Characterization of glutathione proteome in CHO cells and its relationship with productivity and cholesterol synthesis.

Valentine Chevallier[1], Erwin Schoof[2], Laetitia Malphettes[3], Mikael Rørdam Andersen[2,4], and Christopher Workman[2]

[1]UCB NORDIC A/S
[2]Danmarks Tekniske Universitet
[3]UCB Pharma SA
[4]Technical University of Denmark

May 5, 2020

Abstract

Glutathione (GSH) plays a central role in the redox balance maintenance in mammalian cells. The study of industrial CHO cell lines have demonstrated a close link between GSH metabolism and clone productivity. However, a deep investigation is still required to understand this correlation and highlights new potential targets for cell engineering. In this study, we have modulated the GSH intracellular content of an industrial cell line under bioprocess conditions in order to further elucidate the role of the GSH synthesis pathway. Two strategies were used : the variation of cystine supply and the direct inhibition of the GSH synthesis using buthionine sulfoximine (BSO). Cysteine supply modulation have revealed a correlation between intracellular GSH and product titer over time. Analysis of metabolites uptake/secretion rates and proteome comparison between BSO-treated cells and non-treated cells has highlighted a slow down of the TCA cycle leading to a secretion of lactate and alanine in the extracellular environment. Moreover, an adaptation of the glutathione related proteome has been observed with a up-regulation of the regulatory subunit of glutamate cysteine ligase and a down-regulation of a specific glutathione transferase subgroup, the Mu family. Surprisingly, the main impact of BSO treatment was observed on a global down-regulation of the cholesterol synthesis pathways. As cholesterol is required for protein secretion, it can be the missing part of the jigsaw to finally elucidate the link between GSH synthesis and productivity.

Keywords

CHO cells, Glutathione, Buthionine sulfoximine, Cholesterol, Proteomic

Introduction

Under bioprocess conditions, CHO cells can be exposed to oxygen microheterogeneity, free radicals generated by cell culture medium components and high oxidative metabolism, which can lead to oxidative stress. Moreover, product quality can also be affected by reactive oxygen species (ROS) production. For these and other reasons, research related to the control of oxidative stress has been of increased interest. In this context, the controlled modulation of oxidative stress can help scientists to improve bioprocesses.

One of the main targets for modulation of oxidative stress is glutathione (GSH). GSH is a tripeptide (γ-L-glutamyl-L-cysteinyl glycine) which is the cofactor of ROS detoxification enzymes, as well as a direct ROS scavenger. Glutathione can also form conjugates with reactive electrophilic compounds to promote their detoxification (Ketterer et al., 1983). It plays a central role in the detoxification of ROS produced

in the mitochondria, but also in the regulation of disulfide bond formation in the endoplasmic reticulum (Chakravarthi et al., 2004; Ribas et al., 2014).

A potential relationship between GSH and secreted protein productivity has already been suggested in literature. High producing cell lines have been shown to contain more GSH than low producers and an up-regulated GSH metabolism (Chong et al., 2012; Orellana et al., 2015). In order to reproduce this phenomena, cell engineering has been performed to increase GSH synthesis (Orellana et al., 2017). On the one hand, overexpression of the catalytic subunit of glutamate cysteine ligase (GCLc), the rate limiting enzyme in GSH synthesis, did not lead to increased titers despite higher GSH levels. On the other hand, the overexpression of the regulatory subunit of glutamate cysteine ligase also called the glutamate-cysteine ligase modifier subunit (GCLm) led to an increase of productivity.

The uncertainty around the actual role of GSH in CHO bioprocessing led us to investigate the role of this metabolite further. Indeed, if the absolute GSH quantity does not explain productivity between two different clones, can it explain productivity differences between two processes with the same clone? What are the other cellular functions that are directly or indirectly impacted by the intracellular levels of GSH? In this context we have modulated intracellular GSH levels using two approaches: the variation of cystine supply through feed medium composition and direct inhibition of the GCL enzyme using buthionine sulfoximine (BSO). Our goal was to understand which pathways are actually affected by lower levels of GSH. To capture the metabolic adaptations to these two variations we investigated the cell phenotype, measured metabolites involved in the central carbon metabolism, and performed proteomic analysis.

Material and methods

Cell culture

A proprietary DG44 Chinese Hamster Ovary (CHO) cell line engineered to produce a full monoclonal antibody was used. These cells were cultivated for 14 days in 2L glass bioreactors (Sartorius) with dissolved oxygen and pH control. Bioreactors were inoculated on day 0 at a seeding density of $0.35x10^6$ cells/mL. Cells were cultivated in fed-batch mode with addition of feeds from day 3 to day 12. Viable cell concentration (VCC) and viability were measured using a Vi-Cell analyzer (Beckman Coulter). Recombinant protein titer was measured by immunoturbidimetry using a Cedex Bio HT analyzer (Roche) in supernatant.

Two processes have been assessed to produce this recombinant protein: Process 1 and Process 2. In the upstream part, Process 2 has a less concentrated feed medium especially with less cysteine. To mimic an oxidative stress during the culture, L-buthionine sulfoximine (BSO) (Sigma) was spiked on day 3 to a final concentration of 0.5 mM in the bioreactor. All conditions have been performed in triplicates. One bioreactor cultivated with Process 2 and without BSO has been excluded from the data set due to pump failure during the culture.

