Citrate transport and metabolism in mammalian cells

Prostate epithelial cells and prostate cancer

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Citrate, an organic trivalent anion, is a major substrate for generation of energy in most cells. It is produced in mitochondria and used either in the Krebs' cycle or released into cytoplasm through a specific mitochondrial carriers. Citrate can also be taken up from blood through different plasma membrane transporters. In the cytoplasm, citrate can be used ultimately for fatty acid synthesis, which is increased in cancer cells. Here, we review the ways in which citrate can be transported and discuss the changes in transport and metabolism that occur in cancer cells. The primary focus is on the prostate gland, which is known to produce and release large amounts of citrate during its normal secretory function. The significant changes that occur in citrate-related metabolism and transport in prostate cancer are the second focus. This review strives to relate these mechanisms to molecular biology on the one hand and to clinical applications on the other.

Keywords: citrate; membrane transport; prostate

Abbreviations: BPH, benign prostatic hyperplasia; c-ACNT, cytosolic aconitase; c-ICD, cytosolic isocitrate dehydrogenase; CTP, citrate transporting protein; FA, fatty acid; FAS, fatty acid synthase; IRP, iron regulatory protein; KCitT, K^+-dependent citrate transport mechanism; m-ACNT, mitochondrial aconitase; MAS, 'magic angle' spinning; MCT, monocarboxylate transporter; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; MRSI, magnetic resonance spectroscopy imaging; NaCitT, Na^+-dependent citrate transport mechanism; NMR, nuclear magnetic resonance; PCa, prostate cancer; pH_o, extracellular pH; $_pH_1$, luminal $_pH$; PSA, prostate-specific antigen; TCA, tricarboxylic acid cycle; VGSC, voltage-gated Na^+ channel.

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Introduction

A group of organic anions that include dicarboxylates (*e.g.* succinate and α-ketoglutarate) and tricarboxylates (*e.g.* citrate and isocitrate) are intermediates of the Krebs' cycle. Citric acid is a tricarboxylic acid with pK_a values (pH at which dissociation of H^+ occurs) of 2.9, 4.3 and 5.6. Accordingly, in blood, citrate acid exists predominantly as a trivalent anion, $citrate^{3-}$. Change to a divalent anion occurs in acidic media, *i.e.* pH of less than 7.[1] Citrate may play different, tissue-specific metabolic roles, as well as function as a general chelator of physiologically important cations such as Ca^{2+}, Zn^{2+} and Mg^{2+}.

Citrate is a weak acid that plays an important role in metabolic energy production in the mitochondrial Krebs' (or tricarboxylic acid (TCA)) cycle. In addition, citrate is a source of NAD^+ required for the glycolytic pathway. It also provides acetyl-CoA in the synthesis of fatty acids (FAs) and sterols.[2] In most cells, FAs are taken up from circulation, whereas cancer cells synthesise them *de novo* through the overexpressed FA synthase (FAS). Additionally, NADPH necessary for FA production may be supplied from citrate through the action of cytosolic aconitase (c-ACNT) and cytosolic isocitrate dehydrogenase (c-ICD).[3] Finally, citrate is also an allosteric inhibitor of phosphofructokinase-1 that functions as a rate-limiting enzyme in glycolysis.[4] Intracellular citrate levels indicate the energy status of the cell; high levels of intracellular citrate slow down glycolysis by inhibiting phosphofructokinase-1 (thus decreasing energy production) and lead to storage of excess energy in FAs.

In normal cells, the TCA cycle and oxidative phosphorylation are both central to metabolic energy production. Glucose is taken up from blood and degraded to pyruvate through glycolysis. Pyruvate is then introduced into the TCA cycle.[5] The TCA cycle is crucial for normal cellular functioning and its

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dysfunction causes a range of diseases, including neurodegeneration and cancer.[6] In contrast to most normal cells, where mitochondria produce the majority (>90%) of ATP, in cancer cells there is a shift in energy production from oxidative phosphorylation to 'aerobic glycolysis', called the 'Warburg effect'.[7] This change is a fundamental property of cancer cells.[8] Pyruvate induces cell death specifically in cancer cells.[9] There are two ways by which cancer cells can keep pyruvate levels low: oxidation of pyruvate in mitochondria or conversion of pyruvate into lactate. Cancer cells opt for the second pathway, possibly because of the reduction in the mitochondrial TCA cycle associated with malignant transformation. The basic pathways of cellular citrate transport and metabolism, relating to this review, are illustrated in Fig. 1.

In this essay, we (i) focus on known citrate transporters and transport mechanisms expressed in cell membranes and (ii) review the physiological and pathophysiological roles of citrate in different organs. Involvement of citrate in cytosolic metabolism involving FA synthesis is discussed. This is known to be important in many types of cancer, in particular of the prostate, which produces and releases the highest amount of citrate. Prostatic citrate transport has been physiologically and pharmacologically characterised, and molecular aspects are questioned. We also discuss the decrease in citrate that is crucial for prostate cancer (PCa) progression and changes in citrate transport and metabolism in metastatic PCa cells. Finally, we focus on citrate-based imaging methods, aimed at early/functional diagnosis of PCa clinically.

Citrate transporters

In this section, we present an overview of the basic characteristics of citrate transporters operating in mitochondria and plasma membrane.

