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The unbroken Krebs cycle. Hormonal-like regulation and mitochondrial signaling to control mitophagy and prevent cell death

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Title

The unbroken Krebs cycle. Hormonal-like regulation and mitochondrial signaling to control mitophagy and prevent cell death

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Abstract

The tricarboxylic acid (TCA) or Krebs cycle, which takes place in prokaryotic cells and in the mitochondria of eukaryotic cells, is central to the life on Earth and participates in key events such as energy production and anabolic processes. Despite its relevance, it is not perceived as tightly regulated compared to other key metabolisms such as glycolysis/gluconeogenesis. A better understanding of the functioning of the TCA cycle is crucial due to the mitochondrial function impairment in several diseases, especially those that occur with neurodegeneration. This article revisits what is known about the regulation of the Krebs cycle and hypothesizes the need for large-scale, rapid regulation of TCA cycle enzyme activity. Evidence of mitochondrial enzyme activity regulation by activation/deactivation of protein kinases and phosphatases exists in the literature. Apart from indirect regulation via G protein-coupled receptors (GPCRs) at the cell surface, signaling upon activation of GPCRs in mitochondrial membranes may lead to a direct regulation of the enzymes of the Krebs cycle. Hormonal-like regulation by posttranscriptional events mediated by activable kinases and phosphatases deserve proper assessment using isolated mitochondria.

Runnig title

Missing clues in the regulation of Krebs cycle

Keywords

Mitochondrial alterations, anaplerotic, glutamate, glutamine, α-ketoglutarate, signaling in mitochondria, signal transduction, G protein-coupled receptor, neurodegeneration, mitophagy.

Abbreviations

AD: Alzheimer's disease; GPCR: G protein-coupled receptors; FAD: Flavin-adenine dinucleotide; NAD: Nicotine-adenine dinucleotide; PyrDH: Pyruvate dehydrogenase; PD: Parkinson's disease; TCA: Tricarboxylic acid.

Introduction

The TCA cycle is functionally linked to the electron transport chain. As soon as NADH and $FADH_2$ are formed, they are used to pump protons across the inner mitochondrial membrane at the same time that oxygen is reduced to H_2O; proton pumping back serves to synthesize ATP from ADP in a process known as oxidative phosphorylation. The electron transport chain and oxidative phosphorylation are closely coupled and, for decades, were thought to be a single operation. The use of electron transport inhibitors, cyanide, carbon monoxide, etc., served to uncouple the two processes, thus demonstrating that they were different, despite the fact that under physiological conditions they operate in coordination.

TCA cycle regulation appears to be due to allosteric interactions of cycle enzymes through metabolites that reflect the energy state of the cell. If there is enough ATP, the electron transport chain does not function, and nicotinamide nucleotide and flavin-adenine dinucleotide accumulate in their reduced state, NADH and $FADH_2$. Therefore, the cycle would not operate due to the lack of co-substrates, that is, of oxidized nucleotides: NAD^+ and FAD. Furthermore, if the concentration of ATP is high because energy needs are satisfied, the low concentration of ADP would prevent the entry of electrons into the mitochondrial transport chain; the accumulation of NADH/$FADH_2$ would slow down the cycle due to the lack of co-substrates (NAD^+ and FADH). In short, the regulation of TCA cycle is considered automatic since it would depend on the availability of the molecules necessary both for the functioning of the cycle, NAD^+ and FADH, and for the synthesis of ATP**.** In the opposite situation, an ATP decrease would result in increased levels of ADP, which is an allosteric enhancer of the catalytic activity of some TCA cycle enzymes.

Although the need for rapid responses in the regulation of events that take place outside the mitochondria is indisputable, it might seem that all regulations in the mitochondria are dependent on gene transcription, i.e. slow, or automatic/allosteric, i.e. of small magnitude. However, it would be unreliable to think that mitochondrial events cannot change rapidly and by one, two or more orders of magnitude. In fact, there are events in the mitochondria that are similar to those involved in fast-acting regulatory events in the cytosol. In mammals many of these rapid responses originate at the plasma membrane and are a consequence of activation of cell surface receptors by hormones and/or neurotransmitters.

How mitochondrial energy metabolism impacts on cell survival

Energy is critical for homeostasis in multicellular organisms and is provided primarily by mitochondria in well-oxygenated cells. In relation to the emergence of the mitochondrion as a key player in many diseases, one wonders how altered mitochondrial events impact the fate of both mitochondria and cells.

In the nineties it was already evident that mitochondria were at the center stage of diseases related to aging [1–3]. On addressing "*mitochondrial encephalomyopathies*" DiMauro and Moraes [4] described a group of disorders caused by biochemical alterations that included a decrease in the activity of diverse TCA cycle enzymes, aconitase, fumarase or the dihydrolipoyl transferase unit of the α-ketoglutarate dehydrogenase complex. Although the underlying mechanisms are unknown, there is now consensus that the altered functioning of the mitochondria is behind neuronal death in neurodegenerative diseases.

Failure to provide enough reducing power to the electron transport chain leads to oxidative stress. Furthermore, the discovery of an inhibition α-ketoglutarate dehydrogenase complex under oxidative stress conditions is a good example of the impact of oxidative stress in the Krebs cycle. Inhibition of this key TCA cycle enzyme leads to decreased NADH production, which in turn exacerbates stress [5]. This vicious circle can seriously affect mitochondrial function and, consequently, condition cell survival.

TCA cycle balance

Some indirect experimental evidence led to hypotheses about how the Krebs cycle can be accommodated in some situations, physiological or pathological. Illustrative examples come from studies in cancer. Warburg hypothesized about a century ago that, for energy production, some proliferating cancer cells rely more on glycolysis than on TCA cycle [6]. Our recent in silico results [7] do not support this hypothesis thus agreeing with recent studies that challenge Warburg's views, i.e. oxidative phosphorylation is required to maintain energy demands for the anabolic requirement of cancer cells [8–10]. It is quite impossible to significantly reduce TCA cycle flux unless acetyl-CoA availability is reduced or citrate synthase activity is reduced; both of these circumstances are unlikely to occur in cancer cells that, among others, require citrate for anabolic fatty acid synthesis. Furthermore, cancer cells actively expend ATP and produce ADP, which is one of the main positive allosteric regulators of TCA cycle activity. A recent paper shows that the TCA cycle has inertia and is robust in the sense that it is prone to attain a new steady state with simple adjustment of metabolite concentrations and limited changes in flux [7]. If only the activity of one enzyme of the TCA cycle increases/decreases, the change in flux is usually low unless other parameters change substantially, for example, ADP/ATP ratio, NAD^+/NADH ratio and/or acetyl-CoA concentration.

