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Advances in improving mammalian cells metabolism for recombinant protein production

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Abstract

Background: The production of recombinant proteins for therapeutic use represents a great impact on the biotechnology industry. In this context, established mammalian cell lines, especially CHO cells, have become a standard system for the production of such proteins. Their ability to properly configure and excrete proteins in functional form is an enormous advantage which should be contrasted with their inherent technological limitations. These cell systems exhibit a metabolic behaviour associated with elevated cell proliferation which involves a high consumption of glucose and glutamine, resulting in the rapid depletion of these nutrients in the medium and the accumulation of ammonium and lactate. Both phenomena contribute to the limitation of cell growth, the triggering of apoptotic processes and the loss of quality of the recombinant protein.
Results: In this review, the use of alternative substrates and genetic modifications (host cell engineering) are analyzed as tools to overcome those limitations. In general, the results obtained are promising. However, metabolic and physiological phenomena involved in CHO cells are still barely understood. Thus, most of publications are focused on specific modifications rather than giving a systemic perspective.
Conclusions: A deeper insight in the integrated understanding of metabolism and cell mechanisms is required in order to define complementary strategies at these two levels, so providing effective means to control nutrients consumption, reduce by-products and increase process productivity.

Keywords: aerobic glycolysis, cell engineering, CHO cells, glutaminolysis, metabolism

INTRODUCTION

The market of biopharmaceutical products or drugs based on recombinant proteins (r-proteins) is growing rapidly, with total sales reaching more than 138 billion US dollars in 2010 (Visiongain, 2012). Since the first therapeutic application of a r-protein produced in mammalian cells was approved in 1986 (human tissue plasminogen activator, tPA, Genentech), biopharmaceuticals have changed the landscape for the treatment of many diseases, including many types of cancer, rheumatic conditions and orphan diseases. Moreover, the biopharmaceutical market has grown at an annual average rate of 35% since 2001, indicating a financially sound future for the biopharmaceutical industry (Aggarwal, 2007).

Although there are a variety of alternative expression systems available, including those using bacteria and transgenic animals, mammalian cells have remained during the last 25 years as the main expression system for the commercial production of therapeutic proteins, as this system creates

efficient post-translational modifications on the r-proteins (Durocher and Butler, 2009). The Chinese hamster ovary (CHO) cell line is the standard expression system for the production of recombinant products for therapeutic applications. However, other cell lines such as NS0, baby hamster kidney (BHK) and human embryo kidney-293 (HEK-293) cells have also received regulatory approval for r-protein production on a commercial scale (Jayapal et al. 2007; Mohan et al. 2008; Kim et al. 2012). The use of CHO cells has been favoured over other cell lines due to their ability to produce r-proteins with complex post-translational modifications (*e.g.*, glycosylation), which are essential to biologic functionality and security (Wurm, 2004). However, despite the enormous potential of established cell lines, these production systems have inherent technological limitations: low specific growth rate, low cell concentration and high consumption of the main sources of carbon and energy (usually glucose and glutamine). The consequence of the latter aspect is the high generation of undesirable metabolites (mainly lactate and ammonium), limiting the life-span of the culture and the quality of the protein of interest. These limits result in low productivity for the production process (Schröder, 2008; Lim et al. 2010; Omasa et al. 2010; Sunley and Butler, 2010; Dietmair et al. 2011).

Most reports usually include information about glucose and glutamine consumption and lactate and ammonium production, intending to characterise the response of the system in a general way. However, there are rather few studies that provide more insight from a systemic point of view with respect to the metabolic behaviour of mammalian cells cultivated *in vitro*. In these cases, the most commonly used tool is metabolic flux analysis, which allows distinguishing changes in the distribution of intracellular flux as a result of specific perturbations or alternative metabolic routes (Boghigian et al. 2010; Quek et al 2010; Ahn and Antoniewicz, 2012). However, this tool does not provide information concerning changes in genic expression or regulation, existing only few publications regarding this point (Korke et al. 2004; Baik et al. 2006; Gupta and Lee, 2007; Jacob et al. 2010; Becker et al. 2011; Kim, 2012).

Strategies aimed specifically at reducing the production of lactate and ammonium ion have been mainly approached in two ways: the use of slowly metabolised substrates and cell engineering (directed to attenuate or overexpress transport proteins and/or enzymes).

This review presents a description of the main characteristics of the metabolic behaviour of mammalian cells cultivated *in vitro*, mainly referred to CHO cells, and the analyses of medium and host cell engineering strategies to improve the efficiency in the use of glucose and glutamine.

Characteristics of the energetic metabolism of mammalian cell lines

Alterations of the energetic metabolism have been well described and largely documented for the majority of mammalian cells presenting rapid cell growth, such as carcinogenic cell lines (*e.g*. HeLa, MCF-7, hepatomas) and transformed mammalian lines (*e.g*. CHO, BHK, HEK-293, hybridoma). These alterations involve an increase in the glycolytic capacity, even in the presence of high oxygen concentrations (aerobic glycolysis), and glutamine catabolism (glutaminolysis) that exceeds purely biosynthetic requirements. Evidence shows that there is a significant metabolic flexibility between glycolysis, glutaminolysis and oxidative phosphorylation for adapting the energy production mechanisms to microenvironmental changes and/or biosynthetic needs.

