Metabolite Depletion Affects Flux Profiling of Cell Lines

Nilsson, A.; Haanstra, J. R.; Teusink, B.; Nielsen, J.

published in
Trends in Biochemical Sciences
2018

DOI (link to publisher)
10.1016/j.tibs.2018.03.009

document version
Publisher's PDF, also known as Version of record

Forum

Metabolite Depletion Affects Flux Profiling of Cell Lines

A. Nilsson,[1] J.R. Haanstra,[2] B. Teusink,[2] and J. Nielsen[1,3,*]

Quantifying the rate of consumption and release of metabolites (i.e., flux profiling) has become integral to the study of cancer. The fluxes as well as the growth of the cells may be affected by metabolite depletion during cultivation.

Reprograming of metabolism is an emerging hallmark of cancer [1]. This forebodes deep insights to be gained from quantifying the metabolic fluxes of tumors and cell lines. For microbial organisms, intracellular fluxes are routinely estimated by integrating changes in extracellular metabolite concentrations with genome-scale metabolic models, but there are only a few studies of cancer metabolism taking this approach [2].

In an impressive study of the 60 cancer cell lines in the National Cancer Institute (NCI) panel [3], specific exchange fluxes (on a per-cell, per-time unit basis, i.e., in mmol/cell/h) were estimated for 115 metabolites. This study is highly cited and these data are extensively used by others [4–7], for example, to calculate the metabolic flux states for each cell line by mathematical modeling. The study measured metabolite concentrations in the cultivation medium after 4–5 days of growth (S_{Spent}) in duplicates, and the baseline concentrations in ten replicates (S_{Fresh}). To calculate specific exchange fluxes from concentration differences, the amount of cells at each time point must be known, giving the area under the growth curve. In this study, the area was estimated using the final cell count and tabulated doubling times. It has previously been noted that the fluxes calculated in the study may not be comparable between cell types due to the order of magnitude differences in cell volume [6]. Here we discuss how metabolite depletion during the cell cultivation may affect the estimated fluxes.

The area under the growth curve estimate relies on the cells being in the exponential growth phase (Figure 1A). Cell cultures typically undergo several growth phases: a lag phase (L), with very low or even negative growth rates; the first exponential phase (E1), with rapid growth and high absorption rates of metabolites; one or more secondary exponential phases (E2–EN), with slower growth and metabolic rates; and a stationary phase (S), with no growth and low metabolic rates supporting maintenance metabolism.

