Glutamine homeostasis and mitochondrial dynamics

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Glutamine, the most abundant free amino acid in the human body, is described as the main physiological nitrogen vehicle between different mammalian tissues (Krebs, 1980). Although classified as non-essential, recent evidence suggests that glutamine is conditionally essential when it becomes rapidly depleted in the blood in stressful situations. Plasma glutamine concentrations are substantially decreased by clinical trauma such as in major surgery or after prolonged, exhaustive exercise (Castell et al., 1996). Glutamine acts as a nitrogen donor for purine and pyrimidine nucleotide synthesis for new DNA, mRNA repair, synthesis of amino acids, carbamoylphosphate, amino sugars, and other metabolites. It plays a key role in nitrogen metabolism and represents a storage and transport form of glutamate and ammonia. For these reasons, glutamine-derived glutamate and ammonia fulfill crucial physiological roles in many tissues, with particular relevance in brain.

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Abstract
Glutamine is a multifaceted amino acid that plays key roles in many metabolic pathways and also fulfills essential signaling functions. Although classified as non-essential, recent evidence suggests that glutamine is a conditionally essential amino acid in several physiological situations. Glutamine homeostasis must therefore be exquisitely regulated and mitochondria represent a major site of glutamine metabolism in numerous cell types. Glutaminolysis is mostly a mitochondrial process with repercussions in organelle structure and dynamics suggesting a tight and mutual control between mitochondrial form and cell bioenergetics. In this review we describe an updated account focused on the critical involvement of glutamine in oxidative stress, mitochondrial dysfunction and tumour cell proliferation, with special emphasis in the initial steps of mitochondrial glutamine pathways: transport into the organelle and hydrolytic deamination through glutaminase enzymes. Some controversial issues about glutamine catabolism within mitochondria are also reviewed.

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1. Introduction

Glutamine is an essential amino acid for the synthesis of nucleotides, amino sugars, and other metabolites. It is conditionally essential when it becomes rapidly depleted in the blood in stressful situations. Plasma glutamine concentrations are substantially decreased by clinical trauma such as in major surgery or after prolonged, exhaustive exercise. Glutamine acts as a nitrogen donor for purine and pyrimidine nucleotide synthesis for new DNA, mRNA repair, synthesis of amino acids, carbamoylphosphate, amino sugars, and other metabolites. It plays a key role in nitrogen metabolism and represents a storage and transport form of glutamate and ammonia. For these reasons, glutamine-derived glutamate and ammonia fulfill crucial physiological roles in many tissues, with particular relevance in brain.

Abbreviations: GA, phosphate-activated glutaminase; GIP, glutaminase-interacting protein; GSH, glutathione; HE, hepatic encephalopathy; IMM, inner mitochondrial membrane; α-KG, alpha-keto glutarate; MPT, mitochondrial permeability transition; NEM, N-ethylmaleimide; OXPHOS, oxidative phosphorylation; PCIMPS, p-chloromercuriphenylsulphonic acid; PGC-1α, peroxisome proliferator-activated receptor coactivator-1α; ROS, reactive oxygen species; TNF-α, tumour necrosis factor alpha.

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Glutamine homeostasis is likely to increase oxidative stress and might have a prominent role for mitochondrial dysfunction. Special attention is given to mitochondrial glutamine carriers and glutaminase enzymes as initial steps in the glutaminolysis pathway, including some controversial issues about these proteins which need to be addressed to understand the in vivo factors that regulate glutaminolysis, and hence glutamate and ammonia supply, in mammalian tissues.

2. Glutamine impacts mitochondria and metabolic homeostasis

Physiological and/or pathological context can determine the morphology of the mitochondria, suggesting a tight and mutual control between mitochondrial form and bioenergetics (Benard and Rossignol, 2008). Mitochondrial metabolic processes cause reactive-oxygen-species (ROS)-mediated injury to cells that may contribute to the pathogenesis of various diseases including cancer as well as respiratory, cardiovascular, nervous, and gastrointestinal toxicities. Glutamine is an important mitochondrial substrate that is implicated in the protection of cells from oxidant injury. In spite of the mechanisms of its action are incompletely understood, several findings point out outstanding roles for glutathione (GSH) and mitochondrial GA (Matés et al., 2006).

On the other hand, diminished mitochondrial oxidative phosphorylation (OXPHOS) and aerobic capacity are associated with morphofunctional changes of the mitochondria and reduced longevity (Saunders and Verdin, 2009). Antioxidants like the polyphenol resveratrol are associated with an induction of genes for OXPHOS and mitochondrial biogenesis and are largely explained by a resveratrol-mediated decrease in the transcriptional coactivator peroxisome proliferator-activated receptor coactivator-1α (PGC-1α) acetylation and an increase in PGC-1α activity. PGC-1α plays a central role in the regulation of cellular energy metabolism stimulating mitochondrial biogenesis (Lagouge et al., 2006). In addition, incubation of either hepatocytes or heart mitochondria from neonatal rats with ROS, or with cytokines, leads to impairment of mitochondrial oxidative function and NADH/NAD⁺ ratio inside the mitochondria. However, when hepatocytes are incubated with glutamine, there is a restoration of mitochondrial structure and metabolism. In vivo, intraperitoneal injection of glutamine into endotoxic suckling rats partially reverses hypometabolism, markedly reducing the incidence of hypothermia and improving clinical status (Eaton, 2003).

In most human tissues, mitochondria provide the energy necessary for cell growth and biological activities through OXPHOS. For mammals, the respiratory chain consists of four enzyme complexes (I–IV) and two intermediary substrates: coenzyme Q and cytochrome c. Variations in the concentration of these intermediate substrates could play an important role in compensatory mechanisms and the adaptation of metabolic systems to a variety of situations ranging from the physiological to the pathological (Benard et al., 2008). Regarding control of mitochondrial metabolic fluxes, it can be shared among several steps of the OXPHOS process, and this distribution can vary according to the steady state and the tissue (Rossignol et al., 2000).

