

ATP citrate lyase – biology and implication in human pathology

ATP citrat liaza – biologie și implicare în patologia umană

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Abstract

ATP citrate lyase (ACLY) is a cytoplasmic enzyme that catalyzes the cleavage of citrate into oxaloacetate and acetyl-CoA. This reaction provides most of the acetyl-CoA required for the biosynthesis of fatty acids and cholesterol. Since its discovery half a century ago, many of the mechanisms involved in the regulation of ACLY activity have remained unclear. Various attempts to biochemically characterize the enzyme gave highly heterogeneous results. The development of expression systems for the recombinant human protein offered a powerful tool for research on this enzyme. Although the reaction catalyzed by ACLY is not rate limiting in either lipogenesis or cholesterologenesis, its „strategic” metabolic position makes ACLY an appealing target for hypolipidemic intervention. ACLY inhibition experiments showing hypolipidemic and hypocholesterolemic effects in animal models, together with data suggesting the role of the enzyme in cancer biology, cell growth and differentiation, brought it in the attention of scientific community. This paper makes a review of ACLY biology and its potential implication in human pathology.

Keywords: ATP citrate lyase, lipogenesis, enzyme inhibitors, hypolipidemic agents

Rezumat

ATP citrat liaza (ACLY) este o enzimă situată în citoplasmă, care catalizează scindarea citratului în oxaloacetat și acetil-coenzima A. Această reacție oferă majoritatea necesarului de acetil-coenzimă A pentru biosinteza acizilor grași și a colesterolului. Începând cu descoperirea sa în urmă cu o jumătate de secol, mai multe mecanisme implicate în reglarea activității ACLY rămân încă neclare. Încercările de a caracteriza enzima din punct de vedere biochimic au dat rezultate foarte variabile. Dezvoltarea sistemelor de expresie a proteinei umane recombinante au oferit un instrument puternic pentru studierea acestei enzime. Deși reacția catalizată de ACLY nu este limitantă de viteză nici pentru lipogeneză, nici pentru colesterologeneză, poziția ei metabolică strategică face din ACLY o țintă atrăgătoare pentru intervenția hipolipemiantă. Experimente de inhibare a ACLY care au demonstrat efecte hipolipemiente și hipocolesterolemice în modelele animale și date sugerând rolul enzimei în biologia cancerului, în creșterea și diferențierea celulară, au adus-o în atenția comunității științifice. Această lucrare constituie o prezentare generală asupra biologiei ACLY și a implicării sale în patologia umană.

Cuvinte-cheie: ATP citrat liaza, lipogeneză, inhibitori enzimatici, agenți hipolipemianți.

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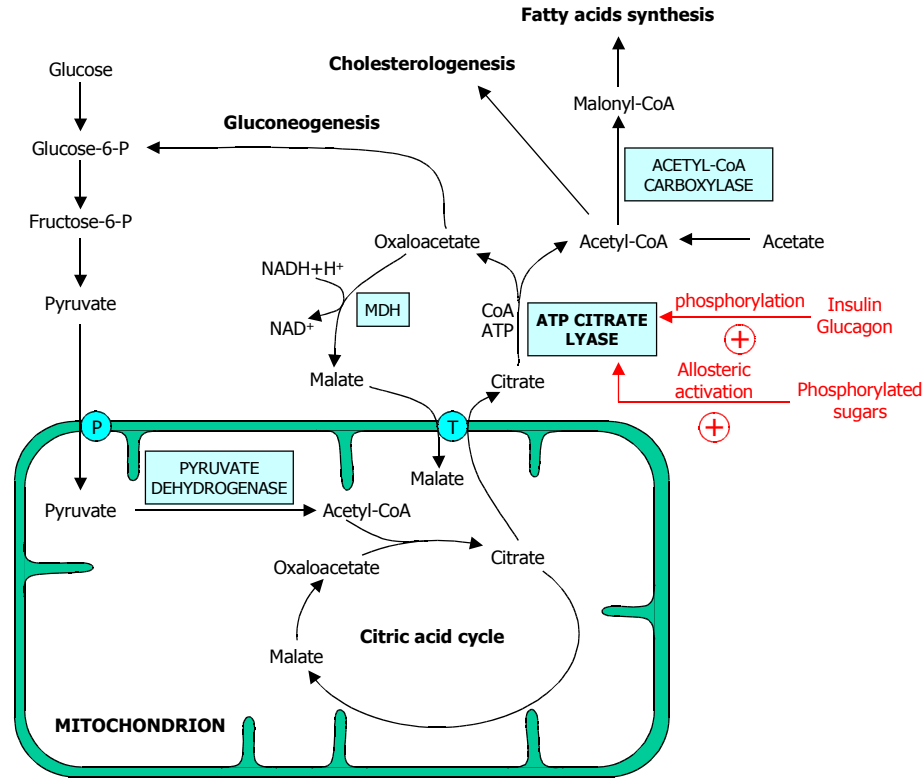


