Engineering cell metabolism for high-density cell culture via manipulation of sugar transport

Katie F. Wlaschin, Wei-Shou Hu *

University of Minnesota Department of Chemical Engineering and Materials Science, 421 Washington Avenue SE, Minneapolis, MN 55455-0132, USA

Received 31 January 2007; received in revised form 1 June 2007; accepted 14 June 2007

Abstract

Transporters mediate the influx of nutrients and excretion of metabolites in mammalian cells, playing a key role in the regulation of metabolism. They are natural targets for cell engineering to alter metabolic characteristics. The GLUT5 fructose transporter was stably expressed in a Chinese hamster ovary cell line, allowing clones to utilize fructose in place of glucose in culture medium. Compared to the ubiquitously expressed GLUT1 glucose transporter, the GLUT5 fructose transporter has a high $K_m$ value for its substrate. Fructose uptake by the GLUT5 transporter should supply sugar to cells at a more moderate rate, even in high fructose concentrations, avoiding the overflow of excess carbon to lactate. When cultured in fructose, selected GLUT5 expressing clones exhibited drastically reduced sugar consumption and lactate production rates. When those same clones were cultured in glucose, high sugar consumption and lactate production rates were observed. GLUT5 transcript expression levels and specific lactate production rates varied among the clones. Clones having a low expression level of the GLUT5 transporter were able to import fructose at more moderate rates in higher sugar concentrations. The reduced lactate production for these clones allowed a significant increase in the final cell concentration in fructose fed-batch processes.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Fed-batch culture; Fructose; Sugar transport; GLUT5; Metabolic engineering; Mammalian cell culture

1. Introduction

In the past decade, many new recombinant antibody therapeutics have reached clinical application. Unlike earlier mammalian cell culture products, such as tissue plasminogen activator (tPA) and erythropoietin (EPO), most antibody therapeutics require higher doses. This increase in demand for production capacity is driving the need to enhance productivity (Seth et al., 2005). The majority of recombinant antibody production processes employ fed-batch culture, which yields increased cell and product concentrations, while maintaining the simplicity and flexibility of batch processes (Wlaschin and Hu, 2006).

The increase in cell and product concentration in fed-batch culture is typically accompanied by the accumulation of inhibitory metabolites, specifically lactate and ammonium. The presence of these metabolites in such high concentrations can limit cell concentrations and product titers (Frame and Hu, 1991; Hu et al., 1987; Hu and Piret, 1992; Ozturk et al., 1992).

The accumulation of lactate results from the consumption of glucose and other nutrients in excess of what is required for cell growth (Reitzer et al., 1979; Zielke et al., 1978). More than two decades ago, process control using online oxygen measurement was implemented to maintain glucose at a very low concentration and resulted in reduced lactate formation by cells cultivated on microcarriers (Fleischaker and Sinskey, 1981). A similar approach was applied for fed-batch culture of hybridoma cells in suspension. The reduced lactate production allowed increased cell concentrations, above that observed in a typical fed-batch culture (Zhou et al., 1995). A highly sophisticated process control scheme, coupling oxygen consumption to nutrient feeding, was required to maintain glucose concentration at a very low level to elicit the metabolic shift. For large scale bioreactors, implementing a similar strategy is technically challenging. Small process perturbations present significant risk for nutrient depletion or reverting metabolism to a high lactate producing state.
This work explores the use of metabolic engineering of transporters to reduce nutrient uptake and metabolite formation in mammalian cells. The GLUT transporters mediate the import of carbohydrates into cells. The 13 members of the GLUT family transporters are highly tissue specific in their expression (Joost and Thorens, 2001). The collection of transporters expressed by a cell dictates its ability to uptake sugars like mannose, fructose, and galactose. The transport rate of various sugars via the GLUT transporters is described using simple Michaelis–Menten enzyme kinetics. The high-affinity glucose transporter, GLUT1, has a very low $K_m$ for glucose. Unlike the other members of the GLUT family, it is expressed in all nearly all cell types (Wood and Trayhurn, 2003) and is likely responsible for the rapid glucose uptake rate observed in cultured mammalian cells.

