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Lysophospholipid acyltransferases and eicosanoid biosynthesis in zebrafish myeloid cells

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ABSTRACT

Eicosanoids derived from the enzymatic oxidation of arachidonic acid play important roles in a large number of physiological and pathological processes in humans. Many animal and cellular models have been used to investigate the intricate mechanisms regulating their biosynthesis and actions. Zebrafish is a widely used model to study the embryonic development of vertebrates. It expresses homologs of the key enzymes involved in eicosanoid production, and eicosanoids have been detected in extracts from adult or embryonic fish. In this study we prepared cell suspensions from kidney marrow, the main hematopoietic organ in fish. Upon stimulation with calcium ionophore, these cells produced eicosanoids including PGE_2, LTB_4, 5-HETE and, most abundantly, 12-HETE. They also produced small amounts of LTB_5 derived from eicosapentaenoic acid. These eicosanoids were also produced in kidney marrow cells stimulated with ATP, and this production was greatly enhanced by preincubation with thimerosal, an inhibitor of arachidonate reacylation into phospholipids. Microsomes from these cells exhibited acyltransferase activities consistent with expression of MBOAT5/LPCAT3 and MBOAT7/LPIAT1, the main arachidonoyl-CoA:lysophospholipid acyltransferases. In summary, this work introduces a new cellular model to study the regulation of eicosanoid production through a phospholipid deacylation–reacylation cycle from a well-established, versatile vertebrate model species.

1. Introduction

Eicosanoids are a wide family of molecules that derive from the oxidation of arachidonic acid (AA) through a variety of metabolic pathways. Many eicosanoids are mediators that act in an autocrine or paracrine fashion to trigger pro-inflammatory, or in some cases anti-inflammatory responses in cells and tissues [1–3]. Biosynthesis of eicosanoids is tightly regulated in cells, and has been extensively investigated for many decades since the elucidation of the chemical structures of prostaglandins (PGs) and leukotrienes (LTs) [4,5]. One of the key factors regulating eicosanoid production is the cellular level of free AA, controlled to a great extent by a deacylation–reacylation biochemical cycle known as the Lands cycle [6]. Considerable effort has been dedicated to investigating the release of AA from membrane phospholipids by the action of phospholipases A_2, particularly by cytosolic phospholipase A_2 alpha (cPLA$_2\alpha$, also known as Group IVA PLA$_2$) [7]. There is also evidence of AA release in some models through the action of monoacylglycerol lipase on AA-containing diglycerides [8]. However, much less is known about the role of reacylation of AA into phospholipids or triglycerides in controlling the levels of free AA and eicosanoid production. This is primarily due to the fact that many of the key enzymes implicated have not been identified until relatively recently, particularly lysophospholipid acyltransferases (LPATs) [9–14]. Some groups, including ours, have now linked two of the LPATs, namely MBOAT5 (also known as LPCAT3) and MBOAT7 (also known as LPIAT1) with regulating AA release and eicosanoid production in human neutrophils and human monocytes [15,16], but not much is known about the potential role of other LPATs or other experimental models.

Despite a sizable body of scientific literature on this subject, there are still many areas that are not well understood about eicosanoid production, and new models of study could prove helpful in providing new insight into their regulation and possible pharmacological targets. Zebrafish (*Danio rerio*) is a unique model in the study of the embryonic development of vertebrates. This animal model is also very prolific, comparatively inexpensive to maintain and amenable to genetic manipulation with a much shorter time frame than other widely used animal models, such as rodents. Like most fish, zebrafish contain abundant *n*-3 fatty acids such as eicosapentaenoic (EPA, 20:5) and docosahexaenoic

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(DHA, 22:6), probably due to a big extent to the composition of their diet [17]. However, they also contain AA (20:4 *n*-6), and AA-derived eicosanoids such as PGs have been observed in whole fish homogenates, where their levels are decreased by pretreatment with cyclooxygenase (COX) inhibitors [18]. In addition, a number of models of inflammation, both local and systemic [19–21], are available in the zebrafish and all of the relevant enzymes that are known to be involved in the Lands cycle in mammals have homologs in zebrafish [22] (also searchable at www.zfin.org). These enzymes include a number of phospholipases A_2 including cPLA$_2\alpha$, acyl-CoA synthases and LPATs including MBOAT5 and MBOAT7. All of these factors make zebrafish an interesting model to study the regulation of eicosanoid production, although there are still some areas where little information is available. For instance, the possible cellular sources of free AA and eicosanoids are not known. Although fish are enriched in polyunsaturated fatty acyl chains, no detailed analysis of the intact phospholipid molecular species that contain esterified arachidonate in the zebrafish has been published. More importantly, a cellular model derived from the zebrafish that would permit facile analysis of different pro-inflammatory stimuli, chemical inhibitors or genetic manipulation and their effect on eicosanoid production is currently not available. The main goal of this research is to present such a cellular model.

Myeloid cells in general, and neutrophils in particular, have been very useful in studying eicosanoid production, since they readily synthesize eicosanoids when challenged with a variety of stimuli. Using the non-specific inhibitor thimerosal, our group showed that inhibition of MBOAT5 and MBOAT7 results in a remarkable increase in the synthesis of leukotriene B_4 (LTB_4) by human neutrophils stimulated by the combination of granulocyte-macrophage colony-stimulating factor (GM-CSF) and formyl-methionyl-leucyl-phenylalanine (fMLP) [23]. Unfortunately, it is not feasible to obtain sufficient numbers of peripheral blood neutrophils from zebrafish. We have previously shown that myeloid cells from the mouse bone marrow cells are able to produce abundant LTB_4 when stimulated by fMLP or zymosan [24]. The functional equivalent of the mammalian bone marrow, the anatomical site of the production of myeloid cells destined to circulate in the blood of zebrafish, is the kidney marrow [25]. Kidney marrow cells from zebrafish were isolated and evaluated as a possible model for eicosanoid production and acyltransferase activity.

