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The olive constituent oleuropein, as a PPAR α agonist, markedly reduces serum triglycerides $\star$

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Abstract

Oleuropein (OLE), a main constituent of olive, exhibits antioxidant and hypolipidemic effects, while it reduces the infarct size in chow- and cholesterol-fed rabbits. Peroxisome proliferator-activated receptor α (PPAR α) has essential roles in the control of lipid metabolism and energy homeostasis. This study focused on the mechanisms underlying the hypolipidemic activity of OLE and, specifically, on the role of PPAR α activation in the OLE-induced effect. Theoretical approach using Molecular Docking Simulations and luciferase reporter gene assay indicated that OLE is a ligand of PPAR α . The effect of OLE (100 mg/kg, p.o., per day, $\times 6$ weeks) on serum triglyceride (TG) and cholesterol levels was also assessed in adult male wild-type and Ppara-null mice.

Molecular Docking Simulations, Luciferase reporter gene assay and gene expression analysis indicated that OLE is a PPAR α agonist that up-regulates several PPAR α target genes in the liver. This effect was associated with a significant reduction of serum TG and cholesterol levels. In contrast, OLE had no effect in Ppara-null mice, indicating a direct involvement of PPAR α in the

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Conflicts of interest

The authors state no conflict of interest.

Study limitations

The investigation of the effect of OLE on serum lipoprotein concentrations was not within the objectives of the present study that focused on the effect of OLE on triglycerides. In addition, the effect of OLE on PPAR β /delta and PPAR γ should be assessed in the framework of future studies.

OLE-induced serum TG and cholesterol reduction. Activation of hormone-sensitive lipase in the white adipose tissue (WAT) and the liver of wild-type mice and up-regulation of several hepatic factors involved in TG uptake, transport, metabolism and clearance may also contribute in the OLE-induced TG reduction. In summary, OLE has a beneficial effect on TG homeostasis *via* PPAR α activation. OLE also activates the hormone sensitive lipase in the WAT and liver and up-regulates several hepatic genes with essential roles in TG homeostasis.

Keywords

Oleuropein; Triglycerides; PPAR α ; Cholesterol; Mice

1. Introduction

Lipid homeostasis is a critical parameter in the physiology and pathophysiology of living organisms because lipids are essential in storing energy, cell signaling and synthesis of steroid hormones, and act as structural components of cell membranes [1]. Disruption of lipid homeostasis results in dyslipidemias, which are etiologically associated with increased risk of developing severe pathologies including atherosclerosis, obesity and cardiovascular disease. A broad and comprehensive meta-analysis study that included 29 prospective studies and 262,525 participants provided a strong and highly significant association between elevated serum triglyceride (TG) concentrations and increased risk for coronary disease [2]. Current guidelines for the treatment and prevention of hypertriglyceridemia recommend the use of fibrates [3, 4]. Fibrates, ligands for the peroxisome proliferator-activated receptor α (PPAR α), are effective in reducing plasma TG levels either as monotherapy or in combination with statins [5, 6]. Several studies reported that even an intensive therapy with statins does not eliminate the residual cardiovascular risk, which is associated with atherogenic dyslipidemia [low high-density lipoprotein (HDL) and high TG]. Instead, it was found that the combined treatment using statins and fibrates is more effective in achieving comprehensive lipid control and reduction of the cardiovascular risk [7]. It should be noted though that co-administration of statins with fibrates is associated with a small but significant increase in mortality due to rhabdomyolysis, with gemfibrozil having a more pronounced effect [8]. Thus, investigation of molecules that reduce essentially TGs may be a future insight into the management of comprehensive lipid control and reduction of cardiovascular risk.

PPAR α is considered as a cellular “lipostat” that transduces alterations in cellular lipid levels to the transcriptional regulation of several PPAR α target genes, which determine the fate of fatty acids [9-11]. Specifically, upon activation, PPAR α heterodimerizes with retinoid X receptor (RXR) and activates the transcription through binding to the PPAR response element (PPRE), which is also called direct repeat 1 (DR1) and is located in the promoter of the target genes. [12]. Thus, PPAR α up-regulates various genes encoding enzymes that are involved in fatty acid uptake, in transport, as well as in peroxisomal and mitochondrial fatty acid β -oxidation and in microsomal fatty acid ω -oxidation. PPAR α also regulates several apolipoprotein (Apo) genes, such as ApoAI, AII and CIII, a fact that indicates a central role for PPAR α in the extracellular transport of fatty acids in the blood [13,14].

Notably, PPAR α agonists, such as fibrates, are used in the treatment of dyslipidemia because they reduce serum triglycerides, essential property for the prevention of atherosclerosis and reduction of the risk for the development of cardiovascular disorders [15-17]. PPAR α -mediated effects on gene expression make this nuclear receptor an ideal pharmacological target for the treatment of hypertriglyceridemias and other dyslipidemias.

Olive oil and olives are integral ingredients of the traditional Mediterranean diet, and several studies attribute many of the healthy advantages of this diet to olive's unique characteristics [18]. Oleuropein (OLE) and hydroxytyrosol (OLE hydrolysis product) are the main polyphenolic substances of the leaves and unprocessed olive drupes of *Olea europaea*, which significantly reduce adiposity and hyperlipidemia in dietary obese rats [19, 20]. Of particular clinical significance is the fact that purified OLE considerably reduced infarct size in normal and hypercholesterolemic rabbits that were subjected to ischemia/reperfusion. OLE protected the reperfused myocardium of rabbits from oxidative damage and reduced total cholesterol and TG serum levels [21].