In a given circumstance TCA cycle is in steady state, i.e., both the flux and the concentrations of metabolites of the TCA cycle remain constant. Dynamics occur from a given steady state to a new one and back to the initial one or, eventually, to a third one. In a resting muscle, the TCA mitochondrial cycle operates in a steady state. Under short exercise, the TCA cycle can undertake dynamic changes without reaching any new steady state but returning to the initial steady state upon recovery. In contrast, over the course of a marathon, the TCA cycle in aerobic muscle cells goes from the resting state to the "marathon" steady state. Although the Krebs cycle can be studied under dynamic conditions [11,12], there are details that can only be understood by considering/analyzing different stationary states.

Taking the steady state as a frame of reference, the variation of the concentration of the metabolites of the TCA cycle transiently disturbs the cycle prior to returning to initial conditions. However, a sustained accumulation of intermediates does not result in large energy output, but rather in severe imbalance that may not be controlled by allostery or long-term mechanisms such as activation/repression of gene transcription [13,14]. Imbalances are probably the cause of mitochondrial malfunction (Figure 1), leading to different pathologies related to mitophagy and cell death. For example, several neurodegenerative diseases show impaired mitochondrial function and energy shortages that correlate with neuronal death. The most extensively studied cases are related to inherited early-onset Parkinson's disease (PD). Mutations in the Parkin and in PTEN-induced putative kinase 1 (PINK1) genes are the two most common causes of inherited PD. In both cases mitochondrial homeostasis is severely affected [15,16]. Mutations of other genes leading related to familial increased risk of suffering the disease also correlate with

mitochondrial function impairment [17]. In Huntington's disease, caused by the aggregation of mutant huntingtin, mitophagy appears important enough to make mitochondria a promising target for therapeutic interventions[18–20]. Finally, in a well characterized transgenic mouse mode of Alzheimer's disease (AD), we showed that the percentage of differentially expressed proteins was much higher for mitochondrial proteins than for cytosolic proteins [21]. Surprisingly, using the same animal model, we later discovered maternal imprinting "*in the wild-type offspring that confers a greater facility to launch an AD-like neurodegenerative cascade*". Mitochondria of mothers carrying the mutant amyloid precursor protein were altered leading to an AD-like phenotype that causes wild-type progeny animals to show cognitive impairment with age (see [22] for details).

Automatic/allosteric metabolic regulation in bacteria and mitochondria

TCA cycle is often described, even in textbooks, as a cycle that operates in automatic fashion. As long as there are intermediaries, and the main "substrates", Acetyl-CoA, NAD^+ and FADH, the cycle can operate at a significant rate. Allosteric regulation is expected because the cycle must adapt to the energetic needs. Accordingly, the cycle operates in faster mode when the ATP level is low than when the ATP level is high. In fact, the ADP levels indicate the energetic status and the compound is a TCA cycle allosteric modulator. This and other allosteric known regulations within the cycle (see [23] for early review) can occur within seconds but are limited in amplitude.

Hormonal regulation that controls, for example, glycolysis/gluconeogenesis in the liver [24] has no equivalence in TCA cycle regulation but in regulation of pyruvate dehydrogenase (PyrDH), the enzyme that provides acetyl-CoA to the cycle [23,25–29]. Yet, it is reasonable to assume that the TCA cycle may be under hormonal-like regulation as marked changes in the activity of enzymes are not possible in the absence of hormonal (or hormonal-like) regulation. In summary, it is likely that the TCA cycle is controlled by hormonal-like mechanisms in both bacteria and mammalian mitochondria.

Hormonal-like regulation of the Krebs cycle

The need of fast-acting regulation mechanisms in mitochondria

TCA cycle is very robust and has inertia; the concentration of cycle intermediates is accommodated to keep the flux with little variations [7]. The cycle is both anabolic and catabolic and, therefore, there are reactions that produce metabolites of the cycle and reactions that extract metabolites from the cycle, they are known as anaplerotic reactions. In muscle mitochondria, during exercise, fumarate supply from an anaplerotic reaction and increased availability of acetyl-CoA lead to a significant increase in cycle flux, when the muscle stops working, the initial steady state is progressively restored [30]. However, as pointed out several years ago such open cycle, i.e. the cycle operating with anaplerotic routes, may be markedly unbalanced to the point of no return unless the carbons that enter the cycle are similar to the carbons that exit the cycle [30]. It is common to find in the literature that anaplerotic reactions are those that refill the cycle thus leading to higher flux and energy production. On the one hand, "anaplerotic" can define both filling and removal reactions. On the other hand, anaplerotic reactions must be contemplated under the perspective that the number of carbons that enter the cycle must leave it by withdrawal reactions and that refilling per se does not lead to significant increases in energy

production by the TCA cycle. It should be also noted that a continuous supply of metabolites to the cycle is incompatible with steady state.

It is assumed that mitochondria of eukaryotic cells derive from bacteria [31,32]. First level metabolic regulation in bacteria is dependent on the energetic needs and on the availability of nutrients. A second level is due to allosteric regulation of key enzymes in the main pathways, glycolysis and TCA cycle. A third level depends on gene transcription regulation, which is triggered by adverse events or a change in the nutrient source. These three levels are incompatible with the possibility of a regulation meeting two criteria: being fast and being of great magnitude. Is there a fourth level of regulation? Generally overlooked, such regulation is evident from data in the literature.

The next sections will cover the most known mechanisms of rapid regulation of TCA cycle enzymes in bacteria and in mitochondria, and the possibility of hormonal-like regulation mechanisms.