Figure 1. Involvement of ATP citrate lyase in several metabolic pathways. MDH = malate dehydrogenase, P = pyruvate transporter, T = tricarboxylate transporter

ATP citrate lyase (ACLY)ⁱ is a cytoplasmic enzyme that catalyses the cleavage of citrate into oxaloacetate and acetyl-CoA, the latter being the major building block for both cholesterologenesis and lipogenesis (*Figure 1*). Acetyl-CoA provided by ACLY is also used in the biosynthesis of acetylcholine. Oxaloacetate enters the gluconeogenic pathway through phosphoenolpyruvate carboxykinase.

In nonruminants, nearly all the acetyl-CoA used in fatty acid synthesis is formed in mitochondria from pyruvate oxidation and from

catabolism of the carbon skeletons of amino acids. The mitochondrial inner membrane is impermeable to acetyl-CoA, so an indirect shuttle transfers acetyl group equivalents across the inner membrane. Intramitochondrial acetyl-CoA first reacts with oxaloacetate to form citrate in the citric acid cycle, reaction catalyzed by citrate synthase. Citrate then passes through the inner membrane on the citrate transporter. In the cytosol, citrate cleavage by ACLY regenerates acetyl-CoA in an ATP-dependent reaction²⁴.

Although the reaction catalyzed by ACLY is not rate limiting in either lipo- or cholesterologenesis, its „strategic” metabolic position upstream of acetyl-CoA carboxylase and hydroxymethyl glutaryl coenzyme A reductase attracted researchers to select this enzyme

ⁱ EC 2.3.3.8 (created in 1965 as EC 4.1.3.8, modified in 1986, transferred in 2002 to EC 2.3.3.8). Systematic name: acetyl-CoA:oxaloacetate C-acetyltransferase [(pro-S)-carboxymethyl-forming, ADP-phosphorylating]. Synonyms: ATP-citrate synthase, ATP-citrate (pro-S-)-lyase, Citrate cleavage enzyme

as a target for hypolipidemic intervention. Results of ACLY inhibition experiments showing hypolipidemic and hypocholesterolemic effects in animal models, together with data suggesting the role of the enzyme in cancer biology, cell growth and differentiation, brought it again in the attention of scientific community.

This paper makes a review of ACLY biology and its potential implication in human pathology.

Reaction mechanism

The reaction catalysed by ACLY is shown in *Equation 1*.

A five-step reaction mechanism was initially proposed, where enzyme was first phosphorylated by ATP and the phosphate was transferred to citrate to form a non-covalent citryl phosphate within the active site. The citrate was then transferred to a thiol nucleophile enzyme active site to form a covalent citryl-enzyme complex which was then attacked by CoA to generate citryl-CoA within the active site. Finally, the enzyme catalysed the retro-Claisen cleavage of citryl-CoA to produce acetyl-CoA and oxaloacetate³⁶.

The primary sequence of rat liver ACLY has been obtained from a cDNA clone and its amino acid sequence was showed to have a high similarity with the alpha chain of succinyl-CoA synthetase of *Escherichia coli*⁷. Both enzymes catalyse similar reactions. The first reaction step for both of them is autophosphorylation of the enzyme by ATP, followed by phosphorylation of the substrate (citrate and succinate, respectively) by the phosphoenzyme formed. CoA reacts with citrate/succinate phosphate to form citryl-/succinyl- CoA. ACLY has one additional step in that citryl-CoA is cleaved into acetyl-CoA and oxaloacetate before release. However, the mechanism of succinyl-

CoA synthetase is not reported to involve the production of a covalent succinyl-enzyme as part of the mechanism³⁶. The structural and functional similarity between the two enzymes led to further investigations which showed that the mechanism of the reaction for ACLY proceeds as far as the production of an enzyme - citryl-phosphate complex. However, the next stage of the reaction is therefore most likely to be the direct attack of CoA on the citrate phosphate. Therefore, only one covalent enzyme-bound intermediate occurs in the ATP-citrate-lyase-catalysed reaction³⁶. The novel proposed mechanism was also confirmed by the failure in the attempts to inhibit enzyme activity using compounds that interacted with the active site thiol nucleophile¹⁰.

Structure

Since the early work of Srere and his collaborators³⁰ who purified ACLY from chicken liver, the methods of production and purification of the active enzyme have evolved, permitting detailed analysis of its structure and function. Cloning and expression of human ACLY have been achieved⁸ and large quantities of active enzyme can now be obtained in baculovirus expression system¹⁹.

