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Review

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Citrate – new functions for an old metabolite

Abstract: Citrate is an important substrate in cellular energy metabolism. It is produced in the mitochondria and used in the Krebs cycle or released into cytoplasm through a specific mitochondrial carrier, CIC. In the cytosol, citrate and its derivatives, acetyl-CoA and oxaloacetate, are used in normal and pathological processes. Beyond the classical role as metabolic regulator, recent studies have highlighted that citrate is involved in inflammation, cancer, insulin secretion, histone acetylation, neurological disorders, and non-alcoholic fatty liver disease. Monitoring changes in the citrate levels could therefore potentially be used as diagnostic tool. This review highlights these new aspects of citrate functions.

Keywords: cancer; citrate; energy metabolism; inflammation; insulin secretion; mitochondrial citrate carrier.

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Introduction

Citrate is found in a critical step at a crossroad in intermediary metabolism of mammalian cells. After its synthesis in mitochondria from acetyl-CoA and oxaloacetate (OAA) by citrate synthase, it becomes a substrate in the tricarboxylic acid (TCA) cycle, and its subsequent complete oxidation provides the major source of cellular ATP production. The citrate level inside the cells exerts a well-known function as a key regulator of energy production because citrate inhibits and induces important strategic enzymes located at the entrance and/or at the exit of glycolysis, TCA cycle, gluconeogenesis, and fatty acids synthesis (Figure 1). In fact, it exerts a negative feedback on glycolysis by inhibiting phosphofructokinase 1 (PFK1) and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatases (PFK2) (Chesney, 2006), which is a bifunctional enzyme that interconverts fructose-6-phosphate in fructose 2,6-bisphosphate (F2,6P), which is a powerful allosteric activator of PFK1 in cancer cells. The level of F2,6P is of particular importance in cancer cells, where it acts as a key intracellular signal. Inhibition of PFK2 greatly slows down cell growth (Yalcin et al., 2009b), whereas overexpression of the nuclear isoform of PFK2 (also named PFKB3) promotes proliferation by modulating the central regulators of the cell cycle (Yalcin et al., 2009a,b), which underlines the importance of glucose uptake and glycolysis to drive cell proliferation (Chesney, 2006). By decreasing the level of fructose-1,6-biphosphate (F1,6P), an allosteric activator of pyruvate kinase (PK), citrate indirectly also inhibits PK (Yalcin et al., 2009b). TCA cycle is inhibited by citrate at the level of pyruvate dehydrogenase (PDH) (Taylor and Halperin, 1973) and succinate dehydrogenase (SDH) (Hillar et al., 1975). On the contrary, it stimulates pathways consuming ATP such as gluconeogenesis and lipid synthesis. It stimulates acetyl-CoA carboxylase, leading to the formation of malonyl-CoA, which, on the one hand, induces fatty acids biosynthesis and, on the other hand, inhibits the mitochondrial transport of fatty acids by carnitine palmitoyltransferase 1 (CPT-1) (Paumen et al., 1997) and consequently β-oxidation. For many years, citrate was therefore associated with an essentially bioenergetic role in cell metabolism. Moreover, in normal prostate cells, citrate is a secretory end product of metabolism rather than a substrate of intermediary metabolism (Westergaard et al., 1994; Costello et al., 1999).

Mitochondrial citrate is transported outside the mitochondria by the citrate carrier (CIC), a member of the mitochondrial carrier family encoded by the *SLC25A1* gene (Bisaccia et al., 1989, 1990; Kramer and Palmieri, 1989; Kaplan et al., 1993; Palmieri et al., 1993, Palmieri, 2013). In the cytosol, citrate is cleaved by ATP-citrate lyase (ACLY) to OAA and acetyl-CoA. Acetyl-CoA is used for fatty acids and sterol biosynthesis, whereas OAA is reduced to malate, which is then converted to pyruvate *via* malic enzyme, which produces cytosolic NADPH and H^+ (necessary for fatty acid and sterol synthesis) (Figure 1).

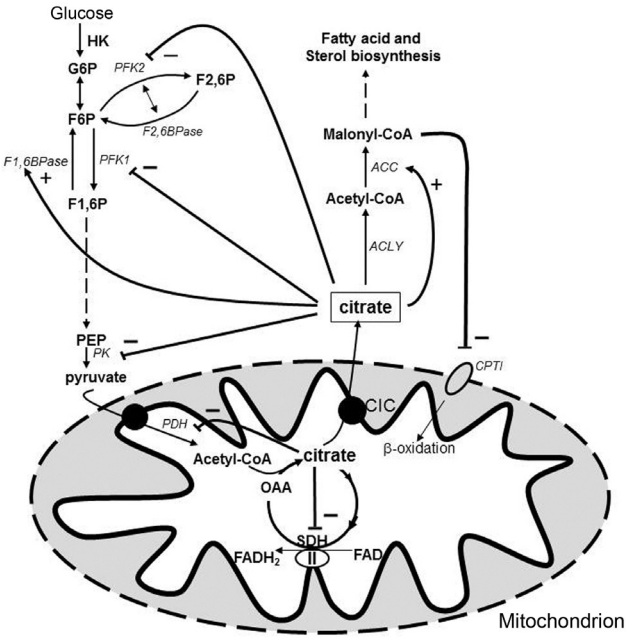

Figure 1 The regulatory role of citrate in the metabolism.
Citrate is synthesized inside the mitochondria by citrate synthase from acetyl-CoA and OAA. It is exported outside the mitochondria by CIC. Citrate inhibits PFK1, PK, PDH, and SDH. Citrate inhibits also PFK2, which produces F2,6P, an allosteric activator of PFK1 in cancer cells. Activation effect is exerted on lipid biosynthesis through ACC, which produces malonyl-CoA, the first product of lipid biosynthesis, which, in turn, inhibits the CPT-1, the first enzyme of β-oxidation process. Through F1,6BPase, citrate stimulates gluconeogenesis. ACC, acetyl-CoA carboxylase; CS, citrate synthase; F1,6BPase, fructose 1,6 bisphosphatase; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; HK, hexokinase; PEP, phosphoenolpyruvate. Symbols + and – indicate stimulation and inhibition, respectively.

