



Review

Understanding the central role of citrate in the metabolism of cancer cells

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ABSTRACT

Cancers cells strongly stimulate glycolysis and glutaminolysis for their biosynthesis. Pyruvate derived from glucose is preferentially diverted towards the production of lactic acid (Warburg effect). Citrate censors ATP production and controls strategic enzymes of anabolic and catabolic pathways through feedback reactions. Mitochondrial citrate diffuses in the cytosol to restore oxaloacetate and acetyl-CoA. Whereas acetyl-CoA serves *de novo* lipid synthesis and histone acetylation, OAA is derived towards lactate production via pyruvate and / or a vicious cycle reforming mitochondrial citrate. This cycle allows cancer cells to burn their host's lipid and protein reserves in order to sustain their own biosynthesis pathways. *In vitro*, citrate has demonstrated anti-cancer properties when administered in excess, sensitizing cancer cells to chemotherapy. Understanding its central role is of particular relevance for the development of new strategies for counteracting cancer cell proliferation and overcoming chemoresistance.

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1. Introduction

Increasing data are currently published in the literature on the altered metabolism which leads to the Warburg effect, focusing attention on the signaling pathways that conduct such reprogramming. Although the precise biochemical pathways involved in the reprogramming of the metabolism of cancer cells remain largely deductive and sometimes hypothetical, understanding biochemical routes is fundamental for the development and proposal of new strategies for counteracting cancer cell development. The aim of this work

is to emphasize the central role of citrate in the metabolism, which can be used to counteract cancer cell proliferation.

1.1. I. The special metabolism of cancer cells

Cancer cells synthesize great quantities of macromolecules and lipids to proliferate and build new cells, while requiring to continuously produce ATP and cofactors (NAD⁺, NADPH, H⁺) in order to sustain synthetic pathways. These cells consume glucose in excess [1–5], large quantities of amino acids (in particular glutamine derived from muscle proteolysis) [6–11], whereas the use of lipids through mitochondrial β -oxidation remains to be investigated [10–12] (Fig. 1). Glucose metabolism in cancer cells most often results in the formation

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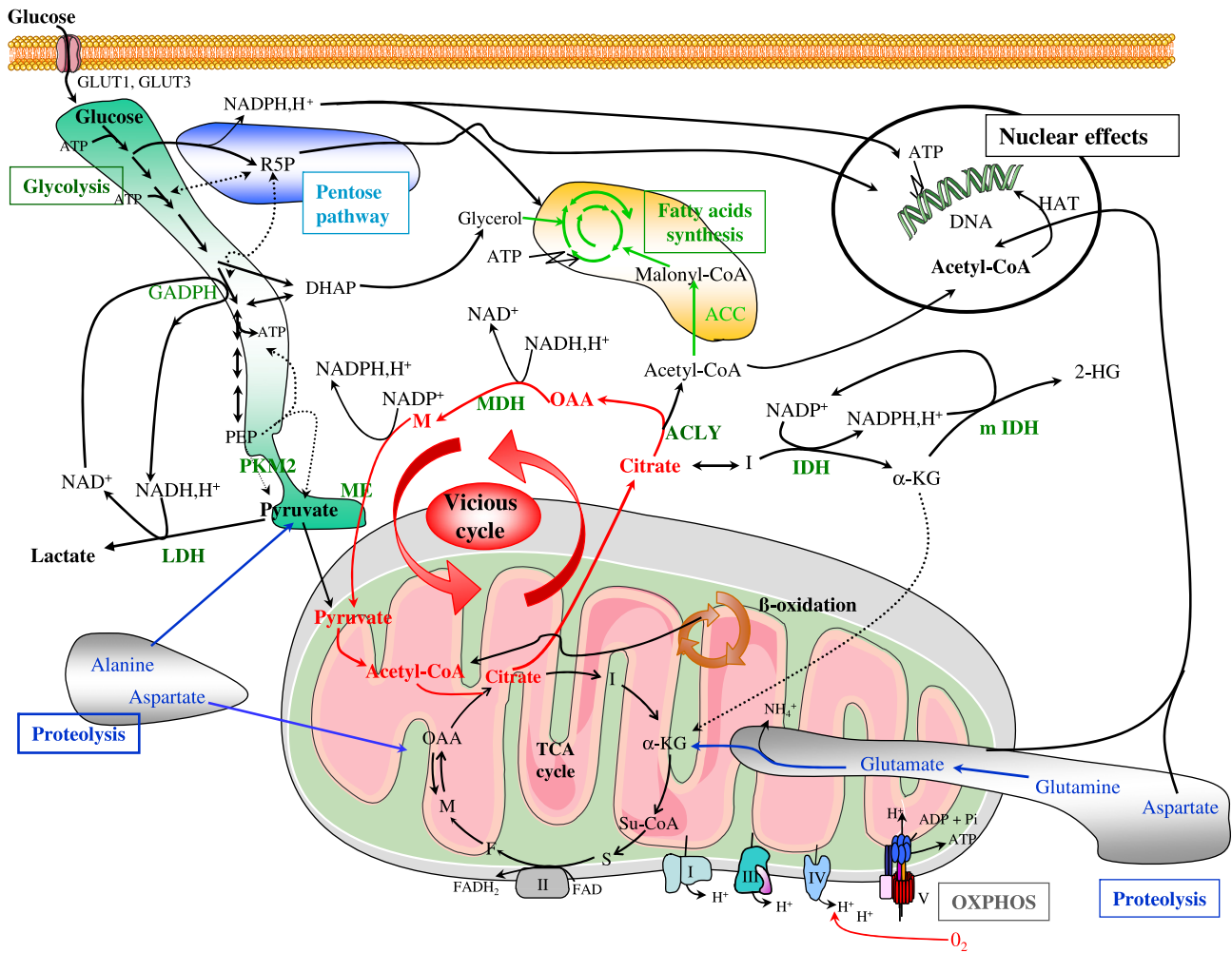