In the light of the above considerations and the increasing interest in Mediterranean diet, the present study focuses on the role of OLE in lipid homeostasis. Emphasis was given in the involvement of PPAR α in the OLE-induced effect. To address this question, wild-type (WT) and *Ppara*-null mice were employed and fed a regular rodent diet for 6 weeks with OLE in the food. The findings of this study indicate that OLE markedly reduces serum TG levels *via* PPAR α activation. The luciferase reporter gene assay and the data from the molecular docking simulation study indicated that OLE is a PPAR α agonist, which up-regulates a broad array of PPAR α target genes related to lipid homeostasis. It appears that the up-regulation of several hepatic factors involved in the uptake, metabolism, transport and clearance of TGs, and the activation of the hormone-sensitive lipase (HSL) in the white adipose tissue (WAT) and the liver, potentially contribute in the OLE-induced suppressive effect on serum TGs.

2. Methods

2.1. Molecular docking simulations

2.1.1. Protein and ligand preparation—Crystal structure of PPAR α in complex with indiglitazar, a PPAR pan-agonist, was retrieved from PDB entry 3ET1. The protein was prepared for the docking calculations using the Protein Preparation Workflow [22] implemented in Schrodinger Suite 2015 and was accessible from the Maestro software [23,24]. OLE was prepared using the Ligprep Workflow as implemented in Maestro software [25]. Three different protonation states were predicted at pH 7.0 $\pm$ 2.0, one neutral and two negative charged, based on deprotonation of 3OH or 4OH group on the phenyl ring.

2.1.2. Computational solvent mapping—Potential hydration sites of the protein–ligand complex were identified using the SZMAP algorithm as previously described [26].

2.1.3. induced Fit Docking (IFD)—Molecular docking was performed using the IFD protocol (Schrödinger Suite 2011, Induced Fit Docking protocol), which is intended to circumvent the inflexible binding site and accounts for the side chain or backbone

movements, or both, upon ligand binding. In the first step of the IFD protocol, docking calculations were performed using the Glide 5.7, and 20 poses per ligand were retained. In the second step, for each docking pose, a full cycle of protein refinement was carried out using the Prime 1.6 on all residues within 8 Å of a ligand pose. The Prime refinement starts with a conformational search and minimization of the side chains of the selected residues. The obtained complexes were ranked according to Prime calculated energy (molecular mechanics and solvation), and those within 25 kcal/mol of the minimum energy structure were used in the last step of the process. Finally, the ligand orientations used in the first docking step were redocked into each of the receptor structures retained from the refinement step. The final ranking was completed by a composite score, which accounts for the receptor–ligand interaction energy (GlideScore), the receptor strain and solvation energies (Prime energy).

2.2. Animals

Adult male SV129 WT and *Ppara*-null mice [27, 28] were used in this study. The WT and *Ppara* null mice received *ad libitum* the standard rodent chow diet (diet 1324 TPF, Altromin Spezialfutter GmbH & Co. KG). All animals had continuous access to drinking water and were housed up to five per cage under a standard 12-h light, 12-h dark cycle. Each mouse was monitored daily for outward signs of distress or adverse health effects. All procedures involving animals were reviewed and approved by the ethics committee of the Faculty of Medicine of the University of Ioannina. They conformed to the International European Ethical Standards (86/609-EEC) for the care and use of laboratory animals and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health.

2.3. Drugs, cell lines and plasmids

The OLE used in this study was isolated from the leaves of *Olea europaea* as previously described [29]. The PPAR α agonist, WY-14643, was obtained from Sigma-Aldrich. The human hepatic cell line, HepG2, was purchased from ATCC. Cell culture medium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics, penicillin and streptomycin, L-glutamine and nonessential amino acids were from Gibco BRL Life Technologies. Charcoal-stripped FBS was obtained from Life Technologies. Luciferase assay system was obtained from Promega, and chemiluminescent reporter gene assay system for detection of β -galactosidase was from Applied Biosystems.

Plasmids, p(A-OX3)-TKSL containing three copies of the DR1 PPRE from the mAcyl CoA oxidase (ACO) gene linked to TK luciferase [30] and PPAR α expression plasmid (PPAR cDNA cloned in pCMX vector), were a generous gift from Dr. M. Ricote. Construct containing the full-length cDNA for RXR α in the expression vector of pMT2 was described previously [31] and was a generous gift from Dr. M. Hadzopoulou-Cladaras.

2.4. In vivo experiment

Adult male (SV129) WT mice were randomized in two groups as follows: control group (n=6) and OLE group (n=7). OLE (100 mg/kg) was administered daily in the food pellets for

6 consecutive weeks. *Ppara*-null mice were randomized in two groups: control (n=6) and OLE group (n=6). OLE was administered at the same dose as mentioned above.

