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Long-chain fatty alcohols from evening primrose oil inhibit the inflammatory response in murine peritoneal macrophages

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ABSTRACT

Ethnopharmacological relevance: Evening primrose (*Oenothera biennis* L., Onagraceae) is a wild medicinal plant of Central American origin that is now one of the most widely used herbal medicines in different parts of the world. Oil extracted from it seeds is traditionally used in the treatment of eczema, asthma, rheumatoid arthritis, breast problem, premenstrual and menopausal syndrome, all they have an inflammatory component. The present study demonstrates the in vitro anti-inflammatory effect of long-chain fatty alcohols, minor compounds isolated from Evening primrose oil (EPO).

Material and methods: A mixture of long chain fatty alcohols (LCFAs) was isolated from the non-triacylglycerol fraction of the EPO. Hexacosanol (C_{26}OH: 38.65%), tetracosanol (C_{24}OH: 31.59%), docosanol (C_{22}OH: 11.36%) and octocosanol (C_{28}OH: 7.64%), were the major constituents, identified and quantified by GC and GC–MS. LCFA was tested with LPS stimulated murine peritoneal macrophage. This fraction, significantly and dose-dependently decreased nitric oxide production induced by LPS ($P < 0.001$) and the inhibitory effect seems to be consequence of an action at the level of the inducible nitric-oxide synthethase (iNOS) gene enzyme expression rather than to a direct inhibitory action on enzyme activity. The release of PLA2 and TXB2 also was significantly inhibited by LCFAs ($P < 0.001$) although LCFAs did not affect to PGE2 generation, however the western blot assay showed that LCFAs reduced cyclooxygenase-2 enzyme gene expression at all doses assayed. In the same way, the secretion of inflammatory cytokines interleukin 1β (IL-1β) and tumour necrosis factor α (TNF-α) from LPS-stimulated murine macrophage, were also significantly reduced ($P < 0.001$).

Conclusion: These results demonstrates the anti-inflammatory activity of LCFAs, providing an additional value about the role of bioactive minor compounds in the beneficial effect of EPO and supports its traditional uses in inflammatory processes management.

1. Introduction

Evening primrose oil (EPO) is one of the most widely used herbal medicines in different parts of the world. EPO is extracted from the seeds (*Oenothera biennis* L., Onagraceae) and it is found in fields and roadsides in the Unites States and Canada (Wettasinghe et al., 2002).The seed oil of evening primrose has attracted interest due to its high content of γ-linolenic acid (≈ 10% w/w) and linoleic acid, precursors of the series-1 prostaglandins. Beneficial effects have been reported in several ailments as eczema, asthma, rheumatoid arthritis, breast problem, premenstrual and menopausal syndrome, (Rodgers et al., 2009; Ola and Omran, 2012).These effects relies mainly on its content in polyunsaturated fatty acid. However, more recently it has been demonstrated that other specific compounds presents in the non-triacylglycerol fraction of EPO could have an important role in these beneficial effects. (Knorr and Hamburger, 2004; Zaugg et al., 2006). In the course of our investigation on the detection and isolation of secondary metabolites potentially actives from vegetables (Puerta et al., 2009; Fernandez-Arche et al., 2009; García-Jiménez et al., 2010), we have carry out a study on the phytochemical composition and the possible bioactive effect of minor compounds isolated from non-fat matter of EPO and we have found that content interesting

Abbreviations: EPO, evening primrose oil; LCFAs, long chain fatty alcohols; LPS, lipopolysaccharide; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; NO, nitric oxide; iNOS, inducible nitric oxide; TXB_2, thrombaxane E_2; PGE_2, prostaglandine E_2; TNF-α, tumor necrosis factor alpha; COX_2, cyclooxygenase 2; PLA_2, phospholipase A_2; $sPLA_2$, secretory phospholipase A_2; IL-1β, interleukin-1β; EIA, enzimoinmunoanalisis; ELISA, enzyme-linked immunosorbent assay; DMSO, dimethyl sulfoxidem; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide; SNP, sodium nitroprusside; NF-κB, nuclear factor kappa B

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molecules as sterols and long-chain fatty alcohols (LCFAs) (Montserrat-de la Paz et al., 2012, 2013a, 2013b).

LCFAs are aliphatic compounds usually found in waxes of common foods and have been isolated from sugarcane, spinach and beeswax by saponification and solvent extraction. A number of beneficial physiological activities have been attributed to LCFAs, such as reducing platelet aggregation, endothelial damage and cholesterol-lowering effects (Shinbori et al., 2007). All these studies have been carried out using "Policosanol", a commercialized mixture of LCFAs (Affuso et al., 2012), whose major components are octocosanol (C_{28}OH), triacosanol (C_{30}OH) and hexacosanol (C_{26}OH) valued nutraceutical by its protective effects on cardiovascular disease (Marazzi et al., 2011). In previous work, we have also studied a similar LCFAs mixture isolated from olive pomace oil, a virgin olive oil by-product, whose non-fat matter is also a source of actives compounds. (Marquez-Martín et al., 2006; Fernandez-Arche et al., 2009)

