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Expression of rat liver long-chain acyl-CoA synthetase and characterization of its role in the metabolism of *R*-ibuprofen and other fatty acid-like xenobiotics

Roland Brugger[a,1], Christine Reichel[a], Begonia Garcia Alia[a], Kay Brune[a], Tokuo Yamamoto[b], Irmgard Tegeder[c], Gerd Geissinger[c,*]

[a]*Institut für Experimentelle Pharmakologie and Toxikologie, Universität Erlangen, Universitätsstr. 22, 91054 Erlangen, Germany*
[b]*Tohoku University Gene Research Center, 1-1 Tsutsumidori-Amamiya, Aoba, Sendai 981, Japan*
[c]*Zentrum der Pharmakologie, Klinikum der Johann Wolfgang Goethe-Universität Frankfurt, Theodor Stern Kai 7, 60590 Frankfurt am Main, Germany*

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Abstract

Our investigations of fatty acid metabolism and epimerization of the 2-arylpropionic acid derivative, *R*-ibuprofen, resulted in the successful purification of an acyl-CoA synthetase from rat liver microsomes that catalyzes the formation of both palmitoyl-CoA and *R*-ibuprofenoyl-CoA. To investigate whether *R*-ibuprofenoyl-CoA synthetase and long-chain acyl-CoA synthetase (LACS) are identical enzymes, we cloned the cDNA from LACS into the pQE30 expression vector and transformed the construct into *Escherichia coli* M15[pREP4]. Induction of the bacterial protein synthesis with 0.2 mM isopropyl-beta-D-galactoside resulted in a strong, time-dependent increase in LACS protein as determined by Western blot analysis using a polyclonal rabbit anti-LACS antibody. Incubations of the recombinantly expressed protein with palmitic acid as physiological LACS substrate or *R*-ibuprofen in the presence of Mg^{2+}, ATP, and CoA resulted in a 5-fold increase in the thioesterification of both substrates. Western blot analysis using tissue homogenates of rat liver, heart, kidney, lung, brain, and ileum showed that LACS was found in every tissue investigated, with the greatest expression in the liver. Similar results were obtained with activity measurements using *R*-ibuprofen and palmitic acid as substrates. Northern blot analysis revealed a hybridization with a 3.8-kb mRNA transcript in rat liver, heart, and kidney, but no signal was observed in lung, brain and ileum, suggesting the expression of different LACS isoform(s) in these organs. In summary, our results further show that *R*-ibuprofenoyl-CoA synthetase and long-chain acyl-CoA synthetase are identical enzymes that are involved in the metabolism of various xenobiotics.

Keywords: Ibuprofen; Long-chain acyl-CoA synthetase (EC 6.1.2.3.); Chiral inversion; NSAIDs; Palmitoyl-CoA

1. Introduction

Acyl-CoA synthetases (ACS) catalyze the formation of acyl-CoA thioesters from fatty acids in the presence of Mg^{2+}, ATP, and CoA. This activation of fatty acids is the critical step in fatty acid utilization and degradation via β-oxidation. Acyl-CoA esters produced by ACS are required for the synthesis of cellular lipids and acylation of many membrane proteins (for review see [1,2]).

It is now well established that hepatic microsomal, mitochondrial, and peroxisomal acyl-CoA synthetases, e.g. the medium and the long-chain acyl-CoA synthetase, are involved in the thioesterification of xenobiotics containing a carboxylic acid moiety [3–6]. The formation of xenobiotic-CoA esters by acyl-CoA synthetases has been discussed as a prerequisite for the activity of a variety of sulfur- and oxy-substituted fatty acid analogs [7]. Therefore, recent studies have focused on the regulation of acyl-CoA synthetase expression induced by amphipathic carboxylic peroxisomal proliferators [8,9].

In addition, peroxisomal and microsomal acyl-CoA syn-

* Corresponding author. Tel.: +49-69-6301-7620; fax: +49-69-6301-7617.

E-mail address: geisslinger@em.uni-frankfurt.de (G. Geisslinger).

