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Extra virgin olive oil phenols down-regulate lipid synthesis in primary-cultured rat-hepatocytes☆

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Abstract

Hydroxytyrosol, tyrosol, and oleuropein, the main phenols present in extra virgin olive oil, have been reported to exert several biochemical and pharmacological effects.

Here, we investigated the short-term effects of these compounds on lipid synthesis in primary-cultured rat-liver cells. Hydroxytyrosol, tyrosol and oleuropein inhibited both *de novo* fatty acid and cholesterol syntheses without an effect on cell viability. The inhibitory effect of individual compounds was already evident within 2 h of 25 μM phenol addition to the hepatocytes. The degree of cholesterogenesis reduction was similar for all phenol treatments (−25/30%), while fatty acid synthesis showed the following order of inhibition: hydroxytyrosol (−49%) = oleuropein (−48%) > tyrosol (−30%). A phenol-induced reduction of triglyceride synthesis was also detected.

To clarify the lipid-lowering mechanism of these compounds, their influence on the activity of key enzymes of fatty acid biosynthesis (acetyl-CoA carboxylase and fatty acid synthase), triglyceride synthesis (diacylglycerol acyltransferase) and cholesterogenesis (3-hydroxy-3-methyl-glutaryl-CoA reductase) was investigated *in situ* by using digitonin-permeabilized hepatocytes. Acetyl-CoA carboxylase, diacylglycerol acyltransferase and 3-hydroxy-3-methyl-glutaryl-CoA reductase activities were reduced after 2 h of 25 μM phenol treatment. No change in fatty acid synthase activity was observed. Acetyl-CoA carboxylase inhibition (hydroxytyrosol, −41%, = oleuropein, −38%, > tyrosol, −17%) appears to be mediated by phosphorylation of AMP-activated protein kinase. These findings suggest that a decrease in hepatic lipid synthesis may represent a potential mechanism underlying the reported hypolipidemic effect of phenols of extra virgin olive oil.

Keywords: Acetyl-CoA carboxylase; AMP-activated protein kinase; Extra virgin olive oil; Lipid synthesis; Phenols; Rat-hepatocytes

1. Introduction

The Mediterranean diet is associated with a low incidence of various chronic degenerative pathologies such as atherosclerotic cardiovascular diseases, neurological disorders and cancer [1–3]. Extra virgin olive oil (EVOO), the main fat source of this diet, is generally considered to be a major contributor to human health in the Mediterranean area [4,5]. New studies are extending EVOO action to the prevention of the metabolic syndrome, a cluster of risk factors that includes hypercholesterolemia, hypertriglyceridemia, high blood pressure, obesity, fatty liver and insulin resistance, all closely linked to diabetes and coronary heart disease [6,7].

The beneficial effects of EVOO have historically been attributed to its elevated oleic acid content; more recently, converging evidence indicates that the EVOO non-saponifiable fraction, rich in phenols, significantly promotes human health [8,9]. This fraction contains a number of phenols ranging from the simple compounds hydroxytyrosol (2,(3,4-dihydroxyphenyl)-ethanol, HTyr, Fig. 1A) and tyrosol (p-hydroxy-phenyl ethanol, Tyr, Fig. 1B), to several products of conjugation of these phenols with elenolic acid, such as oleuropein (Ole, Fig. 1C) [10].

Studies on the mechanism of action of EVOO phenols have mainly focused on their antioxidant and anti-inflammatory properties [11–13]. However, in the last decade several lines of evidence indicate that EVOO phenolic compounds may also possess anti-atherosclerotic and anti-diabetic characteristics [5,9,14,15]. *In vivo* experiments have demonstrated that oral administration of HTyr [16] or Ole [17] to hypercholesterolemic rats significantly lowers the serum levels of total cholesterol, triglycerides (TG) and low density lipoprotein (LDL)-cholesterol, and increases the serum level of high density lipoprotein (HDL)-cholesterol. Diabetic rats fed a diet supplemented with EVOO showed lower serum levels of TG when compared to

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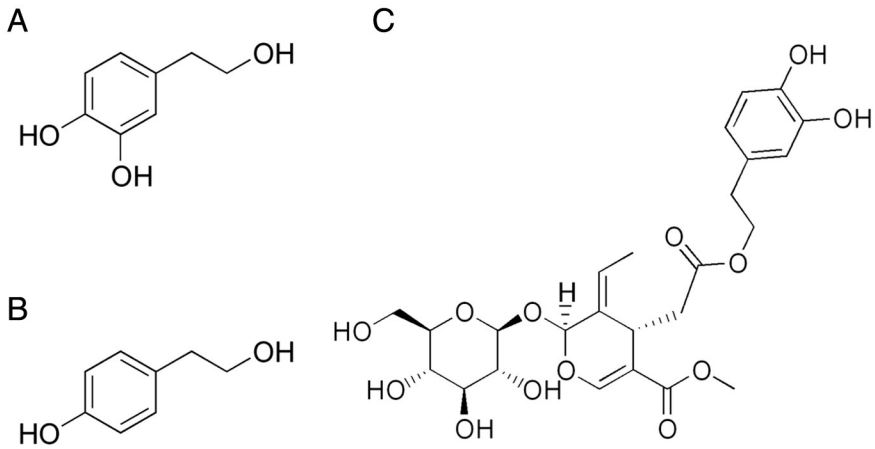

Fig. 1. Chemical structures of EVOO phenols. Chemical structures of hydroxtyrosol (A), tyrosol (B) and oleuropein (C).

animals fed a sunflower oil-enriched diet, suggesting that EVOO provides better control of the hypertriglyceridemia accompanying diabetes [18]. Similarly, administration of Ole- and HTyr-rich extracts from olive leaves, during four weeks significantly reduced serum glucose and cholesterol levels in alloxan-induced diabetic rats [15]. Ole supplementation can attenuate liver steatosis induced by high-fat diets in mice by decreasing hepatic concentrations of cholesterol [19]. In an international study with 200 healthy male volunteers, Covas et al. [20] showed that EVOO phenols are able to increase plasma HDL-cholesterol levels as well as to reduce TG level.

