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Inhibition of inflammatory response in LPS induced macrophages by 9-KOTE and 13-KOTE produced by biotransformation

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ABSTRACT

Lipid mediators such as the leukotrienes, resolvins and protectins have been considered excellent models for the development of new anti-inflammatory drugs, due to their high potentiality. Nevertheless, only tiny amounts are available from natural sources and they have to be prepared by total synthesis. It is known that besides chemical reagents, microorganisms can also promote fatty acid oxygenation, via enzymatic reactions. In this context, the aim of this work was to produce oxylipids analogues in structure to lipid mediators employing microbial biotransformation. To this end, α-linolenic acid (ALA) was biotransformed by the fungi *Aspergillus niger* into oxylipids with different levels of oxygenation within 24 h or 48 h. The anti-inflammatory potential of products were evaluated by means of NO and TNF-α quantification in LPS-stimulated RAW264.7 macrophage cell line which guided the isolation of the regioisomers at *m/z* [M-H]$^-$ 291, 9-keto-10*E*,12*Z*,15*Z*-octadecatrienoic acid (9-KOTE) and 13-keto-9*Z*,11*E*,15*Z*-octadecatrienoic acid (13-KOTE). We showed that biotransformation represents a powerful strategy for the production of potentially interesting candidates for development of anti-inflammation therapies.

1. Introduction

It is known that inflammation is a central process in the pathogenesis of many diseases including diabetes [1], cardiovascular diseases [2], arthritis [3], neurodegenerative diseases [4] and cancer [5].

Lipid mediators, like leukotrienes [6], resolvins [7] and protectins [8] comprehends endogenous anti-inflammatory metabolites which plays a major role in the body's regulation of the immune response during infection and disease [9].They are produced via enzymatic or non-enzymatic oxygenation of arachidonic acid (AA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), polyunsaturated fatty acid (PUFA) that are present in phospholipids from cell membranes.

Fig. 1 illustrates the chemical structure of different bioactive lipid mediators.

Due to their potentiality, the lipid mediators have been considered excellent models for the development of new anti-inflammatory drugs [10]. However, they are found in only tiny amounts in natural sources and they have to be prepared by synthetic methods in order to allow further biological and pharmacological testing.

Abbreviations: PUFA, polyunsaturated fatty acid (s); ALA, α-linolenic acid; LNA, linoleic acid; ALA24 h, biotransformation crude extract of ALA in 24 h; ALA48 h, biotransformation crude extract of ALA in 48 h; LOX, lipoxygenase; COX, cyclooxygenase; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor; 9-KOTE, 9-keto-10E,12Z,15Z-octadecatrienoic acid; 13-KOTE, 13-keto-9Z,11E,15Z-octadecatrienoic acid; 9-KODE, 9-keto-10E,12Z-octadecadienoic acid; 13-KODE, 13-keto-9E,11Z-octadecadienoic acid, 5-oxo-ETE,5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid; 11-oxo-ETE, 11-oxo-5*E*,12*Z*,11*E*,14*Z*-eicosateraenoic acid; 15-oxo-ETE, 15-oxo-5Z,8Z,11E,13E-eicosateraenoic acid; 5-HETE, 5*S*-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid; 5-HEDH, 5-hydroxyeicosanoid dehydrogenase; Lipoxin, A4,6*S*,7*R*,15*S*-trihydroxy-6*E*,8*Z*,10*E*,12*E* eicosatetraenoic acid; Lipoxin B4, 5*S*,14*R*,15*S*-trihydroxy-6*E*,8*Z*,10*E*,12*E* eicosatetraenoic acid; RvE1, Resolvin E1, 5*S*,12*R*,18*R*-trihydroxy-6*Z*,8*E*,10*E*,14*Z*,16*E*-eicosapentaenoic acid; Protectin D1, 10*R*,17*S*-dihydroxy-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-docosahexaenoic acid, 5*S*,6*R*,15*S*-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid; ESI-MS, electrospray ionization mass spectrometry; HPLC-MS, liquid chromatography-mass spectrometry; MS/MS, tandem mass spectrometry; HPLC-MS/MS, liquid chromatography-tandem mass spectrometry; HPLC, high performance liquid chromatography; UHPLC, ultra high performance liquid chromatography; CID, collision induced dissociation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dymethylsulfoxide; RPMI, Medium Roswell Park Memorial Institute; AA, arachidonic acid, 5Z,8Z,11Z,14Z-eicosatetraenoic acid; LTB4, leukotriene *B4*, 5S,12R-dihydroxy-6*Z*,8*E*,10*E*,14*Z*-eicosatetraenoic acid, 15-deoxy-δ-12,14-PGJ2,11-oxo-5Z,9,12E,14E-prostatetraenoic acid.

