Long-chain acyl-CoA synthetase isoforms differ in preferences for eicosanoid species and long-chain fatty acids[S]

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Abstract **Because the signaling eicosanoids, epoxyeicosatrienoic acids (EETs) and HETEs, are esterified to membrane phospholipids, we asked which long-chain acyl-CoA synthetase (ACSL) isoforms would activate these molecules and whether the apparent FA substrate preferences of each ACSL isoform might differ depending on whether it was assayed in mammalian cell membranes or as a purified bacterial recombinant protein. We found that all five ACSL isoforms were able to use EETs and HETEs as substrates and showed by LC-MS/MS that ACSLs produce EET-CoAs. We found differences in substrate preference between ACS assays performed in COS7 cell membranes and recombinant purified proteins. Similarly, preferences and Michaelis-Menten kinetics for long-chain FAs were distinctive. Substrate preferences identified for the purified ACSLs did not correspond to those observed in ACSL-deficient mouse models.[F] Taken together, these data support the concept that each ACSL isoform exhibits a distinct substrate preference, but apparent substrate specificities depend upon multiple factors including membrane character, coactivators, inhibitors, protein interactions, and posttranslational modification.—** Klett, E. L., S. Chen, A. Yechoor, F. B. Lih, and R. A. Coleman. **Long-chain acyl-CoA synthetase isoforms differ in preferences for eicosanoid species and long-chain fatty acids.** ***J. Lipid Res.*** **2017.** 58: **884–894.**

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The arachidonate (AA) metabolites, epoxyeicosatrienoic acids (EETs) and HETEs, are potent mediators of vasodilation, ion channel activation, anti-inflammatory effects, angiogenesis, mitogenesis, and polypeptide hormone secretion in multiple cell types (1, 2). Both EETs and HETEs are synthesized by cytochrome P450 (CYP450) monooxygenases, but their presence in cells does not entirely depend on acute de novo synthesis. Although initially believed to be transiently acting short-lived metabolites, both EETs and HETEs can replace acyl groups in membrane phospholipids; thus, the cellular content of these eicosanoids may also be influenced by reciprocal esterification and phospholipase pathways that have not been well-characterized (3–6). We reported that long-chain acyl-CoA synthetase (ACSL) isoform 4 (ACSL4) can use EETs as a substrate (7), but because EET- and HETE-containing phospholipids are present in tissues that express low levels of ACSL4 (8, 9), we wondered whether one or more of the other ACSL isoforms might also activate these molecules to form EET- or HETE-CoAs that could be esterified to specific phospholipids.

Five ASCLs (ACSL1, -3, -4, -5, and -6) (EC 6.2.1.3) convert FAs of 12–20 carbons to their corresponding FA-CoAs. Each ACSL isoform has been functionally differentiated by its FA chain length preference, tissue distribution, and subcellular location, attributes that alter the ability of an individual ACSL to channel FAs toward disparate metabolic fates (10).

Once activated, acyl-CoAs have multiple metabolic fates, including the synthesis of triacylglycerol, cholesterol esters, retinal esters, and phospholipids, β-oxidation, FA elongation and desaturation, protein acylation, and use as signaling molecules (10). Members of the ACS family require

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Abbreviations: AA, arachidonate; ACS, acyl-CoA synthetase; ACSL, long-chain acyl-CoA synthetase; CYP450, cytochrome P450; EET, epoxyeicosatrienoic acid; F-ACSL, FLAG-long-chain acyl-CoA synthetase; MBOAT, membrane-bound O-acyltransferase.

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Co-A and substrates with a carboxylic acid group in order to catalyze the thioesterification reaction. The thioesterification two-step reaction is energetically costly, using the equivalent of two high-energy bonds (11, 12). This high energetic cost emphasizes the importance of the ACS-catalyzed reaction as a regulatory node for the metabolism of FAs.

The five ACSL isoforms were identified, cloned, and characterized by Yamamoto and his colleagues (8, 13–15), and most of our knowledge regarding their substrate preferences comes from these initial studies. However, the substrate preferences reported in these studies cannot be directly compared with each other because the enzymes were expressed differently and the methods used for activity assays varied. For activity studies of ACSL1, ACSL4, ACSL5, and ACSL6 [initially known as ACSL2 (16)], the enzymes were expressed in and purified from *Escherichia coli*, whereas ACSL3 was expressed and studied in COS7 (monkey kidney) cells, but in each instance, the substrate concentration ranges and the specific V_{max} and K_m values were unclear (8, 13–15). Although substrate specificities for the ACSL isoforms were reported, direct and systematic comparisons of substrate preferences for each ACSL isoform were not performed.

The importance of substrate preference for a specific ACSL isoform suggests how and where a specific substrate will be metabolized. We report here the ACS enzyme kinetics with different FA and eicosanoid substrates of the rat ACSL isoforms overexpressed in bacterial and mammalian cells. Further, we provide validation of the indirect spectrophotometric ACS activity assay by showing LC-MS/MS evidence that the product of the reaction produces an acyl thioester.

MATERIALS AND METHODS

Materials

AA, (±)-8,9-EET, (±)-11,12-EET, and (±)-14,15-EET were purchased from Cayman Chemical (Ann Arbor, MI). Arachidonoyl-CoA (20:4-CoA) was purchased from Avanti Polar Lipids (Alabaster, AL). All other FAs and reagents were purchased from Sigma (St. Louis, MO).

Construction of recombinant pFLAG-ACSLs and mammalian ACSL plasmid

cDNA was synthesized from either rat liver or brain total RNA (extracted using TRIzol; Invitrogen) and used as a template to amplify the ACSL open reading frames (high capacity cDNA reverse transcription kit; Applied Biosystems, Foster City, CA). Primers for amplification of ACSL1, ACSL3, ACSL4, ACSL5, and ACSL6 were designed to include the entire open reading frames, based on nucleotide sequences obtained from the GenBank database (supplemental Table S1). ACSL amplification was performed by PCR with the designed primers. The amplified ACSL PCR products were ligated into either pFLAG-CTC vector (Sigma) or pcDNA3.1 vector (Invitrogen) digested with the same restriction enzymes. The sequences of pFLAG-ACSL and pcDNA3.1-ACSL fusion constructs were verified by the University of North Carolina Automated Sequencing Facility.

