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A Rational Design Approach for Amino Acid Supplementation in Hepatocyte Culture

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ABSTRACT: Improvement of culture media for mammalian cells is conducted via empirical adjustments, sometimes aided by statistical design methodologies. Here, we demonstrate a proof of principle for the use of constraints-based modeling to achieve enhanced performance of liver-specific functions of cultured hepatocytes during plasma exposure by adjusting amino acid supplementation and hormone levels in the medium. Flux balance analysis (FBA) is used to determine an amino acid flux profile consistent with a desired output; this is used to design an amino acid supplementation. Under conditions of no supplementation, empirical supplementation, and designed supplementation, hepatocytes were exposed to plasma and their morphology, specific cell functions (urea, albumin production) and lipid metabolism were measured. Urea production under the designed amino acid supplementation was found to be increased compared with previously reported (empirical) amino acid supplementation. Not surprisingly, the urea production attained was less than the theoretical value, indicating the existence of pathways or constraints not present in the current model. Although not an explicit design objective, albumin production was also increased by designed amino acid supplementation, suggesting a functional linkage between these outputs. In conjunction with traditional approaches to improving culture conditions, the rational design approach described herein provides a novel means to tune the metabolic outputs of cultured hepatocytes.

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KEYWORDS: hepatocyte; urea; amino acids; hormone; flux balance analysis (FBA); mixed integer linear programming (MILP)

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Introduction

The metabolic capabilities and constraints of cultured hepatocytes are of great interest for the development of extracorporeal bioartificial liver (BAL) devices to support and extend the life of critically ill patients until a liver transplantation becomes available (Allen and Bhatia, 2002; Matthew et al., 1996; Tilles et al., 2002). Cultured hepatocytes are core components of BAL devices, which have been developed rapidly in recent years (Strain and Neuberger, 2002). However, hepatocytes are anchorage-dependent cells, and they rapidly lose liver-specific functions once they are removed from their host and cultured in an artificial environment (Allen et al., 2001). Some culture techniques have been introduced to help stabilize liver-specific in vitro functions, which include culturing hepatocytes in sandwich collagen gels (Dunn et al., 1991, 1992), co-culturing hepatocytes with other types of cells such as 3T3 fibroblasts (Bhatia et al., 1997), culturing hepatocytes on extracellular matrix (Page et al., 2007), and addition of insulin to the culture medium (Li et al., 2004). Maintenance of liver-specific functions in hepatocytes during plasma exposure is very important to BAL devices. Effects of short- or long-term exposure of hepatocytes to plasma, as occurs during clinical application, have been studied recently. Hepatocytes exposed to plasma in vitro exhibit a progressive accumulation of lipid droplets, accompanied by a decrease in functional markers such as

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urea and albumin synthesis (Matthew et al., 1996; Stefanovich et al., 1996). Subsequent studies demonstrated that supplying amino acids and hormones (insulin and hydrocortisone) during plasma exposure improves synthetic functions as well as cytochrome P450 (detoxification) activities of the rat hepatocytes (Washizu et al., 2000a,b, 2001).

Computational modeling of hepatic metabolism is increasingly being utilized in tandem with experimental measurements to understand and control the performance of hepatocytes under adverse culture conditions. Metabolic flux analysis (MFA), which provides a comprehensive view of intracellular metabolism based on measurements of extracellular fluxes and stoichiometric mass balances at pseudo-steady-state, has been utilized to investigate the effects of insulin preconditioning and plasma amino acid supplementation on cultured hepatocytes. It was found that a combination of these two treatments promotes hepatic-specific functions (urea and albumin secretion), mediated by an increase in gluconeogenesis and a reduction in the intracellular triglyceride accumulation (Chan et al., 2003a,b,c). Flux balance analysis (FBA) is based on the utilization of linear programming and the pseudo-steady-state assumption to optimize a specific objective function, while satisfying mass balances for all intracellular metabolites and other imposed constraints (Kauffman et al., 2003; Varma and Palsson, 1994a). Depending on the system of interest, various objectives may be employed in FBA, including maximization of growth rate (Edwards et al., 2001; Mahadevan et al., 2002; Varma and Palsson, 1994b), maximization of specific fluxes in mammalian cells (Marin et al., 2004; Sharma et al., 2005), or minimization of metabolic adjustment (MOMA) (Segre et al., 2002) when experimental data are available for comparison. In order to extend the understanding of hepatocyte function relevant to the BAL, constraint-based techniques have been used to characterize the capabilities of the hepatocyte metabolic network. Some recent studies have focused on the overall flux distribution in the central metabolism of primary rat hepatocytes using FBA (Nagrath et al., 2007; Sharma et al., 2005; Uygun et al., 2007).

We present an approach that uses FBA to design an amino acid supplementation to achieve a specific cellular objective in cultured hepatocytes exposed to plasma, in this case urea production. Urea secretion is easily measured to high accuracy using commercial kits and is a well-established baseline marker of hepatocyte culture, making it a suitable model objective. From the amino acid fluxes corresponding to urea output, a novel amino acid supplementation was designed and used in the culture of cryopreserved hepatocytes with varying insulin levels during preconditioning and hormone levels during plasma exposure. Hepatocyte morphology was observed and specific cell functions (urea, albumin production) and lipid accumulation were measured in order to validate our hypothesis that urea secretion would be augmented using amino acids supplementation calculated theoretically from a mathematical model.

Methods

Flux Balance Analysis (FBA)

The basic hepatic network used in this analysis (Appendix A) is based on the network developed for MFA of cultured hepatocytes (Chan et al., 2003a; Lee et al., 2000a; Sharma et al., 2005). The hepatic network considered here involves 45 intracellular metabolites and 76 reactions (33 irreversible reactions and 43 reversible reactions, labeled in Appendix A), including gluconeogenesis, tricarboxylic acid cycle (TCA), urea cycle, amino acid uptake and catabolism, oxygen uptake, electron transport system, pentose phosphate reactions (as a lumped group), ketone body synthesis, fatty acid, triglycerol (TG) and glycerol metabolism. Although this hepatic network does not account for protein degradation, energy balance, nucleotide and transport metabolism, it covers a majority of central carbon and nitrogen metabolism and has been used extensively for analysis of hepatocyte metabolism both in vitro and in vivo (Chan et al., 2003a,b,c; Lee et al., 2003; Nagrath et al., 2007; Sharma et al., 2005; Uygun et al., 2007).

