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Acetate as a Metabolic and Epigenetic Modifier of Cancer Therapy

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ABSTRACT

Metabolic networks are significantly altered in neoplastic cells. This altered metabolic program leads to increased glycolysis and lipogenesis and decreased dependence on oxidative phosphorylation and oxygen consumption. Despite their limited mitochondrial respiration, cancer cells, nonetheless, derive sufficient energy from alternative carbon sources and metabolic pathways to maintain cell proliferation. They do so, in part, by utilizing fatty acids, amino acids, ketone bodies, and acetate, in addition to glucose. The alternative pathways used in the metabolism of these carbon sources provide opportunities for therapeutic manipulation. Acetate, in particular, has garnered increased attention in the context of cancer as both an epigenetic regulator of posttranslational protein modification, and as a carbon source for cancer cell biomass accumulation. However, to date, the data have not provided a clear understanding of the precise roles that protein acetylation and acetate oxidation play in carcinogenesis, cancer progression or treatment. This review highlights some of the major issues, discrepancies, and opportunities associated with the manipulation of acetate metabolism and acetylation-based signaling in cancer development and treatment. J. Cell. Biochem. 117: 574–588, 2016. © 2015 Wiley Periodicals, Inc.

KEY WORDS: ACETYL-CoA; ACETYL COENZYME A; ACETYL-CoA SYNTHETASE; ACETYLATION; DEACETYLATION; FATTY ACID; GLYCERYL TRIACETATE; KETOGENIC DIET; KETONE BODY; METABOLISM; REVIEW

For a century it has been debated whether metabolic or genetic alterations were the predominant basis for carcinogenesis since Peyton Rous proposed that caloric restriction could reduce tumor growth and Theodor Boveri suggested that cancer could arise from chromosomal segregation defects during cell division. The metabolic approach to cancer therapy gained support when Otto Warburg noted that blocking both respiration and fermentation killed cancer cells for want of energy and that most cancer cells displayed high rates of glycolysis and lactate production, even in the presence of adequate oxygen (i.e., aerobic glycolysis, Warburg effect) [Koppenol et al., 2011]. Despite Warburg's seminal findings, cancer metabolism research became marginalized by the discoveries of oncogenes and tumor suppressors. That is, until the recent discovery that a single mutation in the gene encoding the mitochondrial enzyme isocitrate dehydrogenase (IDH) is an early transformative event that results in the generation of the oncometabolite 2-hydroxyglutarate. This observation not only has significantly renewed interest in cancer metabolism, it also has focused efforts to develop interventions that

Abbreviations: ACC, acetyl-CoA carboxylase; acetyl-CoA, acetyl coenzyme A; ACLY, ATP-citrate lyase; ACOT, acyl-CoA thioesterase; ACSS1, mitochondrial acyl-CoA short chain synthetase; ACSS2, nuclear-cytoplasmic acyl-CoA short chain synthetase; ALC, acetyl-L-carnitine; ASPA, aspartoacylase; CBP, CREB binding protein; CRAT, carnitine acetyltransferase; EPO, erythropoietin; FASN, fatty acid synthase; GBM, glioblastoma; GSC, glioma stem cell; GTA, glyceryl triacetate; HAT, histone acetyltransferase; HDAC, histone deacetylase; HMG, 3-hydroxy-3-methyl-glutaryl; HIF, hypoxia-inducible factor; IDH, isocitrate dehydrogenase; KAT, lysine acetyltransferase; LCFA, long-chain fatty acid; LDH, lactate dehydrogenase; NAA, N-acetylaspartate; PDH, pyruvate dehydrogenase; SCFA, short-chain fatty acid; SIRT, sirtuin; TCA, tricarboxylic acid cycle.

Protein and gene designations are a work in progress. For the purposes of this review, we will refer to the mitochondrial isoform of acetyl-CoA synthetase by its gene designation, ACSS1, rather than by its enzyme designation, AceCS2. Similarly, we will refer to the nuclear-cytosolic isoform of acetyl-CoA synthetase as ACSS2, rather than the original enzyme designation of AceCS1.

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reverse the metabolic derangements of cancer cells [Galluzzi et al., 2013].

Acetate is uniquely positioned at the intersection of metabolism and genetics in that, as the substrate for acetyl coenzyme A (acetyl-CoA) synthesis, it promotes oxidative phosphorylation via the tricarboxylic acid (TCA) cycle and regulates gene expression epigenetically via acetylation of histone and non-histone proteins. Interest in the role of acetate and acetyl-CoA in carcinogenesis has been recently piqued by several prominent reports that acetate-derived acetyl-CoA fuels cancer growth. Historically, glucose has been viewed as the primary energy source for cancer cells. However, glutamine is an important alternative nutrient source and many cancer cell types exhibit significantly increased glutaminolysis. In fact, proliferating cells undergo apoptosis upon glutamine, but not glucose, deprivation. Cancer cells also readily metabolize fatty acids since they represent the richest energetic nutrient (i.e., provide up to 129 ATP molecules per palmitate molecule). Inasmuch as acetate-mediated acetyl-CoA synthesis is an ATP requiring process and cancer cells have abundant access to alternative energy rich substrates, the role of acetate metabolism in carcinogenesis remains uncertain and warrants review.

This prospective will first provide a foundational review of the cellular sources of acetate and its utilization. We will then present both negative and positive consequences of acetate utilization by tumor cells. Finally, we propose that acetate supplementation may actually serve as an efficacious therapeutic approach to attack cancer on both fronts—metabolism and epigenetics.

SOURCES OF ACETATE

As a potential carbon substrate for cancer growth, interest in the systemic and intracellular sources of acetate has grown in recent years. Acetate is a two-carbon monocarboxylic acid, qualifying it as the shortest chain fatty acid. Acetate is produced in the liver from acetyl-CoA, but is not extensively metabolized there; rather, acetate is released into the circulation similar to the production and release of ketone bodies [Yamashita et al., 2001]. Therefore, hepatic mitochondria are a major systemic source of both acetate and ketone bodies under ketogenic conditions (Fig. 1). Acetate and ethanol are also produced by microbial fermentation in the intestine. Ethanol is then converted to acetate in two enzymatic steps catalyzed by alcohol dehydrogenase (EC 1.1.1.1) and aldehyde dehydrogenase 2 (EC 1.2.1.3). The acetate is either utilized locally within the intestine or released to the general circulation. At destination tissues, acetate is taken up through cell surface monocarboxylate transporters and converted back into acetyl-CoA by acetyl-CoA synthetase enzymes.

All cells can generate acetate intracellularly from a number of sources. Enzymes traditionally referred to as acetyl-CoA hydrolases (EC 3.1.2.1) act to release acetate from existing acetyl-CoA (Fig. 1). At least two acetyl-CoA hydrolase isoforms (i.e., cytoplasmic and mitochondrial) are known. Both forms generate acetate in a subcellular compartment-specific manner. A newer nomenclature has been proposed for acyl-CoA hydrolase enzymes, grouping them with acyl-CoA thioesterases, or ACOTs [Hunt et al., 2005]. ACOTs are critical enzymes involved in mobilizing fat reserves by hydrolyzing fatty acyl-

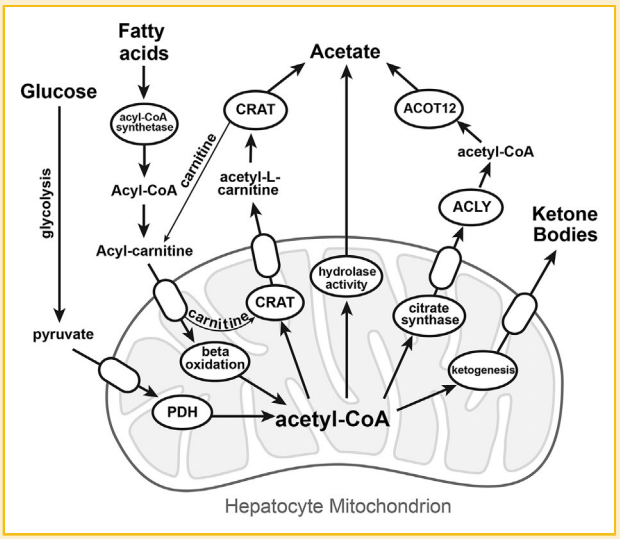

Fig. 1. Schematic of acetate and ketone body production in hepatocyte mitochondria. Fatty acids and glucose are major carbon inputs to mitochondria, and citrate, acetate and ketone bodies are major carbon outputs. The acyl-carnitine system moves fatty acyl groups into the mitochondrial matrix, whereas acetyl-carnitine moves acetate out of the matrix. ACOT12, acyl-CoA thioesterase 12; CRAT, carnitine acetyltransferase; PDH, pyruvate dehydrogenase complex.