A logical approach to reduce carbohydrate consumption is to decrease expression of the GLUT1 transporter using siRNA. Stable expression of anti-GLUT1 siRNA in hybridoma cells yielded a single clone that exhibited reduced glucose consumption. Unfortunately, instability of the phenotype was reported (Paredes et al., 1999). Early cell culture work showed that many cultured cell lines can survive and sometimes grow in medium with glucose completely replaced by alternative sugars, such as galactose, fructose, sucrose, ribose, and mannose (Eagle et al., 1958; Low and Harbour, 1985; Petch and Butler, 1996). In one case, cell growth rates in galactose and fructose were similar to growth rates in glucose, even though these sugars were consumed at slower rates and lactate production was markedly reduced (Reitzer et al., 1979). These results have influenced work to reduce lactate formation by cultivating cells in galactose (Altamirano et al., 2000, 2001, 2004) and fructose (Barngrover et al., 1985; Low and Harbour, 1985; Mochizuki et al., 1993).

In this study, the combined approach of transporter engineering and use of alternative sugars was taken to reduce sugar consumption and metabolite production in CHO cells. Stable expression of the GLUT5 fructose-specific transporter conferred cells with the ability to grow using fructose as the sole source of carbohydrate. Since the GLUT5 transporter has a much higher $K_m$ value for fructose than the GLUT1 transporter has for glucose, sugar consumption was reduced even when cells were cultured in moderate concentrations of fructose. Such metabolic engineering of transporters may provide an effective means to control nutrient uptake, constrict metabolite formation, and increase cell and product concentrations in mammalian cell bioprocesses.

2. Materials and methods

2.1. Cells and maintenance

The Chinese hamster ovary (CHO) cells have been described previously (Wlaschin et al., 2005). The parental CHO cells and their transfected clones were maintained in T-flasks and spinner flasks in DMEM/F12 (1:1) media with the following components added or adjusted: glucose (17.5 mM) or fructose (16.67 mM), glutamine (4–6 mM), sodium bicarbonate (29 mM), ascorbic acid (0.11 mM), putrecine (6.2 µM), penicillin G (0.17 mM), streptomycin (68.6 µM), pluronic F68 (0.01%), phenol red (19.9 µM), apotransferrin (63.7 nM), and Intralipid (0.01%) (Sigma–Aldrich, St. Louis, MO).

2.2. Fed-batch cultures

The 750 mL glass bioreactor culture system with working volumes of 450–550 mL have been described previously (Zhou and Hu, 1994). Dissolved oxygen was maintained at 30% of air saturation, and pH was maintained at 7.2 by controlling the fraction of oxygen, nitrogen, and CO2 in the inlet air. The oxygen uptake rate (OUR) was estimated online by assuming a constant value for $K_{L,a}$ and calculating the amount of oxygen consumed from the gas phase composition. When required, 1N NaOH was added to maintain pH.

Exponentially growing cells from spinner flask seed cultures were inoculated into the bioreactor at a concentration of 4.0–4.5 × 10^5 cells/mL. Feed medium was added proportionally with the online OUR estimates according to stoichiometric ratios determined in previous cultures. The feed media composition was 15-fold (15×) concentrated DMEM/F12 (1:1) excluding glucose, glutamine, and bulk salts (NaHCO3, NaCl, CaCl2, KCl). Fructose or glucose, and glutamine were added to the feed media according to stoichiometric nutrient consumption data from previous cultures. The fructose feed medium contained 14.5 g/L fructose and 60 mM glutamine. The glucose feed medium included 45 g/L glucose and 60 mM glutamine. Samples were taken at 8–12 h time intervals for the duration of the cultures.

For each sample, cell concentration and viability were determined by counting with a hemacytometer using trypan blue staining. Lactate concentrations were measured using the YSI Model 27 industrial analyzer (Yellow Springs Instruments, Yellow Springs, OH). Glucose and fructose concentrations were determined in duplicate using Infinity Glucose Hexokinase Reagent (Thermo Electron Corporation, Waltham, MA). For glucose, concentrations were determined according to the manufacturer’s protocol. For fructose, phosphoglucose isomerase (PGI) (Roche Applied Sciences, Basel, Switzerland) was added to 35 U/mL, and the incubation time was extended to 20 min. Absorbance was read at 340 nm using a SpectraMax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA).