Expression of recombinant F-ACSL proteins in *E. coli*

Recombinant F-ACSLs were expressed in *E. coli* DH5α after induction with 1 mM IPTG at an *A*600 of 1.0. DH5α was grown in Terrific Broth (Life Technologies, Inc.) supplemented with ampicillin (60 g/ml) at 30°C and shaken at 250 rpm. After a 12 h induction, cells were harvested by centrifuging at 4,800 *g* for 10 min in a Sorvall HS-4 rotor. The cell pellet was resuspended in 10 ml of 10 mM Tris (pH 7.4), 0.5 mM EDTA (TE) buffer. Resuspended cells were incubated with 100 g/ml lysozyme for 30 min on ice and then sonicated with six 10 s bursts, each followed by a 10 s rest on ice. Cellular debris was removed from the cell lysates by centrifugation at 3,000 *g* for 10 min. Part of the supernatant was saved (cell extract), and the remainder was layered over a 2 ml cushion of 55% (w/w) sucrose topped with 0.5 ml of 5% (w/w) sucrose in TE buffer. After centrifuging in a Beckman SW41 rotor at 210,000 *g* for 3 h, the supernatant was removed (soluble fraction). The membrane band at the interface was collected with a 19 gauge needle and syringe. Protein concentrations were determined by the BCA method (Pierce).

Purification of the recombinant F-ACSL proteins

F-ACSLs were purified by Flag M2 column chromatography. The Flag M2 antibody affinity matrix (1 ml) (Sigma) was activated with 0.1 M glycine (pH 3.5), 50 mM Tris (pH 7.4), and 150 mM NaCl (TBS) buffer. DH5α membrane fractions containing overexpressed F-ACSLs were solubilized in TBS containing 1% Triton X-100 and passed over the column four times. The column was washed three times with 12 ml of TBS (pH 7.4), and then eluted with five 1 ml aliquots of 100 g/ml Flag peptide (Sigma) dissolved in TBS buffer (pH 7.4).

Transient transfection of pcDNA3.1-ACSL1 and ACSL4

COS7 cells were routinely cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum. COS7 cells were plated at a cell density of 2.0×10^6 in 10 cm dishes and transfected for ~18 h after plating with 10 μg of plasmid carrying rat ACSL1 or ACSL4 (XtremeGene HP; Roche). Cell homogenates were collected 48 h posttransfection in ice-cold medium A [10 mM Tris (pH 7.4), 250 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol, and protease inhibitor mixture (Sigma)]. Homogenates were then centrifuged at 1,000 *g* for 10 min at 4°C. Total membranes were prepared by subjecting the supernatant to ultracentrifugation at 100,000 *g* for 1 h at 4°C. The resulting supernatant was removed and the membrane pellet was resuspended in ice-cold medium A. Aliquots were stored at −80°C until use.

Spectrophotometric ACS activity assay

Acyl-CoA synthetase (ACS) activity was assayed by coupling the reaction of ACS with those of adenylate kinase, pyruvate kinase, and lactate dehydrogenase and following the oxidation of NADH at 334 nm with a recording spectrophotometer (Beckman DU640) (17). The reaction mixture contained 100 mM Tris-HCl buffer (pH 8.0), 10 mM ATP, 15 mM $MgCl_2$, 5 mM dithiothreitol, 150 mM KCl, 1.0 mM potassium phosphoenolpyruvate, 0.3 mM NADH in 100 mM triethanolamine (pH 8.2), and either FA or eicosanoid (solubilized in 15 mM Triton X-100 and 0.1 mM EDTA). Then 45 μg adenylate kinase, 30 μg pyruvate kinase, and 30 μg lactate dehydrogenase were added in a total reaction volume of 1 ml. Varying amounts of either purified ACSL or COS7 cell membranes were used to create a protein dependence curve such that 1 μg protein yielded ACS activity in a linear range. The reaction mixture was incubated for 1 min at 37°C, and the reaction was initiated by the addition of CoA (final concentration 600 μM). Change in absorbance at 334 nm was measured every 10 s for 5 min. The reaction rate was calculated using the slope and

intercept created from an NADH standard curve with concentrations from 0 to 0.2256 M.

Identification of AA-CoA and EET-CoAs by LC-MS/MS

Methods were adapted from Magnes et al. (18). Solid phase extraction was performed on Hypersep Retain PEP 60 mg, 3 ml cartridges (Thermo Fisher Scientific). Elution solvent was prepared by mixing 50 ml water with 50 ml acetonitrile plus 100 μl 28% NH_4OH solution and 5 μl formic acid. Cartridges were conditioned with 3 ml acetonitrile and then 3 ml water. Two hundred fifty microliters of reaction mixture were diluted with 2 ml water and loaded onto the cartridge. The cartridge was rinsed with 6 ml water before eluting acyl-CoAs in 2.5 ml of elution solvent.

Analysis was performed on an Ultimate 3000 UHPLC and Quantiva tandem mass spectrometer (Thermo Fisher Scientific.) The chromatography column was an Xselect CSH C18, 2.1 × 50 mm, 3.5 μm particles (Waters). Mobile phase A consisted of 1 ml 28% NH_4OH solution, 50 μl formic acid, and 1,000 ml water. Mobile phase B was acetonitrile. The starting mobile phase was 20% B and was held for 0.5 min before ramping to 40% B over 3.5 min. The column was held at 40% B for 2 min and then returned to 20% B for 3 min for re-equilibration. The injection volume was 4 μl.

Samples were introduced to the mass spectrometer via an ESI source. The vaporizer temperature was 300°C and the ion transfer tube temperature was 325°C. Sheath, auxiliary, and sweep gas were set to 35, 20, and 1 arbitrary units, respectively. Spray voltage was 4,250 V for positive ion experiments and −3,750 V for negative ion experiments. Acyl-CoAs were detected via positive ion neutral loss scanning from *m/z* 900 to 1,400 with neutral loss set to 507.1. The collision energy was set to 34 V and collision cell pressure was 2 mTorr. Positive product ion spectra of acyl-CoAs were also obtained.

Western blot

FLAG column purified *E. coli* F-ACSLs (F-ACSLs) (6 μg) and membranes from COS7 cells (50 μg) were separated by electrophoresis on a 10% polyacrylamide gel and transferred to nitrocellulose membranes (Bio-Rad). Protein expression of F-ACSLs was detected by anti-FLAG monoclonal antibody (Sigma; F1804). For ACSL1 and ACSL4 expressed in mammalian cells, rabbit anti-human ACSL1 (Cell Signaling, Danvers, MA; 4047) and rabbit anti-human ACSL4 (19), a generous gift from S. M. Prescott (University of Utah, Salt Lake City, UT), were used to detect ACSL1 and ACSL4, respectively. GAPDH was used as the loading control (Abcam, Cambridge, MA; ab8245). To determine the purity of FLAG-column purified F-ACSLs, both *E. coli* lysates (40 μg) and purified F-ACSLs (6 μg) were separated by electrophoresis on a 10% polyacrylamide gel and stained with Coomassie brilliant blue.

Data analyses

Data are expressed as mean ± SEM from three separate experiments. Michaelis-Menten kinetic enzyme activity curves were drawn and V_{max} and K_m values derived using GraphPad Prism® 6.0 h (San Diego, CA).