Amino acid measurement

The cell culture fluid was centrifuged 30min at 17000g in a 30K Amicon® 0.5mL Filter (Merck). The sample preparation and analysis was performed by reverse phase UPLC as described previously (Mulukutla et al., 2019) using a TUV detector (Waters) set at 254 nm.

Glutathione measurement

After sampling, the cell culture fluid was directly quenched using a -20°C ethanol bath and kept cold in a CoolRack® (Corning) during the sample preparation. A volume containing 10^7 cells was then centrifuged at 1000g, 1 min, -5°C. Cell pellets were washed twice using cold 0.9% NaCl solution and frozen at -80°C. The day of the analysis, the cell pellet was resuspended in a 10mM EDTA solution (pH 8) and a ^{13}C-labelled glutathione internal standard (Buchem) was added. Cells were lysed using a sonifier (Brandson) during for 30 seconds (pulse mode [15sec on / 5sec off]) with a 10% amplitude. In order to measure total glutathione,

a DTT solution was added to the lysate and incubated 30 min at room temperature to reduce glutathione disulfide (GSSG). After the 30 minutes, N-ethylmaleimide stock solution was added and incubated 40 min at room temperature, protected from light. Proteins were then precipitated by addition of -20°C acetone. After precipitation, the sample were centrifuged at 17000g for 5min and the supernatant was transferred in a new tube for overnight evaporation (Speedvac, Supplier). The sample pellet was reconstitute in mobile phase A (0.1% formic acid:water) using sonication. The sample was analyzed by LC-MS using a tandem quadrupole detector (Waters). The liquid chromatography was performed on a HSST 3 column of 10 cm (Waters) at 45°C and under a gradient of mobile phase A and B (0.1%formic acid:acetonitrile). GSH quantity were determined by comparison with the internal standard and normalized by the cell quantity.

Sample preparation for proteomics analysis

Cells (5 x 10^7) were harvested on day 6 and 10 of the culture, quenched using a -20°C ethanol bath and kept cold in a CoolRack® (Corning) during the sample preparation. Cells were spun down at 1000g, 1min, -5°C. The cell pellets were washed two times with cold 0.9% NaCl solution. Proteins were extracted in 1mL of 6M guanidine solution, boiled 5min at 95°C, and vortexed. Samples were then spun down 10 min at 17000g and supernatants were stored at -80°C. Protein concentration was determined in samples diluted 10 times following the protocol of the PierceTM BCA Protein assay (Thermo Scientific). 50µg of each samples was reduced and alkylated using tris(2-carboxyethyl)phosphine and chloroacetamide at a final concentration of 10mM and 40mM respectively. Samples were then digested using LysC (1:50 protein ratio, incubation 3-4h at 37°C) and Trypsin (1:10 protein ratio, incubation overnight at 37°C). Samples were then diluted in trifluoroacetic acid to a final concentration of 1% (v/v) to stop the digestion. Prior to labelling, the peptides were desalted using C18 StageTips. Samples and internal standard were labelled using the 11-plexes Tandem Mass Tag reagents (Thermo Scientific). Each Injection was fractionated using ion exchange column and analyzed on Q-Exactive mass spectrometer (Waters).

Proteomics data analysis

MS spectra were processed using Proteome Discoverer (Thermo Fisher Scientific, version) and the MS Amada identification algorithm (Dorfer et al., 2014). The MS/MS data were queried against the CHO-K1 proteome available from UniProtKB (proteome ID : UP000001075, downloaded Sept 3, 2018) and the "common Repository of Adventitious Proteins" database (cRAP) for contaminants available on the Global Proteome Machine website (downloaded Oct 30, 2018)(Consortium, 2018; Craig et al., 2004). Precursor mass tolerance was set at 10ppm. Fragment mass tolerance was set at 0.02ppm. Methionine oxidation and protein N-terminal acetylation were defined as dynamic modifications. Cysteine carbamidomethylation, TMT adduction on Lysine and on protein N-terminal were defined as a fixed modification. Peptides and assembled proteins were searched at a false discovery rate (FDR) of 1%. A minimum of one unique peptide per protein was required for protein identification. For TMT quantification, the ratios of the TMT reporter ion intensities between samples and the internal standard (label 131C), generated by proteome discoverer for each proteins, were used. These ratios were extracted for the statistical analysis in R.

Statistical analysis

Ratios extracted from Proteome discoverer were log2 transformed and quantiles normalized in R. This data was then used to perform an empirical Bayes moderated t -test using the *limma* package in R (Phipson et al., 2016; Ritchie et al., 2015). Effect of the parameter day, BSO treatment, and process have been included in the design matrix. Differentially expressed proteins were identified using an *adj. p. value* <0.05. To narrow down the analysis, we focused only on proteins differentially expressed because of the BSO treatment and with a log fold change (logFC) threshold of 0.5. A heatmap of the differentially expressed proteins was generated by hierarchical clustering using *pheatmap* package in R (Kolde, 2015). Functional analyses were performed using MetaCore (Clarivate Analytics, version 19.2.69700) after protein ID conversion to the *Mus musculus*equivalents. The ID conversion was performed using InParanoid 8 and UniProtKB BLAST when no matches were found in the first method (Sonnhammer et al., 2015).

