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Extravirgin olive oil up-regulates CB_1 tumor suppressor gene in human colon cancer cells and in rat colon via epigenetic mechanisms

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Abstract

Extravirgin olive oil (EVOO) represents the typical lipid source of the Mediterranean diet, an eating habit pattern that has been associated with a significant reduction of cancer risk. Diet is the more studied environmental factor in epigenetics, and many evidences suggest dysregulation of epigenetic pathways in cancer. The aim of our study was to investigate the effects of EVOO and its phenolic compounds on endocannabinoid system (ECS) gene expression via epigenetic regulation in both human colon cancer cells (Caco-2) and rats exposed to short- and long-term dietary EVOO.

We observed a selective and transient up-regulation of *CNR1* gene — encoding for type 1 cannabinoid receptor (CB_1) — that was evoked by exposure of Caco-2 cells to EVOO (100 ppm), its phenolic extracts (OPE, 50 μM) or authentic hydroxytyrosol (HT, 50 μM) for 24 h. None of the other major elements of the ECS (i.e., CB_2; GPR55 and TRPV1 receptors; and NAPE-PLD, DAGL, FAAH and MAGL enzymes) was affected at any time point. The stimulatory effect of OPE and HT on CB_1 expression was inversely correlated to DNA methylation at *CNR1* promoter and was associated with reduced proliferation of Caco-2 cells. Interestingly, *CNR1* gene was less expressed in Caco-2 cells when compared to normal colon mucosa cells, and again this effect was associated with higher level of DNA methylation at *CNR1*. Moreover, in agreement with the *in vitro* studies, we also observed a remarkable (~4-fold) and selective increase in CB_1 expression in the colon of rats receiving dietary EVOO supplementation for 10 days. Consistently, CpG methylation of rat *Cnr1* promoter, *miR23a* and *miR-301a*, previously shown to be involved in the pathogenesis of colorectal cancer and predicted to target CB_1 mRNA, was reduced after EVOO administration down to ~50% of controls.

Taken together, our findings demonstrating CB_1 gene expression modulation by EVOO or its phenolic compounds via epigenetic mechanism, both *in vitro* and *in vivo*, may provide a new therapeutic avenue for treatment and/or prevention of colon cancer.
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1. Introduction

Cancer is a leading cause of death worldwide, and the number of cases and deaths is expected to increase more than twice in the next 20–40 years [1]. According to current estimates, the disease is 30%–40% preventable over time with appropriate nutrition and regular physical activity [2]. Several bioactive food components (BFCs) endowed with cancer prevention potential have been identified, including methyl-group donors, polyphenols, fatty acids, isothiocyanates and allyl compounds [3].

Olive oil represents the typical lipid source of the Mediterranean diet, an eating habit associated with a low incidence of several disease conditions [4–6]. Among these, convincing evidence suggests that olive oil consumption contributes to a significant reduction of colon cancer risk in Mediterranean populations [7,8].

Type 1 cannabinoid receptor (CB_1) is a tumor suppressor gene that exerts antiproliferative effects on colorectal cancer cells and that plays a major role in the regulation of signaling pathways that control

Abbreviations: Caco-2 cell, CNR1, cannabinoid type 1 receptor gene; CB, cannabinoid receptor, human carcinoma colon cells; EVOO, extravirgin olive oil; ECS, endocannabinoid system; OPE, olive oil phenolic extract; HT hydroxytyrosol; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.

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cell survival and apoptosis [9–11]. The mechanism of the CB_1-mediated induction of tumor cell apoptosis has been investigated in detail by several groups and has been recently reviewed [12]. Type 1 cannabinoid receptor$_1$ is one of the major elements of the "endocannabinoid system" (ECS), an ensemble of receptors (like CB_2, GPR55 and TRPV1) and metabolic enzymes (like NAPE-PLD, DAGL, FAAH and MAGL) of endogenous lipids called "endocannabinoids" [13]. Several studies have documented a dysregulation of ECS in various disease conditions, including cancer [14–19].

