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Expression and Characterization of Recombinant Rat Acyl-CoA Synthetases 1, 4, and 5

SELECTIVE INHIBITION BY TRIACSIN C AND THIAZOLIDINEDIONES*

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Ji-Hyeon Kim, Tal M. Lewin, and Rosalind A. Coleman‡

From the Departments of Nutrition and Pediatrics, University of North Carolina, Chapel Hill, North Carolina 27599-7400

Inhibition by triacsins and troglitazone of long chain fatty acid incorporation into cellular lipids suggests the existence of inhibitor-sensitive and -resistant acyl-CoA synthetases (ACS, EC 6.2.1.3) that are linked to specific metabolic pathways. In order to test this hypothesis, we cloned and purified rat ACS1, ACS4, and ACS5, the isoforms present in liver and fat cells, expressed the isoforms as ACS-Flag fusion proteins in *Escherichia coli*, and purified them by Flag affinity chromatography. The Flag epitope at the C terminus did not alter the kinetic properties of the enzyme. Purified ACS1-, 4-, and 5-Flag isoforms differed in their apparent K_m values for ATP, thermolability, pH optima, requirement for Triton X-100, and sensitivity to *N*-ethylmaleimide and phenylglyoxal. The ACS inhibitor triacsin C strongly inhibited ACS1 and ACS4, but not ACS5. The thiazolidinedione (TZD) insulin-sensitizing drugs and peroxisome proliferator-activated receptor γ (PPARγ) ligands, troglitazone, rosiglitazone, and pioglitazone, strongly and specifically inhibited only ACS4, with an IC_{50} of less than 1.5 μM. Troglitazone exhibited a mixed type inhibition of ACS4. α-Tocopherol, whose ring structure forms the non-TZD portion of troglitazone, did not inhibit ACS4, indicating that the thiazolidine-2,4-dione moiety is the critical component for inhibition. A non-TZD PPARγ ligand, GW1929, which is 7-fold more potent than rosiglitazone, inhibited ACS1 and ACS4 poorly with an IC_{50} of greater than 50 μM, more than 100-fold higher than was required for rosiglitazone, thereby demonstrating the specificity of TZD inhibition. Further, the PPARα ligands, clofibrate and GW4647, and various xenobiotic carboxylic acids known to be incorporated into complex lipids had no effect on ACS1, -4, or -5. These results, together with previous data showing that triacsin C and troglitazone strongly inhibit triacylglycerol synthesis compared with other metabolic pathways, suggest that ACS1 and ACS4 catalyze the synthesis of acyl-CoAs used for triacylglycerol synthesis and that lack of inhibition of a metabolic pathway by triacsin C does not prove lack of acyl-CoA involvement. The results further suggest the possibility that the insulin-sensitizing effects of the thiazolidinedione drugs might be achieved, in part, through direct interaction with ACS4 in a PPARγ-independent manner.

Acyl-CoA synthetase (ACS,[1] EC 6.2.1.3) catalyzes the ligation of long chain fatty acids with coenzyme A (CoA) to produce long chain acyl-CoAs (1). The resulting acyl-CoAs can be further metabolized in pathways of β-oxidation, glycerolipid synthesis, cholesteryl ester (CE) synthesis, desaturation, elongation, and protein acylation and can serve as signaling molecules (2–4). Although ACS was first believed to be a constitutive enzyme because the activity in liver was not altered by changes in nutritional status or hormonal stimuli (5–9), the cloning of ACS1 from rat liver in 1990 disclosed that hepatic ACS1 mRNA expression is sensitively regulated by fasting and refeeding as well as by specific nutrients provided as the energy source (10). Later, four additional rat ACS isoforms from different genes were cloned that differed in tissue distribution of their mRNA expression and in substrate preference (11–14). Of these, ACS2 and ACS3 mRNAs are abundantly expressed in brain, but are not detected in liver (13, 14). The mRNAs of ACS4 and ACS5 are highly expressed in steroidogenic tissues and in intestine, respectively, and are also present in liver (11, 12). ACS1 and ACS5 have a broad substrate specificity for saturated fatty acids of 12–18 carbon atoms and unsaturated fatty acids of 16–20 carbon atoms. In contrast, ACS4 has a marked preference for arachidonic acid and eicosapentaenoic acid. The presence of three ACS isoforms in liver suggests that liver ACS activity does not change with physiological alterations (5–9) because the different ACS isoenzymes compensate for each other.

All ACS isoenzymes are members of the luciferase superfamily and have a common structure that consists of an N terminus, two luciferase-like regions, a linker connecting the two luciferase-like regions, and a C terminus. A highly conserved AMP-binding site and a predicted fatty acid binding site are located in the first and the second luciferase-like regions, respectively (10–14). ACS1, ACS2, and ACS5 are structurally similar, with more than 60% amino acid identity (11). ACS3 and ACS4 form a second subgroup with ~30% homology to ACS1 and 68% identity to each other (12). Despite their high degree of homology, the expression of ACS1 and ACS5 mRNA is regulated independently. In liver, both ACS1 and ACS5 mRNAs are increased by high sucrose refeeding whereas high fat refeeding increases only ACS1 mRNA. ACS5 is the sole member of the ACS family whose mRNA decreases with fasting (11). In 3T3-L1 cells, ACS1 mRNA is detected only after adipocyte differentiation, whereas ACS5 mRNA is consistently expressed independent of differentiation status (10, 11). In the

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‡ To whom correspondence should be addressed: Rosalind A. Coleman, CB 7400, Depts. of Nutrition and Pediatrics, University of North Carolina, Chapel Hill, NC 27599-7400. Tel.: 919-966-7213; Fax: 919-966-7216; E-mail: rcoleman@unc.edu.

[1] The abbreviations used are: ACS, acyl-CoA synthetase; CE, cholesterol ester; IPTG, isopropyl-1-thio-β-D-galactopyranoside; NEM, *N*-ethylmaleimide; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; TAG, triacylglycerol; TOS, troglitazone *O*-sulfate; TZD, thiazolidinedione; TBS, Tris-buffered saline; MES, 4-morpholineethanesulfonic acid.

adrenal, ACS4 expression increases with exposure to adrenocorticotropic hormone and to arachidonate (15). These differences in regulation, tissue distribution, and substrate preference suggest that each ACS isoform might function independently in various tissues for different metabolic purposes.

