



Review

Exploring the effect of mild hypothermia on CHO cell productivity

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ABSTRACT

The CHO cell line is mainly used to synthesize recombinant proteins for human therapeutic use. Recent efforts have focused on improving the productivity of this system by mainly using two tools: genetic manipulation of the cells and/or changing the operating conditions of the cultures. In most cases, these techniques have resulted in significant improvements to the productivity of the system. For example, a slight decrease in culture temperature has been used over the past ten years to increase culture longevity, resulting in an increased productivity of r-proteins. However, despite large numbers of diverse studies, there are currently no unified hypotheses to explain the increased productivity of r-proteins. This is mainly because the magnitude of this effect is dependent on the cell line and the r-protein, and the effects of temperature on cellular processes of protein synthesis and cell growth are difficult to decouple and quantify experimentally.

In this review, we assembled the most relevant backgrounds to address this problem, and we explore a different experimental approach that allowed us to decouple specific cell growth from culture temperature, in order to estimate the net effect of temperature on the productivity of r-proteins and to better understand the molecular and cellular processes involved. The information provided in this study can be used to propose new strategies at an industrial scale for implementing fed-batch or perfusion cultures to improve the production of several important r-proteins.

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1. Introduction

The first therapeutic application of a recombinant protein produced by animal cells was approved in 1986 (human tissue plasminogen activator, tPA, Genentech), which established the origins of biopharmaceuticals from mammalian cells [1]. Since then,

therapeutic biologics have changed the landscape of treatment for numerous diseases, including many cancers, rheumatic conditions and orphan diseases. Sales of these types of compounds reached \$48 billion in the US in 2009, representing an important proportion of the pharmaceutical market. Moreover, the biopharmaceutical market has shown an annual average growth rate of 35% since 2001, which suggests a financially sound future for the biopharmaceutical industry [2].

Although there are a variety of alternative expression systems available, including those using cells from bacteria, insects, transgenic animals and plants, during the past 25 years, mammalian cells

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have remained the principal expression system for the commercial production of therapeutic proteins [3] because this system conducts efficient, post-translational modifications on the produced proteins [4]. The Chinese hamster ovary (CHO) cell line is the gold standard cell culture expression system for the production of recombinant products; 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 recombinant protein (r-protein) production on a commercial scale. The use of CHO cells has been favored over other eukaryotic cell lines because they produce very similar glycan structures as those naturally synthesized by human cells [5]. This could be critical because the biological function of the r-protein can be affected by its oligosaccharide composition [6]. Additionally, CHO cells are considered safe for human use, there are powerful gene amplification systems designed to work with these cells and CHO cells can be easily adapted to suspension cultures, a desired feature for the development of large-scale cultures. Several efforts have been performed to improve CHO cells culture conditions in order to enhance yield and quality of various r-proteins.

While the various aspects of cell culture technology for mammalian cells have not substantially changed in the past two decades, it is true that today's processes are more productive. A decade ago, the usual specific and volumetric productivities for these processes were 10–20 pg/cell/day and 50–100 mg/L, respectively [1]. Currently, these bioprocesses have specific and volumetric productivities in the range of 50–90 pg/cell/day and 1–5 g/L, respectively [7]. These remarkable improvements have been achieved by intensive bioprocess engineering research [8] and sophisticated design/selection techniques by the use of high-producer or strong clones [9,10]. In this way, strategies to improve the productivity of the processes based on CHO cells have focused on the increase in the integral of viable cells and an increase in the specific productivity of the r-protein [11].

Low-temperature cultures have been used extensively as a way to improve cell culture longevity and to increase r-protein production. Most of the studies using the low-temperature cultures have been performed in batch cultures, in which the effect of the temperature on the specific productivity of the protein overlaps with the effects of the temperature on the cell growth rate. Therefore, it has not been easy to establish how much of the increase in r-protein productivity is due to a direct effect of temperature on cellular processes that determine r-protein quantities and how much is due to an effect on the specific growth rate.