[1]Current address: F. Hoffman-La Roche Ltd., Vitamins and Fine Chemicals Division, VFPA 90/318A, CH-4070 Basel, Switzerland. *E-mail address:* roland.brugger.rb1@roche.com

Abbreviations: PCR; polymerase chain reaction; IPTG, isopropyl-beta-D-galactoside; LACS, long-chain acyl-CoA synthetase; and NSAIDs, non-steroidal anti-inflammatory drugs.

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thetases play important roles in the chiral metabolism of 2-arylpropionic acid derivatives [10,11]. 2-Arylpropionic acids, such as ibuprofen, constitute an important group of non-steroidal anti-inflammatory drugs. A common feature of this class of drugs is their unidirectional metabolic chiral inversion from the *R*- to the *S*-enantiomer in a species- and substrate-dependent manner [12]. Formation of the coenzyme A thioester with *R*-enantiomers via an adenylate intermediate in the presence of CoA, ATP, and Mg^{2+} is the stereoselective and first step of inversion [13–15]. Recently, our biochemical investigations of the inversion of *R*-ibuprofen in rat liver microsomes resulted in the successful isolation and characterization of an *R*-ibuprofenoyl-CoA synthetase. This enzyme was found to catalyze both the stereoselective activation of *R*-ibuprofen to *R*-ibuprofenoyl-CoA [16] and the thioesterification of palmitic acid to palmitoyl-CoA.

In an attempt to find out whether *R*-ibuprofenoyl-CoA synthetase and long-chain acyl-CoA synthetase are identical enzymes, we isolated the protein from rat liver microsomes and expressed the cDNA of the long-chain acyl-CoA synthetase in *Escherichia coli*. Furthermore, we assessed the localization of *R*-ibuprofenoyl-CoA synthetase in various rat tissue homogenates and the substrate specificity of the long-chain acyl-CoA synthetase for various 2-arylpropionic acid derivatives and other fatty acid-like xenobiotics.

2. Materials and methods

2.1. Chemicals

ATP Grade I, coenzyme A, dithiothreitol, palmitoyl-CoA (as chromatographic standard), palmitic acid, and Triton X-100 were purchased from Sigma. *R*- and *S*-ibuprofen were supplied by PAZ GmbH. The purity of the enantiomers was >99%. Acetonitrile and other chemicals were obtained from Merck. *R*-Ibuprofenoyl-CoA was synthesized as described previously [16].

2.2. Expression and activity

The expression vector pQE30 and the *E. coli* host strain M15[pREP4] were obtained from Qiagen. The cDNA from LACS was used as described previously [17]. The 5′- and 3′-end of the cDNA was modified by PCR amplification. A *BamHI* site was added in front of the starting methionine codon and a *SacI* site was added 3′ behind the termination codon. The PCR product was ligated directly into the pCR™II vector (TA Cloning Kit, Invitrogen) and transformed into the One Shot™ competent cells. After *BamHI/SacI* digestion, the resulting insert was cloned into the *BamHI/SacI*-digested pQE30 vector. The resulting construct was transformed into *E. coli* M15[pREP4]. Protein synthesis was induced by the addition of 0.2 mM IPTG, and synthetase activity was determined by incubations of the enzyme fractions with 1 mM *R*-ibuprofen and 100 μM palmitic acid in the presence of Mg^{2+}, ATP, and CoA. The formation of CoA thioesters was monitored by HPLC analysis as described previously [16].

2.3. Polyclonal antibodies and immunological techniques

Polyclonal antibodies were raised in rabbits according to standard methods. Purified homogenous protein was separated by SDS–PAGE on a 12% polyacrylamide gel and the acyl-CoA synthetase (molecular weight 72 kDa) was excised. After electroelution of the protein, 0.5 mL (100 μg protein/mL of PBS) was suspended in 0.5 mL complete Freund's adjuvant and injected subcutaneously into a rabbit. Further injections followed 2 and 4 weeks later. The LACS antibodies were analyzed by SDS–PAGE and Western blotting. Tissue homogenates of rat liver, heart, lung, kidney, ileum, and brain were prepared according to standard methods and analyzed by Western blotting as described previously [18].