Aerobic glycolysis and glutaminolysis

The main mechanisms that have been suggested for explaining aerobic glycolysis and glutaminolysis in highly proliferative cells (Figure 1) are related to the overexpression of normal proteins or unusual isoforms (although characteristic of highly proliferative cells), the down-regulation of key proteins for the transport of NADH from the cytosol to mitochondria and pyruvate oxidation through the tricarboxylic acid cycle (TCAc).

In highly proliferative cells, the high glycolytic flux can be explained by an overexpression of the glucose transporters GLUT1 and GLUT3 (Macheda et al. 2005) and all the enzymes associated with the glycolitic pathway (Moreno-Sánchez et al. 2007). In the latter case, the overexpression has been as high as 300-fold for hexokinase II (HK-II) and phosphofructokinase type 1 (PFK-1). The overexpression of HK-II may favour its association with mitochondria and may increase its access to synthesized ATP through oxidative phosphorylation (OXPHOS). The overexpression of PFK-1, together with the PFK-2FB3 version, favours the synthesis of F1. This is explained by the fact that PFK-1 is activated by

fructose-2,6-bisphosphate, and PFK-2FB3 has a low affinity for F2, 6BP (unlike the normal isoform PFK-2), thereby favouring the accumulation of F2, 6BP (Yalcin et el. 2009). The activation of PFK-1 by F2, 6BP can be antagonized bycitrate and ATP. Moreover, it is important to note that PFK-1 is positively modulated by ammonium ion in normal mammalian cells (Sanders and Ezzat, 1971) and CHO cells (Urbano et al. 2000).

A high glycolytic flux may depend on the rapid regeneration of NAD^+ from $NADH^+$ through the conversion of pyruvate to lactate and by NADH shuttle systems. Usually, the regeneration of NAD^+ occurs in the mitochondria. However, because the mitochondrial membrane is impermeable to NADH, the cell uses shuttle systems to indirectly incorporate NADH equivalents in the mitochondria, followed by oxidation through the respiratory chain. The malate-aspartate shuttle (MAS) has been identified in many cell lines (Greenhouse and Lehninger, 1976) (Figure 1). The flux through the MAS under determined conditions has been shown to limit the velocity of NAD^+ regeneration in the cytosol (Eigenbrodt et al. 1985). Although the mechanism of these shuttle systems has not been studied in detail, some reports have shown that in some cases, its efficiency and transport rate could be lower than the NADH production rate in glycolysis (Rubi et al. 2004).

Regarding the key enzymes connecting glycolysis to the TCAc - pyruvate dehydrogenase and carboxylase pyruvate - in two different lines of animal cells (BHK and CHO), neither of them have significant activity (Neermann and Wagner, 1996). Based on this evidence, the authors explain that the flux in the formation of pyruvate is similar to the flux in the formation of lactate. Using spectrophotometric assays, (Fitzpatrick et al. 1993) determined that the activity of PDH was undetectable in batch cultures of B-lymphocyte hybridoma cells. At the mid-exponential phase, they showed that 96% of the glucose was catabolized via glycolysis and 36% of the glutamine was oxidized to CO_2. Interestingly, the energy contribution from the catabolism of these two substrates was similar: 55% by glutamine and 45% by glucose. Such behaviour has been verified already in many studies (Quek et al. 2010), showing a low flux through PDH and insignificant changes, even under very distinct culture conditions. Ahn and Antoniewicz (2011) and Sengupta et al. (2011) have recently reported that the metabolic pattern characteristic of the CHO growth phase in batch culture suffer substantial changes in the stationary phase. In the latter phase, the metabolic pattern is characterized by a low glycolytic flux and the activation of the oxidative pentose phosphate pathway flux. However, the fluxes through PDH were similar in the exponential and stationary phases in both case (Ahn and Antoniewicz, 2011; Sengupta et al. 2011). The activities of TCAc and OXPHOS are maintained due to the expression of unusual mitochondrial proteins, such as $NADP^+$-dependent malic enzyme, glutaminase and glutamine transporter, that have high activity and affinity for their substrates (Figure 1).

Three isoforms of malic enzymes are known, the most common being the type II (EM-II) found in carcinogenic cells (Moreadith and Lehninger, 1984; Sauer et al. 1980). EM-II is a mitochondrial enzyme ($NADP^+$ / NADPH dependent) that irreversibly catalyzes the formation of pyruvate from malate, and its activity is 10- to 20-fold higher in these cells than in normal cells. Sauer et al. (1980) suggested that this enzyme is important for the formation of mitochondrial pyruvate from glutamine by determining that its activity increases simultaneously with glutaminase activity. In carcinogenic cells, glutamine is transported into the mitochondria at a rate 4- to 10-fold higher than in normal cells and is then oxidized to glutamate by a P_i-dependent glutaminase, with a 10- to 20-fold higher activity than the normal enzyme. This P_i-dependent glutaminase may be located in the intermembrane space of the mitochondria and/or in the mitochondrial matrix (Molina et al. 1995).