CoAs to release fatty acids for β-oxidation. Local acetate production is also the result of intracellular deacetylation reactions mediated by numerous deacetylase enzymes. When N-terminal acetylated proteins are degraded through lysosomal or proteasomal pathways, the resultant N^{α}-acetylated amino acids are subsequently deacetylated by amino-acylase enzymes in order for the acetate and amino acids to be further metabolized. Acetylated metabolites also provide local sources of acetate. For example, acetyl-L-carnitine (ALC) is involved in shuttling acetate groups across the mitochondrial membrane (Fig. 1). Long-chain acyl-carnitines are transported into mitochondria to provide intra-mitochondrial fatty acids for β-oxidation. The acyl-carnitines are converted to acyl-CoA plus L-carnitine, and the acyl-CoA moieties then undergo β-oxidation. L-carnitine can be combined with acetyl-CoA by the reversible enzyme carnitine acetyltransferase (EC 2.3.1.7) to form ALC, which can be transported to the cytoplasm. Once in the cytoplasm, the same enzyme, carnitine acetyltransferase, converts ALC to L-carnitine and acetate. This provides a mechanism for moving acetate from the mitochondrial matrix to the cytoplasm, and would shift the fate of acetate from energy derivation through intramitochondrial oxidation to other uses, including lipogenesis and protein acetylation reactions. Because acetate cannot be utilized directly by most enzymes, acetyl-CoA is essential for the vast majority of biological processes utilizing acetate.

ACETYL COENZYME A

Acetyl-CoA is the universal metabolic carbon source linking energy derivation to virtually all major cellular functions [Pietrocola et al.,

2015]. In eukaryotes, acetyl-CoA, which is produced primarily in mitochondria, functionally links carbon metabolism to the regulation of gene transcription, transcription factor signaling, lipid metabolism, protein quality control and turnover, protein-protein interactions, cytoskeletal reorganization, nuclear import and export, enzyme activity, and a multitude of other cellular processes (Fig. 2). Acetate must first be enzymatically "activated" by coupling to coenzyme A in an ATP-dependent process. The thioester bond formed between the acetyl group and coenzyme A is a high-energy bond. Hence, the transfer of acetate to acetylation targets is energetically favorable.

Altered acetyl-CoA synthesis and utilization is a component of the metabolic reorganization in cancer cells known as the Warburg effect. The bulk of acetyl-CoA is not derived from acetate, but comes from other sources such as glucose, many amino acids including glutamine, and fatty acids (Fig. 3). In nutrient rich and normoxic

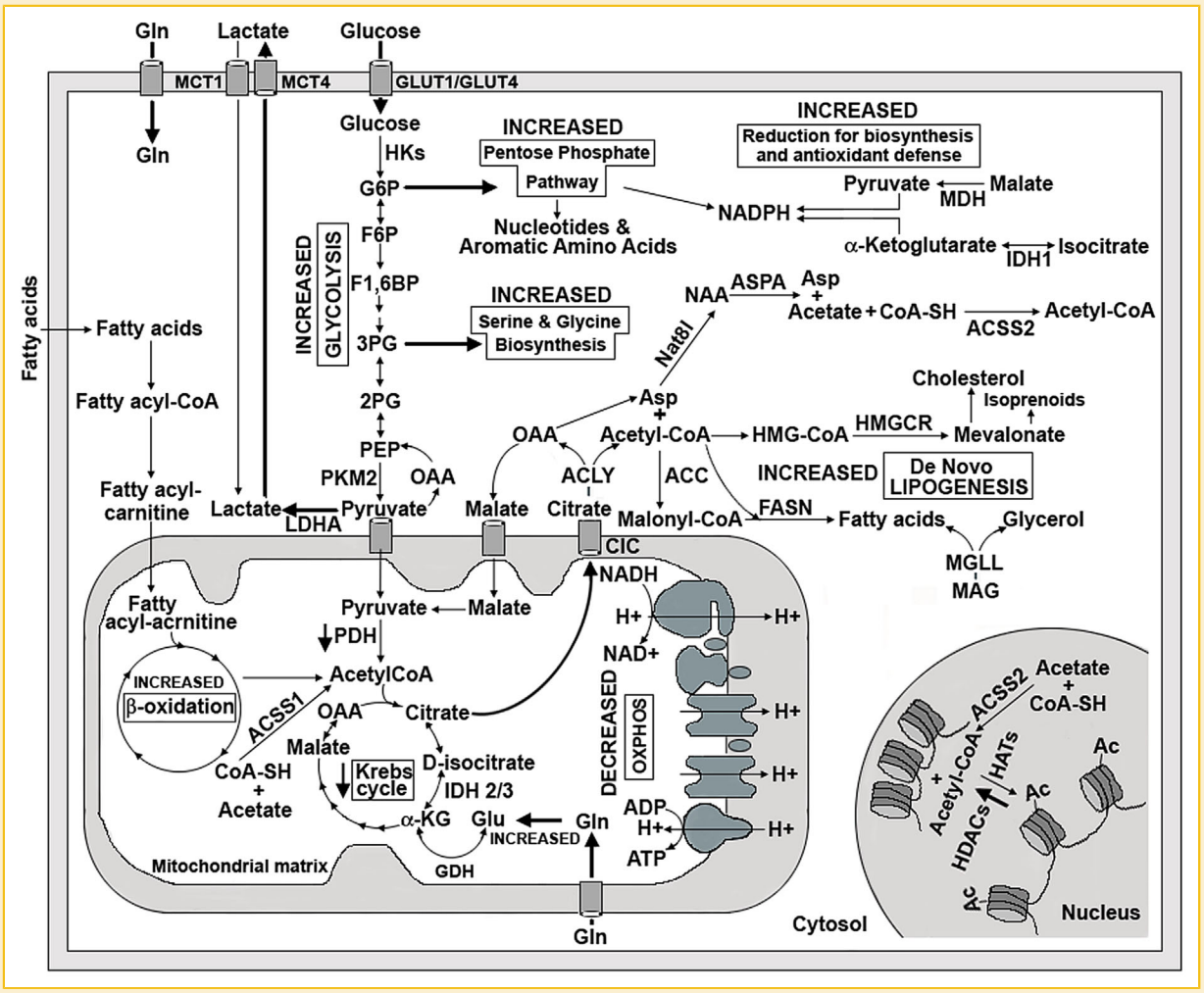

Fig. 2. Metabolic and epigenetic alterations in cancer cells. Cancer cells are typified by increased glucose and glutamine uptake leading to increased glycolysis and biomass accumulation (e.g., amino acid and nucleotide synthesis). Increased lactate dehydrogenase activity results in increased lactate export and extracellular acidification. Mitochondrial acetyl-CoA, primarily produced by β-oxidation, is rapidly converted to citrate and exported to the cytosol to support de novo lipogenesis. Mitochondrial acyl-CoA short chain synthetase (ACSS1) may also contribute to acetyl-CoA synthesis, but this pathway is more active under low nutrient conditions. Cytosolic acetyl-CoA can be derived either from citrate or aspartoacylase (ASPA)-mediated catalysis of N-acetylaspartate (NAA) and subsequent nuclear-cytoplasmic acyl-CoA short chain synthetase (ACSS2) activity. ASCC2 also contributes to nuclear acetyl-CoA synthesis. Cancer cells are characterized by histone hypoacetylation and tightly packed heterochromatin. Increased ACSS2 activity in cancer cells may promote acetylation of histones and transcription factors, thereby regulating expression of genes involved in growth and differentiation. α-KG, α-ketoglutarate; ACC, acetyl-CoA carboxylase; acetyl-CoA, acetyl coenzyme A; ACLY, ATP citrate lyase; ACSS1, mitochondrial acyl-CoA short chain synthetase; ACSS2, nuclear-cytoplasmic acyl-CoA short chain synthetase; Asp, aspartate; ASPA, aspartoacylase; CIC, citrate transporter; CoA-SH, coenzyme A; F1,6BP, fructose-1, 6-bisphosphate; F6P, fructose- 6-phosphate; FASN, fatty acid synthase; G6P, glucose-6-phosphate; GDH, glutamate dehydrogenase; Gln, glutamine; Glu, glutamate; GLUT, glucose transporter; HAT, histone acetyltransferase; HDAC, histone deacetylase; HK, hexokinase; HMG-CoA, 3-hydroxy-3-methyl-glutaryl coenzyme A; HMGCR, HMG-CoA reductase; IDH, isocitrate dehydrogenase; LDHA, lactate dehydrogenase A; MAG, monoacylglycerol; MCT, monocarboxylate transporter; MDH, malate dehydrogenase; MGLL, monoglyceride lipase; NAA, N-acetylaspartate; Nat8l, N-acetyl aspartate synthetase; OAA, oxaloacetate; OXPHOS, oxidative phosphorylation; PDH, pyruvate dehydrogenase complex; PEP, phosphoenolpyruvate; 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; PKM2, pyruvate kinase, muscle, M2 isoform. (adapted from Galluzzi et al., 2013).

conditions, the primary source of acetyl-CoA in most cells, including cancer cells, is glucose, which is converted to pyruvate through glycolysis. The pyruvate is taken up by mitochondria and converted to acetyl-CoA via the pyruvate dehydrogenase (PDH) enzyme complex (Fig. 2). Acetyl-CoA is one of the major carbon sources entering the TCA cycle to be oxidized for the generation of reducing equivalents (NADH, FADH) and driving electron transport. Mitochondrial acetyl-CoA enters the citric acid cycle by combining with oxaloacetate to form citrate through the action of citrate synthase (Fig. 4). Citrate is synthesized in excess of mitochondrial requirements. Because mitochondria do not express acetyl-CoA transporters, citrate provides a ready means for exporting a key carbon source to the cytoplasm. The inner mitochondrial membrane is highly resistant to the movement of charged metabolites such as citrate; therefore, solute movement across this membrane is carrier driven through the mitochondrial citrate carrier, CIC. In the cytoplasm, the enzyme ATP-citrate lyase (ACLY) converts citrate back into acetyl-CoA to be used in fatty acid synthesis or protein and metabolite acetylation reactions. Under hypoxic conditions typical of cancer cells, PDH activity is reduced, which limits oxidative phosphorylation. Instead of being fully oxidized through the TCA cycle to generate reducing power and drive electron transport, pyruvate is converted to lactate via lactate dehydrogenase (LDH) (Fig. 2). Similarly, other substrates entering the TCA cycle in cancer cells such as glutamine, which is imported into cells and converted to glutamate by glutaminase, only make a partial pass through the TCA cycle, and are exported from mitochondria as malate. Malate in the cytoplasm can be converted to pyruvate by malic enzyme, which can then be converted to lactate by LDH. Thus, in cancer cells, acetyl-CoA synthesis from pyruvate and glutamine are reduced, and lactate synthesis is increased (Fig. 2). Acetyl-CoA synthesis from other sources may be increased to compensate.