Fig. 1. A model of biochemical pathways in cancer cells. In cancer cells, a blockage at the end of glycolysis (“the PKM2 bottleneck”) is reinforced by the inhibition of pyruvate dehydrogenase (PD), transforming pyruvate into acetyl-CoA. Intermediates accumulate upstream of the bottleneck and are derived towards glycerol and ribose synthesis (through the pentose phosphate pathway). Due to PD inhibition, pyruvate is transformed into lactate, hence regenerating NAD^+ , which is required for glycolysis function. Due to the PKM2 bottleneck, a large share of pyruvate may derive from other sources than glycolysis. Acetyl-CoA forms citrate after condensation with oxaloacetate (OAA) via citrate synthase (CS). A large share of citrate moves outside mitochondria and reforms OAA and acetyl-CoA in the cytosol. Acetyl-CoA is used for *de novo* lipid synthesis, whereas OAA leads to the formation of pyruvate via two successive reactions regenerating NADPH, H^+ and NAD^+ (vicious cycle). Proteolysis provides alanine and glutamine. Alanine is derived to lactate formation after transamination in pyruvate, whereas glutamine is transformed into glutamate which, along with aspartate (derived from OAA), serves nucleotide synthesis. Glutamate feeds the tricarboxylic acid cycle (TCA). ACC: acetyl-coA carboxylase, ACLY: ATP citrate lyase, DHAP: dihydroxyacetone phosphate, HAT: histone acetyl transferase, 2-HG: 2-hydroxyglutarate, I: isocitrate, IDH: isocitrate dehydrogenase, mIDH: mutant IDH, α -KG: α -ketoglutarate, LDH: lactic dehydrogenase, M: malate, MDH: malate dehydrogenase, ME: malic enzyme, NAD^+ : nicotinamide adenine dinucleotide, NADPH, H^+ : nicotinamide adenine dinucleotide phosphate, PEP: phosphoenolpyruvate, PKM2: embryonic isoform of pyruvate kinase, R5P: ribose 5-phosphate.

of lactic acid, even in the presence of oxygen. This phenomenon, referred to as “aerobic glycolysis” or the “Warburg effect” [1,3], is increasingly studied [11–18]. It is a reversal of the Pasteur effect (inhibition of fermentation by O_2); modifications of the Pasteur effect in cancer cells are linked, among others, to Hypoxia-inducible factor 1 (HIF1) [19]. HIF1 stimulates many target glycolytic enzyme genes, whereas it blocks the use of pyruvate by mitochondrial pyruvate dehydrogenase (PDH). These mechanisms produce a shift from oxidative phosphorylation (OXPHOS) to glycolysis for producing ATP. In normal cells, the Pasteur effect is mediated by ATP and citrate. In hypoxic conditions, full glucose oxidation is decreased, resulting in a decrease in ATP and citrate produced by mitochondria. In turn, the feedback of these molecules on PFK1, the main regulator enzyme for glycolysis, is broken down. Glycolysis is then accelerated, leading to enhanced production of lactic acid. It is interesting to note that the majority of solid tumor cells are hypoxic [20] and glycolysis becomes the fastest way to produce energy and the best way to synthesize several essential metabolic intermediates required for their proliferation, even if relatively