RESULTS

Bacterial and mammalian overexpressed ACSL isoforms are able to activate EETs and HETEs

EETs and HETEs are oxidative products of CYP450 epoxygenases, using AA as the substrate. EETs and HETEs function as autocrine and paracrine signals that regulate vasodilation, ion channel activation, anti-inflammatory effects, angiogenesis, mitogenesis, and polypeptide hormone secretion (2). They are incorporated into phospholipids by a CoA-dependent process that plays a significant role in regulating EET actions on either a membrane receptor or as a direct interactor with intracellular effectors (2). We previously reported that purified bacterial F-ACSL4 can use (±)-8,9-EET, -11,12-EET, and -14,15-EET as substrates in an indirect ACS activity assay (7). Because AA is the precursor for all eicosanoids and because eicosanoids contain a terminal carboxyl group, we hypothesized that the ACSL isoforms would differ in their activation of eicosanoid substrates. Using the sequences of rat ACSL isoforms (supplemental Table S1), we constructed bacterial plasmids containing the cDNA for each isoform linked to a C terminus FLAG tag (pACSL-FLAG). Bacterial pACSL-FLAG plasmids were transfected and induced in *E. coli* and the resulting recombinant proteins were purified by column chromatography. To ensure expression of the recombinant proteins, we resolved 6 μg of FLAG column purified F-ACSLs by PAGE, blotted, and probed with anti-FLAG antibody (**Fig. 1**). Because there were doublet bands observed in the immunoblot, we wanted to ensure the purity of the FLAG-column purified recombinant F-ACSLs and again resolved each purified F-ACSL isoform (6 μg) and the *E. coli* lysates (40 μg) by PAGE and stained with Coomassie brilliant blue (supplemental Figs. S1, S2). Each purified F-ACSL showed a double band with the upper band (∼80–75 kDa) being the predicted molecular mass for each F-ACSL isoform. We suspect that the lower bands (∼60 kDa) are degradation products. Using purified bacterial expressed F-ACSL enzymes, we performed indirect ACS activity assays using 1 μg protein with a fixed (5 μM) final concentration of EETs and HETEs as substrates. To validate our indirect ACS activity assay and ensure that eicosanoid-CoAs were produced, we performed the indirect ACS activity assay with F-ACSL4 using 5 μM AA, 8,9-EET, 11,12-EET, and 14,15-EET as substrates. Upon reaction termination, the reaction products were subjected to LC-MS/MS analyses. Comparing the authentic reference standard, arachidonoyl-CoA (20:4-CoA) (**Fig. 2B**), to the reaction product of F-ACSL4 and AA (Fig. 2C), we found a distinct peak at *m/z* 1,054 corresponding to arachidonoyl-CoA $[M+H]^+$. During positive ion ESI-MS/MS, a distinctive neutral loss of 507.1 Da corresponds to the loss of the phosphoadenosine diphosphate (CoA moiety) that is common to all acyl-CoAs

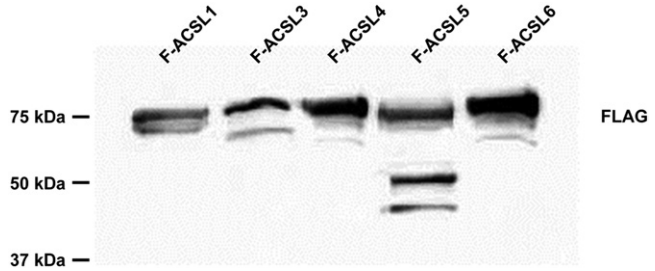

Fig. 1. FLAG column purified F-ACSL isoforms overexpressed in *E. coli* were resolved by PAGE and immunoblotted with anti-FLAG antibody.