The following FBA model is developed for the maximization of urea output, constrained by 45 mass balances for the intracellular metabolites and flux bounds of measured and irreversible reactions based on the hepatic network of Appendix A, as follows:

$$\begin{aligned} &\text{Max}: Z = v_{\text{urea}} \\ \text{Subject to}: \quad &\sum_{j=1}^{N} S_{ij} v_j = 0 \qquad \forall i \in M \\ &v_j^{\min} \le v_j \le v_j^{\max} \quad \forall j \in K \end{aligned} \tag{1}$$

where S_{ij} is the stoichiometric coefficient of metabolite i in the reaction j; v_j refers to the flux of reaction j, v_{urea} is urea flux; M the number of metabolites; K the number of constrained reactions (based on measurements and/or irreversibility), and N the total number of reactions involved in the hepatic network. Model (I) corresponds to a linear programming (LP) problem since the objective and the constraints are linear functions of the involved variables.

The main assumptions for the development of the FBA model are as follows:

(1) Although it is only one of many hepatic functions, urea production is an often used marker of hepatocyte function that typically correlates with a healthy, differentiated phenotype (Chan et al., 2002; Higuchi et al., 2006; Kane et al., 2006; Li et al., 2004; Washizu et al., 2000a,b) and therefore one that hepatocytes may seek to satisfy directly or indirectly; therefore, urea maximization is a reasonable case study for the approach.
(2) One can assume a pseudo-steady-state for the hepatic metabolic network, which means that the rate of change of intracellular compositions is small (Stephanopoulos et al., 1998). This assumption is valid because the

concentrations of intracellular metabolites are very small (~nmol/million cell) compared to their turnover, relative flux (~μmol/million cell/day) × measurement time (1 day) (Marin-Sanguino and Torres, 2000).

(3) We assume that the designed supplementation fine tunes rather than radically alters hepatocyte metabolism. Therefore, we can use a prior set of experimental measurements and calculated fluxes as representative of the physiological space state of hepatocytes. The prior data considered include: high/low insulin preconditioning followed by unsupplemented plasma cultures (HI_NAA_NH/LI_NAA_NH), and high/low insulin preconditioning followed by amino acid-supplemented plasma cultures (HI_RAA_NH/LI_RAA_NH) (see Table I for details of experimental conditions). The range of experimental data (Chan et al., 2003c) defines the minimum and maximum values (bounds) of measured fluxes in the model (Appendix B).

(4) The reversibility of each reaction is determined based on the information given in the metabolic map of KEGG (Kanehisa and Goto, 2000) and is listed in Appendix A. Reversible reactions are allowed to take either positive or negative values, whereas irreversible reactions are restricted to positive values (Appendix B).

The FBA Model (I) leads to one flux distribution that maximizes the objective function and is consistent with the imposed constraints; however, it is possible that alternative flux distribution exists that produces the same maximal output but in which a subset of the reactions exhibits different values or reaction directionalities (Price et al., 2004). The need to search for all alternative solution has been demonstrated previously for the central metabolism of *E. coli* (Lee et al., 2000b). The solution strategy involves a recursive mixed integer linear programming (MILP) to enumerate all solutions. To apply this approach, model (I) is first reformulated to its canonical form (Chvatal, 1983) by introducing slack variable s^L, s^U for the lower and upper bounds of v, respectively.

$$\begin{aligned} &\text{Max}: Z = \alpha^T z \\ \text{Subject to}: \; &Bz = q \\ &z \geq 0 \end{aligned} \tag{2}$$

where $z = \begin{bmatrix} s^L \\ s^U \end{bmatrix}, \alpha = \begin{bmatrix} c \\ 0 \end{bmatrix}, B = \begin{bmatrix} S & 0 \\ I & I \end{bmatrix}, q = \begin{bmatrix} -Sv^{\min} \\ v^{\max} - v^{\min} \end{bmatrix}$; c is an N-dimensional column vector, but only the element related to urea production is equal to 1, and the others are zero.

Next, two types of binary variables are introduced to make sure that all different bases are examined. Binary variable y is used to identify a new basis set in each iteration, whereas another binary variable w represents the variable that does not belong to the basis and takes the value of zero. Therefore, model (II) is rewritten as MILP problem as follows:

$$\begin{aligned} &\text{Max}: Z^P = \alpha^T z \\ &Bz = q \\ &\sum_{i \in NZ^{P-1}} y_i \geq 1 \\ \text{Subject to}: \; &\sum_{i \in NZ^p} w_i \leq |NZ^p| - 1, \; p = 1, 2, \ldots, \; P-1 \\ &0 \leq z_i \leq Uw_i, i \in I \\ &y_i + w_i \leq 1, i \in NZ^{P-1} \\ &z \geq 0 \end{aligned} \tag{3}$$

where p is an iteration counter; y_i is a binary variable that takes the value of 1 if z_i is non-zero basic variable at the previous iteration $p-1$, otherwise it takes the value of 0 and remains in the basis; w_i is a binary variable used to ensure that all non-basic variables are zero; and U is the upper bound of z, chosen arbitrarily to exceed the upper bound of the fluxes. In this model (III), it is chosen to take the value of 1,000, although any value greater than the maximum overall flux is sufficient. There is a new constraint added in each iteration p to eliminate at least one of the non-zero variables of the basis based on the results of the previous iterations,

Table I. Treatment conditions.

Treatment condition	Medium preconditioning	Amino acid supplementation	Hormone supplementation
HI_NAA_NH	High insulin (+)	Unsupplemented (−)	Unsupplemented (−)
LI_NAA_NH	Low insulin (−)	Unsupplemented (−)	Unsupplemented (−)
HI_RAA_NH	High insulin (+)	Reference supplemented (R)	Unsupplemented (−)
LI_RAA_NH	Low insulin (−)	Reference supplemented (R)	Unsupplemented (−)
HI_DAA_NH	High insulin (+)	Designed supplemented (D)	Unsupplemented (−)
LI_DAA_NH	Low insulin (−)	Designed supplemented (D)	Unsupplemented (−)
HI_NAA_WH	High insulin (+)	Unsupplemented (−)	Supplemented (+)
LI_NAA_WH	Low insulin (−)	Unsupplemented (−)	Supplemented (+)
HI_RAA_WH	High insulin (+)	Reference supplemented (R)	Supplemented (+)
LI_RAA_WH	Low insulin (−)	Reference supplemented (R)	Supplemented (+)
HI_DAA_WH	High insulin (+)	Designed supplemented (D)	Supplemented (+)
LI_DAA_WH	Low insulin (−)	Designed supplemented (D)	Supplemented (+)

Three independent variables in the experiments are insulin level (500 mU/mL, HI and, 0.05 mU/mL, LI) during medium preconditioning, amino acid supplementation (unsupplemented, NAA, "reference" supplementation, RAA, "designed" supplementation, DAA), and hormone supplementation (WH) in plasma including 7.5 μg/mL of hydrocortisone and 50 μU/mL of insulin or without hormone supplementation (NH) during plasma exposure.

