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Research Paper

Olive oil polyphenols reduce oxysterols -induced redox imbalance and pro-inflammatory response in intestinal cells

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ABSTRACT

Dietary habits may strongly influence intestinal homeostasis. Oxysterols, the oxidized products of cholesterol present in cholesterol-containing foodstuffs, have been shown to exert pro-oxidant and pro-inflammatory effects, altering intestinal epithelial layer and thus contributing to the pathogenesis of human inflammatory bowel diseases and colon cancer. Extra virgin olive oil polyphenols possess antioxidant and anti-inflammatory properties, and concentrate in the intestinal lumen, where may help in preventing intestinal diseases. In the present study we evaluated the ability of an extra virgin olive oil phenolic extract to counteract the pro-oxidant and pro-inflammatory action of a representative mixture of dietary oxysterols in the human colon adenocarcinoma cell line (Caco-2) undergoing full differentiation into enterocyte-like cells. Oxysterols treatment significantly altered differentiated Caco-2 cells redox status, leading to oxidant species production and a decrease of GSH levels, after 1 h exposure, followed by an increase of cytokines production, IL-6 and IL-8, after 24 h. Oxysterol cell treatment also induced after 48 h an increase of NO release, due to the induction of iNOS. Pretreatment with the phenolic extract counteracted oxysterols effects, at least in part by modulating one of the main pathways activated in the cellular response to the action of oxysterols, the MAPK-NF-kB pathway. We demonstrated the ability of the phenolic extract to directly modulate p38 and JNK1/2 phosphorylation and activation of NF-kB, following its inhibitor IkB phosphorylation. The phenolic extract also inhibited iNOS induction, keeping NO concentration at the control level. Our results suggest a protective effect at intestinal level of extra virgin olive oil polyphenols, able to prevent or limit redox unbalance and the onset and progression of chronic intestinal inflammation.

1. Introduction

One of the main feature of chronic gastrointestinal inflammatory disorders, such as the inflammatory bowel diseases (IBD), is the overproduction of oxidant species, nitric oxide (NO) and pro-inflammatory cytokines and chemokines, secreted by enterocytes and local immune cells, which sustain and amplify inflammation and cause extensive damage to the mucosa [1,2]. Growing evidence is accumulating toward the strong influence of dietary components, whose metabolites exert pro-oxidant or pro-inflammatory features, in the onset and progression of gastrointestinal inflammatory disorders [3]. In this connection, dietary oxidized lipids, such as oxysterols and fatty acids hydroperoxides, together with microbiota, may influence intestinal inflammation through different mechanisms which include direct production of reactive species in the colon [4,5], antigenic effect, alteration of gene expression, changes in the composition of the enteric flora and gut permeability, and immune system deregulation [6].

Oxysterols present in food are the oxidized products of dietary cholesterol and have been reported to reach concentrations ranging from 10 to 100 μM [7,8]. They have been shown to potentially interfere with the homeostasis of the human digestive tract, by promoting and sustaining irreversible damage and dysfunction of the colonic epithelial barrier, as demonstrated in vitro [9,10], which may lead to IBD [11] and colon cancer [12,13]. In previous studies we showed that they may

Abbreviations: H_2-DCFH-DA, 2′,7′-dichlorofluorescein diacetate; iNOS, inducible nitric oxide synthase; MAPK, mitogen activated protein kinase; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; IBD, Inflammatory bowel diseases; NOX-1, NADPH oxidase isoform 1; GSH, reduced gluthatione; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; $O_2^{\bullet -}$, superoxide anion

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contribute to oxidative imbalance of the intestinal epithelium by inducing the generation of oxidant species [9,11], at least in part by up-regulating intestinal NADPH oxidase isoform 1 (NOX-1); they are also able to up regulate interleukin (IL) − 8 and IL-6 expression and synthesis [14]. Their pro-inflammatory action seems to be mediated by the modulation of redox-sensible specific mitogen-activated protein kinase (MAPK) signaling pathways, and by activating redox-sensitive transcription nuclear factor kappa B (NF-kB) [15].

Other dietary components, particularly those with antioxidant and anti-inflammatory properties, such as phytochemicals, may be important in preventing or limiting intestinal barrier alterations [3]. Although there is only a very limited number of human trials that have focused on gastrointestinal inflammatory disorders with respect to polyphenols intervention, several in vitro and animal studies have shown the positive effects that polyphenols may play, in particular in the IBD [16]. Few of them concern olive oil polyphenols and show that an extra virgin olive oil diet enriched with phenolic compounds mitigate the severity of DSS-induced colitis in mice, attenuating clinical and histological signs of damage of colonic segments, suppressing oxidative events and inhibiting pro-inflammatory protein expression [17–21]. The main classes of extra virgin olive oil phenols are phenolic acids, phenolic alcohols, flavonoids, secoiridoids and lignans. Most of them have shown a broad spectrum of antioxidant, free radical scavenger and anti-inflammatory effects [22], which makes them promising dietary supplements in a variety of chronic inflammatory diseases, included IBD [23–25]. The anti-inflammatory activity of olive oil polyphenols seems to be related to their ability to inhibit the pro-inflammatory activity of oxidants-generating enzymes, including cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) and to modulate different intracellular signaling pathways from NF-kB to MAPKs, through the modulation of redox-sensible cellular networks [23].