The enzyme is a tetramer (molecular mass of 440 KDa as determined by sedimentation equilibrium) of four apparently identical subunits²⁹. Each subunit has a sequence of 1101 amino acids and a calculated mass of 120,8 KDa³⁴. Human ACLY amino acid sequence is 96,3% identical to rat ACLY and the inclusion of conservative replacements increases the degree of similarity to 98%⁸.

Studies on this enzyme showed that the isolated protein was a phosphoprotein containing usually two phosphate groups per tetramer¹⁸.



Equation 1. Reaction catalyzed by ATP citrate lyase

Table 1. Features of ATP citrate lyase chain³⁴

Feature Key	Position	Length	Description
Regions			
Nucleotide binding	701-721	21	ATP (by similarity)
Nucleotide binding	752 - 778	27	ATP (by similarity)
Region	779 - 789	11	CoA-binding (potential)
Sites			
Active site	760	1	Tele-phosphohistidine intermediate (by similarity)
Metal binding	718	1	Magnesium (by similarity)
Amino acid modifications			
Modified residue	131	1	Phosphotyrosine
Modified residue	455	1	Phosphoserine
Modified residue	481	1	Phosphoserine
Modified residue	682	1	Phosphotyrosine
Natural variations			
Natural variant	175	1	Glu→Asp

A summary of sequence annotations (features) on the chain of an ACLY subunit as described in UniProtKB (Protein Knowledge-base)³⁴ are presented in *Table 1*.

Enzyme distribution in tissues and cellular localization

ACLY distribution in animal and vegetal tissues has been extensively studied. Extracts of rat, rabbit, chicken, and beef tissues were first compared by Srere³⁰. The greatest enzyme activities were observed in extracts of chicken liver and rat liver. Extracts of spinach and of mushroom had no enzyme activity. Chicken liver frozen for as long as 3 months retained most of its activity. In general, there is little ACLY in ruminants, probably because in these species acetate (derived from the rumen and activated to acetyl-CoA extramitochondrially) is the main source of acetyl-CoA²³.

The liver has been reported to be the organ with the highest ACLY enzyme activity, followed by brain and kidney³⁰. The level of ACLY mRNA in adult male rats has been found to be the highest in liver. The adrenals, lung, brain and large intestine are relatively rich

in ACLY mRNA. In young individuals, ACLY mRNA is increased in brain, as lipogenesis is also an important pathway in the developing brain, particularly with respect to myelination⁷. A diurnal variation in ACLY mRNA in rat liver was demonstrated. However, no diurnal variation in activity of the enzyme was observed⁹.

The activity of ACLY is lower in human tissues compared to rat tissues. However, rat metabolic rates are in general higher than in human. Early studies reported that human adipose tissue, unlike that of the rat, contains little or no ACLY activity. The mean activity of ACLY in subcutaneous adipose tissue in humans was found approximately 50 times lower versus the rat maintained on a normal laboratory diet. It is known that diet influences lipogenic enzymes activity, which is lowered by a high fat intake or by fasting and increased by carbohydrates. The normal diet of laboratory rats contains a high concentration of carbohydrate and a low concentration of fat. The human diet contains a much higher level of fat and most of the studies have been performed on human adipose tissue taken after an overnight fast, which preceded the surgical removal of the tissue. Thus, one can suppose that if rats ate a nor-

Table 2. Reaction conditions used in various experiments for the assessment of ATP citrate lyase activity

Article	Srere, 1959 ³⁰	Linn, 1979 ¹⁸	Gribble, 1996 ⁹	Ki, 2000 ¹⁴
Potassium citrate	21 mM	20 mM	0.1 mM	5 mM
ATP	5 mM	5 mM	0.250 mM	5 mM
CoA	0.3 mM	0.33 mM	0.2 mM	0.33 mM
Malate dehydrogenase	500 U	0.5 U	Present	2 U
NADH	0.1-0.2 mM	0.14 mM	35 mM	0.14 mM
MgCl ₂	10 mM	10 mM	10 mM	10 mM
KCl	-	-	10 mM	-
Tris HCl	100 mM, pH=7.3	100 mM, pH=8.7	100 mM, pH=8.0	100 mM, pH=8.5
Dithiothreitol	10 mM thioethanol	10 mM	10 mM	10 mM
Total volume	1 ml	1 ml		100 µl

mal human diet, their lipogenic enzyme activities would be significantly reduced. Indeed, when rats were fed a high-fat diet and fasted overnight, their ACLY activity in the adipose tissue was only 8 times higher than in humans³².