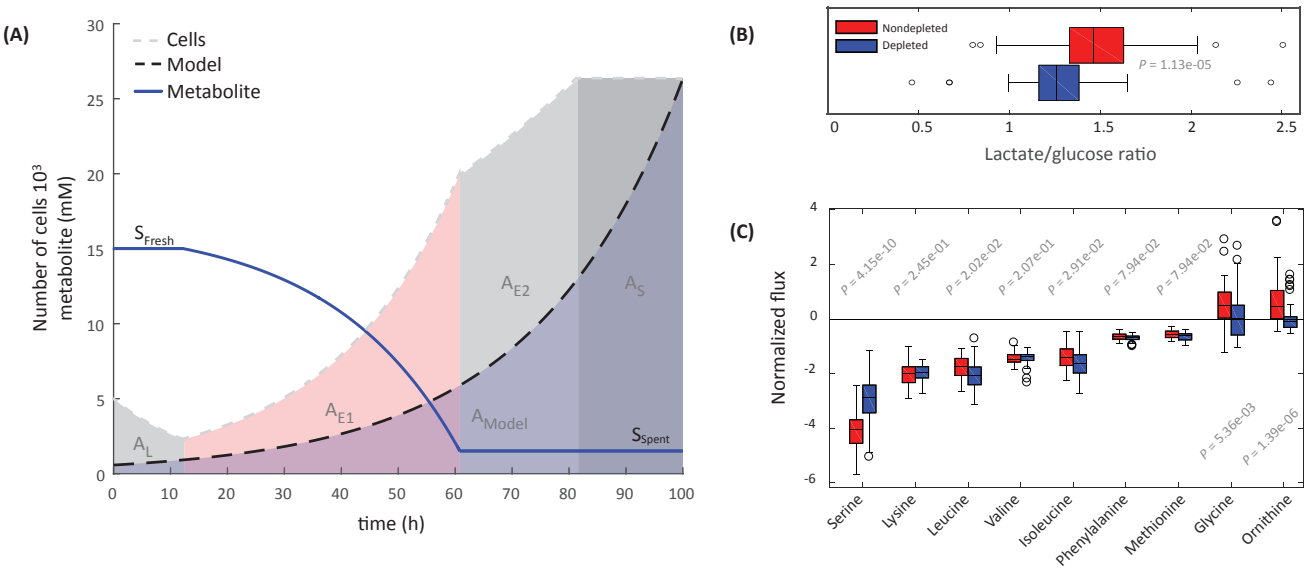

Figure 1. Metabolite Depletion May Trigger a Shift from Fast to Slower Exponential Growth, Which Affects the Estimated Flux Profiles. (A) The model to estimate the area under the growth curve (A_{Model}) using tabulated doubling times will underestimate the true area ($A_L + A_{E1} + A_{E2} + A_S$) if cells grow beyond the first exponential phase. This may be triggered by metabolite depletion. (B) Nondepleted cultures ($N = 36$) show a more glycolytic phenotype, compared with depleted cultures ($N = 84$), defined as the final concentration (S_{Spent}) being below 10% of initial concentration (S_{Fresh}) for at least one metabolite in the culture. Data from [3]. (C) The consumption rate of serine and release rates of glycine and ornithine are markedly higher among the nondepleted cultures, as indicated by normalized flux. *P* values calculated using the two-sample Kolmogorov–Smirnov test.

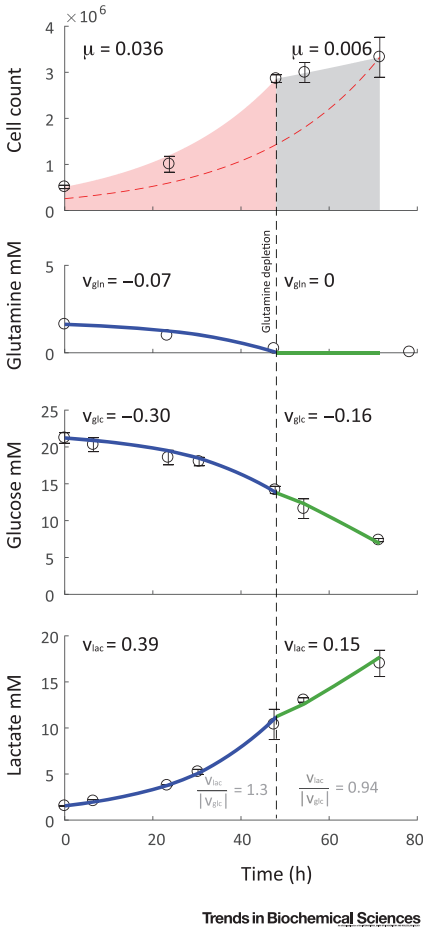

Figure 2. Metabolic Shifts in a Culture of Hepg2Cells Undergoing Metabolite Depletion. The time-resolved cell counts show a shift in growth rate from a first exponential phase with high growth rate to a second exponential phase with markedly lower rate. The switch in growth phase correlates with glutamine depletion (black dotted line). The expected metabolite concentrations (blue lines) are calculated by assuming the indicated specific uptake (mmol/10^6 cells/h) rates and are corrected for volume changes due to repeated sampling (see Supplemental Methods online for details on the experiment). The specific uptake rates are refitted to the indicated lower values after the shift (green lines), revealing a reduced glycolytic phenotype. Calculating fluxes using endpoint measurements and the growth rate from the first exponential phase (red dotted line) underpredicts the observed area under the curve; it would also yield a mixture of the metabolic fluxes from the two growth phases.

The switch between E1 and E2 can be triggered by depletion of a non-essential, but growth-enhancing metabolite. A switch to E2 will affect the area under the growth curve, and is often followed by shifts in the specific exchange fluxes and even reabsorption of excreted products (e.g., the co-consumption of glucose and lactate after glutamate depletion), as seen in the later stages of CHO cell cultivation [8]. The switch to the stationary phase can be triggered by depletion of a growth-essential amino acid.