inefficient in producing ATP (2 versus 36) [14,21,22]. Impairment of mitochondrial respiration can occasionally be observed, resulting in defective OXPHOS and contributing towards tumorigenesis [11,14,16,21,22]. However, in most cases, the cause of the dysregulation of the Pasteur effect appears not to be due to defective respiration and improved understanding of this mechanism remains to be investigated. It is of note that cancer cells tend to focus more on sustaining their metabolic pathways in order to provide the molecules required for biosynthesis, rather than attaining the most efficient energy production pathway, given that they find all of their required nutrients in abundance in their environment [8,10–16,21,22].

Lactate dehydrogenase (LDH) allows the conversion of pyruvate into lactate regenerating NAD^+ , a crucial cofactor for glycolysis function at glyceraldehyde 3-phosphate dehydrogenase (GADPH) level [2,4,5,10,11] (Fig. 1). Consequently, cancer cells reject excessive lactic acid [6–11], which could be used either to reconstruct glucose in the liver via the Cori cycle or taken up by oxygenated tumor cells to reform pyruvate, hence sparing glucose for the most anoxic cells [8,23].

Cancer cells not only consume circulating glucose in excess, but also amino acids, in particular glutamine [7–11], and potentially alanine, both derived from proteolysis, which is particularly enhanced in patients presenting with cachexia. In cells, glutamine, which is the preferential transport mode for blood nitrogen (NH_4^+), is converted into glutamate by glutaminase. Glutamate, which has been reported in increased quantities in tumors [6–9], is then converted into α -ketoglutarate (α -KG) to feed the TCA cycle. Glutamine also provides amine groups for biosynthetic processes, such as purine and pyrimidine synthesis [1–3]. From the intermediate molecules provided by enhanced glycolysis and glutaminolysis, cancer cells synthesize most of the macromolecules required for the duplication of their biomass and genome. Given that inhibition of fatty acid transport into mitochondria induces cancer cell death [24], β -oxidation could provide an important share of acetyl-CoA, NADH^+ , H^+ and ATP, thus sustaining energy production and cancer cell proliferation. However, the contribution of β -oxidation to the metabolism of cancer cells calls for further investigation (Fig. 1).

1.1.1. How do cancer cells favor the production of intermediates required for biosynthesis?

In order to produce many essential intermediates required for biosynthesis, such as ribose, glycerol and serine, glycolysis is slowed down at its end, where pyruvate kinase (PK), which is re-expressed in its embryonic form (PKM2), is less active than the adult form. PKM2 exists either as an active tetramer that promotes ATP production or as an inactive dimer which has a low affinity for PEP and redirects glycolytic intermediates towards biosynthesis, such as the pentose phosphate pathway (PPP) for DNA synthesis, and the amino-acid biosynthetic pathway. As a metabolic sensor, PKM2 is under the positive allosteric regulation of F1,6BP and under the negative allosteric regulation of downstream biosynthetic products such as ATP, alanine, other amino acids and lipids [25]. This re-expression of PKM2 (mainly in its dimeric form) causes a “bottleneck” at the end of glycolysis, which appears to be a typical feature of cancer cells [11,25–27] (Fig. 1). Via this re-expression of PKM2, most cancer cells acquire a finely regulated switch to promote ATP and/or biosynthesis: when the tetrameric form is activated, production of ATP is switched on; when the dimeric form of PKM2 is activated, biosynthesis is enhanced [23,26,27]. Due to this dimeric PKM2 bottleneck, glucose-derived pyruvate derived is reduced. Thus, it is likely that pyruvate, which is required in abundance to sustain LDH activity, is formed by other sources: alanine after transamination and malate derived from cytosolic citrate through the action of malate dehydrogenase (see below—central role of citrate). Furthermore, an alternative pathway could enable cells to bypass the PKM2 bottleneck, producing pyruvate when PEP accumulates above the bottleneck, hence transforming PEP into pyruvate without generating ATP [28].