In human liver, ACLY activity per gram of tissue is approximately 10 times higher than in adipose tissue, due to a higher total protein concentration. Corrected for the total protein concentration, liver and adipose tissue ACLY activities are very similar³².

At cellular level, ACLY is a cytoplasmic enzyme bound to the endoplasmic reticulum¹⁷.

Assessment of ACLY activity

ACLY enzymatic activity can be assayed by trapping acetyl-CoA as acetylhydroxamate and measuring the latter by the color produced with FeCl₃, but this assay is useful only for a small range of enzyme concentration. A

larger range of enzyme activities can be assayed when oxaloacetate is reduced with malate dehydrogenase (MDH) and NADH while monitoring the decreasing NADH level at 340 nm using a spectrophotometer³⁰.

The two linked reactions used in the latter model of assay are presented in *Figure 2*.

The reaction medium contains TrisHCl as a buffer, dithiothreitol, potassium citrate, coenzyme A, magnesium chloride, ATP, NADH and MDH. The reaction temperature used by most of the authors is 25°C. A pH of 8 or above is used to favorize the dissociation of citrate salt²⁶. The reducing agent dithiothreitol is needed for the protection of sulfhydryl groups of the enzyme⁶. Optical density decreases linearly with time when ATP is used to start the reaction (the last component added). When citrate or the enzyme preparation is the last addition, a lag phase occurs. The rate of NADH oxidation is proportional to enzyme concentration³⁰. *Table 2* shows the reaction conditions

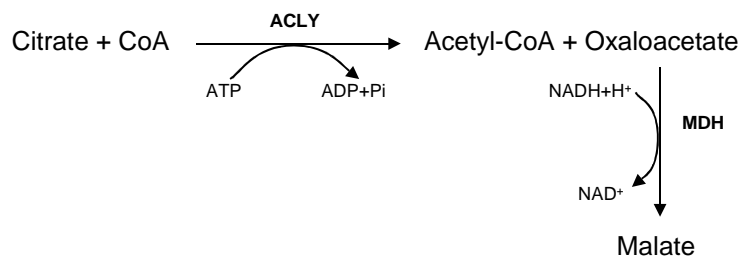


Figure 2. Principle of the ATP citrate lyase activity assay. ACLY = ATP citrate lyase, MDH = malate dehydrogenase, CoA = coenzyme A

used in various experiments.

Attempts to use the MDH-coupled method in the assessment of ACLY activity in renal cortex lysate were unsuccessful because of high native renal NADH dehydrogenase activity. In this case, hydroxamate assay was performed²⁰.

The presence of the chloride ion in the reaction mixture is necessary and its concentration influences the kinetic behavior of ACLY²⁶.

The enzyme partially loses its activity by oxidation upon storage and it is reactivated by dithiothreitol⁶. Some authors incubated the enzyme with 10 mM 2-mercaptoethanol before the assay, because under normal conditions it may take up to 15 minutes to fully reactivate³⁶. Concentrated lyase solutions tend to polymerize after a few days, probably due to sulfhydryl group oxidation. This phenomenon is more likely to occur at pH values above 7,5 and is accompanied by considerable loss of activity even when the enzyme is redissolved in the presence of additional dithiothreitol¹⁸.

Regulation of enzyme activity

Enzyme levels in liver and adipose tissue vary over a wide range under the control of nutrients and hormones¹³, declining sharply in starved animals and rising many fold when the animals are fed a high carbohydrate/ low fat diet¹⁴. These changes in ACLY activity were attributed mainly to alterations in the rate of ACLY biosynthesis. In fasted rats refed a carbohydrate/ protein diet, the transcriptional rate was reported to increase 4,5-fold above the fasted level within 2h and mRNA concentration reached a maximum level of approximately 30-fold increased in 16h⁹.

ACLY has been purified from a variety of tissues and expression systems and the phosphorylation of the enzyme has been shown to be enhanced by glucagon, insulin, vasopressin and transforming growth factor β ¹⁸. Further investigation has identified Thr446, Ser450, and

Ser454 residues to be specifically phosphorylated *in vitro*¹⁹. These sites are different from the catalytic autophosphorylation site His760, but their physiological role is not completely understood. Ser454 is phosphorylated by cAMP-dependent protein kinase (PKA) and an insulin-stimulated kinase. Thr446 and Ser450 are phosphorylated by glycogen synthase kinase-3 (GSK-3)^{19, 27}. Thr446 and Ser450 phosphorylation is decreased by insulin^{4, 27}.