Results

Delayed impact of BSO on growth

In order to study effect of glutathione on recombinant mAb production, GSH was depleted using BSO. A sterile BSO solution was spiked on day 3 of the fed batch culture to a final BSO concentration of 0.5mM. BSO-treated bioreactors and control bioreactors were monitored by daily measurement of viable cell concentration (VCC), intracellular GSH and product titer. Surprisingly, despite a depletion of GSH already observed on day 4, i.e. 24 hours after BSO addition, the growth and production profiles only started to differ from day 6 (Figure 1). The average cell diameter started to increase from day 6 in the BSO condition instead of day 8 in control condition (Figure S1). Furthermore, the decrease in viability was only observed from day 9 in BSO conditions despite a treatment on day 3 (Figure S1). These results suggest that the CHO cell line phenotype was unaffected by glutathione depletion during the three first days after BSO addition. From day 6, their growth rate was gradually reduced and their cellular volume is increasing until day 10. The viable cell concentration decreased after day 10 due to cell death until the end of the culture.

Cysteine supply modulates intracellular GSH and product titer

Since cysteine is a precursor of glutathione, we also investigated if this lower concentration of cysteine influenced the content of intracellular glutathione during the cultivation using a feed (Process 2) containing lower levels of cysteine (Figure 1C). A correlation between the increase of total glutathione and the increase in specific productivity was observed over time.

Except for product titer, no other significant differences between the two feeding strategies were observed in growth profile, proteomic data and metabolites uptake and production rate. As a result, data from the two feeding strategies (processes 1 and 2) were combined to study BSO effect in the next analysis.

BSO treatment affects metabolite secretion and uptake rates

To further characterize the impact of glutathione depletion on CHO cell line metabolism, daily extracellular concentrations of selected metabolites were measured and associated specific uptake and production rate were calculated. Uptake/secretion rates of glucose, lactate and amino acids are shown in Figure 2 and Figure S2. Glucose uptake rates were similar between control and BSO conditions until day 10. Glucose uptake was slightly faster in the BSO condition compared to the control when viability and cell diameter started to decrease. Similar profiles were also observed for histidine, asparagine, and tyrosine uptake rate from day 10. On the contrary, hydroxyproline and aspartic acid were produced/released from this point. These metabolic changes seem to be more related to cell death than to the BSO stress itself. The production of cystine observed from day 8 suggests a cysteine secretion, but high variability was observed for this amino acid in the BSO conditions.

Lactate uptake/secretion rate already started to differ from day 6. Indeed lactate is produced by BSO-treated cells and consumed by non-treated cells. Regarding amino acid uptake/secretion rates, alanine was the only amino acid that displayed a similar profile to lactate in response to the BSO treatment. Since lactate and alanine can be produced from pyruvate, these profiles suggest a failure or slowdown of the TCA cycle.

Proteome related to TCA cycle, GSH metabolism and cholesterol biosynthesis are modulated by BSO treatment

As glutathione metabolism is seemingly linked to the clone productivity, we evaluated the impact of glutathione depletion on host cell protein expression in the studied cell line. For this purpose, we sampled $50x10^6$ cells on day 6 and 10 to perform proteomics analysis using TMT labelling (see Materials and Methods).

Across all samples, 3,281 proteins were identified with the identification criteria defined in the material and method section.

Differentially expressed proteins in the BSO-treated culture have been identified using an empirical Bayes moderated t -test (*adj. p. value* <0.05, LogFC threshold : 0.5). This analysis was done on data from day 6 and day 10, i.e. 3 and 7 days after treatment (Supplementary table S1). 63 proteins were differentially expressed in response to BSO; 47 proteins were down-regulated and 16 proteins were up-regulated. A heatmap of the differentially expressed proteins is shown in Figure 3. No pattern can be observed between data from process 1 versus process 2. The global protein expression was not impacted by a low cysteine supply in contrast to the product titer and intracellular glutathione content. Overall, except for a few proteins, the BSO impact on proteins levels observed on day 6 was amplified on day 10.

In order to identify the cellular functions most impacted by the BSO treatment, enrichment analysis on differentially expressed proteins was performed in Metacore software based on GO annotations and Pathway maps (Supplementary table S2). Based on this results, each cluster identified on the heatmap has been associated with overrepresented functions (Figure 3). In the first cluster, protein expression increased significantly between day 6 and day 10 for the control condition, while proteins expression remained low at day 10 for the BSO condition. This cluster primarily represented cell matrix adhesion proteins. This observation can be associated to cell growth differences observed on day 10 between the two conditions. Likewise, the opposite response observed for CYR61 protein can also be linked to cell growth response as this protein is a known regulator of apoptosis (Lau, 2011).

