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Effects of amino acid transport limitations on cultured hepatocytes

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ABSTRACT

Amino acid supplementation has been shown to enhance the liver-specific functions of cultured hepatocytes during plasma exposure. However, their transport through the cell membrane may restrict their availability for hepatic metabolism. Here, we focus on transport constraints related to uptake of the neutral amino acids and their impact on hepatic metabolism and liver-specific functions. Under varying combinations of their medium concentrations, we found that transport competition exists among the three amino acids alanine, serine and glutamine and that the resulting capacity constraints affect the urea and albumin production of cultured hepatocytes. Regression equations were developed to quantify these constraints and were incorporated with other constraints (mass balance, measured flux data and reaction directionality) within a multi-objective flux balance framework to understand how amino acid transport constraints propagate through central hepatic metabolism and to predict refined amino acid supplementations for specific hepatocyte design objectives.

1. Introduction

The performance of cultured mammalian cells is tightly connected to the amino acid supplementation employed in the culture medium. For industrial cell culture processes, this supplementation can be adjusted using a factorial design methodology. However, such an approach does not provide understanding into the effects of various supplementations and thus must be restarted if the operating conditions or desired outputs change. In the case of primary hepatocytes being cultured for use in a bioartificial liver (BAL) device environment, an empirically derived amino acid supplementation has been shown to counteract the detrimental effects of plasma exposure [1]. Furthermore, we have recently shown that an amino acid supplementation designed using a rational optimization approach can lead to improvements in targeted metabolic objectives [2].

Metabolism of amino acids is tied intimately to their transport into or out of the cell, utilizing specific transporters that bind the amino acids and mediate their passage across the cell membrane. Transporters with the same recognition properties form a transport system. Amino acid transport is generally based on their ionic charge—neutral, cationic and anionic—and is mediated by independent transport systems with overlapping specificities as summarized in Table 1 [3–5]. Since several amino acids share a particular transporter, its capacity affects the transport of each amino acid that utilizes it. Thus, an excess of one amino acid may inhibit the uptake of the other amino acids that are mediated by the same transport system [6]. For hepatocytes, there is evidence to suggest that amino acids sharing System A may be susceptible to competitive capacity constraints. For example, it has been shown that the transport rate is a controlling step for alanine metabolism [7], and a similar conclusion was drawn for glutamine [8]. Alanine and glutamine transport is mediated by the same transport system A with serine [9].

In order to assess the influence of shared system A transport on hepatocyte metabolism, the effect of varying combinations of alanine, serine and glutamine on hepatocyte metabolism was measured. Indeed, competitive relationships were observed, and these were captured mathematically using least-square regression equations, which successfully explain the variance and dependence in amino acids transport fluxes (alanine, serine and glutamine). Furthermore, these amino acid transport constraints are incorporated together with other constraints including mass balances, measured data and reaction directionality within a multi-objective metabolic flux balance model to investigate the effect of transport constraints and to predict the amino acid supplementation of alanine, serine and glutamine for maximum urea production and fatty acid oxidation.

2. Materials and methods

2.1. Design of experiments: amino acids transport

Hepatocytes were isolated from adult male Fisher F344 rat (150–200 g) based on the two-step collagenase-perfusion method via the portal vein described previously [10]. Cells were cultured in a collagen sandwich configuration at a density of 1×10^6 cells/mL in six-well plates. Initially, hepatocytes were cultured in the standard hepatocyte C + H medium, which consists of DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS), 7.0 ng/mL glucagon, 7.5 g/mL

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Table 1
Amino acid transport systems [3–5].

Transport system	Name	Amino acid	Ion sensitive	Others
Neutral	A	ALA, SER, GLN	Na^+	pH-sensitive
	ACS	ALA, SER, CYS	Na^+	pH not affect
	N	GLN, ASN,HIS	Na^+	A/N transport [25,26]
	L	Branched-chain and other aromatic amino acids	–	Poorly metabolized and not accumulated in hepatocytes
Gly	Gly	Glycine	Na^+, Cl^-	
Cationic	y+	ARG, LYS, ORN	Na^+, Cl^-	CAT2 in hepatocytes
Anionic	X^-_{AG}	GLU, ASP	Na^+, K^+	EAAT 1 to 5

hydrocortisone, 20 g/L epidermal growth factor, 200 U/mL penicillin, 200 g/mL streptomycin, and 500 mU/L insulin. Hepatocytes were exposed to this medium for three days with daily medium change. We determined in pilot experiments that this provided sufficient time for fluxes of glucose, urea, glycerol, free fatty acids and the protein albumin to stabilize and remain constant (data not shown).

RPMI_1600, plus 10% FBS and the same concentrations of glucagon, EGF, insulin, and glucose as C + H medium, was used to wash hepatocytes three times at 1 hour intervals. Next, cells were cultured in media with varying combinations of alanine, serine and glutamine supplementation (total 33 combinations, in Appendix A), for an additional three days with daily medium change. At the end of the experiment, the supernatant was collected and stored at 4 °C for later assay.

