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R. A. Coleman.

Rat long-chain acyl-CoA synthetase mRNA, protein, and activity vary in tissue distribution and in response to diet.

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Abstract Distinct isoforms of long-chain acyl-CoA synthetases (ACSLs) may partition fatty acids toward specific metabolic cellular pathways. For each of the five members of the rat ACSL family, we analyzed tissue mRNA distributions, and we correlated the mRNA, protein, and activity of ACSL1 and ACSL4 after fasting and refeeding a 69% sucrose diet. Not only did quantitative real-time PCR analyses reveal unique tissue expression patterns for each ACSL isoform, but expression varied markedly in different adipose depots. Fasting increased ACSL4 mRNA abundance in liver, muscle, and gonadal and inguinal adipose tissues, and refeeding decreased ACSL4 mRNA. A similar pattern was observed for ACSL1, but both fasting and refeeding decreased ACSL1 mRNA in gonadal adipose. Fasting also decreased ACSL3 and ACSL5 mRNAs in liver and ACSL6 mRNA in muscle. Surprisingly, in nearly every tissue measured, the effects of fasting and refeeding on the mRNA abundance of ACSL1 and ACSL4 were discordant with changes in protein abundance. These data suggest that the individual ACSL isoforms are distinctly regulated across tissues and show that mRNA expression may not provide useful information about isoform function. They further suggest that translational or posttranslational modifications are likely to contribute to the regulation of ACSL isoforms.—Mashek, D. G., L. O. Li, and R. A. Coleman. Rat long-chain acyl-CoA synthetase mRNA, protein, and activity vary in tissue distribution and in response to diet. J. Lipid Res. 2006. 47: 2004–2010.

Supplementary key words coenzyme A • acyl-CoA synthetase • fatty acid metabolism

After long-chain fatty acids enter cells, long-chain acyl-CoA synthetases (ACSLs) convert them to acyl-CoAs in an ATP-dependent reaction. The resulting acyl-CoAs have numerous metabolic fates within cells, including incorporation into triacylglycerol (TAG) and membrane phospholipids, use as substrates for β-oxidation and protein acylation, and function as ligands for transcription factors. We have hypothesized that these various fates depend on the action of specific ACSLs. The family of mammalian ACSLs consists of five members, ACSL1, ACSL3, ACSL4, ACSL5, and ACSL6 (1). Two ACSL subfamilies exist. ACSL1, ACSL5, and ACSL6 constitute one family, and ACSL3 and ACSL4 constitute the other. ACSL isoforms within and between subfamilies share ~60% and ~30% homology, respectively, and several of the proteins are expressed as splice variants (1, 2).

The five ACSL isoforms differ in their substrate preferences, enzyme kinetics, and intracellular locations (2–4), suggesting that each has a distinct function, and studies of the outcome of overexpression show that the fates of fatty acids diverge. For example, overexpression of ACSL6 in PC12 neurons increases the uptake and incorporation of docosahexaenoate and arachidionate into phospholipids, but not TAG, during neurite outgrowth (5). In contrast, although it was initially suspected that ACSL5 activated fatty acids destined for β-oxidation (4), overexpressed ACSL5 in hepatoma cells revealed that it activates exogenous fatty acids destined for TAG synthesis (6). The differential regulation of other ACSL isoforms in tissues also supports distinct roles in fatty acid partitioning. An anabolic role for ACSL1 is suggested because it is highly upregulated during 3T3-L1 adipocyte differentiation (7), causes TAG accumulation when overexpressed in the heart (8), and in adipose tissue is a target of peroxisome proliferator-activated receptor γ (PPARγ), which increases genes involved in TAG synthesis (9). However, ACSL1 in liver is a target of PPARα, which enhances the expression of genes involved in β-oxidation (10). These discrepant findings suggest that the function of the ACSLs may differ in different tissues.

Alterations in fatty acid partitioning characterize numerous metabolic diseases such as diabetes and obesity. The members of the ACSL family initiate fatty acid channeling toward both synthetic and degradative metabolic pathways, but the mechanism by which this is accomplished remains unclear. The objective of this study was to determine the tissue distribution and regulation of mRNA abundance for

Abbreviations: ACSL, long-chain acyl-coenzyme A synthetase; PPAR, peroxisome proliferator-activated receptor; SREBP, sterol response element binding protein; TAG, triacylglycerol.