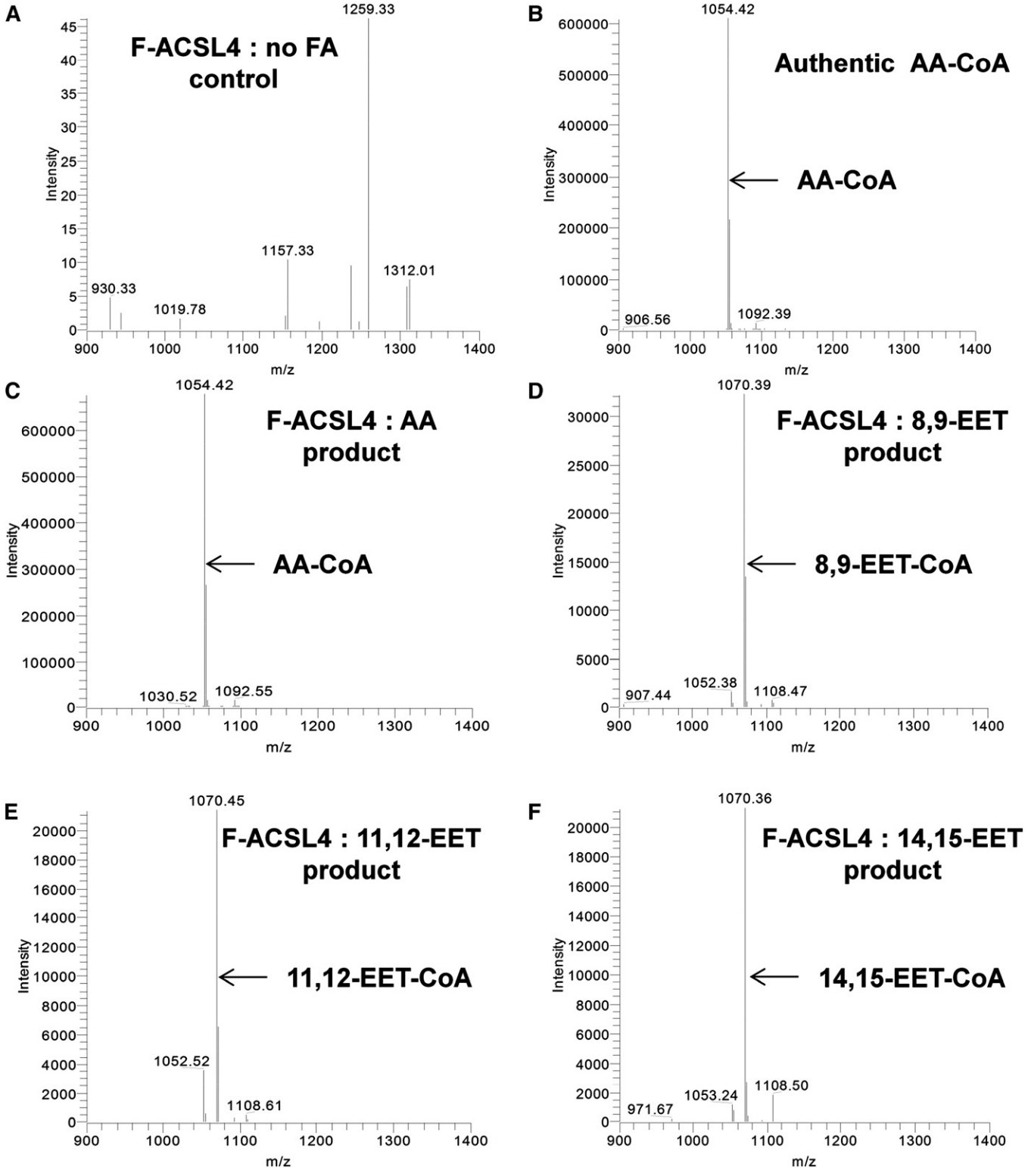

Fig. 2. Neutral loss of phosphoadenosine diphosphate (507.1 Da) scan of the $[M+H]^+$ ions from F-ACSL4 no FA blank control (A), authentic AA-CoA positive control (Avanti Polar Lipids) (B), reaction product from F-ACSL4 and AA (C), reaction product from F-ACLS4 and 8,9-EET (D), reaction product from F-ACSL4 and 11,12-EET (E), and reaction product from F-ACSL4 and 14,15-EET (F). AA-CoA 1,054.3 Da and EET-CoAs 1,070.4 Da.

(supplemental Fig. S3) (18). We next analyzed the reaction products of F-ACSL4 and the EETs, with the expected *m/z* of the EET-CoAs to be 1,070 (AA → 1,054 plus oxygen → 16). Indeed, for each of the EETs tested, we observed strong peaks at *m/z* 1,070 corresponding to EET-CoAs (Fig. 2D–F). Further, positive product ion scans of reaction products showed ion peaks that were consistent with the formation of EET-CoAs (supplemental Fig. S4). Taken

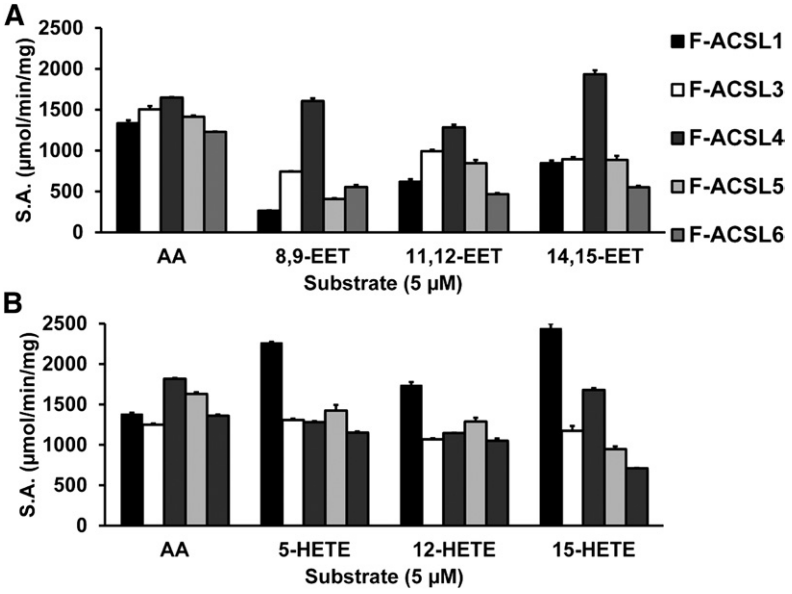

Fig. 3. Purified F-ACSLs can activate either EETs (A) or HETEs (B) as substrates. ACS activity with different substrates at 5 μM (final concentration). Error bars reflect SEM from three separate experiments. S.A., specific activity.

together, the LC-MS/MS analyses of the indirect ACS activity assay reaction products provide strong evidence that EET-CoA products are formed.

Following validation that the indirect ACS activity assay produced EET-CoA products, we assayed each of the F-ACSL isoforms with EETs and HETEs as substrates. Each of the F-ACSL isoforms was able to convert both substrates to acyl-CoA products, but we observed differences in substrate preference among the isoforms (**Fig. 3**). For example, F-ACSL4 exhibited the greatest activity with EETs, whereas F-ACSL1 showed the greatest activity with HETEs (Fig. 3A, B). Interestingly, compared with the parent molecule, AA, F-ACSL1 had greater activity with all three HETE species tested (Fig. 3B). ACS activity with F-empty vector using the substrates tested was not observed (data not shown).

Previous reports describing the substrate preferences of ACSL isoforms were based on proteins expressed and purified from recombinant bacteria (8, 14, 15). Because ACSLs are normally membrane bound proteins, we wondered whether there were differences between purified ACSL isoforms expressed in bacteria versus the ACSL isoforms overexpressed in membranes of mammalian cells. To answer this question, we cloned rat ACSL1 and ACSL4 into the mammalian pcDNA3.1 expression vector and transformed COS7 cells to overexpress ACSL1 and ACSL4 isoforms (supplemental Table S1). We selected ACSL1 and ACSL4 because they had the greatest ACS activity from the bacterial expressed F-ACSLs using the eicosanoids as substrates. To ensure overexpression, we resolved 50 μg of membranes by PAGE, blotted and probed with anti-ACSL1 or anti-ACSL4 antibody (**Fig. 4**). Both ACSL1 and ACSL4 were overexpressed compared with control COS7 cells transfected with the empty vector. Interestingly, in COS7 cells, the basal expression of ACSL4 appeared higher than the overexpressed ACSL1. Despite the high amount of basal ACSL4 protein expression, the indirect ACS activity using palmitate was 1,000-fold lower compared with what we observed with the overexpressed ACSL isoform indirect ACS activities (nanomoles per minute per milligram protein vs. micromoles per minute per milligram protein, respectively) (supplemental Fig. S7). We performed indirect ACS activity assays using 1 μg protein from total membrane preparations using the same fixed (5 μM) final concentration of EETs and HETEs as substrates. To validate that EET-CoAs were produced with COS7 overexpressed ACSL4, we again performed indirect ACS activity assays and subjected the reaction products to LC-MS/MS analyses. As with the F-ACSL4 reaction products, the COS7-ACSL4 reactions with 5 μM AA, 8,9-EET, 11,12-EET, and 14,15-EET as substrates produced AA-CoA and EET-CoA products, respectively (**Fig. 5**). Further, chromatographic retention times of the COS7-ACSL4 reaction products for AA and EETs corresponded to those from the F-ACSL4 reaction products (supplemental Figs. S5, S6). However, the *m/z* 1,054 and 1,070 peaks were much less intense compared with the F-ACSL4 reaction products, probably because the F-ACSLs were purified enzymes, unlike the