All these studies highlight the effect of EVOO phenolic compounds on serum levels of cholesterol and TG in animals under particular nutritional and hormonal conditions. However, the EVOO hypolipidemic action has not been closely investigated. Liver is a key organ in the synthesis, the storage and the excretion of lipids through very low-density lipoprotein (VLDL); alterations in hepatic lipogenesis will have an impact on serum lipid levels.

To date, there have been no reports in the literature that have assessed the direct effects of EVOO phenolic compounds on hepatic lipid metabolism under regular hormonal and nutritional conditions. In the present study we investigated this aspect showing that addition of EVOO phenols to hepatocytes from normal, untreated rats, induced short-term reduction of fatty acid, cholesterol and TG syntheses. Moreover, acetyl-CoA carboxylase (ACC), 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) and diacylglycerol acyltransferase (DGAT) activities as well as AMP-activated protein kinase (AMPK) were involved in tuning down lipid synthesis.

2. Methods and materials

2.1. Materials

HTyr (≥90% purity), Tyr (≥95% purity) and Ole (≥98% purity) were obtained from Extrasynthese (Genay Cedex, France). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, phosphate buffered saline (PBS) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Gibco-Invitrogen (Paisley, UK); [1-^{14}C]acetate was obtained from GE Healthcare (Little Chalfont, UK); [1-^{14}C]acetyl-CoA, [3-^{14}C]HMG-CoA and [1-^{14}C]palmitoyl-CoA were obtained from PerkinElmer (Boston, MA, USA). Primary antibodies for ACCα, pACCα, AMPKα, pAMPKα, and α-tubulin as well as horseradish peroxidase conjugated IgGs were obtained from Cell Signaling Technologies (Boston, MA, USA). All other reagents, obtained from Sigma-Aldrich, were of analytical grade.

2.2. Animals and Ethical Statement

Male Wistar rats (200–250 g) were used throughout this study. Animals had free access to tap water and were fed *ad libitum* with a chow diet consisting of: 18.6% crude protein, 44.2% carbohydrate, 6.2% crude fat with adequate amounts of essential fatty acids, 3.5% crude fiber, 14.7% neutral detergent fiber, 5.3% ash, and a salt and vitamin mixture. The rats were housed individually in a temperature- (22 ± 1°C) and light-controlled (light on 08:00–20:00) environment. All rats received care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, published by the National Institutes of Health (NIH Publication No. 86–23, revised 1985), as well as in accordance with Italian laws on animal experimentation (art. 4 and 5 of D.L. 116/92).

2.3. Preparation of rat-liver cells

Rat-liver cells were isolated by perfusing the liver with collagenase as previously described [21]. Cells were suspended in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2. Unless specified otherwise, primary hepatocytes were seeded at a density of 7×10^5 cells per 35 mm diameter Petri dishes; 2 h after plating, the medium was refreshed and different phenols (HTyr, Tyr and Ole dissolved in dimethyl sulfoxide, DMSO) were added to the serum-rich (10% FBS) medium for a period of 2 h. To determine the putative effects of the phenolic compounds on cell viability, a colorimetric MTT assay was performed as described in [22]. In each experiment and for each determination, control dishes incubated with DMSO were used.

2.4. Determination of fatty acid and cholesterol synthesis

Lipogenic activity was determined by monitoring the incorporation of [1-^{14}C] acetate (16 mM, 0.96 mCi/mol) into fatty acids and cholesterol essentially as reported [23]. Cells were incubated for 0.5, 1 and 2 h with the indicated phenol concentration (2.5–100 μM).

To terminate the lipogenic assay, the medium was aspirated, cells were washed three times with ice-cold PBS to remove unreacted labelled substrate and the reaction was stopped with 1.5 ml of 0.5 M NaOH. The cells were scraped off with a rubber policeman and transferred to a test tube; 100 μl of cells were reserved for a protein assay [23]. The remaining cells were saponified with 4 ml of ethanol and 2 ml of double-distilled water for 90 min at 90°C. Non-saponifiable sterols and fatty acids (after acidification with 1 ml of 7 M HCl) were extracted with 3×5 ml of petroleum ether. The extracts were collected, dried under a stream of nitrogen, and counted for radioactivity.

2.5. Incorporation of radiolabelled acetate into lipid fractions

Newly synthesized labelled fatty acids are mainly incorporated into complex lipids, therefore, neutral lipid analysis was also carried out. Experimental conditions were the same as those for fatty acid and cholesterol synthesis assays. After 2 h of incubation with 25 μM EVOO phenols, the reaction was stopped by washing the cells three times with ice-cold PBS and treating them with 2 ml of KCl/CH_3OH (1:2, v/v). Total lipids were extracted according to Giudetti et al. [23].

Neutral lipids were resolved by thin layer chromatography (TLC) on silica gel plates by using hexane:ethylether:acetic acid (70:30:10, v/v/v) as a developing system. Lipid spots, visualized by iodine vapour, were individually scraped from the plate into counting vials for radioactivity measurement.

2.6. Assay of enzymatic activities of de novo fatty acid synthesis

ACC activity was determined by measuring the incorporation of radiolabelled acetyl-CoA into fatty acids in an assay coupled with the fatty acid synthase (FAS) reaction in digitonin-permeabilized hepatocytes essentially as described by Priore et al.