$p = 1,2,\ldots, p-1$. The algorithm stops when the objective function in the current iteration is less than that of the previous iteration, which means all solutions with the same objective function value have been found.

All mathematical models in this study are implemented using GAMS version 22.4 and solved by CPLEX 9 on a Dell PC (3 GHz, 1 GB of RAM).

Hepatocyte Culture Media

Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Invitrogen Corporation (Carlsbad, CA). Heparinized human plasma was obtained from Innovative Research, Inc. (Novi, MI). Glucagon, hydrocortisone, epidermal growth factor, insulin, Modified Eagle's Medium (MEM) vitamin solution, Eagle's Basal Medium (BME) amino acid solution, RPMI 1640 solution, and all other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Standard hepatocyte C + H medium consists of DMEM supplemented with 10% heat inactivated FBS, 7.0 ng/mL glucagon, 7.5 μg/mL hydrocortisone, 20 μg/L epidermal growth factor, 200 U/mL penicillin, 200 μg/mL streptomycin, and 500 mU/mL insulin (high insulin, HI) or 0.05 mU/mL insulin (low insulin, LI). Type I collagen was prepared by extracting acetic acid-soluble collagen from rat tail tendons as described by Dunn et al. (1991). A collagen gelling solution is prepared by mixing rat type I collagen with DMEM to a final concentration of 1 mg/mL.

L-arginine free medium consists of L-arginine free RPMI 1640 plus 10% FBS, and 500 mU/mL insulin with concentrations of glucagon, hydrocortisone, epidermal growth factor, penicillin, streptomycin identical to those in standard hepatocyte C + H medium.

Plasma solution was prepared by mixing 81 mL of heparinized human plasma with 2 mL MEM vitamin solution, 1 mL streptomycin–penicillin solution, and 5 mL buffer solution with 2.56% (w/v) sodium monophosphate and 7.5% (w/v) sodium bicarbonate. Two types of amino acid supplemented plasma were prepared—a "reference" supplementation (RAA) based on previous published work, and a "designed" supplementation (DAA) based on our FBA predictions. To prepare RAA plasma, 2 mL of 200 mM glutamine, 8 mL (50×) BME amino acid solution were added to the media. To prepare DAA plasma, amino acids were added to the media according to the final compositions based on the calculation from flux balance (see Table II in Results Section).

Design of Experiment: Amino Acid Supplementation

Cryopreserved rat hepatocytes were purchased from Xenotech LLP (Lenexa, KS). The Xenotech protocol for thawing cryopreserved hepatocytes was followed. The viability of reconstituted cells was estimated by trypan blue exclusion.

The experimental timeline is shown in Figure 1. A quantity of 0.4 mL of the mixed collagen solution was poured in each well of 6-well plates and incubated at 37°C and 10% CO_2 for at least 90 min until the gel solidified. Suspended cryopreserved hepatocytes were seeded in each collagen-coated well at a density of 1×10^6 cells/mL. After 24 h incubation, culture medium was aspirated and 0.4 mL of collagen solution was added, which gelled to form a collagen sandwich. After 90 min gelation time, 0.8 mL fresh culture medium was added. Fresh medium was exchanged with spent medium daily for 6 days prior to plasma exposure. The 6-day-old sandwiched hepatocyte cultures

Table II. Composition of designed amino acid supplementation.

	Reference AA	Designed AA
Serine	0.10	0.36
Glycine	0.17	0.41
Tyrosine	0.40	0.72
Valine	0.87	1.49
Alanine	0.29	0.46
Threonine	0.79	1.18
Arginine	0.42	0.56
Histidine	0.35	0.44
Proline	0.18	0.22
Leucine	0.83	1.02
Isoleucine	0.78	0.89
Lysine	0.75	0.79
Aspartate	0.01	0.01
Cysteine	0.13	0.13
Methionine	0.20	0.14
Glutamine	6.85	6.12
Phenylalanine	0.42	0.32
Glutamate	0.11	0.09
Asparagine	0.09	0.01

"Designed" amino acid supplementation (mM) based on flux balance analysis with linear extrapolation between concentration and flux (see text for details), compared with "reference" amino acid supplementation of Yarmush and coworkers (Chan et al., 2003b,c).

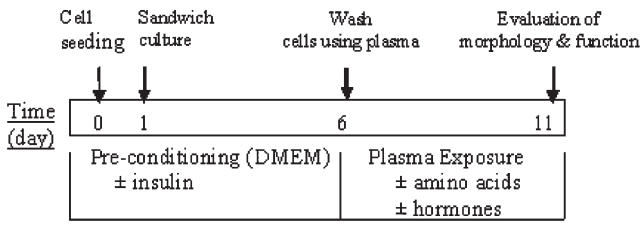

Figure 1. Experimental timeline. Immediately following thawing, cryopreserved hepatocytes were seeded on a layer of collagen at a density of 1×10^6 cells/mL, and a second layer of collagen was added one day later. Then normal C + H culture medium with either physiological or supraphysiological insulin levels was added and exchanged daily for 6 days of preconditioning. Hepatocytes were subsequently exposed to plasma in the presence of varying amino acid and hormone supplementations for an additional 5 days.

were subsequently exposed to either unsupplemented (NAA) plasma, RAA plasma or DAA plasma for an additional 5 days. At the end of the experiment, culture supernatants were collected and stored at 4°C prior to analysis.

The three independent variables used in the design of the experiments are: (1) the insulin level (500 mU/mL, HI and, 0.05 mU/mL, LI) during preconditioning culture with DMEM, (2) amino acid supplementation in the plasma (unsupplemented, NAA, "reference" supplementation, RAA, "designed" supplementation, DAA) and (3) hormone supplementation in plasma including 7.5 μg/mL of hydrocortisone and 0.05 mU/mL of insulin (WH) or without hormone supplementation (NH) during plasma exposure. Twelve treatment combinations are listed in Table I.

After 5 days exposure to the various plasma conditions, the morphology of the hepatocytes cultured in a collagen sandwich medium was captured using an Olympus CKX41 microscope. Urea was measured using a standard, commercial kit (Sigma Chemical). Albumin concentrations were determined by enzyme-linked immunosorbent assay (ELISA) using purified rat albumin (MP Biomedicals, Solon, OH) and peroxidase-conjugated antibody for detection (MP Biomedicals). Free fatty acids were measured in the presence of the enzyme acetyl-COA synthetase using a commercial enzymatic colorimetric kit (Roche, Indianapolis, IN). Triglyceride levels were measured with a commercial kit (Stanbio, Boerne, TX) based on the release of glycerol catalyzed by lipoprotein lipase. β-hydroxybutyrate was quantified using a commercial kit (Stanbio) based on the appearance of NADH in the conversion to acetoacetate by adding β-hydroxybutyrate dehydrogenase, and acetoacetate was quantified based on the disappearance of NADH in the conversion to β-hydroxybutyrate by adding the same enzyme in a different pH buffer (Chan et al., 2003a). Each flux value was calculated by subtracting the supernatant concentration from the unconditioned medium concentration and dividing by the cell number and the time interval over which the medium was exposed to cells.