Results reported on kinetic properties of the enzyme and a possible regulatory role of its phosphorylation have been variable. A possible reason for this is the fact that ACLY was isolated from mammalian tissues or eukaryote systems that were able to phosphorylate the enzyme prior to extraction and purification. Thus, variable phosphorylation and/or degradation status may have conducted to highly heterogeneous results. In this case, the use of an expression system that is unable to posttranslationally modify the enzyme perfectly makes sense. Potapova et al.²⁷ obtained an unphosphorylated human recombinant ACLY using a bacterial recombinant system which relied on the lack of posttranslational processing of eukaryotic proteins expressed in protease-deficient bacteria. It has been shown that unphosphorylated ACLY cannot be the substrate of GSK-3 alone. Phosphorylation of ACLY by PKA introduces a phosphate group on one of the 4 subunits of the enzyme. This first step allows further phosphorylation of the enzyme by GSK-3, which further incorporates another 2 phosphate groups in the same subunit as PKA. Phosphorylation of one subunit of ACLY inhibits the phosphorylation of the other 3 subunits²⁷.

Phosphorylation was found to increase 6-fold the activity of ACLY, without affecting its Michaelis-Menten constants for citrate, ATP or coenzyme A. The unphosphorylated ACLY tetramer shows a negative cooperativity with respect to citrate. This means that binding of the first molecule of citrate to one of the four potential catalytic sites decreases the affinity of

the other 3 catalytic sites for citrate. This phenomenon allows a constant ACLY activity over a wide range of cytoplasmic citrate concentrations. Phosphorylation of the enzyme eliminates the negative cooperation and transforms ACLY into a noncooperative form which is active even at low concentrations of citrate²⁷.

It seemed paradoxal that insulin and glucagon both promote phosphorylation of ACLY at the same serine residue³¹ (Ser454). During fasting state, glucagon activates ACLY by phosphorylation of Ser454 via PKA, thus increasing oxaloacetate availability for gluconeogenesis. Glucagon also inhibits acetyl-CoA carboxylase via its phosphorylation, preventing the use of acetyl-CoA in the synthesis of fatty acids²⁴. The rise of acetyl-CoA levels, a potent activator of pyruvate carboxylase, activates gluconeogenesis from oxaloacetate. On the other hand, during the fed state, insulin activates ACLY via phosphorylation of Ser454 by a reported „insulin-stimulated protein kinase”, which has been shown to be Akt (PKB, cAMP-independent protein kinase)⁵. The identity between the „insulin-stimulated protein kinase” and Akt had been presumed previously by Potapova et al²⁷. The mechanism proposed by Berwick et al. is that insulin receptor activation leads to the phosphorylation of insulin receptor substrates, recruitment of phosphatidylinositol 3'-kinase (PI3K), production of plasma membrane phosphoinositide (3,4,5)-trisphosphate, activation of Akt, and finally phosphorylation of ACLY Ser454⁵. Insulin also promotes the phosphorylation and subsequent activation of acetyl-CoA carboxylase⁵, leading the acetyl-CoA generated by the activated ACLY to the pathway of fatty acids synthesis.

The ability of Akt to phosphorylate and activate ACLY makes the latter potentially responsive to cytokine signalling. In a study performed on cultured IL-3-dependent cells, IL-3 signaling led to a dose and time-dependent increase in ACLY Ser454 phosphorylation. A PI3K inhibitor reduced IL-3-dependent ACLY

phosphorylation, and Akt expression stimulated this phosphorylation event in those cells³.

Human ACLY activity was also found to be regulated allosterically *in vitro* by phosphorylated sugars. Fructose 6-phosphate was found to be a potent activator of the unphosphorylated recombinant enzyme at concentrations close to physiological intracellular levels²⁷.

Experiments performed on rats showed that chronic metabolic acidosis increases cortical renal ACLY activity and protein abundance as determined by immunoblotting, but does not affect its mRNA abundance. Chronic K⁺ deficiency also increases the enzyme activity. These studies suggest that intracellular, rather than extracellular, acidosis is responsible for the increase in ACLY activity in the kidney. Chronic metabolic acidosis did not influence the enzyme activity in liver and did not affect ACLY mRNA abundance in either organ²⁰.

Regulation of gene expression

De novo transcription and translation appears to be one major mode of regulation of ACLY activity, as well as for other lipogenic enzymes^{7, 9}. Diet manipulation of rats demonstrates that ACLY mRNA can be highly induced, the liver mRNA concentration in fasted/refed rats being much higher than in fasted⁹.

The ACLY gene is expressed in most tissues⁷. In fact, this gene has a character of a housekeeping gene; that is, the TATA box is absent in the ACLY promoter and it would be a reason that transcription of ACLY is initiated over several bases¹⁵.

Nutrients and hormones

It was suggested that ACLY gene expression is strongly carbohydrate-dependent and glycolytic metabolites primarily stimulate transcription⁹.