The largest cluster of proteins were down-regulated after BSO treatment on both days 6 and 10 (Figure 3, Table 1). These included three main functions: cholesterol biosynthesis, carboxylic acid metabolism, and aminoacyl-t-RNA biosynthesis in mitochondria. These cellular processes were interpreted to be at least partially down-regulated in the BSO conditions relative to the control. All detected enzymes involved in cholesterol biosynthesis were down-regulated with an average logFC between -0.34 to -1.21 (Table 1). Interestingly the related transcription factor SREBP2 was also down-regulated but to a lesser extent (logFC: -0.17 / *adj. p. value* <0.05).

When we specifically considered mitochondrial proteins related to the TCA cycle, a global down-regulation was observed where 12 mitochondrial proteins were observed to have logFC in the interval [-1.09; -0.13] and *adj. p. value* <0.05 (Table 1). However, glycolysis-related proteins were not differentially expressed (Supplementary table 1).

In the last cluster (Figure 3), the main response to oxidative stress was observed, as this contains the majority of BSO up-regulated proteins, including three proteins were related to heme metabolism. Heme oxygenase had the strongest signal with a logFC of 1.50 and has also been associated with oxidative stress (Hedblom et al., 2019).

Proteins specifically involved in glutathione metabolism can be found in the two last described clusters (Figure 3). More details about proteins detected related to glutathione are presented in Figure 4. Glutathione synthesis-related proteins were overall up-regulated, especially the glutamate cysteine ligase regulatory subunit and the S-formylglutathione hydrolase. In contrast, some proteins involved in the consumption of glutathione, e.g. for the detoxification or catabolism of glutathione, were overall down-regulated. A good example of this was the down-regulation of glutathione S-transferases from the Mu family. However, GSTs from other families (omega, alpha and pi) were not down-regulated (Figure 4).

Discussion

In this study, in order to characterize oxidative stress response in CHO cells, we have modulated the GSH synthesis through two approaches: by the reduction of cysteine supply and by the inhibition of GSH biosyn-

thesis. Reducing cysteine concentration in the feed by 50% did not appear to influence cell growth, but led to a decrease of 24% in titer and of 50% in intracellular glutathione. However, inhibition of GCL activity by BSO led to significant depletion of GSH as well as a reduced cell growth and titer. In addition to classical physiological characterization and metabolite profiling, proteomics was performed at different stages of the culture. Interestingly, no significant differences in protein expression were observed in the reduced cysteine feed condition, whereas 63 proteins displayed different expression levels in the BSO-treated conditions suggesting an adaptation to oxidative stress.

In a recent study, it was shown that a decrease of cysteine supply led to a depletion of glutathione (Ali et al., 2019). As a consequence, the oxidative stress generated led to cell death, titer decrease and differential expression of proteins. In our study, the decrease of cysteine supply did not lead to intracellular GSH depletion. Therefore, we did not observe cell death and significant protein differential expression (adjusted p value$<$0.05, LogFC threshold : 0.5), while specific productivity was substantially reduced. Indeed, a clear correlation over time between specific productivity and GSH intracellular concentration was observed. It can thus be suggested that in response to a reduced cysteine supply and as a consequence a reduce GSH availability, the cell metabolism initially decreases the recombinant protein production to reduce ROS production. The regulation of this phenomena can be due to a differential expression of non-detected host cell proteins such as transcription factors. It could also be due to other regulatory mechanisms such as protein phosphorylations, which are not detected in this type of proteomic analysis.

Partial inhibition of GCL using BSO has been suggested as a selection system to enrich for a cell population with higher productivity (Feary et al., 2017). In our case, specific productivity is not increased by the BSO treatment, because the concentration used appeared to fully inhibit the GCL enzyme. However, the increase in cell diameter and the overexpression of GCLc and GCLm were consistent with previous observations (Feary et al., 2017). Interestingly, in the present study, GCLm was more overexpressed than GCLc in BSO-treated cells. In the control condition, GCLm expression decreases over time following the intracellular GSH increase. However, a constitutive expression of GCLc was observed (Figure S3). Despite a likely higher level of GCLc protein in CHO cells, it looks as though dynamic expression of GCLm is the most important parameter in the regulation of *de novo* synthesis of GSH during the process. In a previous study in CHO cells, it has been demonstrated that up-regulation of GCLm by cell engineering increase GSH content, but surprisingly not the GCL activity (Orellana et al., 2017). In the present study, in the presence of BSO, CHO cells try to compensate for GCL inhibition by producing more GCLm. However, this response was not able to restore normal intracellular GSH levels in our experimental conditions. As cystine cannot be used for GSH synthesis, it is possible that the potential secretion observed from day 8 happened to avoid accumulation of this amino acid in the cell. Indeed, cysteine/cystine accumulation can potentially influence the intracellular redox potential.