Growth curves were reported for six of the 60 cell lines in the study [3], and several showed indications of a switch to secondary exponential phases. As a proxy for cultures transcending the E1 phase, we looked for metabolite depletion in the data. In 70% of the cultures the concentration of at least one metabolite was low at the endpoint measurement, including several growth-enhancing or growth-essential metabolites, for example, glutamine, serine, tryptophan, and valine (see Supplemental Figure S1 online).

Cultures sampled before metabolite depletion (nondepleted) showed a more pronounced glycolytic phenotype (Figure 1B), characterized by a higher molar ratio between lactate produced and glucose consumed compared with the cultures with depleted metabolites (depleted). This is consistent with a more active metabolism in the E1 phase.

Because of differences in cell size and growth rate, the measured exchange flux of a metabolite may differ by an order of magnitude between different cultures [6]. To adjust for this, we divided each flux by the median of the flux magnitude (absolute value) from that culture. These normalized flux profiles were similar between the two groups of cell cultures, but the calculated serine absorption and release of glycine were markedly higher for the nondepleted cells (Figure 1C, Supplemental Figure S2). This suggests that glycine may have been reabsorbed to compensate for serine depletion.

The specific growth rate was lower for the nondepleted cells (0.020 vs. 0.023 h^{-1}, $P = 0.02$), which may be expected since fast growing cells are likely to consume metabolites faster. No difference was, however, observed in the glycolytic phenotype and serine flux between cultures with specific growth rates below and above the median (Supplemental Figure S3).

As metabolite depletion and reabsorption may affect both the area under the curve estimate and the metabolic fluxes, caution is recommended when interpreting the flux data reported in [3] as specific fluxes. We provide a list of the nondepleted cultures, which are more likely to have accurate flux estimates (see Supplemental Table S1 online).

To avoid metabolite depletion in metabolic studies of cancer, we recommend greater medium volumes or shorter culture times. Ideally, metabolite levels and cell numbers should be determined at several time points to get better estimations of specific fluxes and the growth rate. An example of the added value of a time-resolved metabolite experiment in hepatocellular carcinoma cells (Hepg2) is given in Figure 2. In this experiment, we monitored cell numbers and medium metabolites over time, in a similar interval as the NCI-60 study. The growth curve showed a shift in growth phase at 50 h, with continued growth at a lower rate until 80 h. Within this timeframe, glutamine was depleted. The area under the growth curve and the exchange fluxes would have been underestimated if only two time points were used. Time-resolved data not only solve this, but may also reveal important dynamics of the metabolic fluxes.

Supplemental Information

Supplemental information associated with this article can be found, in the online version, at https://doi.org/10.1016/j.tibs.2018.03.009.

[1]Department of Biology and Biological Engineering, Chalmers University of Technology, Gothenburg SE41296, Sweden
[2]Systems Bioinformatics, Amsterdam Institute for Molecules, Medicines and Systems (AIMMS), Vrije Universiteit Amsterdam, 1081 HV Amsterdam, The Netherlands
[3]Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, DK2800 Kongens Lyngby, Denmark

*Correspondence: nielsenj@chalmers.se (J. Nielsen).
https://doi.org/10.1016/j.tibs.2018.03.009

References
1. Hanahan, D. and Weinberg, R.A. (2011) Hallmarks of cancer: the next generation. *Cell* 144, 646–674
2. Nilsson, A. and Nielsen, J. (2016) Genome scale metabolic modeling of cancer. *Metab. Eng.* 43, 103–112
3. Jain, M. *et al.* (2012) Metabolite profiling identifies a key role for glycine in rapid cancer cell proliferation. *Science* 336, 1040–1044
4. Fan, J. *et al.* (2014) Glutamine-driven oxidative phosphorylation is a major ATP source in transformed mammalian cells in both normoxia and hypoxia. *Mol. Syst. Biol.* 9, 712
5. Zielinski, D.C. *et al.* (2017) Systems biology analysis of drivers underlying hallmarks of cancer cell metabolism. *Sci. Rep.* 7, 41241
6. Dolfi, S.C. *et al.* (2013) The metabolic demands of cancer cells are coupled to their size and protein synthesis rates. *Cancer Metab.* 1, 20
7. Aurich, M.K. *et al.* (2017) A systems approach reveals distinct metabolic strategies among the NCI-60 cancer cell lines. *PLoS Comput. Biol.* 13, e1005698
8. Zagari, F. *et al.* (2013) Lactate metabolism shift in CHO cell culture: the role of mitochondrial oxidative activity. *Nat. Biotechnol.* 30, 238–245