This PKM2 bottleneck is reinforced by a second inactivation, which occurs at PDH level [11,29], a mitochondrial enzyme converting pyruvate into acetyl-CoA [2,4,5]. By phosphorylating PDH, pyruvate dehydrogenase kinase 1 (PDK1) inactivates this enzyme and prevents the import of pyruvate into the mitochondria [14,30,31]. As mentioned above, this switch avoids OXPHOS stimulation when limited amounts of O_2 are present, and is induced by HIF-1 α , which up-regulates the expression of both PDK and LDH [14,30,31]. It is interesting to note that acetyl-CoA, NADH and ATP, which could be provided by enhanced glutaminolysis and perhaps β -oxidation, inhibit PDH [1–3]. Therefore, these normal biochemical retroactions could have a synergetic effect on the PKM2 bottleneck and PDK1 activation. These mechanisms could result in a disconnection between glycolysis and the TCA cycle.

1.2. II. The central role of citrate

1.2.1. Citrate as an essential intermediate of biochemical pathways

Due to the aforementioned bottleneck, mitochondrial acetyl-CoA derives rather from glutaminolysis, than from pyruvate produced by

glycolysis. Acetyl-CoA condensates with OAA, forming citrate through citrate synthase (CS). In cancer cells, *de novo* lipid and sterol synthesis are enhanced, supported by citrate, which moves outside mitochondria, in exchange with malate. In the cytosol, citrate is restored into OAA and acetyl-CoA by ATP citrate lyase (ACLY) [1–3]. While acetyl-CoA feeds fatty acid synthesis, OAA is converted into pyruvate via two successive reactions: the first converts OAA into malate by MDH, a reaction producing NAD^+ , whereas the second reaction converts malate into pyruvate by the malic enzyme (ME), hence generating NADPH, H^+ . NAD^+ is not only crucial for glycolysis function, but also for Poly ADP-Ribose Polymerase (PARP) function, which participates towards enhanced nucleotide synthesis [15]. Similarly, NADPH, H^+ is required for several biosynthetic pathways (nucleotides and lipid biosynthesis, redox system, see below) (Fig. 1). It is of note that CS activation drives the flux towards lipid and triglyceride synthesis, whereas ketone body formation is repressed [32], since cancer cells favor lipid synthesis over the ketogenic route. This could result in a lack of β -hydroxybutyrate, an HDAC inhibitor, hence favoring histone deacetylases (HDAC) activity. Therefore, citrate provides acetyl-CoA in abundance for lipid synthesis, whereas it promotes this route by activating acetyl-CoA carboxylase (ACC), the first enzyme for fatty acid synthesis: This key enzyme regulates the entrance of this pathway which consumes large quantities of ATP and NADPH, H^+ [1–3]. To promote this lipid route, it is likely that the TCA cycle below CS is slowed down. For that purpose, two key regulator enzymes of the TCA cycle (isocitrate dehydrogenase IDH and α -keto dehydrogenase) could be inhibited. We must bear in mind that these enzymes are inhibited by NADH, H^+ and ATP, which are produced in abundance when oxidation of fatty and or amino acids is activated [1–3]. While citrate moves outside mitochondria to sustain the lipid route and pyruvate-lactate formation, glutamine replenishes the TCA cycle in intermediates such as α -KG, which are depleted by biosynthetic reactions [14].

Finally, a specific cycle is generated in cancer cells where citrate derived from glutamine oxidation serves lipid synthesis and lactic acid production through several reactions which produce cofactors (NAD^+ and NADPH, H^+). Via this cycle, glycolysis and biosynthetic cancer cell pathways are sustained, whereas host reserves are consumed (Fig. 1). Over and above PPP, this cycle restores NADPH, H^+ , either via the malic enzyme (ME) reaction or cytosolic isocitrate dehydrogenase (IDH) reaction [33,34]. This reduced cofactor is required for the biosynthesis of fatty acids, nucleotides and amino acids [8,10–15]. It is also a key cellular anti-oxidant, which maintains the pool of reduced glutathione (GSH), a key molecule, together with thioredoxin (TRX) system, for preventing oxidative damage induced by reactive oxygen species (ROS). NADPH, H^+ is also crucial for the redox state of cytochrome c, the lack of oxidized cytochrome c preventing apoptosis induction [35]. NADPH, H^+ is also involved in the cytosolic IDH reaction converting isocitrate in α -KG, which could replenish the TCA cycle. However, α -KG could be converted into 2-hydroxyglutarate (2-HG) by IDH mutant (m IDH) consuming NADPH, H^+ (Fig. 1). In reducing the availability of α -KG for reactions that methylate DNA and histones, 2-HG could present oncogenic properties by modifying the epigenome [36,37]. It is likely that a cytosolic regulation diverts citrate either through lipid synthesis or towards the c-IDH pathway producing α -KG and or 2-HG [18,37].