Thus, the availability of citrate depends on the amount of citrate transported from the mitochondria, which, in turn, depends on the regulation of CIC gene expression in different cellular and metabolic conditions. Different regulatory transcription factors of the CIC gene have been identified so far: Sp1 is involved in basal and epigenetic regulation (Iacobazzi et al., 2008; Infantino et al., 2011b); FOXA1 is a gene activator in liver and pancreatic cells (Iacobazzi et al., 2009a; Menga et al., 2013); insulin activates and fatty acids repress gene expression through SREBP-1 (Infantino et al., 2007); ZNF224 acts as a strong repressor (Iacobazzi et al., 2009b); NF-κB mediates CIC overexpression in inflammation (Infantino et al., 2011a); PPARs are gene activators (Damiano et al., 2012; Bonofiglio et al., 2013). These findings support the view of a complex regulation of the *CIC* gene expression related to different biological processes in which citrate is involved.

Studies performed in recent years have indicated that citrate is not only a powerful sensor and regulator of metabolic pathways but also plays other important roles in different biological processes. This review will focus on newly discovered features of the physiological and pathological role of citrate inside the cell and its potential use as a therapeutic drug and biomarker.

The multifunctional role of citrate

Beyond the well-known role as a metabolic sensor, citrate is involved in the following biological processes: inflammation, cancer, insulin secretion, and histone acetylation. Two approaches have been used to identify these new functions. The first is based on the inhibition of mitochondrial CIC activity by the specific substrate analogue 1,2,3-benzene-tricarboxylate (BTA) (Joseph et al., 2006; Infantino et al., 2011a; Cappello et al., 2012; Catalina-Rodriguez et al., 2012) and the second by siRNA gene silencing (Joseph et al., 2006; Morciano et al., 2009; Infantino et al., 2011a; Catalina-Rodriguez et al., 2012). Recently, by mutational analysis of the *SLC25A1* gene (Edvardson et al., 2013; Nota et al., 2013), different mutations were identified in patients with agenesis of corpus callosum and hydroxyglutaric aciduria. Furthermore, direct measurements by liquid chromatography-tandem mass spectrometry (van de Wier et al., 2013) and nuclear magnetic resonance-based methods (Hricak, 2005; Kline et al., 2006) highlighted altered citrate levels in non-alcoholic fatty liver disease (NAFLD) and prostate cancer, respectively. Finally, the use of citrate as a therapeutic drug was also tentatively tested (Zhang et al., 2009).

Citrate in inflammation

During inflammation, cells undergo significant metabolic changes mainly consisting of a downregulation of bioenergetic pathways. A genome-wide expression analysis performed on human leukocytes, from subjects treated with bacterial endotoxin (LPS), identified a significant decrease in the expression of genes involved in mitochondrial energy metabolism (such as genes encoding respiratory chain complex subunits, adenine nucleotide carrier, and PDH) and protein synthesis machinery (such as the elongation initiation factor complex) (Calvano et al., 2005). A downregulation of mitochondrial bioenergetic processes has also been observed in LPS-activated macrophages. These cells rapidly switch from resting-state metabolism, which uses oxidative phosphorylation to generate ATP, to an activated state in which glycolysis is

greatly induced. Upregulated glycolysis is not only used to generate more ATP per time unit but also to generate other intermediates from the pentose phosphate pathway. In fact, macrophages show a high requirement for such intermediates to produce a whole range of inflammatory mediators. Mitochondrial enzymes involved in the Krebs cycle are also inhibited, indicating a shift of the TCA cycle from being a purely catabolic pathway generating ATP to being, at least in part, an anabolic pathway. Citrate is withdrawn from the cycle and exported by CIC from mitochondria to the cytosol, where it is cleaved by ACLY into acetyl-CoA and OAA. Acetyl-CoA provides units to synthesize lipids, including arachidonic acid, which is needed for the production of prostaglandin, an intermediate of inflammation. The second product of citrate metabolism, OAA, through cytosolic malate dehydrogenase and malic enzyme, produces NADPH, which can be used to produce ROS through the action of NADPH oxidase, and NO through inducible NO synthase (iNOS) (Figure 2). The cytosolic supply of citrate is provided by the mitochondrial carrier (CIC), which plays a regulatory role in the production of inflammation intermediates. Experimental evidence for this regulatory role comes from the specific ablation of CIC using siRNA and CIC activity inhibition, thus limiting the export of citrate from mitochondria and greatly decreasing the production of proinflammatory prostaglandins, ROS and NO. Moreover, two functional NF-κB-responsive elements in the CIC promoter are involved in the upregulation of LPS-triggered macrophages. These findings indicate a key role of mitochondria-derived citrate in LPS induction (Infantino et al., 2011a; O'Neill, 2011; O'Neill and Hardie, 2013). Furthermore, the early gene activation of ACLY in LPS as well as in cytokine-induced macrophages suggests a primary role for cytosolic citrate, a substrate of ACLY, which could be a signal molecule in inflammation (Infantino et al., 2013).