Regulation of TCA cycle enzyme activities by post-translational modifications

Experimental evidence of regulation by post-translational modifications

Post-translational modifications such as phosphorylation of enzymes in serine and threonine residues are suggestive of regulation of catalytic activity. Many of the Krebs cycle enzymes has been found with residues, serine and threonine, susceptible to be phosphorylated suggesting a regulation of catalytic activity by serine/threonine kinases.

As early as 1993 Muñoz-Dorado and colleagues posed a seminal question related to the control of bacterium Biology: *"Does Myxococcus xanthus have signal transduction cascades with protein serine/threonine kinases involved?"* [33]. Two-component regulators in bacteria consist of i) a membrane receptor kinase that senses the "first messenger" and ii) a regulator whose activity changes upon phosphorylation by the receptor kinase [34]. One wonders whether the regulatory mechanisms mediated by the variety of bacterial kinases/phosphatases [35,36] are fast or slow, i.e. regulation occurs in seconds or it requires regulation of gene transcription and translation of mRNA into protein. Fast-acting mechanisms are indirectly demonstrated by the fact that enzymes of the TCA cycle and of other central metabolism may become phosphorylated, thus suggesting that such phosphorylation is key for catalytic activity regulation. *Staphylococcus aureus* has enzymes of central metabolism that become phosphorylated upon incubation of cells with ^{32}P [37]. Serine/threonine-protein kinase PrkC is one of the enzymes identified in *Bacillus subtilis* that are capable of phosphorylating enzymes of essential metabolic pathways [38]. Serine/threonine-protein kinase SpkD of *Synechocystis sp.* modulates the pool of TCA cycle metabolites depending on the availability of inorganic carbon [39]. S-adenosylhomocysteine hydrolase of *Mycobacterium tuberculosis* is regulated by phosphorylation by the serine/threonine-protein kinase PknB [40]. Penicillin-binding-protein and serine/threonine associated kinase PrKA (also known as phosphoribulokinase) of *Listeria monocytogenes* participates in phosphorylation events involved in every aspect of the biology of the bacteria, from virulence to metabolism [41].

Protein phosphatase 2C homolog 7 (Ptc7p) a mitochondrial matrix enzyme regulates Cit1P, the "*canonical citrate synthase of the tricarboxylic acid (TCA) cycle, that diminishes its activity*" [42]. Another Krebs cycle enzyme, malate dehydrogenase, can be phosphorylated to serine/threonine by several of the 11 eukaryotic Ser/Thr kinases of *Mycobacterium tuberculosis*; phosphorylation resulting in regulation of catalytic activity

[43]. It is tempting to speculate that α-ketoglutarate dehydrogenase complex as a regulation similar to that of the multienzyme complex, PyrDH, produces Acetyl-CoA from pyruvate [25]. PyrDH is tightly regulated by the action of PyrDH kinases and PyrDH phosphatases whose expression varies when comparing different bacteria or different tissues in higher organisms. In mammals, there are at least two PryDH phosphatase and four PyrDH kinase isozymes [44]. In the seventies and eighties, it seemed that activity of these specific kinases and phosphatases was due to structural changes induced by allosteric modulation and, eventually by insulin hormonal action [26]. In the nineties a family of enzymes were discovered, named as "mitochondrial protein kinases" and shown to have sequence homology with prokaryotic histidine protein kinases; notably, these enzymes may in vitro phosphorylate both PyrDH and α-ketoglutarate dehydrogenase (see [27] and [45] for details).

Phylogenetic evidence of regulation by post-translational modifications

In the absence of research on mechanisms of regulation of TCA cycle enzymes by post-translational modifications, it is convenient to look for phylogenetic evidence. We have considered serine and threonine residues whose phosphorylation may lead to change in enzyme activity [46,47], with a subsequent selection of those that are conserved across species. The results in Table 1 show that several TCA cycle enzymes have serine residues that are highly conserved when comparing the >150 species considered by Ensembl [48] (https://www.ensembl.org/; accessed on July 23, 2022) (for details see Supplementary File 1). It is particularly relevant the fact that in sequences whose homology is near 30% we find that a given serine (Ser^{82} in humans and Ser^{102} in Streptococcus mutans) appears conserved from bacteria to humans. Importantly, phosphorylation of this specific serine regulates the catalytic activity of isocitrate dehydrogenase [47]. In some cases, it has been directly shown that Ser phosphorylation affects the catalytic activity [47][49]. Due to the presence in mitochondria of Src kinases [50,51], which phosphorylate tyrosines, we have done a similar approach to detect conserved tyrosines in TCA cycle enzymes. At least succinate dehydrogenase, has a conserved tyrosine susceptible of phosphorylation by Src kinases (Table 1). The highly conserved tyrosine (99%) in succinate dehydrogenase is important for efficacious electron transfer through complex II and for preventing ROS generation [49]. Although this post-translational modification could be indirectly affecting the TCA cycle flux, we have not followed further this finding; regulation mechanisms involving tyrosine phosphorylation do not usually require modification of the catalytic activity of a metabolic enzyme, which is the main focus of this article.

Table 1

Signal transduction in bacteria and mitochondria

Another of the seminal question posed by Muñoz-Dorado and cols. in 1993 was: *"Does Myxococcus xanthus have a signal transduction system consisting of a receptor, a G protein, an effector, and a protein kinase as found in eukaryotes?"* [33]. On the one hand, there are heptaspanning membrane proteins that share the structure of G protein-coupled receptors (GPCRs) but not their function (Figure 2). The most relevant is the light-driven hydrogen-ion pump, whose structure consists of seven transmembrane domains [52], which is the common structure of the most populated family of the mammalian proteome:

GPCRs. Hence, a relevant question is whether Evolution has allowed the mitochondrion to incorporate GPCRs into its membranes. Evidence of the presence in mitochondria of heterotrimeric G proteins whose genes are not found in prokaryotic genomes is also relevant [53]. As commented below there are reports that identify GPCRs in mitochondrial membranes.