1.2.2. Citrate: an essential donor for protein acetylation

The accessibility of DNA in eukaryotic cells is determined by the organization and dynamic modification of histone proteins forming chromatin [38–40]. Histone acetylation is dynamically regulated by several classes of histone deacetylases (HDACs) and by families of histone acetyltransferases (HATs), which both act on targeted regions of chromatin to regulate specific gene transcription [38–40], in particular in genes involved in glucose metabolisms such as hexokinase 2, phosphofructokinase-1 (PFK-1) and lactate dehydrogenase A

(LDH-A) [41]. HATs appear to be regulated by the ACLY-dependent production of acetyl-CoA [42], the level of cytosolic acetyl-CoA reflecting the nutrient uptake and the metabolites required for proliferation.

Interestingly, cytosolic acetyl-CoA also serves as a signaling molecule that couples apoptotic sensitivity to metabolism by regulating protein N-alpha-acetylation [43,44]. Addition of citrate to Bcl-x_L-expressing cells leads to increased protein N-alpha-acetylation and sensitization of these cells to apoptosis [45]. Thus, the basal level of cytosolic acetyl-CoA might influence the apoptotic threshold in multiple oncogenic contexts. In turn, Bcl-x_L may be able to control the levels of acetyl-CoA and protein-N-acetylation, providing a clear example of a linkage between metabolism and apoptotic sensitivity.

1.3. IV. The use of citrate to counteract cancer cell proliferation

1.3.1. Citrate is a crucial sensor of the energy level and an intermediate located at the crossroads of metabolic pathways

Intracellular citrate levels are both an essential metabolic intermediary and a key regulator of energy production. Normally, when

citrate, the first product of the TCA cycle, is sufficiently produced by this cycle, it exerts a negative feedback on glycolysis (Pasteur effect) and the TCA cycle itself [1–3], slowing down or arresting these pathways, whereas it stimulates gluconeogenesis and ATP-consuming lipid synthesis (Fig. 2). Indeed, citrate inhibits the first regulator enzyme of glycolysis, PFK1, and this inhibition is total when citrate is abundant [2]. Citrate also inhibits PFK2 [46], which produces fructose 2,6-bisphosphate (F2,6BP), a powerful allosteric activator of PFK1 in cancer cells. PFK2 is a 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFKFB) bi-functional enzyme, that interconverts fructose-6-phosphate in F2,6BP. The elevation of PFK2 enables the inhibition of PFK1 by ATP and citrate to be physiologically overridden when glucose uptake is high. This is the case in cancer cells, since membrane glucose transporters (in particular GLUT1 and GLUT3 which have a high affinity for glucose) are overexpressed and under the control of several proteins such as HIF-1 α , c-Myc, Ras activation and p53 mutant [10,11,17,25,30,31,47,48]. High levels of F2,6BP could reflect the importance of glucose uptake and glycolysis and efficiently drive cells towards proliferation [46,49,50]. Overexpression of the nuclear isoform of PFK2 also referred to as PFKB3,