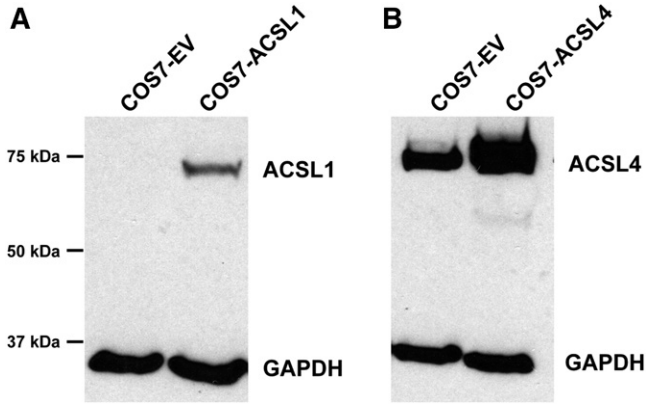

Fig. 4. Overexpressed ACSL1 and ACSL4 in COS7 cells. Immunoblots of membranes (50 μg) from COS7 cells overexpressing ACSL1 (A) and ACSL4 (B). GAPDH served as loading control. EV, empty vector.

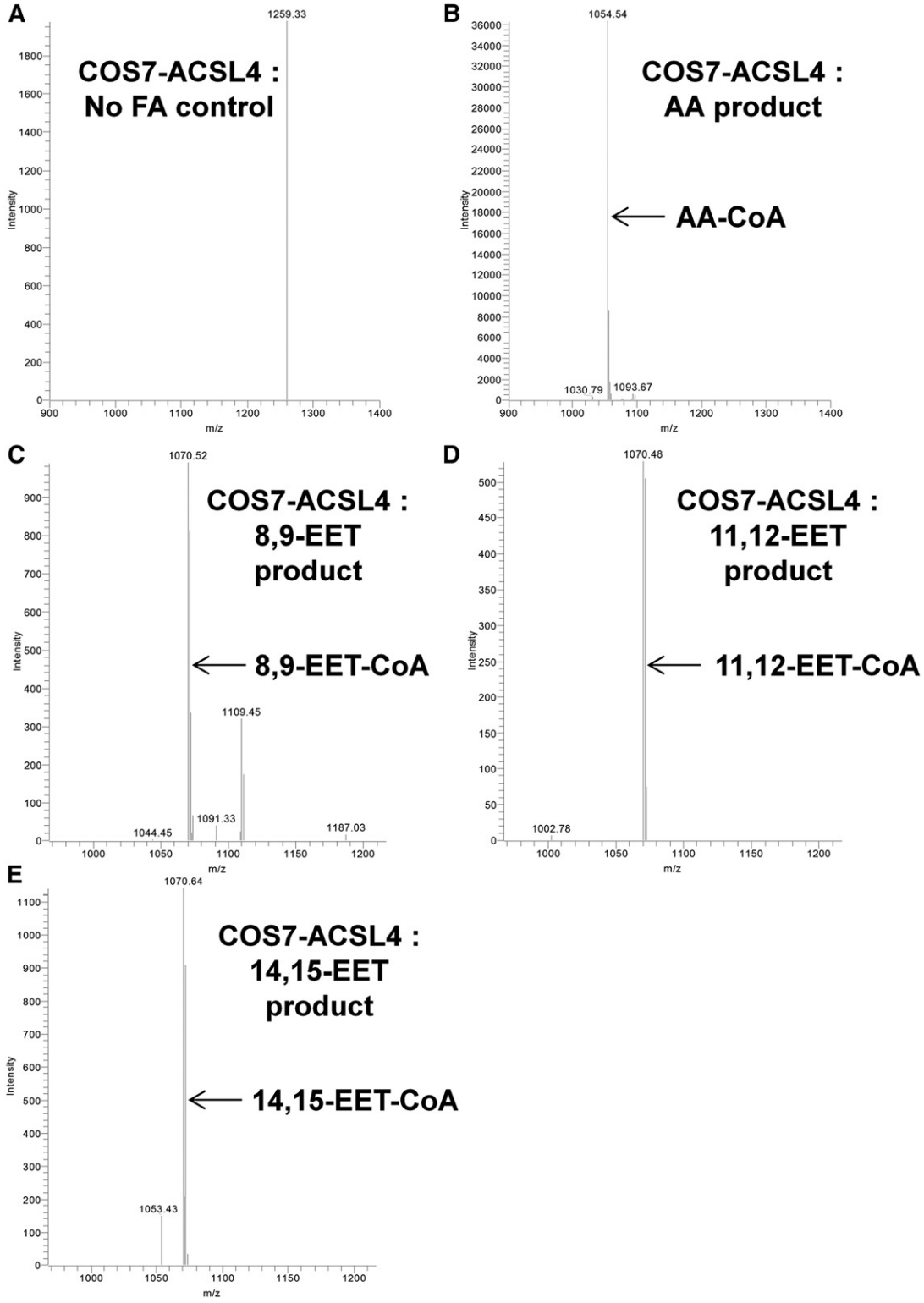

Fig. 5. Neutral loss of phosphoadenosine diphosphate (507.1 Da) scan of the $[M+H]^+$ ions from COS7-ACSL4 and no FA control (A), reaction product from COS7-ACSL4 and AA (B), reaction product from COS7-ACSL4 and 8,9-EET (C), reaction product from COS7-ACSL4 and 11,12-EET (D), and reaction product from COS7-ACSL4 and 14,15-EET (E). AA-CoA 1,054.3 Da and EET-CoAs 1,070.4 Da.

COS7-ACSLs, which were membrane bound and surrounded by lipids, proteins, and cofactors. It is likely that these membrane constituents impact the activity of the ACSL. Despite these less intense peaks, overexpressed COS7-ACSL4 was able to synthesize EET-CoAs.