The emerging role of citrate in immune cells is part of a growing body of literature that focuses on the interplay among immunity, inflammation, and metabolic changes. In fact, there is also evidence that other mitochondrial metabolites such as NAD^+ and succinate are signals regulating the immune system. In LPS-activated macrophages, although there is an overall decrease in TCA cycle activity, there is a marked increase in the TCA cycle intermediate succinate, in addition to citrate. The increase in succinate is from glutamine metabolism by glutamine-dependent anaplerosis and 'GABA shunt' pathway (Tannahill et al., 2013). Importantly, succinate acts as a signal in LPS-activated macrophages, leading to hypoxia inducible factor 1α activation under normoxia (Tannahill et al., 2013). LPS also leads to an increase in protein succinylation, although the consequence of these post-translational modification proteins is not yet understood. Another metabolite related to mitochondrial activity, NAD^+, seems to be an important signal of inflammation acting through NAD^+-dependent sirtuin deacetylases. In activated macrophages, NAD^+ decreases *via* mitochondrial damage, limiting the activity of the NAD^+-dependent deacetylase SIRT2 (Misawa et al., 2013). Recently, it has been shown that reduced levels of NAD^+

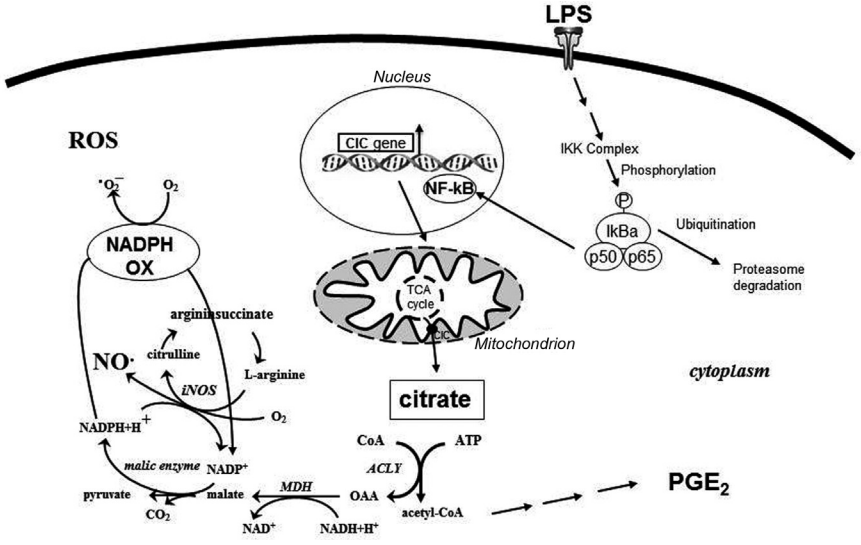

Figure 2 Role of citrate in inflammation.
Following LPS stimulation, activated IκB (inhibitory κB) kinases (IKKs) induce the phosphorylation of IκBs and the release of NF-κB. In the nuclei, NF-κB binds to NF-κB-responsive elements and activates target genes such as CIC, iNOS, NADPH oxidase (NADPH OX), COX2, and phospholipase A2 (PLA2). MDH, malate dehydrogenase; PGE2, prostaglandin E_2.

also controls inflammatory response by regulating SIRT1 activity. Anti-inflammatory action of SIRT1 is exerted by promoting a switch from glycolysis to fatty acid oxidation and by deacetylating and inactivating the p65 subunit of NF-κB, thus limiting the expression of NF-κB-dependent proinflammatory genes (Yeung et al., 2004; Liu et al., 2012). Altogether, these observations strongly indicate that the metabolic state of immune cells is critical to their function: proinflammatory cells such as M1 macrophages, activated dendritic cells, and T_H17 cells are more glycolytic, whereas anti-inflammatory cells such as T_{reg} cells and M2 macrophages have more oxidative phosphorylation (McGettrick and O'Neill, 2013). The meanings of these metabolic changes are not fully understood. The metabolites are not simply consequences of catabolism or anabolism, but they act as specific cell signals. Thus, by studying metabolic alterations in immune cells, we can better understand the pathogenesis of inflammatory diseases and consider novel treatments.

Citrate in cancer

In cancer cells, a reprogramming of cellular metabolism toward macromolecules synthesis is critical to supply enough nucleotides, proteins, and lipids to proliferate and build new cells. Due to cell cycle and proliferative signaling such as the PI3K/AKT pathway, glycolysis and mitochondrial enzymes are induced for the synthesis of anabolic precursors (Figure 3). Activated PI3K/Akt induces glucose uptake and promotes glucose carbon flux into biosynthetic pathways. Thus, glycolysis together with the pentose phosphate pathway becomes the best way to synthesize some metabolic intermediates for biosynthesis, such as ribose, glycerol, serine, NADPH, etc. However, the central aspect of the PI3K/AKT pathway oncogenic activity is the reprogramming of citrate metabolism (Bauer et al., 2005) because continuous export of citrate, together with other anaplerotic substrates, outside the mitochondria appears to be essential for cancer cell proliferation.