Undoubtedly, the molecular mechanisms and the cell physiology associated with low temperatures and the specific production of the r-protein need to be studied in order to develop genetic tools for host cell engineering. However, the molecular mechanisms governing r-protein production in mild hypothermic conditions are currently unknown. Moreover, whether or not it is possible to discriminate targets triggered by low temperatures aimed to improve specific production of the r-protein is yet to be determined. Different hypotheses have attempted to explain the increase in r-protein specific production rate at low temperatures. Here, we review these hypotheses (see Table 1) in an attempt to explain the increase in r-protein productivity and to uncouple the effect of temperature on cellular processes that determine both r-protein quantity and CHO cell growth rate.

2. Use of temperature as a tool for improving the specific productivity of r-proteins

In the past ten years, there have been frequent reports indicating that a decrease of a few degrees in temperature from 37 °C, the usual culture temperature, until 28 °C, which is called mild hypothermia,

Table 1

Outline of some of the possible mechanisms involved in improving r-protein production under mild hypothermia in CHO cell cultures.

Possible mechanism	r-Protein	Source
Cell cycle arrest		
Increase of cell percentage in G1 phase	SEAP N.R. Chimeric IgG4	[12] [13] [14]
Decrease of specific growth rate	SEAP	[15]
	t-PA	[16]
	IgG against the glycoprotein receptor of human platelets	[17]
miRNA synthesis	SEAP	[18]
Apoptosis delay		
Bcl-2 increase	N.R.	[19]
Decrease of importin- α (and subsequently of p21)	N.R.	[20]
Reorganization of the cytoskeleton	EPO	[21]
	IgG	[22]
Increase in the amount and/or stability of r-protein mRNA		
Increase in the amount of mRNA-r-protein	EPO	[23]
Increase in the stability of mRNA-r-protein	TNFR-Fc	[24]
Increase in amount and stability of mRNA-r-protein	IFN- γ	[25]
Changes in protein synthesis	IFN- γ	
CIRP overexpression		[26]

NR: not reported.

allows an incremental increase in the specific productivity of r-proteins in CHO cell culture (Table 2).

With regard to the quality of the r-protein, it seems that in most cases, there are no significant changes in the glycosylation profiles, including the degree of terminal sialylation. Accordingly, these r-proteins produced under mildly hypothermic conditions show the same *in vitro* biological activity as the r-proteins produced at 37 °C [13,23,27]. In addition, the specific growth rate decreases considerably with decreasing temperature, and cell viability increases [23,27,28]. Thus, decreasing the culture temperature has been suggested as a tool for the improvement of the production process, essentially defining biphasic culture strategies. During the first stage, normal cell growth is established at 37 °C, and once the expected cell density is reached, the temperature is decreased to increase r-protein productivity and extend culture viability [12,27–31,43–45]. The connection between decreased cell culture temperatures and the increase in the specific r-protein production rate in mammalian cell cultures is not fully understood. Despite the recent application of “omics” techniques and the development of genomic platforms for CHO cells [46] (Table 3), there is still no unified theory that explains the molecular phenomena responsible for the increase in the specific production rate of r-proteins when cells are cultivated under mild hypothermia conditions. A general analysis of the background shows that the magnitude of the effect of reducing the culture temperature over the specific production rate depends markedly on the cell line and the r-protein itself. Different authors attribute the increase in specific r-protein productivity at mild hypothermia to different events, such as cessation of the cell cycle/low specific growth rate [12,13,16,28,32–34], reorganization of the cytoskeleton [22], an increase in the synthesis of the r-protein mRNA [23,25,30,47], an increase in the average life span of the mRNAs [25], preferential translation of CAP-independent proteins mediated by IRESs [48–50] and/or a widespread reduction in protein synthesis, which, paradoxically, does not affect the r-protein [48,51].