Macrophages play a key role in the inflammatory response and serve as an essential interface between innate and adaptive immunity (Bowdish et al., 2007). In presence of pathogens, macrophages are stimulated to secrete a battery of proinflammatory mediators, including, nitric oxide (NO), eicosanoids and inflammatory cytokines, that are well-known to be involved in the pathogenesis of inflammatory response (Gomez et al., 2005). Arachidonic acid is released from phospholipds by the action of the secretory phospholipase A_2 ($sPLA_2$), thereby providing the substrate for the biosynthesis of proinflammatory eicosanoids. $sPLA_2$ is released in plasma and other biologic fluids of patients with systemic inflammatory, autoimmune and allergic diseases. Different isoforms of $sPLA_2$ are expressed and released by several cells types, such as neutrophils, basophils, T cells, monocytes, macrophages and mast cells, which promotes cytokine and chemokine production. Thus, $sPLA_2$ might play important roles in the initiation and amplification of inflammatory reaction (Triggiani et al., 2005). Therefore, the inhibition of $sPLA_2$ could result in down-regulation of the inflammatory response, acting at an early step of several inflammatory mediator biosynthesis.

Nitric oxide (NO) has been shown to play a central role in inflammatory and immune reaction activities. Macrophages appear to be the main cellular source of NO since these cells significantly contribute to inducible NO synthetase (iNOS) induction after lipopolysaccharide (LPS) incubation (Puerta et al., 2009). NO is also able to enhance the production of tumor necrosis factor-α (TNF-α) and interleukin 1β cytokines, which participate in the macrophage-dependent inflammation. Activation of macrophages also leads to cyclooxygenase-2 (COX-2) stimulation with consequent eicosanoids overproduction (Kang et al., 1996).

In order to better understand the role of minor compounds and its possible involving in the beneficial effects of EPO on inflammatory diseases, this study has been focused to analyze the composition of a LCFAs mixture, isolated from non acylglycerol matter of EPO and to examine its ability to inhibit $sPLA_2$, nitrites, TXB_2, PGE_2, TNF-α and IL-1β generation in LPS-stimulated murine peritoneal macrophages.

2. Materials and methods

2.1. Sample preparation

EPO was purchased from MARNYS® (Cartagena, Spain). Non-acylglycerol matter of EPO was isolated following conventional procedures. In brief, 5 g EPO was saponified with 50 ml of 2N KOH in ethanol/water (80:20, v/v). After heating at 70 °C for 60 min, 100 ml of water and 80 ml of ethyl ether were added. The unsaponifiable fraction (1.9%) was collected by decantation and its components were analyzed following the IUPAC method (Paquot, 1992). Non-acylglycerol matter was fractioned on a silica gel preparative chromatography with a mixture of hexane and ethyl ether (65:35, v/v). The same procedure was used to obtain the LCFAs fraction that was added to the cell culture medium at appropriate concentrations. For LCFAs analysis by gas chromatography, the isolated LCFAs were transformed into trimethylsilyl ethers by adding 200 μl of a mixture of 9:3:1 v/v/v of pyridine–hexamethyldisilazane–trimethylchlorosilane (Cert et al., 1997).

2.2. Chormatography conditions

LCFAs fraction was analyzed with a Chrompack (Middelburg, The Netherlands) CP900 gas chromatography equipped with a capillary column SGL-5, of 30 m length, 0.25 mm i.d. and 0.25 μm film thickness of 5% phenyl methylpolysiloxane stationary phase. Carrier gas was hydrogen, with a head pressure of 110 kPa and 1:60 split ratio. Injector temperature was 280 °C and detector temperature 290 °C. The oven temperature was programmed from 215 °C to 290 °C with a rate of 2 °C/min. The analysis was carried out following the amending Regulation (EC) 2568/91 on the characteristics of olive and the relevant method of analysis. The MS analyses were performed using Kratos MS 80 mass spectrometer equipped with a NBSLIB2 data system. Total LCFAs concentration in EPO was calculated as the sum of individual LCFAs concentrations.

2.3. Reagents

$sPLA_2$, PGE_2 and TXB_2 EIA Kits were from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). Mouse IL 1-β ELISA was from eBioscience (Vienna, Austria) Mouse TNF-α ELISA were from Thermo Scientific (Rockford, IL, U.S.A). Thioglycolate was from Scharlau Chemie S.A. (Barcelona, Spain). The rest reagents were purchased from Sigma Aldrich Chem. (St. Louis, MO, USA). Stock solutions of compounds were prepared in DMSO and later dissolved in ethanol. The final concentration of DMSO or ethanol in the culture medium did not significantly influence cell response. The quantities of the compounds are expressed as their final concentration in the culture medium.

2.4. Isolation and culture of murine peritoneal macrophages

Peritoneal exudates cells (1×10^6 cells/well) from thioglycolate-induced mice were collected from the peritoneal cavities of female Swiss mice and were suspended in culture medium RPMI 1640 supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml), and pre-cultured in 24-well plates at 37 °C in 5% CO_2 in air for 2 h. Nonadherent cells were removed and the adherent cells were cultured in 1 ml of fresh medium (5% FBS) containing 5 μg/ml of *Escherichia coli* (Serotype 0111:B_4) LPS at 37 °C for 24 h in the presence of LCFAs fraction at different doses (25, 50 or 100 μg/ml) or vehicle.

2.5. Cell viability

The mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan was used to assess the possible cytotoxic effect on the murine peritoneal macrophage (Mosmann, 1983).

2.6. Nitrites, $sPLA_2$, TXB_2, PGE_2, IL-1β and TNF-α production in murine peritoneal macrophages

Nitrites, as index of NO generation, were detected in culture supernatants by a fluorimetric method (Griess reaction method)