Eukaryotic cells sense the environment via cell surface receptors. Several first hormones and some neurotransmitters are sensed by cell surface GPCRs and signal transduction includes heterotrimeric G proteins (Figure 2) that are coupled to adenylate cyclase or to endoplasmic reticulum ion channels. Upon receptor activation hormonal action leads to the modification of the level of the so-called second messengers, cAMP and Ca^{2+} that in turn lead to the regulation of the activity of, respectively, protein kinase A and protein kinase C [54]. They are Ser-Thr kinases that participate in the regulation of the activity of key enzymes in the cytosolic metabolism of carbohydrates and lipids [54]. In addition, proteins phosphorylated by protein kinase A and/or C may enter the nucleus and regulate gene transcription. One relevant example is the cAMP response element-binding protein (CREB), a transcription factor of interest in diseases of the central nervous system [55–59], that can translocate from the cytoplasm to the nucleus but, it is also reported that CREB can translocate from the cytosol to the mitochondria [60].

Considering the tight control of mitochondrial membrane permeability for charged molecules, variation of second messengers (cAMP/cGMP and Ca^{2+}) in the cytosol cannot directly affect TCA cycle enzyme activity. The question is whether these second messengers can be produced in situ in the mitochondria and, if so, whether concentration variation in response to hormonal-like actions is involved in controlling the operation of the TCA cycle.

Interest in GPCRs in mitochondria has been scant, and in fact mitochondrial membranes are unlikely to contain the hundreds of existing GPCRs. However, the occurrence of GPCRs in mitochondrial membranes has been already reported for: angiotensin II AT_1 and AT_2, serotonin 5-HT_3, 5-HT_4 and 5-HT_7, melatonin MT_1, cannabinoid CB_1 and purinergic P2Y-like GPCRs [61–72] (Figure 3). GPCRs for compounds that are key in mitochondrial function, for example succinate and oxoglutarate [73,74], are also found in the plasma membrane of mammalian cells. In our opinion, it is worth to check whether these receptors are also present in mitochondrial membranes (Figure 2 and Figure 3).

While the relevance of cytosolic protein kinase A (PKA), which is activated by cAMP, is out of question, less is known on the expression and function of PKA in mitochondria. Interestingly cAMP-dependent PKA-like activity in mitochondria was already reported in 1977 [75]. The study identified in mitochondria 3 protein kinase enzymes: two that were cAMP-dependent but structurally diverse and a third that was cAMP-independent. In tissues such as in ovaries mitochondrial PKA is very active.

Possibilities for adaptation of Krebs cycle to changing scenarios

Imbalances leading to sustained accumulation of TCA cycle intermediates may lead to aberrant sequestering of carbons. TCA cycle must be under a strict control to both respond to energetic demands and to prevent major disturbances. Conceptually, such kind of regulation must be hormonal-like. We here present all the possibilities that directly and indirectly would allow fast and strong regulation of the TCA cycle flux and the TCA cycle metabolite concentrations.

Hormonal-like control of Acetyl-CoA production

The tight regulation of PyrDH makes possible to accelerate or decelerate acetyl-CoA production. The increase in acetyl-CoA leads to increase the flux through the cycle and the production of NADH/$FADH_2$; and the opposite if the production of acetyl-CoA is decreased. However, availability of acetyl-CoA would not restore steady state in a fully unbalanced TCA cycle. Neither, major alterations in PyrDH activity would lead to a TCA cycle imbalance.

Hormonal-like control of enzymes related to anaplerotic reactions

Oxalacetate entrance to TCA cycle may occur via pyruvate carboxylase (requires ATP). As far as we know, regulation by phosphorylation/dephosphorylation has not been reported, but allosterically by acetyl-CoA and aspartate [76]. From our point of view, the enzyme is expected to be regulated by post-translational modifications driven by hormonal-like mechanisms. In 2010 J.C. Wallace wrote: "*with only a little over 2,000 papers listed by PubMed in response to a search for "pyruvate carboxylase," it is not surprising that there remain many questions to be answered about this complex enzyme and its diverse functions*" [77]. In 2022 we still lack relevant information on the regulation of this enzyme.

The entrance via α-ketoglutarate must be tightly controlled to keep a balanced Krebs cycle. This is unlikely to occur via the reaction involved in such event: glutamate transaminase (also known as aspartate/glutamate transaminase). Transaminases catalyze reversible reactions and, accordingly, the flux towards the cycle must be controlled via the α-ketoglutarate dehydrogenase complex. There are early papers that demonstrate that the enzyme, in *Corynebacterium* actinomycetes has the "oxoglutarate dehydrogenase inhibitor" (OdhI) that may be phosphorylated by multiple protein kinases, including protein kinase G[78–82]. Therefore, avoiding the conversion of α-ketoglutarate to succinyl-CoA could resolve imbalances due to accumulation of cycle intermediates.

Hormonal-like regulation of TCA cycle enzymes

Functionally and structurally, the α-ketoglutarate dehydrogenase complex is similar to the PyrDH complex [83]. In an elegant study, Harris and colleagues demonstrated in 1990 that, relative to fasting conditions, the activity of the kinase of α-ketoglutarate dehydrogenase increased 2.5 and 8.6-fold in rats that were fed with, respectively, standard diet of low-protein diet [84]. The same laboratory carried out further investigations related to the mechanism of regulation of the enzyme by protein kinases [27,28]. Interestingly, α-ketoglutarate dehydrogenase is regulated by Ca^{2+}, suggesting that G_q and/or protein kinase C are involved in a hormonal-like type of regulation (see [85,86] and references therein). It is worth hypothesizing that mitochondrial protein kinases and phosphatases are also involved in the regulation of the enzyme activity. In our opinion, the regulatory mechanisms of mammalian mitochondrial α-ketoglutarate dehydrogenase deserve to be explored in detail.

Hormonal-like control of mitochondrial membrane transporters

The precursor of acetyl-CoA, pyruvate, requires a transporter in mitochondrial membranes to act as substrate of mitochondrial PyrDH. Carbons of TCA cycle intermediates may, at some point, enter and/or exit the mitochondria using transporters.

Is the apparent regulation by phosphorylation of, for example, ATP/ADP translocase or cytochrome c oxidase triggered by hormonal-like mechanisms? (see [87] for review). It is likely that the entry/exit of the main substrates (NADH, ADP) and products (NAD+, ATP) of the cycle are regulated. However, an imbalanced cycle would not be restored simply by acting on the transport of metabolites across mitochondrial membranes.