Mitochondrial transport of citrate

Citrate, produced in mitochondria, can either be used for ATP production or transported out of mitochondria into cytoplasm and used in FA and sterol synthesis (Fig. 1). Citrate transport across membranes is strictly regulated. Citrate flow through the inner mitochondrial membrane occurs *via* a citrate transporting protein (CTP) belonging to the SLC25 gene family.[10] All ~20 members of this family have similar structures[11] and are thought to be derived from the same gene by duplication of an internal domain of 100 amino acids.[12] CTP consists of 298 amino acids and a 'presequence' of 13 amino acids, with a total molecular mass of ~32.5 kDa.[12]

CTP catalyses electroneutral exchange of citrate or isocitrate for a dicarboxylate or phosphoenolpyruvate. In an intact cell, the exchange involves the efflux of the divalent citrate (H-citrate^{2-}) from the mitochondrial matrix and the influx of the divalent malate into the matrix.[11] CTP can be inhibited by benzenetricarboxylate and other agents targeting specific residues, including pyridoxal-5-phosphate (for lysine), diethyl pyrocarbonate (for histidine) and mersalyl acid (for sulphydryl groups).[13] Once exported from

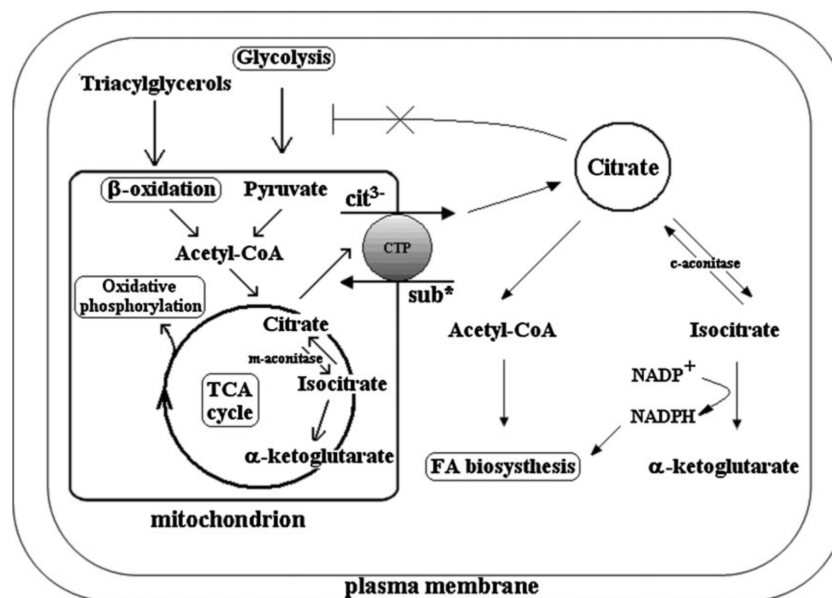

Figure 1. Schematic model of a composite cell showing the various components of the citrate system. TCA (tricarboxylic acid cycle) operates within the mitochondrial matrix whilst CTP (citrate transporting protein) operates in the inner mitochondrial membrane (sub*, exchangeable anion such as malate). Citrate from the pool can be used in many metabolic pathways shown on the figure and described in the Introduction; FA stands for fatty acids. Line with a cross shows inhibiting effect of citrate on glycolysis.

mitochondria, citrate serves as a substrate to ATP-citrate lyase, which converts it into oxaloacetate and acetyl-CoA. In turn, acetyl-CoA is an obligatory substrate for FA and sterol syntheses.[11]

Plasma membrane transporters

Citrate and other TCA cycle intermediates can be taken up from blood *via* transporters of the SLC13 gene family. Such transporters have been found in many different organs including kidney, intestine, liver, placenta and brain.[14–17] In mammals, there are three main plasma membrane transporters: NaDC1, NaDC3 and NaCT.[18] These transporters have broad substrate specificities for a wide range of TCA cycle intermediates.[18] The cellular uptake of di/tricarboxylates is 'energised' by the inward electrochemical gradient of Na^+. There is a relatively high concentration of citrate in blood ($\sim$135 μM) compared to dicarboxylates like succinate ($\sim$40 μM), while the concentrations of other Krebs' cycle intermediates are even lower.[2]

NaDC1 and NaDC3 transport mainly dicarboxylates with low and high affinity, respectively; tricarboxylates can be transported in protonated (*i.e.* divalent) form.[19] Tissue expression of NaDC1 and NaDC3 depends on the functional role of the transported intermediate. The high-affinity Na^+/dicarboxylate co-transporter (NaDC3) is found on the basolateral membrane of rat renal proximal tubules,[20] human and rat placental brush border,[16,21] rat and mouse brain synaptosomes[22] and chick intestinal cells.[23] The low-affinity Na^+/dicarboxylate co-transporter (NaDC1) is expressed mainly in the apical membrane of the tubular epithelial cells in kidney.[24]

NaCT, the only membrane transporter specifically recognising the trivalent form of citrate, was originally cloned from rat and mouse brain[25,26] and the human liver cell line HepG2.[17] Its expression is most predominant in liver and testis. In human brain, NaCT expression is restricted to neurones.[27] In liver, NaCT is expressed in sinusoidal membranes of hepatocytes.[28] NaCT can also transport divalent intermediates of the TCA cycle but with lower affinity.[17,25]