Glutamate can follow two alternative pathways, either via glutamate deshydrogenase or via transaminases (TA). In the GDH pathway, glutamate is deaminated into a-ketoglutarate, releasing an additional ammonium ion, resulting in 2 moles of ammonium ion being generated from 1 molecule of glutamine. Through this route, the carbon skeleton of glutamine can be completely oxidised to CO_2 due to the participation of EM-II or part of it can be released from the mitochondria as malate to be later converted into pyruvate (Bonarius et al. 2001; Chong et al. 2010). Usually, the conversion from malate to pyruvate in the cytosol can be catalysed by phosphoenolpyruvate carboxykinase (PEPCK) or by cytosolic malic enzyme type I (EM-I). Many studies have shown a deficiency of PEPCK in hybridomas and in the CHO and BHK cell lines (Fitzpatrick et al. 1993; Neermann and Wagner, 1996). However, metabolic flux analysis using isotopic labelling with ^{13}C revealing a significant activity of EM-I (Bonarius et al. 2001; Quek et al. 2010).

Through the TA pathway, glutamate is converted into α-ketoglutarate via aspartate transaminase or via alanine transaminase. Because oxaloacetate or pyruvate, which are transaminated, are obtained from

the glutamate channelled through the TCAc, TA pathways have a closed loop structure. They can be used by cells as an internal mechanism to balance the overproduction of ammonium ion due to the rapid consumption of glutamine.

As previously described, the metabolism of glutamine can be channelled through different pathways that share many metabolites and transport systems in the mitochondria. In terms of energy, the GDH pathway is more efficient than the TA pathway (27 ATP moles vs. 9). This pathway is usually preferred when the energy demand is not fulfilled because of glucose exhaustion (Häggström, 1991; Martinelle et al. 1998) or because of the use of alternative, slowly metabolisable substrates (*e.g.*, glutamate) (Altamirano et al. 2001; Bonarius et al. 2001).

The metabolic flexibility observed in various cell lines shows that the cells have the ability to adjust the metabolic flux depending on the specific culture conditions. This adjustment is a result of both regulatory systems that act on the allosteric modulation of enzyme activity and the transcription of one or more enzymes. The latter is probably the most robust regulatory system in terms of control.

One of the most studied regulatory mechanisms regarding transcription and translation, as far as aerobic glycolysis and glutaminolysis are concerned, is the hypoxia inducible factor 1 (HIF-1) pathway. HIF-1 is a transcription dimeric factor, which is highly stable under aerobiosis. Under such condition, one of its units is degraded by the process of ubiquitin-mediated proteolysis (Wood et al. 1998), causing HIF-1 inactivation. HIF-1 has been shown to promote the expression of many glycolytic enzymes, leading to a stimulation of glycolytic flux (Jose et al. 2011). HIF-1a also favours glycolytic flux by increasing the expression of pyruvate dehydrogenase kinase 1 (PDHK-1), which inhibits the complex activity of PDH by phosphorylation (Kim et al. 2006; Hitosugi et al. 2011). Unfortunately, there are very few studies connecting HIF-1 to the regulation of aerobic glycolysis and glutaminolysis in CHO cells (Wood et al. 1998).

The c-Myc transcription factor exhibits similar characteristics to those of HIF-1 because it activates glucose transporter genes (GLUT-1) and glycolytic genes in several carcinogenic cells (Moreno-Sánchez et al. 2007). Moreover, c-Myc has been determined to potentially activate the transport of mitochondrial glutamine and glutaminase, its overexpression being correlated with an increase in the lactate production generated by glutaminolysis (Wise et al. 2008; Gao et al. 2009). c-Myc is also a factor participating in the regulation of cell proliferation and apoptosis (Mulukutla et al. 2010; Jose et al. 2011), which has been described to play that role in CHO cells (Kuystermans et al. 2010).

Lactate and ammonium ion: Undesirable metabolites

One of the main consequences of the metabolic behaviour of animal cells cultivated *in vitro* is the exacerbated formation of lactate and ammonium ion (Lao and Toth, 1997; Sanfeliu et al. 1997; Wagner, 1997). When these products exceed certain critical concentrations, they inhibit cell growth and decrease r-protein production (Lao and Toth, 1997; Wagner, 1997; Gambhir et al. 2003).

Lactate can cause a significant reduction of the pH of the medium, which inhibit the cell growth, but this effect can be avoided with a controlling system that maintains the pH within an optimal range. However, the principal mechanism by which lactate appears to negatively affect cultures is by an increase in the osmolarity of the culture media (Cruz et al. 2000). In general, concentrations lower than 20 mM lactate do not affect cell growth or productivity. A lactate concentration of 20-40 mM impairs the productivity and more than 40 mM inhibits cell growth (Wagner, 1997).

Ammonium ion has a stronger impact on cell culture than lactate because concentrations between 2 and 5 mM ammonium ion can inhibit growth and impair productivity. The precise mechanism of ammonium ion toxicity in cells is not clear, even though this effect has been shown to depend on pH (Schneider et al. 1996). There is evidence that the increase in the ammonium ion concentration in the culture medium produces an increase in the activated N-acetylhexosamine pool (UDP-N-acetylglucosamine and UDP-N-acetylgalactosamine; UDP-GNAc). This increase involves a decrease in the UTP concentration and subsequent growth inhibition (Ryll et al. 1994). Ammonium ion also appears to compete with cations, such as potassium, for transport inside the cell through the Na^+/K^+-ATPase and Na^+K^+2Cl-cotransporter (Martinelle et al. 1998). Moreover, ammonium ion may adversely affect glycosylation in mammalian cell cultures since it causes a decrease of sialic acid (NeuAc) and galactose (Gal) (Gawlitzek et al. 2000; Yang and Butler, 2002), variations of site occupancy (Borys et