At the end of experiment, live cells were distinguished by green fluorescence generated by the fluorogenic reagent calcein AM (Molecular Probes, Eugene, OR), and dead cells were identified by red fluorescence resulting from nucleic acids bound with ethidium homodimer (Molecular Probes). Cell viability was calculated as the ratio of the number of live cells to the total number of cells, which was quantified after killing all of the cells using 75% methanol at the end of the experiment.

Design of Experiment: Supplementation of Arginine

Hepatocytes isolated from adult F344 male rat were suspended in standard C + H medium and cultured in a collagen sandwich configuration at a density of 1×10^6 cells/mL for two days. Hepatocytes were subsequently switched to media with varying L-arginine concentration (0.2, 0.4, 1, 2, 4, and 8 mM) for 3 days. Fresh medium was exchanged with spent medium and supernatant was collected daily. At the end of the experiment, urea production was measured using a standard commercial kit (Sigma Chemical).

Statistical Analysis

The experimental results are presented as mean ± standard deviation for three replicate culture wells. Comparison among groups was performed using analysis of variance (ANOVA) followed by Tukey's studentized range test, performed with SAS software (SAS Institute, Inc., Cary, NC).

Results

Rational Design of Amino Acid Supplementation

The FBA model predictions maximize urea secretion while maintaining exchange fluxes within bounds based on prior experimental observations and satisfying the constraints of material balance at pseudo-steady-state. The solution of model III (Appendix C) offers insights into the flux distribution of exchange reactions (primarily amino acid transport) required for optimization of urea production and allows analysis of the flexibility of the hepatic network via the computation of multiple solutions. With the resulting distribution of exchange fluxes, a level of urea production is predicted (6.81 μmol/million cells/day) that is increased more than twofold compared with urea production reported in the literature for hepatocytes exposed to plasma (Chan et al., 2003a,c). Eight fluxes distributions are found using model (III) that each satisfies all the metabolic constraints and results in the same maximal urea flux. Eleven out of 76 reactions in the overall network illustrate some flexibility, including gluconeogenesis (R1-8), palmitate uptake (R72), production of glyceraldehyde-3-P (R73) and cholesterol eater consumption (R75). These are summarized in Appendix C. Further analysis illustrates that in all cases the difference between the flux value of reaction 6 (oxaloacetate transformation to phosphoenolpyruvate) and reaction 7 (pyruvate transformation to oxaloacetate) is identical, so that the net flux of oxaloacetate entering the TCA cycle and urea cycle is constant. Each of the remaining fluxes has the same value in all eight flux distributions, suggesting that overall the network is not very flexible in attaining the theoretically optimal urea secretion.

The amino acid uptake/secretion rates (v_{18}, v_{22}, v_{24}, v_{26}, v_{29}, v_{35}, v_{37}, v_{44}, v_{58}–v_{68}) associated with theoretically optimal urea production (Appendix C) constitute target fluxes, v_{target}, which are used to calculate the necessary concentration of amino acids in the media (i.e., supplementation) by assuming a linear relationship between amino acid supplementation and corresponding fluxes in the model (Sharma et al., 2005). This extrapolation is likely

to be a reasonable first-order approximation because the designed amino acid supplementation (DAA) is a perturbation from the reference supplementation (RAA), and amino acid flux data are available for both the non-supplemented (NAA) and RAA states as well as for culture in medium only (Chan et al., 2003b,c) (Fig. 2).

Comparing the reference supplementations of amino acids (Table II), three groups of amino acids are identified. Compared with reference supplementation (Chan et al., 2003b), the designed supplementation levels of eleven amino acids are greater by at least 10%. In particular, serine and glycine supplementation levels are 260% and 140% increased, respectively, compared to reference levels. The designed arginine supplementation is a more modest one-third higher than reference level. As arginine is directly transformed to urea production in the urea cycle, it is clear that increasing arginine supplementation should increase the urea production, and this would be a trivial route to increased urea production. However, the other amino acids collectively promote urea production via the TCA cycle, which is linked to the urea cycle by the metabolite fumarate, and an increased turnover of the TCA cycle can help to balance production of fumarate in the state of high urea cycle activity. Thus, optimization of urea production requires activation of multiple, independent pathways that require amino acids as substrates, supporting the hypothesis that amino acid supplementation can directly modulate urea production capabilities in hepatocytes.

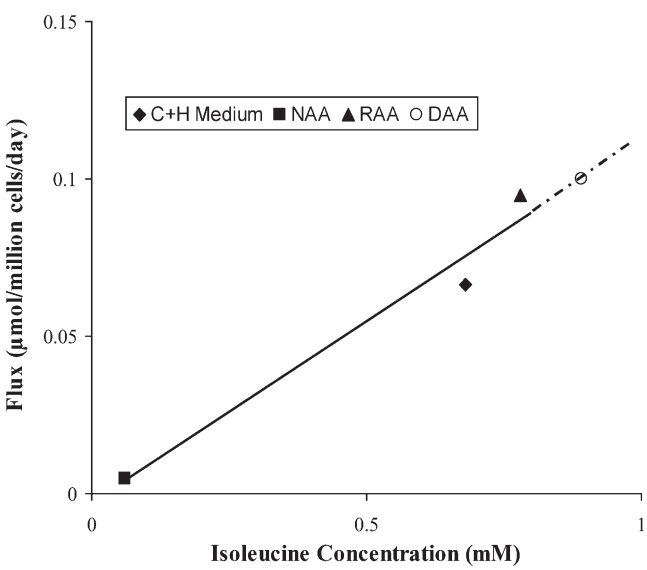

Figure 2. Designed amino acid supplementation levels are calculated by assuming a linear relationship between amino acid supplementation (concentrations) and fluxes (uptake or secretion rates). Previous data for the media concentrations and average fluxes in normal C + H medium, as well as in plasma-containing media with or without amino acid supplementation were utilized (Chan et al., 2003a,c). For example, isoleucine concentration in the designed amino acid supplementation is calculated as an extrapolation point of the linear relation between isoleucine supplementation in the medium (axis x) and isoleucine uptake fluxes (axis y) ($y = 0.1159x - 0.0032$, $R^2 = 0.9656$).