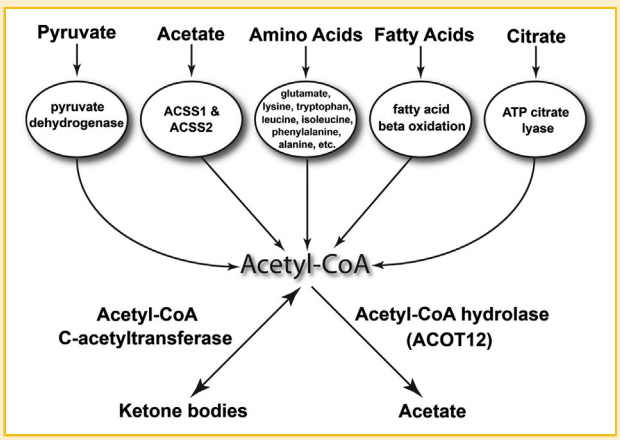

Fig. 3. Schematic of major acetyl-CoA sources. Acetyl-CoA is at the hub of the carbon metabolism network, providing a universal currency for energy derivation, utilization and storage. Ketone bodies reversibly exchange with acetyl-CoA depending upon the activity of the enzyme acetyl-CoA C-acetyltransferase. Acetyl-CoA production from pyruvate and amino acids is reduced in cancer cells, and as a result, acetyl-CoA production from other sources may be increased.

ACYL-CoA SYNTHETASES

Acetyl-CoA is synthesized from a number of substrates in addition to intramitochondrial pyruvate and cytoplasmic citrate. These include fatty acids via β-oxidation, a number of amino acids, ketone bodies, and acetate (Fig. 3). Fatty acids, including short-chain fatty acids (SCFAs) like butyrate, propionate, and acetate are processed enzymatically as variable length acyl (hydrocarbon) chains. They must be activated by using ATP to create a high-energy thioester bond via enzymes known as acyl-coenzyme A synthetases, of which at least 26 family members are known [Watkins et al., 2007]. Most of these enzymes act on fatty acids of various chain lengths and types to generate fatty acyl-CoA products that can be used for lipid synthesis or β-oxidation.

Acyl-CoA short-chain synthetases have a high specificity for acetate, and much lower affinity for longer chain length substrates. These enzymes have been known in much of the previous literature as acetyl-CoA synthetases (AceCS, EC 6.2.1.1) since they preferentially convert acetate and coenzyme A into acetyl-CoA. Unfortunately, the enzyme names were not taken into consideration when gene designations were assigned, resulting in mismatched nomenclature (i.e., AceCS1 is encoded by gene *ACSS2*, and AceCS2 is encoded by gene *ACSS1*) (see Table I). Recent reports have utilized the ACSS terminology to refer to the enzymes. To avoid confusion in the literature, we will use the ACSS designations, which are non-italicized for the enzymes and italicized for the genes (e.g., ACSS1 and *ACSS1*, respectively). There are currently three known ACSS enzyme isoforms encoded by the genes *ACSS1*,*ACSS2*, and *ACSS3* [Watkins et al., 2007]. The enzyme encoded by the *ACSS3* gene is a poorly characterized isoform that is thought to be mitochondrial. The other two isoforms have been more thoroughly characterized, and are differentially expressed both in terms of subcellular localization and tissue distribution.

ACSS1 is a mitochondrial matrix enzyme abundantly expressed in heart, skeletal muscle and brown adipose tissue where it primarily generates acetyl-CoA for energy derivation (Figs. 2 and 4) [Fujino et al., 2001]. In brown adipose tissue, ACSS1 is involved in non-shivering thermogenesis under ketogenic conditions. In fasted *Acss1*-deficient mice there was a 50% reduction in skeletal muscle ATP, and the mice were hypothermic [Sakakibara et al., 2009]. The mice were also hypothermic when maintained on a low-carbohydrate diet. These findings highlight the link between ACSS1 and thermogenesis under ketogenic conditions and show that acetate is a vital energy-deriving metabolite when carbohydrate availability is low, for example during periods of malnourishment or starvation. ACSS1 may contribute to the growth of a subset of cancer cells, but the roles this enzyme plays in cell proliferation and carcinogenesis are not well understood. ACSS1 was highly expressed in cancer cells with low glycolysis, and was well correlated with ^{11}C-acetate uptake [Yun et al., 2009]. *ACSS1* silencing in these cells significantly decreased ^{11}C-acetate uptake and cell viability, suggesting ACSS1 inhibition as a therapeutic approach. However, most cancer cells proliferate in a nutrient-rich environment characterized by high glycolytic rates and negligible ACSS1 expression. So ACSS1 may only become an important energy derivation pathway in some cells when glucose availability is low, as with normal cells during fasting

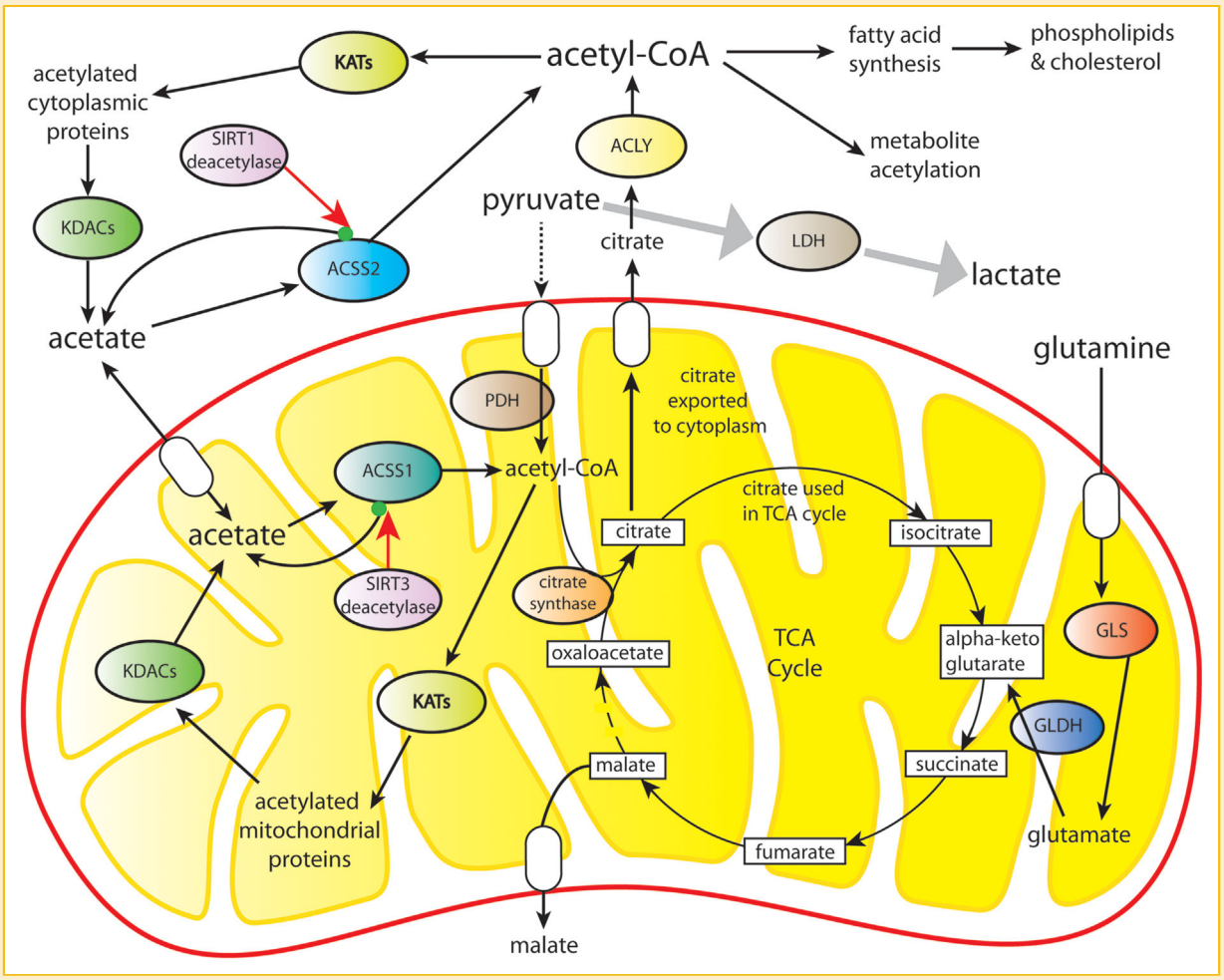

Fig. 4. Diagram highlighting some of the relationships between acetate and acetyl-CoA metabolism and the TCA cycle in mitochondria. Acetyl-CoA production in cancer cells from pyruvate and glutamine is reduced, and lactate and malate production are increased. Acetate utilization in mitochondria and cytoplasm is controlled by the acetylation status of ACSS enzymes. Acetylation of ACSS enzymes inactivates them, whereas deacetylation by SIRT enzymes activates them. ACLY, ATP citrate lyase; ACSS1, mitochondrial acyl-CoA short chain synthetase; ACSS2, nuclear-cytoplasmic acyl-CoA short chain synthetase; GLDH, glutamate dehydrogenase; GLS, glutaminase; KATs, lysine acetyltransferases; KDACs, lysine deacetylases; LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase complex; SIRT1, sirtuin 1; SIRT3, sirtuin 3.

or starvation. But ACSS1 would not contribute significantly to cancer cell energy when glucose was abundant.