2.3. Vector construction, transfection, and clone isolation

The full-length intestinal/kidney isoform of the mouse GLUT5 (Scl2a5) sugar transporter (Catalog # MM1013) was obtained in the pCMV-Sport6 vector (Open Biosystems, Huntsville, AL). The GLUT5 gene was moved into the pcDNA3.1(+) expression vector (Invitrogen, Carlsbad, CA) using common NotI and EcoRI restriction sites. Forty micrograms of plasmid (pcDNA3.1(+) or pcDNA3.1-GLUT5) was linearized by digestion with the Scal restriction enzyme, and purified using QIAQuick PCR purification spin columns (Qiaagen, Valencia, CA). Ten million exponentially growing cells were washed twice in 10 mL of cold Opti-MEM medium (Invitrogen), and electroporated with 20 µg of linearized DNA in 1 mL of cold Opti-MEM in 4 mm electroporation cuvettes (Bio-
Rad, Hurcules, CA). Electroporation was performed in the BioRad Gene Pulser XcelII (BioRad). The electroporation condition used for CHO cells was a single pulse, voltage = 160 V, pulse length = 15 ms, resistance = $\infty$. Transfected cells were transferred into pre-warmed DMEM/F12 (1:1) medium supplemented with 10% FBS (Atlas Biological, Ft. Collins, CO). Efficiencies were determined using FACS by parallel transfection with an EGFP-containing plasmid.

The pools of cells transfected with either pcDNA3.1(+), pcDNA3.1-GLUT5, or water were diluted in 96-well plates at 2000 cells per well, in 0.2 mL of maintenance medium supplemented with 400 $\mu$g/mL G418. Plates were incubated for 10–12 days in a 37 °C, 5% CO2 environment. Clones were expanded for characterization in their selective medium.

2.4. Quantitative real-time PCR

RNA was isolated using RNEasy columns according to the manufacturer’s protocol, with on-column DNase digestion (Qiagen). cDNA synthesis was performed from 2 $\mu$g of total RNA using Superscript III Reverse transcriptase (Invitrogen). Primers for the mouse GLUT5 and the Chinese hamster Cdc42 gene were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3) with a specified product size of 200–250 bp and melting temperature of 60 °C. Real-time PCR was performed using the Stratagene Mx3000P (Stratagene) with SYBR Green I dye chemistry using the Full Velocity SYBR green QPCR kit (Stratagene, La Jolla, CA). PCR conditions were: 94 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 30 s. Dissociation curves were determined after PCR by complete dissociation at 95 °C, 1 min, followed and 30 s annealing at 55 °C and a rapid temperature ramp to 95 °C. The $C_T$ (threshold cycle number) values were determined at 0.2 of the reference dye normalized baseline value. Triplicate cDNA samples, a non-RT-reaction controls, and a non-cDNA template control were run for each sample/primer pair. All PCR products were run on 0.1% agarose gels to confirm expected product sizes.

3. Results

3.1. Growth characteristics of CHO cells in glucose and fructose

Fig. 1 shows the kinetics of growth, sugar consumption, and lactate production of the CHO cell line cultured in fructose as the sole carbohydrate source in the medium. For comparison, data for the same cell line cultured in 3.15 g/L glucose are also shown. A range of fructose concentrations of 2 g/L, 6 g/L, and 10 g/L were used. Cell growth and lactate production showed a concentration dependent response to the amount of fructose in the medium. At 2 g/L fructose, cells maintained a high viability (>90%) for 3 days, but did not grow appreciably. In the 6 g/L and 10 g/L concentrations of fructose, cell growth was observed, but at a markedly slower rate and to a lower maximum cell concentration than in the 3.15 g/L glucose culture. Additionally, lactate production was significantly less in all three fructose cultures.
than in the glucose culture. Similar to cell growth, the total lactate production increased with higher fructose concentrations.

Fructose or glucose consumption was also measured and are shown for the 3.15 g/L glucose culture, and the 2 g/L and 6 g/L fructose cultures. Only a small amount of fructose consumption was detected in the fructose cultures compared with the amount of glucose consumed in the control culture. For the 6 g/L and 10 g/L (data not shown) fructose cultures, the change in fructose concentration relative to the initial concentration was very small, making quantification of fructose consumption subject to significant error. Even though fructose consumption could not be accurately quantified, the production of lactate and the observation of cell growth suggest that fructose was consumed in these cultures. Furthermore, cells died quickly in the complete absence of a carbohydrate source (data not shown).