The designed supplementation of three amino acids, namely lysine, aspartate, and cysteine, is similar to reference values. Moreover, supplementations for methionine, glutamine, phenylalanine, glutamate, and asparagine are predicted to be lower than the experimental value. Many of these amino acids also can feed the TCA cycle; thus, their lower values should not be interpreted as meaning that they cannot lead to urea production but rather that they are either less efficiently transformed to urea or are already present in sufficient quantities for a balanced metabolism. For those amino acids, the DAA was formulated with identical amounts to the RAA.

Effect of Amino Acid Supplementation on Hepatocyte Exposed to Plasma

The behavior of cryopreserved rat hepatocytes exposed to heparinized plasma was compared among groups receiving no amino acid supplementation (beyond what is normally in plasma, labeled NAA), reference supplementation (RAA) and designed supplementation (DAA) of amino acids. Each of these amino acid supplementations was evaluated for cells preconditioned in either high (HI) or low (LI) levels of insulin. Furthermore, groups were evaluated with (WH) and without (NH) hormone supplementation consisting of hydrocortisone and insulin during plasma exposure (Table I). The preconditioning and hormone additions have been found previously to influence the metabolism of fresh, primary rat hepatocytes exposed to plasma (Chan et al., 2003a,c).

First, the morphology of hepatocytes was examined over the course of a 5-day exposure to heparinized plasma under each treatment condition for the appearance of intracellular lipid droplets, a manifestation of stored triglycerides (TG) (Stefanovich et al., 1996). Control cells, which were preconditioned in low-insulin medium and never exposed to plasma, did not exhibit any change in morphology, even after 11 days of culture (Fig. 3g). During unsupplemented plasma exposure, the nuclei of hepatocytes became enlarged and were obscured by lipid droplets (Fig. 3a and d). However, after plasma exposure with RAA supplementation (Fig. 3b and e) or DAA supplementation (Fig. 3c and f), the boundaries between hepatocytes were more clear and bright, formed fewer large lipid droplets, and exhibited similar morphology as in the standard medium of low-insulin precondition (Fig. 3g). This suggests that the amino acid supplementation drives metabolism in a way that leads to reduced lipid accumulation. Hormone (insulin and hydrocortisone, designated as WH) addition along with amino acids during plasma exposure augmented the ability of amino acid supplementation to maintain native hepatocyte morphology (compare Fig. 3e and f to Fig. 3b and c). The morphological trends after high insulin preconditions were very similar to those after low insulin preconditioning (data not shown).

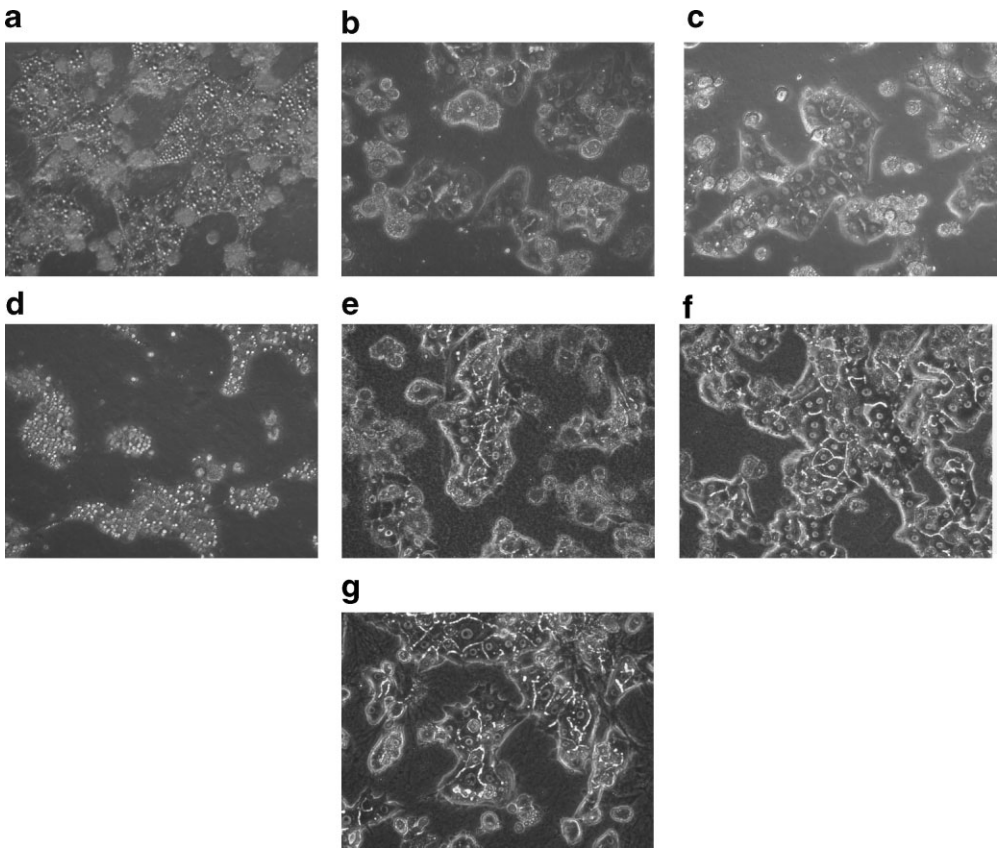

Figure 3. The morphology of hepatocytes was examined on day 11, following 6 days of preconditioning and 5 days of plasma exposure under each treatment condition: (**a**) LI_NAA_NH (**b**) LI_RAA_NH (**c**) LI_DAA_NH (**d**) LI_NAA_WH (**e**) LI_RAA_WH (**f**) LI_DAA_WH (**g**) control cells, which were preconditioned in low-insulin medium and never exposed to plasma. Scale Bar, 100 μm.

A major concern in the use of cryopreserved hepatocytes is loss of viability following the thawing process. To ensure that the various culture conditions, that is, amino acid and hormone supplementation regimens, did not impact differentially on viability, we measured the cell viability using calcein/ethidium staining at the end of the experiment, day 11, corresponding to 6 days of preconditioning and 5 days of plasma exposure. After this extended period of culture, much of it in the challenging conditions of plasma exposure, a significant loss of viability at the end of our experiments was observed compared with 75% viability immediately after thawing cryopreserved rat hepatocytes (Fig. 4). However, cell viabilities were similar in all of the exposure conditions at the end of our experiments, simplifying interpretation of changes in metabolic outputs. Nonetheless, the values of all functional markers are normalized to the number of viable cells in a given sample.

