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Research Article

Elevated pCO₂ affects the lactate metabolic shift in CHO cell culture processes

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The shift from lactate production to consumption in CHO cell metabolism is a key event during cell culture cultivations and is connected to increased culture longevity and final product titers. However, the mechanisms controlling this metabolic shift are not yet fully understood. Variations in lactate metabolism have been mainly reported to be induced by process pH and availability of substrates like glucose and glutamine. The aim of this study was to investigate the effects of elevated pCO₂ concentrations on the lactate metabolic shift phenomena in CHO cell culture processes. In this publication, we show that at elevated pCO₂ in batch and fed-batch cultures, the lactate metabolic shift was absent in comparison to control cultures at lower pCO₂ values. Furthermore, through metabolic flux analysis we found a link between the lactate metabolic shift and the ratio of NADH producing and regenerating intracellular pathways. This ratio was mainly affected by a reduced oxidative capacity of cultures at elevated pCO₂. The presented results are especially interesting for large-scale and perfusion processes where increased pCO₂ concentrations are likely to occur. Our results suggest, that so far unexplained metabolic changes may be connected to increased pCO₂ accumulation in larger scale fermentations. Finally, we propose several mechanisms through which increased pCO₂ might affect the cell metabolism and briefly discuss methods to enable the lactate metabolic shift during cell cultivations.

Keywords: CHO cell culture / Lactate metabolic shift / Metabolic Flux Analysis / Process parameter / Scale-up

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1 Introduction

Monoclonal antibody (mAb)-based products nowadays hold a strong share of overall biopharmaceutical product approvals, with Chinese hamster ovary cells (CHO) as their predominant expression system [1]. Media development, process optimization as well as the development of high and stable producer cell lines have led to optimized high-titer production processes [2].

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Abbreviations: akdh, alpha-ketoglutarate dehydrogenase; CHO, Chinese hamster ovary; g6pdh, glucose-6-phosphate dehydrogenase; GPI, glucose-6-phosphate isomerase; icdh, isocitrate dehydrogenase; mAb, monoclonal antibody; maldh, malate dehydrogenase; pdh, pyruvate dehydrogenase; pepck, phosphoenolpyruvate carboxykinase; pk, pyruvate kinase; R, redox variable; VCD, viable cell density

Despite their extensive usage in the biopharmaceutical industry various challenges exist, especially during process scale-up [3–5]. One prominent example is the accumulation of CO₂ during large-scale and perfusion processes, which can result in reduced cell growth and productivity [6–9]. A common feature of mammalian cell culture processes is the metabolic shift from lactate production to lactate consumption, which has been shown to be beneficial for culture viability and final product titer [10, 11]. However, lactate production often increases in large-scale and the metabolic shift to lactate consumption can be reduced or completely absent in comparison to small-scale processes [10, 12]. Several methods have been developed to reduce lactate accumulation during processes, including the usage of alternative carbon sources, modified cell lines with e.g. lactate dehydrogenase-downregulation and specialized process control strategies [13–17]. Nevertheless, most of these methods are difficult to implement for large-scale bioproduction processes [15]. Moreover, the lactate metabolic shift has

been shown to vary with media composition and cell lines [16, 18, 19].

Process pH has been shown to be capable of inducing the lactate metabolic shift [20] and the timing of pH shift is known to influence the time point of lactate consumption as well [21]. However, reliable control of the metabolic shift throughout different scales, cell lines and processes is not possible until today, due to missing understanding of the mechanisms leading to net lactate consumption [16, 22]. Process $p\text{CO}_2$ has been shown before to potentially influence specific lactate production [23], but has not been attributed so far to be a critical parameter with direct influence on the lactate metabolic shift phenomena.

In this study, we aimed at analyzing whether elevated $p\text{CO}_2/\text{HCO}_3^-$ concentrations during batch and fed-batch cultures can affect the lactate metabolic shift. Through time resolved metabolic flux analysis, it was further possible to correlate the onset of lactate consumption to an intracellular NAD^+/NADH ratio, supporting the hypothesis that this metabolic shift is connected to an intracellular redox equivalent balance. The presented results are of high importance for large-scale and perfusion processes, where increased $p\text{CO}_2/\text{HCO}_3^-$ concentrations are most likely to occur and subsequently might lead to a different process performance than expected from small-scale studies.