In addition to the attempt to up-regulate GSH production when inhibited by BSO, cells recycled GSH through the overexpression of*S* -formylglutathione and GSH reductase. Likewise, GSH catabolism through the gamma-glutamyl cyclotransferase was down-regulated. Interestingly, the GST Mu enzymes 1, 5 and 6 were down-regulated. However other GSTs from other families (omega, alpha and pi) have been detected and were not interpreted as down-regulated. This can be due to the difference of substrate selectivity. For example, GSTs Mu are in general more efficient for nucleophilic aromatic substitution and less selective than GSTs Alpha (Eaton et al., 1999; Salinas et al., 1999). GSTs Alpha is the only family able to reduce hydroperoxides. They are also involved in lipid peroxidation by product detoxification such as Acrolein and 4-hydroxy-2-nonenal (Stevens et al., 2008; Yang et al., 2016). Moreover, some GSTs can have additional activities to conjugation such as GST Pi 1 which can bind to c-Jun N-terminal kinase (JNK) and GST Mu 1 which can bind to apoptosis signal-regulating kinase 1 (ASK1), and modulate apoptosis signaling pathways (Allocati et al., 2018; Armstrong, 2010).

Beside GSH metabolism, other responses to oxidative stress were observed. The main one was the overexpression of the heme oxygenase 1 already observed on day 6 and amplified on day 10. Increase of the free heme detoxification is usually observed under oxidative stress (Gozzelino et al., 2010). However, we also

observed an up-regulation of the transferrin receptor protein 1 and the 5-aminolevulinate (ALA) synthase. The first is involved in iron transport and the second is the rate-limiting enzyme in heme synthesis. However, there is an inconsistency with the regulation of heme biosynthesis described in literature as ALA synthase is usually down-regulated when the heme oxygenase 1 is up-regulated (Ajioka et al., 2006; Fujii et al., 2004).

The heme oxygenase 1 gene expression is regulated by the Nuclear factor E2-related factor 2 (Nrf2). This factor is retained in the cytoplasm through a complex with Keap1 under normal conditions. Under oxidative stress, it is translocated to the nucleus and binds to the antioxidant response element (ARE). The overexpression of Gclm and heme oxygenase 1 in the BSO-treated cells suggests an activation of the Keap1-Nrf2 pathway. Moreover Sequestosome 1, also called p62, is also overexpressed (Supplementary material 1). This proteins is known to compete with Nrf2 for the interaction with Keap1 leading to a stabilization of free Nrf2 (Wei et al., 2019). Others proteins related to the antioxidant defense (catalase, superoxide dismutase [Mn], thioredoxin 1, glutathione reductase) and NADPH regeneration through the oxPPP pathway (Glucose-6-phosphate 1-dehydrogenase, 6-phosphogluconate dehydrogenase) have been measured as differentially expressed in the BSO-treated cells, but with a lower logFC magnitude (Tonelli et al., 2018) (Supplementary Table 1). This observation can support the hypothesis of an Nrf2 activation. One of the limitations with this explanation is that GSTs Mu genes have also been described as Nrf2 target and are significantly down-regulated.

Another response to oxidative stress is the down-regulation of intracellular ROS production. The main source of ROS within the cell is the respiratory chain (Turrens, 2003). In this context, mitochondria proteins should be the main targets of activity reduction. The general down-regulation of proteins involved in the oxidative phosphorylation and the TCA cycle observed in BSO conditions confirm this hypothesis. Acyl-CoA synthetase family member 2, involved in the activation of fatty acid is also down-regulated (LogFC =-0.79, *adj. p. value*=1.11x10^{10}). This observation suggested a decrease of Acetyl CoA supply to the TCA through the beta oxidation pathway. This hypothesis was supported by the down-regulation of other enzymes involved in the beta oxidation process such as the Carnitine O-palmitoyltransferase 2 or the Acetyl-CoA acetyltransferase (Supplementary table 1). Consequently, carbon fluxes through the TCA cycle were reduced and pyruvate accumulated in the cells. Indeed, glycolysis enzymes were not down-regulated and the glucose uptake was constant. Other enzymes involved in pyruvate production such as the malic enzyme [NAD] were down-regulated. Therefore, pyruvate surplus was converted to lactate and alanine which are produced from day 6 in BSO-treated cells.

Another down-regulated process in response to glutathione depletion was lipid metabolism and especially the cholesterol *de novo* synthesis pathway. Cholesterol plays a major role in membrane fluidity regulation. Moreover, cholesterol regulation may also play a role on protein secretion reduction as it is an essential building block of secretion vesicles (Wang et al., 2000). Recently, it has been shown that the increase of cholesterol synthesis with the up-regulation of a micro RNA can increase the productivity of CHO cell lines by increasing their secretion capacity (Loh et al., 2017). It is then possible that the increase of productivity and glutathione content observed over time during the process are also link to cholesterol regulation.

One hypothesis that can be proposed to explain the down-regulation of cholesterol synthesis under glutathione depletion is the accumulation of oxysterols in the ER. The expression of enzymes involved in cholesterol synthesis is regulated by a common transcription factor SREBP2. SERBPs are retained in the ER membrane by forming a complex with the SERBP cleavage-activating protein (SCAP) and the insulin-induced gene protein (Insig). The retention of the complex is controlled by cholesterol and by oxysterol concentrations (Howe et al., 2016). As oxysterol is a byproduct of cholesterol biosynthesis, it is a signal for cholesterol overproduction for the cell. Oxysterols can be enzymatically derived, especially by the cytochrome P450 reductase, or direct products of cholesterol autoxidation (Olkkonen et al., 2012). Hence, it could be hypothesized that the BSO treatment led to an increase of oxysterols in the ER (Micheletta et al., 2006).