Stoichiometry

NaDC1, NaDC3 and NaCT are all electrogenic, with net movement of one positive charge across the membrane into cells per transport cycle.[29] The stoichiometry depends on the valency of the transported carboxylate; three Na^+ to one dicarboxylate for NaDC1 and NaDC3 and four Na^+ to one citrate for NaCT.[25]

Sensitivity to H^+ and Li^+

Di/tricarboxylate transporters exhibit differences in their response to changes in extracellular pH (pH_o), which could affect transport activity by changing the valency of the substrate. Human NaDC3 increases citrate transport severalfold when pH_o is acidified to 6.5.[1,15,30] On the other hand, succinate uptake *via* NaDC3 exhibits a pH_o optimum of 7.0–7.5, consistent with NaDC3 recognising succinate and citrate in divalent forms. NaDC1 also shows similar differential dependence on pH_o for citrate *versus* succinate.[19,20] NaCT is influenced by pH_o, but in a species-dependent manner. Rodent NaCT activity is reduced when pH_o is acidified, while alkalisation has no effect.[25] This could be due to change in the valency of citrate in acidic pH_o. In contrast, citrate transport by human NaCT is stimulated by acidification of pH_o despite the fact that this transporter normally prefers the trivalent citrate. A similar species-specific effect is seen with Li^+, which inhibits rodent NaCT but stimulates human NaCT.[30] It is likely that H^+ and Li^+ compete for the same modulatory site and increase the affinity of the transporter to citrate.[30] Li^+ is also an inhibitor of NaDC1 and NaDC3 in all animal species studied.[19,29]

Unlike for the mitochondrial and plasma membrane di/tricarboxylate transporters, the molecular nature of the mechanisms for the prostatic citrate transport is not known. The available physiological and pharmacological data are discussed in the following and a subsequent section, with a view to gaining an insight on the molecular biology.

Cellular physiology of citrate in prostate

The prostate is a unique organ that produces, accumulates and releases large amounts of citrate into prostatic fluid, the concentration of which can reach up to 180 mM.[31] This supplies sperm with the source of energy necessary for their vitality and motility. Sperm have a significantly increased production of ATP when incubated with citrate.[32] NaCT mRNA is present at high levels in testis and it is possible that expression in this tissue is restricted to germ cells. The unusually high level of citrate production in prostatic epithelial cells is due to the low activity of mitochondrial aconitase (m-ACNT), which prevents metabolism of citrate *via* the TCA cycle.[33] m-ACNT expression is upregulated by testosterone and prolactin, whereas its activity is suppressed by Zn^{2+}.[33]

Citrate transport mechanisms in prostatic epithelial cells

In this review, 'transport mechanism' is used as a term (rather than 'transporter') where the gene responsible for the transport process has not yet been identified.

Using a whole-cell patch clamp, in which citrate was introduced into cells from the recording pipette and allowed to efflux naturally, we have characterised electrophysiologically an electrogenic citrate transport mechanism in human

normal prostate epithelial PNT2-C2 cells.(34) Unlike the Na^{+}-dependent transporters of the SLC13 gene family, the citrate transport mechanism in the plasma membrane of prostatic cells is coupled to K^{+} and, indeed, high extracellular K^{+} reduces citrate efflux and increases citrate uptake.(34) This mechanism is designed primarily to transport citrate in outward direction.(34,35) This putative transporter has been called 'KCitT1' (K^{+}-dependent citrate transport mechanism) (Figs. 2a). An assessment of the stoichiometry suggests a coupling ratio of four K^{+} to one $citrate^{3-}$, consistent with the electrogenic nature of the transport mechanism.(34) Earlier recordings from rat prostate *ex vivo* revealed the presence of a negative (~ -10 mV) lumen potential, which was particularly sensitive to citrate, and could be related to KCitT1 activity.(36) It is not known if a similar lumen potential exists in human prostate. Using chamber recordings from monolayers of PNT2-C2 cells support the K^{+} dependence of citrate transport and, in addition, suggest that the transport mechanism is localised on the membrane (presumed apical) opposite to that containing the aspartate transporter (Fig. 2b).(35)

System organization and ionic cycling

From the studies on citrate metabolism and ionic activity in human and rat prostate secretory epithelia, a functional cellular model can be put forward (Figs. 2b): Aspartate necessary for the citrate production is absorbed from blood into cells by a Na^{+}-coupled co-transporter–EAAC1.(37) The excess Na^{+} is dissipated *via* a Na^{+}/K^{+}-ATPase; EAAC1 and Na^{+}/K^{+}-ATPase are located in the basolateral membrane.(38) Citrate is produced in mitochondria and released into cytoplasm by CTP.(11)

Citrate is transported out of cells into prostatic fluid using the electrochemical energy present in the transmembrane K^{+} concentration gradient. This gradient is maintained by the Na^{+}/K^{+}-ATPase in the basolateral membrane.(38) The K^{+}-citrate co-transport mechanism is electrogenic and hyperpolarises the apical membrane.(39) The inside-negative membrane potential is expected to impede the efflux of citrate *via* this K^{+}-citrate co-transport mechanism, but the involvement of four K^{+} in the transport process will overcome this negative effect of membrane potential and actively pump citrate out of the cell against a concentration gradient. This process is facilitated further by the re-entry of K^{+} back into the cells *via* a H^{+}/K^{+}-ATPase localised in the apical membrane.(40) The accompanying H^{+} efflux causes some acidification of the luminal fluid (luminal pH (pH_l) $\sim$6.5).(41)