ACLY appears to be induced by feeding with a carbohydrate-only diet, primarily at

the transcriptional level⁹. In cultured rat hepatocytes it was found that high concentrations (25 and 50 mM) of glucose without insulin could increase the ACLY protein level. ACLY mRNA level was increased from 6h after the addition of glucose in the medium and then its levels were maintained until 48h¹⁵. Activation of the ACLY promoter by glucose may be an effect in the changes in the expression of Sp1 family proteins²¹.

The fructose diet in diabetic rats, even without insulin treatment, increased the transcriptional rate more greatly and the mRNA concentration, comparably to levels reached by insulin treatment, but it did not increase enzyme activity⁹.

Fat present in the diet of the rats markedly decreases the transcriptional rate, mRNA concentration and enzyme activity of ACLY, suggesting a primary decrease at the transcriptional level⁹.

It has been reported that insulin activates expression of mRNA for ACLY as well as other lipogenic enzymes. Insulin treatment to the diabetic rats increased the ACLY mRNA fourfold in 4 h and induced enzyme activity eightfold within 48 h⁹. Insulin added to culture medium of rat hepatocytes had no effect alone, but in the presence of glucose, it increased the ACLY protein level further than glucose alone¹⁵. Several investigations have demonstrated that insulin transcriptionally stimulates SREBP-1c expression and that the insulin-dependent hepatic expression of lipogenesis-related genes including fatty acid synthase, acetyl-CoA carboxylase, and L-pyruvate kinase is mediated by SREBP-1c. It is not clear, however, if hepatic ACLY gene expression is under the control of SREBP-1c, a predominant form of SREBP in the liver and mediator of insulin action²⁸.

Transcription factors

The transcription of a number of genes encoding enzymes and proteins involved in

cholesterol or fatty acid metabolism is regulated by two sterol regulatory element - binding proteins (SREBPs), designated SREBP-1 and -2. SREBP-1 mainly regulates lipogenic gene expression and SREBP-2 regulates cholesterol gene expression. Two isoforms of SREBP-1 (SREBP-1a and SREBP-1c) are produced by alternate promoters. SREBP-1a has a longer and therefore more potent transactivation domain than SREBP-1c, but is less abundant in mouse and human tissues²⁸.

The amounts of SREBP-1c protein and mRNA, but not those of SREBP-2, dramatically increase when mice are placed on a high carbohydrate diet or after refeeding fasted mice, in a similar manner to changes in mRNAs of lipogenic enzymes².

In experiments with transgenic mice, ACLY gene was profoundly activated in the presence of SREBP-1a overexpression and, to a lesser extent, in the case of SREBP-1c and SREBP-2². Further investigations, conducted on Hep G2 cell cultures, showed that expression of ACLY mRNA is regulated by sterols: under the sterol-depleted conditions, expression of ACLY mRNA was up-regulated 2–3-fold²⁸.

Studies with SREBP-1 knockout mice demonstrated that SREBP-1 plays a crucial role in nutritional induction of lipogenic enzymes including ACL. In the absence of SREBP-1, the ACLY mRNA level was profoundly suppressed. However, SREBP-2 has some significant contribution to ACLY gene transcription in a nonfasted state. As a cytosolic enzyme that provides acetyl-CoA for both cholesterol and fatty acid *de novo* synthesis, ACLY is transcriptionally maintained in a sterol-regulatory fashion under the control of SREBP-2, even though the main regulation of ACLY is mediated by SREBP-1 in a lipogenic regulatory manner²⁸.

The analysis of the promoter of ACLY gene showed that its sequence is highly similar in human and rat, suggesting that the regulation at transcriptional level takes place in the same manners in these two species²¹. In the region

from transcription start site to -419 bp region, six Sp1 binding sites and a CCAAT box exist. NF-Y (a ubiquitous transcription factor) binds to CCAAT box of ACLY²². It is likely that transcription of ACLY gene is regulated by a combination of SREBPs and NF-Y across species. Several genes, including SREBP-2, farnesyl pyrophosphate synthase, squalene synthase, and HMG-CoA synthase have been shown to be regulated by the same combination of transcription factors²⁸. It has been also suggested that ACLY transcriptional activity during ACLY induction by refeeding low fat/high carbohydrate diet may be regulated by Sp1 and/or the ratio of Sp1/Sp3²².

Viral infections

ACLY gene expression seems to be altered in liver cells presenting an active replication of various types of hepatitis viruses. ACLY gene (along with other genes involved in lipid biosynthesis: fatty acid synthase, SREBP-2, and retinol binding protein 1) has been found to be upregulated in the liver of hepatitis B virus (HBV) transgenic mice that replicate HBV¹². ACLY mRNA was induced 3-fold in Huh-7 cells containing the full-length genotype 1b hepatitis C virus (HCV) replicon, as compared with IFN-cured replicon cells, which were negative for HCV RNA¹⁴.