Are mitochondrial GPCRs involved in balancing the cycle?

Reportedly, activation of GPCRs in the cell plasma membrane may affect energetic metabolism in the mitochondrion. One relevant example is provided by the SUCNR1/GPR91succinate receptor that, via cytosolic G_i proteins, leads to regulation of TCA cycle and electron chain operation and mitochondria ROS production. The signaling mechanisms underlying this regulation are currently unknown. [88]. A receptor for another molecule that is key in mitochondrial function is the α-ketoglutarate (oxoglutarate) receptor (previously known as either GPR99 or P2Y purinoceptor 15) [73,89]. One specific question that, to our knowledge has not yet been addressed, is whether succinate and α-ketoglutarate receptors are expressed in mitochondrial membranes.

Receptor-mediated regulation of mitochondrial metabolism has been demonstrated for some GPCRs identified in mitochondrial membranes (Figure 2). The coupling of these receptors to mitochondrial (heterotrimeric) G proteins may be one underlying mechanisms [90]. $G\alpha_i$ was the first subunit of a heterotrimeric G protein identified in mitochondria; the study also reported association of $G\alpha_s$ with mitochondrial components [90]. More recently, other components of the family have been described in mitochondria [91,92], thus indicating that these organelles may contain all the main classes of heteromeric G proteins, namely G_s, G_i and G_q. Hence, mitochondria have all the components to locally produce cAMP, and Ca^{2+} via signal transduction mechanism similar to those mediated by mammalian cell surface receptors.

Predictions of a model considering hormonal-like regulation of TCA cycle operation

The word “broken” is misleading and the mitochondrial oxalacetate concentration is negligible

Studies that combine transcriptomics, metabolomics, (radioactive) label distribution, and systems biology software have reinforced the term "broken" being coined to describe a Krebs cycle that does not function as such [93]. Implicitly, it is considered that there is some TCA cycle reaction that is not taking place, which is why the word broken is used. We believe that the reaction catalyzed by malate dehydrogenase (mitochondrial) is not well valued in the concept of a "broken" cycle. Despite being far from equilibrium, TCA cycle operation, in particular the irreversible reaction that drives the cycle, catalyzed by citrate synthase, necessarily imply that oxalacetate concentration is very low. This is basically due to the thermodynamic parameter (K_{eq}) of the reversible reaction catalyzed by malate dehydrogenase: oxalacetate + NADH = malate + NAD^+. The reaction can only go in the direction oxalacetate to malate if the oxoacid is quickly removed by means of an irreversible reaction. Any acute increase in the concentration of oxalacetate is quickly reduced and “absorbed” by mass distribution among all intermediates of the cycle [7]. Actually, the view that increases in oxaloacetate would interrupt/stop the cycle by converting oxaloacetate to malate, fumarate, and succinate (ie, part of the cycle goes counterclockwise) is not correct. In summary, the determination of the real concentration

of oxalacetate in the mitochondria under a given steady state condition should be, at the very least, attempted.

Addressing the increases in citrate and succinate level in the "broken" cycle

An article on metabolic reprogramming in macrophage cell polarization indicates that the broken cycle is accompanied by accumulation, in mitochondria, of citrate and succinate [94]. Increase in citrate is, supposedly, due to a cycle broken at the level of isocitrate dehydrogenase and to an increased conversion of citrate to itaconate. The increase of citrate and of succinate in the "broken" cycle is explained by the impossibility in such a broken cycle to go from citrate to α-ketoglutarate [95]. According to the model described elsewhere [7], the reported decrease of isocitrate dehydrogenase in such "broken" Krebs cycle in M1 cells [95] does not prevent the cycle to be fully functional. Even magnifying the reported reduction of the activity of isocitrate dehydrogenase, citrate does not accumulate in the "open" TCA cycle model described elsewhere [7]. In fact, a one-fold decrease in the activity of the enzyme leads to an approximately 50% reduction of flux without significant variation in [citrate]. The reported increased drainage from citrate to itaconate would also go against citrate accumulation [95]. The prediction is anaplerosis at the level of oxalacetate (see below).

Validation of interpretations from omics and systems biology approaches require preregistered studies

The use of TCA cycle intermediates for anabolic purposes, as assumed in a broken Krebs cycle, would deplete the cycle very soon, thus stopping anabolism but also making substantial energy production impossible. As for the Warburg effect, the hypothesis of using citrate for anabolic processes in M1 cells require an intact Krebs cycle, not a broken one. Importantly, an increase of citrate requires acetyl-CoA and oxalacetate and, therefore, an alternative explanation to citrate accumulation is the increase in oxalacetate input together with a reduction in the flux of anabolic route starting in citrate and/or with a reduction in the activity of an enzyme of the first half of the TCA cycle. Fine tuning control of Krebs cycle to accommodation diverse circumstances or to fulfill the requirements of specific cells (e.g. M1 versus M2 macrophages) require, in our view, hormonal-like regulation. It is also relevant to consider the multiple factors that affect a certain experimental result and whose neglect leads to incorrect/doubtful interpretations. An example in radiotracer tracking experiments is the underestimation of the amount of radiolabeled CO_2 that is produced in the cycle and is lost as a gas. Real-time measurement of CO_2 production would be essential to bring all the data together and provide models that more reliably reflect the operation of the TAC cycle.