The aim of the present study was to investigate extravirgin olive oil (EVOO) effects on modulation of ECS gene expression via epigenetic regulation in human colon cancer (Caco-2) cells, as well as in colon mucosa of rats exposed to dietary EVOO. It should be recalled that epigenetic silencing of tumor suppressor genes due to site-specific promoter hypermethylation is a common feature of the cancer epigenome and represents an alternative to mutations as a cause of loss in gene function [20]. It has been reported that numerous BFCs can interfere with DNA methylation and histone modifications, thus affecting the expression of genes involved in cancer development [21–24]. However, there is a lack of knowledge on regulation of epigenetic mechanisms by EVOO. Moreover, both EVOO phenolic and lipid fractions contain a variety of antioxidant and antitumoral substances, and due to mutual interactions among them, besides measuring protective effects of each individual component, it is of clear relevance to measure possible antitumoral activity of naive EVOO. So far, the properties of olive oil phenolic extract (OPE) and of naive EVOO have been poorly investigated, and based on all the above observations, our study on their roles on regulation of genes potentially involved in cell proliferation, such as those belonging to ECS, might be of help to clarify the mechanisms behind EVOO beneficial effects.

2. Materials and methods

2.1. Reagents and biological materials

Dulbecco's modified Eagle's medium (DMEM) with 4.5 mg/ml glucose, fetal calf serum (FCS), nonessential amino acids, *N*-(2-ydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid (HEPES), glutamine, penicillin, streptomycin and phosphate-buffered saline were from Gibco Life Science Technologies (S. Giuliano Milanese, Milan, Italy). 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 5-aza-deoxycitidine (5-aza-dC) were from Sigma-Aldrich (Milan, Italy). *N*-Piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazolecarboxamide (rimonabant) was a kind gift from Sanofi-Aventis Recherche (Montpellier, France).

2.2. Olive oil and phenolic extracts

Extravirgin olive oil was obtained using the local Leccino monocultivar fruits (the most common olive cultivar currently used around the world) and was a kind gift of "Frantoio Montecchia, Morro d'Oro, Teramo." In order to obtain a rectified olive oil (ROO) devoid of phenolic compounds, EVOO samples were purified by a solid phase extraction procedure. Extravirgin olive oil and its ROO derivatives were stored in dark bottles at 4°C until use. Afterwards, EVOO was filtered (with filters up to 0.22-mm pore size) and was diluted first in dimethyl sulfoxide (DMSO) and then in culture media to the desired concentrations. Extravirgin olive oil phenolic extracts (OPE) and hydroxytyrosol (HT) were first dissolved in DMSO and then in culture media to obtain a final concentration of 50 μM.

2.3. Extraction of phenolic compounds and their analysis

Commercially available octadecyl C18 cartridges (1 g, 6 ml) (International Sorbent Technology, UK) were used for the extraction of the phenolic fraction, according to the following protocol: 1 g of olive oil was dissolved in 5 ml of hexane, and the obtained solution was loaded onto a column previously conditioned with 2×5 ml of methanol and 2×5 ml of hexane. The column was eluted with 2×5 ml of hexane to completely remove the lipophilic fraction, and the retained polar compounds were recovered by eluting with 2×10 ml of methanol. Subsequently, the eluate was placed in a conical flask and evaporated to dryness at room temperature (30°C, 150 rpm) in a rotary evaporator. The extract was recovered with 0.5 ml of DMSO. The total phenolic content (TP) of the DMSO extracts (and standards) was evaluated colorimetrically using the Folin–Ciocalteau reagent. A volume (20 or 10 μl) of extract or phenolic standard was mixed with 20 μl of Folin–Ciocalteau reagent and shaken for 3 min. Afterwards, 400 μl of a solution of 7.5% Na_2CO_3 was added, and then it was brought to a final volume of 1000 μl with deionized water. Solutions were maintained at room temperature in agitation for 60 min, and the total phenolic content was determined colorimetrically at 760 nm. Gallic acid standard solutions were used to calibrate the method. The single phenolic compounds of the olive oil were determined according a reversed-phase high-performance liquid chromatography (HPLC) procedure [25]. An HPLC Perkin Elmer 200 Series instrument equipped with a UV–Vis detector set at 280 nm was used. A column Kinetex C18 (Phenomenex) of 5-μm particle size and 250-mm, 4.6-mm ID was used. The mobile phase flow rate was 1.0 ml/min. The injection volume was 10 μl. Analyses were carried out at room temperature. The gradient elution was carried out using water/formic acid (99.5:0.5, v/v) as mobile phase A and acetonitrile as mobile phase B of the solvent system. The total run time was 75 min. Phenolic compounds were quantified using a calibration curve made with 3,4-dihydroxyphenylacetic acid (Sigma-Aldrich) (r^2=0.999). The average was calculated by tree replications for each sample. Finally, a portion of the olive oil methanolic extract was dried and successively recovered with DMSO to obtain OPE to a concentration 1 mM considering an average molecular weight of 170 g/mol.