Use of two inhibitors of ACS has suggested the presence of different acyl-CoA pools within cells, because these inhibitors are effective in blocking only some of the metabolic pathways that require long chain acyl-CoAs. For example, in human fibroblasts, the fungal metabolite triacsin C almost completely inhibits *de novo* synthesis of triacylglycerol and phospholipid from glycerol, but does not prevent phospholipid synthesis via reacylation of lysophospholipids with oleate or arachidonate (16). Similar results are observed in rat hepatocytes in which triacsin C blocks *de novo* synthesis of TAG 73%, but inhibits [^{14}C]oleate incorporation into phospholipid and CE, or metabolism via β-oxidation only 30–40% (17). In HepG2 cells, triacsin D inhibits oleate-induced TAG synthesis 80% without affecting CE synthesis (18), and in rat primary hepatocytes, troglitazone at 100 μM inhibits [^{14}C]oleate incorporation into TAG and oxidation products 50% and 20%, respectively, whereas incorporation into phospholipid remains unaffected (19). The sulfo-conjugate of troglitazone appeared to be the likely inhibitory metabolite (19).

These differing effects of ACS inhibitors on metabolic end products of acyl-CoA-dependent pathways suggest the presence of inhibitor-sensitive and -resistant ACSs that are linked to specific pathways. If so, ACS isoforms might play a critical role in partitioning acyl-CoAs toward lipid synthesis *versus* oxidation. In order to determine whether this hypothesis was correct, we needed to examine the effects of the ACS inhibitors on each of the three ACS isoforms expressed in liver and fat cells. To do this, we expressed ACS1, ACS4, and ACS5 as recombinant ACS-Flag fusion proteins, and purified each ACS by Flag antibody column chromatography.

EXPERIMENTAL PROCEDURES

Materials—RT-for-PCR kit and Advantage 2 PCR kit were purchased from CLONTECH. Bacterial Flag expression kit including pFlag-CTC vector, Flag M2 affinity column, and Flag peptides, aminophenylboronate, phenylglyoxal, *N*-ethylmaleimide, coenzyme A, ATP, palmitate, xenobiotic carboxylic acids, and Triton X-100 were from Sigma. HMS174(DE3) and pET21a(+) expression vector were from Novagen. [9,10-^{3}H]Palmitic acid was from PerkinElmer Life Sciences. Triacsin C was from Biomol. The thiazolidinediones, GW1929 and GW4647 were a gift from Dr. Steven Jacobs, GlaxoSmithKline.

Construction of Recombinant pACS-Flag Plasmids—cDNA was synthesized from rat liver total RNA and used as a template for amplification of the ACS open reading frames (CLONTECH, RT-for-PCR kit). The primers for amplification of ACS1, ACS4, and ACS5 were designed to include the entire open reading frames based on nucleotide sequences obtained from the GenBank™ data base (accession nos. D90109, D85189, and AB012933 for ACS1, ACS4, and ACS5, respectively) and specific restriction sites. The upper primer for ACS1 was 5′-ACAGA**CTCGAG**GAGGTCCATGAATTGTTCCGTA-3′ (recognition site indicated in boldface type) and the lower primer was 5′-CCTC**GGTAC-C**AATCTTGATGGTGGAGTACAG-3′. The upper and lower primers for ACS4 were 5′-AA**AAGCTT**GCAAAGAGAATAAAGGCTAAGC-3′ and 5′-TTGC**GTCGAC**TTTGCCCCCATACATC-3′. The upper and lower primers for ACS5 were 5′-TATTC**CTCGAG**CTTTTTATTTTTAACTTCTTGTTTTC-3′ and 5′-CTT**GGTACC**CTCTTCGATGCTCATAGAG-3′. ACS amplification was performed by PCR with the designed primers and Advantage®2 PCR kit (CLONTECH). The amplified ACS1 PCR product was digested with *Xho*I and *Acc*65I and ligated into pFlag-CTC vector (Sigma) digested with the same restriction enzymes. The ACS4 PCR product and the pFlag-CTC vector were digested with *Hin*dIII and *Sal*I and then ligated. To obtain ACS4 without the Flag sequence, a primer was designed by adding a stop codon in the position just before the Flag sequence. The upper primer used was the same as for ACS4-Flag fusion protein, and the newly designed lower primer was 5′-ACAGTGTCGACTTATTTGCCCCCATACAT-3′ (stop codon is underlined). The pFlag-ACS plasmid constructs contain a *tac* promoter, a ribosome binding site, the entire coding region of each ACS isoforms, and C-terminal Flag sequences with pBR322 origin. For ACS5, the pET-Flag vector was used. The plasmid pET-Flag was constructed by cloning the *Sph*I (Klenow-filled)-*Nhe*I fragment from pET-21a(+) (Novagen) into pFlag-CTC (Sigma) digested with *Bam*HI, treated with Klenow, and then digested with *Nhe*I. In the pET-Flag vector, the *tac* promoter region in pFlag-CTC was replaced with the T7 promoter region from pET21a(+) (Novagen). ACS5 PCR products and pET-Flag vector were digested with *Xho*I and *Acc*65I and then ligated. The sequences of ACS-Flag fusion constructs were verified by the University of North Carolina Automated Sequencing Facility.

Expression of Recombinant ACS1- and ACS4-Flag Proteins in Escherichia coli—Recombinant ACS1-Flag, ACS4-Flag, and ACS4 were expressed in *E. coli* JM109 after induction with 1 mM IPTG at an A_{600} of 1.0. JM109 was grown in Terrific Broth (Life Technologies, Inc.) supplemented with ampicillin (60 μg/ml) at 30 °C and shaken at 250 rpm. After a 12-h induction, cells were harvested by centrifugation at 5,000 rpm 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. The resuspended cells were incubated with 100 μg/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 $\times$ *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 centrifugation in a Beckman SW41 rotor at 35,000 rpm 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) with bovine serum albumin as the standard.

Purification of the Recombinant ACS1- and ACS4-Flag Proteins—ACS1-Flag and ACS4-Flag 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. JM109 membrane fractions containing overexpressed ACS1-Flag or ACS4-Flag 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 μg/ml Flag peptide (Sigma) dissolved in TBS buffer (pH 7.4). Eluates were run on a 10% polyacrylamide gel containing 1% SDS and stained with Gel Code® blue stain reagent (Pierce). ACS4 and ACS1 were purified to near homogeneity and migrated as single bands. Compared with the cell extracts the activities of purified proteins increased 19-fold for ACS1 and 10-fold for ACS4.