Urea and albumin synthesis were measured after 5 days exposure to plasma using each of the supplementation protocols (Fig. 5a and b). Hepatocytes exposed to plasma without amino acid supplementation suffered significant impairment of urea and albumin production regardless of their preconditioning or hormone supplementation status. When either the RAA or DAA supplementation was employed, preconditioning of rat hepatocyte cultures in high insulin medium prior to plasma exposure increased albumin production, but did not significantly change urea production. Reference amino acid supplementation results in a marked increase in urea and albumin production compared to unsupplemented plasma, and this increase is augmented further by the use of the designed amino acid supplementation. Hormone plus amino acid supplementation during plasma exposure resulted in comparable urea and further increased albumin production compared with the corresponding conditions without any hormone.

Because of the strong role for lipid metabolism in mediating the effects of various modifiers (i.e., hormones and amino acids) on differentiated functions of cultured hepatocytes exposed to plasma (Chan et al., 2002; Li et al., 2004), we measured lipid uptake and ketone body formation in this system. Total lipid uptake (TG and palmitate) was not significantly affected by either insulin preconditioning prior to plasma exposure or amino acids/hormone supplementation during plasma exposure. Ketone body synthesis (ketogenesis) is a recognizable step of lipid metabolism, and its final products include β-hydroxybutyrate

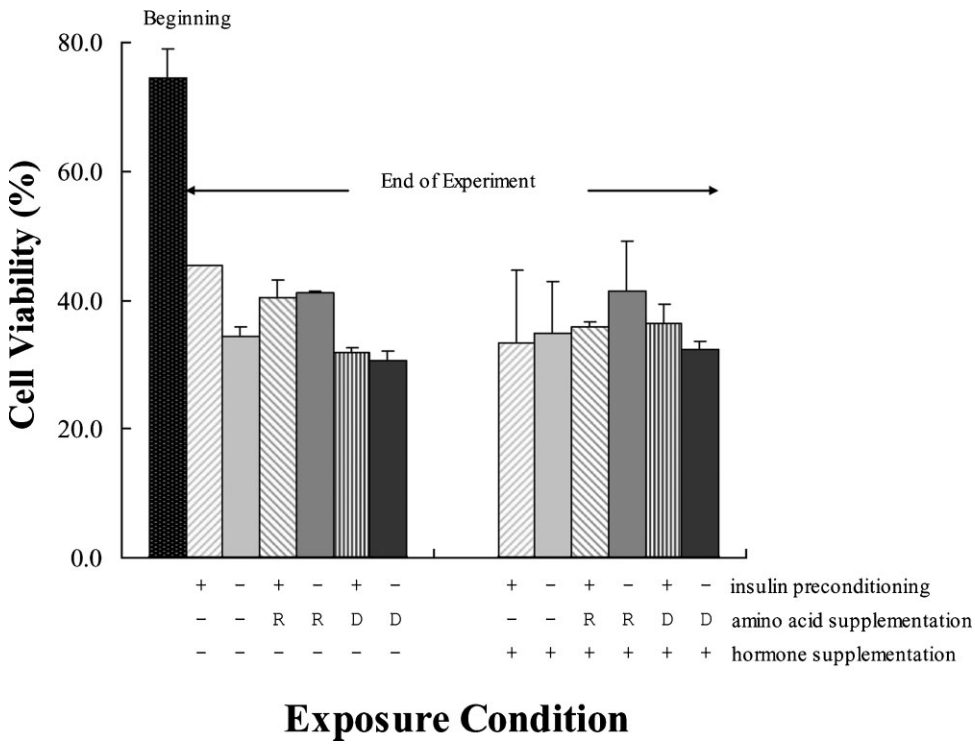

Figure 4. After 11 days of culture, the hepatocyte viability was quantified using calcein/ethidium staining for all 12 culture conditions. The percentage of viable cells is calculated as the ratio of live cells to total cells. The viability after thawing of the cryopreserved hepatocytes, which was determined using trypan blue exclusion assay, is also indicated.

and acetoacetate. The β-hydroxybutyrate production was reduced by supplementing amino acids to plasma independent of hormone supplementation (Fig. 6a). Both the RAA and DAA supplementations were equally effective in reducing β-hydroxybutyrate production. The acetoacetate production was reduced by supplementing hormone to plasma independent of insulin precondition or of amino acid supplementation during plasma exposure (Fig. 6b).

Effect of Arginine Supplementation on Urea Production

In order to determiner whether the observed increase in urea production in supplemented media is due simply to channeling of arginine, rat hepatocytes were cultured in media supplemented with a wide range of arginine levels, holding all other medium components constant. Urea production was found to be linearly increased with increase of arginine supplementation (0.2–4 mM) in the medium (Fig. 7). However, the rate of increase in urea production becomes smaller as arginine concentration increases beyond 4 mM and reaches a maximum peak.

Using arginine as supplementation alone, urea production could only increase by 0.052 μmol/million cells/day when arginine concentration is increased from 0.42 to 0.56 mM (Fig. 7A and B), which is arginine concentration in RAA and DAA design, respectively. Instead, FBA model shows urea production could be increased more than twofold, and experimental data of amino acid supplementation show that urea production could increase more than 0.3 μmol/million cells/day for same addition of arginine supplementation, but with the addition of other amino acid supplementation.

Discussion

Cryopreservation is a necessary step for the practical application of hepatocytes as a resource for in vitro analysis (Garcia et al., 2003) and bioartificial liver devices (Park and Lee, 2005; Watanabe et al., 1997), since cryopreserved hepatocytes can be stored indefinitely, transported to any site, and utilized when desired. In our study, the metabolic state of cryopreserved rat hepatocytes was evaluated under plasma exposure utilizing various cell culture supplementation conditions. A negative consequence of utilizing cryopreserved hepatocytes is the loss of viability from the summed processes of freezing, storage, thawing and culturing (Lloyd et al., 2003). Indeed, we observed a significant loss of viability (32–45%) at the end of our experiments compared with 75% viability at the beginning (Fig. 4). However, we find that the surviving hepatocytes are