Glutamine is similar to glucose in that proliferating cells metabolize it using a variety of pathways that support bioenergetics and biosynthesis. Of interest, cultured tumour cells require at least 10 times as much glutamine as any other amino acid (DeBerardinis et al., 2007). Cell proliferation in normoxia was dependent on glutamine, and glutamine deprivation markedly accelerated cell death in hyperoxia. Cellular ATP content in glutamine-supplemented cells is greater than that in glutamine-deprived cells. Mitochondrial membrane potential is increased by glutamine in hyperoxia-exposed cells (Ogunlesi et al., 2004). Glutamine-supplemented, hyperoxia-exposed cells have a higher O2 consumption rate and GSH content. In hyperoxia, glutamine protected cellular structures, especially mitochondria, from damage. In these conditions, activity of the tricarboxylic acid cycle (TCA) enzyme α-ketoglutarate dehydrogenase is partially protected by this indirect substrate, stating a mechanism of mitochondrial glutamine-dependent protection (Rossignol et al., 2004).

2.1. Mitochondrial network morphology

An important aspect of mitochondrial dynamics is its morphology (Soubannier and McBride, 2009). Recent findings show that the mitochondrial morphology is a function of multiple factors, including energetic status of the cells (Frey and Sun, 2008).

When producing energy by OXPHOS, both HeLa and fibroblast mitochondria adopt the condensed configuration, whereas during glycolysis, they maintain an orthodox state (Lyamzaev et al., 2004; Tolstonog et al., 2005). Mitochondria often appear as a continuously branched reticulum of connected tube-like filaments (Knowles et al., 2002). The ramification of the mitochondrial reticulum for OXPHOS may be important in generating ATP at all parts of the cell. When forced to use OXPHOS, cancer cells that also present a deficient mitochondrial system compared with their tissue of origin could have been expected to lead to the same adaptable modifications of the mitochondria (John et al., 2005). On the contrary, it is the condensation, thinning, and ramification of the mitochondrial reticulum that dominates the changes (Koopman et al., 2005). The signaling pathways by which this is accomplished must include transcription factors that alter expression of OXPHOS components. The factors determining mitochondrial structure in tissues could be substrate nature and availability, oxygen partial pressure, energy demand, and mitochondrial respiratory steady state. Mitochondrial network remodeling could be of particular importance for the survival of large tumours (Benard and Rossignol, 2008).
Complex I \( (\text{H}^+\text{-pumping NADH:quinone oxidoreductase}) \) is an essential component of the mitochondrial respiratory chain, participating not only in cell respiration, but also in cellular/organismal reactive oxygen species homeostasis, apoptosis initiation or modulation, and \( O_2 \) sensing (Piruat and López-Barneo, 2005). This complex is vulnerable to oxidative stress, hence it is one of the factors that determines lifespan, pace of aging, susceptibility to oxidative stress-related diseases, including atherosclerosis, hypertension, ischemia-reperfusion injury, inflammation, cystic fibrosis, cancer, type-2 diabetes, and neurodegenerative diseases. The excess of superoxide production is low in glucose-cultivated cells, but doubled after cultivation with galactose/glutamine (i.e. at obligatory oxidative phosphorylation) (Dlasková et al., 2008).

Mitochondria of cells grown in glutamine/galactose show the condensed configuration when examined by electron microscopy, whereas only a small fraction was in this state in cells grown on glucose. When compared with cells grown in high glucose medium, glutamine/galactose-grown cells showed consistent increases in the amount of cristal membranes, as well as alterations in the mitochondrial reticulum, but not more mitochondrial mass. In HeLa cells grown in glucose medium, the mitochondria appeared as a reticulum mostly clustered in the perinuclear region and not typically extending far out into the cells. In contrast, in glutamine/galactose-grown cells, the mitochondrial reticulum extended outward much more into the additional processes of the cell and appeared to be more elaborately interconnected and ramified, displaying rings joined with one another by short filaments. Although such rings are seen in cells grown in glucose, they were very much less frequent. These rings are not observed in glutamine/galactose grown MRC5 fibroblasts. Consistent with the restructuring of the mitochondrial reticulum, electron microscopy of glutamine/galactose-grown HeLa cells show more heterogeneity of the size of mitochondrial profile than glucose-grown cells because of sectioning through long stretches of reticulum, as well as heavily looped regions (Rossignol et al., 2004).

Sections of human pulmonary epithelial-like (A549) cells grown in normoxia reveal that in the absence of glutamine, the cells have dense but normal-appearing mitochondria. With 1 mM glutamine supplementation mitochondria are abundant in these cells, and they appear larger and have a less dense matrix than those in glutamine-unsupplemented cells. Hyperoxia-exposed cells grown in glucose-free medium show extensive mitochondrial disintegration and injury. Cells exposed to hyperoxia and supplemented with glutamine (1 mM or 4 mM) show some appearing relatively normal, some appearing distended and empty, and some with very few cristae apparent. Additional morphological abnormalities also are detected in hyperoxia-exposed cells. Mitochondria in normoxia-exposed control cells with or without glutamine show thin filamentous structures. In the presence of glutamine and hyperoxia, the mitochondria appear more round in shape and densely packed around the nucleus. Hyperoxic exposure of glutamine-unsupplemented cells results in a complete loss of the filamentous structure. Lastly, in glutamine-supplemented cells, the mitochondria are predominantly enlarged and granular, suggesting the formation of megamitochondria (Ahmad et al., 2001). A549 human airway epithelial cells have been shown to express GA and to generate substantial amounts of ammonia from glutamine alkalizing their culture medium (Hunt et al., 2002).