ACSS1 expression in the brain is low, suggesting that it has a limited capacity to utilize acetate for energy derivation [Fujino et al., 2001]. Enzyme assays confirmed that total ACSS activity was lowest in the mitochondrial subcellular fraction. Immunohistochemistry revealed that ACSS1 expression was associated with the main glial support cell of the brain, astrocytes [Moffett et al., 2013]. Astrocytes have processes that contact vascular endothelial cells and oligodendrocytes, the myelin producing cells associated with neurons. This places astrocytes in a position to take up blood-borne acetate or acetate released from oligodendrocytes for use in the TCA cycle. It is well documented that acetate applied to the brain is avidly taken up and metabolized by astrocytes for energy [Rae et al., 2012]. However, the role of ACSS1 in astrocyte-derived cancers (i.e., astrocytomas) has not been extensively investigated.

ACSS2 is the most broadly distributed ACSS enzyme isoform. ACSS2 is highly expressed in the liver, kidney and heart, and moderately expressed in the brain, testes and other tissues [Luong et al., 2000]. It also exhibits a dual subcellular localization in the nucleus and cytoplasm (Fig. 2) [Wellen et al., 2009; Ariyannur et al., 2010]. In the developing rat brain, ACSS2 expression was observed in the cytoplasm and nucleus of most cells, but the nuclear staining was predominant. In the adult rat brain, ACSS2 expression was almost exclusively nuclear. The expression in neuronal and glial cell nuclei was substantially increased after experimental brain injury [Ariyannur et al., 2010]. The nuclear localization of ACSS2 may play a role in the generation of acetyl-CoA for histone [Wellen et al., 2009] or transcription factor [Xu et al., 2014] acetylation (discussed in detail below). ACSS2 is most recognized for its role in lipid synthesis (Fig. 2), and this is the primary role ascribed to the enzyme in the context of cancer. Sterol regulatory element-binding proteins, transcription factors that regulate cholesterol and unsaturated fatty acid synthesis, induce ACSS2 expression [Luong et al., 2000], probably as a mechanism to recycle acetate released by cytoplasmic deacetylation reactions. However, as will be discussed in more detail

TABLE I. The Acetyl-CoA Synthetases are Members of the Acyl-CoA Synthetase, Short Chain Family of Enzymes that Activate Fatty Acids by Combining Them With Coenzyme A Utilizing ATP to Generate a High-Energy Thioester Bond

Enzyme name	Acronym	Gene	Protein localization	Acetate fate
Acyl-CoA short chain synthetase-1 (also called acetyl-CoA synthetase-1)	AceCS1	*Acss2*	Cytoplasm and nucleus	Protein and metabolite acetylation, lipid synthesis
Acyl-CoA short chain synthetase-2 (also called acetyl-CoA synthetase-2)	AceCS2	*Acss1*	Mitochondria	Oxidation in TCA cycle for energy derivation
Acyl-CoA short chain synthetase-3		*Acss3*	Mitochondria	Uncertain

below, the role of ACSS2 in protein acetylation may be as or more important in cancer cell proliferation than fatty acid synthesis.

The contribution of ACSS2 in carcinogenesis has been highlighted by recent studies suggesting that several cancer cell lines utilize cytosolic ACSS2-mediated acetate metabolism to fuel cancer growth. Expression of cytosolic ACSS2 is up-regulated in multiple cancers and is involved in the incorporation of [1-^{14}C] acetate into acetyl-CoA [Yoshii et al., 2009b]. This observation led to the use of [^{11}C]-acetate positron emission tomography as a non-invasive cancer diagnostic tool. *ACSS2* inhibition in cancer cells enhanced cell death under long-term hypoxia in vitro [Yoshii et al., 2009a] and *Acss2*-deficient mice exhibited significant reduction in tumor growth and burden [Comerford et al., 2014]. Under metabolic stress (i.e., hypoxia and low serum), ACSS2 promoted the biosynthesis of fatty acids and membrane phospholipids and increased proliferation [Schug et al., 2015]. Taken together, these data suggest that cytosolic ACSS2-mediated acetate metabolism promotes carcinogenesis by promoting lipogenesis.

ACETATE METABOLISM MAY PROMOTE LIPOGENESIS AND CARCINOGENESIS

Lipogenesis, which provides the plasma membrane components required to support biomass expansion necessary to sustain proliferation, is increased in cancer cells [Baenke et al., 2013]. Most normal cells take up free fatty acids and lipoproteins from the bloodstream to support lipogenesis. In contrast, cancer cells preferentially use de novo fatty acid synthesis even though it, like aerobic glycolysis, is energetically expensive. De novo fatty acid synthesis not only redirects acetyl-CoA from oxidative phosphorylation and ATP production, but also requires expenditure of ATP and NADPH. Nonetheless, cancer cells divert acetyl-CoA from the TCA cycle to support cholesterol and fatty acid synthesis (Fig. 2).

Cholesterol is synthesized from acetyl-CoA via the mevalonate pathway. Two acetyl-CoAs undergo condensation to form acetoacetyl-CoA by the enzyme thiolase. Another acetyl-CoA condenses with acetoacetyl-CoA to form 3-hydroxy-3-methyl-glutaryl (HMG)-CoA by the enzyme HMG-CoA synthase. In the cytosol, NADPH reduces HMG-CoA to mevalonate by the enzyme HMG-CoA reductase. Finally, mevalonate is converted to cholesterol and cholesterol esters through the coordinated action of numerous enzymes. Inasmuch as HMG-CoA reductase represents the rate-limiting step for cholesterol synthesis, the cholesterol-depleting drugs known as statins, which act by inhibiting HMG-CoA reductase, have been tested as cancer therapies [Matusewicz et al., 2015]. Statins have also been tested as cancer preventative agents, but results suggest that statins exert a greater effect on cancer progression than initiation.

Under normoxia, fatty acid synthesis is initiated by the generation of acetyl-CoA from cytoplasmic citrate by the enzyme ACLY (Figs. 2 and 4). Acetyl-CoA is then converted to malonyl-CoA by the enzyme acetyl-CoA carboxylase (ACC) (Fig. 2). Acetyl-CoA and malonyl-CoA are then coupled by the multifunctional enzyme complex fatty acid synthase (FASN). FASN catalyzes the ligation and reduction of 8-acetyl units to generate palmitate, the first long-chain fatty acid (LCFA) generated through de novo synthesis. Triglycerides and phosphoglycerides are subsequently generated from fatty acids via the glycerol phosphate pathway, which uses the glycolytic intermediate glycerol-3-phosphate to form the glycerol backbone of these lipids. ACLY, ACC, and FASN are overexpressed in cancer and increased glycolysis in cancer cells provides abundant glycerol-3-phosphate; collectively, increasing lipogenesis in and proliferation of cancer cells (Fig. 2). Hence, these enzymes serve as therapeutic targets. Numerous ACLY inhibitors have been developed; unfortunately, reduced cell permeability of many of these compounds limits their in vivo use [Zu et al., 2012]. Metformin, a drug used in the treatment of type-2 diabetes, reduces malonyl-CoA levels and lipogenesis via 5′ adenosine monophosphate-activated protein kinase-mediated phosphorylation of ACC [He et al., 2015]. Similar to statins, metformin exerts a greater effect on cancer progression than initiation. Since FASN is increased in cancer cells, but relatively low in normal cells, inhibiting FASN has garnered the most attention [Flavin et al., 2010]. Several compounds are known to inhibit FASN, including cerulenin, C75, orlistat, C93, and several natural plant-derived polyphenols. One of the best-characterized polyphenol FASN inhibitors is epigallocatechin-3-gallate, a natural component of green tea.

