The Unsaponifiable Fraction of Virgin Olive Oil in Chylomicrons from Men Improves the Balance between Vasoprotective and Prothrombotic Factors Released by Endothelial Cells

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ABSTRACT Minor components of virgin olive oil (VOO) may play a key role in the beneficial effects of VOO on atherosclerosis. In the present study we evaluated the influence of the unsaponifiable fraction of VOO on the production of eicosanoids and nitric oxide (NO) by endothelial cells (HUVECs). Triglyceride-rich lipoprotein (TRLs) were isolated from human serum after the intake of meals enriched in 3 high-oleic acid oils, i.e., high-oleic sunflower (HOSO), VOO, or enriched-virgin olive (EVO) oils, the last-mentioned containing 2.4% of unsaponifiable matter. HOSO induced a greater accumulation of triglycerides (TGs) in the postprandial serum than VOO or EVO, as measured by calculating the area under the curve. The incubation with TRLs increased NO release by endothelial cells compared with untreated control cells, but the effects of the various TRLs did not differ. EVO-derived TRLs reduced the production of prostaglandin E2 (PGE2) and thromboxane B2 (TxB2) (the stable metabolite of TxA2) compared with VOO- or HOSO-derived TRLs. The release of PGI2 (as 6-keto PGF1α) was similarly diminished by all TRLs compared with the control. In conclusion, the unsaponifiable fraction of VOO does not affect postprandial triglyceridemia, but it has favorable effects on endothelial function, mainly by reducing proinflammatory and vasoconstrictor eicosanoid synthesis (PGE2 and TxB2). J. Nutr. 134: 3284–3289, 2004.

KEY WORDS: • unsaponifiable matter • virgin olive oil • triglyceride-rich lipoprotein • endothelial 
• eicosanoids

Because postprandial hyperlipidemia has been identified as a potential independent cardiovascular risk factor, different components of this complex process were investigated to clarify the mechanisms underlying its atherogenicity (1–3). The relation of triacylglycerol-rich lipoprotein (TRL)3 particles, including chylomicrons (CMs), VLDL, and their remnants, with atherosclerosis was demonstrated. TRL can cross the endothelial barrier and enter into the vascular wall (4) where, without the need of prior oxidation, they can affect endothelial function, and receptor-mediated lipid accumulation in the macrophage, leading to foam cell formation (5,6).

The vascular endothelium is an active organ that responds to vasodilator and vasoconstricting agents, regulating the balance between thrombosis and fibrinolysis. Production of nitric oxide (NO) by nitric oxide synthases is believed to be integral to many of these functions, by inhibiting platelet aggregation and adhesion, modulating smooth muscle cell proliferation, and reducing leukocyte adhesion (7). Eicosanoids also play an important role in vascular homeostasis. Prostacyclin (PGI2) causes vasodilation and inhibits platelet aggregation, whereas thromboxane A2 (TxA2) counteracts that effect by increasing vasoconstrictions and aggregation (8). The imbalance between these anti- and proaggregating molecules is associated with the pathophysiologic conditions underlying thrombotic and ischemic events (9,10).

High consumption of monounsaturated fatty acids (MUFA) is characteristic of the Mediterranean diet; after the Seven Countries Study, it was proposed as a healthy dietary standard because of its association with a low rate of cardiovascular mortality (11). Among other effects, the substitution of dietary SFA by MUFA was shown to improve thrombotic response in humans (12). However, there seem to be differences between diets enriched in 2 different MUFA sources. Indeed, virgin olive oil (VOO), but not high-oleic sunflower oil (HOSO), reduces blood pressure in healthy subjects and in hypertensive patients (13). Muriana et al. (14) also showed that VOO restores both the impaired membrane cholesterol distribution and the activity of Na+/Li+ countertransport in erythrocytes of patients with untreated essential hypertension. In addition, we demonstrated that VOO and HOSO produce

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2 To whom correspondence should be addressed. E-mail: valruz@ig.csic.es.
3 Abbreviations used: apo, apolipoprotein; CHD, coronary heart disease; CM, chylomicron; EVO, enriched virgin olive oil; FCS, fetal calf serum; HOSO, high-oleic sunflower oil; HUVEC, human umbilical vein endothelial cell; MUFA, mono-unsaturated fatty acids; PG, prostaglandin; TG, triacylglycerol; TRL, triacylglycerol-rich lipoprotein; TX, thromboxane; VOO, virgin olive oil; vWF, von Willebrand factor.
different effects on the magnitude and duration of the postprandial triglyceridemia in normolipidemic subjects (15). Because a delayed clearance of postprandial TRL was observed in patients with atherosclerosis compared with normolipidemic men (16), and postprandial triglyceridemia is suggested to be an independent risk factor for coronary heart disease (CHD) (1,3), the effects of VO0 on postprandial TRL may have clinical importance.