### 3.2. Growth characteristics of GLUT5 transfected clones

The CHO cell line was transfected with the pcDNA3.1-GLUT5 plasmid, for constitutive expression of the mouse intestinal isoform of the GLUT5 fructose transporter and the neomycin resistance gene. Two control transfections were also performed in parallel: transfection with the pcDNA3.1 plasmid, containing only the neomycin resistance gene, and electroporation in the absence of DNA. Stably transfected clones were isolated by limiting dilution in 96-well plates in 3.15 g/L glucose-containing maintenance medium, supplemented with 400 mg/L G418 for selection. After 2 weeks, the pcDNA3.1-GLUT5 transfection yielded 129 clones (6.7% of 1920 wells). The pcDNA3.1 control transfection yielded 183 clones (21.1% of 864 wells). No clones were found in 672 wells screened for the no-DNA control electroporated cells. The pcDNA3.1-GLUT5 transfected clones (1–11) were expanded and transferred into glucose-free medium, supplemented with 3 g/L fructose. The pcDNA3.1 transfected clones grew poorly in 3 g/L fructose medium, and were maintained in 3 g/L glucose medium.

Quantitative real-time PCR (Q-RT-PCR) was used to quantify the expression level of the GLUT5 transcript in these clones. Table 1 shows the ratio of expression of the GLUT5 transcript in each clone to its expression level in clone 1, which had the lowest level of GLUT5 expression among the clones. GLUT5 expression was detected in all pcDNA3.1-GLUT5 transfected clones (1–11, FSA and FSB). After 35 cycles, no product was amplified from the untransfected cells or the pcDNA3.1 transfected clone. The GLUT5 primers were designed to amplify both the CHO plasmid and the CHO genomic DNA. The band amplified from the CHO genomic DNA was shifted up by approximately 100 bp. This corresponds to the presence of a known 97 bp intron within this segment of the GLUT5 gene in the mouse genome. The presence of this intron is likely conserved in the CHO genome. The GLUT5 primers were indeed capable of amplifying both the CHO and mouse form of the GLUT5 transcript. The Q-RT-PCR results demonstrate that the native CHO GLUT5 transporter was not expressed at a significant level in untransfected CHO cells or pcDNA3.1 transfected CHO clones. In contrast, a sig-

<table>
<thead>
<tr>
<th>Clone</th>
<th>Expression level of GLUT5 transcript (fold expression, clone #/clone 1)</th>
<th>Doubling time (h)</th>
<th>Lactate to sugar molar ratio (mol/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fructose</td>
<td>Glucose</td>
</tr>
<tr>
<td>G418 selected clones (1–11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.00</td>
<td>19.0</td>
<td>19.5</td>
</tr>
<tr>
<td>2</td>
<td>10.60</td>
<td>22.9</td>
<td>26.1</td>
</tr>
<tr>
<td>3</td>
<td>11.47</td>
<td>22.0</td>
<td>24.3</td>
</tr>
<tr>
<td>4</td>
<td>10.15</td>
<td>27.4</td>
<td>26.4</td>
</tr>
<tr>
<td>5</td>
<td>5.02</td>
<td>23.1</td>
<td>21.4</td>
</tr>
<tr>
<td>6</td>
<td>5.95</td>
<td>24.8</td>
<td>26.8</td>
</tr>
<tr>
<td>7</td>
<td>4.60</td>
<td>24.3</td>
<td>24.6</td>
</tr>
<tr>
<td>8</td>
<td>4.30</td>
<td>24.7</td>
<td>23.9</td>
</tr>
<tr>
<td>9</td>
<td>10.43</td>
<td>20.5</td>
<td>20.9</td>
</tr>
<tr>
<td>10</td>
<td>9.71</td>
<td>23.8</td>
<td>23.6</td>
</tr>
<tr>
<td>11</td>
<td>20.40</td>
<td>18.3</td>
<td>17.0</td>
</tr>
<tr>
<td>Control cell lines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pcDNA3.1</td>
<td>–</td>
<td>30.00</td>
<td>20.00</td>
</tr>
<tr>
<td>CHO cells</td>
<td>–</td>
<td>–</td>
<td>17.60</td>
</tr>
<tr>
<td>0.3 g/L fructose selected clones</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSA</td>
<td>24.20</td>
<td>19.9</td>
<td>21.2</td>
</tr>
<tr>
<td>FSB</td>
<td>3.80</td>
<td>23.9</td>
<td>27</td>
</tr>
</tbody>
</table>