2 Materials and methods

2.1 Cell line, seed train, and fermentation processes

An industrial CHO cell line producing a monoclonal antibody (mAb) was cultivated in chemically defined media. Precultures for fermentation processes were cultivated in shake flasks and incubated at 10% (75 mmHg) $p\text{CO}_2$ and 36.5°C temperature. Exponentially growing cells were transferred into 3 L glass bioreactors resulting in an inoculation density of 3×10^5 cells/mL. A specific control strategy was used to individually control $p\text{O}_2$, $p\text{CO}_2$ and process pH as described earlier in Brunner et al. [8]. pH was measured by an in-line probe (EasyFerm, Hamilton, USA) and regulated via addition of HCL and NaOH respectively. $p\text{O}_2$ was controlled by an in-line probe (VisiFerm, Hamilton, USA). $p\text{CO}_2$ was measured and controlled by use of an off-gas sensor (BlueInOne, Bluesens, Germany). Batch cultivations were part of a previous Design of Experiments study [8] and processed at 37°C and different set-points of pH at 7.0 or 7.2, $p\text{CO}_2$ at 12.5 or 20% (94–150 mmHg) and $p\text{O}_2$ at 10 or 25%. Fed-batch cultivations were performed at 36.5°C and pH set-point was set to pH 7.0 (pH deadband 0.03), whereby temperature was shifted after 60 h to 33.0°C. Control cultivations were conducted at $p\text{CO}_2$ 12.5% (94 mmHg) and $p\text{O}_2$ 40%. Fermentation runs at elevated $p\text{CO}_2$ were cultivated at $p\text{CO}_2$ 20% (150 mmHg) and $p\text{O}_2$ 25 or 40%. Feed A was added continuously to processes starting on day 4 until day 10 and on day 12. Feed B was added continuously starting on day 6 until day 10 and on day 12. Glucose was added to processes as soon as its concentration dropped below 2 g/L with bolus addition to 2 g/L.

2.2 In-process analytics, mAb determination, and amino acid measurement

Cultivation samples were taken every 12 h and cell counting/viability determination was performed using the automatic picture analyzer Cedex HiRes Analyzer (Roche, Germany). Osmolality of supernatant was determined via freeze point depression (Mikro-Osmometer TypOM806, Löser, Germany). Analyses of the metabolites glucose, lactate and ammonium were performed using the Cedex Bio HT Analyzer (Roche, Germany). Antibody titer determination was carried out by HPLC (Ultimate 3000, Dionex, USA) with a Protein A sensor cartridge (Applied Biosystems, The Netherlands). Amino acid concentrations were determined by HPLC measurement (Ultimate 3000, Dionex, USA; ZORBAX Eclipse Plus C18 column, Agilent Technologies, USA).

2.3 Time resolved metabolic flux analysis

Metabolic flux analysis was performed as described before in detail by Zalai et al. [21]. Briefly, intracellular metabolic rates were calculated for every point in time of the cultivation using a previously published metabolic network of the central carbon metabolism of CHO cells [24, 25]. Biomass composition of CHO cells was taken from literature [24, 26]. Specific rates of uptake and production of metabolites as well as the specific oxygen uptake rate at sampling time points were used as model inputs and detection of gross measurement errors was performed via data reconciliation as described in literature [27, 28]. Flux constraints and the stoichiometric matrix of the model are presented in the Supporting Information.

2.4 Calculation of specific rates and standard deviations

Calculation of specific cell growth rates and metabolite production or consumption rates was performed for every sampling interval and similar to Sauer et al. [29]. Chemical degradation of glutamine to ammonia was considered [30]. The specific oxygen uptake rate was calculated as described in Ruffieux et al. [31]. Standard deviations of the calculated mean specific rates were determined by Monte-Carlo parameter estimation to generate multiple time-courses for each fermentation similar to Murphy and Young [32] using MATLAB® (The MathWorks, Inc., USA). Error in prime variables (viable cell density, metabolite and product concentrations) for Monte-Carlo simulation were derived from manufacturer specifications, repeated measurements and published studies that used the same analytical devices [33–35]. Errors of the intracellular fluxes were assumed to be similar to the closely related greater extracellular fluxes as shown by Goudar et al. [36].