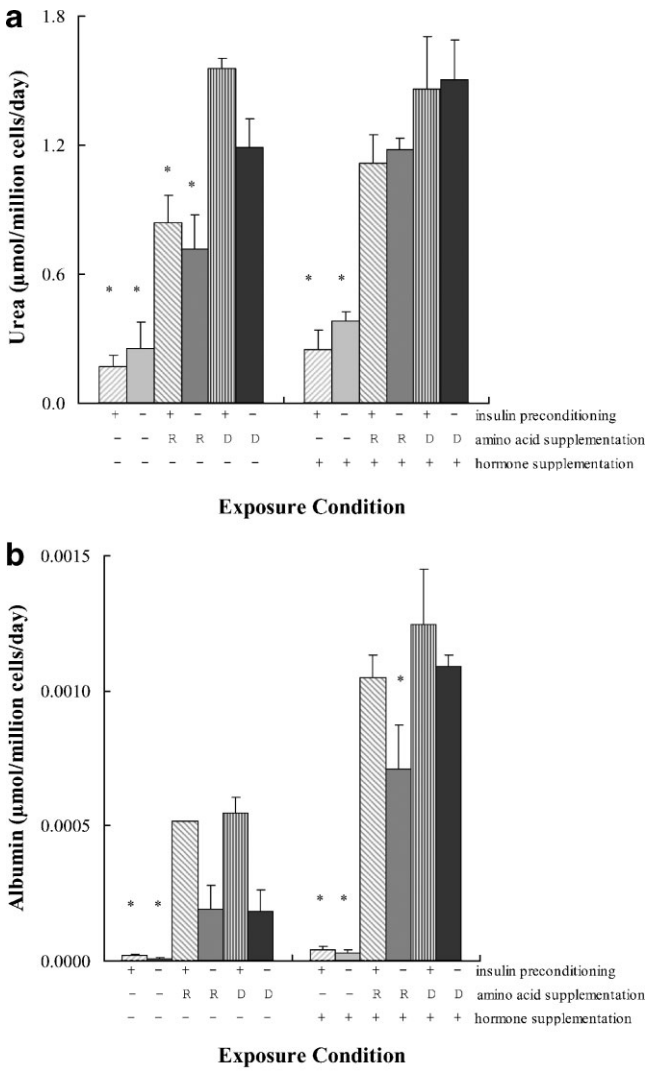

Figure 5. Urea (**a**) and albumin (**b**) production under the twelve experimental conditions: insulin preconditioning level (+, supraphysiological insulin; −, physiological insulin), plasma amino acid level (−, without supplementation; R, "reference" supplementation; D, "designed" supplementation) and hormone supplementation (−, without hormone; +, with hormone) during plasma exposure. Values shown represent means ± standard deviation of three independent measurements. Asterisks (*) indicate significant difference ($P < 0.05$) between the designed supplementation and either the reference supplementation or non-supplemented control for the same insulin precondition and the same hormone supplementation during plasma exposure.

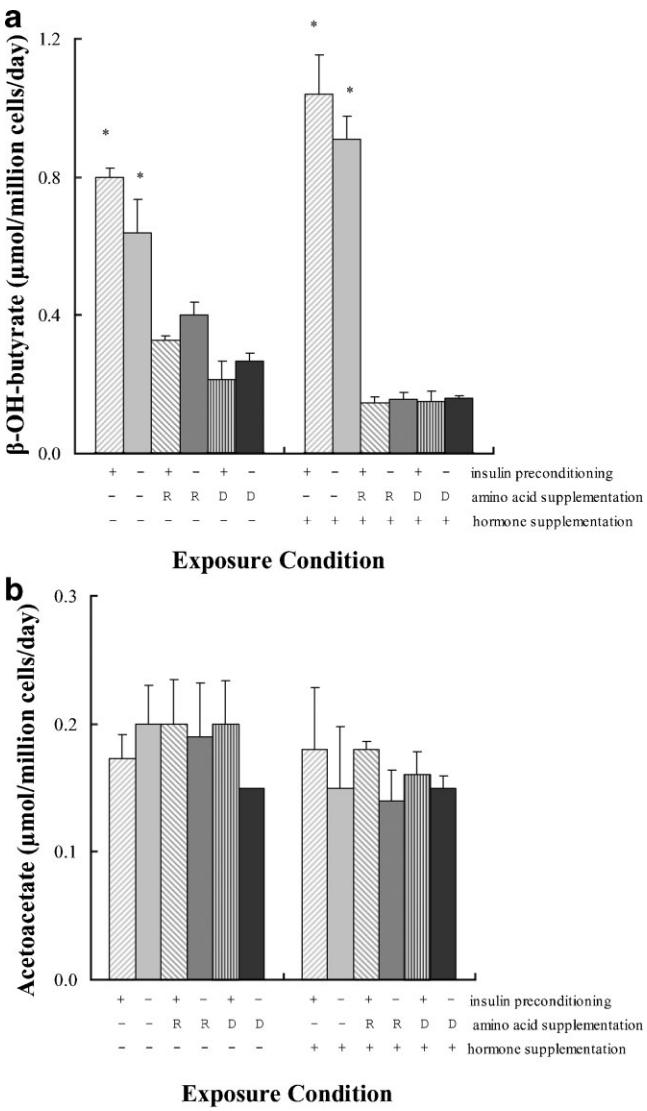

Figure 6. β-hydroxybutyrate (**a**) and acetoacetate (**b**) production under each of the experimental conditions as indicated in Figure 5. Values shown represent means ± standard deviation of three independent measurements. Asterisks (*) indicate significant difference ($P < 0.05$) between the designed supplementation and either the reference supplementation or non-supplemented control for the same insulin precondition and the same hormone supplementation during plasma exposure.

robust in their ability to adopt morphologies akin to freshly isolated hepatocytes (Fig. 3) and to produce levels of differentiated markers only slightly reduced from their freshly isolated counterparts (Chan et al., 2003a).

The effects of supplemented amino acids and hormones to plasma on liver-specific functions have been investigated in previous studies using freshly isolated rat hepatocytes (Chan et al., 2003a); however, the exact composition of the supplementation was derived empirically. The RAA supplementation was derived by addition of glutamine, deemed to be a critical amino acid for hepatocyte metabolism, mixed with a commercial amino acid supplementation, Basal Medium Eagle (BME). This has proven to be an effective starting point; however, we hypothesized that it could be fine-tuned using a rational design strategy based on FBA. It is not feasible to merely supplement all amino acids at arbitrarily high levels, as such a concentrated medium with amino acid supplementation would become harmful to cells due to high osmolarity (Washizu et al., 2000a). On the other hand, it has been shown that addition to plasma of single amino acids, such as glycine or glutamine, is insufficient to maintain hepatocyte-specific functions (Washizu et al., 2000a). We found here that addition of arginine is not

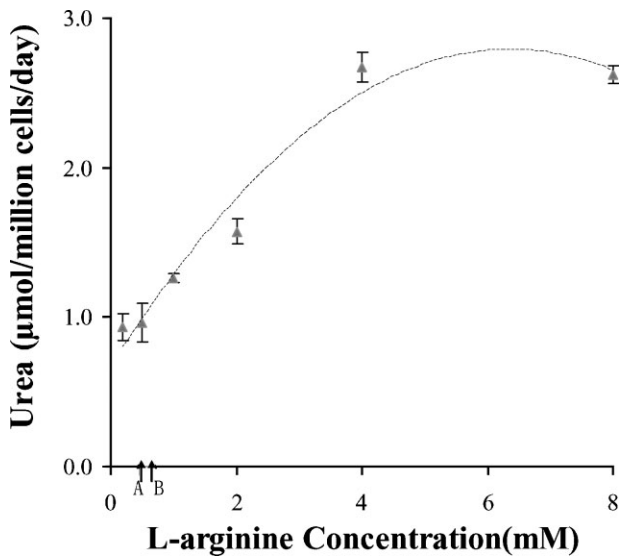

Figure 7. Urea production under arginine exclusive supplementation. Hepatocytes were cultured in L-arginine free medium with the addition of L-arginine at the concentrations specified. Arginine concentration in RAA and DAA is represented in A, and B, respectively.

enough to significantly improve urea production. These studies suggest that a balanced supplementation is necessary to improve liver-specific functions, but it is not clear from such studies how to design an effective amino acid supplementation from data on addition of a single amino acid. As such, a quantitative, rational design methodology for amino acid supplementation would be very useful for metabolic engineering. Our studies represent a first attempt to use FBA to quantitatively design amino acid supplementation to the plasma for cultured hepatocytes.