The dose of OLE was based on our previous findings [21,29] and on the literature [18]. OLE was administered in the food pellets because it has been shown that, even under normal iso-osmotic luminal conditions, OLE is poorly absorbed. Its absorption can be significantly improved by solvent flux through paracellular junctions, made possible by hypotonic conditions in the intestinal lumen [32]. The presence of glucose or amino acids in the intestinal lumen after a meal stimulates water flux *via* the opening of paracellular junctions, and it may be that this mechanism has a similar effect on the absorption of OLE as does the use of a hypotonic solution [33]. Although the OLEs' pharmacokinetic profile has not been determined in mice, Boccio and colleagues indicated that a single oral dose of OLE (100 mg/kg) is absorbed in rats, reaching 200 ng/ml in tmax of 2 h [34]. Therefore, 2 h after the last drug treatment, mice of all groups were killed by CO₂ asphyxiation, and trunk blood was collected in BD Microtainer Serum Separator Tubes (Becton, Dickinson and Company, USA) for biochemical analyses. Liver and white adipose tissue samples were dissected for total RNA, nuclear/cytosolic and total cellular protein extraction. All tissue and serum samples were kept at — 80°C until assayed.

2.5. Quantitative real-time polymerase chain reaction (qPCR)

Total RNA from liver and white adipose tissue from WT and *Ppara*-null mice was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The concentration of total RNA was determined spectrophotometrically. qPCR was performed with cDNA generated from 1 µg total RNA with an Invitrogen SuperScript II reverse transcriptase kit (Thermo Fisher Scientific, USA). The sequences for the forward and reverse gene-specific primers used are shown in Table S1. SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was used for the real-time reactions, which were performed by using a C1000 Touch thermal cycler with a real-time detection system (Bio-Rad Laboratories, Hercules, CA, USA). Relative mRNA expression levels were normalized to β -actin levels (QuantiTect primer assay; QIAGEN, Valencia, CA, USA), and values were quantified by using the comparative threshold cycle method.

2.6. Western blot analysis

Immunoblot analysis of PPAR α protein expression was carried out using nuclear extracts of liver samples from WT and *Ppara* null mice. For the preparation of the nuclear extracts, the NE-PER nuclear extraction kit (Pierce, Rockford, IL, USA) was used. Alterations in HSL phosphorylation, total ATGL, perilipin 5 (PLIN5) and total perilipin apoprotein levels were assessed in total cellular proteins extracted from the WAT. Protein concentrations were determined by the BCA protein assay (Pierce, Rockford, IL, USA). Proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting using the rabbit monoclonal anti-mouse PPAR α IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The rabbit polyclonal phospho-HSL (Ser563, Ser565, Ser660) IgGs, the rabbit polyclonal total HSL and Perilipin IgGs were analyzed using the Lipolysis Activation Antibody Sampler kit (Cell Signaling). The goat polyclonal PLIN5 and ATGL IgGs (Santa Cruz Biotechnology) were also used. Immunoblotting with mouse either β -actin or histone-

H3 antibodies (Santa Cruz Biotechnology) was used as loading control. For secondary antibodies, the anti-rabbit, anti-goat or anti-mouse IgG horseradish-peroxidase-conjugated antibodies (Cell Signaling) were used, and the proteins were detected using an enhanced chemiluminescence detection kit (Thermo Scientific-Pierce, Rockford, IL, USA).

2.7. Determination of total hepatic TG content

Total TG content was determined in the liver of mice using a method previously described by Karavia et al. [35]. The results are expressed as mg per gram of liver tissue (mean±S.E.).

2.8. In vitro experiment

2.8.1. Preparation of hepatocyte cultures—Hepatocytes were prepared following a modified method based on a previous report [36]. In brief, *in situ* perfusion of the murine liver was used for the isolation of parenchymal hepatocytes. The isolated hepatocytes were suspended in Williams' Medium E supplemented with L-glutamine, penicillin and streptomycin and were plated at a density of 0.80– 1.0×10⁶ cells in 60-mm diameter collagen-type-I-coated dish (BIOCOAT, Cell Environment, Becton Dickinson Labware, UK). Trypan blue dye exclusion was used to check the viability of isolated cells, and those cells with viability higher than 85% just before plating were used. Hepatocytes were cultured at 37°C for 24 h under an atmosphere of humidified 5% CO₂ in order to allow the cells to adhere to the dish. Time response experiments started 24 h later. Primary hepatocytes were cultured in the presence of OLE at a final concentration of 10 µM or DMSO. The strongest OLE effect on PPARα was observed following 2 h incubation.

2.8.2. Cell culture and transient co-transfection assay—HepG2 cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, 0.25 µg/ml streptomycin, 2 mM L-glutamine and 0.1 mM nonessential amino acids at 37°C in a humidified atmosphere of 5% CO₂. The cell cultures were routinely passaged while growing exponentially. On day 0, 5×10⁵ cells were seeded in cell culture dishes (60-mm diameter) in DMEM containing 10% FBS. On day 2, cells were switched to medium containing 5% charcoal-stripped FBS in order to deplete PPAR ligands and serum growth factors. On day 3, transient transfections were performed using the calcium phosphate DNA co-precipitation method in fresh DMEM containing 5% charcoal-stripped FBS. Briefly, HepG2 cells were co-transfected using PPRE firefly luciferase construct p(A-OX)₃-TKSL (1.5 µg) with the constructs of PPARα (1.5 µg) and/or RXRα (1.5 µg) along with 0.5 µg β-galactosidase plasmid. Sixteen hours after transfection, cells were rinsed twice with PBS, and fresh DMEM with 5% charcoal-stripped FBS was added. Test compounds, OLE and the PPARα agonist WY-14643, were added 24 h after transfection in fresh DMEM containing 5% charcoal-stripped serum. The tested compounds OLE and the PPARα agonist were dissolved in DMSO prior to appropriate dilution with cell culture medium, so the final DMSO concentration never exceeded 0.5% (v/v) in the culture. Control cells received DMSO without the test compounds. Cells were harvested 48 h after transfection, lysed and assayed for luciferase and β-galactosidase activities using the respective assay kits according to the manufacturer's instructions. Luciferase activities were normalized to β-galactosidase activities (internal control).