3 Results

3.1 Batch cultivations: initial observations

In a previous study, batch fermentations were performed according to a Design of Experiments approach at different constant

Figure 1. Viable cell density (VCD) and metabolite concentrations over process time of the batch fermentation processes. Processes at elevated $p\text{CO}_2$ started to consume lactate only after glucose depletion, whereas processes at lower $p\text{CO}_2$ values consumed lactate early in the process after glutamine depletion. Error bars indicate range of analytical accuracy of measurements.

pH, $p\text{CO}_2$ and $p\text{O}_2$ set-points to investigate the effects of process parameter interactions on cell physiology and process performance [8]. Besides the reported effects, further investigation of the data set with focus on the lactate metabolic shift revealed that out of the 14 different set-point variations in pH, $p\text{CO}_2$ and $p\text{O}_2$, only two processes did not switch from lactate production to consumption after glutamine was depleted in the media. Remarkably, both processes were conducted at high $p\text{CO}_2$ levels (Fig. 1). The presented data-set consists of processes at pH 7.0 and pH 7.2 with variations in high (20%) and low $p\text{CO}_2$ (12.5%) as well as variable $p\text{O}_2$ set-points. Cell growth was reduced at increased $p\text{CO}_2$, resulting in a lower maximum VCD at these conditions (Fig. 1A). We found that processes at elevated $p\text{CO}_2$ did not follow a lactate metabolic shift until both glutamine and glucose depleted, whereas processes at lower $p\text{CO}_2$ already switched from lactate production to consumption right after glutamine depletion (Fig. 1B–D). However, at pH 7.2 and $p\text{CO}_2$ 12.5% the data set is not that clear since glutamine and glucose depletion occurred close to each other. To further investigate how elevated $p\text{CO}_2$ levels might affect the lactate metabolic shift, additional fed-batch cultivations were conducted and metabolic flux analysis was used to reveal intracellular flux distributions.

3.2 Fed-batch cultivations: verification of batch findings

Fed-batch control experiments were conducted at a constant pH of 7.0, $p\text{CO}_2$ 12.5% and $p\text{O}_2$ 40%. In parallel to each control run one fed-batch culture was performed at the same pH but at elevated $p\text{CO}_2$ levels of 20% and at a $p\text{O}_2$ set-point of 25 or 40%, respectively. The viable cell densities were comparable within the two control runs and within fermentation processes

at elevated $p\text{CO}_2$ concentrations (Fig. 2A). However, viable cell densities were reduced at high $p\text{CO}_2$ in comparison to the control cultures. Due to the applied feeding strategy, no depletion of glutamine nor glucose occurred during the processes (Fig. 2B and C). Cultures at high $p\text{CO}_2$ did produce lactate throughout the entire process, whereas both control cultures started to consume lactate around day 5 of the process (Fig. 2D). Early in culture, all conditions stayed at the low end of the pH control range (6.97). After day 5 the conditions with elevated $p\text{CO}_2$ remained at the low range whereas control cultures began to consume lactic acid (the metabolic shift, Fig. 2D) and moved to the upper end of the pH control range (7.03). The average specific growth as well as metabolite production/consumption rates and average specific oxygen consumption of all fed-batch cultures before and after lactate uptake are presented in Fig. 3, whereby the separation between those two phases is indicated by the dotted line in Fig. 2. All cell-specific rates out of Fig. 3 after lactate uptake were calculated until the maximum viable cell density (VCD) of the respective process. Maximum viable cell densities were reached in between day 7.5 to day 8.5 for all processes. The average rates of the controls and the respective cultures at elevated $p\text{CO}_2$ in Fig. 3 were tested for significant differences with a Student's *t*-test and results are presented in the Supporting Information. Average rates of glucose consumption, lactate production as well as specific growth, oxygen uptake rates and specific IgG productivity at elevated $p\text{CO}_2$ were all decreased in comparison to the control cultures before lactate uptake occurred (Fig. 3A). After the occurrence of the metabolic shift in the control cultures, specific glucose consumption was higher at elevated $p\text{CO}_2$ than in the respective controls, whereas specific cell growth and specific oxygen consumption became more similar (Fig. 3B). Specific productivity stayed at lower values for cultures at increased $p\text{CO}_2$ after the metabolic shift. Final

Figure 2. Viable cell density (VCD) and metabolite concentrations over process time of the fed-batch fermentation processes. Cultures at $p\text{CO}_2$ 20% did not switch from lactate production to consumption, in contrast to the control cultures at 12.5% $p\text{CO}_2$. No depletion of glucose or glutamine occurred during the processes. The time of the metabolic shift is indicated by the dashed line. Error bars indicate range of analytical accuracy of measurements.