Comparing the ACS activities between COS7-ACSL1 and COS7-ACSL4 with EET and HETE as substrates, we found that ACSL4 had greater activity with both EETs and HETEs compared with ACSL1 (**Fig. 6**). Further, the mammalian expressed ACSL1 and ACSL4 had greater activity with the

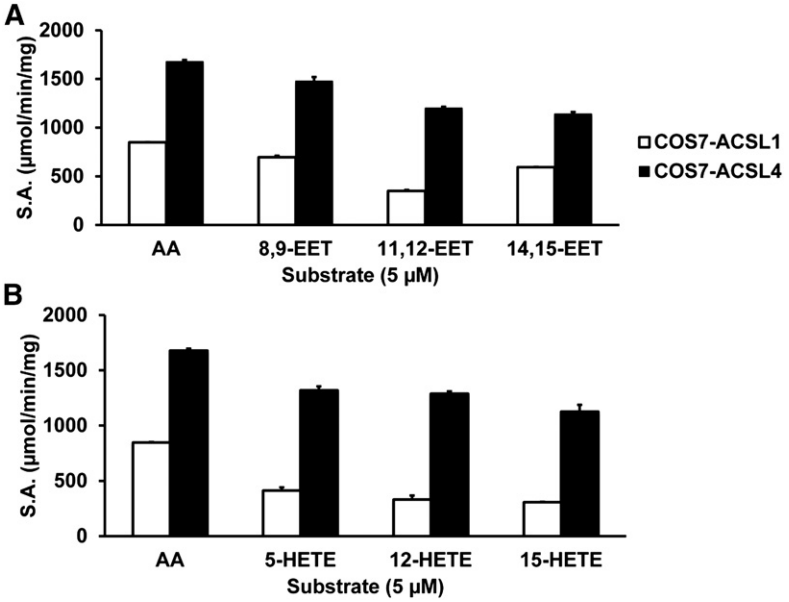

Fig. 6. ACSL1 and ACSL4 overexpressed in COS7 cells use both EETs and HETEs as substrates. ACS activity with 5 μM final substrate concentrations of AA, 8,9-EET, 11,12-EET, 14,15-EET, 5-HETE, 12-HETE, and 15-HETE. Error bars reflect SEM from three separate experiments. S.A., specific activity.

parent AA than with EETs or HETEs, which was not seen with the bacterial F-ACSL1 and F-ACSL4 assays. Again, these differences in ACS activity are likely due to the membrane constituents retained in mammalian preparations, but not in the purified F-ACSLs.

FA substrate preferences of ACSL isoforms expressed in mammalian cells differ from those expressed in bacteria

Because of previous reports describing the substrate preferences of ACSL isoforms from purified recombinant bacteria and because we identified substrate preference differences using EETs and HETEs between bacterial and mammalian expressed ACSL isoforms, we wondered whether there were differences in substrate preference for FAs based on whether the ACSL isoform was expressed in bacteria or expressed in mammalian membranes. Using FAs of differing chain length and degree of unsaturation (palmitate, 16:0; oleate, 18:1; stearate, 18:0; linoleate, 18:2; AA, 20:4) at concentrations ranging from 2.5 μM to 100 μM, we performed indirect ACS activity assays (17) using the purified F-ACSL isoforms to characterize the enzyme kinetics. The plotted Michaelis-Menten kinetic

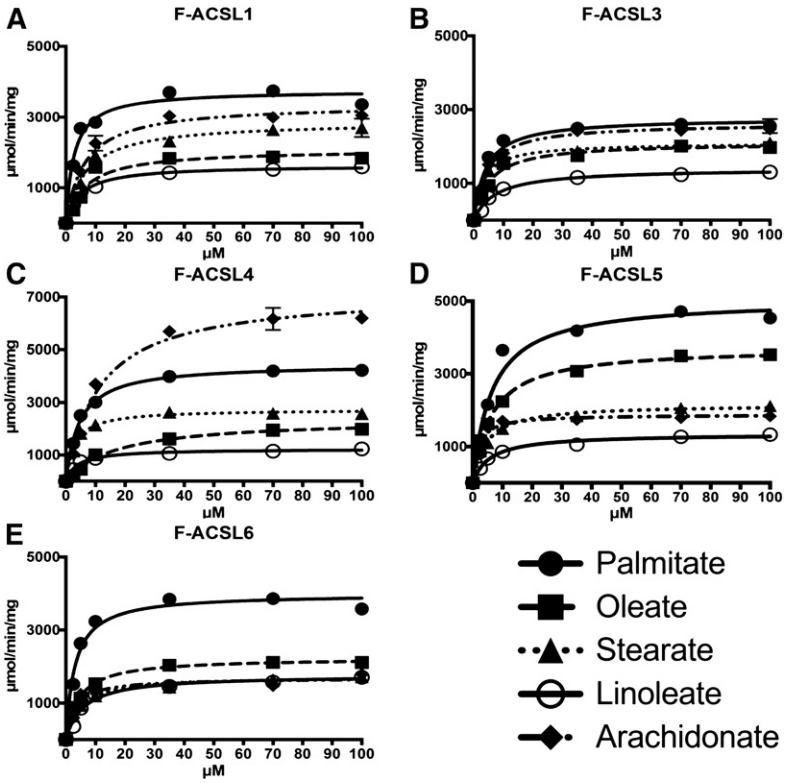

Fig. 7. Michaelis-Menten kinetic enzyme activity curves from purified F-ACSL isoforms assayed spectrophotometrically with different FAs. F-ACSL1 (A), F-ACSL3 (B), F-ACSL4 (C), F-ACSL5 (D), and F-ACSL6 (E). Data points represent the mean of determinations from proteins obtained in three independent experiments.

TABLE 1. V_{max} values from purified F-ACSLs with various substrates

	V_{max} (μmol/min/mg)				
	16:0	18:0	18:1	18:2	20:4
F-ACSL1	3,754 ± 100[a]	2,874 ± 74	2,089 ± 108	1,635 ± 33	3,363 ± 104
F-ACSL3	2,763 ± 98[a]	2,099 ± 33	2,109 ± 52	1,394 ± 31	2,627 ± 44
F-ACSL4	4451 ± 60	2,737 ± 45	2,366 ± 79	1,231 ± 44	7,180 ± 229[a]
F-ACSL5	5,053 ± 209[a]	2,164 ± 39	3,726 ± 44	1,351 ± 35	1,880 ± 49
F-ACLS6	3,993 ± 105[a]	1,725 ± 31	2,238 ± 35	1,763 ± 55	1,682 ± 69

V_{max} values derived from kinetics data from Fig. 7 where data points are the mean of determinations from proteins obtained in three independent experiments.

[a]Indicates the substrate with which the greatest ACS activity is achieved for each F-ACSL.

enzyme activity curves (**Fig. 7**) showed that each F-ACSL isoform had activity with each substrate, but that substrate preferences were distinct. For F-ACSL1, -3, -5, and -6, the apparent V_{max} values were highest with palmitate (**Table 1**); however, similar to a previous report (8), the V_{max} and K_m values of F-ACSL4 were greatest with AA [7,180 ± 229 μmol/min/mg and 11.4 ± 1.3 μM, respectively (Table 1)]. The apparent V_{max} and K_m values obtained from recombinant proteins show different orders of FA substrate preferences in Table 1 and **Table 2**.

Because we noted differences in substrate preference between loss-of-function rodent models of ACSL1 (20) and the purified bacterial expressed ACSLs, we suspected that substrate preference would differ in the mammalian expressed ACSL isoforms. Indeed, similar to the overexpressed purified F-ACSL isoforms in bacteria, the substrate preferences, based on the apparent V_{max} and K_m values, differed between COS7 cell overexpressed ACSL1 and ACSL4 isoforms (**Fig. 8**, **Tables 3, 4**).