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individual ACSL isoforms and, because many studies examine only mRNA as a measure of ACSL activity, determine whether mRNA, protein, and enzyme activity are related. Thus, we decided to 1) quantify the mRNA of the five ACSL isoforms in numerous tissues; 2) measure changes in mRNA in all five isoforms in liver, adipose tissue, and muscle in response to fasting and to fasting followed by refeeding a high-sucrose diet; 3) measure diet-induced changes in ACSL1 and ACSL4 protein; and 4) characterize diet-induced changes in ACS specific activity in selected tissues.

METHODS

Animals, diets, and tissue harvesting
Male Sprague-Dawley rats weighing ~130 g were fed a chow diet (catalog number 611000; Purina), fasted for 48 h, or fasted for 48 h and then re-fed for 24 h that was 69% sucrose (catalog number 111780; Dyets, Inc.). Food was removed or changed and animals were anesthetized at 9:00 AM. After anesthesia with tribromoethanol, tissues were immediately harvested. To obtain duodenal mucosa, the first one-third of the intestine was washed with cold PBS and then a glass microscope slide was used to scrape the mucosa from the muscularis layer. Tissues were placed in RNAlater (Qiagen) for future RNA analysis or frozen in liquid nitrogen for protein or ACS activity assays.

Quantitative real-time PCR
RNA was extracted from samples using the Qiagen RNeasy Kit, and RNA integrity was verified by formaldehyde gel electrophoresis. Samples were quantified in duplicate (TaqMan® procedure) using the Applied Biosystems 7700 sequence detection system (Foster City, CA). Samples that varied by more than one-half threshold cycle were repeated. Primers and probes used for all of the ACSL isoforms and β-actin are shown in Table 1. All data were quantified using the relative standard curve methods as described in User Bulletin 2 from Applied Biosystems.

Cell homogenization, protein determination, and Western blot analysis
Tissues were homogenized in buffer containing 250 mM sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA, and 1 mM dithiothreitol on ice with 10 up-and-down strokes in a Teflon glass motor-driven homogenizing vessel, and aliquots were stored at −80°C. For adipose tissue, the homogenate was centrifuged at 500 g for 5 min, and the fat cake was removed from the supernatant. Homogenates were separated by electrophoresis on an 8% polyacrylamide gel containing 1% SDS, transferred to a polyvinylidene fluoride membrane (Bio-Rad), and incubated with polyclonal peptide antibodies against either ACSL1 or ACSL4 (4) and β-actin (Abcam). For chemiluminescent detection, immunoreactive bands were visualized by incubating membranes with horseradish peroxidase-conjugated antibodies followed by PicoWest reagents (Pierce). NIH Image J software was used for densitometry analysis.

ACS activity assays
Initial rates of ACS activity in tissue homogenates were measured in the presence of 175 mM Tris, pH 7.4, 8 mM MgCl2, 5 mM dithiothreitol, 10 mM ATP, 250 μM CoA, 50 μM [1-14C]palmitic acid in 100 μM Triton X-100, and 10 μM EDTA in a total volume of 200 μl. The assays were performed in triplicate at 37°C for 5 min with shaking. The reaction was started by adding the homogenized sample and terminated by adding 1 ml of Dole’s reagent (isopropanol-heptane-1 M H2SO4, 80:20:2, v/v). Fatty acid was removed with two ml heptane washes, and the labeled acyl-CoA produced by the reaction was measured by scintillation counting.

Statistics
One-way ANOVA was performed to test the significance of treatment effects. If the F-test was significant, Fisher’s protected least significant difference was used for pair-wise comparisons. All data are presented as means ± SEM.

RESULTS

Tissue distribution of ACSL isoform mRNA levels
Because limited and only qualitative characterizations of the tissue expression patterns for the ACSL isoforms had been reported, we performed an extensive investigation of expression. The mRNA abundance of individual ACSL isoforms differed greatly between tissues (Fig. 1). ACSL1 mRNA has been shown to be present in liver, heart, and adipose tissue (11), but its relative abundance in other tissues was unknown. Our results show that ACSL1 mRNA is highest in brown adipose tissue, gonadal adipose tissue, heart, and liver. In addition, ACSL1 mRNA was also expressed at lower levels in soleus and gastrocnemius muscles and in inguinal adipose tissue, where the expression was 75% lower than in gonadal adipose. The highest expression of ACSL3 was in brain followed by testis, but low expression was observed in all tissues examined. ACSL4 was expressed abundantly in adrenal gland and liver, with considerably lower expression in brain. ACSL4 expression is of particular interest because human mutations in ACSL4 are associated with a sex-linked mental retardation syndrome (12), but there are no reports of liver or adrenal abnormalities in the affected boys. ACSL5 was primarily expressed in brown adipose tissue, duodenal mucosa, and liver. In agreement with previous data (13), ACSL6 was expressed at high levels in brain and testis; however, we also