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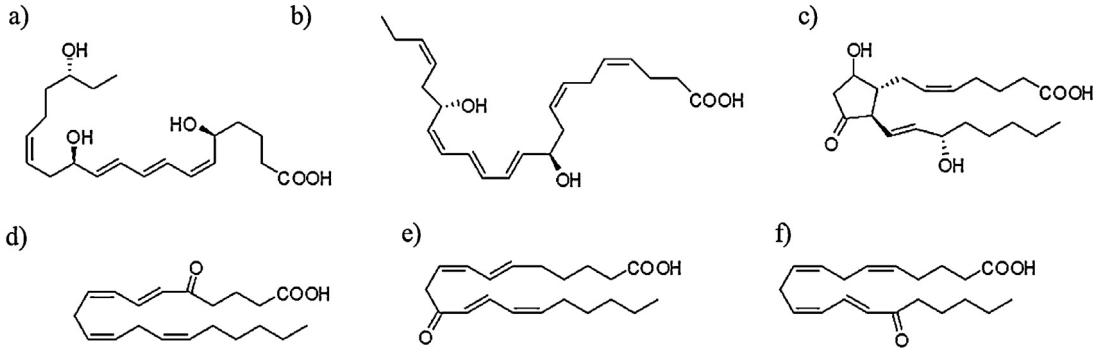

Fig. 1. Structures of bioactive eicosanoids: (a) 5*S*,12*R*,18*R*-trihydroxy-6*Z*,8*E*,10*E*,14*Z*, 16*E*-eicosapentaenoic acid [7]; (b) 10*R*,17*S*-dihydroxy-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-docosahexaenoic acid [8]; (c) 9S,15S-dihydroxy-11-oxo-5Z,13E-prostadienoic acid [38]; (d) 5-oxo-6*E*,8*Z*,11*Z*,14*Z*-eicosateraenoic acid [32]; (e) 11-oxo-5*E*,12*Z*,11*E*,14*Z*-eicosateraenoic acid [33]; (f) 15-oxo-5*Z*,8*Z*,11*E*,13*E*-eicosateraenoic acid [34].

In the last decades, microorganisms and their enzymes have been employed as exceptionally versatile catalysts for the conversion of a variety of PUFA to oxylipids of relevance to the flavor, fragrance, pharmaceuticals and fine-chemicals industries [11–13].

Oxygenative biotransformation has demonstrated unique advantages over chemical approaches. Firstly, enzymes can catalyze many types of chemical reactions, affording a wide variety of compounds that are difficult to obtain by organic synthesis. In addition, such reactions are highly stereo-regioselective, a crucial aspect when it comes to the production of bioactive compounds [14].

In this context, the aim of the present study was to produce of oxylipids using biotransformation process in order to generate molecular structures analogous to lipid mediators with potential for application as anti-inflammatory drugs. The biotransformation substrate α-linolenic acid (ALA) is an abundant omega-3 fatty acid found in many common vegetable oils. It is considered essential in mammalian diet for prevention and modulation from certain diseases such as coronary heart disease and stroke, retinal and brain development, mild hypertension, rheumatoid arthritis [15,16]. Thus, whether ALA can be biotransformed into potent anti-inflammatory compounds is of considerable interest to pharmaceutical industry and public health.

To evaluate whether the biotransformation products presented any anti-inflammatory potential, we investigated their capacity to inhibit nitric oxide (NO) and inflammatory cytokine tumor necrosis factor (TNF-α) production in the RAW 264.7 macrophage cell line stimulated with LPS. The analysis of the course of biotransformation reactions and the characterization of bioactive molecules were performed by HPLC-MS/MS, a powerful technique for characterization and quantification of oxylipis in biological samples [17,18]. Furthermore, HPLC-MS/MS have been used to differentiate the regioisomers, due to their intrinsic fragmentation spectra [19].

2. Materials and methods

2.1. Solvents and reagents

ALA (99%, Acros) was used as substrate in the biotransformation assay (Fig. 2). For the HPLC-MS analysis, methanol (JT Baker HPLC grade), ultrapure water (Milli-Q, Millipore), and formic acid (Sigma-Aldrich) were employed. LPS, a lipopolysaccharide used in the inflammation assay, was an *Escherichia coli* LPS purchased from Sigma-Aldrich. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich. All the other chemicals were analytical grade.