Table 2

The effect of mild hypothermia on specific r-protein productivity in CHO cell culture.

r-Protein	T (°C)	Productivity (fold change)	Culture modality	Promoter	Acclimated/supplement	Source
SEAP	30	+1.7	Batch	P hCMV	Not acclimatized/FCS (10%)	[12]
Chimeric Fab	30	ND	Batch	NR	NR/WO	[13]
tPA	33	+1.5	Batch	CMV	Acclimatized/WO	[16]
Antibody	33	+1.7	Batch	NR	Not acclimatized/WO	[22]
EPO	33	+4	Batch	CMV	Not acclimatized/WO	[23]
EPO	30	+5.6	Batch	CMV	Not acclimatized/WO	[13]
TNFR-Fc	30	+5	Batch	NR	Not acclimatized/WO	[24]
hGM-CSF	33	+2.1	Batch	CMV	Not acclimatized/FCS (1–5%)	[27]
C-terminal alpha-amidating enzyme	35	ND	Batch	SV40	Not acclimatized/bovine insulin	[28]
Chimeric Fab	28	+14	Batch	CMV	NR/FCS (10%)	[29]
IFN- γ	32	+2	Batch	SV40	Acclimatized/WO	[30]
EPO-Fc	30	+1.85	Batch	NR	Not acclimatized/peptone (0,25%)	[31]
TIMP	34	+4	Batch	hCMV	Not acclimatized/FCS (8%)	[32]
tPA	32	+1.7	Batch	SV40	Not acclimatized bovine insulin	[33]
Anti-4-1BB antibody	33	+1.2	Batch	CMV	Not acclimatized/WO	[34]
Anti-4-1BB antibody	30	NC	Batch	CMV	Not acclimatized/WO	[34]
Antibody-IL-2 fusion	30	+2.8	Batch	SV40	NR/WO	[35]
Pro-urokinase	31	+1.3	Batch	NR	Not acclimatized/FCS(1%)	[36]
IFN- γ	30	+2	Batch	CMV	NR/NR	[37]
IgG	30	+2	Batch	NR	Not acclimatized/WO	[38]
IFN- γ	32	+4	Fed-batch	SV40	Not acclimatized/FCS or Insulin	[25]
Not disclosed	32	+6	Continuous (immobilized cells)	NR	Acclimatized/WO	[39]
Pro-urokinase	34	+1.5	Perfusion	NR	Not acclimatized/FCS (1%)	[36]
EPO	32	+4	Perfusion	CMV	Not acclimatized/WO	[40]
tPA	33	+1.7	Perfusion	CMV	Not acclimatized/WO	[41]
FSH	32	+13	Perfusion	NR	Not acclimatized/WO	[42]

WO: without.

NR: not reported.

Supplement: FCS, insulin, peptone.

As shown in Table 2, most experiments have been performed in batch cultures, where it is not easy to evaluate the effect of the operational variables independently from the specific growth rate. Furthermore, the results obtained can vary substantially depending on whether the mild hypothermia is induced by means of a sharp temperature change or by means of acclimatizing cells to the operational temperature. On the other hand, in the majority of the experiments, culture medium supplemented with serum has been used, which contains mitogenic agents that may have an influence on the global effect observed [25,30]. Thus, experimental data assessments and comparisons among different published results are not a simple task. Some examples are shown below.

For example, adaptation of CHO cells to low-temperature cultures should ideally have increased the specific growth rate and the specific production rate; however, it unexpectedly resulted in a decrease in the specific production rate, and the effects were cell-line specific [53]. On the other hand, adaptation of CHO cells producing IFN- β to a low temperature (32 °C) allowed the growth rate to increase two-fold without changing the specific productivity of the r-protein, which resulted in an increase in volumetric productivity [53]. Fox et al. [25,30] showed that the addition of mitogenic compounds to a culture of CHO cells producing

IFN- γ at 32 °C allowed these cells to reach a growth rate similar to the one observed at 37 °C, maintaining high productivity. Performing cell engineering on CHO cells, Hong et al. [54] showed that down-regulation of CIRP (a cold-shock protein involved in growth arrest under hypothermia conditions) did not recover the cells from the growth arrest under mildly hypothermic conditions, and its over expression had no effect on r-protein productivity at 37 °C. Together, these results suggest that the application of low temperatures affects the specific growth rate and the specific productivity by different mechanisms. Interestingly, we maintained continuous cultures of CHO cells producing t-PA at 37 °C and 33 °C and both at the same dilution rate (and, therefore, the same specific growth rate), and we observed a significant decrease in the t-PA specific productivity at 33 °C (unpublished data). These results indicate that there is a direct relationship between the culture temperature and the specific productivity of the r-protein in this range.