2.4. Northern blot analysis

Total RNA was prepared from various rat tissues using guanidinium isothiocyanate [19]. The 2097-bp *BamHI/SacI* fragment containing the full-length LACS cDNA was labeled by incorporation of digoxigenin-labeled UTP (DIG-11-UTP) (Boehringer) during PCR amplification. The labeled fragment was hybridized to total RNA of rat liver, heart, lung, kidney, ileum, and brain. The hybridization and washing conditions were as described by the manufacturer (Boehringer).

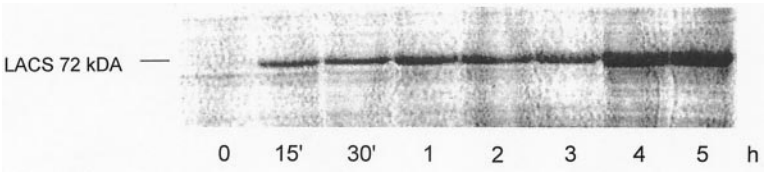

Fig. 1. Induction of the heterologous protein expression of rat long-chain acyl-CoA synthetase (LACS) in *E. coli* M15 after incubation with 0.2 mM IPTG. LACS cDNA had been cloned into the pQE30 expression vector, and the construct was transformed into *E. coli* M15 containing the repressor plasmid pREP4.

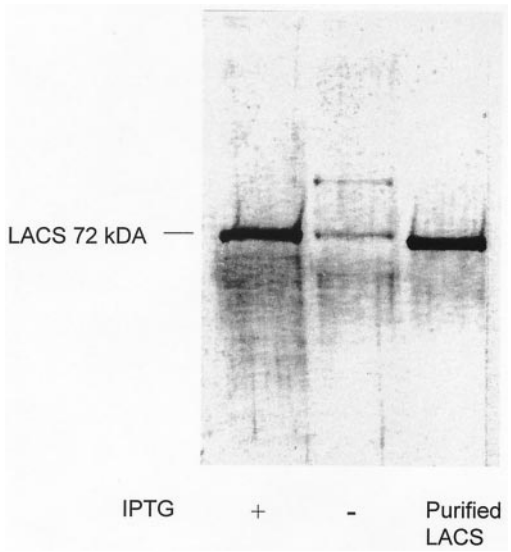

Fig. 2. Western blot analysis of LACS protein expression in IPTG-induced and non-induced *E. coli* cultures using a polyclonal antibody raised in rabbits and directed against purified microsomal rat liver LACS.

3. Results

Following the induction of bacterial protein synthesis by addition of 0.2 mM IPTG, the expression of recombinant LACS protein increased in a time-dependent manner (Fig. 1). No heterologous protein was detected in non-induced cultures containing the vector construct. The 72 kDa band of purified microsomal LACS as well as a protein of approximately 72 kDa in IPTG-induced *E. coli* cultures were detected by the LACS antiserum (Fig. 2). No signal was observed in non-induced cultures containing the LACS vector. The minor difference in the molecular weight of the recombinant and microsomal rat liver proteins is due to the additional expression of 6 histidines at the N-terminal region of the engineered enzyme.

The specific activities for palmitoyl-CoA and *R*-ibuprofenoyl-CoA formation were 68 and 3.8 nmol/min/mg protein, respectively (Table 1). Purification of the recombinant LACS protein resulted in approximate 4-fold increases in specific activity for palmitoyl-CoA (280 nmol/min/mg protein) as well as *R*-ibuprofenoyl-CoA (17 nmol/min/mg protein) synthesis. The specific activities of purified heterologously expressed LACS were lower than those of purified LACS from rat liver microsomes, suggesting that the heterologous enzyme was partially inactive.