### 3. Mitochondrial glutamine transport

Glutamine has to be transported through both plasma and inner mitochondrial membranes before glutaminolysis can take place. Glutamine transport across cell membranes has been extensively studied. The transport of glutamine at the plasma membrane level is out of the scope of this article, but readers interested may find excellent recent reviews on the topic (see for example McGivan and Bungard, 2007). The transport of glutamine across the membrane of mammalian mitochondria has been investigated although in a lesser extent than its plasma membrane counterpart, probably due in part to the peculiar environment of the inner mitochondrial membrane (IMM) and mitochondrial matrix, where high protein concentrations can be achieved. Moreover, the characterization of mitochondrial glutamine carriers has been hampered by the very active glutamine metabolism in this organelle, which overlaps with the transport process posing severe methodological shortcomings when measuring transport rates.

Studies on mitochondrial glutamine transport using isolated mitochondria from various mammalian tissues have been done since 1970. Although the existence of a mitochondrial glutamine carrier was widely postulated, data concerning the structure and function of the transport system are scarce and only one mitochondrial glutamine carrier has been purified so far (Indiveri et al., 1998), which can give us an idea of the difficulty of the task. On the other hand, mitochondrial transport studies have led to the characterization of about 25 distinguishable inner membrane transport systems (Porter, 2000).

Kovacevic et al. (1970) firstly postulated the existence of a neutral uniport mechanism for L-glutamine in liver mitochondria; the uptake was inhibited by mersalyl (Simpson and Adam, 1975). Since the mitochondrial transport of metabolites is usually 1–2 orders of magnitude faster than the plasma membrane related transport (Klingenberg, 1979), special problems arise in mitochondria transport studies. So, several experimental approaches have been attempted to study transport in intact mitochondria, including the inhibitor stop technique and rapid centrifugation and filtration procedures (La Noue and Schoolwerth, 1979). Using the latter technique, Goldstein and Boylan (1978) were able to obtain initial velocity data in the 1-second range for glutamine transport in rat kidney mitochondria. However, a major drawback in this study was the strong interference due to the very active mitochondrial glutamine metabolism. To avoid this problem, Kovacevic and Bajić (1982) loaded rat liver mitochondria with \([14C]\)-glutamine and the efflux rates were determined at 0–1 min in an inorganic phosphate (Pi)-free medium to block GA activity. A more effective method to study mitochondrial transport in the absence of further metabolism of the molecule transported is the use of sealed vesicles. Thus, submembranous particles prepared by sonication or inner-membrane vesicles obtained by mild treatment with the detergents digitonin/Lubrol have been employed to characterize glutamine transport. The glutamine transporter from Ehrlich ascites carcinoma cells was studied using this last method (Molina et al., 1995). The native IMM vesicles were optimized to allow transport measurements without any interference by the mitochondrial glutamine metabolism. Conveniently, the absence of any GA activity in the vesicles was critical, since this process interferes notably with glutamine transport studies (La Noue and Schoolwerth, 1979). L-glutamine transport showed a curvilinear time course, but the initial rate approximated to linearity for the first 60s (Fig. 1). The L-glutamine uptake reached a steady state within 120 min; at this time an accumulative transport value of 726.2 ± 68 pmol/mg was calculated. The transport capacity of tumour cells was one order of magnitude greater than the value reported for submembranous particles of rat kidney, where
plots were non-linear. The curve drawn through the experimental data represents the best fit to the Hill equation. (Reproduced with permission, from Molina et al., 1995, Biochemical Journal, 308, 629–633. ©the Biochemical Society.)

Mitochondrial L-glutamine transport in Ehrlich tumour cells showed an allosteric kinetic with positive cooperativity and a Hill coefficient of 2.2 (Fig. 1). The kinetic parameters $S_{0.5}$ and $V_{\text{max}}$ had values of 5 mM and 26 nmol/30 s per mg of protein, respectively (Molina et al., 1995). Sigmoidal kinetics were also observed in rat kidney submitochondrial particles, with $S_{0.5}$ and $V_{\text{max}}$ values of 19.5 mM and 3.76 nmol/15 s per mg of protein, respectively (Sastrasinh and Sastrasinh, 1989). The comparison of the normalized tumour data with rat kidney data shows that the ratio $V_{\text{max}}/S_{0.5}$, a criterion of the transport efficiency, was about 16-fold greater in tumour mitochondria. Compared with hyperbolic kinetics, a protein following sigmoidal kinetics would allow a more sensitive control of the glutamine transport rates. This mechanism could avoid a rapid depletion of cytosolic glutamine essential for biosynthetic purposes. The pH optimum of the tumour mitochondrial carrier was about 8.0, in excellent agreement with the value reported for GA (Quesada et al., 1988). When the mitochondrial electron transport is activated, the matrix pH becomes slightly alkaline, and both the glutamine carrier and GA can be fully operative. Actually, glutamine is a good respiratory substrate for tumour cells, even in the presence of glucose. In contrast, a pH optimum of 6.5 has been reported in rat kidney submitochondrial particles (Sastrasinh and Sastrasinh, 1990). In this system, the glutamine uptake increased more than 2-fold when the pH of the medium was decreased from 8.5 to 6.5, in accordance with the physiological role of renal glutaminolysis: the neutralization of $H^+$ excess.