Another possible explanation is that the reduction of the cholesterol synthesis could be an attempt to decrease the use of NADPH. Indeed, the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase use two NADPH molecules to reduce HMG-CoA to mevalonate (Burg et al., 2011). Moreover, NADPH electrons are

transferred by the cytochrome P450 reductase to squalene monooxygenase and lanosterol demethylase during cholesterol synthesis (Porter, 2015). In total, four NADPH molecules are used to produce one cholesterol molecule from acetyl-CoA, which is stoichiometrically a lot. Decreasing this activity may also help to maintain NADPH/$NADP^+$ redox homeostasis and indirectly counteract oxidative stress.

Another hypothesis is the down-regulation of cholesterol to favorize GSH import in the mitochondria. Indeed, cholesterol has been reported as a mitochondrial GSH transport regulator (Ribas et al., 2016). Accumulation of cholesterol in the mitochondria membrane has been shown to impair the activity of some membrane proteins such as the 2-oxoglutarate carrier which exports 2-oxoglutarate in the cytosol in exchange of the import of GSH in the mitochondria. Moreover, it has been shown that accumulation of mitochondrial cholesterol can damage the respiratory chain complexes assembly (Solsona-Vilarrasa et al., 2019). Under GSH depletion, the cells potentially tried to stabilize the mitochondria membrane and favorize GSH import in the mitochondria matrix by lowering cholesterol.

We showed that reducing GSH intracellular content by half led to a decrease productivity of heterologous protein production despite a modest number of changes in the host cell protein expression profile. However, GSH depletion resulted in an adaptation of GSH metabolism and triggered an oxidative stress response. In addition, cells died and recombinant protein was completely stopped. Thanks to these extreme conditions, this study have lighted up that the modulation GSH thanks to BSO also impacted lipid biosynthesis, especially cholesterol which play a role in protein secretion. Thus, in order to finally figure out how glutathione metabolism is linked to productivity , further work should include a control of cholesterol metabolism.

References

Ajioka, R. S., Phillips, J. D., & Kushner, J. P. (2006). Biosynthesis of heme in mammals. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1763* (7), 723-736. doi:https://doi.org/10.1016/j.bbamcr.2006.05.005

Ali, A. S., Raju, R., Kshirsagar, R., Ivanov, A. R., Gilbert, A., Zang, L., & Karger, B. L. (2019). Multi-Omics Study on the Impact of Cysteine Feed Level on Cell Viability and mAb Production in a CHO Bioprocess.*Biotechnol J, 14* (4), 1800352. doi:10.1002/biot.201800352

Allocati, N., Masulli, M., Di Ilio, C., & Federici, L. (2018). Glutathione transferases: substrates, inihibitors and pro-drugs in cancer and neurodegenerative diseases. *Oncogenesis, 7* (1), 8. doi:10.1038/s41389-017-0025-3

Armstrong, R. N. (2010). Glutathione Transferases. In*Comprehensive Toxicology, Second Edition* (Vol. 4, pp. 295-321).

Burg, J. S., & Espenshade, P. J. (2011). Regulation of HMG-CoA reductase in mammals and yeast. *Progress in lipid research, 50* (4), 403-410. doi:10.1016/j.plipres.2011.07.002

Chakravarthi, S., & Bulleid, N. J. (2004). Glutathione is required to regulate the formation of native disulfide bonds within proteins entering the secretory pathway. *J Biol Chem, 279* (38), 39872-39879. doi:10.1074/jbc.M406912200

Chong, W. P., Thng, S. H., Hiu, A. P., Lee, D. Y., Chan, E. C., & Ho, Y. S. (2012). LC-MS-based metabolic characterization of high monoclonal antibody-producing Chinese hamster ovary cells. *Biotechnol Bioeng, 109* (12), 3103-3111. doi:10.1002/bit.24580

Consortium, T. U. (2018). UniProt: a worldwide hub of protein knowledge.*Nucleic Acids Research, 47* (D1), D506-D515. doi:10.1093/nar/gky1049

Craig, R., Cortens, J. P., & Beavis, R. C. (2004). Open Source System for Analyzing, Validating, and Storing Protein Identification Data.*J Proteome Res, 3* (6), 1234-1242. doi:10.1021/pr049882h

Dorfer, V., Pichler, P., Stranzl, T., Stadlmann, J., Taus, T., Winkler, S., & Mechtler, K. (2014). MS Amanda, a universal identification algorithm optimized for high accuracy tandem mass spectra. *J Proteome Res, 13*

(8), 3679-3684. doi:10.1021/pr500202e

Eaton, D. L., & Bammler, T. K. (1999). Concise review of the glutathione S-transferases and their significance to toxicology. *Toxicol Sci, 49* (2), 156-164. doi:10.1093/toxsci/49.2.156

Feary, M., Racher, A. J., Young, R. J., & Smales, C. M. (2017). Methionine sulfoximine supplementation enhances productivity in GS-CHOK1SV cell lines through glutathione biosynthesis. *Biotechnol Prog, 33* (1), 17-25. doi:10.1002/btpr.2372

Fujii, H., Takahashi, T., Matsumi, M., Kaku, R., Shimizu, H., Yokoyama, M., . . . Morita, K. (2004). Increased heme oxygenase-1 and decreased delta-aminolevulinate synthase expression in the liver of patients with acute liver failure. *Int J Mol Med, 14* (6), 1001-1005.