Implication of ACLY in human pathology and its role as a potential therapeutic target

ACLY role in development

In order to further elucidate the *in vivo* role of ACLY and its expression patterns in tissues, ACLY-deficient mice have been created, in which a β -galactosidase marker was expressed under the control of ACLY regulatory sequences⁴. Homozygous ACLY knockout mice died early in development. It was concluded that ACLY expression in embryos was required

for development and that other enzymes potentially capable of supplying cytosolic acetyl-CoA, such as acetyl-CoA synthetase, were unable to functionally complement ACLY³. Heterozygous mice had half-normal amounts of ACLY mRNA and appeared healthy. ACLY was expressed at high levels throughout embryonic development and β -galactosidase staining revealed that ACLY was expressed at high levels in the neural tube. In the adult brain, ACLY expression was in general much lower, consistent with reduced levels of lipogenesis; however, it remained very high in cholinergic neurons, likely reflecting the importance of ACLY in generating acetyl-CoA for acetylcholine synthesis.

The surprise was that adult heterozygous (ACLY^{+/-}) mice had the same body weight and the same levels of plasma cholesterol, triglycerides and fatty acids as mice normally expressing ACLY (ACLY^{+/+}). Diet manipulations meant to stimulate the activity of ACLY did not result in any differences between ACLY^{+/-} mice and ACLY^{+/+} mice. That means that either ACLY is synthesized in abundant quantities and half-normal levels are not limiting for the downstream reactions, or the activation of the enzyme produced in low quantities rises its activity enough for the metabolic needs³.

Obesity and hyperlipidemia

Since ACLY is the primary enzyme responsible for the production of cytosolic acetyl-CoA, a precursor required for *de novo* biosyntheses of cholesterol and fatty acids, inhibition of ACLY has the potential to reduce cholesterol and triglyceride levels and possibly exert an impact on obesity via reduction of lipogenic factors. Several theoretical and experimental aspects sustain this theory.

The importance of the cholesterol synthesis pathway in controlling plasma lipid levels has been clearly demonstrated with 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) re-

ductase inhibitors, statins. Their hypocholesterolaemic activity results from an increase in hepatic LDL receptors as a consequence of the inhibition of cholesterol synthesis.

ACLY inhibition would theoretically decrease the amount of substrate for acetyl-CoA carboxylase and lower malonyl-CoA levels, the product of this enzyme. Malonyl-CoA negatively regulates the entry of acyl moieties into the mitochondrial β -oxidation spiral (at the level of carnitine-palmitoyl transferase I) and a decrease in malonyl-CoA levels can thus lower the amount of liver free fatty acids available for VLDL synthesis. As VLDL are the precursor for LDL, this is another mechanism of lowering circulating LDL levels, supplementary to the increase in LDL receptor numbers. Experimental evidence sustains this mechanism: inhibitors of fatty acid synthesis like RO-22-0654 have been shown to decrease hepatic VLDL output and plasma triglyceride levels.

Studies on (-)-hydroxycitrate, a natural citrate analogue and potent ACLY inhibitor, have proved its ability to inhibit cholesterol synthesis in Hep G2 cells, increase in HMG-CoA reductase activity and up-regulate the LDL receptor activity in these cells. It is also able to stimulate the oxidation of palmitate and decrease its esterification in pancreatic islets. However, (-)-hydroxycitrate is not the best model of ACLY inhibitor as it poorly penetrates the cellular membrane due to its polarity and is also an allosteric activator of acetyl-CoA carboxylase³⁵.

SB-204990, the γ -lactone prodrug of an ACLY inhibitor caused a dose-dependent decrease in the rates of cholesterol and fatty acid synthesis in Hep G2 cells²⁵.

In another study, a series of (3R*,5S*)- ω -substituted-3-carboxy-3,5-dihydroxyalkanoic acids have been synthesized and evaluated as inhibitors of the recombinant human form of ACLY. A number of these compounds are able to inhibit cholesterol and fatty acid synthesis in HepG2 cells and reduce plasma triglyceride

levels *in vivo*. The best of these is able to induce clear hypocholesterolemic and hypotriglyceridaemic responses when administered orally to rat and dog¹¹.

A novel series of 2-hydroxy-N-arylsulfonamides have been identified as ACLY inhibitors. When administered to mice fed on a high-fat diet, a compound from this class produced an approximate 20%-30% lowering in plasma cholesterol and triglycerides. Chronic administration of this compound showed a gradual inhibition of weight gain along with a reduction in adiposity without apparent changes in food intake¹⁶.