2.9. Biochemical determinations

Serum total cholesterol concentration was measured with the Cholesterol EIA kit (Wako Diagnostics, Richmond, VA, USA). The levels of serum nonesterified fatty acids were determined using the NEFA C, EIA kit (Wako Chemicals GmbH, Neuss, Germany). Serum triglycerides were determined using the ThermoTrace kit (Melbourne, Australia).

2.10. Statistical analysis

The data are presented as the mean $\pm$ S.E. and were analyzed using one-way analysis of variance (ANOVA) followed by multiple comparisons with Bonferroni's and Tukey's least honestly significant difference methods. The significance level for all analyses was set at probability of less than .05.

3. Results

3.1. Assessment of OLE binding to PPAR α

Previous studies indicated that OLE markedly suppressed serum TG levels [19, 20]. The principal role of PPAR α in TG homeostasis is also well established [9-11]. In particular, PPAR α agonists markedly reduce serum TG levels. Therefore, molecular simulation studies and the Luciferase reporter gene assay were conducted in order to obtain an insight about a potential interaction between OLE and PPAR α .

3.1.1. Molecular simulation-based assessment of OLE binding to PPAR α —

The crystal structures of PPAR α in complex with a variety of PPAR α ligands, such as fibrates and the PPAR pan agonist indeglitazar (Fig. 1), are deposited in Protein Data Bank [37]. Primarily, we examined the PPAR α binding site starting from its crystal structure in complex with indeglitazar (PDB entry 3ET1) [38]. Using SZMAP application (Open-Eye Inc.), possible water-accessible surfaces inside the proteins' binding pocket were investigated. A number of water molecules reside near the entrance of the binding cavity among helices H4, H6 and the (β -sheet between H4 and H7. SZMAP calculations provide thermodynamic information regarding the substitution of those water molecules. As depicted in Fig. 2A, the most thermodynamically stable water molecule (yellow colored) is located between Tyr314 and Tyr464 and is obviously substituted by the hydrophilic carboxylate group of the PPAR α ligand indeglitazar. It is of interest to note that there are several water molecules in the vicinity of the Leu331 carbonyl oxygen (yellow colored; Fig. 2A), which can be substituted by hydrophilic functional groups, thus enhancing the free energy of binding. In contrast, there are several magenta-colored water molecules near the hydrophobic regions of the binding cavity.

Moreover, using the same crystal structure of PPAR α , IFD simulations of OLE were performed. The resulting low-energy structure is shown in Fig. 2B and C (brown) superimposed with the crystal structure in complex with indeglitazar (green) and GW409544 (cyan) for comparison reasons. The methylester group of OLE is placed exactly where the acid group of indeglitazar is found, thus forming a hydrogen bond with the OH group of Tyr464. The glucose residue is placed near the entrance of the cavity, among Met335, Leu331, Asn219 and Thr283 stabilized by hydrogen bonds (Fig. 2D). The catecholic

hydroxyl groups are placed next to Glu269 forming hydrogen bond interactions, while the rest of the hydrophobic core of the molecule has an extended orientation towards the indole moiety of indeglitazar.

Further detailed examination of minimum energy structure reveals that the location of the glucose group next to the cavity entrance could substitute water molecules, which, according to our previous SZMAP analysis, results in increased chemical affinity towards protein through interactions with Asn219, Thr279, Ser280 and Thr283 (Fig. 2A and D).

PPAR α ligands are known to occupy a considerable space, their total surface being ~1300–1500 Å³. They form several interactions with the protein, which extend to the whole surface of the binding cavity, namely, with residues Y314, H440, Y464, S280, C276, C275, L247 and V255 [39]. The spacious size of the binding pocket can be well demonstrated by the distances between residues located at the two edges of the binding pocket, namely, Y464 and V255 (C α carbons), which is 25 Å. OLE pertains the structural characteristics to fit in the PPAR α binding cavity. It consists of three parts, the central elenolic methyl ester, the glucose and the hydroxytyrosol moieties, and according to our theoretical models described above, it can form interactions analogous to those described for known PPAR α inhibitors. In agreement with the experimental evidence provided in this work, only in its integral form OLE can structurally fit in the PPAR α binding site in a similar way as the known PPAR α ligands, not as a metabolite. Hydroxytyrosol, a known OLE metabolite, exhibits a surface of about the one third of OLE and does not show structural similarities to well-known PPAR inhibitors in terms of length, surface or volume. In this aspect, hydroxytyrosol is not expected to interact with the receptor unless weakly. Therefore, we consider that the biological activity of OLE, in relation to PPAR α , as indicated by the present experiments is not likely to be attributed to a metabolite, such as hydroxytyrosol.

3.1.2. Luciferase reporter gene assay—To test whether OLE acts as a PPAR α ligand, we used a reporter gene assay with PPRE-luc plasmid. OLE treatment led to a significant increase in the PPRE-luc activity by 55%, implying activation of the PPAR α -RXR α heterodimer (Fig. 3). Further investigation indicated that OLE treatment in the presence of only RXR α or PPAR α did not activate the PPRE-luc plasmid (Fig. 3). The ligand WY-14643, which was used for comparison, led, as expected, to a significant increase of the PPRE-luciferase activity. From the above data, it is evident that OLE acts as a PPAR α ligand, which though is weaker compared to WY-14643.