The uptake of L-glutamine in mitochondria from Ehrlich tumour cells was stereospecific and not affected by the presence of excess D-glutamine, L-cysteine, L-histidine, L-alanine, L-serine and L-leucine, whereas L-glutamate behaved as a weak inhibitor (Molina et al., 1995). Curiously, histidine has been shown to inhibit Gln uptake in cerebral mitochondria (Albrecht et al., 2000). With regard to inhibition by thiol reagents, vesicles exposed to 1 mM NEM or 0.5 mM mersalyl were unaffected; however, incubation with 0.5 mM PCMPs yielded a full inhibition of the glutamine uptake. The inhibition pattern of the mitochondrial carrier was markedly different from that shown by GA in mitochondria, where 1 mM NEM completely abolished the enzyme activity (see next section). These results, along with the lack of any GA activity or GA protein in the IMM vesicles, clearly discarded the possibility of GA and the carrier forming part of the same protein.

Whether Gln uptake to mitochondria is directly coupled with its intramitochondrial degradation by GA is a matter of dispute. Experimental evidences both in favor and against this concept have been reported. Thus, inhibition of mitochondrial Gln uptake in brain mitochondria by some natural amino acids and their synthetic analogs was found to correlate well with the inhibitory effect of these compounds on Gln degradation (Albrecht et al., 2000). On the other hand, factors such as calcium plus phosphate which stimulate GA inhibit Gln uptake, while the endogenous amino acids taurine and N-acetylaspartic acid inhibit GA but do not affect Gln uptake (Albrecht et al., 2007). A very attractive hypothesis would be the association of GA with the mitochondrial glutamine carrier as a channeling mechanism; some early experimental data suggested that glutamine is not released into the matrix but rather delivered directly to GA (Simpson and Adam, 1975). Alternatively, GA might be also part of the mitochondrial transport system for glutamine (Curthoys and Weiss, 1974). However, the IMM vesicles obtained by digitonin/Lubrol showed a very active L-glutamine transport activity although they were devoid of GA protein. Thus, in Ehrlich mitochondria both activities belong to different proteins, although the possibility of a physical interaction between them cannot be completely ruled out (Matsuno, 1987).

The intracellular glutamine concentration is undetectable during the exponential growth phase of Ehrlich ascites tumour cells (Márquez et al., 1989). The glutaminolysis process seems to be regulated by the GA expression, which also reaches a maximum during the exponential growth of the tumour (Aledo et al., 1994). However, there has been much controversy about the relative importance of the various steps in glutamine degradation. In fact, one of the most conflicting points regarding the mitochondrial glutamine metabolism is to elucidate the rate-limiting step of the process: the carrier, GA or both. Some authors concluded that mitochondrial transport is not rate-limiting for glutamine hydrolysis (Goldstein and Boylan, 1978; Kovacevic and Bajin, 1982; Nelson et al., 1992), whereas others have suggested that transport could be the major site of regulation of the pathway (Haußinger et al., 1985; Lenzén et al., 1987). Comparison of the normalized values of $V_{\text{max}}/S_{0.5}$ for the carrier and GA in tumour cells points out that GA has a 15-fold higher catalytic efficiency than the carrier (Molina et al., 1995). This may indicate a putative regulatory role for the carrier in the glutaminolytic process, characteristic of the neoplastic transformation. A similar result has been reported for rat kidney (Sastrasinh and Sastrasinh, 1989); however, in rat liver cells GA was identified as the major control site of mitochondrial glutamine metabolism (Low et al., 1993). Therefore, we can conclude that glutaminolysis control should be carefully re-evaluated to clarify inconsistencies, although species- and/or tissue-specific differences might also explain these discrepancies on glutaminolysis control.
4. Glutaminases

Mammalian glutaminases catalyze the hydrolytic deamidation of glutamine to glutamate and ammonium ions. Two distinct genes in separate chromosomes encoding GA isozymes have been identified in mammals; in humans, the Glu gene is located in chromosome 2 and encodes the kidney-type (K) isozyme; the second locus is the Gls gene located on chromosome 12 and coding for the liver-type (L) isozyme (Aledo et al., 2000). From each gene, at least two isoforms have been reported so far: two K-type GA isoforms named KGA (Shapiro et al., 1991) and GAC (Elgadi et al., 1999) were derived from the Glu gene, and two L-type GA isoforms termed LGA (Smith and Watford, 1990) and GAB (Gómez-Fabre et al., 2000; de la Rosa et al., 2009) arise from the Gls2 gene. The different isoforms differ in their molecular, kinetic, immunological and regulatory properties (Curthoys and Watford, 1995; Márquez et al., 2006). GA is expressed in most mammalian tissues and cancer cells, but the regulation of its organ- and tumor-specific expression is now starting to be unraveled. The presence of multiple GA transcripts has been demonstrated even in a single cell type (Turner and McGivan, 2003; Pérez-Gómez et al., 2005). The abundance of a particular GA mRNA species may significantly change depending upon the tissue type and the developmental or metabolic state of the tissue; it is tempting to speculate that each transcript may represent a specific target for different stimuli, the overall GA expression being the balance between these stimuli.