The re-entry of K^{+} would prevent the build-up of K^{+} concentration in the lumen, thus maintaining the transmembrane concentration gradient for this ion across the apical membrane as the driving force for the K^{+}-citrate co-transport process. Further, re-absorption of K^{+} by the cells could occur *via* K^{+} channels controlled by membrane voltage and/or Ca^{2+}.(34,42) Indeed, the K^{+} channel blockers 4-aminopyridine has been found to suppress the citrate-induced membrane current.(34)

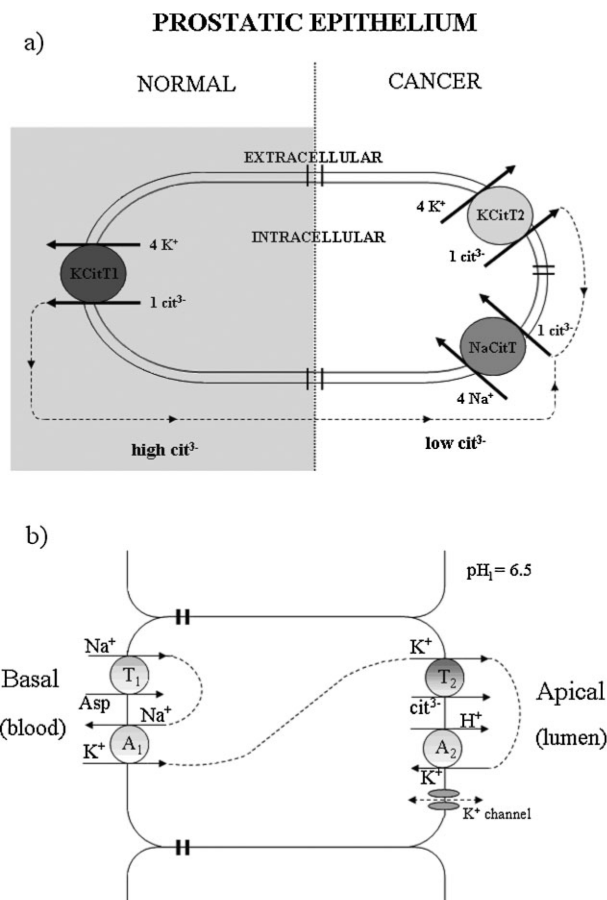

Figure 2a. Schematic comparison of intracellular and extracellular citrate levels and transporter mechanisms expressed in normal and cancerous/metastatic prostatic epithelial cells, modelled by PNT2-C2 and PC-3M cells (gray and white areas, respectively). Normal cells release citrate *via* KCitT1 and the extracellular level of citrate is high (~13,000 nmol/g wet weight). Cancer cells, which may release less citrate (*via* KCitT2), take it up from extracellular space *via* NaCitT. Consequently, the extracellular level of citrate in prostatic tumour tissue is much lower (<500 nmol/g wet weight). The citrate released by KCitT1/2 into the system may be taken up by NaCitT (dashed lines). The proposed transport mechanisms may be expressed in separate cell populations within the same tumour mass.
b. A model of possible ionic 'recycling' pathways associated with K^{+}-dependent release of citrate in normal prostatic epithelial cells. The system incorporates activities of citrate and aspartate transporters, Na^{+}/K^{+}- and H^{+}/K^{+}-ATPases and K^{+} channels. The proposed ionic cycling is consistent with the electrolytic characteristics of blood and prostatic fluid. The data have been integrated from published works(31, 34, 35, 38, 45). A1, Na^{+}/K^{+}-ATPase. A2, K^{+}/H^{+}-ATPase. T1, Na^{+}-coupled aspartate transporter EAAT3 (also known as EAAC1). T_2, K^{+}-coupled citrate transport mechanisms (KCitT1/2). pH_l, lumen pH. Na^{+}/K^{+}-ATPase may also be expressed basolaterally.

The level of citrate found in prostate is significantly reduced in PCa and drops to a level similar to that found in blood when the cancer becomes metastatic. In the following, we discuss the changes in citrate metabolism and transport in PCa cells, compared with normal prostatic epithelia.

Prostate cancer

It is likely that the drop in prostatic citrate precedes malignant transformation.[33] It has been suggested that, due to a metabolic switch, neoplastic cells would become citrate oxidizing, unlike normal prostatic cells that show a low citrate-oxidizing capability.[43] Reduced level of Zn^{2+}, which would relieve m-ACNT from inhibition, has been proposed as one of the reasons for the decreased level of citrate in PCa.[33] We should note, however, this role of citrate may not be the same in all animal (*e.g.* mouse) models of PCa.[44]

We have examined citrate transport in the strongly metastatic human PCa PC-3M cells.[45] In addition to a K^+-dependent citrate release mechanism KCitT2, similar to KCitT1, these cells express a novel Na^+-dependent transport mechanism (NaCitT) designed for taking up citrate.[45] The main characteristics, including the value of 'reversal potential', inhibitor profile and pH sensitivity of NaCitT differ from those of KCitT1/2.[45] No physiological differences are observed between KCitT1 and 2. In the human prostate gland, production and release of citrate occur mainly in the peripheral zone (comprising ~50% of the gland), whereas citrate depletion is associated with malignant loci.[46] Prostate malignancies also originate mainly from the peripheral zone.[47] Thus, it is possible that the citrate released by one cell population within this zone is taken up by another subpopulation during development and progression of malignancy, as illustrated in Fig. 2a.