Forum

Long-Term Transcriptional Gene Silencing by RNA Viruses

Juozas Baltusnikas,[1,*] Saulius Satkauskas,[1] and Kenneth Lundstrom[2]

Long-term transcriptional gene silencing has been hampered by delivery issues. A potential solution is the application of RNA viruses that generate small RNAs without any DNA intermediate. Long-term therapy for various diseases is expected after a single administration.

Long-Lasting Therapeutic Effect after Single Therapy

Many chronic, incurable, and fatal diseases provide no options for efficient therapy, and long-term consumption of drugs results in harmful side effects. Currently, small RNA [sRNA; small interfering RNA (siRNA) and/or microRNA (miRNA)] delivery strategies exploit sRNA-mediated post-transcriptional gene silencing (pTGS) as a therapeutic approach to treat various diseases. However, pTGS is short-term and requires continuous sRNA supply to maintain its effect. This problem has been addressed by employing viruses that provide long-term sRNA expression. However, the drawback of this approach is that long-term expression of sRNAs may oversaturate the cellular machinery, affect expression levels of nontarget genes, and cause toxicity. Moreover, currently popular viral delivery vehicles, such as adeno-associated viruses and lentiviruses, have the capacity to randomly integrate their viral sequences into the host genome, which poses a risk of oncogenesis. For additional information about viral and sRNA therapy, see Supplemental Information Box S1 online.

Here, we propose an alternative that allows a single administration of drugs to safely induce long-term therapeutic efficacy. Single-stranded self-replicating RNA viruses can effectively transduce cells and produce functional sRNAs [1,2]. These viruses present a great potential for short-term sRNA delivery [3] and do not generate any DNA intermediates, thus providing a potentially safer approach than adeno-associated viruses and lentiviruses. In addition, instead of using sRNA-mediated short-term pTGS, a better solution would be to use sRNA mediated long-term transcriptional gene silencing (TGS). In the long run, self-replicating RNA viruses could cause short-term sRNA expression that would be sufficient to induce long-term TGS. This approach could lead to long-term therapeutic effect induced by a single administration, which could be especially useful for patients with various chronic, incurable, and fatal diseases (Figure 1, Key Figure).

Long-Term TGS

The mechanism for long-term TGS is not well understood, yet some important details have already been revealed (Figure 2). At the active promoter target site (Figure 2A), sRNA guides the gene silencing complex with Argonaute 1 (AGO1), DNA methyltransferase 3a (DNMT3a), and histone deacetylase 1 (HDAC1) serving as core elements (Figure 2B). It is postulated that sRNAs guide the gene silencing complex to the promoter by complementary binding of promoter-associated RNA, which remains in close proximity to the promoter during the transcription (Figure 2C) [4]. Thereafter, this complex increases dimethylation at H3K9 (lysine 9 of histone H3) and trimethylation at H3K27 (lysine 27 of histone H3), decreases acetylation at H3K14 (lysine 14 of histone H3), and induces TGS (Figure 2D). However, DNA methylation and a long-term TGS require maintaining exogenous sRNA levels in the cell for 7 days [4,5].

The fact that the gene silencing complex is required to bind to promoter-associated RNA indicates that TGS could be applied only for those genes whose promoters are transcribed. It appears that the promoter transcription is a general feature in human cells [4], suggesting that TGS could be universally applied for many genes.

Epigenetic changes can occur downstream from a targeted sRNA locus without changes in upstream sites [5]. Therefore, sRNAs targeting upstream