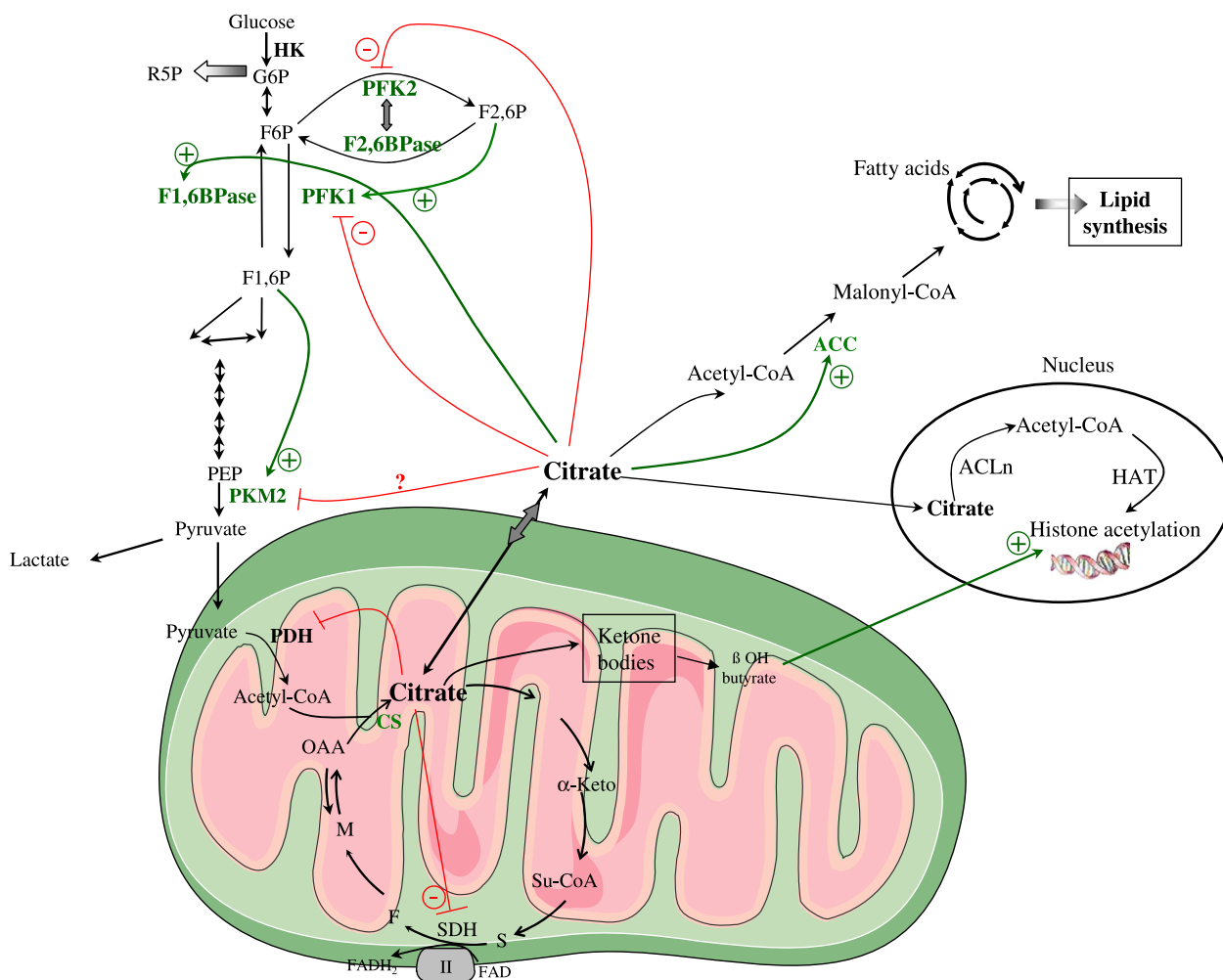


Fig. 2. The central and regulating role of citrate. Citrate inhibits PFK1, PK, PDH and SDH, and these inhibitions allow an immediate adjustment of glycolysis and TCA cycle fluxes, adapted to ATP production. Concurrently, citrate stimulates gluconeogenesis (through F1,6BPase) and lipid synthesis through ACC. Citrate also inhibits PFK2 which produces fructose 2,6-bisphosphate, a powerful allosteric activator of PFK1 in cancer cells. Citrate inhibits PK at least indirectly since PFK1 inhibition decreases the level of fructose 1,6-bisphosphate (F1,6P), which is a powerful allosteric activator of PK. In mitochondria, CS condenses OAA with acetyl-CoA to form citrate, which then moves outside mitochondria. In the cytosol, ATP citrate lyase restores acetyl-CoA, which sustains the lipid route or histone acetylation. Citrate leads to the formation of malonyl-CoA, the first product of lipid synthesis, which in turn inhibits the mitochondrial transport of fatty acids by carnitine acyl transferase I (CPT I). ACC: acetyl-coA carboxylase, ACLY: ATP citrate lyase, CPT: carnitine palmitoyl transferase, CS: citrate synthase, F1,6BPase: fructose 1,6 biphosphatase, F2,6BPase: fructose 2,6 biphosphatase, G6P: glucose 6-phosphate, F6P: fructose 6-phosphate, F1,6P: fructose 1,6 diphosphate, HAT: histone acetyl transferase, HK: hexokinase, α -KG: α -ketoglutarate, OAA: oxaloacetate, PEP: phosphoenolpyruvate, PDH: pyruvate dehydrogenase, PFK: phosphofructokinase, PKM2: embryonic isoform of pyruvate kinase, SDH: succinate dehydrogenase.

