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Role of long-chain acyl-coenzyme A synthetases in the regulation of arachidonic acid metabolism in interleukin 1β-stimulated rat fibroblasts

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ABSTRACT

Acyl coenzyme A synthetase long-chain family members (ACSLs) are a family of enzymes that convert long-chain free fatty acids into their acyl-CoAs and play an important role in fatty acid metabolism. Here we show the role of ACSL isozymes in interleukin (IL)-1β-induced arachidonic acid (AA) metabolism in rat fibroblastic 3Y1 cells. Treatment of 3Y1 cells with triacsin C, an ACSL inhibitor, markedly enhanced the IL-1β-induced prostaglandin (PG) biosynthesis. Small interfering RNA-mediated knockdown of endogenous Acsl4 expression increased significantly the release of AA metabolites, including PGE_2, PGD_2, and $PGF_{2\alpha}$, compared with replicated control cells, whereas knockdown of Acsl1 expression reduced the IL-1β-induced release of AA metabolites. Experiments with double knockdown of Acsl4 and intracellular phospholipase A_2 (PLA_2) isozymes revealed that cytosolic $PLA_2\alpha$, but not calcium-independent PLA_2s, is involved in the Acsl4 knockdown-enhanced PG biosynthesis. Electrospray ionization mass spectrometry of cellular phospholipids bearing AA showed that the levels of some, if not all, phosphatidylcholine (PC) and phosphatidylinositol species in Acsl4 knockdown cells were decreased after IL-1β stimulation, while those in control cells were not so obviously decreased. In Acsl1 knockdown cells, the levels of some AA-bearing PC species were reduced even in the unstimulated condition. Collectively, these results suggest that Acsl isozymes play distinct roles in the control of AA remodeling in rat fibroblasts: Acsl4 acts as the first step of enzyme for AA remodeling following IL-1β stimulation, and Acsl1 is involved in the maintenance of some AA-containing PC species.

1. Introduction

Arachidonic acid (AA) is a precursor of a wide variety of eicosanoids, such as prostaglandin (PG) and leukotrienes, and its level is stringently controlled by the deacylation/reacylation pathway. The enzymes that control these pathways involve phospholipase A_2 (PLA_2), acyl-CoA synthetase, and acyl-CoA acyltransferase [3–8]. AA released by the action of PLA_2 is metabolized to various PGs and leukotrienes via cyclooxygenase (COX) or 5-lipoxygenenase, depending on the type of cells. The unmetabolized free AA is converted rapidly into arachidonoyl-CoA by acyl-CoA synthetase(s) long-chain family member (ACSL[2]) and re-esterified into glycerophospholipids by lysophospholipid acyl-CoA acyltransferase(s).

Currently, more than 20 PLA_2 isoforms have been identified. They are divided into several classes: cytosolic PLA_2 ($cPLA_2$), secreted PLA_2 ($sPLA_2$), Ca^{2+}-independent PLA_2 ($iPLA_2$), and platelet-activating factor acetylhydrolase [3–5]. Each PLA_2 isoform is believed to have their unique functions. Among PLA_2 family proteins, $cPLA_2\alpha$ has a marked preference for AA and is known to be an important PLA_2 enzyme for the stimulus-dependent liberation of AA from cellular phospholipids [9,10]. The elevation of intracellular Ca^{2+} and its own phosphorylation by mitogen-activated protein kinase are critical for the activation of $cPLA_2\alpha$ [11,12]. It was recently revealed that mice deficient in $cPLA_2\alpha$ dramatically attenuate stimulus-dependent eicosanoid biosynthetic reactions [10]. In addition, it has been reported that several $sPLA_2$s, such as group IIA $sPLA_2$ ($sPLA_2$-IIA), $sPLA_2$-V, and $sPLA_2$-X, can facilitate stimulus-dependent eicosanoid production [13–16].

Acyl-CoA synthetases, especially ACSL, catalyze the production of long-chain acyl-CoA species from long-chain fatty acids, CoA, and ATP. In humans and rodents, five ACSL isoforms have been identified: ACSL1, ACSL3, ACSL4, ACSL5, and ACSL6 [1]. Each ACSL isoform has a distinct substrate preference, subcellular localization, and tissue distribution [17–20], and has been suggested to be involved in the modulation of various pathophysiological events via the generation of long-chain acyl-

Abbreviations: AA, arachidonic acid; PG, prostaglandin; PLA_2, phospholipase A_2; COX, cyclooxygenase; ACSL, acyl-CoA synthetase long-chain family member; $cPLA_2$, cytosolic PLA_2; $sPLA_2$, secreted PLA_2; $iPLA_2$, Ca^{2+}-independent PLA_2; IL, interleukin; cPGES, cytosolic PGE synthase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; HETE, hydroxyeicosatetraenoic acid; HHT, hydroxyheptadecatrienoic acid; $AACOCF_3$, arachidonyl trifluoromethyl ketone; ESI, electrospray ionization; MS, mass spectrometry; LC, liquid chromatography; MRM, multiple reaction monitoring; PI, phosphatidylinositol

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[1] Deceased.