The origin of fatty acid carbons differs under hypoxic conditions. Under normoxia, up to 25% of fatty acid carbons are derived from glutamine, while up to 80% are glutamine-derived under hypoxia. Reductive carboxylation of glutamine-derived α-ketoglutarate mediated by IDH enzymes contributes to acetyl-CoA synthesis for lipogenesis [Metallo et al., 2011]. However, further analysis demonstrated that fatty acid labeling from glutamine could be explained by isotope exchange without net reductive IDH flux [Fan et al., 2013]. Under hypoxia, it has been reported that up to 50% of the carbons in fatty acids are not derived either from glucose or glutamine, but are derived from acetate, suggesting that acetate represents a major previously unrecognized carbon donor to fatty acids under hypoxia [Kamphorst et al., 2014]. In contrast, another

study reported that, in breast cancer cells, anoxia increased glycolytic activities 2–3 fold compared to normoxia, yet there was no concomitant increase in ^{14}C-acetate incorporation into total lipids, nor a change in lipid composition [Hopperton et al., 2014]. Further, these investigators found that cancer cells excreted endogenously synthesized fatty acids. Taken together, the results suggest that cancer cells may synthesize fatty acids in excess of their requirements, and that increased de novo lipogenesis is not required to maintain increased glycolytic activity. Therefore, even though FASN expression is increased in many cancer cells, it does not automatically lead to enhanced lipogenesis. Importantly, ACSS2 utilization of acetate for fatty acid synthesis would be minimal when glucose was available as a carbon source.

BENEFICIAL EFFECTS OF ACETATE—FATTY ACIDS AS CANCER THERAPEUTIC AGENTS

Although lipogenesis is primarily thought to promote biomass expansion and cancer cell growth, a subset of lipids including certain fatty acids exert well-accepted anti-neoplastic effects. Fatty acids are monocarboxylic acids categorized based on the number of carbon atoms in the aliphatic chain, including short-chain (<C8:0), medium-chain (C8-14:0) and long-chain (>C16:ω3-9), and on their degree of unsaturation. SCFAs are of particular significance in cancer therapy. SFCAs, including acetate (C2:0), propionate (C3:0) and butyrate (C4:0), are produced by bacteria through anaerobic fermentation of dietary fiber in the intestine, some of which is used in the colon, with the remainder released to the bloodstream. β-Oxidation of fatty acids in the liver leads to a significant degree of acetogenesis, with most of the acetate being released to the general circulation. Fatty acids exert diverse biological functions in addition to serving as an energy source [Baenke et al., 2013].

Fatty acids are efficacious cancer therapeutic agents. The SCFAs acetate, propionate and butyrate induce high rates of apoptosis, presumably via epigenetic modifications that promote differentiation, cell cycle arrest and activation of pro-apoptotic genes. Longer unsaturated fatty acids also exhibit anti-neoplastic effects, although via different mechanisms, including induction of oxidative stress, mitochondrial membrane permeabilization, and activation of the caspase cell death cascade. Both fatty acid chain length and the degree of unsaturation contribute to the extent of toxicity (i.e., longer chained and more unsaturated fatty acids are more toxic to cancer cells).

The 4-carbon SFCA butyrate, not to be confused with the ketone body β-hydroxybutyrate, is the best-characterized fatty acid used as a cancer therapy. In the 1980s, sodium butyrate was found to potentiate ionizing radiation-induced cell death. Butyrate is thought to exert its anti-proliferative effects primarily by inhibiting histone deacetylase (HDAC) activity [Davie, 2003]. In fact, butyrate is the smallest known HDAC inhibitor. Although not as potent as butyrate, other SCFAs (e.g., valproate and 4-phenylbutyrate) also have HDAC inhibitory activity. HDAC inhibition leads to hyperacetylation of core histone proteins H3 and H4 and transcriptional regulation of genes inducing growth inhibition, differentiation, and apoptosis. HDAC inhibitors not only modulate the acetylation state of histones, but also non-histone proteins. Butyrate treatment increases expression of the cell cycle inhibitors p21 and p27, and decreases cyclin B1 expression leading to growth inhibition. Cell cycle arrest, which occurs both in the G_0/G_1 and G_2/M phases in a cell type-dependent manner, leads to apoptosis. In addition, butyrate inhibits cellular migration and metastasis, suggesting interference with cell adhesion molecules and/or proteolytic enzymes. Butyrate also leads to increased reactive oxygen species that, at high levels, can damage organelles, particularly mitochondria. Tributyrin, a pro-drug of butyrate, suppresses the production of pro-inflammatory cytokines. Thus, butyrate exerts pleiotropic anti-neoplastic effects.

Acetate, the smallest SCFA, also exerts anti-proliferative effects. Physiological concentrations of sodium acetate induced apoptosis of colorectal carcinoma cells by inducing DNA fragmentation, caspase activation, lysosomal membrane permeabilization, and the appearance of a sub-G_1 population [Marques et al., 2013]. Similarly, we recently demonstrated that sodium acetate induced a dose-dependent growth arrest of glioma stem cells (GSCs) in vitro by an as yet undetermined mechanism [Long et al., 2013b, 2015].

Interestingly, the anti-proliferative effects of butyrate are dependent on the cell's metabolic state. Butyrate stimulated proliferation of normal and cancerous colonocytes when the Warburg effect is prevented, but inhibited cancerous colonocyte proliferation undergoing the Warburg effect [Donohoe et al., 2012]. Similarly, we showed that acetate supplementation was most effective at growth arrest of GSCs with a more glycolytic metabolism [Long et al., 2013b, 2015]. Collectively, these data support the use of acetate-derived fatty acids as a therapeutic approach in a wide variety of tumor types and confirm a relationship between metabolism and epigenetics mediated by protein acetylation/deacetylation.

ACETATE METABOLISM CONTRIBUTES TO PROTEIN ACETYLATION

Acetylation is a ubiquitous protein modification that requires acetyl-CoA for almost all acetylation reactions. Two types of protein acetylation can be distinguished. N^{α}-terminal acetylation, mediated by N-terminal acetyltransferase enzymes, occurs co-translationally and involves amino acids at the N-termini of proteins. N-terminal acetylation, which occurs on approximately 85% of eukaryotic proteins, protects proteins from proteases, thereby increasing protein stability and facilitating protein-protein interactions [Persson et al., 1985]. Because lysine N^{ε}-acetylation is reversible, it may be of greater functional significance in cancer cells than N^{α}-terminal acetylation. Lysine N^{ε}-acetylation is mediated by lysine acetyltransferase enzymes (KATs) and occurs post-translationally at any exposed lysine in a protein. N^{ε}-acetylated proteins are abundantly expressed in all subcellular compartments [Choudhary et al., 2009]. A subset of nuclear lysine acetyltransferases, referred to as histone acetyltransferases (HATs), is recruited to the transcriptional machinery to acetylate histone and non-histone proteins, including transcription factors, structural proteins and proteins involved in signal transduction [Singh et al., 2010]. Because acetyl-CoA availability profoundly influences acetyltransferase catalytic activity, subtle differences in acetate levels may significantly impact

protein acetylation; therefore, acetyltransferase activity must be tightly regulated.

Protein deacetylation is mediated by HDACs, more generally known as KDACs, which target histone and non-histone proteins, and other deacetylase enzymes. In the human genome, eighteen HDAC family members, grouped into four classes, have been identified. Class I HDACs (i.e., 1, 2, 3, and 8) are mainly located in the nucleus. Class II HDACs (i.e., 4–7 and 9–10) are primarily localized in the cytoplasm; however, depending upon the phosphorylation status they can be shuttled between the cytoplasm and nucleus. HDAC11 is the only member of class IV family and is localized in the nucleus. Aberrant HDAC activity has been documented in several types of cancers and HDAC inhibitors have been utilized as therapeutic approaches [West and Johnstone, 2014]. Whereas class I, II, and IV HDACs are Zn^{2+}-dependent metalloproteins, class III HDACs are NAD^{+}-dependent enzymes known as sirtuins (SIRTs 1–7) [Feldman et al., 2012]. Although SIRTs are located in the nucleus, cytoplasm and mitochondrion, the mitochondrial expression is of particular relevance to cancer (Fig. 4). Mitochondrial protein expression levels are only modestly changed in response to caloric restriction, while mitochondrial protein acetylation profiles change dramatically, indicating that protein acetylation plays an important role in adjusting metabolism to caloric intake. SIRT3 serves as the major protein deacetylase within the mitochondrial matrix where it increases LCFA oxidation and energy derivation during fasting by deacetylating and activating long-chain acyl-CoA dehydrogenase [Hirschey et al., 2011]. SIRT3 also increases oxidative phosphorylation by deacetylating complex I of the electron transport chain. SIRT3-mediated deacetylation of other key mitochondrial enzymes, such as IDH2 and HMG-CoA synthase, act to increase energy derivation from fatty acids, ketone bodies and acetate [Martinez-Pastor and Mostoslavsky, 2012]. Most importantly, the activities of ACSS1 and ACSS2, the primary acetate activating enzymes, are regulated by sirtuins. Acetylation inactivates ACSS enzymes, whereas deacetylation activates them. SIRT1 deacetylates nuclear-cytoplasmic ACSS2, while SIRT3 deacetylates mitochondrial ACSS1 (Fig. 4). Interestingly, SIRT1 and SIRT3 act as tumor suppressor genes; SIRT1 contributes to the repair of double- and single-strand DNA breaks, while SIRT3 represses the Warburg effect. Therefore, sirtuin-mediated deacetylation, in conjunction with ACSS-mediated acetyl-CoA synthesis, increases the conversion of acetate to acetyl-CoA in all cellular compartments.