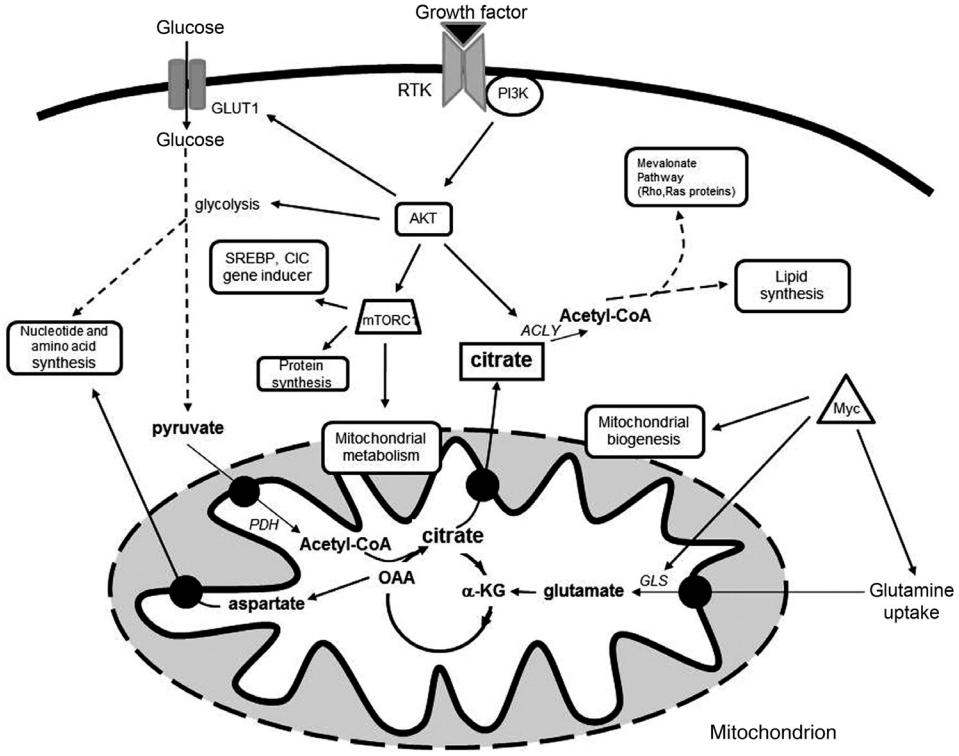

Figure 3 Central role of PI3K/Akt signaling pathway in reprogramming cancer cells metabolism.
PI3K/Akt signaling activates GLUT1, increases glucose uptake, stimulates glycolysis, and branches glycolytic metabolism (pentose pathway) to produce substrate for nucleotide, and amino acid synthesis. Akt stimulates ACLY, promoting a rapid conversion of citrate to acetyl-CoA needed for lipid biosynthesis. Downstream of PI3K/Akt pathway, mTORC1 promotes protein synthesis and mitochondria metabolism. Myc increases glutamine uptake and its conversion into mitochondrial carbon source (glutamate and α-KG) by promoting expression of glutaminase. Myc also promotes mitochondrial biogenesis and nucleotide and amino acid synthesis. RTK, receptor tyrosine kinase; GLUT1, glucose transporter 1; GLS, glutaminase.

Recently, Catalina-Rodriguez et al. (2012) demonstrated that CIC is much more expressed in cancer cells, suggesting an increased need for citrate in cancer cells. This metabolic requirement protects the cancer cells' mitochondria through the inhibition of glycolysis, promotion of mitochondrial OXPHOS and ATP production, and stabilization of the mitochondrial membrane potential. On the contrary, when the CIC gene is silenced by siRNA or the CIC activity is inhibited by BTA, its adaptive protection activity is abolished, the respiratory capacity is compromised, the ROS level increases, and the mitochondria are subjected to autophagy (Catalina-Rodriguez et al., 2012). As a consequence of CIC inhibition, the tumor cells die. Therefore, it is conceivable that CIC inhibitors could provide a platform for the design of a new class of anticancer drugs. Diversion of citrate from the TCA cycle to acetyl-CoA production is also induced by AKT, which activates ACLY (Bauer et al., 2005). Citrate-derived acetyl-CoA is also used in the mevalonate pathway leading to prenylation, a process required for the ability of Ras and Rho proteins to induce malignant transformation invasion and metastasis (Armstrong et al., 1995; Sebti, 2005). Increased production of acetyl-CoA does not promote ketogenesis. This would result in a lack of β-hydroxybutyrate, an inhibitor of HDAC, hence favoring histone deacetylase activity.

Glutamine has an important role in cancer metabolism (Figure 3). It is a carbon unit supplier in proliferating cancer cells because it provides both OAA and acetyl-CoA for citrate production (Reitzer et al., 1979; DeBerardinis and Cheng, 2010). The transcriptional factor Myc increases glutamine uptake and its conversion into glutamate by promoting expression of glutaminase. Glutamine also provides amine groups for other biosynthetic processes such as pyrimidine and purine synthesis.

Downstream of the PI3K/Akt pathway, mTORC1, a cell-growth regulator, promotes protein synthesis and mitochondria metabolism. Interestingly, SREBP, a key regulator of CIC gene expression (Infantino et al., 2007) that mediates lipogenesis, is a critical component of mTORC1-driven cell proliferation (Kim et al., 2006; Duvel et al., 2010). Another major target of mTORC1 is the hypoxia inducible factor 1 (HIF1), which is not crucial in mTORC1-driven cell proliferation. HIF1 stimulates the expression of many target glycolytic enzymes and blocks the use of pyruvate by mitochondrial PDH. By diverting pyruvate into lactate, the so-called aerobic glycolysis or Warburg effect (Warburg, 1956; Israel and Schwartz, 2011), HIF1 blocks carbon incorporation into mitochondrial citrate, which is critical for lipid biosynthesis. Therefore, the HIF1 conditions may exert some anti-proliferative effect as observed in hematopoietic and renal cells (Lum et al., 2007), which fits recent findings suggesting a tumor suppression action of HIF1 in some cancers (Shen et al., 2007).

Interestingly, an alternative pathway to maintain the level of citrate in cells proliferating in hypoxia and/or with HIF1 has been proposed. When glucose is diverted from mitochondrial acetyl-CoA and citrate production, proliferating cancer cells may use a reductive and carboxylating biosynthetic reaction from glutamine instead of the oxidative metabolism of both glucose and glutamine (Le et al., 2012; Metallo et al., 2012). In this pathway, glutamine provides a maintained citrate level *via* transformation of glutamine-derived α-ketoglutarate (α-KG) to isocitrate and citrate by means of mitochondrial isocitrate dehydrogenase 2 (IDH2) (Figure 4). This reaction is accomplished by NADH conversion to NADPH by mitochondrial transhydrogenase, and the resulting NADPH is used in α-KG carboxylation. The citrate exported to the cytosol may in part be metabolized in the oxidative direction by isocitrate dehydrogenase 1 (IDH1) and contribute to a shuttle that produces cytosolic NADPH (Ward and Thompson, 2012) (Figure 2). This shuttle promotes electron transport from mitochondria when the activity of electron transport chain is inhibited as in hypoxic conditions.