[2] In the present study, the human ACSL enzymes that we used are entirely capitalized and the rodent enzymes are lowercase with the exception of the first letter, according to the guidelines for human and rodent gene nomenclature [1,2].

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CoA [21,22]. Among ACSL family proteins, ACSL4 (also known as ACS4 or FACL4) has a strong preference for polyunsaturated fatty acids, such as AA and eicosapentaenoic acid [18]. The substrate preference of this isoform supposes that ACSL4 is an important enzyme for controlling the levels of free AA.

Recently, it has become clear that PG released from fibroblasts exerts a wide variety of functions in pathophysiological events, such as induction of vascular endothelial growth factor expression in mouse and human fibroblasts [23,24], mast cell maturation and anaphylaxis [25] and Th17 cell-mediated synovial inflammation [26]. Thus, dysfunction of PG synthesis in fibroblasts may trigger various undesired biological responses. Our previous studies revealed that a synergistic action of $sPLA_2$-IIA and $cPLA_2\alpha$ contributes to the release of AA for PG biosynthesis in interleukin (IL)-1β-treated rat fibroblastic 3Y1 cells [13,27]. However, little is known about the roles of ACSL enzymes in AA metabolism in these cells. The aims of this study are to assess the role of the remodeling pathway for polyunsaturated fatty acid in the regulation of eicosanoid production in rat fibroblasts.

2. Materials and methods

2.1. Materials

Mouse IL-1β and human IL-1β were purchased from R & D Systems (Abingdon, UK). Lipopolysaccharide (LPS) and mouse monoclonal antibody against β-actin were purchased from Sigma (St. Louis, MO, USA). Goat polyclonal antibodies against COX-1, COX-2, and ACSL4, and mouse monoclonal antibodies against $cPLA_2\alpha$ and ACSL3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibody against ACSL1 was purchased from Abcom (Cambridge, UK). Rabbit polyclonal antibody against rat cytosolic PGE synthase (cPGES) was prepared as described previously [28]. Triacsin C was purchased from Biomol (Plymouth Meeting, PA, USA). Lipofectamine RNAiMAX reagent, Opti-MEM medium, and TRIzol reagent were obtained from Invitrogen. Phenol red-free Dulbecco's modified Eagle's medium (DMEM) was purchased from Life Technologies (Rockville, MD, USA). 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (14:0/14:0 phosphatidylcholine (PC)), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (14:0/14:0 phosphatidylethanolamine (PE)), and 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (14:0/14:0 phosphatidylglycerol) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). PGB_2, PGD_2, PGE_2, $PGF_{2\alpha}$, 11-hydroxyeicosatetraenoic acid (11-HETE), 15-HETE, 12-heptadecatrienoic acid (12-HHT), arachidonyl trifluoromethyl ketone ($AACOCF_3$), and NS-398 were purchased from Cayman Chemicals (Ann Arbor, MI, USA).

2.2. Cell culture and treatment

Maintenance of 3Y1 cells was described previously [13]. Human fetal lung fibroblast WI-38 cells were obtained from RIKEN Cell Bank and maintained with Eagle's medium supplemented with 10% (v/v) fetal calf serum (FCS), penicillin/streptomycin (100 units/ml and 100 μg/ml, respectively), and 2 mM glutamine (Invitrogen, Carlsbad, CA, USA). Primary mouse embryonic fibroblasts (MEF) were prepared as described previously [29]. The media of these cells that had attained 80% confluence in 24-well plates were replaced with 0.5 ml of phenol red-free DMEM supplemented with 2% (v/v) FCS. After 24 h culture, 1 ng/ml mouse IL-1β (3Y1 cells and MEF), 1 ng/ml human IL-1β (WI-38 cells) was added to the cultures to assess the PG biosynthetic response. Human THP-1 monocytes were kindly provided by Dr. Hayashi (Nagoya City University, Japan) and maintained with RPMI 1640 with the same supplements. THP-1 cells were seeded on day 0 (2×10^5 cells/well in 24-well plate), and then cultured with 100 nM phorbol 12-myristate 13-acetate (PMA) to induce macrophage differentiation. After 3 days of differentiation, the media of cells were replaced with 0.5 ml of phenol red-free DMEM supplemented with 2% (v/v) FCS containing 100 ng/ml LPS, and the amounts of PG released into the supernatant were measured. In other sets of experiments, these cells were pretreated with various inhibitors for 1 h and then treated with IL-1β or LPS for 24 h. The PGs released into the culture medium and cellular phospholipid levels were determined by LC–ESI-MS/MS (see below).