Figure 3. Average specific rates of several metabolites (glucose consumption (q_{gluc}), lactate production (q_{lac}), glutamine consumption (q_{gln}), ammonia production (q_{amm}), specific productivity (q_p), cell growth (μ) and oxygen consumption (q_{O_2}) of the fed-batch cultures (A) before and (B) after the metabolic shift. The average rates of the controls and the respective cultures at elevated $p\text{CO}_2$ were tested for significant differences with a Student's t -test and results are presented in the Supporting Information. (A) q_{gluc} , q_{lac} as well as q_p , μ and q_{O_2} were reduced under elevated $p\text{CO}_2$ conditions in comparison to the control cultures. (B) After the metabolic shift q_{gluc} was lower in the control cultures than in processes at $p\text{CO}_2$ 20%. In contrast to fermentation processes at elevated $p\text{CO}_2$, control cultures switched from lactate and ammonia production to consumption of these metabolites.

product titers were strongly reduced at high $p\text{CO}_2$ conditions, 1.4 and 1.1 g/L compared to 2.2 and 2.0 g/L respectively, in consequence of the reduced integral viable cell density and specific productivity. During the control cultures a switch from ammonia production to consumption occurred, which was absent in the other fermentations. Moreover, although metabolic rates for glucose consumption and lactate consumption of the control cultures after the metabolic shift seemed to be similar, the specific rates of these metabolites for the cultivations at high $p\text{CO}_2$ and different $p\text{O}_2$ set-points showed several significant differences. Since glutamine has been shown before to be

connected to the lactate metabolic shift (Fig. 1) and ammonia profiles of the fed-batch cultures differed strongly (Fig. 4A), the specific glutamine consumption was investigated in more detail. Shortly after cells started to consume lactate in the control cultures, ammonia concentrations started to decline in contrast to the cultures at high $p\text{CO}_2$ (Fig. 4A). The specific ammonia production correlates well with the specific glutamine uptake rate of the cells (Fig. 4B), regardless of the individual fed-batch conditions. As soon as glutamine consumption rates dropped below approx. 0.12 [pmol/(cell*day)], indicated by the horizontal dashed line in Fig. 4C, ammonia concentrations decreased.

Figure 4. (A) Ammonia concentration over process time of the fed-batch processes. Shortly after the metabolic shift occurred in the control cultures, indicated by the dashed line, processes started to show a decline in ammonia concentrations. (B) The relation between specific ammonia production (q_{NH_4}) and specific glutamine consumption (q_{gln}). The data-set consists of data from all fed-batch processes from inoculation until maximum viable cell densities were reached. (C) Specific glutamine consumption (q_{gln}) over process time of all fed-batch processes. Only slight differences in the glutamine consumption rates between processes at pCO_2 20% and the control cultures could be observed. It seemed that q_{gln} decreased in the control cultures after the metabolic shift occurred, indicated by the vertical dashed line. As soon as glutamine consumption rates dropped below approx. 0.12 [pmol/(cell*day)], indicated by the horizontal dashed line, ammonia concentrations decreased, except from day 9 on in the control cultures when alanine is consumed. (D) Osmolality profiles of the fed-batch processes. Osmolality values were comparable between all processes until the timepoint of the lactate metabolic shift (dashed line). Error bars indicate range of analytical accuracy of measurements.

After the metabolic shift occurred, it seemed that cultures at high pCO_2 showed a slightly higher specific glutamine uptake rate than the control cultures (Fig. 4C), however no significant differences could be derived through comparison of the average rates under consideration of the calculated standard deviations (Fig. 3). Subsequently metabolic flux analysis was used to generate a more detailed understanding of the metabolic behavior of the cells under high and low pCO_2 conditions.

3.3 Metabolic flux analysis

Metabolic flux analysis was performed for the fed-batch experiments. A simplified metabolic network is presented in Fig. 5. The metabolic model consists of fluxes of the central carbon metabolism of glycolysis, pentose phosphate pathway and TCA cycle. Analogous to the extracellular rates in Fig. 3 average intracellular specific metabolite rates were calculated before the uptake of lactate occurred (Fig. 6A) and after the metabolic switch until the end of the growth phase of the respective process (Fig. 6B). The average rates of the controls and the respective cultures at elevated pCO_2 were tested for significant differences with a Student's *t*-test and results are presented in the Sup-