Directly addressing the potential of hormonal-like mechanisms in regulating TCA cycle enzyme activities

A change in enzyme activity results from at least 3 factors: a change in enzyme concentration, a change in allosteric regulation and a change in posttranslational modifications that affect enzyme activity. As evidence indicates that many TCA cycle enzymes are susceptible to phosphorylation and that bacteria and mitochondria have components of the machinery that allows signal transduction to take place, the hormonal-like regulation of TCA cycle enzyme activity needs to be addressed. The prediction is

that many TCA cycle enzymes may be regulated by phosphorylation-dephosphorylation events originated by some regulatory compound acting on membrane receptors. The main interest is in the GPCRs already detected in the mitochondrial membranes. Does activation of these receptors rapidly alter the catalytic activity of TCA cycle enzymes? α-ketoglutarate dehydrogenase is the most important enzyme to pay attention to, and for many reasons: because i) it is a macromolecular complex similar to the highly regulated PyrDH, ii) several Ser residues are phylogenetically conserved, and iii) it is positioned in a key step in the first part of the Krebs cycle. Isocitrate dehydrogenase and succinate dehydrogenase are also of interest regardless of whether their activity at any time depends on the concentration of the cosubstrates (NAD^+ and NADH). Furthermore, succinate dehydrogenase is predicted to be key in controlling fumarate concentration and this may be relevant in view of the results that make succinate and fumarate now considered oncometabolites [96–98]. Despite the first enzyme of the cycle, citrate synthase, may be considered an enzyme to be put the focus on, our view is that it is not tightly regulated because the first TCA cycle reaction is mainly dependent on acetyl-CoA availability and such availability is controlled by tight regulation of PyrDH. Is it necessary a tight hormonal-like regulation of citrate synthase further to that of PyrDH? Although PyrDH cannot be considered a TCA cycle enzyme, we suggest that a study of how signaling in mitochondria would affect PyrDH activity be performed. In summary, we suggest that hormonal-like regulation of PyrDH and α-ketoglutarate dehydrogenase activities would be relevant to controlling Krebs cycle functioning, while hormonal-like regulation of isocitrate and succinate dehydrogenase activities would be relevant for events other than those related to NADH/$FADH_2$ production.

Conclusions

As detailed in previous sections, compelling evidence suggests that the regulation of the TCA cycle is far from being fully understood. First, in silico modeling shows that automatic and allosteric regulation of the TCA cycle cannot maintain homeostasis and, therefore, further levels of regulation may exist [7]. Second, there are highly conserved residues in TCA cycle enzymes amenable to phosphorylation/dephosphorylation by mitochondrial kinases/phosphates [35–38]. At least one of the TCA cycle enzymes, α-ketoglutarate dehydrogenase, must be subject to tight regulation, as is the case with the dehydrogenase complex that produces acetyl-CoA, PyrDH [83]. Finally, circumstantial but strong evidence comes from the fact that almost all components of the machinery required for GPCR-dependent hormonal mechanism of action are present in mitochondria [61–65,67–74]; it would be unlikely to have the machinery but not the function of a GPCR-mediated hormonal-like action.

An unbalanced Krebs cycle may lead to severe alterations to mitochondria and result in cell death. There is indirect evidence that TCA cycle is regulated by hormonal-like mechanisms, that is, rapid and leading to large changes in the enzyme's catalytic activity. Mitochondria have all the components that participate in cytosolic signaling cascades triggered by activation of cell surface receptors. Despite the technical challenges, some cell surface GPCRs have been discovered in mitochondrial membranes. Potential signaling upon activation of GPCRs in mitochondrial membranes may lead to direct regulation of Krebs cycle enzymes. Hormonal-like regulation by post-transcriptional events mediated by activatable kinases and phosphatases deserves proper evaluation using isolated mitochondria. It should be noted that cell surface GPCRs exist for relevant intermediates of the Krebs cycle, e.g. succinate. It would be important to establish

whether the receptors for Krebs cycle intermediates are expressed in mitochondrial membranes and whether their activation gives rise to signaling cascades that end up regulating the cycle to keep it balanced, i.e. under tight control.

Graphical Abstract text

The TCA cycle is a key metabolic pathway in mammals. It is necessary to revisit its regulation to understand how mitochondria are at the center of the scene in various diseases, with special relevance in those caused by the death of neuronal cells. This review explores the possibility that the regulation of the TCA cycle is strongly orchestrated by hormonal-like events involving signal transduction and the activities of mitochondrial protein kinases and phosphatases that, by balancing the degree of Ser-Thr phosphorylation, would regulate the activity of the enzymes of the TCA cycle.

Figure Legends

Figure 1. Balanced and unbalanced Krebs cycle. A TCA cycle in steady-state (left) maintains a constant flux and a constant concentration of metabolites (value of reference: 100 for each intermediate). The increase in succinyl-CoA and succinate concentrations can be achieved by anaplerosis at the oxaloacetate level. A rapid increase in their concentration cannot be sustained over time (center) without removing these metabolites from the mitochondrial matrix (rescue route). If anaplerosis is sustained over time, the expected enormous excess of some of the metabolite concentrations (right) would lead to osmotic problems that would compromise mitochondrial structure and function. Excess metabolite concentrations can be addressed through metabolization and/or flux of compounds to the cytoplasm (Rescue routes). OMM: outer mitochondrial membrane. IMM: Inner mitochondrial membrane. TCA cycle enzymes: CS: citrate synthase; Aco: aconitase; IDH: isocitrate dehydrogenase; αKDH: α-ketoglutarate dehydrogenase; SuS: succinyl-CoA synthetase; SDH: succinate dehydrogenase; FuH: fumarate hydratase; MDH: malate dehydrogenase.

Figure 2. Signal transduction mediated in mammals by cell surface GPCRs. Cell surface GPCRs couple to heterotrimeric G proteins (among others G_q, G_s and G_i) that, upon signal transduction, alter the levels of second messengers (cAMP, Ca^{2+}) thus leading to regulation of protein kinase A and protein kinase C. Phosphorylation of the substrates of these kinases contribute to the regulation of enzymes that are key for cytosolic metabolic processes and also to factors that may enter the nucleus and regulate gene transcription. The question is whether similar events may take place in the mitochondria via GPCRs located in outer or inner mitochondrial membranes. AC: Adenylate cyclase; CAMK: Ca^{2+}/calmodulin kinase; CREB: cAMP-response element binding protein; GPCR: G-protein coupled receptor; PLC: phospholipase C, PKA protein kinase A; PKC, protein kinase C; TCA: Tricarboxylic acid; other abbreviations are indicated in Figure 1 legend.