Northern blot analysis of total RNA from rat liver, kidney, lung, heart, brain, and ileum revealed a hybridization with a 3.8-kb mRNA transcript in liver, kidney, and heart, but no signal was observed in lung, brain, or ileum (Fig. 3). Western blots, however, showed that LACS protein was expressed in every tissue investigated, with a strong signal in the liver (Fig. 4). In addition, *R*-ibuprofenoyl-CoA (Fig. 5) and palmitoyl-CoA (not shown) were formed in all tissue homogenates, predominantly in the liver, indicating LACS activity in all organs tested.

Table 1
Activity of purified and recombinantly expressed long-chain acyl-CoA synthetase to form either *R*-ibuprofenoyl-CoA or palmitoyl-CoA

Long-chain acyl-CoA synthetase	Activity for the formation of: (nmol/min/mg protein)		
	R-Ibuprofenoyl-CoA	Palmitoyl-CoA	Ratio
Crude liver homogenate	1.9	22	1:11.6
Purified microsomal protein from rat liver	71	901	1:12.7
Recombinant protein	3.8	68	1:17.9
Purified recombinant protein (Ni-NTA-resin)	17	280	1:16.5
M15-pQE30 control	not detected	23	

NI-NTA, nickel-nitrilotriacetic acid.

The relative activity of LACS for various substrates is shown in Table 2. The highest specific activity was observed with palmitic acid (901 nmol/min/mg protein), and was defined as 100%. High activities were also obtained with arachidonic acid (85%) and octanoic acid (20%). Hexanoic acid (4%), the preferred substrate of medium-chain acyl-CoA synthetase, showed only a low affinity to LACS. The fatty acid-like xenobiotics, valproic acid (1%), salicylic acid (1%), clofibric acid (<1%), and pivalic acid (<1%), were very poor substrates of LACS. Incubation of LACS with the enantiomers of the 2-arylpropionic acid derivatives, i.e. ibuprofen, ketoprofen, and flurbiprofen, resulted in an exclusive CoA thioesterification of the *R*-enantiomers,

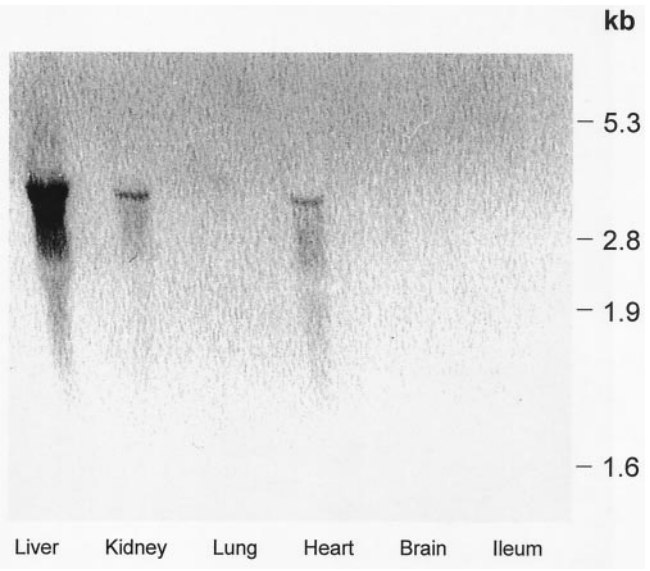

Fig. 3. Northern blot analysis of total RNA from rat liver, kidney, lung, heart, brain, and ileum hybridized with digoxigenin-labeled full-length LACS cDNA.