Tumor-specific mutations in IDH1 and mitochondrial IDH2 genes give rise to loss of their normal enzymatic activity, which interconverts isocitrate and α-KG. Mutations determine a novel reductive activity to convert α-KG to 2-hydroxyglutarate (2-HG), a rare metabolite found in only trace amounts in mammalian cells under normal conditions but in high concentrations in cancer cells (Dang et al., 2009). Although it still remains unclear if 2-HG is truly a pathogenic oncometabolite resulting from IDH mutations or if it is just the byproduct of a loss-of-function mutation, its measurement in primary tumors with unknown IDH mutation status could serve as a biomarker for both IDH1 and IDH2 mutations (Ward and Thompson, 2012). Concerning the biological role of 2-HG, some hypothesis have been proposed. Evidence from myeloid cells overexpressing IDH mutants (Figueroa et al., 2010) supports the hypothesis that accumulation of 2-HG may impair cellular differentiation. Other reports highlight the inhibitory role of 2-HG on the α-KG-dependent dioxygenase enzymes such as the TET family, which hydroxylates 5′-methylcytosine (Figueroa et al., 2010; Xu et al., 2011) and the Jumonji C-domain histone demethylases (Chowdhury et al., 2011), thereby impairing normal epigenetic regulation. This results in altered histone methylation marks and dysregulated cellular differentiation. Taking into consideration that the CIC gene has previously been implicated in epigenetic and cancer biology (Morciano et al., 2009; Wellen et al., 2009; Catalina-Rodriguez et al.,

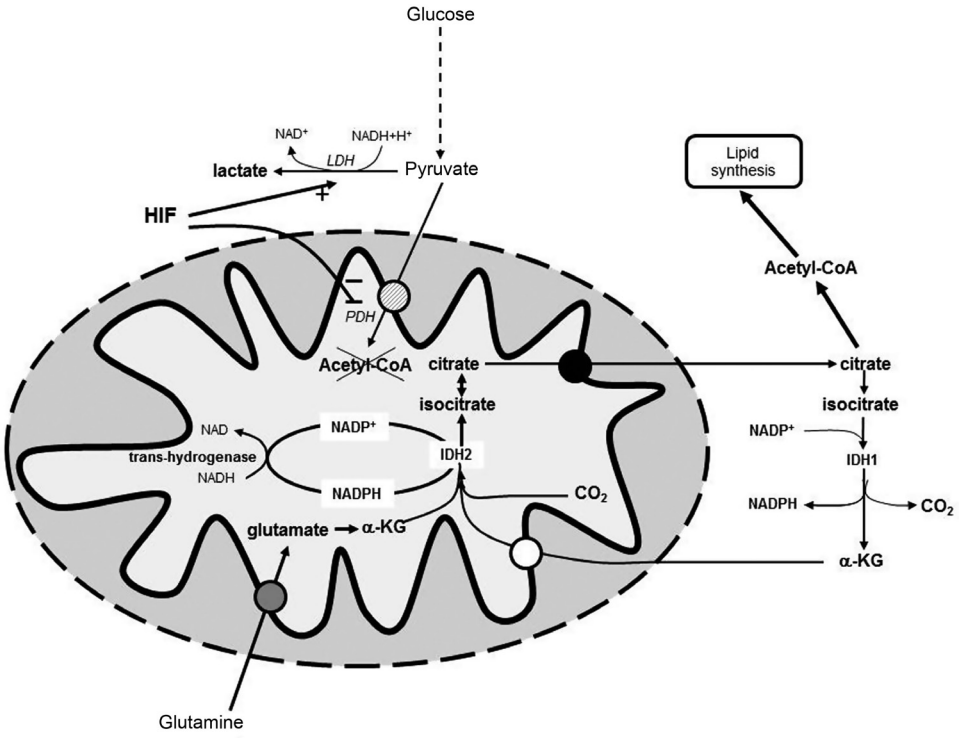

Figure 4 Citrate synthesis *via* reductive carboxylation.
In proliferating cells and/or HIF-1 activation, glucose is diverted from acetyl-CoA and citrate production. Citrate levels can be maintained through the flux of glutamine-derived α-KG, which is carboxylated to isocitrate and citrate by the mitochondrial isoform of IDH2. This mitochondrial flux can be accomplished by NADH conversion to NADPH by mitochondrial transhydrogenase and the resulting NADPH use in α-KG carboxylation. Citrate is exported to the cytosol, where it is in part used for lipogenesis, and some may be metabolized in the oxidative direction by IDH1 and contribute to a shuttle that produces cytosolic NADPH.

2012) and that CIC gene defect causes elevation of 2-HG (see section Citrate in Neurodevelopmental Syndromes and Hydroxyglutaric Aciduria), the previous hypothesis needs to be further focused and investigated. It is interesting to note that 2-HG can also increase in the absence of metabolic enzyme mutations. For example, in human cells proliferating in hypoxia, α-KG can accumulate and be metabolized by the action of mitochondrial IDH2, leading to 2-HG accumulation in absence of IDH mutations (Wise et al., 2011). The ability of 2-HG to alter epigenetics may reflect its evolutionary ancient status as signal for elevated oxygen deficiency.