In batch cultures, we previously determined a substantial increase in the t-PA specific productivity at 33 °C compared to the value reached at 37 °C; likewise, we observed an inverse correlation between specific growth rate and specific t-PA production [16]. The latter finding illustrates that in batch cultures, there is an overlap effect of these factors over the specific production of this r-protein.

Table 3

The “omics” studies used to understand temperature shifts in CHO cell cultures.

Source	Approach	
	Transcriptomics	Proteomics
[12]		✓
[20]		✓
[18]	✓	
[22]	✓	
[44]	✓	
[52]	✓	
[21]	✓	✓
[38]	✓	✓

3. Low temperature and cell growth

3.1. Cell cycle

Jenkins and Hovey [32] were pioneers in the use of temperature as a tool to control cell growth and r-protein productivity in CHO cell cultures. They developed a batch culture process in which they alternated culture temperature between 39 °C and 34 °C, which allowed them to maintain high culture viability for 170 h, and this process resulted in a 35% increase in r-protein yield compared to that in a control culture at 37 °C. Moreover, they noticed arrest of the cell cycle at the G1/G0 phase. Since then, arrest of the cell

cycle promoted by temperature changes has been a strategy used to increase productivity of a protein of interest in CHO cell cultures [45].

Specific r-protein production rate increases during the G1 phase have been studied not only by using cultures under mild hypothermia [12,13,28,33,34] but also by using chemical compounds (mainly sodium butyrate and DMSO) and via genetic manipulation of crucial elements that control the transition of the cell cycle (see these reviews on these topics [55,56]). From a biotechnological point of view, it is important to bear in mind that any of these modifications can cause failures in normal cell functions, leading to cell death, either from apoptosis or necrosis [57]. Therefore, when cells are arrested in the G1 phase, environmental conditions must be able to maintain the balance of cellular functions; otherwise, the cell culture could rapidly lose its viability.

At the molecular level, arrest of the cell cycle at the G1 phase (i.e., block of the transition G1-S) is regulated by the E cyclin-linked CDK2 protein (CDK, cyclin-dependent kinase). Genes involved in this regulation are *p21* and *p27*, which are regulated at a higher level by *p53* and *c/ebpα* [57,58]. Besides its regulation effect on the cell cycle, *p53* participates in the activation of the apoptotic process in several animal cell lines [59]. In these same cell lines, the expression of the *bcl-2* gene inhibits the apoptotic process [60]. The expression level of these genes can vary from one cell line to another, which means that the cell cycle response to the arrest of G1 could also vary.

The productivity increase during the arrest of the G1 phase of the cell cycle is suspected to be associated with the maintenance of cell viability, leading to a delayed start of apoptosis [13]. The latter has been explained by the fact that several genes involved in ribosomal biogenesis and protein translation are highly expressed in that phase [12,14,61]. It has also been reported that cells arrested at the end of the G1 phase are metabolically more active and of a larger size in comparison to the non-arrested cells [14,15]. For these reasons, the G1 phase has been considered the ideal phase for obtaining high productivity of a protein of interest [12,17,62,63]. Nevertheless, there are some researchers who do not follow this hypothesis. Kubbies and Stockinger [64] stimulated t-PA production in dhfr⁺ CHO cells by using MTX and reported an increase in the production of the r-protein at all stages of the cell cycle: 100% during the G1 phase, up to 159% in the S phase and 118% in the G2/M phase. Subsequently, Fox et al. [25] showed that the addition of mitogenic compounds to a culture of CHO cells producing IFN-γ at 32 °C allowed the cells to reach a specific growth rate similar to the one observed at 37 °C. These cells maintained high productivity in normal division cycles instead of arresting at the G0/G1 phase. This resulted in a higher cell density in low temperature cultures and an increase in the volumetric productivity (up to 40%) during the S phase of the cell cycle at both temperatures, resulting in higher levels of IFN-γ mRNA. Kaufmann et al. [12], working with CHO cells producing an alkaline phosphatase (SEAP) cultivated at 30 °C, proposed that an increase in the phosphorylation of protein kinases, key molecules in the regulation of the cold response, could explain the alteration in the regulation of the cell cycle. In the same paper, the authors correlated the decrease in culture growth rate with an increase in the productivity of the r-protein. They demonstrated that a reduction in the specific growth rate produced an increase in the productivity of the SEAP protein in CHO cell cultures at 30 °C. Yoon et al. [23,34] showed that the productivity of erythropoietin and an antibody increased in CHO cell cultures when the temperature was reduced, and, consequently, there was a decrease in cell growth. Berrios et al. [16] showed an inverse relationship between the specific cell growth rate and specific t-PA productivity in cultures maintained under three different concentrations of mannose and at different temperatures.