al. 1993) and increase of branching (Gawlitzek et al. 1998). However, it is not clear if these effects are due to alterations in glucosamine and galactosamine levels (Koch et al. 1979), the UDP-GNAc pool (Ryll et al. 1994) or changes in the intracellular pH (Grammatikos et al. 1998). An increase in glycosilation branching is observed when the UDP-GNAc extracellular level increases, without affecting the NeuAc content of the glycoprotein (Grammatikos et al. 1998; Baker et al. 2001), whereas high ammonium ion concentrations lead not only to an increase in glycosylation branching but also to a loss of NeuAc in the glycoproteins (Grammatikos et al 1998; Gawlitzek et al. 2000; Yang and Butler, 2002). In contrast, an increase in ammonium ion generates an increase in glucosamine and galactosamine levels. However, the impact of these aminated sugars on glycosylation is quite different. Glucosamine feeding causes a reduction in glycosylation (Koch et al. 1979), whereas galactosamine feeding increases both the mannosylation and sialylation of proteins (Koch et al. 1979; Pels-Rijcken et al. 1995) suggested that the negative impact of glucosamine on glycosylation might be due to glucosamine itself rather than a nucleotide sugar, as the reversibility of the glucosamine effect was correlated with glucosamine concentration during the period when UDP-Glc, UDP-GlcNAc and GDP-Man did not change significantly. This observation might lead to the hypothesis that the use of other carbon sources (galactose or mannose) instead of glucose can have different and potentially favourable consequences on the protein glycosylation pattern. In this sense, supplementation of the culture media with 10 mM galactose has been shown to produce a 5-fold increase in the UDP-Gal levels and a slight increase in the galactosylation level (Hills et al. 2001). However, it is interesting to note that in this study the carbon source was glucose, with galactose added as a supplement. No reports have been found on the effect on glycosylation when galactose is the only carbon source and on the effect of mannose under the same conditions.

In the majority of studies that have assessed the effect of ammonium ion on the culture of mammalian cells, particularly on CHO cells, the experimental model studied has used exogenous ammonium ion sources (*e.g*. NH_4Cl) (Ryll et al. 1994; Lao and Thoh, 1997; Cayli et al. 1999; Yang and Butler, 2002). However, the effect of endogenous ammonium ion could produce a different response in cell metabolism (Schneider et al. 1996). Such effect has not been analysed yet.

Due to the impact that lactate and ammonium ion have on the production of r-proteins in mammalian cells, multiple strategies have been designed to reduce their negative effects. These strategies are discussed below.

Improving metabolism: Alternative metabolisable substrates and cell engineering

There are two main approaches that have been used to limit glucose and glutamine consumption (Figure 2). One is based on substrates that, because of their nature, are less available or slowly metabolised. The other one is based on using genetic modifications (cell engineering) for changing the metabolic behaviour by attenuating or stimulating the overexpression of key metabolic proteins. Strategies that involve the controlled feeding of glucose and glutamine such as fed-batch cultures are also possible, but are out of the scope of this review.

Reducing lactate production

The use of galactose or fructose instead of glucose has been shown to be an alternative to prevent lactate overproduction because the affinity of hexokinase for these sugars is approximately 20-fold lower than for glucose (Wagner, 1997). In particular, the use of galactose has been studied as an alternative for reducing lactate production levels (Wagner et al. 1991; Altamirano et al. 2000; Altamirano et al. 2004; Wilkens et al. 2011). Galactose is metabolised by a series of reactions that constitute the Leloir pathway, which consists of three enzymatic activities. UDP-glucose pyrophosphorylase (Lai and Elsas, 2000) also participates in the metabolism of galactose, which is part of other metabolic pathways. Galactose metabolism is slower than glucose, allowing a reduction in lactate generation. Galactose metabolism has also been shown to increase cell viability and culture duration (Altamirano et al. 2000). However, a significant reduction of cell growth occurs. Noticeably, lactate produced when growing on galactose is re-metabolized by the cells. These results have been used as a basis for the study of fed-batch (Clark et al. 2005; Altamirano et al. 2006) and perfusion cultures (Vergara et al. 2012).

On the other side, various cell engineering strategies have been tested to reduce both glucose consumption and lactate generation. Paredes et al. (1999) reported that knocking down the glucose

transporter GLUT1 in hybridoma cells reduced glucose uptake by almost 50% and lactate/glucose yield by 12%. Recently, tests have been conducted regarding the transfection of CHO cells with the fructose transporter GLUT5 to enable growth on fructose (Wlaschin and Hu, 2007). The clone with the lowest GLUT5 expression had the lowest lactate/hexose yield (ΔL/ΔHexose), whereas the clone with the highest GLUT5 expression had the highest ΔL/ΔHexose. This result suggests that the abundance of fructose transporter is important and that limiting sugar uptake may increase metabolic efficiency. The clone with the lowest lactate production achieved 3-fold higher cell densities in fructose fed-batch cultures compared with glucose. Using a similar approach, the recombinant IgG production of human myeloma cells cultured in a fructose-based medium exhibited an approximately 2-fold increase compared with that in a glucose-based medium (Inoue et al. 2010).