Finally, beyond the 2-HG involvement as oncometabolite, others Krebs cycle-related enzymes have previously been identified as oncometabolite. Mutations in SDH and fumarate hydratase (FH) give rise to increase of succinate and fumarate levels in renal carcinoma, pheochromocytoma, and paraganglioma (Isaacs et al., 2005; Lee et al., 2005). However, the role of these metabolites in tumorigenesis is not clear. It is not excluded an involvement of alteration of histone demethylase activity, as found by Smith et al. (2007) in yeast. However, these findings highlight an increasing involvement of TCA cycle enzymes and metabolites in tumor formation.

Citrate in insulin secretion

In β cells, glucose generates stimulus/secretion factors that are responsible for the control of insulin secretion. One component of the signaling pathway involves glucose-induced increase in the cytosolic ATP/ADP ratio, which suppresses ATP-sensitive K^+ channels and activates voltage-gated Ca^{2+} channels (MacDonald et al., 2005). In addition to this mechanism, different studies have demonstrated the important role of pyruvate metabolism in cyclic pathways, in particular, the pyruvate/isocitrate cycle in the control of insulin secretion (Jensen et al., 2008). Studies performed on CIC in rat insulinoma-1 (INS-1) cells indicate the important role played by the isocitrate/pyruvate cycle in the control of glucose stimulated insulin secretion (GSIS) (Figure 3). This cycle involves the exit of citrate and isocitrate from the mitochondria *via*

CIC. In the cytosol, citrate can be converted to isocitrate by cytosolic aconitase. Isocitrate is then transformed to α-KG by a cytosolic NADP-dependent isocitrate dehydrogenase and can be cycled back to pyruvate by mitochondrial and cytosolic pathways (Palmieri, 2004; Castegna et al., 2010). Inhibition of CIC activity by BTA in INS-1-derived 832/13 cells or primary rat islets and suppression of CIC expression *via* a recombinant adenovirus significantly inhibit GSIS in 832/13, whereas overexpression of CIC enhances GSIS and increases cytosolic citrate levels (Ronnebaum et al., 2006). α-KG derived from isocitrate can serve as a direct signal for insulin secretion. In fact, the silencing of isocitrate dehydrogenase, which transforms isocitrate into α-KG, impairs GSIS in INS-1 derived 832/13 cell line as well as in primary rat islets (Ronnebaum et al., 2006).

From studies performed on abnormal insulin secretion induced by the antipsychotic drug clozapine, we have further demonstrated the involvement of citrate as a signaling molecule and CIC in insulin secretion through its transcriptional regulator FOXA1 (Menga et al., 2013). Silencing of FOXA1 significantly reduces CIC expression at mRNA and protein levels and abolishes abnormal insulin secretion induced by clozapine at low glucose in INS-1.

Recently, a regulatory role of CIC in GSIS in human-ejaculated spermatozoa has been demonstrated by Cappello et al. (2012). Incubation of spermatic cells with a high glucose concentration (16.7 mM) and 2.0 mM of the CIC inhibitor BTA significantly inhibited GSIS. Interestingly, CIC inhibition reduced sperm hyperactivated motility and acrosome reaction. Moreover, incubation of sperm cells with citrate induced insulin secretion and triggered the activity of these sperms.

Citrate in acetylation of histones

Chromatin remodeling through histone modification greatly affects the accessibility of DNA and regulates specific gene expression (Sterner and Berger, 2000; Kurdistani and Grunstein, 2003; Li et al., 2007). Among the modifications, acetylation and deacetylation processes, catalyzed by classes of histone acetyltransferases and histone deacetylases, respectively, are the most common modifications. The acetylation process is dynamically regulated by physiological changes in concentration of acetyl-CoA derived from the citrate exported by CIC outside the mitochondria and produced by ATP citrate lyase. Experimental evidence indicates a significant link between cellular metabolism and histone acetylation. Wellen et al. (2009) demonstrated that acetyl-CoA derived from glucose metabolism and ACLY generate a chromatin modification and a signal for activating a glycolytic enzymes, such as hexokinase 2, phosphofructokinase, and lactate dehydrogenase. Inhibition of ACLY significantly decreases the amount of histone acetylation (Wellen et al., 2009). However, the acetylation of histones does not depend on ACLY activity but is linked to the cytosolic citrate pool, suggesting that a crucial role is performed by the CIC as a citrate supplier. Morciano et al. (2009) demonstrated that mutations in CIC, which impairs transport of citrate from mitochondria, cause a very significant reduction of histone acetylation. Moreover, the use of siRNA reduces CIC gene expression by 70% and leads to chromosome breaks in *Drosophila* and human primary fibroblasts. Such breakage is rescued by treating mutant cells with citrate and TSA, an inhibitor of HDAC (Morciano et al., 2009).

Citrate in neurodevelopmental syndromes and hydroxyglutaric aciduria

Different defects of early brain development have been associated with defects of mitochondrial respiratory chain and Krebs cycle, including PDH deficiency, *SLCA25A19* defect, aconitase deficiency, fumarate deficiency, and complex I assembly defect (Rosenberg et al., 2002; Miller et al., 2004; Sugiana et al., 2008; Mroch et al., 2012). Recently, agenesis of the corpus callosum, a birth defect that occurs in different human congenital syndromes, and optic nerve hypoplasia have been associated with mutations in the *SLC25A1* gene (MIM 190315) (Edvardson et al., 2013). Two mutations (G130D and R282H), located in highly conserved positions of CIC through evolution, significantly affect protein function, as demonstrated by experiments performed with a yeast strain harboring human CIC mutations at equivalent positions in the orthologous yeast protein, which exhibits a growth defect under stress condition and a marked loss of citrate transport activity in reconstituted liposomes (Edvardson et al., 2013). As consequence of loss or marked reduction of CIC activity, citrate/isocitrate cytosolic level, TCA cycle flux, and NADPH content decreased, but oxidative stress and ROS toxicity increased. The metabolic derangement was also shown by the organic acid profile of patient's urine, which showed an increased amount of Krebs cycle intermediates (α-KG, malate, fumarate, succinate) accompanied by decreased levels of citrate/isocitrate and prominent presence of 2-HG. Increased 2-HG is most likely caused by the inability of

