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Microalgae-derived oxylipins decrease inflammatory mediators by regulating the subcellular location of NFκB and PPAR-γ

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ABSTRACT

Oxylipins (OXLs) are bioactive molecules generated by the oxidation of fatty acids that promote the resolution of acute inflammation and prevent chronic inflammatory processes through molecular mechanisms that are not well known. We have previously reported the anti-inflammatory activity of microalgae-derived OXLs and OXL-containing biomass in two inflammatory bowel disease (IBD) models: 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced acute colitis and TNBS-induced recurrent colitis. In this study, we examined the in vitro anti-inflammatory mechanism of action of the most abundant OXLs isolated from *Chlamydomonas debaryana* (13*S*-HOTE and 13*S*-HODE) and *Nannochloropsis gaditana* (15*S*-HEPE). These OXLs decreased IL-1β and IL-6 pro-inflammatory cytokines production as well as iNOS and COX-2 expression levels in THP-1 macrophages. In addition, OXLs decreased IL-8 production in HT-29 colon cells, the major chemokine produced by these cells. The interaction of OXLs with NFκB and PPAR-γ signaling pathways was studied by confocal microscopy. In THP-1 macrophages and HT-29 colon cells, stimulated by LPS and TNFα respectively, a pre-treatment with 13*S*-HOTE, 13*S*-HODE and 15*S*-HEPE (100 μM) resulted in a lower nuclear presence of NFκB in both cell lines. The study of the subcellular localization of PPAR-γ showed that the treatment of THP-1 and HT-29 cells with these OXLs caused the migration of PPAR-γ into the nucleus. Colocalization analysis of both transcription factors in LPS-stimulated THP-1 macrophages showed that the pre-treatment with 13*S*-HOTE, 13*S*-HODE or 15*S*-HEPE lowered nuclear colocalization similar to control value, and increased cytosolic localization above control level. These results indicate that these OXLs could act as agonist of PPAR-γ and consequently inhibit NFκB signaling pathway activation, thus lowering the production of inflammatory markers, highlighting the therapeutic potential of these OXLs in inflammatory diseases such as IBD.

1. Introduction

In the last years, the interest of microalgae as sources of a vast number of new biomolecules useful in pharmaceutical and food industries [1], among others, has increased. Microalgae are producers of n-3 polyunsaturated fatty acids (PUFAs) such as α-linoleic acid (ALA), eicosapentanoid acid (EPA) and docosahexaenoic acid (DHA) [2]. Currently, these PUFAs are being investigated to be used as therapeutic or nutraceutical agents, because PUFAs and their derivates play important roles as signaling molecules. The oxidative transformation of PUFAs results in a diverse family of lipid mediators named OXLs, which play a pivotal role in different methabolic responses, including their function as inter-kingdom mediator molecules [3].

Recently, it has been demonstrated that OXLs can resolve acute inflammatory processes [4] having a role in chronic evolution and tissue remodeling [5,6]. In a previous study, we showed that an oxylipin-containing lyophilised biomass from the microalga (OLM) *C. debaryana*, and its main constituent 13*S*-HOTE, decreased inflammatory parameters in a 2,4,6-trinitrobenzenesulfonic acid (TNBS)-experimental induced ulcerative colitis, by down-regulation of ciclooxigenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) expression [7]. More recently, we have shown that this OLM, orally administered, was capable to ameliorate the acute inflammation parameters in a TNBS-induced recurrent colitis model in mice by decreasing

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pro-inflammatory cytokines production and modulating inflammatory and oxidative gene expression responses through Nuclear factor κB (NFκB) and nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) signaling pathways [8].

The aim of this study was to investigate the molecular mechanism responsible for the anti-inflammatory effects of the major OXLs produced by *C. debaryana* (13*S*-HOTE and 13*S*-HODE), and by *N. gaditana* (15*S*-HEPE). These three OXLs and four additional ones had previously shown anti-inflammatory activity by decreasing TNFα production [9]. The first objective of this study was to evaluate the effect of these OXLs on the production of cytokines in macrophages THP-1 stimulated by LPS and in the adenocarcinoma colon cells HT-29 stimulated by TNFα. iNOS and COX-2 are the main pro-inflammatory genes whose expression is raised in IBD; moreover, NFκB is a transcriptional factor that controls multitude of pro-inflammatory cytokines and genes expression in IBD [10,11], while the activation of PPAR-γ by lipid agonists has been shown to have anti-inflammatory actions [12]. Thus, the effects of 13*S*-HOTE, 13*S*-HODE and 15*S*-HEPE on iNOS and COX-2 expression and on the subcellular localization of NFκB and PPAR-γ were evaluated. In addition, the effect of the pretreatment with OXLs on the colocalization of both transcriptional factors was monitorized in LPS-stimulated THP-1 macrophages. The results show that three OXLs decrease the production of inflammatory molecules (IL-1β, IL-6, IL-8) and inflammatory genes expression (iNOS, COX-2) by interacting with NFκB and PPAR-γ signaling pathways, inactivating the first one and activating the second one, and thus changing the nucleus-to-cytoplasm ratio of both transcriptional factors and thus collaborate to the recovery of cellular homeostasis.