However, it has been recently reported that cDNA CHO cell microarray results [22] suggest that the genes involved in the cell cycle control and apoptosis, which are differentially expressed in CHO cell cultures stimulated with sodium butyrate, did not seem to be significantly altered under conditions of mild hypothermia (33 °C), and for this reason, it would be rather unlikely that the genes involved in such functional types are directly related to a higher specific productivity. It is important to point out the possible role that miRNA (noncoding RNA molecules) have in the control of the cell cycle through regulating gene expression at the translational level. It has been reported [18,44] that low temperatures produce changes in the expression of the miRNAs involved in the regulation of cell growth and, at the same time, in r-protein expression in CHO cells.

3.2. Apoptosis delay

The apoptosis delay in CHO cell cultures cultivated under mild hypothermia conditions is a phenomenon that has been reported by several authors [13,19,23,35]. This phenomenon could be a consequence of the cell cycle arrest or a delay in the signals associated with the exhaustion of key nutrients, the accumulation of toxic substances or the expression of new proteins, such as antiapoptotic proteins.

Moore et al. [13,57] cultivated CHO cells at 30 °C and 33 °C and demonstrated that a significant suppression of cell death due to apoptosis was induced at low temperatures. However, once apoptosis was triggered, the death rate of the cells at all temperatures was the same; however, it has not been possible to associate this fact with the arrest of the cell cycle at the G0/G1 phase. Kumar et al. [20] reported that the down-regulation of the importin-α protein in CHO-K1 cell cultures at 31 °C, compared with that in the culture at 37 °C, could have an impact on proliferation and protection against apoptosis because they observed that a decrease in importin-α correlated with a decrease in the levels of the *p21* (*waf1/cip1*) transcript and, therefore, apoptosis. Slikker et al. [19] investigated the effect of mild hypothermia on the damage caused by the oxidative stress response in CHO cell cultures cultivated between 37 °C and 30 °C. They showed that a decrease in the culture temperature improved the activity of the glutathione peroxidase antioxidant enzyme and also increased the expression of the *bcl-2* antiapoptotic protein, when compared with a culture cultivated at the normal temperature of 37 °C. The authors concluded that mild hypothermia delayed the apoptotic pathway by strengthening the cell defense mechanisms. Despite the evidence discussed above, there is no research that clearly links these observations to an increase in the specific productivity of the r-protein.

4. Low temperature and other cell mechanisms

A significant number of studies have suggested that an increase in the specific production rate of the r-protein under mild hypothermia conditions could occur as the result of several different cellular events, and this increase is not necessarily related to a decrease in the specific growth rate.

4.1. Reorganization of the cytoskeleton

Stapulionis et al. [65] investigated the importance of the cytoskeleton structure on protein synthesis over a temperature range of 0–28 °C. These temperatures were lower than those shown to provide an adequate growth rate for CHO cells to conduct bioprocessing of r-proteins on an industrial scale. Nevertheless, this study confirmed that the organization of the protein synthesis machinery needed to perform an efficient translation is important. They suggested that even though the individual components