Gozzelino, R., Jeney, V., & Soares, M. P. (2010) Mechanisms of cell protection by heme Oxygenase-1. In*: Vol. 50. Annu Rev Pharmacol Toxicol* (pp. 323-354).

Hedblom, A., Hejazi, S. M., Canesin, G., Choudhury, R., Hanafy, K. A., Csizmadia, E., . . . Wegiel, B. (2019). Heme detoxification by heme oxygenase-1 reinstates proliferative and immune balances upon genotoxic tissue injury. *Cell Death Dis, 10* (2), 72. doi:10.1038/s41419-019-1342-6

Howe, V., Sharpe, L. J., Alexopoulos, S. J., Kunze, S. V., Chua, N. K., Li, D., & Brown, A. J. (2016). Cholesterol homeostasis: How do cells sense sterol excess? *Chem Phys Lipids, 199* , 170-178. doi:10.1016/j.chemphyslip.2016.02.011

Ketterer, B., Coles, B., & Meyer, D. J. (1983). The role of glutathione in detoxication. *Environmental health perspectives, 49* , 59-69. doi:10.1289/ehp.834959

Kolde, R. (2015). pheatmap: Pretty heatmaps [Software]. In.

Lau, L. F. (2011). CCN1/CYR61: the very model of a modern matricellular protein. *Cell Mol Life Sci, 68* (19), 3149-3163. doi:10.1007/s00018-011-0778-3

Loh, W. P., Yang, Y., & Lam, K. P. (2017). miR-92a enhances recombinant protein productivity in CHO cells by increasing intracellular cholesterol levels. *Biotechnol J, 12* (4). doi:10.1002/biot.201600488

Micheletta, F., & Iuliano, L. (2006). Free Radical Attack on Cholesterol: Oxysterols as Markers of Oxidative Stress and as Bioactive Molecules. *Immunology, Endocrine & Metabolic Agents - Medicinal Chemistry, 6* , 305-316. doi:10.2174/187152206777435618

Mulukutla, B. C., Mitchell, J., Geoffroy, P., Harrington, C., Krishnan, M., Kalomeris, T., . . . Hiller, G. W. (2019). Metabolic engineering of Chinese hamster ovary cells towards reduced biosynthesis and accumulation of novel growth inhibitors in fed-batch cultures. *Metabolic Engineering, 54* , 54-68. doi:10.1016/j.ymben.2019.03.001

Olkkonen, V. M., Béaslas, O., & Nissilä, E. (2012). Oxysterols and their cellular effectors. *Biomolecules, 2* (1), 76-103. doi:10.3390/biom2010076

Orellana, C. A., Marcellin, E., Gray, P. P., & Nielsen, L. K. (2017). Overexpression of the regulatory subunit of glutamate-cysteine ligase enhances monoclonal antibody production in CHO cells. *Biotechnol Bioeng, 114* (8), 1825-1836. doi:10.1002/bit.26316

Orellana, C. A., Marcellin, E., Schulz, B. L., Nouwens, A. S., Gray, P. P., & Nielsen, L. K. (2015). High-antibody-producing Chinese hamster ovary cells up-regulate intracellular protein transport and glutathione synthesis. *J Proteome Res, 14* (2), 609-618. doi:10.1021/pr501027c

Phipson, B., Lee, S., Majewski, I. J., Alexander, W. S., & Smyth, G. K. (2016). Robust hyperparameter estimation protects agaisnt hypervariable genes and improves power to detect differential expression. *The annals of applied statistics, 10* (2), 946-963. doi:10.1214/16-AOAS920

Porter, T. D. (2015). Electron Transfer Pathways in Cholesterol Synthesis. *Lipids, 50* (10), 927-936. doi:10.1007/s11745-015-4065-1

Ribas, V., García-Ruiz, C., & Fernández-Checa, J. C. (2014). Glutathione and mitochondria. *Frontiers in pharmacology, 5* , 151-151. doi:10.3389/fphar.2014.00151

Ribas, V., García-Ruiz, C., & Fernández-Checa, J. C. (2016). Mitochondria, cholesterol and cancer cell metabolism. *Clinical and translational medicine, 5* (1), 22-22. doi:10.1186/s40169-016-0106-5

Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W., & Smyth, G. K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Research, 43* (7), e47-e47. doi:10.1093/nar/gkv007

Salinas, A. E., & Wong, M. G. (1999). Glutathione S-transferases–a review. *Curr Med Chem, 6* (4), 279-309.