porting Information. In agreement with the results presented in Fig. 3A reduced glycolytic influx via glucose-6-phosphate isomerase (GPI) and finally pyruvate kinase (pk) could be observed at increased pCO_2 levels in comparison to the control cultures before the lactate metabolic shift (Fig. 6A). Moreover, TCA cycle fluxes (isocitrate dehydrogenase (icdh), alpha-ketoglutarate dehydrogenase (akdh), malate dehydrogenase (maldh)) were significantly reduced at high pCO_2 . Interestingly several fluxes with CO_2 as a reaction product (glucose-6-phosphate dehydrogenase (g6pdh), pyruvate dehydrogenase (pdh), icdh, akdh, maldh, phosphoenolpyruvate carboxykinase (pepck)), indicated by the highlighted arrows in Fig. 5, were lower at elevated pCO_2 conditions, resulting in reduced specific carbon dioxide production rates (Fig. 6A). After the metabolic shift, average glycolytic fluxes were higher at processes with elevated pCO_2 set-points, whereas all other intracellular fluxes out of Fig. 6A became similar to the control reactors (Fig. 6B). Besides the general reduction of the cells metabolism (glycolytic fluxes, TCA cycle fluxes and respiration) at increased pCO_2 before the metabolic shift, we wanted to further investigate if the activity of certain metabolic pathways changed in relation to others (Table 1). The activity of the TCA cycle was defined as the cisaconitase flux similar to Wahrheit et al. [37] and the glycolytic activity was defined by the pyruvate

Figure 5. Simplified version of the metabolic network used for the metabolic flux analysis. Main intracellular fluxes that produce pCO_2 (G6P to R5P, OAA to PEP, Pyr to AcoA, Cit/Icit to aKG and aKG to Suc) are highlighted in red, derived from Goudar et al. [55].

kinase flux. Respirational activity was represented by the specific oxygen uptake rate. It became evident that not only the overall glycolytic flux decreased under elevated pCO_2 , but furthermore the ratio of lactate per pyruvate kinase flux, leading to less lactate production per consumed glucose. Moreover, the ratio of flux entering the TCA cycle per glycolytic flux, represented by the yield of pdh versus pyruvate kinase flux, was increased at high pCO_2 . However, respirational activity in comparison to the TCA cycle activity was around 10% higher in the control reactors. Since differences in intracellular flux ratios can strongly affect intracellular metabolic equilibria and the redox variable R has been shown before to be connected to lactate production [21], intracellular $NADH$ and NAD^+ production/regeneration were investigated in more detail. The redox variable R is defined as the ratio of the cytosolic $NADH$ produced during the glycolysis via glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the mitochondrial capacity for NAD^+ regeneration via the respiratory chain (Fig. 7). Due to a limited capacity of the cell to regenerate NAD^+ via the respiratory chain, possibly caused by $NADH$ transfer limitations through the malate-aspartate shuttle [38], surplus cytosolic NAD^+ can be regenerated via lactate formation. R values above 1 imply that the cells keep producing lactate to regenerate cytosolic NAD^+ , whereas an R value below 1 would lead to the complete regeneration of NAD^+ via the respiratory chain and even subsequent consumption of lactate leading to additional production of cytosolic $NADH$.

The cytosolic $NADH$ producing flux via GAPDH, the mitochondrial NAD^+ regeneration and the redox variable R over process time are presented for all fed-batch fermentations in Fig. 8. The cytosolic $NADH$ producing flux before the lactate metabolic shift is higher in the control cultures, compared to the fermentation runs at increased pCO_2 which is in accordance with the previously investigated specific glucose uptake rates (Figs. 8A and 3). After the metabolic shift, the control cultures showed a reduced cytosolic $NADH$ producing flux due to

Figure 6. Average specific rates of several intracellular fluxes of the glycolysis (GPI, pk , pdh), pentose phosphate pathway ($g6pdh$) and TCA cycle ($icdh$, $akdh$, $maldh$, $pepck$) as well as specific carbon dioxide production (q_{CO_2}) of the fed-batch cultures before (A) and after the metabolic shift (B). The average rates of the controls and the respective cultures at elevated pCO_2 were tested for significant differences with a Student's t -test and results are presented in the Supporting Information. (A) Glycolytic and TCA cycle fluxes were reduced under elevated pCO_2 conditions in comparison to the control cultures, resulting in lower carbon dioxide production rates. (B) After the metabolic shift glycolytic influx via GPI was lower in the control cultures than in processes at pCO_2 20%. TCA cycle fluxes and q_{CO_2} became similar within all fed-batch processes after the metabolic shift.