TABLE 1. Primer and probe sequences used for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACSL1</td>
<td>GCAAGAACAGCTGAAGCCCA</td>
<td>AGGTGCACATTGGGCAGCCA</td>
<td>CCAATGTCCCCCGTGTCGTAACCA</td>
</tr>
<tr>
<td>ACSL3</td>
<td>CAAATTACAGAAGTGGGAGACT</td>
<td>CACCCCTCTCCCAAGTTTCTTT</td>
<td>TACCCGGAGACTGGAGACGCCCA</td>
</tr>
<tr>
<td>ACSL4</td>
<td>TATGCGTCAGACAGAATGTG</td>
<td>CAAGCTTTCCACATTGAGGAT</td>
<td>TAAACTCCAGTAACTGCTCCAGCCC</td>
</tr>
<tr>
<td>ACSL5</td>
<td>GGCCAAACAGAATGGCAGA</td>
<td>GGAGTTCCACGATTGACCTG</td>
<td>TGGCTCCAGTCCACGATTGAAAT</td>
</tr>
<tr>
<td>ACSL6</td>
<td>TGAATGCGACAGTGCGGTTGA</td>
<td>ATGTGGTTTCGACGGCCAGG</td>
<td>TGGCTCCAGTCCACGTTGAGAA</td>
</tr>
<tr>
<td>β-Actin</td>
<td>TGCCCTAGCTCAGGCTCA</td>
<td>CAGGAAAGAGAGCTGGAAG</td>
<td>GACCTATCGGCAATGCAGCGGTTCGG</td>
</tr>
</tbody>
</table>

ACSL, long-chain acyl-coenzyme A synthetase.
found abundant expression in both soleus and gastrocnemius muscles. ACSL6 mRNA was not detected in liver and was expressed only at very low levels in white adipose tissue, duodenal mucosa, kidney, and lung.

**Nutritional regulation of ACSL mRNA abundance**

To determine the responsiveness of ACSL isoforms to dietary alterations, we measured the mRNA abundance of individual ACSL isoforms in rats after a 48 h fast or in rats that were fasted for 48 h and then refed a 69% sucrose diet for 24 h (Fig. 2). These dietary changes were designed to induce the use of acyl-CoAs for fatty acid oxidation or for the synthesis of TAG, respectively. Although ACSL1 has been the best studied ACSL isoform, its regulation is perhaps the most ambiguous. We found that ACSL1 mRNA increased in response to fasting in inguinal adipose tissue, gastrocnemius muscle, and liver but not in gonadal adipose. In addition, compared with fasted animals, refeeding with a high-sucrose diet to induce lipogenesis decreased ACSL1 mRNA abundance in gastrocnemius and liver. Changes in the expression of ACSL3 and ACSL5 were observed only in liver, where both ACSLs decreased after fasting, but only ACSL5 mRNA increased after refeeding. Like ACSL1, the mRNA abundance for ACSL4 consistently increased after fasting in all tissues examined and declined after refeeding. Fasting caused ACSL6 mRNA abundance to decline in gastrocnemius, and refeeding did not further alter expression. ACSL6 mRNA, which was not detected in liver and was detected only at very low levels in adipose tissues, did not respond to fasting or refeeding in these tissues.
Nutritional regulation of ACSL protein

To determine whether the amount of ACSL1 protein was concordant with diet-induced changes in mRNA abundance, we quantified ACSL1 protein in liver, gonadal and inguinal adipose tissue, and gastrocnemius muscle after fasting and refeeding (Fig. 3A, C). All data are normalized to β-actin protein abundance, which was not changed in response to dietary treatments. In contrast to the fasting-induced increases in mRNA, a 48 h fast resulted in a decrease in ACSL1 protein abundance in liver and in both gonadal and inguinal adipose tissues. Refeeding increased ACSL1 protein in the same tissues, although the absolute increase varied and the most pronounced increase occurred in gonadal adipose. In contrast, in gastrocnemius muscle, fasting increased ACSL1 protein as it had mRNA, but refeeding did not alter protein abundance. In gastrocnemius and adipose tissue, the amount of ACSL4 protein was insufficient for detection with the ACSL4 peptide antibody. Hepatic ACSL4 protein abundance did not change after rats were fasted for 48 h but nearly doubled after rats were refed the high-sucrose diet (Fig. 3B, C). In general, the changes measured in ACSL1 and ACSL4 proteins are opposite to the changes seen in mRNA abundance under the same dietary regimens. This divergence between mRNA and protein levels suggests that ACSL isoforms are regulated by translational and/or posttranslational mechanisms.