Solsona-Vilarrasa, E., Fucho, R., Torres, S., Nunez, S., Nuno-Lambarri, N., Enrich, C., . . . Fernandez-Checa, J. C. (2019). Cholesterol enrichment in liver mitochondria impairs oxidative phosphorylation and disrupts the assembly of respiratory supercomplexes. *Redox Biol, 24* , 101214. doi:10.1016/j.redox.2019.101214

Sonnhammer, E. L., & Ostlund, G. (2015). InParanoid 8: orthology analysis between 273 proteomes, mostly eukaryotic. *Nucleic Acids Res, 43* (Database issue), D234-239. doi:10.1093/nar/gku1203

Stevens, J. F., & Maier, C. S. (2008). Acrolein: sources, metabolism, and biomolecular interactions relevant to human health and disease.*Mol Nutr Food Res, 52* (1), 7-25. doi:10.1002/mnfr.200700412

Tonelli, C., In, C. C. I., & Tuveson, D. A. (2018). Transcriptional Regulation by Nrf2. *Antioxid Redox Signal, 29* (17), 1727-1745. doi:10.1089/ars.2017.7342

Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. *J Physiol, 552* (Pt 2), 335-344. doi:10.1113/jphysiol.2003.049478

Wang, Y., Thiele, C., & Huttner, W. B. (2000). Cholesterol is required for the formation of regulated and constitutive secretory vesicles from the trans-Golgi network. *Traffic, 1* (12), 952-962.

Wei, R., Enaka, M., & Muragaki, Y. (2019). Activation of KEAP1/NRF2/P62 signaling alleviates high phosphate-induced calcification of vascular smooth muscle cells by suppressing reactive oxygen species production.*Sci Rep, 9* (1), 10366. doi:10.1038/s41598-019-46824-2

Yang, Y., Huycke, M. M., Herman, T. S., & Wang, X. (2016). Glutathione S-transferase alpha 4 induction by activator protein 1 in colorectal cancer. *Oncogene, 35* (44), 5795-5806. doi:10.1038/onc.2016.113

Table

Table 1 : Cholesterol and TCA cycle related proteins expression in BSO-treated cells. The logFC and the adjusted p. value have been generated using limma empirical Bayes moderated t-test. The BSO-treated condition (n=10) have been compared to control conditions (n=12).

Pathway	Uniprot Accession	Protein names
TCA cycle	G3H5K6	Pyruvate dehydrogenase E1 component subunit alpha
	G3HRP3	Citrate synthase
	G3II47	Aconitate hydratase, mitochondrial (Aconitase) (EC 4.2.1.-)
	G3H450	Isocitrate dehydrogenase [NAD] subunit, mitochondrial
	G3H0B5	Isocitrate dehydrogenase [NAD] subunit, mitochondrial
	G3HSW9	Isocitrate dehydrogenase [NAD] subunit, mitochondrial
	G3HU51	Isocitrate dehydrogenase [NADP]
	G3IHC0	Isocitrate dehydrogenase [NADP]
	G3HMB4	2-oxoglutarate dehydrogenase E1 component, mitochondrial
	G3IP00	Succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial

Pathway	Uniprot Accession	Protein names
	G3HZ50	Succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial
	G3HQ05	Succinate–CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial
	G3GS40	Succinyl-CoA ligase [GDP-forming] subunit beta, mitochondrial
	G3IFX1	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial
	G3IEY0	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial
	G3H6M5	Fumarate hydratase, mitochondrial
	G3HA23	Malate dehydrogenase (Fragment)
	G3HDQ2	Malate dehydrogenase
	G3HTR9	Malic enzyme (NAD)
Cholesterol biosynthesis	G3HMY0	3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-CoA synthase)
	G3HP76	3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-CoA synthase)
	G3GRT8	Diphosphomevalonate decarboxylase
	G3HC39	Farnesyl pyrophosphate synthetase
	G3H0L7	Squalene synthetase

Figure Legends

Figure 1 : Effect of BSO treatment on cell growth, productivity and GSH content. Gray and blue indicates the feed medium used – control feed medium or low cysteine feed medium, respectively. Circles indicates non-treated bioreactors and triangles indicates BSO-treated bioreactors. The red dotted lines represent the timing of BSO addition to a medium concentration of 0.5mM. (A) viable cell concentration (VCC) profile. (B) Product titer in the supernatant over time. (C) Intracellular GSH concentration overtime. (D) mAb Specific productivity over time.

Figure 2 : Effect of BSO treatment on glucose, lactate and alanine uptake/secretion rates. Gray and blue indicates the feed medium used – control feed medium or low cysteine feed medium, respectively. Circles indicates non-treated bioreactors and triangles indicates BSO-treated bioreactors. The red dotted lines represent the timing of BSO addition to a medium concentration of 0.5mM. (A) Glucose rate (B) Lactate rate (C) Alanine rate.

Figure 3 : Heatmap of differentially expressed proteins under BSO treatment in CHO cells. Main cellular function of proteins cluster has been added in the figure.

Figure 4 : Glutathione metabolism related proteins expression in BSO-treated cells. The logFC and the *adj. p. value*are represented by the color from green to red. Red indicates a logFC>0.5 and a *adj. p. value* <0.05 ; ligh red indicates logFC<0.5 and a *adj. p. value*<0.05; green indicates a logFC<-0.5 and a *adj. p. value* <0.05; light green indicates logFC>-0.5 and a *adj. p. value* <0.05 and grey *adj. p. value*>0.05. Statistical data have been generated using limma empirical Bayes moderated t-test. The BSO-treated condition (n=10) have been compared to control conditions (n=12).