the mitochondria to export citrate and isocitrate into the cytosol, giving rise to increased mitochondrial concentration and subsequent higher excretion of intermediates downstream isocitrate. Although the source of 2-HG is not fully understood, experimental evidences indicate that IDH1 and IDH2 have a role in the production of D-2-HG (Matsunaga et al., 2012). D-2-HG and its enantiomer L-2-HG are linked to the Krebs cycle (Raimundo et al., 2011) and are found to be elevated in certain types of cancer (Dang et al., 2009; Rakheja et al., 2011) and in different variants of the neurometabolic 2-hydroxyglutaric aciduria. In relation to the latter defect, a recent study from Nota et al. (2013) reported 12 recessive mutations (Ser193Trp, Arg282Gly, Arg282Cys, Gly167Arg, Pro45Leu, Glu144Gln, Met202Thr, Tyr297Cys, Tyr256*, Ala9Profs*82, A274Ilefs*24, and Arg-173Glyfs*2) found in the CIC gene of three individuals and their families that cause combined D-2- and L-2-hydroxyglutaric aciduria. It is known that D-2-hydroxyglutaric acid is caused either by mutations in D-2-HG dehydrogenase (type I, MIM 6000721) or by recurrent *de novo* dominant gain-in-function mutations in IDH2 (type II, MIM 613657). Based on these findings, in addition to detection of mutations in the D-2-HG dehydrogenase gene and isocitrate dehydrogenase, detection of CIC gene mutations should be included in the analysis of patients with neurometabolic diseases, such hydroxyglutaric aciduria. All patients with CIC gene defects showed an impaired mitochondria citrate efflux, demonstrated by stable isotope labeling experiments and the absence or marked reduction of CIC activity in fibroblasts. Moreover, significant reduction of citrate/isocitrate accompanied by increased level of several Krebs cycle intermediates and D,L-2-hydroxyglutaric acid were found in the patient' urinary organic acid profile. This new finding provides new basis in understanding the pathophysiology of this disease.

Citrate in NAFLD

The plasma level of citrate is increased in patients with NAFLD (101–210 vs. 52–106 μM). This effect is a consequence of high levels of free fatty acids and glucose associated with metabolic syndrome. In combination with hydrogen peroxide, elevated citrate levels promote oxidative stress, whereas citrate alone has no effect on oxidative stress, suggesting that citrate acts only indirectly (van de Wier et al., 2013). The molecular mechanism of this interaction is not clear. It is likely that citrate stimulates hydroxyl radical formation from hydrogen peroxide in the presence of iron through the formation of an iron-citrate complex (Gutteridge, 1990). Because iron is stored as ferritin, it has to be released to promote radical formation. Release requires chelation of iron with citrate (or other chelating agents), suggesting that citrate could be an iron recruiter rather than a direct radical inducer (Goddard et al., 1992; Reif, 1992).

Interestingly, citrate levels are also high in patients with aspartate/glutamate carrier deficiency (Kobayashi et al., 2003; Wibom et al., 2009). The increase of citrate levels may be due to an upregulation of CIC to compensate for the aspartate/glutamate carrier impairment (van de Wier et al., 2013).

Citrate as a therapeutic drug

Because citrate is an essential intermediate located at the crossroad of metabolism and a key regulator of energy production, its use in cancer treatment has been hypothesized and tentatively tested. An excess of citrate in cancer cells would inhibit all PFK isoforms, leading to an arrest of glycolysis, inhibition of all ATP production pathways, and stimulation of ATP-consuming pathways. This metabolic condition would result in ATP depletion, leading to arrest of cancer cell growth and cell death (Zhang et al., 2009; Lu et al., 2011). MSTO-211H mesothelioma cells continuously exposed to citrate show a down-proliferation or cell death, depending on the citrate concentration. When citrate is removed from the medium, culture cancer cells restart to grow. Interestingly, this regrowth is hampered in presence of the drug cisplatin, suggesting a synergistic effect between citrate and cisplatin (Zhang et al., 2009). The mechanism of this effect is not completely known. It could be that depletion of ATP, required for DNA repair, could lead to persistent DNA damage and to the sensitization of cells to cisplatin (Zhang et al., 2009; Lu et al., 2011). Moreover, citrate could also sensitize cells to cisplatin through the inhibition of the anti-apoptotic protein Mc1-1 and Bcl-x_L, which are overexpressed in mesothelioma cells (Willis et al., 2005; Warr and Shore, 2008). Recently, a lethal effect of citrate in different cell lines (Tet21N, Sk-N-SH, and U1810), through the activation of caspases 8 and 2, has been demonstrated (Kruspig et al., 2012).

For this reason, administration of citrate is also considered as a 'new targeting metabolism strategy' (Vander Heiden, 2011).

Citrate as secretory end product

Among all mammalian organs, the prostate possesses the particular feature of accumulating high amounts

of citrate, reaching up to 180 mM in the prostatic fluid (Kavanagh, 1994; Mazurek et al., 2010). This capability, which does not exist in other mammalian cells, is referred to as 'net citrate production'. Therefore, the intermediary metabolism of prostatic cell is modified by their specialized function of net citrate production. Whereas two of the six carbons from citrate are stored as OAA and two are lost as CO_2 in the normal mammalian metabolism, all six carbons are removed from the metabolic pools as an end product of metabolism in the citrate-producing prostatic cells. Consequently, a continuous supply of acetyl-CoA and OAA is requested. Acetyl-CoA derives from glucose oxidation and OAA from aspartate *via* transamination. The high aspartate level is maintained in cells by the contribution of the aspartate transport process from the blood by a Na^+-coupled co-transporter EAAC1 (Franklin et al., 2006). However, to maintain this high cellular level, citrate oxidation must be inhibited. This is achieved by the mitochondrial accumulation of zinc (3000–5000 nmol/g compared with 200–400 nmol/g in other tissues), which inhibits *m*-aconitase activity, and further oxidation of citrate in the Krebs cycle. Furthermore, to conserve cytosolic citrate for secretion, the ACLY activity is low, considering also that lipogenesis and cholesterologenesis are quite low in prostatic cells.