Expression and Purification of Recombinant ACS5-Flag Protein—Unlike ACS1- or ACS4-Flag, ACS5-Flag protein could not be expressed under the *tac* promoter in JM109 or DH5α. To express ACS5-Flag, we used the pET-Flag vector because the T7 promoter regulates the expression of foreign proteins more tightly than does the *tac* promoter. Overexpressed ACS5-Flag was present in the membrane fraction of HMS174(DE3). ACS5-Flag was maximally expressed at 30 °C after a 12-h induction with 1 mM IPTG beginning at an A_{600} of 0.4 in HMS174(DE3), which provides the T7 polymerase. Purification of active ACS5-Flag was similar to that described for ACS1 and ACS4, except that 0.1% Triton X-100 was required in the elution buffer that contained 100 μg/ml Flag peptide. When Triton X-100 was omitted, only inactive ACS was eluted. ACS5-Flag migrated as a single band on a 10% polyacrylamide gel containing 1% SDS and stained with Gel Code® blue stain reagent. Compared with the cell extracts, the activities of purified proteins increased 29-fold for ACS5. The purification yield was relatively low because excess protein applied to the column was eluted in the flow-through fraction and during the washing procedure.

Assay for ACS Activity—The ACS assay contained 50 μM [^{3}H]palmitate in Triton X-100, 1 μM EDTA, 10 mM ATP, 250 μM CoA, 175 mM Tris (pH 7.5), 8 mM $MgCl_2$, and 5 mM dithiothreitol in a total of 200 μl (20, 21). The maximum concentration of Triton X-100 in the assay was 0.03% (0.5 mM). All comparisons of the recombinant ACS isoenzymes employed identical concentrations of substrates and Triton X-100. The reaction was initiated with 0.1 μg of protein for ACS1 and ACS4, and 0.5–1.0 μg of protein for ACS5. ACS activity was measured after a 5-min incubation at 37 °C, except for the inhibition kinetic studies, which were only incubated for 1 min (20). Substrate concentrations and time allowed measurement of initial rates. The specific activities of recombinant ACS1-, ACS4-, and ACS5-Flag proteins from different preparations were in the ranges of 939–1472, 1670–2200, and 98–166 nmol/min/mg, respectively. All the experiments were repeated three

TABLE I
Kinetic constants for ACS1, ACS4, and ACS5

Recombinant, purified enzymes were assayed as described under "Experimental Procedures." Substrate concentrations for these studies were held to between 0.3 and 2.0 times the K_m value (30).

Substrate	ACS1	ACS4	ACS5
ATP			
K_m (μM)	649	34	666
V_{max} (nmol/min/mg protein)	1012	1050	287
CoA			
K_m (μM)	6.4	4.1	2.4
V_{max} (nmol/min/mg protein)	1072	2092	75
Palmitate			
K_m (μM)	5.0	5.4	8.6
V_{max} (nmol/min/mg protein)	1695	2800	130

times, and virtually identical results were obtained from each experiment.

RESULTS

Expression and Purification of Recombinant ACS-Flag Proteins—Recombinant rat ACS1, ACS4, and ACS5 had previously been purified by column chromatography (10–12). To obtain purified ACS isoenzymes more efficiently, we designed plasmid constructs to produce ACS proteins with Flag epitopes at their C termini. After induction with IPTG, 10% of the ACS activity was found in the soluble fraction. This soluble activity is surprising because ACS4 is an integral membrane protein, which remains associated with rat liver microsomes after washing with 0.5 M KCl (data not shown). Nevertheless, soluble ACS4 (21%) was also obtained when ACS4 was overexpressed in DH5α (12). ACS1- and ACS5-Flag were also overexpressed and identified in the membrane fraction. Recombinant ACSs were purified to near homogeneity by one-step Flag-antibody affinity chromatography. To ensure that the Flag epitope did not alter ACS function, we compared ACS activity in membrane fractions from JM109 that overexpressed ACS4 or ACS4-Flag. The kinetic constants for palmitic acid, CoA, and ATP were virtually identical, and the sensitivity to heating at 43 °C was similar, indicating that the Flag epitope at the C terminus does not alter the catalytic properties of ACS and supports the use of ACS-Flag recombinant proteins for comparative study (data not shown).

Characterization of ACS1, ACS4, and ACS5—To compare the purified ACS isoenzymes, we studied the kinetics of the three substrates required for ACS catalysis. The three ACS isoenzymes had similar apparent K_m values for CoA between 2.4 and 6.4 μM and for palmitic acid between 5.0 and 8.6 μM (Table I). ACS4, on the other hand, had an apparent K_m value for ATP that was almost 20-fold lower than that of ACS1 or ACS5 (34 μM *versus* 649 and 666 μM). To test sensitivity to heating, ACS isoenzyme activities were measured after heating at 43 °C for various times (Fig. 1). ACS1 was most stable to heat with a half-life of about 10 min whereas ACS4 had a $t_{½}$ shorter than 2 min, and ACS5 had a $t_{½}$ of ~4 min. To find the optimum pH for each ACS isoenzyme, MES was used as a buffer for ACS reaction in the range of pH 6–7, Tris for pH 7–9, and glycine for pH 9 and 9.5. ACS1 and ACS4 had broad pH optima between pH 7.4 and 9.0, but the optima for ACS5 was more narrow (pH 7.4–8.0) (data not shown). The three purified ACS isoenzymes were differently affected by Triton X-100 (Fig. 2). ACS4 gradually lost activity at Triton X-100 concentrations above 0.5 mM, the concentration normally used for assay, whereas ACS1 maintained maximal activity up to 1.5 mM Triton X-100. In contrast, ACS5 activity dramatically increased at Triton X-100 concentrations above 0.5 mM, and maintained maximal activity at 3 mM. ACS5 remained highly active even at 10 mM Triton X-100 (data not shown).

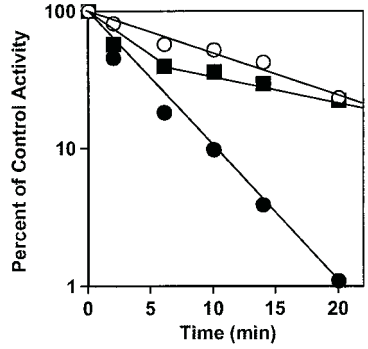

FIG. 1. **ACS1-, ACS4-, and ACS5-Flag proteins differ in thermolability.** Purified ACS1-Flag (○) and ACS4-Flag (●) (each 4 μg/ml) and ACS5-Flag (■) (40 μg/ml) were heated at 43 °C in TE buffer. Samples containing 0.1 μg of protein were removed at the times indicated for immediate assay at 37 °C. Initial activities were 873 for ACS1, 1491 for ACS4, and 97 nmol/min/mg for ACS5.

