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Rosemary polyphenols induce unfolded protein response and changes in cholesterol metabolism in colon cancer cells

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ABSTRACT

Several studies have demonstrated that rosemary polyphenols exert changes in the lipid metabolism in adipose and hepatic cells. In this work, the effects of a polyphenol-enriched supercritical rosemary extract (SC-RE) and carnosic acid (CA) on the transcriptome and cholesterol metabolism in HT-29 colon cancer cells were examined using a Foodomics approach. Targeted metabolomics analysis indicated that the SC-RE treatment induced cholesterol accumulation after 24 h. Transcriptomic analysis suggested that most of the changes induced by the SC-RE and CA were orchestrated by unfolded protein response (UPR) and triggered by endoplasmic reticulum stress. Results suggested up-regulation of VLDLR gene as the principal contributor to the observed cholesterol accumulation in SC-RE-treated cells. In addition, the SC-RE attenuated the activity of *E2F* transcription factor, down-regulating several genes involved in G1–S transition of the cell cycle.

1. Introduction

Rosemary (*Rosmarinus officinalis L.*) polyphenols possess numerous desirable characteristics, including antioxidant, anti-inflammatory and chemoprotective activities for the prevention or treatment of diseases (Ben Jemia et al., 2013; Johnson, 2011; Xiang et al., 2013). In the last few years, many reports have demonstrated the *in vitro* antiproliferative activity of rosemary polyphenols in several cancer cell models (Petiwala, Puthenveetil, & Johnson, 2013; Tsai, Lin, Lin, & Chen, 2011; Valdés et al., 2012). Regarding colon cancer, despite investigations on rosemary polyphenols in the last years, most *in vitro* studies use targeted traditional biological approaches (biochemical analysis of specific compounds) trying to understand the molecular mechanisms of their antiproliferative activities.

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Abbreviations: CA, carnosic acid; DCF-DA, 2′,7′-dichlorofluorescin diacetate acetyl ester; DEGs, differentially expressed genes; ER, endoplasmic reticulum; ERAD, ER-associated degradation; FC, free cholesterol; FDR, false discovery ratio; IPA, ingenuity pathway analysis; ROS, reactive oxygen species; SC-RE, supercritical rosemary extract; SIM, selected ion monitoring; TC, total cholesterol; UPR, unfolded protein response; UR, upstream regulator

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Following this common strategy, a broad range of cellular and molecular effects of these compounds have been described in the literature (Barni, Carlini, Cafferata, Puricelli, & Moreno, 2012; Yana, Lia, Petiwala, Householter, & Johnson, 2015). In addition, the application of novel Foodomics approaches (García-Cañas, Simó, Herrero, Ibáñez, & Cifuentes, 2012) for the comprehensive analysis of different *in vitro* cancer cell models have demonstrated that rosemary polyphenols exert substantial modulation of the transcriptome and metabolome (Ibáñez, Simó et al., 2012; Ibáñez, Valdés et al., 2012). This general conclusion in all these studies supports the idea that the underlying mechanisms of action of rosemary extracts are diverse and complex, not only because of the reported pleiotropic cellular and molecular effects of some of the major constituents, but also for the presence of other compounds with unknown bioactivities.