2. Materials and methods

2.1. Compounds

13*S*-HOTE, (9*E*,11*E*,13*S*,15*Z*)-13-hydroxyoctadeca-9,11,15-trienoic acid; 13*S*-HODE, (9*Z*,11*E*,13*S*)-13-hydroxyoctadeca-9,11-dienoic acid; 16-HOTE, (9*Z*,12*Z*,14*E*)-16-hydroxyoctadeca-9,12,14-trienoic acid; 11-HHT, (7*Z*,9*E*,13*Z*)-13 hydroxyhexadeca-7,9,13-trienoic acid; 9-HOTE, (10*E*,12*Z*,15*Z*)-9-hydroxyoctadeca-10,12,15-trienoic acid and 11*S*-HHTT, (4*Z*,7*Z*,9*E*,11*S*,13*Z*)-11-hydroxyhexadeca-4,7,9,13-tetraenoic acid were isolated from the microalga *Chlamydomonas debaryana* and 15*S*-HEPE, (5*Z*,8*Z*,11*Z*,13*E*,15*S*,17*Z*)-15-hydroxyeicosa-5,8,11,13,17-pentaenoic acid was isolated from the microalga *Nannochloropsis gaditana* as previously reported [7]. These oxylipins were dissolved in dimethylsulfoxide (DMSO) and added to the cultures at indicated concentration.

2.2. Cell lines

Human acute monocytic leukemia cell line THP-1 was obtained from the American Type Culture Collection (TIB-202, ATCC, USA) and cultured in RPMI 1640 media (GIBCO®, Life Technologies, NY, USA); human adenocarcinoma colon cell line HT-29 was obtained from the European Collection of Cell Cultures (ECACC) and cultured in McCoy's 5A media (PAA®, Pasching, Austria), both containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin (PAA®, Pasching, Austria), in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Cell proliferation assay

Viability of THP-1 macrophages and HT-29 cells upon exposure to OXLs was determined by the sulforhodamine B (SRB) assay [13] (Sigma-AldrichR®, St Louis, MO). Briefly, for differentiation into macrophages THP-1 cells were seeded into 96-well plates (100 μL/well) at a density 10^4 cells per well in presence of phorbol 12-myristate 13-acetate (PMA) (DMSO-dissolved, 0.8 mM) for a final concentration of 0.2 μM, incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 72 h. The HT-29 cells were seeded into 96-well plates (100 μL/well) at a density 5×10^3 cells per well and incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 24 h. After that, in both cell lines the medium was removed, cells were washed with phosphate saline buffer (PBS, 4 °C), and then incubated for 24, 48 and 72 h with OXL solutions (6.25, 12.5, 25, 50, and 100 μM) that were freshly prepared in DMSO (Panreac, Barcelona, Spain) stock solutions at a concentration of 10 mM and diluted to desired concentration directly in the culture medium. Controls were incubated in fresh medium containing DMSO (1%, v/v) which did not affect cell viability. After incubation time, the cells were fixed with 50 μL of cold trichloroacetic acid (TCA, 50%, v/v) and processed as described in the literature.

2.4. Determination of pro-inflammatory cytokines levels

Differentiation into macrophages was achieved by incubating THP-1 cells (10^4 cells/well) with PMA 0.2 μM in 96-well plates (100 μL/well) for 72 h, as described above. HT-29 cells were seed at 10^4 cells/well (100 μL/well) for 24 h to ensure the correct adhesion. After that, the medium was removed, the cells were washed twice with ice-cold PBS, and then an incubation with OXL solutions (25, 50, and 100 μM) was performed for 1 h. Positive references were incubated with a dexamethasone solution (1 μM) that was prepared by dilution of a stock solution (20 mM in DMSO) with fresh medium. Control groups, unstimulated (Control) and stimulated (LPS or TNFα), were incubated with growth medium containing DMSO (1%, v/v). The inflammatory response was induced by addition of lipopolysaccharide from *E. coli* (LPS, 1 μg/mL on THP-1 cells, or TNFα, 50 ng/mL on HT-29 cells) by dilution of a stock solution (5 mg/mL) prepared in DMSO and then diluted with fresh completed medium. After 24 h, the supernatants were collected and stored at −80 °C until cytokines (IL-1β, IL-6 and IL-8) measurements by ELISA kits (Diaclone GEN-PROBE, France) according to the manufacturer's protocol. The absorbance was measured with a microplate reader (Labsystems Multiskan EX, Thermo Scientific) at 450 nm.

2.5. Western blot assay

THP-1 cells (10^6 cells/mL) were transformated into macrophages by incubating with PMA (0.2 μM) for 72 h in a final volume of 2 mL in 6-well plates. Then, the medium was removed, the cells were washed with ice-cold PBS, and incubated with compounds 13*S*-HOTE, 13*S*-HODE and 15*S*-HEPE solutions (50 μM) for 1 h. Control groups, unstimulated and stimulated, were incubated with growth medium containing DMSO 1% (v/v). The inflammatory response was induced by addition of LPS (1 μg/mL). After 24 h, cell pellets were mixed with cold lysis buffer containing a cocktail of protease inhibitors (50 mM Tris HCl, pH 7.5, 8 mM $MgCl_2$, 5 mM EGTA, 0.5 mM EDTA, 0.01 mg/mL leupeptin, 0.01 mg/mL pepstatin, 0.01 mg/mL aprotinin, 1 mM PMSF and 250 mM NaCl) (50 μL per sample). After incubation on ice for 30 min the protein extracts were spined (12,000g, 4 °C) for 10 min, cell debris and DNA form a pellet at the bottom of the tube allowing the proteins to be suspended in the supernatant that were collected and stored at −80 °C. Protein concentration of the homogenate was determined following Bradford colorimetric method. Aliquots of supernatants containing equal amounts of protein (50 μg) were separated on 10% acrylamide gel by SDS-polyacryamide-gel electrophoresis. In the next step, the proteins were electrophoretically transferred onto a nitrocellulose

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