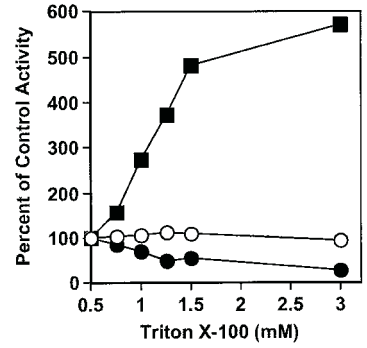

FIG. 2. **Triton X-100 activates ACS5.** The activities of purified ACS1-Flag (○) and ACS4-Flag (●) (each 0.1 μg) and ACS5-Flag (■) (1 μg) isoenzymes were measured in the presence of different concentrations of Triton X-100. ACS activity at 0.5 mM Triton X-100, the concentration regularly used in the assay, represents 100%. Activities with 0.5 mM Triton X-100 were 1340 for ACS1, 2220 for ACS4, and 100 nmol/min/mg for ACS5.

Triacsin C Strongly Inhibited ACS1 and ACS4, but Did Not Affect ACS5—Triacsin C, an alkenyl-*N*-hydroxytriazene fungal metabolite, was reported to be a potent competitive inhibitor of ACS (22, 23). Studies in fibroblasts, HepG2 cells, and rat hepatocytes, however, suggest that triacsin C does not equally inhibit all the pathways in which acyl-CoAs are used. To test the effects of triacsin C on the purified ACS isoenzymes, triacsin C was added directly to the reaction mixture (Fig. 3). Triacsin C selectively and strongly inhibited ACS1 and ACS4 in a dose-dependent manner, but had little effect on ACS5. The IC_{50} for ACS1 and ACS4 was 4–6 μM.

Thiazolidinediones Are Specific Inhibitors of ACS4—Thiazolidinediones are oral antidiabetic drugs, generally believed to act as insulin sensitizers through activation of PPARγ (24–26). Because the sulfo-conjugate of troglitazone was reported to directly inhibit ACS activity in both microsomes and mitochondria from rat liver (19), we tested purified recombinant ACS1, ACS4, and ACS5 to determine which isoform was inhibited by thiazolidinediones. Troglitazone at 1 μM decreased ACS4 activity 50%; with 10 μM troglitazone, the activity was almost completely blocked (Fig. 4*A*). In contrast, ACS1 and ACS5 activities were unaffected by troglitazone at 10 μM. Because troglitazone contains the ring structure of α-tocopherol linked to the thiazolidinedione moiety, we determined which part of the molecule was critical for inhibition by testing the effect of α-tocopherol itself and of other thiazolidinediones on each of the purified ACS isoenzymes. α-Tocopherol had little effect on three ACS isoforms (Fig. 4*B*). On the other hand, both pioglitazone and rosiglitazone, which contain the common thiazo-

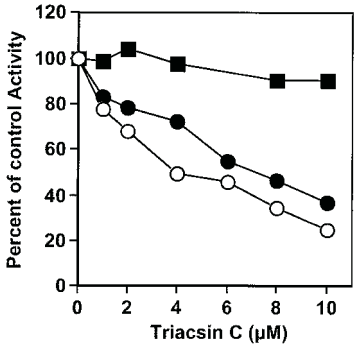

FIG. 3. **Triacsin C inhibits ACS1 and ACS4.** Purified ACS1-Flag (○) and ACS4-Flag (●) (each 0.1 μg) and ACS5-Flag (■) (0.5 μg) were assayed for ACS activity in the presence of varying concentrations of triacsin C dissolved in Me_2SO (2.5% of final assay reaction). The values are from an experiment that is representative of three independent determinations. Activities in the absence of triacsin C were 763 for ACS1, 1378 for ACS4, and 63 nmol/min/mg for ACS5.

lidinedione group but lack the α-tocopherol moiety, showed virtually the same effect as troglitazone on the three ACS isoenzymes. At 10 μM both drugs inhibited ACS4 85–95%, inhibited ACS5 less than 20%, and did not alter ACS1 activity (Fig. 4, *C* and *D*). At concentrations up to 10 μM, troglitazone had little effect on ACS5 activity, even after full activation by 3 mM Triton X-100 (data not shown). Of the thiazolidinediones, rosiglitazone was the strongest inhibitor of ACS4. The IC_{50} was 0.5 μM for rosiglitazone and 1.5 μM for troglitazone and pioglitazone (Table II). These data indicate that the thiazolidine-2,4-dione moiety is the critical moiety for ACS4 inhibition.

Troglitazone exhibited a mixed type inhibition of ACS4. With respect to palmitate, the reciprocal plots intersected above the 1/palmitate axis (Fig. 5*A*) and the K_i was 0.1 μM as calculated from the K_m/v_{max} *versus* inhibitor replot (Fig. 5*B*). Since the 1/*v* replot intersected with the K_m/v_{max} replot near the *y* axis, we can characterize the system as exhibiting linear non-competitive inhibition. Linear mixed inhibition also arises when an inhibitor binds at mutually exclusive sites. Therefore, we examined troglitazone inhibition kinetics of CoA and ATP, the other two substrates of ACS4. Troglitazone exhibited partial uncompetitive inhibition with respect to CoA (Fig. 5*C*). For ATP, the reciprocal plots intersected below the 1/palmitate axis (Fig. 5*D*), another linear mixed-type inhibition system.

Non-thiazolidinedione PPARγ and PPARα Activators Do Not Inhibit ACS4—*N*-(2-Benzoylphenyl)L-tyrosine (GW1929) is a newly identified non-thiazolidinedione PPARγ activator with a high affinity for human PPARγ (27). The glucose-lowering effect of GW1929 in rats is 100-fold more potent than that of troglitazone (27). In contrast, however, GW1929 was a poor inhibitor of ACS4 with an IC_{50} of 50 μM *versus* 1.5 μM for the TZDs) (Table II). PPARα activators such as clofibric acids or GW4647 up to 50 μM had no effect on ACS1, ACS4, or ACS5 (Table II) despite the ability of PPARα ligands to improve insulin sensitivity and reduce adiposity (28). We also tested a variety of xenobiotic carboxylic acids, which contain hydrophobic and carboxylic acid moieties, are known to be incorporated into complex lipids, and either resemble natural fatty acids or are aromatic derivatives of short chain fatty acids (29). The long chain cyclopropane (bridged) analogue of oleic acid, (±)-*cis*-9,10-methylene octadecanoic acid, inhibited ACS1 and ACS4 activities 50% at 25 and 30–40 μM, respectively, but had no effect on ACS5. Other xenobiotic carboxylic acids, 3-phenoxybenzoic acid, *p*-coumaric acid, ibuprofen, ferulic acid, and firefly luciferin, at concentrations up to 50 μM had no effect on three ACS isoenzymes.