Nutritional regulation of ACS activity

Because one cannot distinguish the individual activities of the five ACSL isoforms either by assay conditions or by isolating different membrane fractions, we measured total ACS activity in homogenates from liver, gonadal and inguinal adipose tissue, and gastrocnemius muscle under different dietary conditions (Fig. 4). Fasting or refeeding did not alter total ACS activity in rat liver or in gastrocnemius, which had the lowest activity of all tissues examined. In contrast, activity in gonadal adipose tissue decreased 52% after a 48 h fast, and refeeding the high-sucrose diet for 24 h normalized the activity to chow-fed levels. Surprisingly, in contrast to gonadal adipose tissue, ACS specific activity in inguinal adipose was 80% lower and was insensitive to changes in diet.

DISCUSSION

ACSLs catalyze the initial step for both anabolic and catabolic pathways of fatty acid metabolism and are key determinants in regulating the metabolic fate of fatty acids within cells. To gain insight into their regulation and function, we measured mRNA, ACSL1 and ACSL4 protein, and ACSL activity in numerous tissues and followed their changes in response to fasting, which promotes the partitioning of fatty acids into oxidative pathways, and fasting followed by refeeding a high-sucrose diet, which promotes the incorporation of fatty acids into TAG. The tissue distribution of the ACSL isoforms revealed distinct patterns. ACSL1 was highly expressed in major energy-metabolizing tissues such as adipose, liver, and muscle. Surprisingly, ACSL3 and ACSL6, both of which were reported to be expressed primarily in brain and testis (13–15), were also expressed at moderate levels in muscle. Together with ACSL1, these three isoforms appear to constitute most of the ACS activity in muscle. The tissue expression pattern also revealed that ACSL1 mRNA is expressed at very different levels in gonadal and inguinal adipose tissue. It has been well documented that fat depots show significant differences in their gene expression pattern, metabolism, and relationship to insulin resistance (16). Thus, by altering fatty acid activation and trafficking, ACSL1 could contribute to the innate physiological differences between adipose tissue depots.

In addition to the heterogeneity of their expression patterns, individual ACSL isoforms also responded differently to dietary changes. When rats were fasted for 48 h, ACSL1 mRNA increased in all tissues with the exception of...
gonadal adipose. ACSL1 is a PPARα target gene (17); thus, the increase in ACSL1 mRNA in liver could reflect the regulation of PPARα in response to a fast (9). These data contrast with a previous Northern analysis in older and heavier Wistar rats showing that fasting decreased and sucrose refeeding increased ACSL1 mRNA abundance in rat livers (11). In the liver, the role of ACSL1 is not known, but changes observed in mRNA abundance generally coincide with reported changes in oxidative genes. For example, dietary supplementation with polyunsaturated fatty acids, overexpression of hepatocyte nuclear factor 4-α, and several PPARα agonists all increase the mRNA of ACSL1 as well as that of oxidative genes such as carnitine palmitoyl transferase-1 and acyl-CoA oxidase (9, 18–20). It seems likely that ACSL1 mRNA is under a complex regulatory control that is probably tissue specific.

Similar to ACSL1, fasting also increased ACSL4 mRNA in all tissues measured. Although the exact mechanism responsible for ACSL4 mRNA changes is not known, it has been shown to be regulated at the level of transcription. Administering the PPARα agonist GW9578 to rats increased hepatic ACSL4 mRNA (21). Compared with control cells, adenoviral overexpression of PPARγ in PPARα knockout mice increases hepatic ACSL4 mRNA by >5-fold (22). Additionally, sterol-response element binding protein-1α (SREBP-1a) transgenic mice have increased ACSL4 mRNA in the liver, but SREBP cleavage-activating protein knockout mice have diminished expression (23). These findings suggest that ACSL4 is regulated by both SREBP-1 and PPARγ in liver. However, ACSL4 is not upregulated during adipocyte differentiation (5), suggesting that the mechanisms controlling ACSL4 expression are different in liver and adipose tissue.

As reported previously, fasting decreased and refeeding increased ACSL5 mRNA in liver (7). These data are consistent with the finding that hepatic ACSL5 expression increases after insulin treatment or SREBP-1c overexpression (24) as well as with the observation that overexpression of ACSL5 increases oleate incorporation into TAG (6). Finally, we found that fasting decreased ACSL3 mRNA abundance in liver and ACSL6 mRNA abundance in gastrocnemius muscle. ACSL3 and ACSL6 have not been studied in these tissues, and the regulation and specific roles of these isoforms remain to be determined.