The above-mentioned studies highlight the fact that other factors such as triacylglycerol (TG) species composition, minor fatty acids, and nonfatty acid constituents (unsaponifiable fraction), rather than the content of oleic acid, might be responsible for the long-term and postprandial response of VO0. The aim of the present study was to evaluate the influence of the unsaponifiable fraction of VO0 on postprandial TRL composition and its effect on eicosanoid and NO production by human endothelial cells.

SUBJECTS AND METHODS

Diet study. Men (n = 10) aged 26.5 ± 3.6 y with BMI 24.9 ± 1.4 kg/m² participated in the study. They had mean fasting serum TG concentrations in the normal range (1 ± 0.2 mmol/L) and did not suffer from any digestive or metabolic disease as verified by medical history. The subjects gave written, informed consent to a protocol approved by the Institutional Committee on Human Research (Hospital Universitario Virgen del Rocío, Sevilla).

The test meal consisted of 1 slice of brown bread (28 g); 100 g of plain pasta (cooked with 200 mL water); 130 g of tomato sauce, and 1 skimmed yogurt, providing 2102 kJ of energy. HOSO, VO0, or enriched-virgin olive (EVO) oils (70 g) were supplied mixed with the tomato sauce (total energy 4523 kJ). The enrichment of EVO was carried out by isolating the unsaponifiable fraction from VO0 and adding it to another aliquot of VO0 from 1.2% (wt:wt) to a final concentration of 2.4% (wt:wt). The unsaponifiable fraction of VO0 was isolated following conventional procedures, as described elsewhere. In brief, oils were saponified with 25 mL of 2 mol/L KOH in methanol:water (80:20, v:v). After heating at 70°C for 30 min, 50 mL of water and 50 mL of ethyl ether were added. The unsaponifiable fraction was collected by decantation. Table 1 shows the fatty acid composition of the oils (VO0 and HOSO) (v. cornicabra) (Aceites Toledo SA). Unsaponifiable components of the oil were also analyzed and are presented in Table 2.

Participants were asked to refrain from smoking and drinking during the day preceding the study because these activities might affect lipid metabolism. During the course of the study, the partici-pants were allowed to drink water and/or black coffee without sugar and undertook only light activities. A baseline blood sample was collected from fasting subjects into vacutainer tubes immediately before they consumed the test meal. The meals were given in sequential order, HOSO, VO0, and EVO, to the 10 men, with a 2-wk washout period between test meals. Blood samples were drawn hourly for 7 h during the postprandial period.

Lipoprotein determination. Serum was recovered rapidly by centrifugation (16200 × g, 20 min, 12°C). Sodium azide (1 mol/L), phenylmethylsulfonyl fluoride (10 mmol/L in isopropanol) and aprotonin (1400 mg/L) were added to the serum to a final concentration of 1 mol/L, 10 μmol/L, and 28 mg/L, respectively. Serum total and HDL-cholesterol and TG concentrations were measured by conventional enzymatic kits (cholesterol and free glycerol determination kits, Sigma-Aldrich). LDL cholesterol concentration was calculated according to the Friedewald equation. TRLs collected 2 h after the meal intake were isolated from 4 mL of serum layered with 6 mL of a NaCl solution (d = 1.066 kg/L) by a single ultracentrifugation spin (281000 × g, 18 h, 15°C). Ultracentrifugation was performed using a SW 41 Ti rotor in a Beckman L8–70M preparative ultracentrifuge (Beckman Instruments).

TRL lipid composition. Total lipids were extracted from TRLs following a modification of the method of Folch et al. (17), using 2,6-di-tert-butyl-p-cresol as antioxidant. The lipids extracted were redissolved in 1 mL of chloroform:methanol (2:1, v:v) and preserved at −20°C until used. The lipid composition was determined by HPLC as described by Perona and Ruiz-Gutierrez (18). Proteins were measured using the method of Bradford (Bio-Rad Laboratories).