N-Ethylmaleimide Strongly Inhibited ACS4 and Weakly Inhibited ACS1, but Had No Effect on ACS5—To further characterize the ACS isoenzymes, the effects of amino acid-reactive compounds were investigated. Before assay, each purified ACS isoenzyme was pre-incubated with various concentrations of NEM, a sulfhydryl-reactive compound, for 10 min on ice (Fig. 6). NEM at 3 mM inhibited ACS4 and ACS1 activities 83% and 25%, respectively, but had no effect on ACS5, indicating that amino acids containing a sulfhydryl group were more critical

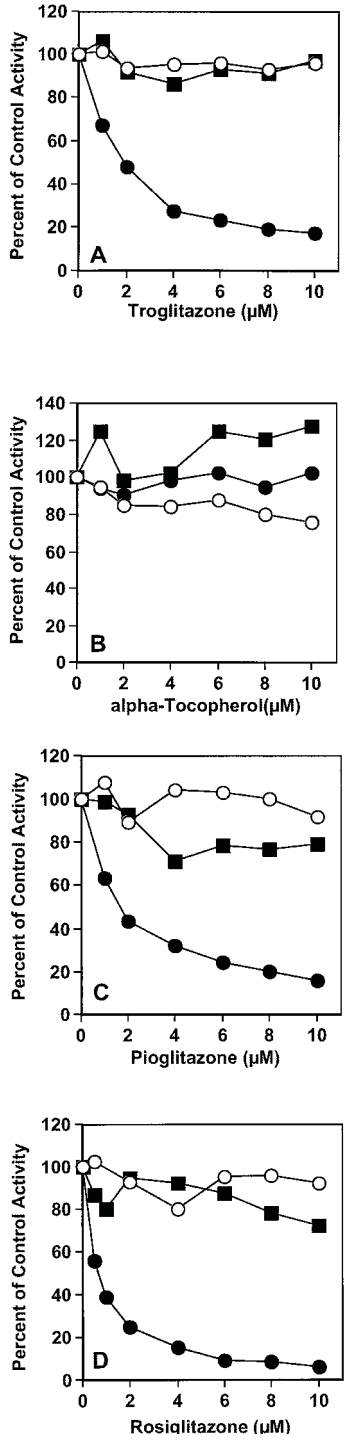

FIG. 4. **Thiazolidinediones inhibit ACS4.** Purified ACS1-Flag (○) and ACS4-Flag (●) (each 0.1 μg) and ACS5-Flag (■) (1 μg) were assayed for ACS activity. Troglitazone, pioglitazone, rosiglitazone, and α-tocopherol were dissolved in Me_2SO (2.5% of final assay reaction). The values shown are from an experiment that is representative of two independent determinations. *A*, troglitazone; *B*, α-tocopherol; *C*, pioglitazone; *D*, rosiglitazone.

TABLE II

Effect of PPARγ and PPARα activators and of selected xenobiotic carboxylic acids on purified ACS1-, ACS4-, and ACS5-Flag activities

Purified ACS1-Flag and ACS4-Flag (each at 0.1 μg of protein) and 0.5 μg of ACS5-Flag were assayed for ACS activity in the presence of up to 50 μM test compound. Chemicals were dissolved in Me_2SO, and the amount of Me_2SO was consistently maintained as 2.5% of the total assay reaction. The reaction was initiated by the addition of enzyme. NE, no effect.

Chemicals	IC_{50}		
	ACS1-Flag	ACS4-Flag	ACS5-Flag
		μM	
Troglitazone	1.5	NE	NE
Pioglitazone	1.5	NE	NE
Rosiglitazone	0.5	NE	NE
GW1929	>50	50	NE
GW4647	NE	NE	NE
Clofibrate	NE	NE	NE
(±)-*cis*-9,10-Methylene octadecanoic acid	25	30–40	NE
3-Phenoxybenzoate	NE	NE	NE
p-Coumaric acid	NE	NE	NE
Ferulic acid	NE	NE	NE
Ibuprofen	NE	NE	NE
Firefly luciferin	NE	NE	NE

for ACS4 catalysis compared with ACS1 or ACS5 (Fig. 5). After a 10-min preincubation, the arginine-reactive compound, phenylglyoxal, at 25 mM inhibited ACS1 38%, ACS4 78%, and ACS5 65%, suggesting the importance to each isoform of an arginine residue for catalysis (data not shown). *m*-Aminophenylboronate (0–20 mM), a serine-reactive compound, however, had little effect on ACS1, ACS4, and ACS5 (data not shown).

DISCUSSION

We focused on ACS1, ACS4, and ACS5 because these three isoforms are present in liver, a tissue that metabolizes long chain fatty acids via a variety of synthetic and degradative pathways. To characterize ACS1, ACS4, and ACS5 and test the effects of inhibitors, we purified each ACS isoform as a ACS-Flag fusion protein after confirming that the Flag sequence at the C terminus does not alter enzymatic kinetic properties. The ACS5-Flag fusion protein was unique with regard to the conditions required for its expression, purification, and full recovery of activity. Although others have expressed ACS5 without reported difficulty in DH5α with a vector containing the *lac* promoter (11), our successful ACS5 expression required a vector containing the T7 promoter, suggesting that the ACS5 protein may be toxic to *E. coli* since the T7 promoter-driven vector suppresses the expression of foreign proteins in uninduced states more completely. In addition, elution of active ACS5-Flag from the Flag antibody column required the presence of 0.1% Triton X-100 in the elution buffer, whereas active ACS1 and ACS4 could be eluted without adding detergent. Furthermore, the loss of 50% of ACS5 activity, merely in the process of obtaining bacterial membranes, suggests that detergent might be helpful at every stage of preparation. Although 0.5 mM Triton X-100 is regularly used in the ACS assay, we found that maximal activation of ACS5 demanded an ~6-fold higher concentration of Triton X-100. Thus, optimal assay conditions required the presence of ~500 Triton X-100 micelles/molecule of ACS1 or ACS4, and about 3000 micelles/molecule of ACS5. The critical micellar concentration of Triton X-100 is 0.3 mM, and, although it remains unclear why ACS5 requires such a large number of Triton X-100 micelles, the detergent may be essential to reconstitute or stabilize purified ACS5 or to relieve product inhibition by palmitoyl-CoA. In order to be consistent in our comparisons, we used identical concentration of Triton X-100 (0.5 mM) and substrates to assay all recombinant ACS isoenzymes.