The down regulation of α-enolase in hybridoma cells, which converts 2-phosphoglycerate to phosphoenolpyruvate (Figure 1) through iRNA has been assessed. This intervention allowed reducing glucose uptake by 22% but did not significantly reduce the lactate/glucose yield (Paredes et al. 1999). Works using that approach have not been reported since.

Sanfeliu et al. (1997) used oxamate, a specific lactate-dehydrogenase (LDH) inhibitor, to modulate the activity of this enzyme in hybridoma cells. They concluded that this activity is fundamental for the maintenance of cell viability because it helps recycling the NADH produced and accumulated during glycolysis. However, it should be considered that oxamate inhibits any LDH isoform. Most recently, hybridoma cells were genetically manipulated to obtain mutants having a reduction of 50% or more in LDH activity (Chen et al. 2001). It has been shown that the LDH isotype of CHO cell exists as A3B and A2B2 tetramers (Jeong et al. 2001). Viability and growth was improved when the cells were genetically manipulated, and the production of the r-protein increased by a factor of 3. Similarly, Kim and Lee (2007) found that LDH knockdown in CHO cells reduced specific lactate production by as much as 79% and improved product titer up to 2.2-fold.

The genetic manipulation of cells has also improved the connection between glycolysis and TCAc by the inclusion of pyruvate carboxylase (PC), which has reduced lactate levels considerably (Irani et al. 1999; Elias et al. 2003). The expression of PC in BHK cells reduced glucose and glutamine consumption and decreased lactate production up to 2.6-fold. In perfusion cultures, a 2-fold increase in the erythropoietin titer was obtained (Irani et al. 2002). Similarly, the overexpression of PYC in HEK293 cells decreased glucose and glutamine consumption, with a reduction in lactate and ammonia production up to 4- and 3-fold, respectively (Elias et al. 2003). Although CHO cells overexpressing PYC did not exhibit significant decrease in glucose uptake rate, lactate production decreased by 1.2- to 1.5-fold, and the specific rate of oxygen consumption increased by up to 1.9-fold (Fogolin et al. 2004). Additionally, the culture duration was extended up to 5 days, resulting in a 2.5-fold improvement in the yield of rhGM-CSF (recombinant human granulocyte macrophage colony stimulating factor).

Wilkens and Gerdtzen (2011) transfected CHO cells with GLUT5 and PYC genes (CHO-PYC-Slc2a5). These cells had the highest specific growth rate among all the tested clones (CHO-PYC, CHO-Slc2a5 and CHO wild type). The CHO-PYC-Slc2a5 cells had a ΔL/ΔHexose 50% lower than wild type cells and 35% more than CHO-Slc2a5 cells (cells only transfected with GLUT5). Using a similar approach, Zhou et al. (2011) attenuated the expression of LDH-A and PDHK in CHO cells through iRNA. In fed-batch cultivation of the genetically modified cells, a strong reduction in lactate production (90%), and increase in specific and volumetric productivity of r-protein (approximately 75% and 68%, respectively) was obtained, without a significant impact on cell growth. These results indicate that the cellular system is highly flexible and additional improvements in metabolism are feasible.

Reducing ammonium ion production

The efforts to avoid the effects of an increase in ammonium ion concentration have basically focused on the substitution of glutamine by another compound able to enter the TCAc. The replacement of glutamine by glutamate is an example of this approach, having the following advantages (Altamirano et al. 2000): glutamate has a higher chemical stability than glutamine, glutamine can be directly synthesized from glutamate in CHO cells because they express glutamine synthetase (GS), and glutamate is less ammoniagenic because it has only one amine group. Indeed, the replacement of glutamine by glutamate in the culture media has proved to be a good strategy for CHO cells, resulting in a cell growth improvement of 17% and reduction in the accumulated ammonium ion of 70% (Altamirano et al. 2000). Hong et al. (2010) recently showed that this substitution caused the

concentration of an IgG produced in CHO cells to increase by 1.7-fold. In contrast, the level of r-protein galactosylation increased depending on the proportion of galactosylated glycans.

The substitution of glutamine by dipeptides such as alanyl-glutamine and glycyl-glutamine has also been used to reduce ammonium ion production. These dipeptides, that are more stable than the amino acids in the culture medium, are intra or extracellularly cleaved by peptidases, thereby releasing glutamine and either alanine or glycine. The availability of glutamine is therefore dependent on the peptidase activity. In hybridoma cell cultures, high cell yields were obtained in the presence of 6 mM ala-gln or 20 mM gly-gln (Christie and Butler, 1994). The final cell yield in gly-gln was 14% higher than in gln. However, the productivity of monoclonal antibody was comparable in gln, ala-gln or gly-gln. The specific consumption rates of glucose and six amino acids were reduced, and additionally the accumulation of ammonia and lactate was significantly lower (Christie and Butler, 1994). In all these cases, a significant reduction in specific glucose consumption and specific lactate production was observed. The reason for slower glucose consumption is still unclear. One explanation could be related to the participation of PFK-1, which has been determined to be activated by ammonium ion in CHO K1 cells (Urbano et al. 2000). Therefore, in agreement with Schneider et al. (1996), a decrease in the intracellular ammonium ion concentration could reduce the glycolytic flux and thus the lactate production.