Glutaminases have been traditionally considered as mitochondrial enzymes (Curthoys and Watford, 1995), although novel subcellular locations have been recently reported for L-type GA in mammalian brain (Ollalla et al., 2002) and cells of the immune system (Castell et al., 2004). The submitochondrial localization of GA has been a very controversial issue with profound physiological implications. To illustrate the discrepancies, we can say that almost all possible submitochondrial localizations have been proposed for GA, including matrix soluble, matrix-side of the IMM and simultaneous presence in both halves of the IMM. Thus, previous studies on kidney GA have reported that the enzyme is located in the matrix region (Kalra and Brosnan, 1974), matrix side of the IMM (Shapiro et al., 1985) and in both halves of the IMM, existing two populations of GA: one oriented in the intermembrane space (c-side); the other in the matrix space (m-side) (Kvamme and Olsen, 1979). The last authors observed an incomplete blockade of GA activity in brain mitochondria by glutamate, ammonia and the SH-group reagent N-ethylmaleimide (NEM) (Kvamme and Olsen, 1981; Kvamme et al., 1983) and used this evidence as the basis to propose that GA exists in two forms located in separate sites: the NEM-sensitive form was located at the outer surface of the IMM (c-side), whereas the insensitive form was situated at the inner surface (m-side) of the IMM. Later on, Kvamme and associates have suggested predominant c-side localizations for the K-type isoenzymes from kidney and brain, based on the inactivation by non-permeant SH-reagents and the lack of mixing between the glutamine-derived glutamate and the endogenous matrix glutamate (Kvamme et al., 1991; Roberg et al., 1995).

We studied the submitochondrial localization and membrane topography of the Ehrlich ascites tumour cells GA. The GA enzyme from Ehrlich cells was essentially a K-type isoform as judged by kinetic and immunological approaches (Quesada et al., 1988). The topography of GA in the mitochondria has important implications in tumour cells, since they maintain a high glutaminolysis related to the operativity of glutamine, glutamate and phosphate carriers. We found convergent evidence from enzymatic, immunological and chemical modification studies which discard a transmembrane topography and strongly support a peripheral location for GA on the inner surface of the IMM; the protein has its functionally relevant domain in the matrix space and the lipid environment may be important in regulating GA activity (Aledo et al., 1997). For example, the inhibition pattern of GA observed with SH-reagents of different permeability clearly indicated that the IMM becomes a barrier to access to essential GA residues; in particular fractions, mitochondria and mitoplasts (mitochondria devoid of outer membrane), the membrane-permeant reagent NEM strongly inhibited GA activity but almost no effect was detected with membrane-impermeant reagents such as mersalyl and p-chloromercuriphenylsulphonic acid (PCMS). However, at the same concentrations, mersalyl and PCMS, as well as NEM, were very effective inhibitors of the soluble GA (Aledo et al., 1997).

Moreover, GA was fully inactivated when mitoplasts were treated with either phospholipase A2 or C, suggesting important lipid–protein interactions needed for proper enzyme function (Fig. 2A). The orientation of GA in the IMM was also studied by proteolytic digestions with trypsin on both sides of the IMM (Fig. 2B). The results strongly support that most of the functionally sensitive domain of Ehrlich GA is located on the m-side of the membrane, and do not agree with the orientation reported for the kidney and brain K-type enzymes, where a main external (c-side) location was postulated (Kvamme et al., 1991; Kvamme et al., 2001). This last orientation would allow the enzyme being regulated by a great variety of compounds present in the cytosol, as well as direct access of the glutamine-derived glutamate to cytosolic metabolism. However, our results are only compatible with glutamine-derived glutamate
being generated into the matrix and having direct access to mitochondrial metabolism.

Recently, additional experimental evidence has been reported demonstrating that the active site of GA must be facing the matrix side. Metabolic studies employing [U-13C]glutamine and NMR spectroscopy in cultured neurons have established that almost 50% of the glutamine metabolism involves TCA reactions (Waagepetersen et al., 2005). This result means that part of the neurotransmitter glutamate could be ascribed to transamination pathways but, at the same time, it is incompatible with most of the GA-derived glutamate being generated outside the matrix. Similar findings were obtained using isolated rat brain mitochondria (Bak et al., 2008).

However, an interesting observation also emerged from this last study: a 50% decrease in the mitochondrial glutamate level was detected in the presence of histidine, previously characterized as an inhibitor of glutamine uptake in cerebral mitochondria (Albrecht et al., 2000). The authors interpreted these results as evidence of GA acting from the mitochondrial matrix; furthermore, they show that glutamine transport is a prerequisite for glutamate formation and further metabolism in the TCA cycle (Bak et al., 2008). In fact, the location of GA’s active site in the c-side of the IMM, being accessible to the cytosol, poses several questions difficult to ascertain, such as the necessity for very active mitochondrial glutamine carriers, as those reported for rat kidney and tumour cells (see previous section). In contrast, the generation of glutamate into the matrix would facilitate their further metabolism for bioenergetics or biosynthetic purposes.

5. Glutamine in brain cells

Mitochondria are particularly important in the nervous system due to the peculiar metabolic profile of neurons and to the need of neurotransmitter synthesis, release and re-uptake. Mitochondria are essential to the functions of neurons because their limited glycolytic capacity makes them highly dependent on aerobic OXPHOS. Mitochondrial decay plays a key role in aging and colytic capacity makes them highly dependent on aerobic OXPHOS, are essential to the functions of neurons because their limited glyco-

neurotransmitter synthesis, release and re-uptake. Mitochondria facilitate their further metabolism for bioenergetics or biosynthetic processes. In addition, ammonia has been shown to induce the MPT in mitochondria. The key piece of this hypothesis is the existence of GA activity in astrocytes by proposing a toxic role: the “Trojan horse”, glutamine, ends up causing neurotoxicity through deamination by GA, thereby generating very high levels of ammonia in mitochondria. The key piece of this hypothesis is the existence of GA activity in astrocytes by proposing a toxic role: the “Trojan horse”, glutamine, ends up causing neurotoxicity through deamination by GA, thereby generating very high levels of ammonia in mitochondria.