Cancer

Growing cells face a high biosynthetic challenge; at each division they must double their macromolecular mass, including phospholipids, sphingolipids, cholesterol, and lipid moieties for protein modification that are essential for membrane integrity, permeability, and signal transduction³. Transformed cells autonomously activate growth factor signaling pathways involved in this process. The phosphatidylinositol 3'-kinase/protein kinase B (PI3K/Akt) pathway is autonomously upregulated in many cancer cells and stimulates glucose metabolism in excess of bioenergetic demand. This signaling pathway stimulates lipogenic gene transcription through activation of the lipogenic transcription factor SREBP-1 and directly activates lipogenic enzymes such as ACLY³³. In cells that have undergone a glycolytic conversion, i.e., have shifted from an oxidative to a glycolytic mode of metabolism under aerobic conditions, much of the bioenergetic supply is provided through glycolytic ATP production, and glycolytically-derived pyruvate enters a truncated tricarboxylic acids cycle where citrate is preferentially exported to the cytosol via the tricarboxylate transporter¹³. Once in the cytoplasm, citrate is cleaved by the stimulated ACLY, providing a high amount of acetyl-CoA for *de novo* lipid synthesis. This

phenomenon links the upregulation of lipogenesis in cancer cells to the well known tumor-associated increase in glycolysis³³. It has already been shown that glucose-dependent lipogenesis is an important component of cell growth. Experiments with IL-3-dependent hematopoietic cells showed that knocking-down the ACLY led to a significant impairment of glucose-dependent lipogenesis³.

Due to its key position in several metabolic pathways, ACLY is an appealing target for the treatment of malignancy. Stable knockdown of endogenous ACLY levels by shRNA has been found to suppress tumor growth *in vivo* and to induce glandular differentiation in A549 lung adenocarcinoma cells. It also induced differentiation in the K562 chronic myelogenous leukemia cell line *in vitro*. Pharmacologic inhibition of ACLY activity in several cancer cell lines resulted in a dose-dependent inhibition of cell proliferation *in vitro* and tumor growth *in vivo*¹³.

In another study, hematopoietic cells stably expressing an activated form of Akt known to predispose to tumorigenesis, have been injected to athymic mice and produced leukemia³. The same type of cells, but in which ACLY gene had been knocked-down by shRNA, either failed to or needed a longer time to induce leukemia than control cells expressing ACLY.

The ability of ACLY inhibition to suppress tumor growth correlates with the glycolytic phenotype of the tumor. Cancer cells displaying high rates of glucose metabolism are more severely affected, whereas those displaying a low rate of aerobic glycolysis are largely unaffected. An explanation may be the fact that there are alternative salvage lipogenic pathways that tumor cells can use to produce acetyl-CoA independently of ACLY, and cytosolic acetyl-CoA production from acetate via acetyl-CoA synthetase is one of them¹³.

Renal lithiasis

It has been found that ACLY mediates hypocitraturia in rats²⁰. Urinary citrate plays an important role in preventing formation of calcium-containing kidney stones and low urinary citrate occurs in approximately half of patients with renal stone disease. Citrate is freely filtered by the glomerulus and reabsorbed in the renal proximal tubule, the rate of the reabsorption being the major determinant of urinary citrate excretion. Once reabsorbed across the apical membrane, citrate is either transported into mitochondria and metabolized in Krebs cycle or is cleaved by ACLY to oxaloacetate and acetyl-CoA. As kidney tissue homogenates have been found to possess the third highest activity of ACLY after liver and brain, it has been thought that the modulation of ACLY might influence citrate renal excretion. Indeed, specific inhibition of ACLY with 4S-hydroxycitrate in a rat model attenuated hypocitraturia of chronic metabolic acidosis and K⁺ depletion²⁰, two conditions known to be associated with hypocitraturia.

Albeit not a rate-limiting enzyme, ACLY is an interesting metabolic target as it is known that small changes in the rates of enzymes at the beginning of a biochemical pathway may produce significant changes in the rate of production of the end product of a pathway composed of many enzymes²⁷.

Abbreviations

ACLY = ATP citrate lyase

Akt = cAMP-independent protein kinase, protein kinase B

CoA = coenzyme A

GSK-3 = glycogen synthase kinase-3

HBV = hepatitis B virus

HCV = hepatitis C virus

HMG-CoA = 3-hydroxy-3-methylglutaryl-CoA

IL-3 = interleukin 3

LDL = low density lipoproteins

MDH = malate dehydrogenase
 PI3K = phosphatidylinositol 3'-kinase
 PKA = cAMP-dependent protein kinase
 shRNA = short hairpin RNA
 SREBP = sterol regulatory element binding protein
 VLDL = very low density lipoproteins

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