Regulation of NaCitT expression

The relative levels of expression of the Na^+- and K^+-dependent components of citrate transport in PC-3M cells are modified by long-term (24–48 hour) preincubation with tetrodotoxin (TTX), a specific blocker of voltage-gated Na^+ channels (VGSCs).[45] Functional VGSC activity has been previously shown to occur specifically in strongly metastatic PCa cells of rat and human.[48,49] Thus, VGSC activity could regulate citrate transport mechanism in PCa, especially expression of NaCitT. Interestingly, VGSC expression itself can be controlled by steroid hormones, including androgen.[50] This could suggest a possible loop between VGSC and NaCitT expression in prostate tissue where lack of androgen signalling could upregulate VGSC activity and, by doing so, enhance citrate uptake in PCa.[45] VGSC upregulation may also be induced by epidermal growth factor,[51] also known to be associated with metastatic PCa.[52]

Citrate metabolism in prostate cancer

Although use of cytoplasmic citrate as a source of acetyl-CoA for FA and sterol production occurs in physiological conditions, it is particularly important in cancer cells, especially PCa.[53] The possible cytoplasmic components are FAS, c-ICD, cytosolic malic enzyme and c-ACNT.

Fatty acid synthase

Cytoplasmic citrate comes either from mitochondria or blood. It is converted by ATP-citrate lyase to acetyl-CoA and then catalysed by acetyl-CoA carboxylase to malonyl-CoA. Acetyl-CoA and malonyl-CoA are used for production of palmitate by FAS, with NADPH as the reducing agent. Mitochondrial β-oxidation of FAs represents a physiological response to tissue energy depletion (*e.g.* during fasting, febrile illness or increased muscular activity) and provides ~80% of the energy for heart and liver functions.[54] FA synthesis in cytoplasm and β-oxidation within mitochondria are tightly regulated so that they do not occur simultaneously. Malonyl-CoA, a substrate for FAS and an inhibitor of carnitine–palmitoyl CoA transferase (an enzyme involved in the transfer of long-chain FA into mitochondria), is primarily responsible for the co-ordinated regulation of these two metabolic processes. High levels of malonyl-CoA facilitate FA synthesis and block β-oxidation.

Cytosolic ICD and malic enzyme

FAS-mediated FA synthesis is a process that requires NADPH as the source of reducing power in the cytoplasm. The pentose phosphate pathway is principally responsible for the NADPH production. However, NADPH can also be generated by two cytosolic enzymes: $NADP^+$-dependent malic enzyme and $NADP^+$-dependent c-ICD, both involving cytosolic citrate. When citrate is cleaved into acetyl-CoA and oxaloacetate by ATP-citrate lyase, oxaloacetate can be converted into malate, which can serve as a substrate for malic enzyme to generate NADPH. Citrate can also be converted to isocitrate by c-ACNT. Isocitrate can serve as a substrate for c-ICD to generate NADPH.

Cytosolic aconitase

Also known as the 'iron regulatory protein' (IRP), c-ACNT produces isocitrate from citrate in the cytoplasm. The activity of this enzyme depends on the availability of intracellular iron. When intracellular iron is low, IRP binds to the mRNAs for a number of proteins involved in iron metabolism. When iron is available, it facilitates the formation of iron–sulphur clusters and IRP acquires enzymatic activity.[55]

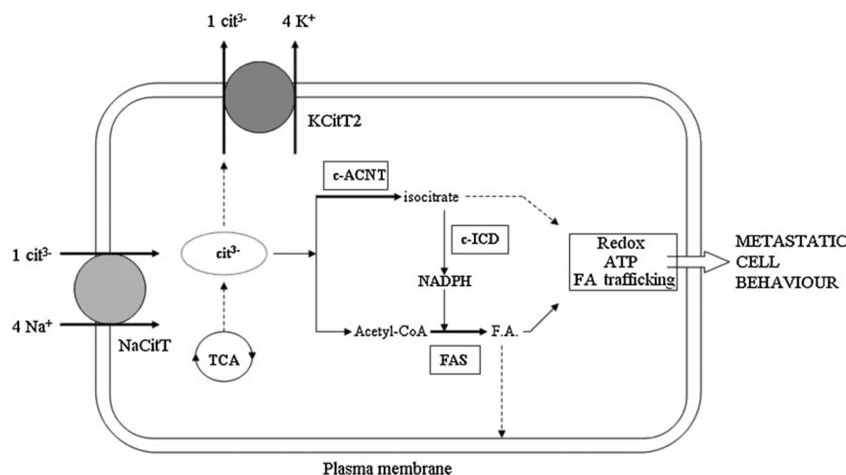

Figure 3. A model showing possible c-ACNT- and FAS-dependent pathways through which extracellular citrate can be metabolised in metastatic prostate cancer (modelled by PC-3M cells). Citrate is taken up from extracellular medium via NaCitT. Some citrate may be released through a K^+-dependent transporter (KCitT2), depending upon the cells' metabolic status. Intracellular citrate can be converted into isocitrate through the action of c-ACNT and then into α-ketoglutarate (not shown) and NADPH by $NADP^+$-dependent c-ICD. Citrate can also be converted into acetyl-CoA, precursor of the fatty acid (FA) synthesis, through the action of ATP-citrate lyase. Fatty acids produced by the action of fatty acid synthase (FAS) can be used in the biogenesis of plasma membrane and other membranes. Both c-ACNT- and FAS-dependent pathways will control redox balance, energy (ATP) production and FA trafficking[101] thereby facilitating enhanced metastatic cell behaviours.