promotes cancer cell proliferation by modulating central cell cycle regulators [49] and selective inhibition of PFK2 inhibits cell growth [46,50]. It is of note that PFKB3, which sustains a high rate of glycolysis, has elevated PFK2 activity and almost no FBPase activity [51,52]. It is an HIF-1 [53] target and is highly expressed in several forms of human cancer [54,55].

Furthermore, citrate also inhibits PK, at least indirectly, since it decreases the level of fructose 1,6-bisphosphate (F1,6P), a powerful allosteric activator of PK. The decrease in F1,6P is a consequence of citrate inhibition on PFK1 [1–3,56] (Fig. 2).

Citrate inhibits the TCA cycle downstream, both at its entrance—by slowing down PDH [57], and at its exit—by acting on succinate dehydrogenase (SDH) [58]. It also inhibits β -oxidation, at least indirectly, because the stimulation of fatty acid synthesis leads to the formation of malonyl-CoA, the first product of this biosynthesis, which in turn inhibits the mitochondrial transport of fatty acids by carnitine acyl transferase I (CPT I) [1–3,24] (Fig. 2). Furthermore, when present in excess, malonyl-CoA can also form malonate (via malonyl CoA transferase) which is a strong inhibitor of SDH [1,2]. By regulating strategic enzymes located at the entrance and/or at the exit of glycolysis (PFK1, PFK2, PK), TCA cycle (PDH, SDH), gluconeogenesis (F1,6BPase) and fatty acid synthesis (ACC), citrate allows a close adjustment of metabolic flows to ATP production.

1.4. Excess administration of citrate

When citrate is administered in excess to cancer cells, we hypothesize that it may sufficiently inhibit all PFK isoforms to arrest glycolysis and reverse the nuclear action of F2,6P, hence arresting proliferation. The growth of various human cancer cells was stopped when cultured cells were exposed to 10 mM of citrate, and massive cell death was observed at this concentration in certain cell lines [59,60]. Interestingly, citrate sensitized cancer cells to chemotherapy [60]. Citrate may fool the cell's energy level, and could inhibit all ATP production pathways (glycolysis, TCA cycle and β -oxidation), whereas it could stimulate synthesis routes which consume ATP (gluconeogenesis and lipid synthesis). These mechanisms may rapidly result in severe energy depletion inside cells, leading to cell growth arrest and cell death [61,62], the nature of death (apoptosis or necrosis) depending on the severity of ATP depletion and on the resistance of the tested cell lines. The mechanisms linking glycolysis and death pathways remain to be investigated. The link could be located at various levels: HK II and voltage dependant anion channel (VDAC) [63–65], GAPDH [66] and also PFK 1 which has been reported as a Bad-associated protein [67].

Interestingly, citrate leads to an early decrease in the expression of the anti-apoptotic protein Mcl-1, a molecule which plays a key role, together with the protein Bcl-x_L, in the chemoresistance of certain cancers [68,69], in particular mesothelioma [70]. As previously mentioned, the addition of citrate to Bcl-x_L-expressing cells may lead to increased protein N-alpha-acetylation and sensitization of these cells to apoptosis [45]. The mechanism explaining the sensitization to chemotherapy of citrate-exposed cells [60] remains to be investigated.

In fact, citrate is not only an essential intermediate located at several branch points of biochemical pathways [33], but it also acts as an “energy gauge”, a powerful sensor and regulator of cell energy production, adjusting both production and need. Since citrate promotes histone acetylation, it could also play a role in adjusting the level of a number of key regulator enzymes.

Interestingly, oral administration of citrate may generate a decrease in tumoral markers [71]. Since citrate can sensitize cancer cells to chemotherapy, administration of this molecule at high dosage should be considered as a new “targeting metabolism strategy” as recently described by Vander Heiden [72].

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