2. Materials and methods

2.1. Reagents

Salts, HPLC-grade solvents, calcium ionophore A23187, and dihydroxyacetophenone (DHAP) were purchased from Fisher Scientific (Pittsburgh, PA). Bovine serum albumin (BSA), ATP, and thimerosal were purchased from Sigma–Aldrich (St. Louis, MO). Lysophospholipids, fatty acyl-CoA esters and deuterated phospholipid internal standards were obtained from Avanti Polar Lipids (Alabaster, AL). Protease inhibitor cocktail tablets were obtained from Roche Molecular Diagnostics GmbH (Mannheim, Germany). Standard and stable isotope-labeled eicosanoids were purchased from Cayman Chemical Co. (Ann Arbor, MI).

2.2. Zebrafish kidney dissection and kidney marrow cell isolation

Adult and larval (3 dpf) zebrafish were obtained from the zebrafish facility at the University of Colorado Center for Comparative Medicine. They were maintained at 28.5 °C with a light/dark cycle of 10/14 h according to established procedures [26]. All procedures were performed following guidelines approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver. Kidneys were dissected from adult fish and kidney marrow cells isolated according to LeBlanc et al. [27], with minor modifications. Briefly, after euthanizing the zebrafish by submersion in iced water, kidneys from 20 to 25 zebrafish were isolated into 10 mL of 0.9× PBS supplemented with 5% FCS and 1% penicillin/streptomycin solution. The kidneys were then broken apart using first a 1 mL pipet and then a 10 mL syringe with an 18G needle. After centrifuging at 500 × *g* for 8 min, the pellet was resuspended and filtered through a 40-μm cell strainer. The filtrate was centrifuged and the kidney marrow cells resuspended in Hanks Balanced Salt Solution without calcium, magnesium or phenol red ($HBSS^=$). The cellular distribution was verified by cytospin on microscopy slides and staining using a Hema 3 Stain Set (Fisher Scientific), following the manufacturer's instructions. Identification of the different cell types was carried out by comparison with published cytological data [25].

2.3. Lysophospholipid acyltransferase activity assay

Microsomes from zebrafish kidney marrow cells were prepared by resuspending the cells in homogenization buffer (250 mM sucrose, 50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 20% glycerol, and protease inhibitor cocktail) and disrupting them by sonication. The homogenate was centrifuged at 15,000 × *g* for 15 min at 4 °C to pellet unbroken cells and nuclei. The supernatant was then centrifuged at 100,000 × *g* for 1 h at 4 °C and the microsome pellet was resuspended in assay buffer (150 mM NaCl, 10 mM Tris/HCl, pH 7.4, 1 mM EDTA). Protein content was determined by the bicinchoninic acid assay (Pierce, Rockford, IL), using BSA as standard.

Zebrafish kidney marrow cell microsomes were tested for acyltransferase activity as described by Martin et al. [28]. Microsomes (10 μg protein) were incubated with 3 μM each of eight acyl-CoAs (14:0, 16:0, 18:0, 18:1, 18:2, 20:4, 20:5, and 22:6), 3 μM each of six lysophospholipids (LPA, LPC, LPE, LPG, LPI, and LPS), and 12.5 μM BSA, in the presence or absence of thimerosal (50 μM). The reaction was allowed to proceed for 10 min at 37 °C. Reaction was stopped by addition of 500 μL of methanol, prior to phospholipid extraction.

2.4. Phospholipid extraction and liquid chromatography/mass spectrometry

After addition of the deuterated internal standards [$^2H_{31}$]16:0/18:1-PA, [$^2H_{31}$]16:0/18:1-PC, [$^2H_{31}$]16:0/18:1-PE, [$^2H_{31}$]16:0/18:1-PG, [$^2H_{31}$]16:0/18:1-PI and [$^2H_{31}$]16:0/18:1-PS (25 ng each), samples were extracted according to the method of Bligh and Dyer [29]. The organic phase was dried under a stream of nitrogen gas and resuspended in 100 μL of a mixture of 75% HPLC solvent C (hexanes/isopropanol 30:40, v/v) and 25% solvent D (5 mM ammonium acetate in hexanes/isopropanol/water 30:40:7, v/v/v). Samples were injected into an HPLC system connected to a triple quadrupole mass spectrometer (API3200, AB SCIEX, Framingham, MA) and normal-phase chromatography was performed using a silica HPLC column (Ascentis, 150 × 2.1 mm, 5 μm, Supelco, Bellefonte, PA) at a flow rate of 200 μL/min. Solvent D was maintained at 25% for 5 min, increased gradually to 60% in 10 min and then to 95% in 5 min, and was held for 20 min before re-equilibration for 15 min. Mass spectrometric analysis was performed in the negative-ion mode using multiple-reaction monitoring (MRM) of the forty-eight molecular species potentially generated during the enzymatic assay, plus the six deuterated standards [28]. The precursor ions monitored were the molecular ions $[M-H]^-$, except for PC in which case the acetate adducts $[M+CH_3COO]^-$ were monitored. The product ions analyzed after collision-induced decomposition were the carboxylate anions corresponding to the acyl chains. Results are reported as the ratio