Endothelial cell culture. Primary cultures of human umbilical vein endothelial cells (HUVECs) were obtained by the collagenase digestion method as originally described by Jaffe et al. (19) with minor modifications (20). Cells were cultured in Medium 199 (M199) supplemented with 20% fetal calf serum (FCS), 30 mg/L endothelial cell growth supplement (Sigma), 100 mg/L heparin, t-glutamine (2 mmol/L), sodium pyruvate (10 mmol/L), and antibiotics (100 KU/L penicillin and 0.1 g/L streptomycin).

HUVEC cultures were characterized by immunocytochemistry using antibodies against human von Willebrand factor (vWF) (clone F8/86, Dako). HUVECs were grown on gelatin-coated cover slips, cells were fixed with 4% (v:v) paraformaldehyde/PBS for 15 min at room temperature, and permeabilized with 0.4% (v:v) Triton X-100 for 4 min. Cells were incubated with the mouse monoclonal antibody against vWF and fluorescein isothiocyanate-conjugated goat anti-mouse IgG as secondary antibody. For all studies, HUVECs were used at passages 3–5. HUVECs were seeded into multiwell plates, grown to semiconfluent density (80–90%) and arrested with medium containing 0.4% (v:v) FCS for 16 h before treatment with TRL (1.50 mg cholesterol/L, for 14 h) in serum-free medium. After incubation, supernatants and cell extracts were collected.

NO synthesis and eicosanoid determinations. Synthesis of NO was determined in culture supernatants measuring NO− (nitrite), as previously described (21). NO− concentrations were calculated by comparison with a standard curve prepared using NaNO2 diluted in culture medium. Culture media from cell cultures treated with TRL

<table>
<thead>
<tr>
<th>Component</th>
<th>HOSO</th>
<th>VOO</th>
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<tbody>
<tr>
<td>mg/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squalene</td>
<td>ND</td>
<td>6621 ± 235</td>
</tr>
<tr>
<td>Total sterols</td>
<td>1497 ± 54</td>
<td>1558 ± 33</td>
</tr>
<tr>
<td>Total tocopherols</td>
<td>393 ± 25</td>
<td>207 ± 48*</td>
</tr>
<tr>
<td>Erythrodiol + uvaol</td>
<td>ND</td>
<td>20.98 ± 1.07</td>
</tr>
<tr>
<td>Waxes</td>
<td>30.0 ± 4.8</td>
<td>83.5 ± 7.9*</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 10. * Different from HOSO, P < 0.05. ND, not detected.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>HOSO</th>
<th>VOO</th>
</tr>
</thead>
<tbody>
<tr>
<td>g/100 g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>4.2  ± 0.1</td>
<td>13.5 ± 0.4*</td>
</tr>
<tr>
<td>16:1(n-7)</td>
<td>0.1  ± 0.0</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>18:0</td>
<td>0.1  ± 0.0</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>18:1(n-9)</td>
<td>4.4  ± 2.3</td>
<td>2.3 ± 0.3*</td>
</tr>
<tr>
<td>18:1(n-7)</td>
<td>0.1  ± 0.0</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>18:2(n-6)</td>
<td>10.4 ± 0.7</td>
<td>5.8 ± 0.7*</td>
</tr>
<tr>
<td>18:3(n-3)</td>
<td>0.1  ± 0.0</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>20:1(n-9)</td>
<td>0.1  ± 0.0</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>22:0</td>
<td>0.7  ± 0.2</td>
<td>0.2 ± 0.1*</td>
</tr>
</tbody>
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1 Values are means ± SEM, n = 10. * Different from HOSO, P < 0.05. ND, not detected.
were collected and kept at −80°C. Prostacyclin (PGI2) [as its stable metabolite (6-keto-PGF1α)], prostaglandin E2 (PGE2) and thromboxane B2 (TXB2) levels in these media were determined by commercial enzyme immunoassays (Cayman Chemical). Untreated cells were also cultured for every experiment to serve as controls.

Cell viability. TRL did not affect cell morphology, cell apoptosis (assessed by staining with Hoech 33258 colorant), or cell viability analyzed measuring the mitochondrial dehydrogenase activity by a commercial kit (XTT-based assay for cell viability, Roche) or by trypan blue exclusion test.