Activities with 16:0 were highest for both purified recombinant F-ACSL1 and the COS7 cell overexpressed ACSL1, but marked differences were observed for substrates with chain lengths longer than 16 carbons and for unsaturated FAs. For example, recombinant F-ACSL1 preferred 20:4, whereas COS7-ACSL1 did not (Tables 1, 3). Major differences in substrate preference were not observed between recombinant F-ACSL4 and COS7-ACSL4, but we confirmed the previous findings that ACSL prefers 20:4 (8), a preference that was not affected by ACSL4 expression in bacteria compared with expression in mammalian cells. Taken together, these data suggest that ACSL activity and substrate preference depend on multiple factors, including the substrate, membrane character, coactivators, inhibitors, interactions with other enzymes, ACSL cellular location, and ACSL tissue expression.

DISCUSSION

AA is converted to potent eicosanoid signaling molecules by the cyclooxygenase, lipoxygenase, and CYP450 monooxygenase pathways, which produce both EETs and HETEs by CYP epoxygenases and CYP ω-oxidases, respectively (21). EETs and HETEs have multiple biologic functions that have been primarily defined in vascular and renal systems, including vasodilation, ion channel activation, anti-inflammatory effects, angiogenesis, mitogenesis, regulation of coagulation, and polypeptide hormone secretion. Despite the focus on the free eicosanoids, most EETs and HETEs are in cell membranes, esterified in glycerophospholipids (4, 5, 22). The mechanism by which EETs and HETEs exert their biological effects is not entirely known, but the presence of esterified EETs and HETEs in phospholipids suggests that they might either alter membrane dynamics that change receptor or channel activity or serve as a preformed reservoir that can be released by activated phospholipases potentially leading to downstream receptor activation (1, 2, 23). Further, the esterification of EETs and HETEs into glycerophospholipids could serve as a means to "de-activate" these signaling molecules. The incorporation of EETs and HETEs into phospholipids requires a CoA-dependent process (5, 24). Once the EET or HETE is activated by an ACS, it can be esterified by a phospholipid acyltransferase, such as membrane-bound O-acyltransferase (MBOAT)5 (MBOAT5/LPCAT3) or MBOAT7 (MBOAT7/LPIAT1) (**Fig. 9**) (25). Indeed, we show here that each of the ACSL isoforms is able to activate

TABLE 2. K_m values from purified F-ACSLs with various substrates

	K_m (μM)				
	16:0	18:0	18:1	18:2	20:4
F-ACSL1	2.7 ± 0.4	6.6 ± 0.7	6.7 ± 1.4	5.0 ± 0.4	6.3 ± 0.8
F-ACSL3	4.0 ± 0.7	3.1 ± 0.2	5.3 ± 0.6	7.3 ± 0.6	4.3 ± 0.3
F-ACSL4	4.5 ± 0.3	2.9 ± 0.2	16.7 ± 1.9	4.0 ± 0.6	11.4 ± 1.3
F-ACSL5	6.5 ± 1.1	4.4 ± 0.4	6.3 ± 0.3	5.8 ± 0.6	1.8 ± 0.3
F-ACLS6	3.0 ± 0.4	4.4 ± 0.4	4.4 ± 0.3	6.0 ± 0.8	2.9 ± 0.6

K_m values derived from kinetics data from Fig. 7 where data points are the mean of determinations from proteins obtained in three independent experiments.

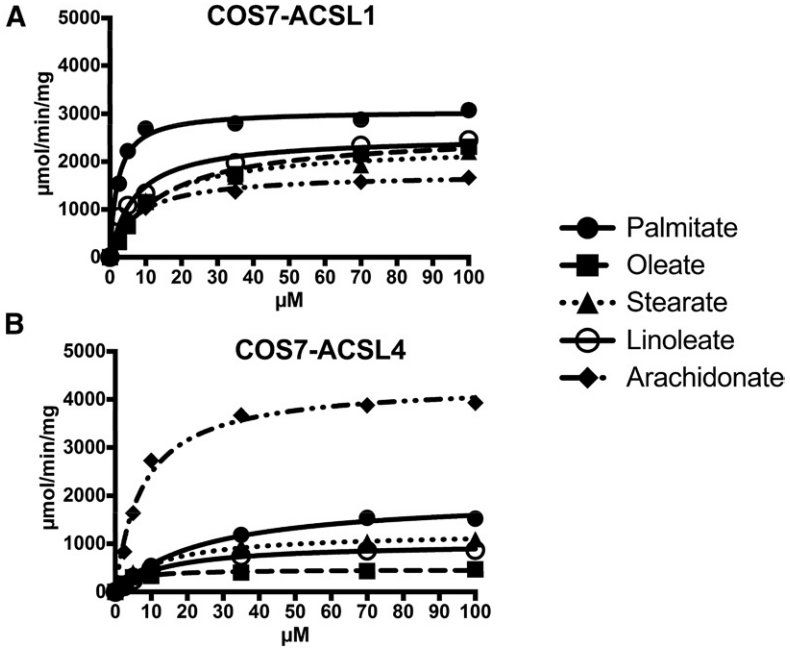

Fig. 8. Michaelis-Menten enzyme activity curves from membranes of COS7 cells overexpressing ACSL1 (A) or ACSL4 (B) with different FAs. Data points represent the mean of determinations from three independent experiments.

EETs and HETEs to produce EET-CoAs and HETE-CoAs, respectively.

The CYP450 monooxygenases that produce EETs and HETEs are expressed in the heart, vasculature, kidney, brain, liver, lung, spleen, blood cells, testis, and pancreas (26). Because the different ACSL isoforms differ in their EET and HETE substrate specificities, the expression of specific ACSL isoforms (27) may directly influence EET and HETE metabolism and action in a tissue-specific manner and suggest why these eicosanoids might differ in their biological functions and metabolism.

ACSs catalyze the thioesterification of FAs with CoA to yield fatty acyl-CoAs. After activation, the fatty acyl-CoA products have multiple metabolic fates, including membrane synthesis, energy production, storage, and the production of signaling molecules. Despite the fact that each ACSL isoform performs the identical reaction, the isoforms differ in their tissue and subcellular distribution and likely substrate preference. We show here that FA preferences of the ACSL isoforms are similar, but not identical, when determined with purified *E. coli* preparations versus COS7 cell membranes. The reason for the differences in substrate preference between the purified bacterial and membrane bound mammalian expressed ACSL isoforms is multifactorial, including membrane character, coactivators, inhibitors, interactions with other enzymes, specific ACSL cellular location, specific ACSL expression, and posttranslational modification (6). Indeed, we have previously shown that ACSL1 is differentially phosphorylated and acetylated in different cell types (28). Furthermore, in cardiac muscle, where the ACSL1 isoform predominates, ACSL activity is 3.5 times greater with 18:2 than with 16:0, 18:0, 18:1, or 20:4 (20), strongly suggesting that tissue-specific composition of membranes may also influence ACSL substrate preference. Collectively, the substrate preferences presented here and those from previous animal model experiments indicate that when describing ACSL substrate preference, it must be placed into the context of the model system.