PROTEIN ACETYLATION AND CARCINOGENESIS

N^{ε}-acetylation can both positively and negatively regulate cancer cell growth and survival. Acetyl-CoA functions as a nutrient rheostat to control cell cycle entry. Elevated acetyl-CoA levels promote histone acetylation of more than 1,000 growth genes and enhance cell proliferation [Cai et al., 2011]. For example, in the presence of high glucose, ACLY becomes acetylated at lysine residues (designated K, the amino acid symbol for lysine) K^{540}, K^{546}, and K^{554}, resulting in inhibition of ubiquitylation-dependent ACLY degradation, and enhanced lipogenesis, cell proliferation and tumor growth [Lin et al., 2013]. However, other acetylation reactions negatively affect cell growth. Under stressed conditions, acetylation of the tumor suppressor p53 is increased, leading to its activation. Acetylation of p53 at K^{373} by p300/CREB binding protein (CBP) induces apoptosis, whereas acetylation at K^{320} by p300/CBP associated factor promotes cell cycle arrest [Marouco et al., 2013]. Acetylation of p53 is also controlled at the deacetylation level by HDAC-1, HDAC-3, and SIRT1.

Protein acetylation also plays a role in the regulation of autophagy, a lysosomal-mediated recycling of defective cellular organelles to make use of their constituents in the presence of limited external resources. Cytosolic accumulation of the acetyl-CoA precursor acetate led to hyperactivation of nuclear-cytosolic ACSS2, increased histone acetylation, and p300-mediated repression of autophagy-associated proteins; culminating in a reduced lifespan [Eisenberg et al., 2014; Mariño et al., 2014].

Virtually every metabolic enzyme is regulated by acetylation and deacetylation [Zhao et al., 2010]. Enzymes that participate in intermediate metabolism are preferentially acetylated. For example, LDH-A acetylation at K^{5} reduces LDH activity, promotes lysosomal degradation, and impairs pancreatic cancer cell proliferation [Zhao et al., 2013]. A recent study using ^{13}C-nuclear magnetic resonance analysis showed that acetate metabolism in mitochondria was increased in human glioblastoma tumors and brain metastases [Mashimo et al., 2014]. This study also indicated that mitochondrial acetate metabolism was correlated with ACSS2 levels. However, it remains unclear how the nuclear-cytoplasmic enzyme ACSS2 contributes to mitochondrial acetate metabolism because ACSS2 generates acetyl-CoA in the cytoplasm and nuclei of cells, rather than within mitochondria. Acetyl-CoA is impermeable to the mitochondrial membrane, and therefore it seems unlikely that cytoplasmic acetyl-CoA could contribute significantly to energy derivation in mitochondria. It is more likely that ACSS2 is involved in the regulation of metabolism in cancer cells by acetylation of key enzymes and transcription factors.

A large proportion of acetylated proteins are either localized to the nucleus or shuttle between the cytosol and nucleus [Choudhary et al., 2009]. The nuclear-cytosolic localization of ACSS2 becomes important in this context. As with ACSS1, the function of ACSS2 is tied to caloric intake. When nutrients are limiting or absent, ACSS2 recycling of acetate by conversion to acetyl-CoA, which requires ATP, becomes more critical. The cell must sacrifice existing ATP to reclaim carbon in the form of acetate. This reaction is not unlike the action of ACLY, an ATP requiring enzymatic process that generates acetyl-CoA from citrate. Under hypoxia, ACSS2 catalyzes the reverse reaction of acetyl-CoA to acetate to allow cancer cells to excrete excess acetate and buffer the intracellular acetyl-CoA pool [Yoshii et al., 2009a]. Hence, judicious regulation of acetylation and deacetylation reactions permits eukaryotic cells to differentially utilize nutrients in a hierarchical manner (e.g., energy > acetylation > lipogenesis) to best fit their physiological requirements.

ACETATE AS A SIGNALING AGENT

Acetate is more than just a substrate for acetyl-CoA generation; it also acts through ACSS2 as a signaling agent. One acetate-mediated

signaling pathway of particular relevance for carcinogenesis is the induction of erythropoietin (EPO) transcription in response to hypoxia. EPO, a hormone produced in the liver and kidneys in response to hypoxia, stimulates red blood cell production. Experimentally induced anemia increases acetate levels in the kidneys and liver of mice that leads to hypoxia-inducible factor-2α (HIF-2α) acetylation in wild-type mice, but not in *Acss2*-deficient mice, indicating a requirement for ACSS2 in HIF-2α acetylation [Xu et al., 2014]. Other SCFAs did not induce HIF-2α acetylation. Acetylation required the CBP acetyltransferase, which forms a complex with HIF-2α as part of the active transcription factor (Fig. 5). Formation of the HIF-2α/CBP transcription factor complex led to recruitment to the EPO enhancer, induction of EPO transcription, and increased EPO expression. Complex recruitment and increased EPO expression were dependent upon ACSS2-mediated acetylation of HIF-2α. The source of the intracellular acetate resulting from hypoxia is not known. Glucose deprivation also triggers ACSS2-HIF-2α complex formation [Chen et al., 2015]. In HT1080 cells, hypoxia or glucose deprivation led specifically to ACSS2- and CBP-dependent HIF-2α acetylation. Moreover, ACSS2 was found to rapidly translocate to the cell nucleus within 2 h of hypoxia onset [Xu et al., 2014; Chen et al., 2015]. These findings demonstrate that acetate release and/or generation occurs under conditions common in cancer (e.g., hypoxia and glucose deprivation) and that the acetate is utilized by ACSS2 to acetylate HIF-2α via CPB and facilitate transcription factor complex formation and translocation to the nucleus. SIRT1 acts to deacetylate HIF-2α providing a means for terminating EPO transcription and allowing for the cycle to repeat. Taken together, these findings implicate acetate mobilization by unknown factors, and acetate utilization by ACSS2 in transcription factor acetylation and activation in the stress response to the lowered oxygen and glucose levels associated with cancer.

BENEFICIAL EFFECTS OF ACETATE—THE KETOGENIC DIET AS A CANCER THERAPY

Attempts to starve cancer cells via glucose deprivation have proven unsuccessful because, when glucose deprived, cancer cells simply utilize alternative fuel sources to support anabolic metabolism and proliferation. Fasting not only decreases glucose levels, but also increases ketone body levels in the blood. In contrast to most normal cells, cancer cells are incapable of ketone metabolism, which relies on intact mitochondrial function. Thus, consumption of a glucose-restricted, ketone-rich diet could support normal cell functions while constraining cancer cell growth [Maurer et al., 2011].

The ketone bodies acetoacetate and β-hydroxybutyrate are synthesized in the liver from acetyl-CoA generated by β-oxidation of fatty acids when acetyl-CoA levels exceed the TCA cycle's utilization capacity. The ketogenic diet, a high fat and low carbohydrate/protein diet, mimics fasting and significantly raises serum β-hydroxybutyrate and acetoacetate concentrations. The

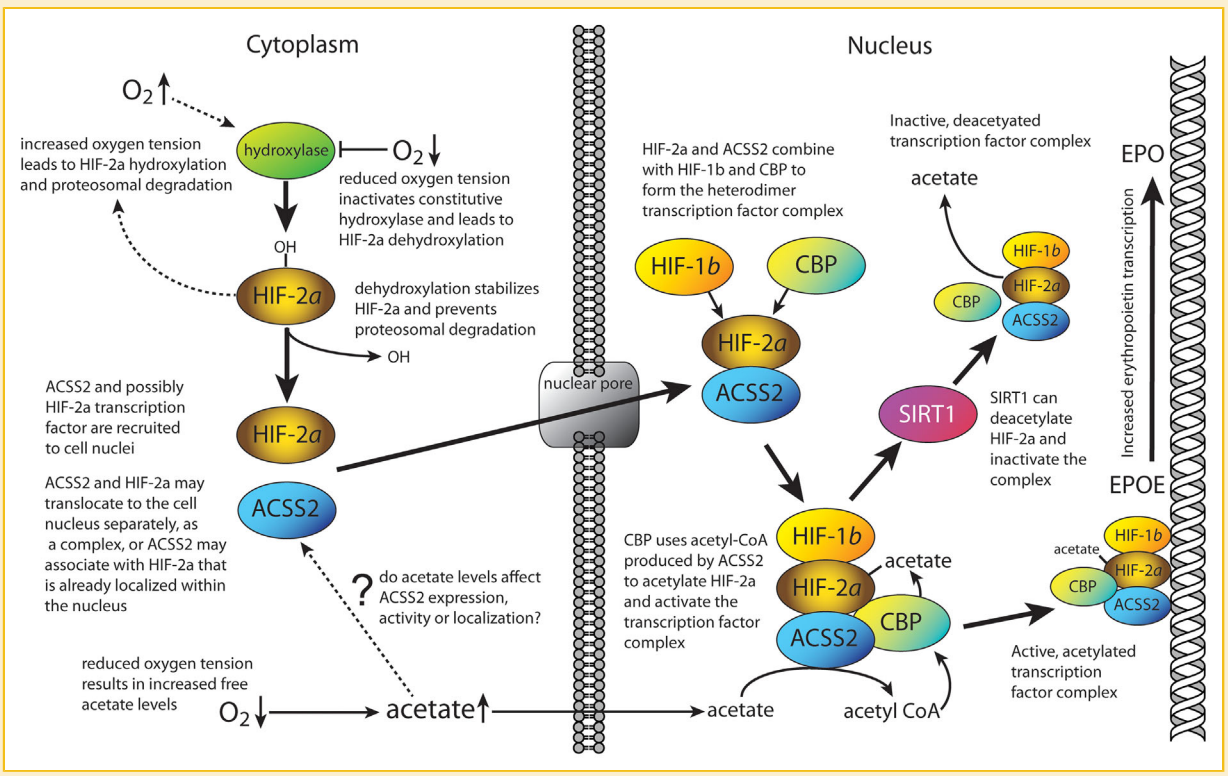

Fig. 5. Schematic of proposed interactions in the regulation of the HIF-2α signaling pathway of hypoxic stress response and the roles played by acetate and protein acetylation. See text for additional details (adapted from Chen et al., 2015).

ketogenic diet is regarded as safe because ketone body levels are self-limiting since excess ketone bodies are excreted in the urine.