Thirteen pcDNA3.1-GLUT5 transfectants, a pcDNA3.1 transfectant, and the untransfected cells were cultured in either 3 g/L fructose or 3 g/L glucose. The kinetics of cell growth, sugar consumption and lactate production were monitored. Cell growth, sugar consumption and lactate production are shown along with the ratio of the moles of lactate produced to the moles of sugar consumed. The relative expression level of the GLUT5 transcript was also assayed by Q-RT-PCR.
significant level of the GLUT5 transcript was detected in every pcDNA3.1-GLUT5 transfected clone assayed. All of the GLUT5 transfected CHO clones grew with comparable specific rates and to similar maximum cell concentrations in both fructose-containing medium and glucose-containing medium. In glucose-containing medium, each clone accumulated lactate at a similar rate; however, in fructose-containing medium there was a prominent difference in the amount of lactate produced by each clone. This difference was readily observed during clone expansion and maintenance by the noticeable range of colors of the spent media using phenol red as a pH indicator.

Fig. 3 illustrates the variability in lactate production of two representative GLUT5 transfected clones, clone 1 (Fig. 3A1–A3) and clone 11 (Fig. 3B1–B3), cultured in 3 g/L fructose and 3 g/L glucose medium. The average data from three replicate cultures are plotted, with error bars indicating one standard deviation of the average value. Clone 1 had a similar growth rate and reached a similar maximum cell concentration in 3 g/L fructose and 3 g/L glucose medium; however, it produced significantly less lactate in the fructose medium. Coordinate, far less fructose was consumed than glucose. Clone 11 also had similar growth rates and grew to similar maximum cell concentrations in both sugars. In addition to having similar growth characteristics, clone 11 consumed both fructose and glucose in similar amounts and coordinately produced a similar amount of lactate. During the exponential growth phase, the molar stoichiometric ratio of lactate produced to fructose consumed was 0.6 for clone 1 and 1.4 for clone 11. When cultured in glucose, the molar ratio of lactate produced to glucose consumed was 1.5 for clone 1 and 1.2 for clone 11. The fraction of sugar converted into lactate was significantly lower for clone 1 cultured in fructose.

Table 1 also summarizes the growth and metabolic characteristics of the GLUT5 transfected clones cultured in 3 g/L fructose and 3 g/L glucose. Data for a pcDNA3.1 transfected clone and for the untransfected cell line cultured in glucose are also included. The GLUT5 transfected clones exhibited a similar growth rate (shown as doubling time) when cultured in either fructose or glucose medium. For comparison of nutrient utilization among clones, molar ratios of lactate production to sugar consumption during exponential growth are shown. When grown in glucose medium, this ratio (L/G) was relatively invariant among clones, ranging from 1.0 mol lactate/mol glucose to 1.6 mol lactate/mol glucose. For nine of the clones, the ratio in fructose medium (L/F) was lower than the corresponding ratio in glucose (L/G). Additionally, a much broader range of values of the lactate/fructose ratio was observed, ranging from 0.3 mmol lactate/mmol fructose to 1.7 mmol lactate/mmol fructose. The clones that accumulated less lactate in fructose medium also had a lower lactate/sugar ratio.

3.3. Fed-batch cultures

The GLUT5 transfected CHO cell, clone 1, had a substantially lower specific lactate production rate in fructose medium, and was further characterized in fed-batch cultures. With its reduced lactate production in fructose medium, a higher final cell concentration may be attainable without controlling sugar concentration at a low level. Two fed-batch cultures were run under similar conditions, except the carbohydrate source was either glucose or fructose. The concentrations of all nutrients were maintained throughout the culture without accumulating to great excess by continuous feeding of a concentrated feed medium. This was executed by online estimation of metabolic demand by OUR measurements coupled to stoichiometric feeding of nutrients based on oxygen consumption. Amino acid and carbohydrate concentrations were monitored every 8–12 h throughout the cultures and the stoichiometric feeding ratio was adjusted as needed. Sugar concentration was maintained between 2 g/L and 3.5 g/L and glutamine concentration was maintained between 0.45 g/L and 1.15 g/L throughout both cultures. For the glucose culture, 1N NaOH was used to maintain pH when reducing CO2 in the air stream was inadequate for pH control. Base addition was not required in the fructose culture.