Figure 3. GPCRs identified in mitochondria and potential mitochondrial signaling mechanisms contributing to the regulation of TCA cycle enzyme activity. SUCN and α-ketoglutarate receptors are in a grey box as their presence in mitochondria has not been unequivocally demonstrated. A signaling pathway similar to that described for cell surface GPCRs in the cell surface may happen inside the mitochondria because several

molecules related to GPCR-mediated signaling have been also identified in the mitochondria. In addition, mitochondrial protein kinases/phosphatases may use TCA cycle enzymes as substrates. The scheme depicts a hypothetical hormonal-like mechanism that would lead to the regulation of the α-ketoglutarate dehydrogenase complex, whose structure is similar to the tightly regulated enzyme that provides acetyl-CoA to the TCA cycle, pyruvate dehydrogenase. In addition, receptors for two of the TCA cycle intermediates have been identified on the cell membrane; however, their identification in mitochondrial membranes has not yet been addressed. 5-HT: 5-hydroxytryptamine; AT: Angiotensin; CB: Cannabinoid; MT: Melatonin; P2: Purinergic P2; R: receptor; SUCN: Succinate; ECM: Extracellular matrix; PKA: Protein kinase A; PKC: Protein kinase C; other abbreviations are indicated in Figure 1 legend.

Conflict of interests

The authors declare no conflicts of interest.

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Data availability statement

Data is available from the corresponding author upon reasonable request.

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Table 1. Highly conserved residues found in the TAC cycle enzymes. The number in each residue corresponds to the protein sequence. In parentheses the percentage of identity of the residue among all the assessed species (>150).

	Serine	Tyrosine
Citrate synthase	-	-
Aconitase	Ser^{471} (98%) Ser^{690} (98%)	-
Isocitrate dehydrogenase	Ser^{82} (90%)	-
α-ketoglutarate dehydrogenase E1	Ser100 (50%)	-
α-ketoglutarate dehydrogenase E2	Ser81 (94%) Ser285 (43%) Ser297 (46%) Ser502 (85%)	-
Succinyl-CoA synthetase	-	-
Succinate dehydrogenase	-	Tyr^{215} (99%)
Fumarase	-	-
Malate dehydrogenase	Ser^{246} (85%) Ser^{326} (80%)	-

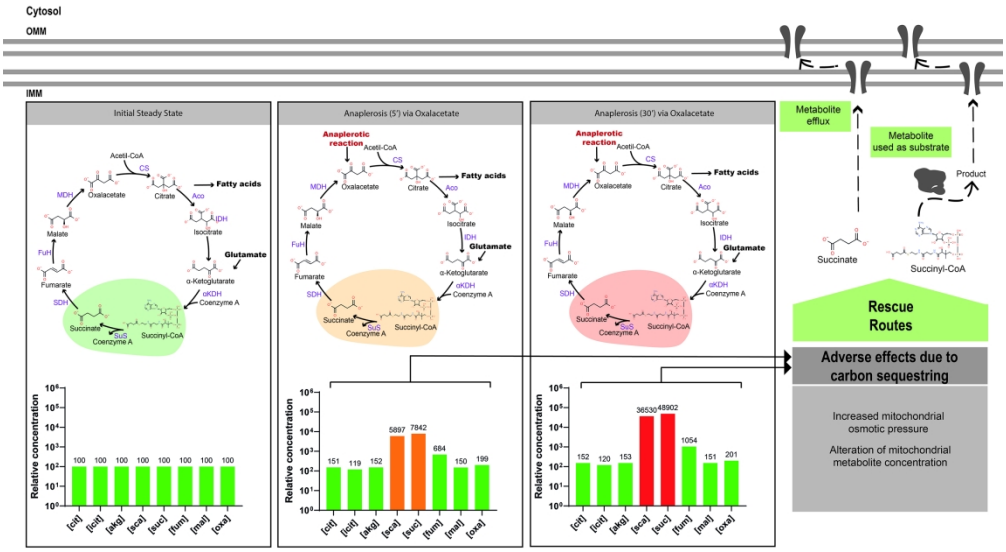

Figure 1. Balanced and unbalanced Krebs cycle. A TCA cycle in steady-state (left) maintains a constant flux and a constant concentration of metabolites (value of reference: 100 for each intermediate). The increase in succinyl-CoA and succinate concentrations can be achieved by anaplerosis at the oxaloacetate level. A rapid increase in their concentration cannot be sustained over time (center) without removing these metabolites from the mitochondrial matrix (rescue route). If anaplerosis is sustained over time, the expected enormous excess of some of the metabolite concentrations (right) would lead to osmotic problems that would compromise mitochondrial structure and function. Excess metabolite concentrations can be addressed through metabolization and/or flux of compounds to the cytoplasm (Rescue routes). OMM: outer mitochondrial membrane. IMM: Inner mitochondrial membrane. TCA cycle enzymes: CS: citrate synthase; Aco: aconitase; IDH: isocitrate dehydrogenase; αKDH: α-ketoglutarate dehydrogenase; SuS: succinyl-CoA synthetase; SDH: succinate dehydrogenase; FuH: fumarate hydratase; MDH: malate dehydrogenase.

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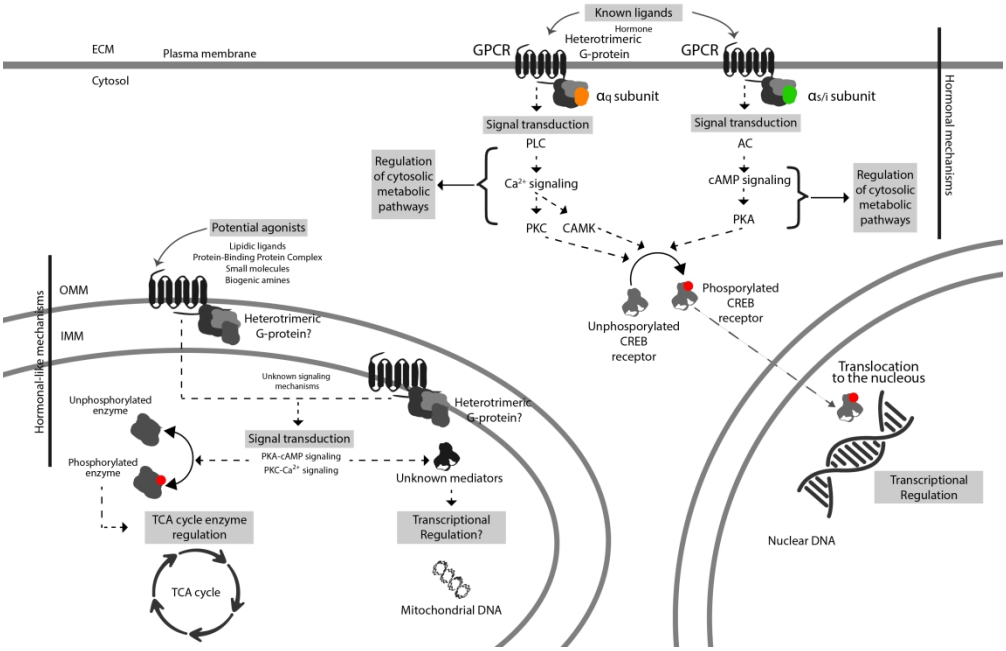