3.2. Effect of OLE on serum lipid levels

Chronic treatment of WT mice with OLE resulted in significant reduction of serum TG levels and liver TG content (Table 1), whereas OLE did not affect them in *Ppara*-null mice (Table 1). These findings indicate the involvement of PPAR α in the OLE-mediated TG reduction. Total cholesterol concentration was also reduced by OLE in the serum of WT mice (Table 1), an effect that was not evident in *Ppara*-null mice (Table 1). Notably, OLE had no effect on the relative liver weight (liver weight/body weight; control vs. OLE; 0.0483 ± 0.0017 vs. 0.044 ± 0.0025).

3.3. OLE-induced Ppara expression in vivo

OLE administration for 6 weeks led to a significant induction of PPAR α at a level of mRNA and protein in the liver of WT mice (Fig. 4A). The OLE-induced up-regulation of PPAR α was followed by up-regulation of several PPAR α target genes including *Acox1*, *Cyp4a10*, *Cyp4a14*, *Acot1*, *Acot4*, *Lipin 1*, *Lipin2* and *Scd1*. Hepatic *Rxra* and *Pgc1a* mRNA expressions were also increased by OLE in WT mice compared to controls (Fig. 4B). The induction of RXR α by OLE is of particular significance since this nuclear factor is required for high-affinity DNA binding of the heterodimer PPAR α -RXR on the PPREs of target genes, serving as an auxiliary DNA binding factor [40]. Notably, *Fas* mRNA expression, which is not a PPAR α regulated gene, was not significantly affected by OLE in the liver and WAT of mice [relative *Fas* mRNA expression, mean $\pm$ S.E., control vs. OLE, 1 $\pm$ 0.54 (liver) and 1 $\pm$ 1.16 (WAT)].

It should be also noted that the OLE-induced increase in PPAR α activation is not connected to any effect of the drug on the animals' eating behavior, as no fasting or overeating was observed. The animals' body weights ranged within comparable levels in OLE-treated mice and controls at the beginning and the end of the treatment (Table S2).

3.4. OLE-induced PPAR α expression in primary hepatocytes

Treatment of primary hepatocytes with OLE increased *Ppara* mRNA expression (Fig. 5A). The OLE-induced up-regulating effect on hepatic *Ppara* mRNA expression was accompanied with up-regulation of several PPAR α target genes including *Acox1*, *Cyp4a14*, *Lipin 1* and *Acot4* (Fig. 5B).

3.5. Effect of OLE on hepatic factors, critical in TG homeostasis

Treatment of WT mice with OLE resulted in increased mRNA expression of several genes involved in the synthesis, transport, metabolism and clearance of TGs in the liver. Specifically, OLE increased *Dgat1*, *Lpl*, *Atgl/Pnlpa2* and *Nr4A* mRNAs in the liver of treated mice compared to controls (Fig. 6A). *Dgat2*, *Aadac*, *Mttp*, *Ces/Tgh*, *Cd36* and *Angptl4* mRNA expression was also increased following treatment with OLE (Fig. 6B). Notably, OLE also increased *Hsl* mRNA expression and HSL phosphorylation at Ser565 and Ser660 in the liver of WT mice compared to controls (Fig. 6A and C, respectively).

3.6. Effect of OLE on factors expressed in the white adipose tissue, critical in TG homeostasis

No effect was observed on genes encoding factors with essential roles in TG synthesis, transport, metabolism and clearance in the WAT, including *Dgat1*, *Dgat2*, *Atgl*, *Nr4A*, *Mttp*, *Ces/Tgh* and *Cd36* (Fig. 7A), with the exception of *Aadac* (Fig. 7B), *Ppara* and *Acox1* (Fig. 7C) that were up-regulated by OLE. The OLE-induced reduction in serum TG levels could be attributed in part to activation of the HSL in the WAT, as OLE increased HSL phosphorylation at Ser563 and Ser565 (Fig. 7D).

3.7. In vivo assessment of the OLE-mediated effects on *Acadm*, *Ldl-r* and *Pcsk9* expression

OLE up-regulated acyl-coenzyme A dehydrogenase (*Acadm*) mRNA expression in the murine liver (Fig. 8), but it did not affect it in the WAT. OLE also markedly increased the low-density lipoprotein receptor (*Ldl-r*) mRNA expression in the liver (Fig. 8). In contrast, the hepatic mRNA expression level of the proprotein convertase subtilisin/kexin type 9 (*Pcsk9*) was not markedly affected by OLE (Fig. 8).

4. Discussion

The data of the present study using *in silico*, *in vitro* and *in vivo* approaches demonstrate that OLE acts as a PPAR α ligand, which up-regulates a broad spectrum of factors in the liver and WAT that hold essential roles in lipid synthesis, transport, metabolism and clearance, thus leading to the reduction of serum TGs and cholesterol.