The three purified ACS isoenzymes had similar apparent K_m values for CoA and palmitic acid. The apparent K_m for palmitic acid of ACS4 in this study is more than 10-fold lower than the one reported previously (12), probably because the concentrations of palmitic acid we used to study kinetics ranged from 0.33 to 2.0 of the K_m, as recommended (30). ACS4 had approximately a 20-fold higher affinity for ATP than did ACS1 or ACS5. If these isoenzymes compete for the same pool of ATP, ACS4 might remain active during fasting or when cellular energy levels are low. On the other hand, however, the subcellular location of ACS4 and its increase after refeeding (31) suggest that ACS activities are not simply dependent on substrate availability.

Despite their striking homology, the three ACS isoforms exhibited marked differences in their responses to heating and amino acid-modifying reagents. ACS4 was the most sensitive isoform when heated at 43 °C. ACS1 and ACS4 had broad pH optima from pH 7.4 to pH 9.0, whereas the optimum for ACS5 was from pH 7.4 to pH 8.0. ACS4 was more severely inhibited by phenylglyoxal than were ACS1 or ACS5, suggesting that arginine residues may be more critical for ACS4 activity. The marked inhibition of only ACS4 by NEM suggests that sulfhydryl group-containing amino acids may also be important. On the other hand, none of the isoforms was affected by *m*-aminophenylboronate, indicating that serine is not an essential residue for catalysis. Taken as a whole, the responses to site-specific inhibitors probably reflect the relative similarity of ACS1 and ACS5 in amino acid sequence and the relative dissimilarity of ACS4.

ACS inhibitors such as triacsin C or troglitazone differentially affect metabolic end products of ACS-dependent pathways (16–19). Thus, we hypothesized that pathways using acyl-CoA substrates might be initiated by distinct ACS isoforms and that the different degree of inhibition of TAG, phospholipid, and CE synthesis, or fatty acid β-oxidation by ACS inhibitors might result if each ACS isoform had a different sensitivity to the two inhibitors. If this were true, ACS isoforms could play a critical role in partitioning their products toward specific pathways through independent regulation of each ACS isoform. This hypothesis was supported by our data. The current study using purified ACS isoenzymes demonstrates that ACS isoforms can be distinguished by their sensitivity to ACS inhibitors. Triacsin C had little effect on ACS5, but strongly inhibited ACS1 and ACS4 activities in a dose-dependent manner. Because studies with cultured cells show that triacsin C strongly blocks *de novo* synthesis of TAG and has little affect on phospholipid reacylation or CE synthesis, or on β-oxidation (16–18), ACS1 and ACS4 may be closely linked to TAG synthesis.

Troglitazone at 10 μM did not alter ACS1 activity, mildly inhibited ACS5 activity, and almost completely blocked ACS4 activity. α-Tocopherol was not an inhibitor, whereas rosiglitazone and pioglitazone were potent ACS4-specific inhibitors and had little affect on ACS1 or ACS5. Thus, the critical component for ACS4 inhibition appears to be the thiazolidine-2,4-dione moiety rather than the α-tocopherol ring. It is most likely that ACS4 inhibition by thiazolidinediones is more closely related to TAG synthesis rather than to β-oxidation for several reasons. 1) ACS4 protein was most prominently located in the mitochondrial-associated membrane (31), which exhibits the highest specific activities for enzymes of TAG synthesis (32), and was not detected in mitochondria (31). 2) After a 48-h fast followed by 24-h refeeding with chow or sucrose (69%), hepatic ACS4 protein increased 150% (31). 3) The preference of ACS4 for arachidonic acids is consistent with only a weak link to β-oxidation because arachidonic acid is among the least preferred