The time profiles of cell concentration and lactate concentration for the glucose and fructose fed-batch cultures are shown in Fig. 4. To account for the effects of dilution by the feed volume, the data are presented as volume adjusted values by dividing the cumulative amount of cells and lactate produced by the initial culture volume. At the end of the cultivation, 47% and 64% of the initial culture volume was added for the glucose and fructose cultures, respectively. The maximum viable cell concentration achieved was more than threefold higher in the fructose culture.
Fig. 3. Metabolic and growth characteristics of GLUT5-transfected CHO cells. Time course of average values for cell concentration (1), lactate formation (2), and sugar consumption (3) are shown for triplicate T-flask cultures of two GLUT5 transfected CHO clone 1 (A) and clone 11 (B), grown in 3 g/L fructose (♦) or 3 g/L glucose (♦) as the sole carbon source. Error bars are ±1 S.D. of the average value.

than in the glucose culture. The non-volume adjusted maximum cell concentrations were $11.5 \times 10^6$ cells/mL in the fructose culture and $4.4 \times 10^6$ cells/mL in the glucose culture. In the first 60–70 h, the specific growth rate was similar in both cultures. The total amount of lactate accumulated in the fructose culture was substantially lower than in the glucose fed-batch culture.

3.4. Fructose selection

As shown in Fig. 1, the CHO cells used in this study had a limited capability to grow in medium containing fructose as the sole carbohydrate source. Transfection of the GLUT5 transporter may confer the ability to grow in low concentrations of fructose, and potentially be useful as a selection marker. CHO cells transfected with the pcDNA3.1-GLUT5 plasmid were plated using limiting dilution in 0.3 g/L fructose medium without G418. Two clones (FSA and FSB) were isolated from ten 96-well plates. No clones were isolated by limiting dilution of untransfected cells in the same medium. Similar to the clones isolated by selection in G418 containing medium, the two fructose selected clones also grew at similar rates in both glucose and fructose medium (Table 1). Both clones also produced lactate at high levels when cultured in either fructose or glucose medium. RT-PCR was performed to confirm that these clones expressed both GLUT5 and the NeoR gene from the transfected plasmid. Fig. 5 shows the amplified PCR fragments of the GLUT5 and NeoR gene for the two isolated clones. Control RT-PCR reactions were performed for a pcDNA3.1 transfected clone, and the untrans-
Fig. 4. Comparison of glucose and fructose fed-batch processes for clone 1. Time course of volume adjusted cell concentration (A) and lactate product ion (B) are shown for clone 1 fed-batch cultures using either fructose (△) or glucose (○) as the sole carbohydrate source.

Fig. 5. Expression of GLUT5 and NeoR transcripts in fructose selected clones. Expression of GLUT5 and the NeoR transcripts was probed by RT-PCR to verify their expression in the two CHO cell clones (FSA and FSB) isolated by selection in 0.3 g/L fructose medium. For controls, GLUT5 and NeoR transcript expression was also probed by RT-PCR for the untransfected CHO cells and a pcDNA3.1 stable transfectant.

4. Discussion

The high-affinity glucose transporter, GLUT1, has a low \( K_m \) for glucose and is expressed ubiquitously in all tissues. In addition to glucose, GLUT1 also has a low affinity for the transport of galactose. In addition to glucose and galactose, some cell lines are capable of using fructose as the sole carbohydrate source in culture medium; however, the GLUT1 transporter is thought to have no affinity for fructose. In addition to GLUT1, the GLUT2 glucose transporter can transport fructose with a very low affinity. Other GLUT transporters, namely class II transporters, can also mediate fructose uptake; however, their affinities and their expression patterns in various tissues have not been well characterized (Wood and Trayhurn, 2003). As illustrated in the results section, CHO cells could not grow substantially when fructose was used as the sole carbohydrate source at a moderate concentration (2 g/L). Increasing the concentration of fructose to a higher level (6 g/L and 10 g/L) led to more growth, although both the growth rate and the extent of growth were substantially lower than in glucose medium. The endogenous CHO GLUT5 transporter was not expressed at a detectable level in untransfected CHO cells (Table 1) but it is possible that other GLUT transporters with very low affinity for fructose might be expressed in this cell line.

The transport rate of fructose into cells was increased to a level sufficient for rapid cell growth by stable expression of the mouse GLUT5 gene. All GLUT5 transfected CHO clones gained the ability to utilize fructose as the sole carbohydrate source could grow equally well using either fructose or glucose as the sole sugar source. The control pcDNA3.1 transfected clone, expressing only the NeoR transcript, did not grow appreciably in the 3 g/L fructose medium, suggesting that expression of the GLUT5 transporter is indeed responsible for the observed phenotype.