The effects of Mediterranean diet and, specifically, the olive bioproducts consumption have been thoroughly investigated by various clinical trials, which showed a significant reduction of morbidity and mortality in the consuming populations [41]. They were also associated with the prevention of metabolic syndrome, a cluster of risk factors that includes hypercholesterolemia, hypertriglyceridemia, atherosclerosis, high blood pressure, obesity, fatty liver and insulin resistance, all closely linked to coronary heart disease [42]. The ACCORD Trial comparing statin monotherapy with a statin plus fibrate on cardiovascular disease event rate in patients with type II diabetes indicated that fenofibrate plus statin produced an additional protective effect than statin alone. Subgroup analysis showed that patients with TG levels ≥ 204 mg/dl and HDL cholesterol levels ≤ 34 mg/dl benefited the most from fenofibrate therapy. Evaluation of the current literature on this topic suggests that when a patient reaches the maximum benefit on statin therapy, he will further benefit by adding fibrates in the therapeutic scheme provided that the TG serum levels are >200 mg/dl and HDL cholesterol levels are <35 mg/dl. Based on this evidence, in recent years, three of the main extra virgin olive oil's phenolic compounds, OLE and its two major metabolites, hydroxytyrosol and tyrosol, have attracted considerable attention due to their lipid-lowering effects in different hyperlipidemic animal models [21, 43]. It was reported that OLE, when administered in an experimental animal model of metabolic syndrome, improved glucose tolerance and normalized abdominal fat deposition, plasma TG and total cholesterol concentrations [20,44-46]. Several *in vitro* studies also supported the hypolipidemic effects of OLE. According to these studies, treatment of HepG2 and FL83B cells with OLE decreased free-fatty-acid-induced lipid accumulation, lipid droplet size and the intracellular TG content [47], which was also reduced by OLE in primary hepatocytes [48]. Despite intensive investigation, to this date, the biochemical mechanisms underlying the hypolipidemic effects of OLE remain vague, having been attributed mainly to its antioxidant and anti-inflammatory properties [44].

The central hypothesis of the present study was that OLE reduces serum TGs and cholesterol levels *via* PPAR α activation. In order to investigate this hypothesis, firstly, an *in silico* study was conducted using a molecular docking simulation model to assess if OLE is a PPAR α ligand. It was based on the fact that all known PPAR α agonists possess an acid group next to

Tyr464 and His440 to stabilize the AF2 domain in the agonist conformation. The theoretical model resulting from this work suggests that the methyl ester of OLE lies in the same location, forming hydrogen bond with the OH group of Tyr464. It has been previously reported that the presence of esterases and/or alkaline environment could demethylate OLE [49], resulting in a metabolite that could have enhanced its binding affinity. Additionally, the glucose residue is placed next to the entrance stabilized by several hydrogen bonds. The findings of the *in silico* study clearly indicated that OLE is a PPAR α ligand and were further corroborated by reporter gene assays showing that OLE increased PPRE-luc activity, indicating activation of the PPAR α -RXR α heterodimer.

The *in vivo* data of the present study indicated a clear association of PPAR α expression and activation with the OLE-induced reduction in serum TG and cholesterol levels, as OLE reduced them only in WT and not in Ppara-null mice. Notably, the data from the genomic study indicated that OLE induced *Ppara* expression in the liver of WT mice followed by induction of several hepatic PPAR α target genes, including *Acox1*, *Cyp4a10*, *Cyp4a14*, *Lipin1*, *Lipin2*, *Acot1*, *Acot4* and *Scd1*. *Pgc1* and *Rxra* mRNA expressions were also increased by OLE in the liver. These OLE-induced effects though were not observed in *Ppara*-null mice, indicating that *Pgc1* and *Rxra* are PPAR α regulated. Interestingly, the above-mentioned genomic data indicate an autoregulation mechanism for the mouse PPAR α gene. This has been previously demonstrated for the human PPAR α gene [50]. However, although several putative PPRES have been found in the mouse PPAR α promoter region [51], concrete evidence for a functional PPRES/DR1 is still lacking. It should be also noted that a comparative analysis of PPAR α regulated genes, using a whole genome transcriptomics approach, revealed a mostly divergent set of PPAR α -dependent genes in human and mouse hepatocytes [52].

Interestingly, in WT mice, OLE increased the hepatic expression of *Ldl-r*, the main receptor in the uptake and clearance of LDL cholesterol-rich and TG-poor lipoproteins. OLE also up-regulated several genes in the liver of WT mice that hold critical roles in the synthesis, uptake, transport, metabolism and elimination of TGs. In particular, OLE increased the hepatic expression of *Dgat1* and *Dgat2*, which are involved in the formation of TG from diacylglycerol and acyl-CoA [53]. *Atgl*, which encodes an enzyme catalyzing the rate-limiting hydrolysis step of TG in the triacylglycerol lipolysis cascade [54], was also up-regulated by OLE in the liver of WT mice. The hepatic expression of *Aadac* was also increased by OLE. This factor is mainly expressed in the liver displaying a cellular TG lipase activity, thus increasing the concentration of intracellular fatty acids derived from the hydrolysis of newly formed TG stores. It also holds a role in the assembly of very low-density lipoproteins [55]. *Mttp* that encodes the microsomal triglyceride transfer protein residing in the endoplasmic reticulum of hepatocytes and intestinal epithelial cells [56] was also up-regulated by OLE. LPL, which functions as TG hydrolase and ligand/bridging factor of Ldl-r-receptor-mediated lipoprotein up-take, was also up-regulated by OLE [57, 58]. In addition, *Ces3/Tgh*, which encodes enzymes catalyzing the hydrolysis of long-chain fatty acid esters and thioesters [59], and *Cd36*, encoding a scavenger receptor for lipoproteins, oxidized phospholipids and long-chain fatty acids [60-64], were up-regulated by OLE. Recently, it was demonstrated that the nuclear receptor Nr4A (Nur77) modulates lipid metabolism in hepatocytes by repressing the expression of *SREBP-1c*, thus inhibiting TG