Data analysis. Results are expressed as means ± SEM, unless otherwise stated. The Statview 4.1 (Abacus Concepts) statistical package for the Macintosh computer system was used for all of the analyses. Multiple groups were compared by 1-way ANOVA with repeated measures, followed by the Scheffé F-test to assess specific group differences. Differences were considered significant at P < 0.05.

RESULTS

Fatty acid composition of VOO and HOSO oils. Both oils contained a similar amount of MUFA (79.6% for HOSO and 77.4% for VOO) with oleic acid [18:1(n-9)] accounting for 99% of all MUFA (Table 1). Although VOO had a higher SFA content, due to a higher percentage of palmitic (16:0) and docosanoic (22:0) acids (P = 0.001), the content of stearic acid (18:0) was higher in HOSO (P = 0.001). In contrast, HOSO had more linoleic acid [18:2(n-6)] (P = 0.001). The fatty acid composition of EVO was the same as that of VOO because this was not modified when supplemented with the unsaponifiable fraction.

Unsaponifiable fraction composition of virgin olive (VOO) and high-oleic sunflower (HOSO) oils. Squalene and erythrodial were absent from the unsaponifiable fraction of HOSO, but were present in high concentrations in VOO (Table 2). The wax concentration was also higher in VOO (P < 0.05). The sterol concentration did not differ between the oils. In contrast, the tocopherol concentration was higher in HOSO (P = 0.02). The unsaponifiable fraction composition of EVO was the same as that of VOO but the concentrations were double.

Serum TG concentration during the postprandial period. TGs were measured in serum for 7 h after the ingestion of the meals enriched in HOSO, VOO, or EVO (Fig. 1). The highest TG concentration occurred 2 h after the intake of the oils. At time points other than 2 and 3 h, serum TG concentrations were greatest after the men consumed HOSO. Further, the TG area under the curve was significantly higher after the men consumed HOSO (Table 3).

Lipid classes and apolipoprotein (apo)B composition of TRL. The TG, free cholesterol, and phospholipid concentrations did not differ in TRL at 2 h after the intake of the 3 oils tested (Table 3). However, the cholesteryl ester concentration was higher after VOO (P = 0.005) and EVO (P = 0.001) than after HOSO. The apoB concentration was higher in the TRL of subjects after HOSO, and did not differ after the intake of VOO and EVO.

Endothelial effects of TRL. NO production from endothelial cells was not differentially affected by TRL (HOSO, VOO or EVO) (Fig. 2). Treatment of cells with TRL obtained after the men consumed the EVO-diet significantly reduced PGE2 (Fig. 3A) and TXB2 (Fig. 3B) release by HUVECs compared with those obtained after the HOSO- and VOO-enriched diets (Fig. 3). Cells treated with HOSO and VOO lipoproteins (but not with EVO lipoproteins) had higher production of TXB2 than untreated control cells. The TRL did not differ in their effects on PGI2 (Fig. 3C) release, but

FIGURE 1  Serum TG concentration in men during the 7 h after the ingestion of meals enriched in HOSO, VOO, or EVO oils. Results are means ± SEM, n = 10. Means at a time without a common letter differ, P < 0.05.

HUVECs treated with TRL obtained after men consumed the EVO diet released less and those obtained after the HOSO and EVO diets tended to release less (P = 0.05 and 0.01) than control cells.

DISCUSSION

The influence of the unsaponifiable fraction of VOO on the ability of TRL to modify the production of NO and vasoactive eicosanoids by human endothelial cells was investigated in this study. HUVECs were incubated with TRL isolated from the serum of healthy men 2 h after the intake of meals enriched in HOSO, VOO, or EVO.

In a population with a Mediterranean diet background, the intake of HOSO induced a higher accumulation of TG in the postprandial phase than the intake of VOO or EVO (Fig. 1, Table 3). At 2 h after the intake of the meals, when the TG concentration in serum was the highest, the TG concentration in TRL was not significantly different after any of the oleic acid–rich oils, but the apoB concentration was higher after HOSO. MUFA oils were found to increase the number of CMs compared with PUFA, but a background diet based on VOO prevents this effect (22). We previously showed that TRLs derived from HOSO contain higher concentrations of TG, compared with VOO, during the postprandial period (15). Because the oleic acid content in these oils is virtually the same, we proposed that the triglyceride molecular species or other minor oil components might be responsible. In the present study, we observed no significant difference between the serum TG profiles after VOO or EVO. Thus, we suggest that the postprandial TG response is dependent mainly upon the triglyceride molecular species and not on the unsaponifiable fraction. Our present data do not exclude that a higher increase in the unsaponifiable fraction could also affect the TG content of TRL, but it is very unlikely to find concentrations higher than 2.4% (wt:wt).