2.3. Immunoblot analysis

Cell lysates (10^5 cells equivalent) were subjected to SDS-PAGE using 10% (w/v) gels under reducing conditions (for Acsl1, Acsl3, Acsl4, COX-1, COX-2, $cPLA_2\alpha$, and β-actin) or 15% (w/v) gels under nonreducing conditions (cPGES). The separated proteins were electroblotted onto nitrocellulose membranes (Schleicher & Schuell) with a bath-type blotter. After blocking for 1 h with 5% (w/v) skimmed milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (TPBS), the membranes were probed for 2 h with the respective antibodies (1:2000 for Acsl1 and Acsl3, 1:5000 for Acsl4, COX-1, COX-2, and cPGES, 1:10,000 for β-actin), followed by incubation with horseradish peroxidase-conjugated anti-mouse (1:2000 for Acsl3, $cPLA_2\alpha$, and β-actin), anti-rabbit (1:5000 for Acsl1, and cPGES), or anti-goat (1:5000 for Acsl4 and COX-2) IgG. After washing, the membranes were visualized with Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Wellesley, MA, USA).

2.4. Reverse-transcription (RT) PCR

RNA obtained from 3Y1 cells was subjected to reverse transcription using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Subsequent amplifications of the cDNA were performed using 0.5 μl of the reverse-transcribed mixture as a template with specific oligonucleotide primers (Sigma) as follows: rat Acsl1, sense 5′-ATG GAA GTC CAC GAA TTG TTC-3′ and antisense 5′-AAT CTT GAT GGT GGA GTA CAG TTC-3′; rat Acsl6, sense 5′-ATG CAG ACC CAG GAG ATC C-3′ and antisense 5′-CAT GGA GAT TGA GTA CAG CTC TTC-3′; rat Acsl3, sense 5′-ATG AAT AAC CAC GTA TCT TCA ACA C-3′ and antisense 5′-TTT TCT TCC GTA CAT CCG CTC-3′; rat Acsl4, sense 5′-ATG GCA AAG AGA ATA AAA GCT AAG C-3′ and antisense 5′-TTT GCC CCC ATA CAT CCG C-3′. The PCR mixtures were subjected to 35 cycles of amplification at 94 °C for 10 s and 65 °C for 30 s. The PCR products were analyzed by 1% agarose gel electrophoresis with ethidium bromide. The cDNA fragments were subcloned into the pcDNA3.1/V5/His-TOPO vector (Invitrogen) and sequenced.

2.5. Transfection of small interfering RNA (siRNA) into 3Y1 cells

Rat Acsl1, Acsl3, and Acsl4 siRNAs (20 nM) (siRNA ID numbers SASI_Rn01_00073026, SASI_Rn01_00100113, and SASI_Rn01_00041844; Sigma) or rat $cPLA_2\alpha$ siRNA (5 nM) (siRNA ID numbers SASI_Rn02_00267738) as well as negative control siRNA (Sigma) were transfected into 3Y1 cells using Lipofectamine RNAiMAX reagent according to the manufacturer's instructions. Two days after transfection, the media were replaced with phenol red-free DMEM supplemented with 2% FCS. After 24 h culture, 1 ng/ml IL-1β was added to the culture to activate the cells. The efficacy of each siRNA was assessed by real-time PCR or immunoblotting. The siRNA treatment resulted in a marked reduction of each Acsl and PLA_2 expression in comparison with those in the control cells (Figs. 2B and 3D).

2.6. Quantitative real-time PCR

RNA extraction, cDNA synthesis and qPCR were carried out according to standard protocols in our laboratory, as previously described [30]. The following primers were used: Acsl1, sense 5′-CTA CAG GCA ACC CCA AAG GA-3′ and antisense 5′-CCG GAG CAA TCG TTC ATA ATG-3′; Acsl3, sense 5′-AGG CCA ACA TCG CCA TCT T-3′ and antisense 5′- ACG CCT