This cycle assumes that glutamine, in turn, is released from the astroglial glutamine pool (Bröer and Brookes, 2001). Glutamine has been shown to be a primary target before the development of any amyloid deposition or cogni-

Cerebral glutamate and glutamine are predominantly localized in glutamatergic neurons and astroglia, respectively (Shen, 2006). Independent studies using in vivo microdialysis and mass spectrometry to determine the labeling of extracellular glutamate and glutamine have shown that neuronal glutamate (through glutamate–glutamine cycling) is the precursor for 80% to 90% of glial glutamine synthesis. In fact, the reported values of the glutamate–glutamine neurotransmitter cycle flux in humans and the relationship between glutamate–glutamine cycle flux and neuronal TCA cycle flux are in relatively good agreement (Shen et al., 2009). In addition to its interaction with the predominantly neuronal glutamate, the astroglial glutamine is also in exchange with blood, allowing for unlabeled glutamine from blood to be transported into brain, and the glial compartment, effectively diluting the astroglial glutamine pool (Bröer and Brookes, 2001).

Mitochondrial membrane potential, respiratory control ratio, and cellular oxygen consumption decline with age and correlate with increased oxidant production. Chronic, accumulated oxidants most likely also cause increased damage and consume critical resources in neurons and astrocytes.

Glutamine has been implicated in the mechanism of ammonia neurotoxicity and many observations support the view that mitochondria are indeed the critical organelles in which glutamine undergoes its toxic action. Glutamine synthetase (GS; EC 6.3.1.2), an enzyme primarily expressed in brain astrocytes (Norenberg and Martínez-Hernández, 1979), detoxifies ammonia through its incorporation into glutamine. Accordingly, glutamine levels are elevated several fold in the cerebrospinal fluid of patients with HE, as well as in different animal models (reviewed by Albrecht and Norenberg, 2006). Administration of methionine sulfoximine (MSO), an inhibitor of GS, prevented cerebral glutamine accumulation and the increase of brain water (Blei et al., 1994). Furthermore, astrocyte swelling, ammonia-induced MPT and free radical production can be completely blocked by MSO (Bai et al., 2001; Norenberg et al., 2004 and references therein), suggesting that glutamine can mediate ammonia toxicity to the brain. Similar and convergent results were also obtained by inhibiting the mitochondrial transport of glutamine (Pichili et al., 2007) or by blocking the mitochondrial glutamine catabolism with the GA inhibitor 6-diazo-5-oxo-noerulene (DON) (Jayakumar et al., 2004).

A “Trojan horse” hypothesis has been formulated to explain why glutamine is capable of reproducing many of the toxic effects of ammonia on astrocytes (Albrecht and Norenberg, 2006). The authors summarized recent investigations showing that glutamine, when added directly to cultured astrocytes, induces the MPT and promotes the formation of free radicals. Then, they question the benign role of glutamine in astrocytes by proposing a toxic role: the “Trojan horse”, glutamine, ends up causing neurotoxicity through deamination by GA, thereby generating very high levels of ammonia in mitochondria. The key piece of this hypothesis is the existence of GA activity in vivo in astrocytes, a strongly questioned issue and matter of debate and controversy for many years. Conflicting reports have been published about the expression of GA in astrocytes: in vitro experiments reporting GA activity have been largely questioned arguing that GA was induced by the glutamine present in the culture medium (Erecinska and Silver, 1990). Of interest, the expression of L-type GA has been recently demonstrated in rat brain astrocytes by immunocytochemistry (Olalla et al., 2008). The possible occurrence of GA in astrocytes in vivo is an interesting and important issue which demands further investigations.

Although there is more than one pathway for glutamate synthesis in cells, the vesicular glutamate pool in neurons is derived primarily from glutamine through the enzyme GA (Nicklas et al., 1987). Furthermore, neurotransmitter glutamate synthesis has been shown to be also dependent on transamination of α-ketoglutarate involving tricarboxylic acid cycle reactions (Waagepetersen et al., 2005). Most of the released glutamate is taken up by the glial compartment and converted to glutamine. A glutamate–glutamine shuttle between neurons and glial cells has been postulated for neurotransmitter recycling (Hertz, 1979). This cycle assumes that glutamine, in turn, is released from the glial cells, taken up by neurons, and converted back to glutamate. Cerebral glutamate and glutamine are predominantly localized in glutamatergic neurons and astroglia, respectively (Shen, 2006). Independent studies using in vivo microdialysis and mass spectrometry to determine the labeling of extracellular glutamate and glutamine have shown that neuronal glutamate (through glutamate–glutamine cycling) is the precursor for 80% to 90% of glial glutamine synthesis. In fact, the reported values of the glutamate–glutamine neurotransmitter cycle flux in humans and the relationship between glutamate–glutamine cycle flux and neuronal TCA cycle flux are in relatively good agreement (Shen et al., 2009). In addition to its interaction with the predominantly neuronal glutamate, the astroglial glutamine is also in exchange with blood, allowing for unlabeled glutamine from blood to be transported into brain, and the glial compartment, effectively diluting the astroglial glutamine pool (Bröer and Brookes, 2001).
metabolites as small molecular weight antioxidants. Damaged mitochondria are associated with ATP production and increased ROS production. In a very recent experiment, neurons from old control animals show a range of mitochondrial abnormalities: the presence of enlarged (giant) mitochondria, clusters of mitochondria with electron-dense matrices, mitochondrial matrix edema, mitochondrial with partially and/or completely damaged cristae, double membrane surrounding lisosome-like structures, vacuoles, vacuolar lipofuscin, lipofuscin deposits, and membrane disruptions. Mitochondrial damage is not limited to hippocampal neurons. The aged rat hippocampal astrocytes also reveal mitochondria with electron-dense matrices. Neuron from young rats + 0.2% lipoic acid + 0.5% acetyl L-carnitine dietary supplementation stimulates mitochondrial proliferation, showing preservation of cristae, less structure disruption and significant improvement of mitochondrial integrity in comparison with young untreated rats (Aliév et al., 2009).