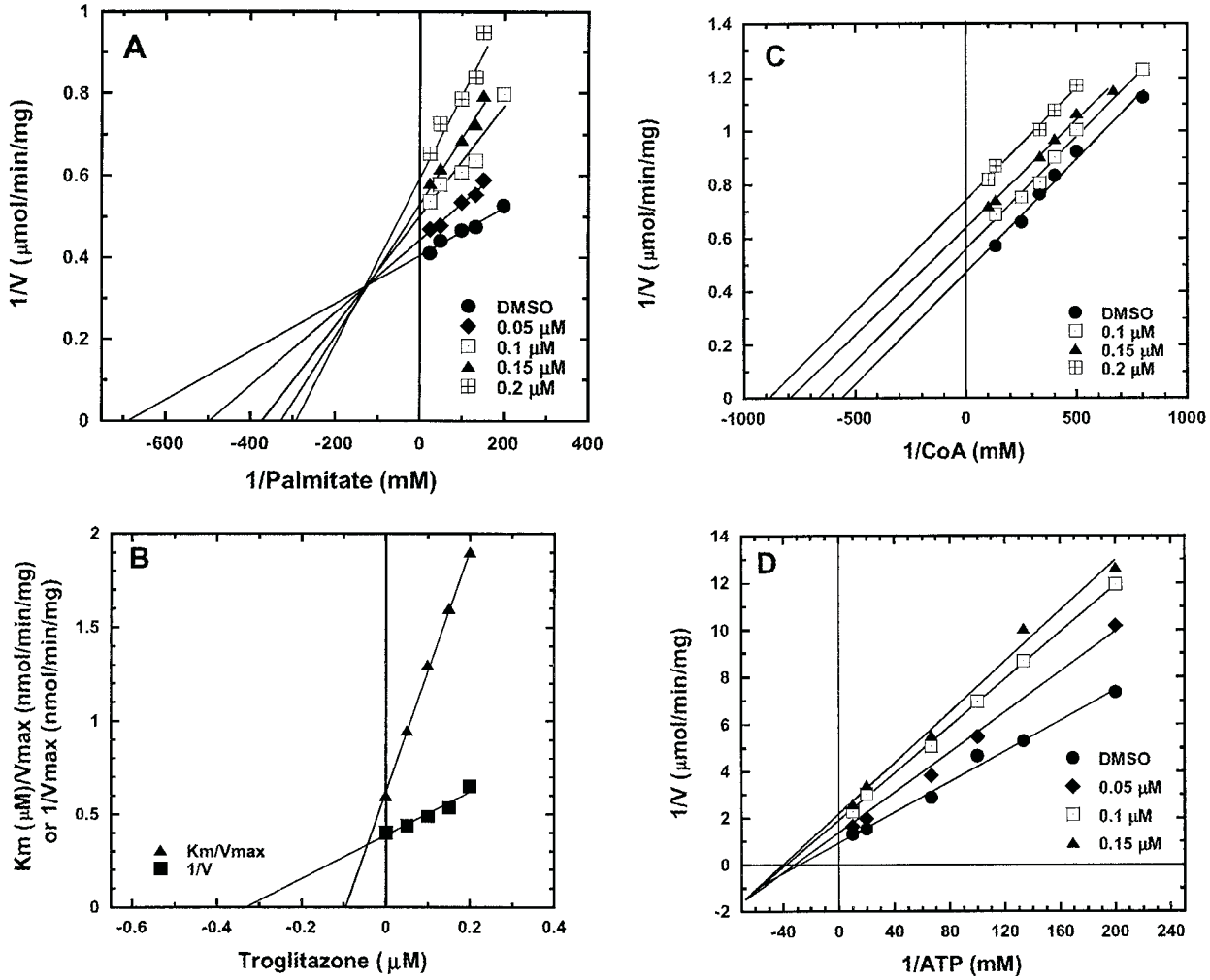

FIG. 5. **Troglitazone is a mixed-type inhibitor of ACS4.** Purified ACS4-Flag (0.1 μg) was assayed for ACS activity for 1 min at 37 °C. Troglitazone was dissolved in Me_2SO (*DMSO*; 2.5% of final assay reaction). *A*, Lineweaver-Burk plot with palmitate; *B*, replots of K_m/v_{max} and $1/v_{max}$ *versus* troglitazone; *C*, Lineweaver-Burk plot with CoA; *D*, Lineweaver-Burk plot with ATP.

fatty acids for β-oxidation during fasting states (33).

Thiazolidinediones appear to be highly specific inhibitors of ACS4. Troglitazone exhibited mixed-type inhibition of ACS4 with respect to all three substrates. Troglitazone is believed to activate PPARγ because it mimics a fatty acid; therefore, we postulated that troglitazone might compete with palmitate in the acyl-CoA ligation reaction. We found that troglitazone displayed linear mixed-type inhibition (Fig. 5*A*). This could arise because the ACS4-troglitazone complex has a lower affinity than ACS4 for palmitate and the enzyme-substrate(s)-inhibitor complex is nonproductive. Another possibility is that troglitazone interacts at more than one site, so we examined troglitazone inhibition kinetics of ACS4 with CoA and ATP. Troglitazone is a partial uncompetitive inhibitor of ACS4 with respect to CoA (Fig. 5*C*), indicating that the affinity for CoA of the ACS4-troglitazone complex increases by the same degree as the inhibitor decreases v_{max}. For ATP, troglitazone displayed linear mixed-type inhibition with the reciprocal plots intersecting below the 1/palmitate axis (Fig. 5*D*), suggesting that the ACS4-troglitazone complex has an increased affinity for ATP, but that the enzyme-substrate(s)-inhibitor complex forms product more slowly than the enzyme-substrate(s) complex. It should be noted that these types of inhibition kinetic systems were initially characterized using soluble single substrate enzymes (30), whereas we are extrapolating them to apply to a membrane-bound enzyme, ACS4, that has three substrates, only two of which are soluble.

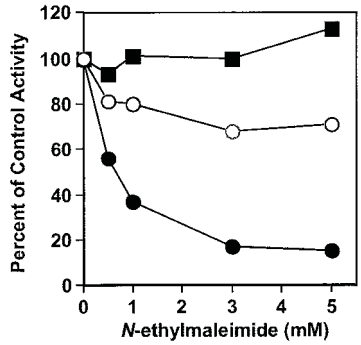

FIG. 6. ***N*-Ethylmaleimide inhibits ACS4.** The reaction was initiated by adding ACS1-Flag (○), ACS4-Flag (●) (each 0.1 μg), and ACS5-Flag (■) (1 μg) that had been incubated with different concentrations of NEM for 10 min on ice. The proteins were preincubated with NEM dissolved in 10 mM Tris, and 0.5 mM EDTA (pH 7.4) buffer. The values are from an experiment that is representative of two independent determinations. Uninhibited activities were 1159 for ACS1, 2477 for ACS4, and 113 nmol/min/mg for ACS5.

GW1929, a potent non-thiazolidinedione PPARγ activator, was a weak inhibitor of both ACS1 and ACS4, with an IC_{50} value for ACS4 more than 30-fold higher than that observed with thiazolidinediones. ACS4 was not affected by the PPARα activators clofibric acid and GW4647 at concentrations up to 50

μM, even though these compounds, like the thiazolidinediones, can markedly lower hyperinsulinemia and hyperglycemia in diabetic animal models (28). Furthermore, ACS4 was unaffected by a variety of xenobiotic carboxylic acids that contain hydrophobic rings and are known to be incorporated into complex lipids.

Although we did not test the effects of troglitazone *O*-sulfate (TOS), a major troglitazone metabolite, 100 μM TOS was reported to inhibit ACS activity 80% in both microsomes and mitochondria from rat hepatocytes, whereas troglitazone itself had no effect (19). The purity of the membrane fractions used in the reported study was not evaluated. We found that troglitazone at 10 and 50 μM inhibited ACS activity 25% and 50%, respectively, in purified mitochondria-associated membrane fractions (31). The reported poor ability of troglitazone to inhibit ACS activity (19) probably occurred because the membrane fractions used in that study contained relatively little ACS4, but may have contained other ACS isoforms that were sensitive to high concentrations of TOS or to an inhibitory factor elicited by TOS.

Thiazolidinediones are powerful oral antidiabetic drugs that act as insulin sensitizers. Although it is believed that their efficacy occurs via activation of PPARγ (24–26), some evidence suggests that some thiazolidinedione actions can occur in a PPARγ-independent manner. In PPARγ-deficient heterozygous mice, for example, glucose disposal rate and suppression of hepatic glucose production are greater than in normal mice, the reverse of what one would expect if thiazolidinediones ameliorate insulin resistance by activating PPARγ (34). Further, some compounds exhibit potent antidiabetic effects despite their weak activation of PPARγ (35). Troglitazone and rosiglitazone decrease serum triacylglycerol even in aP2/DTA mice, which lack adipose deposits, a main site for PPARγ action (36–38); troglitazone has acute effects in rats both on glucose disposal and on hepatic glucose production (39); finally, TZDs can decrease the expression of tumor necrosis factor-α and interleukin-6 in macrophages that contain no PPARγ (40). Our results suggest that ACS4 could contribute to the insulin-sensitizing and anti-inflammatory effects of the thiazolidinediones through direct interaction with ACS4 in a PPARγ-independent manner.