of the translation apparatus are not inactivated as a response to cold shock, the supramolecular organization of the system seems to be altered because levels of F-actin decrease as protein synthesis decreases. Moreover, the microtubules do not appear to be directly needed for translation, but they would likely play a role in determining the location of various components of the translational machinery. In addition, they reported that as a consequence of the decreased culture temperature, changes occurred in the permeability of the plasma membrane, which lead to a reduction in the intracellular pH. Interestingly, the pH reduction had an impact on the interaction of the cytoskeleton with the 1α elongation factor (EF1 α), which directly affected translation. Hudder et al. [66] supported this hypothesis by demonstrating that the efficiency of protein synthesis depends on the supramolecular organization and the organization of the actin cytoskeleton because the components of the translational apparatus, such as tRNA, aminoacyl-tRNA synthetase and EF1 α , are normally linked to the cytoskeleton. Nevertheless, Hayduk et al. [67] reported that an alteration of the actin cytoskeleton had beneficial effects on the secretion of heterologous proteins in CHO cells producing human recombinant alkaline phosphatase (SEAP) with up to 150 times higher productivity. However, they did not study the effect of cytochalasin D (used for the depolymerization of actin) on protein synthesis in the transporter vesicles inside the cell; therefore, it was not possible to determine if the positive effect of the depolymerization was due to the translational machinery or the intracellular transport system. In an effort to characterize the transcriptome and proteome of the CHO cells producing EPO under mildly hypothermic conditions, Baik et al. [21] reported an increase in the level of expression of the vimentin protein (using 2D PAGE), which is a member of the protein family that constitutes the intermediary filaments. They explained that the increase was due to an enhancement in the size of the cells in low-temperature cultures, which may have caused an increase in the amount of vimentin. Interestingly, an increase in vimentin occurs in cultures treated with sodium butyrate that have been shown to overproduce the r-protein [52]. Kumar et al. [20] used 2D PAGE with differential staining to demonstrate that CHO K1 cells cultivated at 31 °C exhibited an increase in the expression of vimentin when compared with CHO cells cultivated at 37 °C. They also reported differential expression of other proteins related to the cytoskeleton, including the up-regulation of β -actin and the down-regulation of CAPZB. Finally, Yee et al. [22] reported that the overexpression of the *Ckap4*, *Arpc5*, *Mid1p1*, *Tmsb10* and *Vim* genes that encode cytoskeleton elements resulted in an increase in the specific productivity of an antibody under the conditions of mild hypothermia (32 °C) in the CHO cell line. This observation suggested that the up-regulation of intermediary filaments was required to resist an increase in secretion traffic. Dinnis et al. [68] reported the same results after studying the behavior of GS-NS0 Murine Myeloma Cell Lines. Yee et al. [22] suggested that vesicle traffic, endocytosis and cytoskeleton elements would be involved in the increased rate of production for the antibody under mildly hypothermic conditions.

4.2. Variation in the synthesis and translation of mRNAs

The stability and processing of mRNAs are important aspects that control gene expression. Changes in the environmental conditions can cause alterations in several factors that control mRNA expression [69]. However, the temperature effect on mRNAs is not fully understood. Moreover, until recently, quantification of RNA was performed using the hybridization technique known as the Northern blot assay [12,17,28], which has quantitative limitations. Nowadays, techniques such as RT-PCR (Real-Time Polymerase Chain Reaction) and microarrays are more suitable to quantify and identify mRNAs, and these methods can be used to obtain comparable results about the behavior of these molecules under mild