Q-RT-PCR revealed that the lowest-lactate producing clone expressed the GLUT5 transcript at a significantly lower level than other higher lactate producing clones. It is plausible that the lower level expression of GLUT5 transporters provided a supply of fructose just sufficient for optimal growth, with little excess overflow of carbon to lactate. A closer examination of the GLUT5 expression levels among all clones fails to establish a clear relationship with lactate production. This is not unusual, as clone-based studies often fail to establish a quantitative relationship between transgene expression level and the desired trait. Aside from clonal variations that may cause differences in metabolic characteristics, lactate production is affected by many factors in addition to sugar uptake. Most obvious is the coupling of the fluxes of fructose \( \rightarrow \) pyruvate and pyruvate \( \rightarrow \) lactate, with the cycling of \( \text{NAD}^+ / \text{NADH} \). Since these two arms of sugar metabolism cannot easily be decoupled, the fluxes affect one another. The flux of pyruvate \( \rightarrow \) lactate is greatly affected by lactate secretion, which is coupled to the transport of a proton. The
difference in extracellular and intracellular pH can induce subtle changes in the balance between lactate and pyruvate, which, in turn may affect fructose consumption.

While the difference in the molar lactate/fructose ratio among the clones expressing GLUT5 at higher levels may be explained by such subtle variation among clones and culture conditions, it is also known that transcript levels are not always directly proportional to protein expression levels. Still, the low level of expression of GLUT5 by clone 1, and more moderate expression of GLUT5 in clones 2–10 suggest that a lower expression level could be a factor in obtaining clones with the desired phenotype of low lactate production. Use of a less strong promoter for expression of the GLUT5 gene could be used as a strategy to obtain clones exhibiting the desired phenotype of reduced sugar consumption and lactate production.

The primary aim of this work was to engineer cell metabolism through the manipulation of transporters to modulate the uptake of carbohydrates. In addition to altering the cell’s metabolic characteristics, expression of the GLUT5 transporter also conferred cells with the ability to grow in medium containing fructose as the sole carbohydrate. This newly acquired growth ability can potentially be useful as a selection marker for co-transfection and expression of another desired gene. Genetic engineering of cell lines for enhanced metabolic properties is becoming an increasingly attractive methodology in cell line development. It is likely that a single cell line will be genetically modified to express multiple genes, in addition to the recombinant product. The availability of an additional selection marker increases the number of repeated genetic modifications that can be performed to a cell lineage.

The slower transport kinetics of the GLUT5 transporter enabled cells to restrict their sugar uptake to more moderate rates, resulting in reduced lactate accumulation. In addition to carbohydrates, many other nutrients are oversupplied to cells under typical culture conditions. Upon metabolic shift from a high lactate producing state to a low lactate producing state, reduced amino acid consumption was observed without affecting growth rates or antibody production rates (Gambhir et al., 2003). It is conceivable that metabolic engineering of transporters responsible for uptake of other nutrients may be a strategy to further reduce metabolite accumulation. As opposed to simply cloning in additional transporters, many strategies will require the suppression of endogenous transporters. Currently, gene suppression technologies, like siRNA, require that the exact sequence of the target gene be known. Most recombinant therapeutically significant proteins are produced in either murine cells (such as NS0) or CHO cells. While the entire mouse genome sequence, along with millions of ESTs, are publicly available for such work in mouse derived cell lines, there are substantially fewer genomic resources available for CHO cells. It is our hope that our nascent effort in CHO EST sequencing (Wlaschin et al., 2005) will facilitate the manipulation of transporters in this important cell line.

Transporters are gatekeepers for nutrient influx and metabolic efflux. Many cell-engineering efforts to alter carbohydrate metabolism have focused on changing expression levels of other metabolic enzymes (Chen et al., 2001; Irani et al., 1999, 2002); however, transporters are a more natural target for changing cell metabolism. In this work, engineering sugar transporters conferred cells with a new ability to grow in a different sugar and, at the same time, reduced lactate accumulation and allowed higher cell concentrations to be achieved in fed-batch processes. Transporter engineering and the development of bioprocesses using such engineered cell lines promise to assist in meeting the growing demand for life-saving therapeutics.

Acknowledgements

The authors thank Dr. R. Scott McIvor for insightful discussions, Dr. Jongchan Lee, Dr. Eyal Epstein and Bhanu Mulukutula for their assistance in clone characterization, and Molly Smith, Cheston Hsiao and Lewis Marshall for their technical assistance. KFW was a recipient of an NSF Graduate Fellowship and the NIH Biotechnology Traineeship (GM08347).

References


