 \)).

To explore the importance of potential predictors, 2 stepwise multiple linear regression models were fitted for FVIIa and PAI-1. After adjustment for HOMA-IR, fasting triacylglycerol, and the AUC for triacylglycerol, only the incremental AUC of nonesterified fatty acids remained significantly associated with FVIIa in the model (\( R^2 = 0.467; \beta: 0.787; SE: 0.02; P < 0.001 \)). A similar strategy was used to assess the predictors of plasma PAI-1 concentrations. After adjustment for HOMA-IR, BMI, and triacylglycerol, plasma adiponectin concentrations remained significantly associated with plasma PAI-1 (\( R^2 = 0.232; \beta: -1.594; SE: 0.629; P < 0.05 \)).

**DISCUSSION**

Our study showed that, in patients with hypercholesterolemia, the consumption of a breakfast containing virgin olive oil with a high content of phenols induces a smaller postprandial increase in the concentration of FVIIa and a greater decrease in PAI-1 plasma activity than the same olive oil with a low content of phenols. It has been suggested that the postprandial hypertriglyceridemia that follows the intake of high-fat meals activates FVII. The intrinsic mechanism of this activation is not clear, although it is known that some of the reactions for activation of hemostatic factors are due to exposure to lipid bilayers with negative charges, such as those of denuded endothelium or the surface of platelets or oxidized LDL (24). Some studies have observed that FVIIa plasma concentrations correlate positively with plasma phospholipid concentrations (25). Our study showed a significant association between the incremental AUC of nonesterified fatty acids and postprandial changes in FVIIa. The hydrolysis of triacylglycerol-rich lipoproteins by lipoprotein lipase may be an important source of elevated concentrations of fatty acid anions near the endothelium. These fatty acids are substrates for the lipoperoxidation produced by the increase in oxidative stress during the postprandial period. Olive oil phenolic compounds have been shown to act as chain-breaking antioxidants for the autocatalytic chain reaction of fatty acid peroxidation (26). The attenuating effect of olive oil–rich test meals on FVIIa was shown previously (6, 27), but these studies focused on the effect of modified test meal dietary fat composition. What proportion of this effect is due to the phenolic or fatty acid profile of virgin olive oil is still unclear.

In the present study, we found a significant decrease in postprandial concentrations of FVIIa, but no significant differences in the effects of these 2 types of olive oil. At the same time, there was a smaller increase in FVIIa after the ingestion of an olive oil with a high content of phenols than after the olive oil with a low content of these compounds. Because the sole difference in the composition of these 2 oils was their phenolic content, these data suggest that the effect of the diet on the decrease in FVIIa is due to the difference in their fat content, and the effect on FVII activation to their content of phenols.

How phenols interfere with this postprandial activation of FVIIa is not known. We previously showed the protective effect of olive oil phenols on the postprandial microvascular endothelial function of hypercholesterolemic persons (28), an effect that is strongly influenced by procoagulant and prooxidant factors. It could be hypothesized that the known antioxidant properties of phenols act as a potent buffer in the vicinity of endothelial cells, thus reducing the activation of FVIIa.

We also observed a significant reduction in PAI-1 concentrations 120 min after the ingestion of olive oil with a high phenolic content. Although changes in fasting PAI-1 concentrations after prolonged dietary intervention periods have been described in several studies, less evidence exists of this effect of the diet in the postprandial state. Furthermore, it is difficult to make a global interpretation of the postprandial effects on PAI-1 on the basis of...
these studies because of the different designs and methods used. Both increases (3, 29) and reductions (9, 30, 31) in PAI-1 concentrations after meals with a high fat content have been described. It has even been suggested that these data might simply be the result of a circadian variation in PAI-1 concentrations, without any direct relation to diet (32, 33). Nor is there a clear explanation of the mechanism by which PAI-1 concentrations would be influenced by the acute intake of different amounts of fat, although it has been suggested that VLDL could up-regulate PAI-1 transcription (34–36), something that can be avoided by antioxidants (37–39).

In our study, PAI-1 activity showed a greater postprandial decrease after the intake of the olive oil with high phenolic content, whereas it correlated negatively with fasting adiponectin concentrations and positively with HOMA-IR. Correlations between HOMA-IR, adiponectin, and PAI-1 were previously reported in both adults (40–42) and children (43). PAI-1 concentrations are directly influenced by insulin (44), even in situations of insulin resistance (45), a fact that has been explained in terms of abdominal fat, via a higher concentration of cytokines in subjects with central obesity (41). Nevertheless, the association of PAI-1 and insulin resistance has also been found in nonobese cohorts (46). These apparently contradictory findings may be partially explained by the identification of a regulatory element (AP-1 response element) in the PAI-1 promoter. This element enhances PAI-1 transcription by a factor of 7 when stimulated by insulin increases mediated by FOX protein transcription factors (47), which may explain the association between insulin resistance and PAI-1. Interestingly, the same element also induces a 3-fold rise in the PAI-1 transcription rate in the presence of oxidative stress (48), as occurs in the postprandial state. The double regulation of the PAI-1 promoter element may explain both the fasting correlations between adiponectin, HOMA-IR, and PAI-1 and the larger postprandial decrease in PAI-1 after the phenol-rich breakfast as a result of its antioxidant properties and the lower activation of nuclear factor-κB that occurs after meals rich in virgin olive oil (49). In line with our findings, Pacheco et al (15) recently found an enhancement in postprandial hemostasis after a very phenol-rich olive oil meal (1125 ppm of phenolic compounds), an enhancement that they evaluated in terms of a greater decrease in PAI-1, smaller tissue factor and fibrinogen AUCs, and a larger postprandial decrease in tissue-type plasminogen activator.

The results of the present study may partly explain earlier contradictory results of studies that tested the effects of olive oil on hemostasis. It is possible that the concentrations of microcomponents of the olive oil used in some of those studies did not reach the levels needed to activate the anti-thrombotic properties of olive oil. However, and although this study deals with the microcomponents of virgin olive oil, we should still think in terms of evaluating the biological properties of complete foods. In this perspective, our findings provide new evidence of the healthy effects of virgin olive oil. In conclusion, the consumption of a breakfast containing olive oil rich in phenolic compounds may improve the thrombogenic postprandial profile of FVIIa and PAI-1 concentrations associated with acute fat intake.

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The contributions of the authors were as follows—JR, JL-M, and FP-J: responsible for the conception and design of the study; JR, FF, CM, and YJ: responsible for the provision of study materials or subjects; JR, JL-M, JC, JF, FF, RdSmart, and CM: responsible for the collection and assembly of data; JR, JL-M, JC, YJ, and FP-J: responsible for the analysis and interpretation of the data; JL-M, CM, FF, and FP-J: provided statistical expertise; JR, PP-M, JL-M, and FP-J: responsible for drafting the manuscript; JD-L, RdSmart, and CM: responsible for the critical review of the manuscript and for important intellectual content; FP-J: obtained funding. None of the authors had any personal or financial conflicts of interest.

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