FAS is highly over-expressed in several different cancers, including those of prostate,[56] breast,[57] ovary,[58] stomach,[59] colon[60] and brain.[61] c-ICD is also upregulated in cancers, including pancreas,[62] colon[63] and gastrointestinal tract.[64] Increased expression of FAS as well as increased expression and activity of c-ACNT have been found in PC-3M cells,[65] and FAS and c-ACNT have also been shown to be partially inter-dependent.[65]

Taking the available evidence together, we propose a model of c-ACNT- and FAS-dependent metabolism of citrate in metastatic PCa cells (Fig. 3). In this model, citrate would enter cytoplasm (i) through NaCitT expressed in plasma membrane or/and (ii) from mitochondria. Citrate would then be converted to acetyl-CoA by ATP-citrate lyase and/or into isocitrate by c-ACNT. Consistent with the latter requiring iron for activity, several studies have shown that cancer cells have elevated levels of iron.[66] Acetyl-CoA is further metabolised into malonyl-CoA and, together with NADPH, can be used in FA synthesis.[3] NADPH is produced from citrate by c-ACNT and c-ICD[3] or by ATP-citrate lyase/$NADP^+$-dependent malic enzyme. α-Ketoglutarate (product of c-ICD) can be converted to glutamate and used in energy production and increase of metastatic cell behaviour, as discussed below.

Use of citrate in enhancement of metastatic cell behaviour in PCa

As most normal cells have the potential to take up citrate present in blood, whether this uptake affects cellular behaviour has been investigated. Citrate has no effect on proliferation, endocytic membrane activity and adhesion of normal cells, including endothelia[67] and prostate epithelial cells.[65] In contrast, long-term (24 hour) preincuabtion of strongly metastatic human PC-3M cells with citrate results in enhancement of metastatic cell behaviour, including increased motility, membrane endocytic activity and decreased adhesion.[65] These effects are reduced by inhibition of FAS and c-ACNT/c-ICD. Interestingly, PC-3M cells have a lower level of intracellular citrate (even after citrate preincubation) compared with normal prostatic epithelial cells (4.0 ± 0.48 mmol/mg).[65] Furthermore, PCa cells possess increased expression and activity of several citrate-related enzymes (including c-ACNT and FAS). It seems possible that the higher citrate sensitivity and lower intracellular citrate content of PCa cells, compared with normal cells, are due to their ability to use citrate for metabolic energy production, mainly FA synthesis, to enhance metastatic cell behaviour.

Pharmacology and further molecular aspects

There are some pharmacological data available on KCitT1/2, NaCitT and members of the SLC13 gene family.[2,19,34,45] These are consistent with KCitT1/2 and NaCitT showing non-ATPase and non-channel like behaviour. Diethyl pyrocarbonate, a histidine-selective reagent, suppresses KCitT1/2 and NaCitT activities.[34,35] This inhibitor is also efficient in blocking rabbit NaDC1[68] and CTP.[13]

The genes for NaDC1, NaDC3 and NaCT occur on human chromosomes 17, 20 and 12, respectively.[18] The

members of this gene family have a conserved 17-amino acid motif sequence (TSFAFLLPVANPPNAIV), the 'sodium sulphate symporter family signature'.[18] Stimulation of human NaCT by Li^+ can be abolished by a single amino acid substitution, Phe500 → Leu.[30] The inhibitory effect of Li^+ on rabbit NaDC1 is lost when amino acids at positions 373 and 475 are mutated.[69] Normally, NaDC1 and NaDC3 occur on apical (brush border) and basolateral membranes of renal proximal tubules, respectively.[19,29] For NaDC3, this positioning is dependent on a short amino acid 'signature' sequence (AKKVWSARR) at the N terminus and involves two particular hydrophobic amino acids (V and W). Addition of this signature to NaDC1 changes its subcellular location to basolateral membrane, similar to NaDC3.[70]

The differential pharmacological characteristics indicate that the molecular natures of KCitT1/2 and NaCitT could be distinct from the known members of the SLC13 family. Further, work is required to elucidate the genes coding for KCitT1/2 and NaCitT.

Citrate in other organs

This section focuses on different citrate transporters and citrate-related functions specific for different organs and their pathophysiological aspects.

Kidney

The basic function of kidney is to produce urine, which facilitates excretion of waste material, while conserving salts, water and metabolic substrates in homeostatic balance. Consequently, kidneys have a high energy demand for active transport.[71] Di/tricarboxylates are transported across the luminal and basolateral membranes of proximal tubules[72] by NaDC1 and NaDC3, respectively,[16,29] which correlate with the transporters' substrate affinity and physiological role. In the basolateral membrane, NaDC3 is functionally coupled to organic anion transporters, which facilitate influx of organic anions in exchange for efflux of dicarboxylates such as α-ketoglutarate.[73] The entry of dicarboxylates *via* NaDC3 is essential for optimal activity of these anion exchangers. Inhibition of NaDC1 by Li^+ has pharmacological and therapeutic relevance and would be expected to interfere with the renal reabsorption of TCA cycle intermediates. This provides a molecular mechanism for the increased urinary excretion of TCA cycle intermediates in patients taking Li^+ for therapeutic purposes.[74] Urinary citrate can chelate Ca^{2+}, thus preventing its precipitation;[75] therefore, low urinary citrate is associated with development of kidney stones.[29] Citrate can also modulate Tamm–Horsfall proteins (the most abundant protein in urine). By chelating Ca^{2+}, citrate reduces grouping of the proteins, thus helping them inhibit crystal aggregation.[76]