hypothermia conditions. Fox et al. [25] used RT-PCR to determine that mRNA levels increased in proportion to the production of IFN- γ at 32 °C. Moreover, they proposed that the regulation of protein synthesis under mild hypothermic conditions for CHO cells producing IFN- γ would be governed at the translational level, mainly due to the increase in recombinant mRNA levels associated with a higher stability of these molecules. The half-life of the recombinant mRNAs increased about 7–8-fold at 32 °C, indicating that the lower culture temperature delayed mRNA degradation. Similar results were obtained by Yoon et al. [23], who showed that the relative content of EPO mRNA increased with a decrease in temperature, suggesting that an increase in EPO transcription levels was partially responsible for an increase in the specific production of EPO under mildly hypothermic conditions. However, Kou et al. [24] demonstrated that when TNFR-Fc was produced at 30 °C, the increase in specific productivity was exclusively caused by an increase in the mRNA-TNFR-Fc stability of TNFR-Fc and not by an increase in mRNA-TNFR-Fc synthesis nor an increase in transcription rate. Further, Nissom et al. [70] more broadly examined the transcriptome and proteome profiles of high- and low-producer CHO cells at 37 °C and concluded that there was no correlation between mRNA levels and protein concentrations. However, no model that explains the behavior of the mRNA molecules under mildly hypothermic conditions has been developed, and what seems to be the general rule—an increase in r-protein mRNA results in a higher specific productivity of the protein of interest—appears to be contradicted in the report by Yee et al. [22]. Even though these authors observed an increase in the specific production rate of the r-protein, they did not detect changes in the quantities of mRNA antibodies in CHO cell cultures at 33 °C. Moreover, using cDNA microarrays, they reported that the initiation factor of eukaryotic translation, 2- α kinase 3, was up-regulated at 33 °C. The product of this gene phosphorylates and deactivates the elongation factor 2 α , which results in a decrease in the translational activity of the mRNAs. Using the same technique at 33 °C as well as adding sodium butyrate to CHO cell cultures producing IgG, Kantardjieff et al. [38] demonstrated that hundreds of genes with differential expression patterns changed in response to the overproduction of these two stimuli, and remarkably, some of those genes are involved in the post-translational modification of proteins. In addition, it was demonstrated that the increase in the specific productivity of r-protein induced by the addition of sodium butyrate was associated with a significant increase in mRNA-IgG. However, when the increase in specific productivity was induced by the lower temperature (33 °C), there was no change in mRNA-IgG levels.

We determined that a more significant increase in the specific productivity of tPA occurred at 33 °C, as compared to the increase obtained at 37 °C in batch CHO cell cultures. However, under both conditions, the tPA mRNA levels did not show a significant difference (unpublished data), and these results were consistent with other reports [22]. Similarly, in continuous cultures maintained at 37 °C and 33 °C with the same dilution rate (and, therefore, the same specific growth rate), we determined that an important decrease in the specific productivity of tPA occurs at 33 °C, but we did not observe any significant differences in the levels of tPA mRNA (unpublished data).

4.3. Changes in protein synthesis

An Arrhenius mathematical model, designed to characterize and improve productive processes under mildly hypothermic conditions, has been proposed by Kretzmer et al. [71]. In this model, they propose that the effect of decreasing the temperature reduces the central metabolism, while protein synthesis is either not affected or in some cases improved. Currently, the consensus in the field suggests that the primary metabolism is considerably reduced by the

decreased temperature. Nevertheless, levels of protein expression vary by cell line and by protein. For example, proteomic studies of CHO cells, in which Baik et al. [21] used transcriptomics and proteomics as a first approach to elucidate the behavior of CHO cells producing EPO at 33 °C, showed that the level of seven proteins increased in response to the decrease in temperature (PDI, vimentin, NDK B, ERp57, RIKEN cDNA, phosphoglycerate kinase and heat-shock cognate 71 kDa protein), while two proteins decreased their expression levels (HSP90- β and EF2) [21]. A study conducted by Kumar et al. [20] reported significant changes in the protein expression profiles of 201 proteins in CHO-K1 cells cultured at 31 °C in comparison with cultures grown at 37 °C. Among the identified proteins, 23 showed significant differences in expression levels because of the reduction in culture temperature, and these included proteins related to diverse cell functions that have been mentioned in this review, such as growth regulation (HNRPC, down-regulated), apoptosis (importin- α down-regulated), cytoskeleton conformation (vimentin up-regulated) and CAP-independent translation (EIF4A, up-regulated).

More generally, attenuation of transcription and translation in response to the condition of mild hypothermia appears to be a universal solution for prokaryotic and eukaryotic systems [72]. However, it has been reported that when CAP-dependent translation is attenuated due to a stress condition, a small percentage of the mRNAs (~5%) are continuously translated and may even increase in quantity due to CAP-independent translation mediated by IRES activity [48,73]. Interestingly, the cold-shock proteins are synthesized as a part of this mechanism, including the best-known and most well-studied cold-shock proteins in mammalian cells: Rbm3 (RNA-binding motif protein 3) and CIRP (cold-inducible RNA-binding protein). These proteins belong to a family of RNA-binding proteins [74], a group of proteins with highly conserved structures the functions of which have not been completely elucidated. Nevertheless, there is a consensus that one of the main functions of these proteins is to act as chaperones to assure and improve the correct translation of specific mRNAs at low temperatures [75–77].