Figure 2. Signal transduction mediated in mammals by cell surface GPCRs. Cell surface GPCRs couple to heterotrimeric G proteins (among others Gq, Gs and Gi) that, upon signal transduction, alter the levels of second messengers (cAMP, Ca2+) thus leading to regulation of protein kinase A and protein kinase C. Phosphorylation of the substrates of these kinases contribute to the regulation of enzymes that are key for cytosolic metabolic processes and also to factors that may enter the nucleus and regulate gene transcription. The question is whether similar events may take place in the mitochondria via GPCRs located in outer or inner mitochondrial membranes. AC: Adenylate cyclase; CAMK: Ca2+/calmodulin kinase; CREB: cAMP-response element binding protein; GPCR: G-protein coupled receptor; PLC: phospholipase C, PKA protein kinase A; PKC, protein kinase C; TCA: Tricarboxylic acid; other abbreviations are indicated in Figure 1 legend.

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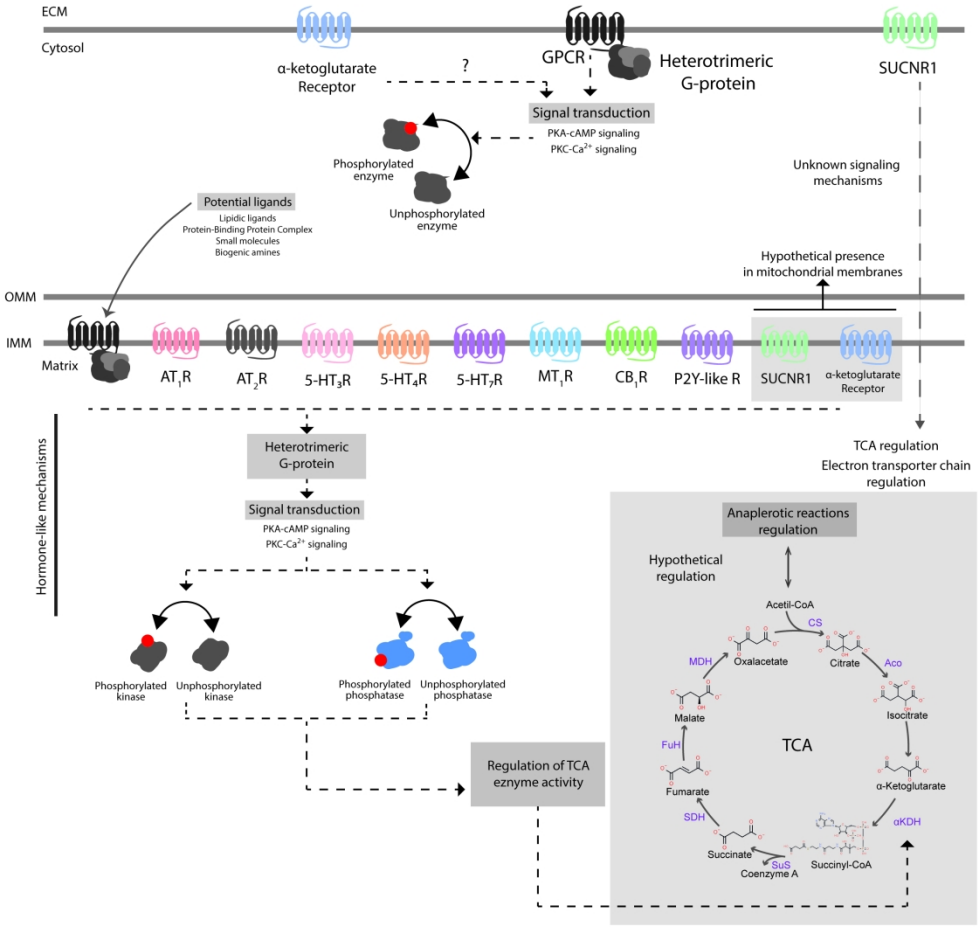

Figure 3. GPCRs identified in mitochondria and potential mitochondrial signaling mechanisms contributing to the regulation of TCA cycle enzyme activity. SUCN and α-ketoglutarate receptors are in a grey box as their presence in mitochondria has not been unequivocally demonstrated. A signaling pathway similar to that described for cell surface GPCRs in the cell surface may happen inside the mitochondria because several molecules related to GPCR-mediated signaling have been also identified in the mitochondria. In addition, mitochondrial protein kinases/phosphatases may use TCA cycle enzymes as substrates. The scheme depicts a hypothetical hormonal-like mechanism that would lead to the regulation of the α-ketoglutarate dehydrogenase complex, whose structure is similar to the tightly regulated enzyme that provides acetyl-CoA to the TCA cycle, pyruvate dehydrogenase. In addition, receptors for two of the TCA cycle intermediates have been identified on the cell membrane; however, their identification in mitochondrial membranes has not yet been addressed. 5-HT: 5-hydroxytryptamine; AT: Angiotensin; CB: Cannabinoid; MT: Melatonin; P2: Purinergic P2; R: receptor; SUCN: Succinate; ECM: Extracellular matrix; PKA: Protein kinase A; PKC: Protein kinase C; other abbreviations are indicated in Figure 1 legend.

214x215mm (300 x 300 DPI)