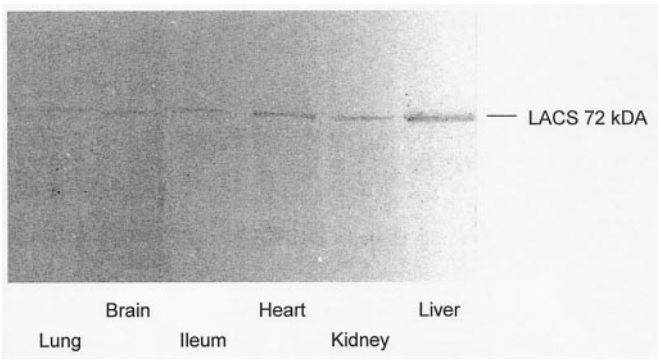

Fig. 4. Western blot analysis of LACS protein expression in rat liver, kidney, lung, heart, brain, and ileum using a rabbit polyclonal antibody raised against purified microsomal rat liver LACS.

with highest LACS activity for *R*-ibuprofen (9% as compared to 100% with palmitic acid).

4. Discussion

In the present work, we assessed the involvement of microsomal rat liver long-chain acyl-CoA synthetase in the CoA-thioesterification of *R*-ibuprofen using heterologously expressed LACS. Although the specific activity of the recombinantly expressed protein for the formation of palmitoyl-CoA was considerably lower than that of the purified microsomal liver synthetase, coincubations of enzyme fractions with *R*-ibuprofen and palmitic acid resulted in the formation of both palmitoyl- and *R*-ibuprofenoyl-CoA. The formation of *R*-ibuprofenoyl-CoA thioester correlated well with the induction of heterologously expressed synthetase and supported our hypothesis that *R*-ibuprofenoyl-CoA synthetase and long-chain acyl-CoA synthetase are identical enzymes. In addition, the lack of *R*-ibuprofen thioesterification in control cells (*E. coli* M15 containing plasmid without LACS cDNA) indicates that in contrast to other microorganisms such as *Cunnninghamella echinulata*, *Mucor hiemalis*, and *Verticillium lecanii* [20,21], bacterial LACS of *E. coli* M15 has no substrate specificity for *R*-ibuprofen. These findings correspond to the observed lack of chiral inversion of 2-arylpropionic acid derivatives in these bacteria [22]. The specific activities of heterologously expressed LACS were substantially lower than those of purified LACS from rat liver microsomes in the present study. This is in line with the findings of Sevoz *et al*., who have recently described two recombinant acyl-CoA synthetase isoforms expressed in *E. coli* [23]. The specific palmitoyl-CoA activity of these LACS isoforms was 3- to 6-fold lower than what was previously reported for the purified native enzyme [24]. These findings might suggest that LACS undergoes some kind of posttranslational modification that influences its activity.

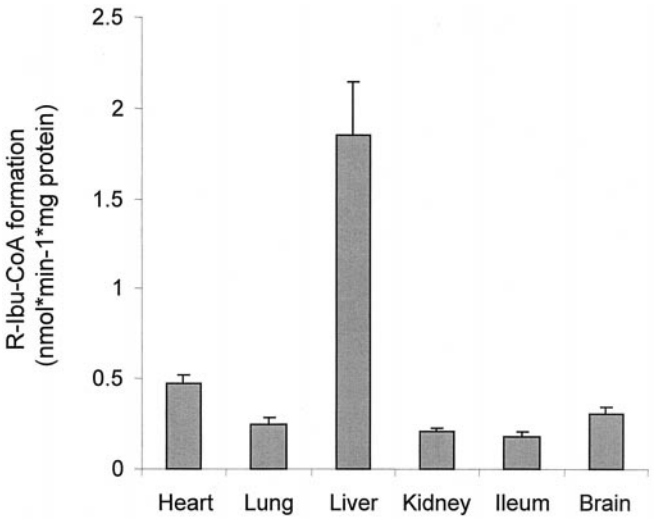

Fig. 5. *R*-Ibuprofenoyl-CoA formation (means ± SEM, N = 3) in tissue homogenates from rat liver, kidney, lung, heart, brain, and ileum. Enzyme fractions were incubated with 1 mM *R*-ibuprofen in the presence of Mg^{2+}, ATP, and CoA. The CoA thioester formed was monitored by HPLC analysis.