Our results with recombinant expressed ACSL isoforms are comparable to substrate preferences that were previously reported (8, 14, 15) in that ACSL1, -5, and -6 preferred 16:0, whereas ACSL4 preferred 20:4. Because the ACS activity of ACSL3 was originally reported from expression in COS7 cells, we were not able to directly compare our substrate preference from bacterial recombinant F-ACSL3. However, our results that F-ACSL3 prefers 16:0 agree with the preferences reported for ACSL3 expressed in COS7 cells (13). We also observed higher ACS specific activities from recombinant expressed ACSL isoforms than previously reported. While there are multiple

TABLE 3. V_{max} values from overexpressed ACSL1 and ACSL4 COS7 membranes with various substrates

	V_{max} (µmol/min/mg)				
	16:0	18:0	18:1	18:2	20:4
COS7-ACSL1	3,068 ± 54[a]	2,342 ± 74	2,607 ± 63	2,525 ± 75	1,745 ± 48
COS7-ACSL4	1,990 ± 69	1,240 ± 29	466 ± 11	1,011 ± 30	4,339 ± 102[a]

V_{max} values derived from kinetics data from Fig. 8 where data points are the mean of determinations from proteins obtained in three independent experiments.

[a]Indicates the substrate with which the greatest ACS activity is achieved for each COS7 ACSL.

TABLE 4. K_m values from overexpressed ACSL1 and ACSL4 COS7 membranes with various substrates

	K_m (μM)				
	16:0	18:0	18:1	18:2	20:4
COS7-ACSL1	2.1 ± 0.2	11.5 ± 1.3	14.7 ± 1.2	7.0 ± 0.8	7.3 ± 0.8
COS7-ACSL4	24.8 ± 2.5	12.5 ± 1.0	3.6 ± 0.4	13.3 ± 1.4	7.5 ± 0.7

K_m values derived from kinetics data from Fig. 8 where data points are the mean of determinations from proteins obtained in three independent experiments.

explanations for these differences, the most likely relate to how we made the ACSL isoform constructs, which contained a FLAG epitope, and our method of purification. We likely obtained purer enzyme preparations because we used FLAG affinity column purification. It seems less likely that the fusion of FLAG to the C terminus enhances ACSL activity.

Our measures of ACS-specific activity between the different ACSL isoforms and substrates have several limitations. First, three different methods have been used historically to measure ACS activity; these include direct HPLC measurements (29) or ^{3}H-labeled CoA (30), and the indirect coupled assay measuring the reduction of NADH (17). Each method has its own specific pitfalls, but in all cases the methods measure only relative activities for a given experimental condition. As a result, it is not possible to compare ACS activities obtained with differing methods. In this study, to allow comparisons of substrate preference, we used the same assay for recombinant and cell expressed ACSL isoforms, and we maintained the same experimental conditions. A second limitation concerns substrate concentration; the amount of substrate used in these assays far exceeds the intracellular concentration of unbound free FA, calculated to be less than 1 μM (31). Furthermore, we cannot know the FA concentration at the enzyme's active site. These difficulties make it difficult to relate in vitro to in vivo ACS activities. Until we have more sensitive methods to measure the in vivo fluxes of free FAs and acyl-CoAs simultaneously, we are forced to rely on the current measures of ACS activity. Finally, our in vitro ACS activity assays do not account for the mixtures of FAs that are normally presented to ACSLs. To define a true substrate preference, an ACSL must select a FA from a cellular pool of FAs and FA metabolites.

We have shown, using a validated indirect assay for ACS activity, that whereas each ACSL isoform catalyzes the same reaction, individual ACSL isoforms differ in substrate preferences that appear to be modified depending on the model system in which the ACSL is expressed. These differences likely allow specificity in FA and FA metabolite partitioning toward different metabolic fates in a cellular or tissue-dependent manner. We have also demonstrated that each of the ACSL isoforms, whether expressed in bacterial or mammalian cells, can use both EETs and HETEs as substrates. Given the importance of these signaling eicosanoids and the ability of the ACSL isoforms to activate them, further efforts should be directed toward determining whether the functions of esterified eicosanoids differ from those that have been newly formed, as well as determining how ACSLs and acyltransferases work together to channel eicosanoids toward membrane storage pools.

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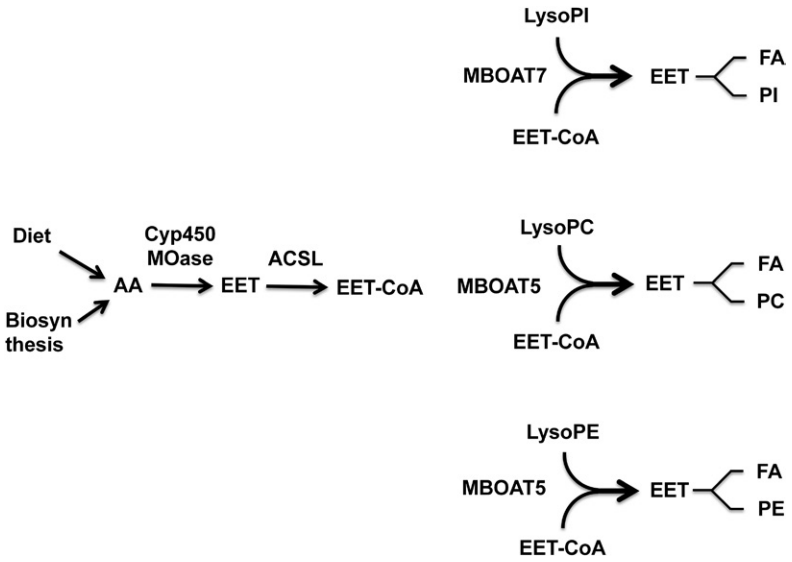

Fig. 9. Scheme of the synthesis of EETs from unesterified AA catalyzed by CYP450 monooxygenase (Cyp450 MOase). EETs can then be activated by ACSL to produce EET-CoA. Once activated, the EET-CoA can be esterified by a phospholipid acyltransferase such as MBOAT5/LPCAT3, specific to lysophosphatidylcholine and lysophosphatidylethanolamine, or MBOAT7/LPIAT1, specific to lysophosphatidylinositol, to produce oxidized phospholipids.

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