Liver

In liver, citrate serves as a regulator of glycolysis and gluconeogenesis, and can thus regulate blood glucose levels.[2] NaDC3 and NaCT, the high-affinity transporters for dicarboxylates and citrate, are expressed in the sinusoidal membrane of hepatocytes[19,28] where they can take up citrate from blood. Thus, the cytosolic levels of citrate in liver cells may be controlled by release of mitochondrial citrate *via* CTP and by uptake of extracellular citrate *via* NaCT. An increase in cytosolic citrate in hepatocytes would facilitate synthesis of FAs and cholesterol, and inhibit glycolysis. Therefore, activation of human NaCT by Li^+ may have clinical implications. Stimulation of NaCT-mediated citrate uptake occurs at therapeutically relevant concentrations of Li^+. Accordingly, chronic use of Li^+ could potentially increase the circulating levels of FAs, triglycerides, low-density lipoproteins and cholesterol. Similarly, inhibitors of NaCT may reduce the synthesis of FAs and cholesterol in liver, and thus affect the levels of triglyceride and cholesterol in blood.

Brain

Citrate is important for normal brain function as a neurotransmitter precursor and substrate for energy production. Release of citrate from astrocytes (~70 nmol/h/mg of protein) results in concentrations of citrate in cerebrospinal fluid (~400 μM) even higher than the concentration in blood (~135 μM).[77] This seems necessary to supply neurones with the carbon skeleton for the synthesis of neurotransmitters such as glutamate and γ-aminobutyric acid. Also, by chelating cations (as Ca^{2+} and Mg^{2+}), citrate is thought to regulate neuronal excitability.[77]

A number of different citrate transporters have been identified in brain tissue. NaCT is mostly detectable in neurones, especially in hippocampus, cerebellum, cerebral cortex and olfactory bulb.[17,25,27] NaDC3 is found in cerebral cortex, hippocampus and cerebellum[78] and is localised mainly to astrocytes.[27]

In pathophysiological conditions, such as diabetes or starvation, monocarboxylates become an important source of energy production in brain. They are transported into neurones by proton-linked monocarboxylate transporters (MCTs), which catalyse the facilitated diffusion of monocarboxylates (*e.g.* lactate, ketone bodies) with a proton. MCT1 and MCT2 isoforms are most abundant in brain and can carry citrate when cation bound.[79] For example, Al^{3+} can be complexed to citrate by two of the carboxylate groups, leaving one carboxylate group free. The latter can then be moved

across the cell membrane by MCTs.[80] However, such a process may have pathological consequences, if the bound cation is cytotoxic.

Although astrocytes produce citrate,[81] the citrate transport mechanism in astrocytes is not known. Citrate synthesis and/or release is sensitive to extracellular K^+.[77,82] Therefore, citrate release from astrocytes may be similar to KCitT1/2. Neurones are dependent on extracellular sources of TCA cycle intermediates, and astrocytes are the main provider of citrate, α-ketoglutarate and malate.[81] Neuronal NaCT mediates uptake of the metabolites.[27] The levels of another metabolite, *N*-acetyl aspartate, are high in brain. It is produced and stored by neurones. It is then released and taken up through NaDC3 and NaCT by astrocytes and other brain cells.[27] There appears to be an astrocyte–neurone metabolic cooperation, analogous to that of the prostate epithelia–sperm, involving release of citrate/*N*-acetyl aspartate from one[34] and uptake/use by the other.[32] Interestingly, Li^+, which enhances human NaCT activity,[30] is used clinically for several neurological disorders including bipolar disease.[83] Furthermore, Li^+ has a protective role against oxidative stress in neurones, but does not exhibit any protective role for astrocytes.[84] It is possible that Li^+ action might be beneficial for neurones by increasing their uptake of metabolic substrates, especially in degenerative brain disorders.

Clinical aspects

The dramatic decrease (by ~20-fold) in citrate levels in prostate gland during malignancy[33,46] has led to the development of magnetic resonance spectroscopy (MRS) to determine the metabolic profile of prostatic tissue ('intact' biopsy), hence helping in diagnosing PCa.[85] MRS combined with conventional magnetic resonance imaging (MRI), *i.e.* 'MRS imaging' (MRSI), enables non-invasive biochemical imaging ('metabolic mapping') of prostate, displaying relative concentrations of chemicals within contiguous small volumes called 'voxels'.[86,87] The MRSI technique offers several advantages over prostate-specific antigen (PSA) measurements, since (i) it is non-invasive and (ii) changes in the citrate level may precede the onset of malignancy so, in principle, MRSI could be an early detector of PCa.[46]

The initial application of MRSI to prostate involved mainly the unique relationship of citrate with differing pathologies of the gland, shown to exist *ex vivo*[87] and *in vivo*[88] even in specimens that contained minimal foci of cancer.[89] This further supported the notion that the metabolic changes in PCa precede the pathology.[43] This set the scene for a novel non-invasive imaging technique without any of the risks associated with exposure to ionising radiation.