synthesis [65]. Treatment of mice with OLE resulted in increased expression of this nuclear transcription factor in the liver. Furthermore, HSL mRNA expression and protein phosphorylation at Ser 565 and Ser 660 were also increased in the liver, indicating a potential increased hydrolysis rate of TGs. ANGPTL4, angiopoietin-like 4, which is a potent inhibitor of serum TG clearance, leading to elevation of serum TG levels *via* inhibition of LPL, is also up-regulated by OLE. Notably, OLE markedly up-regulated ACADM in the liver, which participates in the degradation of medium-chain fatty acids catalyzing the initial step of a mitochondrial fatty acid beta-oxidation pathway [66]. The OLE-induced up-regulation of the above mentioned genes in the liver is potentially mediated by activation of PPAR α as most of them are PPAR α target genes [67-70]. It is of interest also to note that no similar changes were observed in the expression of these factors in the WAT, with the exception of AADAC that functions as a cellular TG lipase. Interestingly though, OLE activated HSL, one of the major enzymes contributing to TG breakdown in the WAT [71]. Although OLE induced the expression of perilipin 5 (Plin5) in this tissue, apparently, this effect was not efficient enough to prevent the action of lipases and hydrolases on TG.

Collectively, based on the above *in silico*, *in vitro* and *in vivo* data, it appears that OLE acts as a PPAR α agonist that reduces serum TG and cholesterol levels. This beneficial effect of OLE on lipid homeostasis could be also attributed to its effect on several factors in the liver and WAT that hold essential roles in lipid up-take, synthesis, transport, metabolism and clearance, and it appears to be mediated mainly by PPAR α activation as most of them are regulated by PPAR α . It is likely though that as-yet-unidentified mechanisms, other than those involving PPAR α activation, LDL-r up-regulation and HSL phosphorylation, contribute to the hypolipidemic effect of OLE. These data and those from previous studies suggest that OLE could be used in the treatment of hypertriglyceridemias and other dyslipidemias and may be the basis for the design of new molecules with improved hypotriglyceridemic and hypolipidemic activities, and reduced side effects compared to statins and fibrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AADAC	arylacetamidedeacetylase
ACADM	acyl-CoA dehydrogenase
ACOX1	acyl-CoA oxidase
ACOT	acyl-CoA thioesterase
ATGL	adipose triglyceride lipase

BAAT	bile acid CoA
Cd36	cluster of differentiation 36
Ces3/tgh	carboxylesterase 3
DGAT	diacylglycerol O-acyltransferase
EIA	Elisa
FFA	free fatty acids
HDL	high-density lipoprotein
HSL	hormone-sensitive lipase
IFD	Induced Fit Docking
LPL	lipoprotein lipase
LDL-r	low-density lipoprotein receptor
MTTP	microsomal triglyceride transfer protein
NEFA	nonesterified fatty acids
Nr4A	nuclear receptor 4a
ANGPTL4	angiopoietin-like 4
SCD1	stearoyl-CoA desaturase-1
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1- alpha
OLE	oleuropein
PCSK9	proprotein convertase subtilisin/kexin type 9
PKA	protein kinase A
PPARα	peroxisome proliferator-activated receptor- α
PCR	polymerase chain reaction
RIA	radioimmunoassay
TG	triglycerides
VLDL	very low density lipoprotein
WAT	white adipose tissue.

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Fig. 1.
Chemical structures of OLE, indeglitazar and GW409544 (PPAR α ligand).

Fig. 2.

(A) SZMAP water analysis of PPAR α . Yellow-colored water molecules indicate hydrophilic regions, while purple-colored water molecules indicate hydrophobic regions. Hydrophilic fragments near yellow regions enhance binding affinity. (B) Superposition of the crystal structure of indeglitazar (green) inside the binding pocket of PPAR α , with the global minimum of OLE (orange) and GW409544 (cyan). (C) Binding mode of OLE minimum energy conformation inside the binding pocket of PPAR α . (D) Interactions of OLE minimum energy conformation inside the binding pocket of PPAR α . For reasons of clarity, only crucial amino acids are shown. Hydrogen bonds are depicted as red dashed lines.

Fig. 3.

HepG2 cells were co-transfected using PPRE firefly luciferase construct p(AOX)₃-TKSL with the plasmids expressing PPAR α and RXR α along with β -galactosidase plasmid. Cells were treated with either OLE (10 μ M) or the PPAR agonist WY-14643 (10 μ M) for 24 h and harvested 48 h after transfection, lysed and assayed for luciferase and β -galactosidase activities. Bars represent luciferase activities normalized to β -galactosidase activities (internal control). Values are expressed as mean $\pm$ S.E., $n=6$ per treatment group. * $P<.05$, ** $P<.01$.

Fig. 4.