Table 1. Average intracellular yields before the metabolic shift

	(i) Yield lactate/pyruvate kinase flux [%]	(ii) Yield pdh/pyruvate kinase flux [%]	(iii) TCA activity/glycolytic activity [%]	(iv) Respirational activity/TCA activity [%]
Control run 1	62	34	40	338
pCO ₂ 20/pO ₂ 25 run 1	52	37	46	298
Control run 2	57	35	49	329
pCO ₂ 20/pO ₂ 40 run 2	51	39	53	298

Control cultures produced more lactate per consumed glucose (i) and showed reduced flux from glycolysis into the TCA cycle via pyruvate dehydrogenase (ii) (pdh). The ratio of TCA activity to glycolytic activity was higher at elevated pCO₂ (iii). Furthermore, the ratio of respiratory activity to TCA cycle flux was increased at the conditions of the control processes (iv).

Figure 7. The redox variable R depends on the ratio of cytosolic NADH via glycolysis and the mitochondrial NAD⁺ via respiration and TCA cycle. The NADH/NAD⁺ ratio is tightly balanced, therefore R -values above 1 lead to an additional production of lactate and concurrent cytosolic NADH consumption. In contrast an R value below 1 would support cytosolic NADH production via lactate consumption.

the lower specific glucose uptake rate in comparison to the processes at elevated pCO₂. The average mitochondrial NAD⁺ regeneration was higher in the control cultures than in the processes at increased pCO₂ before the metabolic shift, however values became more similar after the switch from lactate production to consumption (Fig. 8B). The resulting redox variable out of the cytosolic NADH producing flux and the mitochondrial NAD⁺ regeneration revealed that cells in the control cultures started to consume lactate as soon as the R value became lower than 1 (Fig. 8C). In contrast cultures at elevated pCO₂ stayed above the critical R value of 1 and did not switch from lactate production to consumption.

4 Discussion

4.1 Batch cultivations

The results of the conducted batch fermentation experiments at different pCO₂ set-points demonstrated that processes at elevated pCO₂ conditions of 20% did not switch from lactate production to consumption after glutamine depletion as long as

glucose was available in the media. Zagari et al. [16] reported similar effects when cultivating two different clones in a batch process. One clone switched from lactate production to consumption after glutamine depletion, whereas the other clone did continue to produce lactate until glucose was depleted as well. Interestingly, the general metabolic behavior regarding the lactate metabolic shift did not change by increasing glucose or glutamine concentrations in their study. In contrast to Zagari et al. [16] however the divergences in metabolic behavior in our experiments were observed with the same clone and only by variations of the pO₂ and pCO₂ concentrations. Due to these observations, additional fed-batch experiments were designed to further investigate the effect of elevated pCO₂ on the lactate metabolic shift phenomena.

4.2 Fed-batch cultivations

During the conducted fed-batch processes, control cultures at 12.5% pCO₂ did switch from lactate production to consumption, whereas cultures at elevated pCO₂ of 20% did produce lactate throughout the entire fed-batch process. Similar to Martinez et al. [39] and Mulukutla et al. [11] data evaluation was split into process phases before and after the lactate metabolic shift. The process phase before the lactate metabolic shift displayed reduced glucose uptake, lactate production and specific oxygen consumption at elevated pCO₂ concentrations. Differences in the cellular metabolism occurring before or with the lactate metabolic shift have been hypothesized before to be the major causes for the observed lactate divergences with the observations occurring after the shift being the cellular response [18]. Furthermore, the reduced specific oxygen consumption rate at elevated pCO₂ before the metabolic shift indicates a minor oxidative capacity of the cells under these conditions, which has been linked before to be connected with continuous lactate production [16, 18]. Average rates of specific oxygen consumption and specific cell growth became similar after the shift, whereas control cultures showed a lower glucose consumption and consumed lactate and ammonia. Differences in the second process phase might be for some part a consequence of the variation in lactate metabolism, but slightly different pH profiles and strongly different osmolality values in the second phase (Fig. 4D) have to be considered as well. As can be seen in Fig. 4D the osmolality values of the control cultures and cultures at elevated pCO₂ started to diverge after the metabolic shift and increased osmolality is known to potentially affect cell metabolism. Since

Figure 8. Cytosolic NADH production, mitochondrial NAD⁺ regeneration and the redox variable R over process time of the fed-batch cultivations. The time of metabolic shift is indicated by the dashed line. Presented fluxes are interpolated between the data points via B-splines. (A) The NADH producing flux via glycolysis is higher at the control cultures in comparison to the processes at elevated pCO₂ before the metabolic shift. After the shift fluxes increased at high pCO₂ when compared to the control runs. (B) Mitochondrial NAD⁺ regeneration was higher in the processes at control conditions, however fluxes seemed to become more similar after the metabolic shift. (C) The value of the redox variable R stayed above the critical value of 1, indicated by the horizontal dashed line, for processes at elevated pCO₂ values. Fed-batch cultures at control conditions dropped below an R-value of 1 as soon as the metabolic shift occurred.