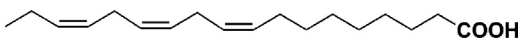

Fig. 2. Chemical structure of α-linolenic acid (ALA), the substrate used in biotransformation assay.

2.2. Biotransformation and products isolation

Aspergillus niger was employed for ALA biotransformation into oxygenated products. The fungi was collected from samples of soil or decomposing organic material from the State of São Paulo (Brazil), as established by the SinBiota-FAPESP program.

The culture medium used in the biotransformation reaction was composed of glucose (10 g/L), $NaNO_3$ (3 g/L), KH_2PO_4 (1 g/L), KCl (0.5 g/L), and MgSO4 (10 mg/L) prepared in 0.1 M sodium phosphate buffer, pH 6.5, supplemented with 0.1% (v/v) of Tween-20 T [20]. The solutions were prepared in 150 mL Erlenmeyer flasks containing 30 mL culture medium. After sterilization at 120 °C, 60 μL of ALA was added to each flask. After inoculation, the microbial culture was incubated at 27 °C in an orbital shaker (TE-420 Technal) working at 130 rpm. After 24 h or 48 h, the fungal mycelia were removed by filtration. The filtrate from each flask (30 mL) was acidified to pH 3.0 with hydrocloridric acid. The biotransformation products were extracted with the aid of SPE cartridges (1 g) ODS-C18 Accubond^{II} (Agilent Technologies) and pre-conditioned with methanol (6 mL) and water (6 mL) prior to loading with the filtrate. After complete elution, the cartridges were washed with water (1 mL) and biotransformation products were extracted with ethyl acetate (2 mL), followed by washing with methanol. Samples were subsequently dried under vacuum and stored in the dark, at −18 °C. The ALA biotransformation extracts obtained at 24 h and 48 h were labeled ALA24 h and ALA48 h, respectively.

For fractionation of the crude extract ALA24 h, six Erlenmeyer flasks containing 30 mL culture media with ALA (60 μL) were prepared. The biotransformation reaction resulted in 160 μg crude extract after a period of 24 h. This extract was fractionated in a preparative HPLC system (Shimadzu) equipped with an LC6AD pump and a UV-Vis SPD-20 detector. To this end, the crude extract was dissolved in methanol and injected into the chromatograph by using a syringe. The chromatographic column consisted of a preparative C18 ODS (H) Shim-pack (Shimadzu) with dimensions of 194 mm × 6 mm, 0.5 μm. Elution was conducted by employing a binary gradient system at a flow rate of 10 mL/min with detection at 234 nm. Phase A was composed of water/formic acid (99.9:0.1, v/v) whereas phase B consisted of methanol/formic acid (99.9:0.1, v/v). Elution started with 60% B, which was increased to 100% B for 60 min and held there for 20 min. Each fraction was collected in one minute in intervals. A total of 62 chromatographic fractions were obtained and dried under vacuum.

2.3. Biotransformation products analysis

The biotransformation reactions were monitored by HPLC-MS. Analysis was carried out on a Varian 1200L HPLC-MS system operating with a triple quadrupole and electrospray ionization source (ESI). The chromatographic separation was conducted by using a C-18 column (Ascentis EXPRESS) with dimensions of 100 mm × 4.6 mm, 2.7 μm. A binary gradient system was employed for elution of extracts. Phase A consisted of was water/formic acid (99.9:0.1, v/v) whilst phase B was composed of methanol/formic acid (99.9:0.1, v/v). Elution started with 65% B from 0 to 2 min and continuously increased to 100% B from 2 to 17 min, which was maintained until 30 min. The injection volume was 10 μL and solvent flow rate was 0.3 mL/min. Data were acquired and analyzed using the Varian Workstation 4.0, using *full scan* mode with negative ionization. The analytical parameters were as follows: source temperature: 250 °C; capillary voltage: 40 eV; needle voltage: 5500 V.

HPLC-MS/MS was employed for the structural characterization of the active compounds. Analyses were performed using an Acquity UPLC (Waters) apparatus equipment with a quaternary pump system and an auto sampler coupled to the mass spectrometer Xevo TQ-S equipped with an orthogonal Z-spray electrospray ionization source (Waters Corp., Milford, MA, USA). Chromatographic conditions