Three-dimensional proton MRSI can generate a metabolic picture of the entire prostate with a resolution of 0.24 mL or less[90] displaying levels of citrate, creatine, choline-containing compounds and polyamines. While the citrate level is reduced in PCa, choline is elevated,[86] which is probably due to high phospholipid turnover in membranes of proliferating cells in malignancy[91] and to the frequent overexpression of choline kinase activity in tumours, including PCa.[92] Hence, the basic method for diagnosing PCa by MRSI is based on an increased total choline/citrate ratio (Fig. 4); less than 0.75 is considered normal, 0.75–0.86 as likely PCa, and $\geq$0.86 as definite PCa.[93] MRSI can be combined with MRI to simultaneously give a corresponding anatomical image. Overlaying the metabolic and anatomical maps improved the accuracy of PCa diagnosis from 71 to 81%, compared with MRI alone.[94]

MRSI can also be used to determine disease stages in PCa. Scheidler *et al*[93] demonstrated a linear correlation between the total citrate/choline ratio and Gleason score. Another advantage of MRSI is improved localisation of tumour within the gland, which can increase accuracy in radiotherapy and better monitoring of the effectiveness of other therapies.[93] Improved localisation can also help in targeted biopsies, especially in those patients with previous 'negative' biopsies but persistently raised levels of PSA.[95] Finally, MRSI also improves estimation of tumour volume, which relates to extracapsular extension.[96] This is particularly important in facilitating the decision making process regarding possible radical surgery.

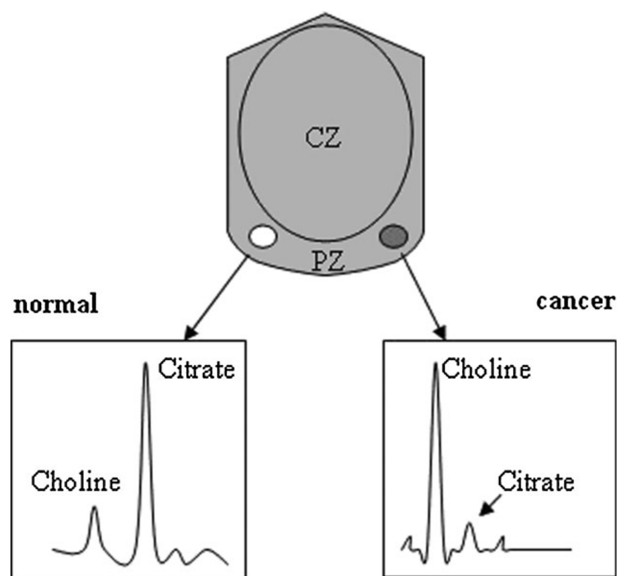

Figure 4. A schematic diagram of magnetic resonance spectral imaging (MRSI) traces from normal and cancerous prostate. CZ, central zone. PZ, peripheral zone. The citrate level is decreased whilst choline level increases markedly during progression from normal to cancer. Modified from Kantoff et al.[102].

Recently, there has been a growing interest in intact tissue analysis by 'magic angle' spinning (MAS) nuclear magnetic resonance (NMR). Swanson *et al.*[85] showed that while presurgical MRI/3D-MSRI can target malignant prostate tissues with an accuracy of 81%, *ex vivo* MAS-NMR can indicate further significant decreases in citrate and polyamine levels in PCa defined as more aggressive by Gleason score. Furthermore, citrate and polyamine levels are correlated directly with the percentage of tumour in excised tissues and are high in normal glandular and non-stromal tissue when compared to tumour.[85] ^{1}H-NMR spectroscopy of seminal or prostatic fluid has been shown to differentiate PCa from benign prostatic hyperplasia (BPH) by comparing levels of metabolites, especially citrate.[97] Measurements of citrate in both semen and expressed prostatic secretion could outperform PSA in detecting PCa.[98] In terms of disease staging, 600 MHz ^{1}H-MAS-NMR revealed significant differences of tumours stages T2ab, T2c and T3.[99] Citrate was among the metabolites associated with discrimination, together with choline, phosphocholine, glycerophosphocholine, polyamines, lactate and mobile lipids. Importantly, the same metabolite pattern was also discriminatory in tissues that were histologically benign, but taken from prostates that contained malignant disease with Gleason scores of 6 or 7. These results have significant implications for post-surgical management of therapy, and for the diagnosis of PCa in patients with repeated 'negative' biopsy.

The electrochemical (electrophysiological and/or metabolic) changes occurring during development and progression of PCa may be exploited clinically in other novel diagnostic procedures, including 'tissue resonance interaction method probe' (TRIMprobe) and positron emission tomography.[100] However, it is not yet clear how these methods may relate specifically to tissue levels of citrate.

Concluding remarks

Citrate is clearly an important anion essential for cellular metabolism and cation chelation. Citrate levels in blood and many organs are strictly regulated and play a significant role in tissue physiology as well as having pathophysiological consequences, as in kidney, liver, brain and prostate. Indeed, cytoplasmic citrate might play a major role in PCa metabolism through overexpression of citrate-related enzymes, like FAS and c-ACNT. Transport of citrate across cell membranes is strictly regulated and mediated by proteins belonging to two different gene families. However, for some transport mechanisms (mainly outward) the molecular nature has not yet been identified, including for those present in prostate and glia. Cloning of the genes responsible for such citrate transport might reveal new transporter families or add new members to the existing ones.

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