2.4. Cell cultures and treatments

Experiments were carried out by using the tumor-derived human colon cancer cell line Caco-2, as well as the epithelial cell line NCM460 derived from the healthy colon mucosa of a 68-year-old Hispanic male [26]. Caco-2 cells were obtained from ATCC (Rockville, MD, USA) and were routinely grown in DMEM supplemented with heat-inactivated fetal bovine serum (10% v/v), 2 mM glutamine, 1% nonessential amino acids, HEPES (25 mM), penicillin (100 U/ml) and streptomycin (100 mg/ml). NCM460 cells were grown as a monolayer culture in M3Base medium (which contains growth supplements and antibiotics) supplemented with 10% heat-inactivated FCS and 2.5 mM of D-glucose (final concentration 5 mM glucose, 2 mM glutamine). Cell cultures were maintained at 37°C in a 95% air, 5% CO_2 and humidified environment. The intake of olive oil in the Mediterranean countries is estimated to be 30–50 g/day [27], corresponding to about 10–15 mg of total phenols/day when considering the olive oil used in this study (320 mg of total phenols/kg olive oil). Taking into account the absorption (around 55%–66% of the ingested olive oil phenols) [27] and the distribution volume in the plasma compartment, it can be estimated that the circulating levels of olive oil phenols were in the μM range.

2.5. Cell growth analysis

To determine cell proliferation, the MTT test was performed as reported [28]. Caco-2 cells were seeded onto 96-well plates (7.5×10^3 cells/well) and were starved by serum deprivation for 24 h. At the beginning of the experiment, this medium was replaced with fresh medium (untreated cultures) or with medium containing increasing dilutions of OPE or HT. The antiproliferative effect of phenolic compounds was also evaluated in the presence of rimonabant (0.1 μM, CB_1 receptor antagonist) incubated 30 min before phenolic OPE or HT. After 24 and 48 h of incubation, the supernatants were replaced with 0.1 ml of fresh medium without phenol red, containing 0.5 mg/ml of MTT. The samples were returned in the incubator for 4 h and gently shaken occasionally. Then, crystals of formazan (MTT metabolic product) were solubilized by 0.1 ml ethanol/DMSO 1:1 lysis buffer and spectrophotometrically quantified (Thermo Fisher Scientific, Waltham, MA, USA) at a wavelength of 570 nm with reference at a wavelength of 695 nm. Differences in cell growth were measured as a percentage of growth rates of treated cells compared to untreated cultures.

2.6. Analysis of gene expression by quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR)

Relative abundance of each mRNA species was assessed by real-time qRT-PCR using SensiFAST SYBR Kit (Bioline, London, UK) on an DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research, Waltham, MA, USA). All data were normalized to the endogenous reference genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin and rRNA 18s. Differences in threshold cycle (Ct) number were used to quantify the relative amount of PCR target contained in each tube. Relative expression of different gene transcripts was calculated by the ΔΔCt method and converted to relative expression ratio ($2^{\wedge-\Delta\Delta Ct}$) for statistical analysis. After qRT-PCR, a dissociation curve was generated in the range of 60°C to 95°C to evaluate the specificity of the amplification products. Human and rat primers used for PCR amplification were designed using Primer 3 software (see Table S1 in supplementary material).

2.7. DNA methylation analysis by methylation-specific PCR (MSP)–qPCR and pyrosequencing

Genomic DNA was extracted from cells or tissues by using TRIzol Reagent (Life Technologies) and was subjected to bisulfite modification by means of a commercially available modification kit (Zymo Research, Irvine, CA, USA). The schematics of CpG island in *CNR1* promoter and regions analyzed by MSP and pyrosequencing are illustrated in supplementary material (Fig. S1). In MSP–qPCR, two pairs of PCR primers were designed to span 15 CpG sites within the *CNR1* promoter region. Methylation

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