6. Glutamine in cancer cells

Tumours generally reveal a strong diminution in mitochondrial content and in OXPHOS capacity (Rossignol et al., 2004). Energy substrate availability could play an important role in this phenomenon. Of particular significance, ability to use glycolysis versus OXPHOS, and the effect that energy substrate type has on mitochondrial composition, structure, and function. Accordingly, mitochondria participate in a variety of cellular processes, like apoptosis, indicating that the control of their dynamic shape is probably multifactorial and their impact on cell activity very diverse. Defective mitochondrial system described in cancer cells can be dramatically improved by solely changing substrate availability. For example, HeLa cells can adapt their mitochondrial network structurally and functionally to derive energy by glutaminolysis only (Benard et al., 2007). This may also provide an explanation for the enhancement of OXPHOS capacity observed after tumour regression or removal.

Cancer cells generate energy by glycolysis in strong preference to OXPHOS (Warburg, 1930; Reitzer et al., 1979). When glucose is no longer available, as can occur in solid tumours, cancer cells are forced to use alternative energy substrates such as the oxidation of glutamine (glutaminolysis), which can produce differences in the number of mitochondria, their ultrastructure, the content and composition in all OXPHOS complexes, ATP synthase, the expression of OXPHOS dependent genes, and levels of mtDNA. In fact, glutaminolysis requires an active OXPHOS for ATP production (Reitzer et al., 1979). In HeLa cells grown in glutamine/galactose, when forced from glycolysis to growth by OXPHOS, mitochondrial respiration is stimulated, matrix redox state is more oxidized, and cells synthesize more respiratory chain proteins like porin, citrate synthase, PDH, and respiratory chain complexes I, II, IV, and ATP synthase. Additional experiments using culture of a primary (lung) fibroblast line (MRC5) also show slower growth in glutamine/galactose than in glucose (Rossignol et al., 2004).

With regard to the energy metabolism of tumour cells, almost one century ago Warburg proposed his early idea that respiration was quantitatively impaired in tumours. He observed that in tumours there is a high glycolytic flux even in the presence of oxygen. This fact has been named aerobic glycolysis and is characteristic in a great variety of tumour cells (Warburg, 1930) and also in normal proliferant cells (Medina and Núñez de Castro, 1990). However, early works showed that the respiration deficit in tumour cells was not manifested in a significant diminution in oxygen consumption of such cells in vitro (Weinhouse, 1955) and the presumed impairment of mitochondrial function was never established in cancer biology (Pedersen, 1978). As a matter of fact, we do not know at present the exact role that mitochondria play in neoplastic transformation, with only one renowned exception: in highly glycolytic hepatoma cell lines their abnormal energetic signature was ascribed to a marked reduction in the cellular content of mitochondria, mimicking the foetal hepatocyte phenotype where an organelle biogenesis programme limits the number of mitochondria per cell (López de Heredia et al., 2000). Interestingly, it has been recently found what seems to be a strong experimental support for Warburg’s hypothesis: in human liver carcinomas there has been a marked depletion of the cellular mitochondrial content, whereas in colon and kidney carcinomas a down-regulation of the β-F1-ATPase concurrent with an overexpression of glycolytic glyceraldehyde-3-phosphate dehydrogenase were detected (Cuevza et al., 2002).

Tumour-derived cells cultured in glucose medium can produce 40 times more lactic acid than normal cells. This reaction can only occur when the cytosolic NADH/NAD+ ratio is high and/or when the pyruvate does not enter the mitochondria for complete oxidation by the PDH, the Krebs cycle and mitochondrial OXPHOS. Several suggestions have been proposed including mutations in mtDNA (Singh et al., 1999). For instance, a point mutation in a gene coding for a respiratory chain complex results in biochemical threshold effect (Rossignol et al., 2000). Actually, variations in the amount of mtDNA which are observed under different metabolic conditions represent an adaptation of the mitochondrial metabolism to cellular metabolism (Rocher et al., 2008). Increased binding of hexokinase to the mitochondrial outer membrane can also occur, reducing Pi and ADP delivery to the OXPHOS system. One interesting difference between a transformed and non-transformed cell line is the change in levels of PDH complex. Of note, in glutamine/galactose medium energy for growth is derived via glutamine, which is catalyzed by mitochondrial glutaminase into glutamate, which is then converted into α-keto glutarate (α-KG) by glutamate dehydrogenase and additionally processed in the Krebs cycle (Rossignol et al., 2004).

Tumour decisions on the type of preferred metabolite to satisfy its energy and biosynthetic needs seem to be dictated by mutations affecting certain oncogenic signaling pathways. Activating mutations in the phosphoinositide 3-kinase (PI3K) and its downstream effector AKT make the cells take up glucose in excess of its bioenergetic needs, and leads mitochondria to support glucose-dependent lipid synthesis and non-essential amino acid production (Elstrom et al., 2004; Wise et al., 2008). Recently, it has been revealed a novel role of the overexpressed oncogene Myc on the tumour glutamine metabolism: Myc represses miR-23a/b microRNAs resulting in greater expression of mitochondrial GA and leading to upregulation of mitochondrial glutamine catabolism (Gao et al., 2009). Myc has been shown to be involved in the glutamine dependence feature of certain tumours, leading to commit Myc dependent apoptosis in a glutamine deficient ambient (Yuneva et al., 2007). To activate glutaminolysis in these tumours Myc seems to induce not only glutamine transportes such as ASCT2 and SN2, but also GA, that deamidates glutamine into glutamate resulting in its intracellular capture, and lactate dehydrogenase (LDH), which converts glutamine-derived pyruvate into lactate (Fig. 3). Glutaminolysis replenishes TCA cycle via α-KG and generates a robust production of NADPH necessary for cell growth. Thus, as a consequence of Myc overexpression, the mitochondria are reprogrammed to depend on glutamine catabolism to sustain cellular viability and TCA cycle anaplerosis (Wise et al., 2008).