The effect of postprandial lipemia on endothelium-dependent dilatation is unclear; in fact, it was shown that it could
inhibit, stimulate, or have no effect (23–27). In our study, TRLs derived from the 3 enriched diets did not differ in NO release, but TRL significantly increased NO release compared with untreated control cells. This increase is in agreement with previous results showing a similar effect when endothelial cells were incubated with LDL (28,29). Although the mechanism is unclear, it could be related to the ability of lipoproteins to increase intracellular calcium, which promotes NO synthesis. In previous studies, oxidized chylomicron remnant-like particles but not native chylomicron remnant-like particles increased the vessel tone of porcine coronary rings and decreased NO release from porcine aortic endothelial cells (30). A number of authors also observed suppression of NO synthesis when endothelial cells were incubated with individual TG species (31), FFA, or TG-rich emulsions (32). However, TRLs are not composed only of triglycerides or fatty acids but also transport other lipid compounds from the diet (33). Dietary oils influence the lipid composition of postprandial TRLs and hence their physiologic effects (33). Among the components of the unsaponifiable fraction, tocopherols were reported to enhance the production of NO (34). However, the tocopherol concentrations in VOO were lower than those in HOSO, and not significantly different than in EVO (data not shown). We demonstrated recently that erythrodiol, a triterpenoid present in the unsaponifiable fraction of VOO, evokes an endothelium-dependent vasorelaxation in rat aorta (35). The mechanism proposed seems to be mediated mainly by endothelial production of NO because the vasorelaxant effect was blocked by the NO synthase inhibitor \( \text{N}^\omega\text{-nitro-L-arginine-methylester} \), although other mechanisms could not be excluded, such as participation of eicosanoids.

In the present study, the main effect of the type of oil consumed was the ability of TRL to modify the production of eicosanoids by endothelial cells. TRL obtained after the intake of EVO reduced the production of PGE\(_2\) and TxB\(_2\) (the stable metabolite of TxA\(_2\)) compared with the other dietary oils. The release of PGJ\(_2\) (as 6-keto-PGF\(_{1\alpha}\)) was similar after all TRL treatments. Although a reduction in a vasoprotective molecule such as prostacyclin could be considered harmful, the balance between vasoprotective and proinflammatory/prothrombotic molecules must be considered. These molecules share key regulatory steps in their biosynthetic pathways (i.e., cyclooxygenase) but they are the result of specific enzymatic
activities that can be regulated independently by active components of the oils. Thus, EVO-derived TRL produced the most favorable vascular protective effects. Although the atheroprotective effects of the olive oil on the plasma lipid profile and oxidative processes have been widely investigated (36,37) very little has been published regarding the effects of its unsaponifiable fraction on the biosynthesis of eicosanoids by the endothelium (38). However, we and others previously described the decreased production of PGE2 and TxB2 by neutrophils (39) and macrophages (40) in rats fed VOO. Among minor components of VOO potentially responsible for these effects are polyphenols; they elicit vasorelaxation in aortic rings (41), which seems to be independent of NO (42). However, although polyphenols are readily absorbed in the intestine, it is unlikely that they are present in postprandial TRL due to their hydrophilic nature (43). On the other hand, in rat neutrophils, VOO polyphenols appear not to affect cyclooxygenase activity and the production of its derived metabolites (44). Other minor components of VOO present in the unsaponifiable fraction, such as β-sitosterol (45), tocopherols (46), and triterpentenoids (47), which were reported to reduce the production of PGE2 by macrophages, could also be involved in the effect observed in endothelial cells. Because no difference was found between men in PGE2 and TxB2 involved in the effect observed in endothelial cells. Because N. Engl. J. Med. 326: 310–318.

The pathogenesis of coronary artery disease and the acute coronary syndromes (2). Of note, the acute coronary syndromes (1).


micron remnant induction of lipid accumulation in J774 macrophages is associated including low-density lipoprotein, high-density lipoprotein, and albumin. Coron. Artery Dis. 5: 695–705.