It is worth mentioning that in HeLa cell cultures, the mRNA and protein levels increase under hypoxia conditions for Rbm3 and CIRP [78], which indicates that the overexpression of these genes is not exclusively triggered under mildly hypothermic conditions. A variety of studies with Rbm3 report several regulatory elements that have been identified, and these respond to the hypothermic conditions in the 5' UTR region [48,79]. As Rbm3 mRNA protein levels increase in cell cultures under mildly hypothermic conditions (33 °C), the IRES activity increased to be up to 5-fold higher than the activity at 37 °C, depending on the cell line [48]. Interestingly, these authors suggest that the effects of mild hypothermia on the translation of Rbm3 was not caused by decreased cell growth, as similar effects were not observed when cells were grown with and without serum. Dressios et al. [74] report a 3-fold increase in the synthesis of this protein when reducing the temperature from 37 °C to 32 °C in mouse neuroblastoma N2a cells cultures. They observed that although decreasing cell culture temperature produced a general decrease in protein synthesis, the presence of Rbm3 improved global protein synthesis, decreasing the magnitude of this effect. However, the mechanism involved in this phenomena is not absolutely clear.

Nishiyama et al. [76] identified and studied the CIRP protein in the nucleoplasm of BALB/3T3 cells and showed that this molecule was a cold-shock protein. They demonstrated that the overexpression of CIRP resulted in a reduced growth rate and prolongation of the G1 phase. It is worth mentioning that in cultures subjected to mildly hypothermic conditions, the temperature was sharply reduced from 37 °C to 32 °C, and by 24 h, CIRP expression was evaluated, with no evidence of expression at extended culture times. Later, Fujita [77] determined that CIRP synthesis is induced in two

ways. The first is by a cold-inducible mechanism, and the second is by a protein-synthesis inducible inhibitor. The increase in the expression of some proteins under mildly hypothermic conditions could be related to a recent finding in CHO cells [80], where there are promoters that are sensitive to this condition, increasing the expression level of the proteins under their control.

Yoon et al. [81] adapted cells at 32 °C and cultivated two CHO cell lines producing EPO and FSH. After adaptation, there was a decrease in the specific productivity of the r-proteins of 49% and 22% for EPO and FSH, respectively, and a decrease in the specific growth rate to around 30%. Under these conditions, CIRP expression did not show significant changes compared to the control for both cell lines. On the other hand, Hong et al. [54] used CHO cells producing EPO to show that unexpectedly, the down-regulation of CIRP under hypothermic conditions did not rescue the cells from growth arrest at the low culture temperature of 32 °C, and its overexpression had no effect on r-protein productivity at 37 °C after 400 h in culture. In contrast, Tan et al. [26] showed that the stable overexpression of CIRP at 37 °C improved the final titer of IFN- γ produced in a CHO cell line by 25% in adherent cultures and up to 40% in suspension cultures. Real-time PCR analysis showed that the increase in recombinant IFN- γ titer could be attributed to increased recombinant IFN- γ mRNA levels, while growth data showed that CIRP overexpression did not result in growth arrest in CHO IFN- γ cells. These results demonstrate that there are additional factors that regulate growth suppression under mildly hypothermic conditions, especially after large periods of cell incubation at low temperatures.

5. Conclusion

As has been discussed in this review, it is important to perform studies that independently assess temperature effects separately from the cell-specific growth rate because that is the only way to identify rational targets in a refined manner without interactions between variables. In this regard, a suitable experimental system for such studies is the chemostat, a continuous culture system that provides a constant milieu. The value of the chemostat in studies of such processes lies in the removal of secondary growth effects that may mask subtle physiological changes, revealing biologically relevant trends and providing confidence in the data obtained.

This operational modality can be tedious to implement, and it demands long experimental time periods due to low specific growth rates of mammalian cells. Nevertheless, it is an operational tool that when combined with robust “omics” methodologies and metabolic models will allow the discrimination of targets directed toward an increasing understanding of the productivity of r-proteins in CHO cells. Based on the information discussed in this review, we believe it is possible to develop strategies at an industrial scale that use fed-batch or perfusion cultures to increase r-protein productivity.

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