The effect of OLE treatment on hepatic *Ppara* expression. (A) PPARα protein levels were determined in liver nuclear fractions by Western blot analysis. Histone H3 served as a loading control. In the bar graph, the quantified data from the Western blot image are presented as the ratio of PPARα/Histone3. Following treatment with OLE, hepatic *Ppara* mRNA levels were analyzed by qPCR in SV129 WT and *Ppara*-null mice. (B) *Acox1*, *Cyp4a10*, *Cyp4a14*, *Acot1*, *Acot4*, *Lipin1*, *Lipin2*, *Scd1*, *Pgc1a* and *Rxra* mRNA levels were also analyzed with qPCR. Values were normalized to β -actin and are expressed as mean $\pm$ S.E., $n=5-7$ mice per treatment group. Comparisons were between controls and OLE-treated mice. Group differences were calculated by one-way ANOVA followed by Bonferroni's test. * $P<.05$, ** $P<.01$, *** $P<.001$.

In vitro experiment

Fig. 5.

(A) *Ppara* mRNA levels were determined by qPCR following treatment of primary hepatocyte cultures with OLE for 2 h. (B) Effect of OLE on *Acox1*, *Cyp4a14*, *Acot4* and *Lipin1* mRNA expression (PPAR α target genes) in primary hepatocytes was also evaluated. Values in panels A and B were normalized to β -actin and are expressed as mean $\pm$ S.E., $n=6$ per treatment group. DMSO-treated primary hepatocytes served as controls, and comparisons were between DMSO and drug-treated hepatocytes. Calculation by Bonferroni's test. * $P<.05$, ** $P<.01$, *** $P<.001$.

Fig. 6.

OLE-induced effect on hepatic TG homeostasis. (A) Effect of OLE on genes involved in TG synthesis and lipolysis in the liver. (B) Effect of OLE on genes involved in TG metabolism and clearance in the liver. (C) Effect of OLE on HSL phosphorylation in the liver.

Comparisons were between controls and OLE-treated mice. *Dgat1*: diacyl glycerol acyltransferase, *Dgat2*: diacyl glycerol acyltransferase 2, *Lpl*: lipoprotein lipase, *Hsl*: hormone-sensitive lipase, *Atgl/Pnpla2*: adipose triglyceride lipase/patatin-like phospholipase domain containing 2, *Nr4A*: orphan nuclear receptor NR4A, *Aadac*: arylacetamide deacetylase, *Cd36*: cluster of differentiation 36 or fatty acid transporter, *Ces3/tgh*: carboxylesterase 3, *Mttp*: microsomal triglyceride transfer, *Angptl4*: angiopoietin-like-4, C: control. Values are expressed as mean±S.E., $n=5-7$ per treatment group. # $P<.025$, ** $P<.01$, *** $P<.001$.

Fig. 7.

OLE-mediated effect on TG homeostasis in the WAT of WT mice fed a regular diet. (A) Effect of OLE on the expression of genes involved in TG synthesis and lipolysis. (B) Effect of OLE on the expression of genes involved in TG metabolism and clearance. (C) Effect of OLE on the expression of *Ppara* and the target gene *Acox1* in the WAT (D) Effect of OLE on factors important in lipid β -oxidation, the clearance of TG rich lipoproteins and the transport of free fatty acids. Comparisons were between controls and OLE-treated mice. *Dgat1*: diacyl glycerol acyltransferase 1 (acyl coenzyme A (CoA), *Dgat2*: diacyl glycerol acyltransferase 2, *Lpl*: lipoprotein lipase, *Hsl*: hormone-sensitive lipase, *Atgl/Pnpla2*: adipose triglyceride lipase/patatin-like phospholipase domain containing 2, *Nr4A*: orphan nuclear receptor, *Aadam*: arylacetamide deacetylase, *Cd36*: cluster of differentiation 36 or fatty acid transporter, *Ces3/tgh*: carboxylesterase 3, *Mttp*: microsomal triglyceride transfer, *Angptl4*: angiopoietin-like-4, PLIN5: perilipin 5, *Ppara*: peroxisome proliferator activated receptor α , *Acox1*: acyl-CoA oxidase, C: control. Values are expressed as mean $\pm$ S.E., * $P < 0.05$, *** $P < 0.001$, $n = 5-7$ per treatment group. Lanes in Western blots correspond to one sample per treatment and represent one sample of five separate samples tested in different blots.

Fig. 8.

OLE-induced effect on *Acadm*: acyl-CoA dehydrogenase, *Ldl-r*: low density lipoprotein receptor and *Pcsk9*: protein convertase subtilisin/kexin type 9 mRNA expression in the liver and WAT of WT mice fed the regular rodent diet. Values were normalized to β -actin and are expressed as mean $\pm$ S.E., $n=6-7$ per treatment group. Comparisons took place between controls and OLE-treated mice. *** $P<.001$.

Table 1

Oleuropein-induced alterations in serum lipid markers and liver TG content

	WT		<i>Ppara</i> null	
	Control	OLE	Control	OLE
TG	79.7±7.9	39.6±2.9 [*]	182.3±6.7	192.7±9.0
FFA	0.3±0.1	0.5±0.1	0.7±0.04	0.9±0.04
t-Cholesterol	139.0±8.3	93.0±6.1 [*]	119.6±8.2	123.2±7.0
Liver TG content	141.3±19.5	108.4±11.5 [*]	161.0±5.0	162.5±0.7

Effect of OLE treatment on serum TG and t-Cholesterol levels, as well as on liver TG content, of SV129 WT and *Ppara*-null mice. Serum TGs in mg/dl; FFA: free fatty acids in mEq/l; t-Cholesterol in mg/dl; liver TG content in mg/g tissue; *n*=5–7 adult male mice per treatment group;

^{*} *P*<.001.