Unlike the level of citrate in normal prostate cells, it is significantly reduced in prostate cancer. This change is due to a metabolic shift of neoplastic cells that become citrate oxidizing with respect to normal prostatic cells that possess a low citrate-oxidizing capability (Costello et al., 1999).

Citrate as a biomarker

The drastic decrease of citrate level (up to 40-fold) in prostate cancer with respect to normal tissue is a hallmark to distinguish between normal and hyperplastic glands (Costello and Franklin, 2000, 2006), suggesting that citrate could be used as biomarker. At present, a simple evaluation of the citrate level is not enough to prove the reliability and specificity of prostatic fluids citrate levels for diagnosis of prostate cancer and more extensive clinical studies are necessary. Instead, prostate metabolic profile methods have been developed. MRSI has been developed to generate a prostate metabolic profile displaying levels of citrate and others metabolites such as creatine, choline-containing molecules, and polyamines (Hricak, 2005). Because citrate levels decrease and choline increase in prostate cancer (Gillies and Morse, 2005), the basic method for diagnosing prostate tumor by MRSI is based on the increase of the total choline/citrate ratio. A ratio ≥ 0.86 is associated with a definite prostate cancer (Scheidler et al., 1999). Another technique, magic angle spinning nuclear magnetic resonance, is more sensitive in detecting significant decrease of citrate and polyamine levels directly in intact tissue (Swanson et al., 2006).

Conclusion and perspectives

Citrate is clearly an important molecule essential for cellular metabolism because it regulates different key metabolic enzymes. The different roles of citrate, described in this review, indicate that metabolism is not a simply way to transform and store energy, but it is an integrated system whose intermediates, such as citrate, are involved in normal and pathological processes, such as inflammation, cancer, epigenetics, insulin secretion, oxidative stress, neurological and neurometabolic defects, etc. Therefore, insight to changes in metabolism that occur in metabolic diseases, inflammation, and cancer may improve the understanding of disease pathogenesis and point out the way for new therapeutic strategies. The different functions ascribed to citrate highlight these aspects and suggest other routes of investigation.

For example, an important question concerns histone acetylation. Experimental evidence indicates a significant link between cellular metabolism and supply of acetyl units derived from citrate for histone acetylation. As an acetyl supplier, citrate could also play a role in adjusting the expression of a number of key regulatory enzymes. Therefore, it would be interesting in the future to establish whether the link among citrate levels, citrate transport, chromatin epigenetic modifications, and genome stability may be pivotal for the understanding of specific human genetic diseases, including cancer.

Another important point concerns the involvement of citrate in inflammation and cancer suggesting that substrates of metabolism may act as signaling molecules in these pathologies. It will be very interesting to investigate on the involvement of citrate (or other metabolites) and CIC (or other mitochondrial carriers) with different human pathogenic conditions hallmarked by mitochondrial oxidative damage and by alteration of mitochondrial turnover. For example, the database derived from Parkinson's disease shows statistically significant alteration of CIC expression levels in various regions of the brain compared with normal healthy control individuals

(Catalina-Rodriguez et al., 2012). The discovery of involvement of the citrate pathway in inflammation and cancer might lead to the development of a new class of drugs that alone or in combination with other metabolic modulators can be used in anti-cancer or anti-inflammatory treatment. Moreover, a close link among citrate, 2-HG, and cancer is emerging, but very few information is still available. Thus, further research is needed to unravel how SLC25A1 defects cause elevation of 2-HG and to elucidate its clear role in cancer.

Considering that TCA metabolites (succinate, fumarate, citrate, α-KG-derived HG), enzymes (SDH, FH, and IDH), and TCA-related proteins (CIC) are involved in tumor formation, many questions remain to be investigated. For example, how the TCA cycle operates in different cells, different tissues, and even under different conditions in the same cell. Genomic and metabolomic methodologies make accessible the study of the TCA cycle metabolites and enzymes occurring in the human body, both physiological and pathological. Moreover, the extra-metabolic role of the TCA metabolites needs to be elucidated. It is possible that TCA cycle metabolites have the ability to modulate the activity signaling cascades or transcription factors, thus affecting regulatory effects on several cellular decisions.

A not fully understood question is the GSIS process in which citrate is involved. Different studies indicate the importance of anaplerotic metabolism for glucose and pyruvate cycling pathways as key mediators of GSIS. This suggests that enzymes, proteins, and metabolites, such as citrate, that regulate pyruvate cycling are evaluated for their potential role in β-cell failure and diabetes or as targets for enhancing β-cell functions. Furthermore, if the pyruvate cycle pathways are as important in the regulation of GSIS in human islets as they appear in rat islets needs to be evaluated.

In addition, other aspects of citrate function, such as its role in NAFLD and its increase in aspartate/glutamate carrier deficiency, need to be further investigated. In fact, patients with aspartate/glutamate carrier deficiency can develop NAFLD and NASH without presenting other features of metabolic syndrome. How these patients develop NAFLD is unknown, but the radical-promoting potential of citrate might come into play in the pathogenesis of this disease.

In conclusion, although some aspects of citrate functions have been elucidated, others need further investigations, especially considering metabolism as an integrated system involving coordinate functions in different tissues.

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