In human colon cancer cell lines, glutamine supplementation stimulates proliferation, promotes a less differentiated phenotype and inhibits cell adhesion to solid matrix, changes expected for a more aggressive tumour behavior in vivo (Turowskij et al., 1994). The authors conclude that nutritional supplementation with glutamine may be deleterious in patients with colon cancer. On the other hand, the high rates of glutaminolysis shown by certain types of tumours...
Glucose to lactate in the presence of oxygen was first reported for tumour cells. Increased glucose consumption may also help supply carbons for proliferation even under 80% O2, termed “HeLa-80,” was derived from wild-type HeLa cells (“HeLa-20”). Surprisingly, antioxidant defenses are nearly identical, indicating that the tolerance of these cells is high oxygen is probably not because of enhanced defenses against ROS. Besides, many of these antioxidant enzymes are known to be induced by ROS (Matés et al., 2008).

The fact that antioxidant enzymes display normal activities even in the tolerant HeLa cells exposed to high oxygen, suggests that these cells somehow manage to avoid exaggerated ROS production even when exposed to high partial pressures of oxygen (Campian et al., 2004). Assuming that hyperoxic cell damage does arise from exaggerated mitochondrial ROS production, the site of electron leak from the electron transport chain becomes an important question (Gerschman et al., 2001). Results point to an increased coupling and efficiency of mitochondrial metabolism in the oxygen-tolerant HeLa-80 cells. Indeed, in these cells both glucose consumption and lactate production are lower under both normoxia and hyperoxia, whereas oxygen consumption does not differ significantly from the wild-type HeLa-20 cells. Possibly reflecting an increased efficiency of mitochondrial metabolism, it was found that the lesser glucose consumption by HeLa-80 cells is paralleled by an increase in glutamine consumption (Campian et al., 2004).

Cellular respiration declines in parallel with the loss of aconitase activity in cells cultured in hyperoxia (Gardner et al., 1994). One mechanism by which cultured cells adapt to the stress of hyperoxic exposure is through increased glycolysis (Allen and White, 1998). Although impairment of the aconitase step occurs, inhibition of subsequent steps in the TCA cycle pathway occurs later and less completely (Joenie et al., 1985). The alternate substrate glutamine enters the TCA cycle subsequent to the aconitase step (Ahmad et al., 2001). As stated before, mitochondria are a potential target of injury by ROS, and an alteration in mitochondrial membrane function is an important component of oxidative stress in cells. It was found that the inner mitochondrial membrane potential is profoundly affected by the presence or absence of glutamine (Matés et al., 2006). Cells cultured in air in the absence of glutamine can survive, but not proliferate, and preserve mitochondrial integrity (Souba et al., 1990). In hyperoxia, these cells can neither proliferate nor survive, and their death is preceded by degeneration of their mitochondria. Paradoxically, cells provided with glutamine utilized the amino acid at a considerably increased rate in hyperoxia compared with cells exposed to normal O2 tensions. Glutamine significantly increased cellular ATP levels in normoxia and prevented the loss of ATP in hyperoxia seen in glutamine-deprived cells. Glutamine supplementation also prevented the loss of ATP observed in the unsupplemented cells (Ahmad et al., 2001).

Glutamine is an important substrate for GSH synthesis and GSH is an important antioxidant in cellular tolerance to oxidative stress (Lora et al., 2004). Total GSH content of hyperoxia-exposed...
glutamine-unsupplemented cells decreased, compared with that in similar cells in normoxia (Schoonen et al., 1990). Two biochemical mechanisms may have contributed to these effects. First, a decline in cell GSH content; and second, a complete inhibition of α-KG dehydrogenase activity in the TCA cycle (Shi et al., 2008).

Intramitochondrial redox status and apoptosis. Pentose phosphate and TCA pathways afford NADPH status, which regulates GSH levels. In most cell types, limiting glutamine supply or inhibiting glutamine metabolism via GA, inhibits the availability of intracellular glutamate available for GSH biosynthesis, which is essential for counteracting against the release of cytochrome c leading to apoptosis. GA: glutaminase, GSH: glutathione, TCA: tricarboxylic acid cycle.

Fig. 4. Intramitochondrial redox status and apoptosis. Pentose phosphate and TCA pathways afford NADPH status, which regulates GSH levels. In most cell types, limiting glutamine supply or inhibiting glutamine metabolism via GA, inhibits the availability of intracellular glutamate available for GSH biosynthesis, which is essential for counteracting against the release of cytochrome c leading to apoptosis. GA: glutaminase, GSH: glutathione, TCA: tricarboxylic acid cycle.

8. Perspective

As detailed in this review, glutaminolysis has relevant implications in cell bioenergetics, signal transduction pathways, oxidative stress, apoptosis and mitochondrial shape and dynamics. In the last years, a growing body of evidence indicates that glutaminolysis may have a profound effect on mitochondrial functionality. The high proliferation rate of transformed cells induces a change in their energy metabolism where glutamine, instead of the glucose-derived pyruvate, is used preferentially for ATP production. The signals and mechanisms underpinning this metabolic shift are largely unknown and not fully understood, but they are certainly involved in the regulation of cell growth, proliferation and apoptosis. Future analysis of inherited defects of glutaminolytic enzymes, molecular characterization of mitochondrial glutamine carriers and glutaminases, as well as generation of transgenic animal models will provide further insights into the specific actions of glutamine and give further rationale for considering glutamine as a therapeutic tool.

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