Table 2
Substrate specificity of long-chain acyl-CoA synthetase (LACS) for various fatty acids and carboxylic acids

Fatty acids and other xenobiotics	Activity in percent
Palmitic acid (C16:0)	100
Arachidonic acid (C20:4)	85
Octanoic acid (C8:0)	20
Hexanoic acid (C6:0)	4
Valproic acid	1
Salicylic acid	1
Clofibric acid	<1
Pivalic acid	<1
2-Arylpropionic acid derivatives	
R-Ibuprofen	9
S-Ibuprofen	0
R-Ketoprofen	4
S-Ketoprofen	<1
R-Flurbiprofen	2
S-Flurbiprofen	0

The activities of microsomal rat liver LACS were determined by a coupled enzyme assay, which monitors the formation of AMP as described by Kasuya *et al*. [4]. Concentration of carboxylic acid was 500 μM. Activities are expressed as percent of the specific activity to form palmitoyl-CoA. Values represent the average of three experiments.

The tissue distribution of LACS was characterized by Western and Northern blot analysis and activity measurements. The Northern blot hybridization analysis showed significant signals in rat liver, kidney, and heart, supporting similar results published by Suzuki *et al*. [8]. In contrast to their results, however, no hybridization was seen in lung, brain, or ileum, and no further band occurred at 2 kb. This may be explained by our use of digoxigenin-labeled full-length LACS cDNA instead of radiolabeled cDNA, which is more sensitive. On the other hand, Western blot analysis and activity measurements revealed that long-chain acyl-CoA synthetase was expressed in every tissue tested, with the strongest expression and activity in the liver. Since

different isoforms of acyl-CoA synthetase have been found in rat brain [25] and ileum [26], the differing Northern and Western blot results might point to additional isoforms of these proteins in the lung.

In another series of experiments, we assessed the substrate specificity of purified microsomal LACS for various fatty acids and xenobiotics containing a carboxylic acid moiety (Table 2). As described previously by Tanaka *et al*. [27], the purified enzyme utilizes palmitic acid (C16:0) most effectively, but has no affinity to saturated fatty acids containing more than 20 or less than 6 carbon atoms. Clofibric, pivalic, valproic, and salicylic acids are all poor substrates for LACS. Valproic and salicylic acid have been previously described as substrates for medium-chain acyl-CoA synthetases [4]. Nevertheless, even the low rate of CoA thioesterification of salicylic acid and valproic acid by LACS might contribute to the previously observed inhibition of the activation and subsequent oxidation of long-chain fatty acids by these compounds [28–30]. Among the 2-arylpropionic acid derivatives, *R*-ibuprofen exhibited the highest affinity to LACS (9%, expressed as percent of the affinity of palmitic acid, which was defined as 100%). There was no activation of *S*-enantiomers of 2-arylpropionic acids, confirming the known stereospecificity of LACS. In addition, the determined minor activation of *R*-flurbiprofen (about 2%) corresponds to its negligible inversion in the rat [31]. The different potential to inhibit the β-oxidation of palmitic acid by ibuprofen and flurbiprofen enantiomers is discussed controversially in the literature [29,30]. While Freneaux *et al*. [32] reported that only the *R*-enantiomer of ibuprofen inhibited the β-oxidation, Zhao and co-workers [33] observed an inhibition of palmitic acid degradation, no matter whether the animals were treated with *R*- or *S*-ibuprofen or flurbiprofen. The latter finding may be due to an observed uncoupling effect of NSAIDs on mitochondrial oxidative phosphorylation [34,35]. The effects of profens on fatty acid β-oxidation and incorporation of acyl-CoA thioesters into triglycerides and phospholipids suggests that hybrid–thioester formation by LACS might contribute to toxicological effects of xenobiotics containing carboxylic acid moieties.

In summary, our results describe the central role of LACS in fatty acid and xenobiotic carboxylic acid CoA activation and the strong involvement of this enzyme in the chiral metabolism of *R*-enantiomers of 2-arylpropionic acids such as ibuprofen. Further investigations are required to determine whether xenobiotics might affect the activity or expression of LACS, thereby influencing fatty acid metabolism.

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