The use of mannose alone has also been used as a strategy to reduce ammonium ion effects (Ryll et al. 1994; Cayli et al. 1999; Berríos et al. 2009; Berríos et al. 2011). Mannose enters the cells using a hexose transporter in mammalian cells (Rodriguez et al. 2005), merging with the glycolytic pathway at the F6P level. In CHO cells, mannose consumption occurs at the same rate as glucose, generating similar levels of lactate (Altamirano et al. 2000; Berríos et al. 2009; Berríos et al. 2011). However, intracellular metabolism has been shown to respond in a very different manner when mannose is in the culture medium (Ryll et al. 1994, Cayli et al. 1999, Berríos et al. 2011), especially with respect to the UDP-GNAc pool levels (Ryll et al. 1994; Cayli et al. 1999), as mentioned above. This phenomenon would be mediated by M6F, which acts as an inhibitor of glucosamine-6-phosphate isomerase (Figure 3). This enzyme catalyses the reaction between F6P and ammonium ion to produce GlcN-6P, a precursor of UDP-GNAc. The use of galactose or fructose instead of glucose has proved to reduce the UDP-GNAc pool, even though this change has been attributed to a low rate of glucose synthesis (Ryll et al. 1994).

Engineering hybridoma cells with GS, which converts glutamate to glutamine, allowed cell growth in glutamine free medium (Paredes et al. 1999), with results similar to those described previously (that is, when glutamine is replaced by glutamate).

The urea cycle is likely to be inactive in CHO cells, since only trace amounts of urea have been detected (Zamorano et al. 2010). In this regard, the expression of the carbamoyl phosphate synthetase I (CPS-I) and ornithine transcarbamoylase (OTC) (that are mitochondrial enzymes that catalyse the first and second steps of the urea cycle) in CHO cells reduced ammonium ion production by 33%, cell growth rate increasing in a similar percentage (Park et al. 2000). In a later study, it was determined that tPA produced in CHO cells overexpressed CPS-I and OTC was highly active which is quite valuable for the biopharmaceutical industry (Kim and Kim, 2006).

CONCLUDING REMARKS

Culture longevity, productivity and quality of the r-protein have been improved by strategies based on modifications of the metabolic behaviour of established mammalian cell lines by using either alternative substrates or host cell engineering. However, success largely depends on the host cell, the produced r-protein and the conditions of the production process (*e.g*. fed-batch or perfusion culture). A deeper insight in the integrated understanding of metabolism and cell mechanisms is required in order to define complementary strategies at these two levels, so providing effective means to control nutrients consumption, reduce by-products and increase process productivity.

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Figures

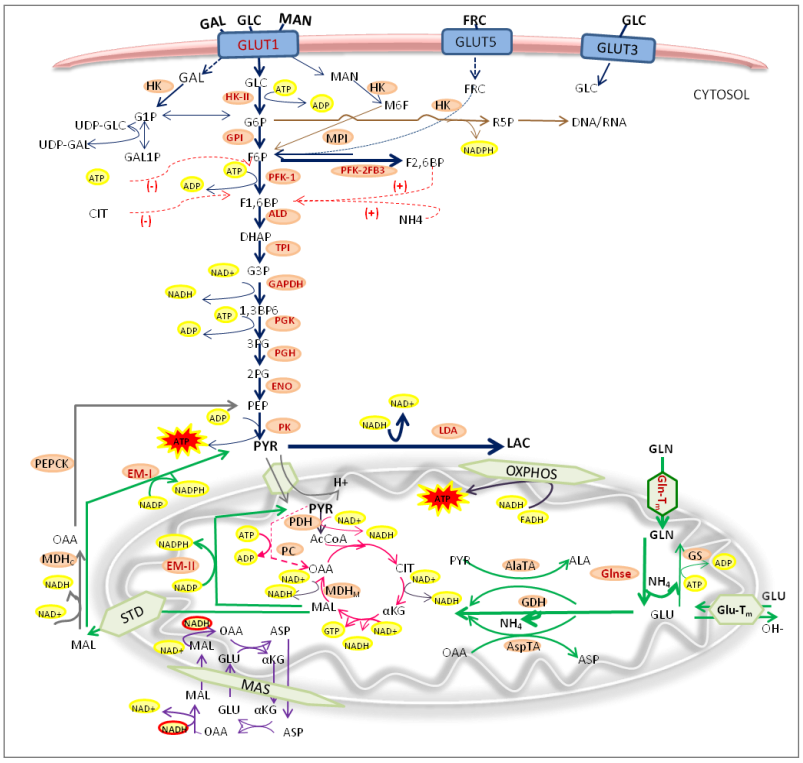

Fig. 1 Scheme of glycolytic and glutaminolysis pathways, tricarboxylic acid cycle and main mitochondrial transport systems. Figure legend: blue lines: reaction steps of glycolysis pathway; green lines: reaction steps of glutaminolysis pathway; pink lines: pyruvate dehydrogenase reaction plus reaction steps of tricarboxylic acid cycle (TCAc); purple lines: reaction steps of the malate aspartate shuttle; red words: overexpressed enzymes in highly proliferative cells; dotted red line: positive (+) or negative (-) regulation.