Ketone body catabolism is essentially a reversal of the synthetic pathway, with one difference. β-hydroxybutyrate is first converted to acetoacetate by the enzyme 3-hydroxybutyrate dehydrogenase. The second step in ketone body utilization is the major difference from ketone body synthesis. Acetoacetate is converted to acetoacetyl-CoA via β-ketoacyl-CoA transferase. Finally, acetoacetyl-CoA generates acetyl-CoA by reversing the enzymatic reaction of acetyl-CoA C-acetyltransferase (Fig. 3). Thus, similar to fatty acid β-oxidation, ketone body metabolism generates the biologically active acetyl-CoA precursors. In contrast to glucose, ketone bodies bypass glycolysis and enter the citric acid cycle directly as acetyl-CoA, thereby sparing cytosolic NAD that is normally consumed during glycolysis. The ketogenic diet is distinct from caloric restriction in that, when administered in unrestricted amounts, the ketogenic diet does not cause decreased serum glucose or weight loss [Seyfried et al., 2003], suggesting that the ketogenic diet is not merely promoting a global state of starvation.

Caloric restriction and the ketogenic diet have shown efficacy as anti-cancer approaches, but the underlying mechanisms are distinct. The anti-proliferative effects of caloric restriction are primarily due to decreased plasma glucose, whereas ketogenic diet therapy effectiveness is most correlated with the degree of increased ketone levels. Multiple mechanisms of action underlie the anti-proliferative effects of ketone bodies, including inhibition of glycolysis, enhanced antioxidant defenses in normal, but not cancer cells, and import of ketone bodies and export of lactate through monocarboxylate transporters [Poff et al., 2014]. Of relevance for the treatment of brain tumors, the ketogenic diet is neuroprotective and lowers brain glucose uptake, even in the presence of normal circulating glucose [Maalouf et al., 2009]. The efficacy of the ketogenic diet may also be ascribed to the accumulation of free, non-esterified LCFAs that limit the availability of glycerol-3-phosphate and insulin required for the esterification of fatty acids into down-stream lipid products. Also, as described earlier, LCFAs exert direct anti-neoplastic effects. Similar to SCFAs, β-hydroxybutyrate inhibits histone deacetylation [Shimazu et al., 2013]. Notably, the ketogenic diet shifts the overall gene expression in tumor tissue to a pattern seen in normal brain [Stafford et al., 2010; Woolf et al., 2015]. Hence, similar to SCFAs, ketone bodies can exert their anti-neoplastic effects, at least in part, via altering metabolism and the epigenome of cancer cells.

The ketogenic diet has long been used to treat refractory pediatric epilepsy. It is well tolerated, can be administered chronically, and is associated with only minor side effects. However, the ketogenic diet has been largely ignored as a cancer therapeutic approach, with the exception of the long-standing, dogged determination of Thomas Seyfried and colleagues. Seyfried's work indicates that the hallmarks of cancer can primarily be linked to impaired mitochondrial function and energy metabolism and not genetic alterations [Seyfried and Shelton, 2010]. Moreover, he and others have demonstrated that the ketogenic diet is an effective cancer therapeutic approach [Seyfried et al., 2015; Woolf and Scheck, 2015]. Nevertheless, the beneficial effects of fatty acids, including acetate, and the ketogenic diet have, until recently, been generally dismissed or ignored.

The ketogenic diet has been successfully used as a chemo-therapeutic adjuvant or radiosensitizing agent in a wide variety of cancer types [Allen et al., 2014]. Based on Seyfried's work, the ketogenic diet has been most extensively studied in adult primary brain tumors (i.e., gliomas) [Seyfried et al., 2011]. Glioblastoma (i.e., GBM, grade IV astrocytoma) is the most prevalent glioma and the subtype associated with the direst prognosis. Despite maximal surgical resection followed by concurrent radiotherapy and chemo-therapy, median survival for patients with GBM is ~14 months. Sadly, this prognosis has not changed in the decade since the current standard of care was established. The ketogenic diet as a glioma therapy was first tested on two pediatric patients with non-resectable advanced-stage tumors [Nebeling et al., 1995]. Both children responded well to the medium-chain triglyceride diet and had long-term tumor management. The ketogenic diet was tested in conjunction with standard of care in a 65-year-old female with multicentric GBM [Zuccoli et al., 2010]. After 2 months on the diet, no tumors were detectable. However, ten weeks after stopping the diet, the tumors recurred and therapy was reinitiated, but the patient succumbed in less than 2 years after diagnosis. The effects of the ketogenic diet are not restricted to brain tumors. Patients with advanced metastatic tumors that followed the ketogenic diet with normal groceries and supplemental food additives showed overall improvement with tumor shrinkage or slowed growth, no severe side effects, and some patients reporting improved quality of life [Schmidt et al., 2011]. Based on these results, several Phase I trials are assessing the ketogenic diet in refractory or recurrent GBM patients and a new study testing the ketogenic diet as up-front concurrent therapy was recently opened.

The ketogenic diet is not without issue. Compliance is a major obstacle. In fact, of the sixteen patients with advanced metastatic tumors enrolled on the ketogenic diet, only five completed the 3-month intervention period [Schmidt et al., 2011]. Patient motivation and discipline are required to maintain ketone body levels within therapeutic ranges. This point is critical since the ketogenic diet is only effective at reducing tumor growth when calories are administered in restricted amounts [Seyfried et al., 2015]. When fed in unrestricted amounts, blood glucose levels remain high and ketones are excreted in the urine, potentially leading to no growth inhibition or, worse yet, growth potentiation. This is reminiscent of the dose-dependent effect of HDAC inhibition (i.e., low doses promote proliferation, while higher doses inhibit proliferation). The administration of ketone esters or other dietary compounds that promote ketosis and inhibit HDAC activity may be an alternative to the ketogenic diet. We propose acetate supplementation using glyceryl triacetate (GTA, Triacetin®), a synthetic short-chain triglyceride with three acetate moieties on a glycerol backbone, as one potential alternative.

BENEFICIAL EFFECTS OF ACETATE—GLYCERYL TRIACETATE AS A CANCER THERAPY

For acetate supplementation to be an effective therapy, it requires an acetate donor that is readily absorbable, metabolizable in all cells, and well tolerated. Studies have used sodium acetate, calcium acetate, or GTA as the acetate source. Thus far, GTA has proven to be the most effective acetate delivery vehicle. Four features of GTA

support its enhanced ability to increase intracellular acetate levels. First, sodium and calcium acetate are not readily absorbed in the gastrointestinal tract, whereas GTA is easily absorbed. Second, as a small triglyceride, GTA is hydrophobic and can penetrate the plasma membrane without the aid of transporters or carriers. Notably, GTA can cross the blood-brain barrier and be used to treat brain cancers. Third, once intracellular, GTA undergoes hydrolysis by ubiquitous lipases and esterases responsible for cleaving SCFAs from triglycerides; thus, GTA can be metabolized in all cells. Finally, upon hydrolysis, three acetate molecules, and a glycerol molecule, are generated per GTA molecule as opposed to the single acetate molecule derived from sodium or calcium acetate. Most importantly, GTA is safe [Fiume and CIRE, 2003]. In fact, GTA is a "generally regarded as safe" Food and Drug Administration food additive. Hence, GTA is an ideal acetate delivery vehicle. However, it should be noted that therapeutic doses of GTA are not achievable simply through consumption of foods containing GTA.

GTA-derived acetate has been shown to exert significant beneficial effects in a variety of model systems. GTA effectively reduced infarct size following cerebral ischemia [Robertson et al., 1992] and improved motor performance following traumatic brain injury [Arun et al., 2010a], suggesting that GTA exerts neuroprotective effects. GTA reduced the percentage of reactive astrocytes and activated microglia in response to lipopolysaccharide-induced neuroinflammation [Reisenauer et al., 2011] and reduced pro-inflammatory cytokine levels in rats subjected to Lyme neuroborreliosis [Brissette et al., 2012], indicating that GTA also exerts anti-inflammatory effects. GTA has been most extensively studied as a treatment for the fatal leukodystrophy Canavan disease. N-acetylaspartate (NAA) is the primary storage form of acetate in the brain. NAA is present in the human brain at concentrations exceeding 10 mM, rivaling glutamate as the most concentrated brain metabolite [Moffett et al., 2007]. NAA, which is synthesized from acetyl-CoA and aspartate (Fig. 2), supplies approximately one-third of the carbon required for myelin lipid synthesis during brain development [Madhavarao et al., 2005]. Canavan disease is caused by mutations in the gene encoding aspartoacylase (ASPA), the only known aminoacylase enzyme to catalyze the deacetylation of NAA [Kaul et al., 1991]. Lack of ASPA enzymatic activity during brain development causes NAA accumulation, reduced myelin formation, vacuolation, and eventually death. In a rat model of Canavan disease, GTA increased myelin-associated lipids, reduced vacuole formation, and improved motor function [Arun et al., 2010b]. Moreover, GTA is well tolerated when administered to infants with Canavan disease [Madhavarao et al., 2009; Segel et al., 2011]. GTA has been tested as a parenteral nutrient and can be administered orally [Madhavarao et al., 2009; Reisenauer et al., 2011], intragastrically [Segel et al., 2011], or intravenously [Bailey et al., 1991]. Thus, GTA is an excellent candidate for systemic acetate delivery and can be administered chronically without side effects.