glutamine metabolism has been shown before to correlate with the lactate metabolic shift in the batch cultivations and other studies [16, 40] and furthermore ammonia metabolite profiles were strongly different between cultures at low and high pCO₂, the role of glutamine was investigated in more detail. Differences in the ammonia profiles could be directly linked to variations in the specific glutamine consumption rate. The specific glutamine uptake rates (q_{gln}) between the conditions at the time of lactate uptake however were not strongly different and q_{gln} continued to decrease throughout processes at high pCO₂ without lactate consumption. Furthermore, complete glutamine depletion did not induce the lactate metabolic shift during the batch cultures at elevated pCO₂. Therefore, we concluded that the observed differences in the lactate profiles between the fed-batch processes at increased pCO₂ and the control processes did not only derive from variations in the glutamine metabolism.

4.3 Metabolic flux analysis

Via time resolved stoichiometric metabolic flux analysis it was possible to investigate intracellular flux distributions inside of the cells throughout the entire fed-batch processes. Average glycolytic fluxes as well as TCA cycle fluxes were reduced under elevated pCO₂ conditions before the metabolic shift and subse-

quently led to a lower specific carbon dioxide production under these conditions. A generally lower TCA cycle activity in cultures that do not switch from lactate production to consumption has been observed before by Luo et al. [18], using a metabolomics approach. Furthermore, the yield of lactate per pyruvate kinase flux was increased and the flux entering the TCA cycle via pyruvate dehydrogenase in relation to the pyruvate kinase flux, decreased under control conditions before the metabolic shift. This led further to an increased ratio of TCA cycle activity to glycolytic activity in both cultures at high pCO₂. Lower fluxes from OAA to PEP via pepck at elevated pCO₂ seemed to contribute to this metabolic divergence. However, reduced yields of lactate from glycolysis most probably derive from a reduced specific glucose consumption as described previously by Konakovskiy et al. [14] and Zalai et al. [21]. Moreover, respirational activity in respect to the TCA cycle flux seemed to be higher in the control cultures, again underlining a correlation of high oxidative capacity and the capability to consume lactate [16, 18]. The equilibrium between cytosolic and mitochondrial reduction equivalents, NADH and NAD⁺, has been hypothesized before to be connected to the lactate production/consumption state [13, 16, 21, 41]. Furthermore, a generally reduced intracellular NADH/NAD⁺ ratio over process time in a process including a lactate metabolic shift was measured in Templeton et al. [42]. NADH and NAD⁺ producing/regenerating fluxes were higher in the control cultures

before the metabolic shift occurred, whereas NAD^+ regeneration became more similar after the shift and NADH producing fluxes became even higher in the cultures at elevated pCO_2 . These findings were in agreement with the observed differences in glycolytic and TCA cycle fluxes. However, the ratio of cytosolic NADH and mitochondrial NAD^+ , expressed by the redox variable R, stayed at lower levels in the respective control cultures throughout most of the process. Furthermore, both control reactors dropped below the critical R-value of 1 at the same time they started to consume lactate, whereas cultures with constant lactate production stayed above R-values of 1. Considering the relationship of R and the glycolytic, TCA cycle and respirational fluxes, it could be derived that the increased R-values at elevated pCO_2 originated mainly from a strongly reduced respirational activity and subsequent decrease of the produced mitochondrial NAD^+ via respiration. Additionally, an increased ratio of TCA cycle activity/glycolytic activity contributed further to a reduced mitochondrial NAD^+ concentration.