Recently, novel bioactivities of rosemary extracts and the two major phenolic diterpenes present in rosemary, i.e., carnosic acid (CA) and carnosol, have been linked to glucose and lipid metabolism. In this regard, studies on different animal models indicate that rosemary polyphenols limit lipid absorption *in vivo* through their inhibitory activity against lipase (Ibarra et al., 2011), and protect against steatosis by reducing fasting plasma triacylglycerol and cholesterol levels (Wang et al., 2011). Different studies, although reporting contrasting results, suggest that rosemary extracts and some of their major compounds regulate the activity of different master transcriptional regulators of cell metabolism such as *PPARγ*, *C/ebpα*, and *C/ebpβ* (Gaya et al., 2013; Ibarra et al., 2011). Despite recent *in vivo* and *in vitro* studies that have provided significant insight into the mechanisms underlying the antiadipogenic effect of rosemary compounds in adipose and hepatic cells, very limited information is available about the effect of these compounds on lipid metabolism in cancer cells. Altered lipid metabolism has long been recognized as an important feature of cancer cell metabolic reprogramming and a prerequisite for the rapid proliferation rates (Santos & Schulze, 2012). Indeed, inhibition of cholesterol and fatty acid biosynthesis pathways constitute an important strategy to block cancer cell growth (Abramson, 2011). Previous results obtained in our laboratory, using gene expression microarrays, have shown that rosemary polyphenols alter expression of various genes involved in cholesterol biosynthesis, trafficking and metabolism in colon cancer cells (data not shown). Thus, it is tempting to speculate (based on these observations) that some rosemary polyphenols may also exert modulating effects on cholesterol metabolism in colon cancer cells. In order to explore this aspect and its potential connection with the antiproliferative activity of rosemary compounds, in this work, colon cancer cells were treated with CA and a SC-RE enriched in CA and their effect on cholesterol metabolism and transcriptome was investigated. The relations between the observed metabolic changes and the transcriptional responses in colon cancer cells after the mentioned treatments can provide crucial information on the molecular mechanisms behind the observed changes in metabolism and cell proliferation. To attain this, a Foodomics approach, based on targeted metabolomics, transcriptomics and bioinformatics tools, is applied to analyze the different levels of information obtained from HT-29 colon cancer cells exposed to CA and the SC-RE.

2. Materials and methods

2.1. Standards and supercritical rosemary extract samples

CA and cholesterol were purchased from Sigma (St. Louis, MO, USA); ergosterol was purchased from Acros Organics (Geel, Belgium). SC-RE was obtained from dried rosemary leaves using supercritical CO_2 and 7% ethanol at 150 bar as reported in Sánchez-Camargo et al. (2014). Chemical characterization of the SC-RE indicated that two main diterpenes, CA and carnosol, were found at high concentrations in the extract, namely, 363.3 and 45.6 μg/mg extract, respectively. The chromatographic analysis of the SC-RE is shown in Supplementary Fig. S1. Dry extract and standards samples were dissolved in dimethyl sulfoxide (DMSO) at 30 mg/mL and 100 mM, respectively, and stored as aliquots at –80 °C until use.

2.2. Cell culture conditions

Colon adenocarcinoma HT-29 cells obtained from ATCC (American Type Culture Collection, LGC Promochem, Middlesex, UK) were grown in McCoy's 5A supplemented with 10% heat-inactivated fetal calf serum, 50 U/mL penicillin G, and 50 U/mL streptomycin, at 37 °C in humidified atmosphere and 5% CO_2. When cells reached ~50% confluence, they were trypsinized, neutralized with culture medium, plated in different culture plates at 10,000 cells/cm^2 and allowed to adhere overnight at 37 °C.

2.3. Cell proliferation assays and cell cycle flow cytometry analysis

Cell viability was determined using the MTT assay. Briefly, cells were treated with the vehicle, CA (12.5 μg/mL) or SC-RE (30 μg/mL) for 24–72 h followed by incubation with 0.5 mg/mL MTT solution at 37 °C for 3 h. The medium was removed, and the purple formazan crystals were dissolved in dimethyl sulfoxide. The absorbance was measured at 570 nm. For cell cycle analysis, cells were treated with the vehicle, CA (12.5 μg/mL) or SC-RE (30 μg/mL) for 24 h, and then trypsinized, washed with PBS, and fixed with 70% cold ethanol at –20 °C for at least 24 h. Fixed cells were resuspended in 0.5 mL of PI/RNase staining buffer (BD Pharmingen, San Jose, CA, USA), incubated for 15 min in the dark, and analyzed on a Gallios flow cytometer equipped with a 0.75 W argon laser set at 488 nm (Beckman Coulter, Miami, FL, USA).

2.4. Cholesterol analysis by GC–MS

Total cholesterol (TC) and free cholesterol (FC) levels were measured on HT-29 cells exposed to 30 μg/mL of SC-RE or 12.5 μg/mL of CA for 0–72 h. After incubation, cells were trypsinized and centrifuged at 300 × *g* for 5 min at 25 °C. The supernatant was discarded and cell pellets were washed with PBS and further centrifuged. To obtain cellular lipids, the pellets were treated according to Folch method (Folch, Lees, & Sloane-Stanley, 1957) using chloroform/methanol (2:1, v/v), and ergosterol was used as internal standard. Each extract was dried under N_2, dissolved