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REFERENCES

1. Kornberg, A., and Pricer, W. E. J. (1953) *J. Biol. Chem.* **204,** 329–343
2. Faergman, N. J., and Knudsen, J. (1997) *Biochem. J.* **323,** 1–12
3. Prentki, M., and Corkey, B. E. (1996) *Diabetes* **45,** 273–283
4. Coleman, R. A., Lewin, T. M., and Muoio, D. M. (2000) *Annu. Rev. Nutr* **20,** 77–103
5. Aas, M., and Daae, L. N. W. (1971) *Biochim. Biophys. Acta* **239,** 208–216
6. Berge, R. K., Flatmark, T., and Christiansen, E. N. (1987) *Arch. Biochem. Biophys.* **252,** 269–276
7. Lawson, N., Pollard, A. D., Jennings, R. J., Gurr, M. I., and Brindley, D. N. (1981) *Biochem. J.* **200,** 285–294
8. Lippel, K. (1971) *Biochim. Biophys. Acta* **239,** 384–392
9. Lippel, K. (1972) *Biochim. Biophys. Acta* **280,** 531–537
10. Suzuki, H., Kawarabayasi, Y., Kondo, J., Abe, T., Nishikawa, K., Kimura, S., Hashimoto, T., and Yamamoto, T. (1990) *J. Biol. Chem.* **265,** 8681–8685
11. Oikawa, E., Iijima, H., Suzuki, T., Sasano, H., Sato, H., Kamataki, A., Nagura, H., Kang, M.-J., Fujino, T., Suzuki, H., and Yamamoto, T. T. (1998) *J. Biochem.* (*Tokyo*) **124,** 679–685
12. Kang, M.-J., Fujino, T., Sasano, H., Minekura, H., Yabuki, N., Nagura, H., Iijima, H., and Yamamoto, T. T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 2880–2884
13. Fujino, T., and Yamamoto, T. (1992) *J. Biochem.* (*Tokyo*) **111,** 197–203
14. Fujino, T., Kang, M.-J., Suzuki, H., Iijima, H., and Yamamoto, T. (1996) *J. Biol. Chem.* **271,** 16748–16752
15. Cho, Y.-Y., Kang, M.-J., Ogawa, S., Yamashita, Y., Fujino, T., and Yamamoto, T. T. (2000) *Biochem. Biophys. Res. Commun.* **274,** 741–745
16. Igal, R. A., Wang, P., and Coleman, R. A. (1997) *Biochem. J.* **324,** 529–534
17. Muoio, D. M., Lewin, T. M., Weidmar, P., and Coleman, R. A. (2000) *Am. J. Physiol.* **279,** E1366–E1373
18. Wu, X., Shang, A., Jiang, H., and Ginsberg, H. N. (1996) *J. Lipid Res.* **37,** 1198–1206
19. Fulgencio, J. P., Kohl, C., Girard, J., and Pegorier, J. P. (1996) *Diabetes* **45,** 1556–1562
20. Banis, R. J., Roberts, C. S., Stokes, G. B., and Tove, S. B. (1976) *Anal. Biochem.* **73,** 1–8
21. Polokoff, M. A., and Bell, R. M. (1977) *J. Biol. Chem.* **252,** 1161–1171
22. Tomoda, H., Igarashi, K., and Omura, S. (1987) *Biochim. Biophys. Acta* **921,** 595–598
23. Tomoda, H., Igarashi, K., Cyong, J. C., and Omura, S. (1991) *J. Biol. Chem.* **266,** 4214–4219
24. Saltiel, A. R., and Olefsky, J. M. (1996) *Diabetes* **45,** 1661–1669
25. Schoonjans, K., Staels, B., and Auwerx, J. (1996) *J. Lipid Res.* **37,** 907–925
26. Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Wilson, T. M., and Kliewer, S. A. (1995) *J. Biol. Chem.* **270,** 12953–12956
27. Brown, K. K., Henke, B. R., Blanchard, S. G., Cobb, J. E., Mook, R., Kaldor, I., Kliewer, S. A., Lehmann, J. M., Lenhard, J. M., Harrington, W. W., Novak, P. J., Faison, W., Binz, J. G., Hashim, M. A., Oliver, W. O., Brown, H. R., Parks, D. J., Plunket, K. D., Tong, W. Q., Menius, J. A., Adkison, K., Noble, S. A., and Willson, T. M. (1999) *Diabetes* **48,** 1415–1424
28. Guerre-Millo, M., Gervois, P., Raspe, E., Madsen, L., Poulain, P., Derudas, B., Herbert, J. M., Winegar, D. A., Willson, T. M., Fruchart, J. C., Berge, R. K., and Staels, B. (2000) *J. Biol. Chem.* **275,** 16638–16642
29. Dodds, P. F. (1995) *Prog. Lipid Res.* **34,** 219–247
30. Segel, I. H. (1975) *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, John Wiley & Sons, Inc., New York
31. Lewin, T. M., Kim, J.-H., Granger, D. A., Vance, J. E., and Coleman, R. A. (2001) *J. Biol. Chem.* **276,** 24674–24679
32. Rusinol, A. E., Cui, Z., Chen, M. H., and Vance, J. E. (1994) *J. Biol. Chem.* **269,** 27494–27502
33. Cunnane, S. C. (1988) *Lipids* **23,** 372–374
34. Miles, P. D. G., Barak, Y., He, W., Evans, R. M., and Olefsky, J. M. (2000) *J. Clin. Invest.* **105,** 287–292
35. Fukui, Y., Masui, S., Osada, S., Umesono, K., and Motojima, K. (2000) *Diabetes* **49,** 759–767
36. Wang, Z., Zhou, Y., Lee, Y., Higa, M., Kalra, S. P., and Unger, R. H. (1999) *Biochem. Biophys. Res. Commun.* **260,** 653–657
37. Burant, C. F., Sreenan, S., Hirano, K., Tai, T. A., Lohmiller, J., Lukens, J., Davidson, N. O., Ross, S., and Graves, R. A. (1997) *J. Clin. Invest.* **100,** 2900–2908
38. Chao, L., Marcus-Samuels, B., Mason, M. M., Moitra, J., Vinson, C., Arioglu, E., Gavrilova, O., and Reitman, M. L. (2000) *J. Clin. Invest.* **106,** 1221–1228
39. Lee, M.-K., and Olefsky, J. M. (1995) *Metabolism* **44,** 1166–1169
40. Chawla, A., Barak, Y., Nagy, L., Liao, D., Tontonoz, P., and Evans, R. M. (2001) *Nat. Med.* **7,** 48–52