4.4 Possible mechanistic explanations for the observed effects of pCO_2 on cell metabolism

Deriving a mechanistic explanation of the observed effects is not easy, since in contrast to microbial cultures, in mammalian cell culture only a few studies exist concerning pCO_2 effects. Moreover, CO_2 in cell culture media readily reacts with water to form HCO_3^- (bicarbonate) which is further dependent from process pH [43, 44]. Furthermore, batch fermentations out of a previous study [8], at pH 6.8 and elevated pCO_2 values (20%) did switch from lactate production to consumption, pointing to the fact that not elevated pCO_2 alone but further increased HCO_3^- at high pH and high pCO_2 might be the dominant factor. Additionally, several pH and pCO_2 interaction effects on CHO physiology have been reported before [8, 43]. $\text{CO}_2/\text{HCO}_3^-$ may act as a substrate and product for enzymes and interacts with intracellular pH in mammalian cells, furthermore effects on membrane permeability in microbial cultures are reported [44, 45]. Regulation of enzymes via $\text{CO}_2/\text{HCO}_3^-$ has been described for yeast by Jones and Greenfield [46]. Their review presents inhibitions of glucose-6-phosphate dehydrogenase (g6pdh), malate dehydrogenase (maladh) and succinate dehydrogenase at elevated HCO_3^- level and they further state that an extreme sensitivity of phosphoenolpyruvate carboxykinase (pepck) to elevated pCO_2 is expected. pCO_2 related product inhibition of enzymes might also occur in mammals. HCO_3^- is one of the key molecules involved in intracellular pH regulation in mammals [47]. Several studies have shown that mammalian cells have efficient mechanisms to regulate their intracellular pH [48, 49], however extreme conditions of elevated HCO_3^- concentrations might affect these regulation mechanisms. Intracellular pH is known to potentially affect phosphofructokinase, a key enzyme of the glycolysis [50]. At last, high concentrations of $\text{pCO}_2/\text{HCO}_3^-$ could react increasingly with hydrogenperoxides to form peroxymonocarbonate ion (HCO_4^-) which is an extremely reactive oxygen species [51, 52]. Since mitochondria are extremely sensitive to oxidative damage [18], the capacity for oxidative phosphorylation could be impaired with increasing HCO_4^- species.

4.5 Methods to induce/facilitate the lactate metabolic shift in mammalian cell culture based on the redox variable R

According to the previously shown results, two general approaches can be stated:

- (i) Strong *reduction of the glycolytic NADH producing flux* will reduce R and consequently might lead to lactate consumption. Low glucose feeding as applied from Konakovsky et al. [14] can induce lactate consumption as shown in their study. Furthermore, pH-shifts to lower values can strongly reduce glucose consumption rates [8, 53] and have been shown before to induce lactate consumption [54].
- (ii) *Increasing the mitochondrial NAD^+ regeneration* would be the second approach to reduce R and initiate lactate consumption. At first the selection of a cell line with a generally high oxidative capacity seems to be beneficial [16, 18]. Furthermore, the respiratory capacity could be improved by supplying the phosphorylation enzymes with needed cofactors as proposed by Luo et al. [18]. In this context copper concentrations have been shown before to correlate with the lactate consumption state [19]. Reduction of the TCA cycle flux without the impairment of glycolytic activity would be another possibility to increase mitochondrial NAD^+ . Glutamine is directly fed into the TCA cycle and significantly contributes to cell metabolism. Reduction of its specific uptake rate could induce lactate consumption and glutamine depletion has been shown to have the potential to induce the lactate metabolic shift in this and other studies [16].

Based on our results it seems further beneficial to reduce $\text{pCO}_2/\text{HCO}_3^-$ concentrations to assure lactate consumption. This is especially interesting for large-scale and perfusion processes where high pCO_2 concentrations are most likely to occur. Usage of optimized buffering agents and base, e.g. Na_2CO_3 instead of NaHCO_3 , can significantly reduce HCO_3^- concentrations [9]. Furthermore, optimized gassing strategies to reduce pCO_2 accumulation should be used [4, 6]. Finally, we have to confine our findings to the used CHO cell line since effects on lactate metabolism may vary with different cell lines as observed before in other studies. However, this publication is to our knowledge the first one to show that elevated $\text{pCO}_2/\text{HCO}_3^-$ concentrations can potentially affect the lactate metabolic shift mechanism and therefore is intended to draw other researchers attention to consider these effects in their experiments.

Practical application

Robust cell culture scale-up is a critical requirement toward large-scale fed-batch manufacturing processes. Inexplicable metabolic deviations between small-scale and large-scale cultures can therefore be crucial for the final success of a product. Large-scale fed-batch processes as well as perfusion processes, which are gaining more attention along the paradigm shift toward continuous bioprocessing, usually exhibit higher dissolved carbon dioxide concentrations than small-scale fed-batch cultures. This article

demonstrates that elevated dissolved carbon dioxide concentrations in batch and fed-batch cultures can lead to the complete absence of a key metabolic shift in CHO cells from lactate production to consumption. This metabolic shift could be further linked to an intracellular ratio of reducing equivalents using metabolic flux analysis. The presented results give new insights into the metabolic behavior of cells under scale-up critical conditions. Finally, potential methods to induce the metabolic shift and therefore potentially increasing scale-up robustness are discussed.

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