Metabolites:	Enzymes:	Transporters:
AcCoA = Acetyl-CoA	AlaTA = Alanine transaminase	Gln-T = Glutamine transporter
ALA = Alanine	ALD = Aldolase	Glu-T = Glutamate transporter
ASP = Aspartate	AspTA = Aspartate transaminase	GLUT1 = Glucose transporter (Isoform 1)
CIT = Citrate	CPS-I = carbamoyl phosphate synthetase I	GLUT1 = Glucose transporter (Isoform 1)
DHAP = Dihydroxyacetone phosphate	EM-I = Malic enzyme type I	GLUT3 = Glucose transporter (Isoform 3)
F1,6BP = Frucote-1,6-biphosphate	EM-II = Malic enzyme type II	GLUT5 = Glucose transporter (Isoform 5)
F2,6BP = Frucote-2,6-biphosphate	ENO = Enolase	MAS = Malate aspartate shuttle
F6P = Fructose-6-phosphate	F6P = Fructose-6-phosphate isomerase	OXPHOS = Oxidative phosphorilation
FRC = Fructose	GAPDH = Glyceraldehyde-3-phosphate dehydrogenase	STD = Standart transport of dicarboxylate
G1P = glucose-1-phosphate	GDH = Glutamate dehydrogenase	
G3P = Glyceraldehyde-3-phosphate	Glnse = Glutaminase	
G6P = Glucose-6-phosphate	GPI = Glucose-6-phosphate isomerase	
GAL = Galactose	GS = Glutamine syntetase	
GAL1P = Galactose-1-phosphate	HK = Hexokinase	
GDP-GAL = UDP-galactose	HK-II = Hexokinase (Isoform II)	
GLC = Glucose	LDA = Lactate dehydrogenase	
GLN = Glutamine	MDHc = Malate dehydrogenase (citoplasmatic)	
GLU = Glutamate	MDHm = Malate dehydroganse (mitochondrial)	
LAC = Lactate	MPI = Mannose-6-phosphate isomerase	
M6F = Mannose-6-phosphate	PC = Pyruvate caboxylase	
MAL = Malate	PDH = Pyruvate dehydrogenase	
MAN = Mannose	PDHK = Pyruvate dehydrogenase kinase	
OAA = Oxaloacetate	PEPCK = Phosphoenolpyruvate carboxykinase	
PEP = Phosphoenolpyruvate	PFK-1 = Phosphofructokinase-1	
PYR = Pytuvate	PFK-2FB3 = Phosphofructokinase-2 isoform FB3	
R5P = Ribose-5-phosphate	PGH = Phosphoglyceromutase	
UDP-GLC = UDP-glucose	PGK = Phosphoglycerate kinase	
1,3BPG = 1,3-biphosphoglicerate	PK = Pyruvate kinase	
2PG = 2-phosphoglycerate	PYC = Pyruvate carboxylase from yeast	
3PG = 3-phosphoglycerate	TPI = Triosephosphate isomarase	
αKG = alfa-ketoglutarate		

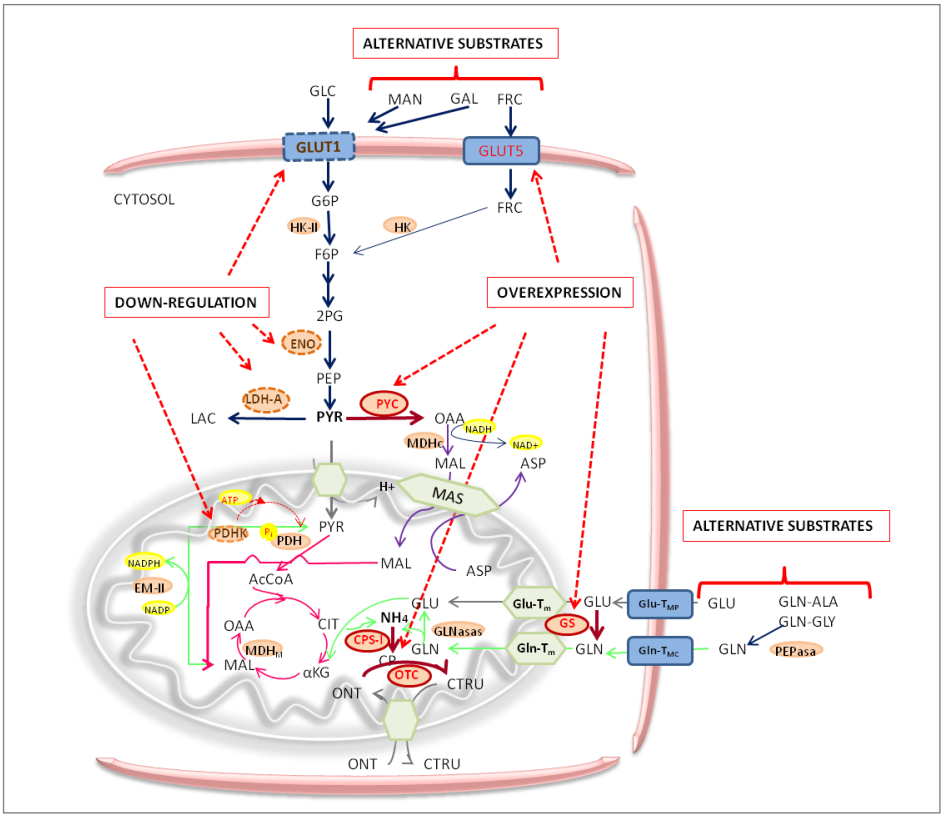

Fig. 2 Schematic representation of the main strategies used to improve cell metabolism. Figure legend: blue lines: reaction steps of glycolysis pathway; green lines: reaction steps of glutaminolysis pathway; pink lines: pyruvate dehydrogenase reaction plus reaction steps of tricarboxylic acid cycle (TCAc); purple lines: reaction steps of the malate aspartate shuttle; red words: overexpressed enzymes/transporter; brown letters: down-expressed enzymes/transporter.