In adipose tissue and liver, we observed protein expression that was almost the opposite of the mRNA data. Fasting decreased and refeeding increased ACSL1 protein abundance in both adipose and liver. Similar results were observed for changes in hepatic ACSL4 protein. These data agree with previously reported changes in hepatic ACSL1 and ACSL4 in microsomal fractions (21, 25). Compared with fasting levels, ACSL1 protein in gonadal adipose increased by 12-fold after refeeding, whereas mRNA did not change significantly under the same conditions. Short-term cafeteria diet feeding is known to preferentially increase gonadal adipose mass (+95%) compared with inguinal adipose (+30%) (26). Thus, the observed increases in ACSL1 may coincide with the acute changes in fat deposition in gonadal adipose. These data further support a role for ACSL1 in channeling fatty acids to TAG in adipose tissue. Unlike inguinal adipose, gastrocnemius, and liver, in which ACS activity was unaltered in response to diet, fasting decreased and refeeding increased ACS activity in gonadal adipose. Thus, in gonadal adipose, ACS activity paralleled increased ACS specific activity (r = 0.40) and may be related to acute changes in TAG content during periods of fat accumulation. Additionally, correlations for ACSL1 protein and ACS activity were also present in muscle (r = 0.52) when data were analyzed across different dietary treatments.

Fig. 4. Fasting and refeeding altered ACS activity. ACS activity was measured in rat tissue homogenates obtained after rats were fasted for 48 h or fasted for 48 h and then refed a 69% sucrose diet. Initial rates of activity were determined with [1-14C]palmitate as the substrate. Data are presented as means ± SEM (n = 4).

* P < 0.05 versus chow-fed, # P < 0.05 versus fasted as determined by ANOVA and Fisher’s protected least significant difference.
We observed that mRNA and protein expression for ACSL1 and ACSLA in adipose tissue and liver were clearly dissociated. In mice, correlations between mRNA and protein abundance for a given gene range from 0.46 to 0.76 (27). Such discrepancies may result from differences in the regulation of mRNA processing and translation or from posttranslational modifications that affect protein turnover. However, neither mRNA nor protein necessarily reflects protein function. Because tissue ACS specific activity is the sum of contributions from all ACSL isoforms plus the activities of medium-chain and very-long-chain ACSs whose substrate specificities overlap with those of ACSL, it is not surprising that dietary treatments had little effect on total ACS specific activity in muscle, liver, or inguinal adipose despite changes in mRNA and/or protein of individual isoforms.

Others have reported changes in ACS activity in different subcellular compartments that may reflect alterations in specific ACSL isoforms. For example, in response to fasting, total ACS activity in adipose tissue decreased slightly but the activity in mitochondria/nuclei fractions increased, whereas the activity in microsomes, plasma membrane, and cytosol decreased (28). Similarly, lipopolysaccharide treatment decreased ACS activity in mitochondria by 61% and increased activity by 55% in microsomes, with only a 20% decline in total hepatic ACS activity (29). Because the intracellular locations of ACSL isoforms are distinct (4), the regulation of individual isoforms likely explains the observed changes in ACS activity from different subcellular fractions.

The hypothesis that ACSL isoforms have distinct functions in different tissues is supported by direct studies. For example, transgenic mice that overexpress ACSL1 in heart cardiomyopathy (8). Yet, overexpression of ACSL1 in rat hepatocytes did not alter TAG accumulation (L. O. Li et al., unpublished data). Although the mechanisms by which different ACSL isoforms can be regulated in a tissue-specific manner are not known, several possibilities exist, including posttranslational modifications that regulate acute changes in enzyme activity or specificity and interaction with proteins that dictate the function of ACSL isoforms within a specific tissue. For example, ACSVL1 (also known as fatty acid transport protein-1) is expressed at high levels in adipose but very low levels in liver (http://symatlas.gnf.org/SymAtlas/); thus, the interaction identified between ACSL1 and ACSVL1 in adipocytes (30) may result in a role that is functionally different in adipocytes compared with liver.

As a whole, these data support the hypothesis that each ACSL isoform plays a unique role in partitioning fatty acids toward distinct pathways. In addition, the disparity between mRNA and protein abundance and activity suggest caution when interpreting the significance of changes in ACSL gene expression.

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