Based on the substantial neuroprotective and anti-inflammatory effects exerted by GTA, we recently tested GTA as a therapeutic approach for glioma. Cancer cells are characterized by a loss of monoacetylated forms of histone H4 [Fraga et al., 2005], suggesting acetate supplementation may be a therapeutic approach. Furthermore, NAA levels [Moffett et al., 2007] and ASPA expression [Tsen et al., 2014] are decreased in glioma. Thus, similar to Canavan disease, glioma may be associated with reduced acetate bioavailability due to defective ASPA-mediated NAA deacetylation. NAA supplementation is not a viable therapeutic approach since we demonstrated that treatment with physiological levels of NAA increased GSC proliferation in vitro [Long et al., 2013a]. In contrast, GTA treatment induced in vitro cytostatic growth arrest of both oligodendroglioma GSCs [Long et al., 2013b] and GBM GSCs [Long et al., 2015] with little to no effect on normal brain cells. Moreover, GTA increased chemotherapeutic efficacy and survival of mice orthotopically engrafted with GSCs [Tsen et al., 2014], suggesting that GTA may be an effective chemotherapeutic adjuvant. Although the mechanism(s) underlying GTA-mediated glioma growth arrest are yet to be elucidated, previous studies provide some potential clues.

GTA exerts effects on brain energy metabolism. Inasmuch as GTA-mediated acetate supplementation and the ketogenic diet share a common metabolic intermediate, acetyl-CoA, it is not surprising that both would exert similar metabolic effects. In fact, GTA promotes ketosis. GTA significantly increased brain acetate [Mathew et al., 2005] and acetyl-CoA levels [Soliman and Rosenberger, 2011], but did not increase NAA levels [Mathew et al., 2005]. That NAA levels are not increased is critical for the use of GTA as a cancer therapy since we showed that NAA accumulation promotes GSC proliferation [Long et al., 2013a]. GTA also decreased plasma lactate and pyruvate concentrations and increased plasma ketone body concentration and resting energy [Bailey et al., 1991]. The mechanism by which GTA increases brain energy utilization has begun to be examined only recently. GTA increased phosphocreatine levels and reduced AMP levels, but did not affect cardiolipin fatty acid composition or whole brain mitochondrial mass [Bhatt et al., 2013]. Therefore, although both acetate and ketone bodies can be utilized for brain energy production, bypass glycolysis, and spare cytosolic NAD, acetate and ketone bodies are distinct in that ketone bodies, but not acetate, promote neuronal mitochondrial biogenesis. Since astrocytes are the primary cellular site of acetate metabolism [Rae et al., 2012], future studies are needed to determine whether GTA promotes astrocytic, and perhaps glioma, mitochondrial biogenesis. The divergent effects of acetate and ketone body on mitochondrial biogenesis may be due to differences in their metabolic compartmentalization. Ketone bodies are metabolized exclusively within mitochondria, whereas acetate-derived acetyl-CoA can be utilized in mitochondria, the cytosol, and nucleus. As described earlier, acetate utilization is dictated not only by its subcellular localization, but also ACSS acetylation status (Fig. 4). KAT-mediated acetylation of the ACSS enzymes inactivates them, and acetate availability may affect KAT activity. *ACSS2* inhibition in cancer cells enhanced cell death and significantly reduced tumor growth [Yoshii et al., 2009a; Comerford et al., 2014]. We hypothesize that some of the reduced tumor growth and increased survival observed in GTA treated mice could be due to the creation of a ketotic metabolic state that is less conducive to glioma growth combined with increased ACSS acetylation resulting in inactivation.

Similar to fatty acids and ketone bodies, GTA promotes histone acetylation. GTA increased brain histone H4K8, H4K16, and H3K9 acetylation [Soliman and Rosenberger, 2011]. No other changes in

brain histone acetylation were found. Also, increased histone acetylation was not observed in the liver. In Canavan disease, H3K9 was found to be hyperacetylated and GTA administration reduced the hyperacetylation at this locus to near normal levels [Moffett et al., 2014]. These findings indicate that acetylation status is not solely due to mass action to indiscriminately promote protein acetylation. Rather, acetate availability regulates acetylation in a complex manner based on which KATs and KDACs are active, and which acetylation targets are available. This is reminiscent of HDAC inhibitors, which do not indiscriminately alter gene transcription, but selectively target less than 10% of the human genome. Acetylation of H4K16 is particularly noteworthy since loss of H4K16 acetylation is a common hallmark of cancer [Fraga et al., 2005]; also, acetylated H4K16 marks actively transcribed euchromatin. GTA also promoted HIF-2α acetylation in response to hypoxia in an ACSS2-dependent manner [Xu et al., 2014]. ACSS2 activity is responsible for the majority of acetate incorporation into lipids and histones. The study highlighting that acetate fuels cancer growth by promoting lipogenesis [Comerford et al., 2014] did not consider altered histone and transcription factor acetylation as a contributing factor to ACSS2-mediated growth promotion.

GTA also inhibits HDAC expression and activity. Short-term GTA treatment inhibited HDAC activity without affecting HAT activity [Soliman and Rosenberger, 2011]. In contrast, long-term GTA treatment increased HAT activity, but had no effect on total HDAC activity [Soliman et al., 2012]. Although GTA inhibited HDAC expression and activity, there are no data to suggest that intact GTA functions as a HDAC inhibitor similar to the SCFA butyrate. HDAC inhibitors reduce proliferation by inducing terminal cell differentiation and apoptosis, neither of which was observed in GTA treated GSCs.

Data suggest that GTA might subserve functions distinct from serving as an acetate delivery vehicle. GTA was more effective at inhibiting GSC proliferation than glycerol and sodium acetate at concentrations generated by complete GTA catalysis [Long et al., 2013b, 2015]. Moreover, GTA induced cytostatic growth arrest without inducing apoptosis, while the glycerol/sodium acetate treatment significantly increased cell death. These differences could be due to differential acetate utilization or that GTA exerts distinct functions as an intact molecule. It is also possible that GTA is able to deliver high levels of acetate to subcellular compartments that are less accessible to acetate. Recent data suggests that GTA may also modulate purinergic signal transduction [Smith et al., 2014]. Whether altered signaling was due to acetylation or GTA-derived acetate functioning as a signaling molecule is yet to be determined. Further analyses are needed to determine whether GTA can evade intracellular catalysis to function as an intact molecule either as an HDAC inhibitor or a signaling molecule.

Collectively, these data support the use of GTA as a therapeutic agent to promote neuroprotection, reduce inflammation, and inhibit tumor growth. Because the effects of short- and long-term GTA treatment differ substantially, future studies on GTA as a chemotherapeutic adjuvant need to assess whether GTA must be administered chronically or only concurrent with chemotherapy. This is an important issue to resolve since continuous histone hyperacetylation potentiates chemotherapy resistance [Kitange et al., 2012]. If GTA enhances chemotherapeutic efficacy without promoting chemotherapy resistance, it could significantly improve standard of care and enhance survival of GBM patients beyond the current ~14 months.

SUMMARY

Acetate, fatty acids, and ketone bodies act to redistribute oxidizable carbon from a source tissue, such as liver, to target tissues such as heart, skeletal muscle and brain. Inasmuch as acetate is a substrate for lipogenesis and the production of metabolites, such as glutamine and glutamate, that promote anabolic cancer cell growth, it seems counter-intuitive that acetate supplementation would decrease tumor growth. Nonetheless, there is a growing body of data supporting the use of fatty acids and the ketogenic diet, both having acetyl-CoA as a metabolic intermediate, as cancer therapeutics. Acetate and GTA share many features of fatty acids and ketone bodies (i.e., promote histone acetylation and increase energy status). Additionally, high levels of acetate are neuroprotective by reducing neuroinflammation. Acetate regulates gene transcription though multiple mechanisms including enzyme acetylation (e.g., KATs) and deacetylation (e.g., sirtuins), transcription factor activation via acetylation, and chromatin remodeling via HATs. Additional areas of interest for future investigation include the role of acetate in cytoskeletal remodeling and protein/protease release to the extracellular matrix involved in cancer metastasis. In conclusion, despite recent reports that acetate promotes cancer growth via acetate oxidation, we believe that the preponderance of evidence supports further pursuit of the ketogenic diet and GTA-mediated acetate supplementation as chemotherapeutic adjuvants and radiosensitizing agents for cancer therapy.

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