2.2. Design of experiments: amino acids supplementation

Hepatocytes were cultured using the collagen sandwich method [10] at a density of 1×10^6 cells/mL in six-well plates. After the second gel layer solidified, 0.8 mL fresh C + H medium (described in Section 2.1) with 0.05 mU/mL insulin (low insulin, LI) or 500 mU/mL (high insulin, HI) was added. The medium was exchanged every day. After 6 days of preconditioning, hepatocyte cultures were exposed to plasma with hormone supplementation including 7.5 g/mL of hydrocortisone and 0.05 mU/mL of insulin (WH) or without hormone supplementation (NH). Treatment groups varied in the amino acid supplementation in the plasma, comparing "designed" amino acid supplementation (DAA) based on a rational design approach described in our previous work [2], and "reference" amino acid supplementation (RAA) based on published data [1]. The medium concentrations corresponding to each amino acid supplementation are given in Appendix B. Medium exchanges were performed daily over the five days of plasma exposure. At the end of the experiment, culture supernatants were collected and stored at 4 °C prior to analysis.

2.3. Biochemical assays

In the conditioned culture medium, urea was measured using a commercial kit (Sigma, St. Louis, MO) which is based on the reaction of diacetyl monoxime with urea. Albumin production was quantified by an enzyme-linked immunosorbent assay (ELISA) using purified rat albumin (MP Biomedicals, Solon, OH) and peroxidase-conjugated antibody for detection (MP Biomedicals, Solon, OH).

Amino acids were labeled with AccQ reagent (Waters Corporation, Milford, MA) and separated using a Beckman Coulter HPLC system with a fluorescence detector (Waters 470, Waters Corporation, Milford, MA) scanning at 250/395 nm excitation/emission. Serial dilutions of standards were used to construct a calibration curve for each analyte, with the linear portion utilized for measurements. The concentrations of amino acid/ammonia in the fresh or spent medium were determined using these standard curves, with sample dilutions performed as necessary to operate within the linear range.

In addition, for the experiment of the amino acid supplementation during plasma exposure, the concentrations of glucose, lactate, glycerol, and glutamine were measured colorimetrically using commercial kits (Sigma, St. Louis, MO). Enzymatic kits were utilized for the measurement of cholesterol (BioAssay System, Hayward, CA), acetoacetate, β-hydroxybutyrate, triglycerides (Stanbio, Boerne, TX), and free fatty acids (Roche, Indianapolis, IN).

2.4. Transport constraints

A quadratic model is used to quantify the transport limitations that exist between amino acid uptake/secretion and their concentrations in the cultured medium, as follows:

$$v_l = \beta_0 + \sum_k \beta_k C_k + \sum_i \sum_j \beta_{ij} C_i C_j + e; \quad (i,j,k,l) = \{\mathrm{Ala}, \mathrm{Ser}, \mathrm{Gln}\}. \quad (1)$$

The regression parameters were estimated from a set of known values of concentrations of alanine (C_{Ala}), serine (C_{Ser}), and glutamine (C_{Gln}) supplied in the medium and their corresponding measured uptake/secretion fluxes (v_l) using the method of least squares regression, as implemented in SAS/STAT (SAS Institute, Inc., Cary, NC). A Student's *t*-test was used to identify which terms contribute significantly to the flux values. Terms with $p<0.05$ were considered to be significant, while the others were excluded from the model.

2.5. Metabolic flux modeling

In this work, we measure and interpret the metabolism of hepatocytes in terms of their fluxes as opposed to metabolite concentrations as would be done in a metabolomics approach. The advantages of utilizing metabolic flux are that the flux, or throughput, is more representative of the metabolic phenotype and that measurement of some flux values can be used to infer the values of additional fluxes through material balances, enabling a more complete view of cellular metabolism. Metabolic flux modeling involves construction of a metabolic reaction network that captures the reactions of interest and the formation of mass balances around each of the internal metabolites, expressed as

$$\frac{d\mathbf{X}}{dt} = \mathbf{S}\cdot\mathbf{v}, \quad (2)$$

where **X** is the vector of metabolite concentrations, **S** the stoichiometric matrix and **v** the vector of fluxes [11]. This equation is applied in the pseudo-steady-state, removing the metabolite concentrations from explicit consideration. The flux vector **v** is partitioned into measured and unknown fluxes and the resulting system of linear equations can be solved algebraically if the number of unknown fluxes is equal to the number of balance equations. Otherwise, optimization approaches are used to solve the equations for an assumed objective function and/or subject to additional physicochemical constraints.

The basic hepatic network used in this work builds upon previously reported hepatic networks [1,12–15] and involves 46 intracellular metabolites, and 78 reactions (30 reactions with measurement data, 48 reactions with unknown fluxes as listed in Appendix C). The pathway for gluconeogenesis, and not glycolysis, is used in this work, based on previous studies of hepatocyte cultures exposed to plasma using amino acid supplementation (Yang et al., submitted for publication). The other reactions included those involved in tricarboxylic acid cycle (TCA), urea cycle, amino acid uptake/secretion and catabolism, oxygen uptake, electron transport system, pentose phosphate reactions (as a lumped group), ketone body synthesis, fatty acid, triglyceride (TG) and glycerol metabolism.

The constraints derived to express the transport limitations of amino acids are incorporated into a flux balance optimization framework to investigate the effects of amino acid supplementation