However, the signaling pathways related to intestinal inflammation that may be modulated by dietary oxysterols and olive oil polyphenols still need clarifications.

In this contest, our study was aimed to evaluate the ability of an extra virgin olive oil phenolic extract to counteract the pro-oxidant and pro-inflammatory effect of oxysterols in intestinal cells, further exploring the molecular mechanism involved. Human colon adenocarcinoma Caco-2 cell line spontaneously undergoes differentiation into normal enterocyte-like cells, at about 21 days after plating [26]. Differentiated Caco-2 cells were treated with an oxysterol mixture composed by the most widely represented oxysterols in processes or/and stored cholesterol-rich foods [27]. The phenolic extract used, was obtained from a monovarietal extra virgin olive oil of Bosana cultivar, one of the most common and widespread varieties in Sardinia (Italy), extracted trough industrial standard procedures [28]. The protective action of the phenolic extract was evaluated as ability to modulate cellular redox status alteration and MAPKs-NF-kB activation induced by oxysterols and the inflammatory mediators acting downstream of this pathway, IL-6 and IL-8, NO and iNOS.

2. Materials and methods

2.1. Reagents

Unless otherwise specified, all reagents and chemicals were from Sigma-Aldrich (Milan, Italy). 5-cholesten-3β,7α-diolo (7α-hydroxycholesterol) and 5-cholesten-3β,7β-diolo (7β-hydroxycholesterol) were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). Cell culture materials were purchased from Invitrogen (Milano, Italy) and from Lonza (Basel, Switzerland). The primary antibodies anti-phospho-JNK 1/2, anti-JNK, anti-phospho-p38, anti-p38 and the enhanced chemiluminescence (ECL) reagent were purchase from Millipore (Watford, UK). Anti-iNOS (C-11), anti-p-IκB (B-9) and anti-IκB (9242) mouse monoclonal primary antibodies where purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Gels and all material for electrophoresis and immunoblotting were purchased from Invitrogen (Milan, Italy). Nitrocellulose membranes were obtained from Amersham (Little Chalfont, UK).

2.2. Cell culture and treatments

Human colon adenocarcinoma Caco-2 cells (ECACC Salisbury, Wiltshire UK) were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated bovine serum, 2 mM L-glutamine, 1% nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin, in monolayers at 37 °C in a humidified atmosphere of 5% CO_2. For experimental studies Caco-2 cells, at passage 45–60, were plated and used 18–21 days post seeding, when fully differentiated. Cells were treated with the oxysterol mixture, composed of 42.96% 7-ketocholesterol, 32.3%, 5α,6α-epoxycholesterol, 5.76% 5β,6β-epoxycholesterol, 4.26% 7α-hydroxycholesterol, and 14.71% 7β-hydroxycholesterol, to a final concentration of 60 μM, at different times to locate the temporal window for investigating the activation of different cell signaling pathways. In a parallel set of experiment, 30 min prior to the oxysterols exposure, cells were treated with different amounts of the phenolic extract in PBS solution (1–25 μg/ml).

2.3. Preparation of the olive oil phenolic extract

The phenolic fraction was extracted from a monovarietal extra virgin olive oil obtained from an olive orchard located in South Sardinia (Villasor, Cagliari, Italy). Olive trees were of Bosana cultivar, and the oil extraction was performed in an industrial oil extraction plant following standard procedures by the AGRIS technicians, as previously reported [28]. The separation of the phenolic fraction was carried out through a liquid–liquid extraction method using MeOH/H_2O 80:20 (v/v) and phenolic components were identified and quantified by LC-DAD analyses as previously described by Incani et al. [28].

2.4. Evaluation of IL-8 and IL-6 protein levels

Differentiated Caco-2 cells seeded in 6-well plates (5×10^4 cells/ml in 2 ml) were treated for 24 h with the oxysterol mixture, and pre-treatment with the phenolic extract in a set of samples; at the end of the incubation time the culture medium was collected and used for ELISA detection. Levels of IL-8 and IL-6 were quantified using the Human IL-8 ELISA kit (Campoverde s.r.l., Milano, Italy) and the Human IL-6 ELISA kit (Pantec s.r.l., Torino, Italy) following the manufacturer's instructions. Sample absorbance values were read at 450 nm with a wavelength correction of 550 nm in a microplate reader (Model 680 microplate reader Bio-Rad), and data analyzed using SlideWrite Plus software (Advanced Graphics Software).

2.5. Measurement of nitric oxide production

In order to analyze the nitric oxide release, differentiated Caco-2 cells, seeded in 6-well plates (5×10^4 cells/ml in 2 ml), were rinsed with PBS and cultured for 24 h in serum-free, phenol red-free DMEM with L-arginine 0.1 mM. A time course analysis of NO production was performed by incubating cells from 18 to 72 h. In another set of experiments cells were also pre-incubated with phenolic extract before for 30 min and then incubated with oxysterols with further 24 h. At the end of different incubation times NO production was evaluated in terms of quantity of nitrite accumulated in the culture medium by using the Griess' reagent. Nitrite concentration was determined by mixing 100 μl of the collected medium with an equal volume of Griess' reagent and incubating at room temperature for 15 min. Absorbance values were read at 540 nm, and nitrite levels were determined with a sodium nitrite standard curve ranging from 0.1 to 10 μM.