Metabolites	Enzymes
AcCoA = Acetyl-CoA	CPS-I = carbamoyl phosphate synthetase I
ASP = Aspartate	EM-II = Malic enzyme type II
CIT = Citrate	ENO = Enolase
CP = Carbamoyl phosphate	F6P = Fructose-6-phosphate isomerase
CTRU = Citrulline	GS = Glutamine syntetase
F6P = Fructose-6-phosphate	HK = Hexokinase
FRC = Fructose	HK-II = Hexokinase (Isoform II)
G1P = glucose-1-phosphate	LDH-A = Lactate dehydrigenase isoform A
G3P = Glyceraldehyde-3-phosphate	MDHc = Malate dehydrogenase (citoplasmatic)
G6P = Glucose-6-phosphate	MDHm = Malate dehydroganse (mitochondrial)
GAL = Galactose	OTC = ornithine transcarbamoylase
GLC = Glucose	PDH = Pyruvate dehydrogenase
GLN = Glutamine	PDHK = Pyruvate dehydrogenase kinase
GLN-ALA = Alanyl-Glutamine	PYC = Pyruvate carboxylase from yeast
GLN-GLY = Glycyl-Glutamine	**Transporters**
GLU = Glutamate	Gln-T = Glutamine transporter
LAC = Lactate	Glu-T = Glutamate transporter
MAL = Malate	GLUT1 = Glucose transporter (Isoform 1)
MAN = Mannose	GLUT1 = Glucose transporter (Isoform 1)
OAA = Oxaloacetate	GLUT5 = Glucose transporter (Isoform 5)
ONT = Ornithine	MAS = Malate aspartate shuttle
PEP = Phosphoenolpyruvate	
PYR = Pytuvate	
2PG = 2-phosphoglycerate	
αKG = alfa-ketoglutarate	

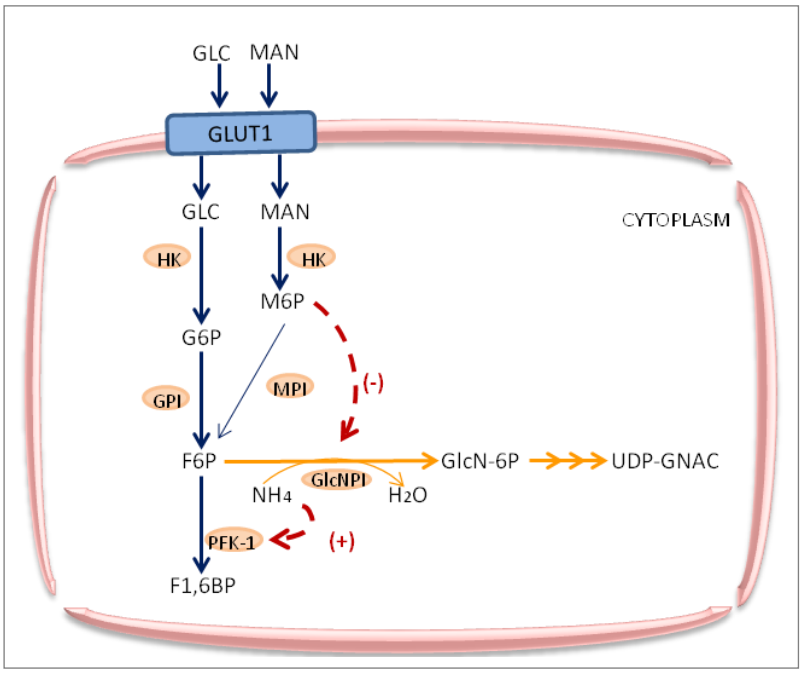

Fig. 3 Possible role of the mannose-6-phosphate (M6P) in the regulation of glucosamine phosphate isomerase (GlcNPI) and generation of activated sugars. Figure legend: blue lines: reaction steps of glycolysis pathway; orange lines: nucleotide-activated sugars pathways (precursors of oligosaccharides); dotted red line: positive (+) or negative (-) regulation.

Metabolites	Enzymes	Transporters
F1,6BP = Frucote-1,6-biphosphate	F6P = Fructose-6-phosphate isomerase	Gln-T = Glutamine transporter
F6P = Fructose-6-phosphate	GlcNPI = Glucosamine phosphoisomerase	Glu-T = Glutamate transporter
GLC = Glucose	HK = Hexokinase	GLUT1 = Glucose transporter (Isoform 1)
GlcN-6P = Glucosamine-6-phosphate	MPI = Mannose-6-phosphate isomerase	GLUT1 = Glucose transporter (Isoform 1)
MAN = Mannose	PFK-1 = Phosphofructokinase-1	
OAA = Oxaloacetate		
UDP-GNAC = UDP-N-acetylhexosamine		