This rational design method accounts for multiple flux variables and their interconnections considering a complex hepatic network of interdependent chemical reactions by using linear programming and MILP. In accordance with a number of previous studies, the network includes those reactions and pathways known to play a significant role in nitrogen metabolism (Chan et al., 2003b,c; Lee et al., 2003; Nagrath et al., 2007; Sharma et al., 2005; Uygun et al., 2007). From this standpoint, the production and degradation of protein is a potentially important consideration. Albumin production, by far the most abundantly produced secreted protein and the only protein included in the network, exhibits a flux that is very small compared to other fluxes. Protein degradation was examined in a previous study by adding breakdown of apolipoprptein B into the system (Chan et al., 2003a), with the result that it was found to play a negligible role in hepatic network. An overall nitrogen balance calculated as the difference between nitrogen uptaken and excreted in the forms of amino acids, ammonia, urea and albumin production closes within 10%, which suggests that other forms of nitrogen not included in the model (mainly protein and nucleic acids) play an insignificant role in the overall nitrogen balance. Although energy balance was not explicitly due to the fact that many ATP consuming reactions were not included in the network, thermodynamic constraints were included by giving positive flux bounds for each irreversible reaction in the FBA model. To confirm that the results are thermodynamically feasible, each flux calculated from FBA model was multiplied by the Gibbs free energy change (ΔG) and the summation $\sum_j \Delta G_j v_j$ was evaluated and found to be negative.

The single objective of urea synthesis was chosen for FBA in this study. In FBA, a single objective can be achieved via multiple, distinct flux distributions. In this case, FBA produced eight different flux distributions that achieve the same urea production and satisfy the same constraints. However, these distributions differed primarily in their glucose utilization and not at all in their amino acid uptake. Therefore, only one designed amino acid supplementation was generated (Table II).

While urea synthesis is only one of many functions that hepatocytes must perform, a high level of urea production is generally regarded as indicative of healthy, metabolically active hepatocytes. Utilizing the idea of multi-objective optimization, previous work has shown that another liver specific function, albumin production, is compromised when reaching the highest possible urea production (Nagrath et al., 2007; Sharma et al., 2005). However, high levels of both functions are quite feasible. In Figure 8, point A presents the result from FBA model which is the global maximum urea production, corresponding to very low albumin production. However, in moving from points A to

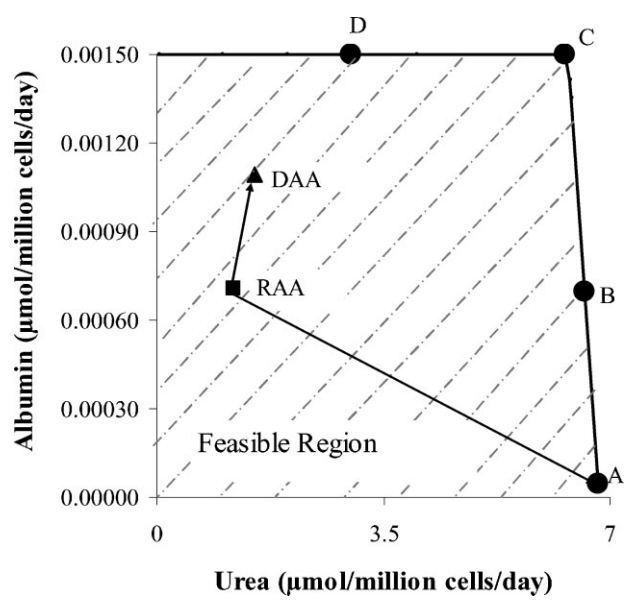

Figure 8. Pareto set for bi-objective problem of urea and albumin synthesis in hepatic network. The black circles are results calculated from multiobjective model, and point A is the result for maximization of urea production in the FBA model. RAA and DAA are experimental results as defined before. The hashed area corresponds to the feasible region of the model.

C through point B, albumin production is increased dramatically with very little decrease in urea synthesis. On the boundary containing points D and C, albumin production is maximal, with a wide range of possible values for the urea production. These points illustrate the Pareto set (Fig. 8), which defines the feasible solution space for the FBA model.

Experimentally, we found that albumin secretion was either unaffected or somewhat increased, depending on the hormone exposure, as urea production increased in going from the RAA to DAA supplementation. This finding remains consistent with the metabolically feasible region shown in Figure 8. Given that the liver performs a myriad of metabolic functions in vivo, it is likely that the regulatory program of hepatocytes is geared towards maintaining many functions at sub-optimal levels rather than any particular function at its apparent maximum.

Hormone levels either during preconditioning of hepatocytes or during plasma exposure may affect their metabolic functions. Previous studies using freshly isolated rat hepatocytes showed that insulin preconditioning does not significantly affect urea synthesis in the absence of amino acids; however, when supplemented with amino acids, low insulin preconditioning resulted in increased urea production compared with that of high insulin preconditioning (Li et al., 2004). In our studies using cryopreserved rat hepatocytes, the level of insulin did not affect urea production significantly regardless of amino acid composition. On the other hand, albumin production was enhanced by supraphysiological insulin preconditioning (Fig. 5b).

Previous studies found that physiological levels of hydrocortisone (50–250 ng/mL) in the culture medium in precondition are necessary to maintain differentiated functions of hepatocytes in sandwich culture (Dunn et al., 1991). However, we found that hydrocortisone supplementation without amino acid supplementation during plasma exposure did not alter the morphological appearance of cryopreserved hepatocytes or their production of urea and albumin. Rather, supplementation of amino acids in combination with hormone preconditioning is required to increase urea synthesis and albumin production. The synergistic effects of hormone addition and amino acid supplementation could be due to hormonal regulation of amino acid transport systems in primary rat hepatocytes. For example, hydrocortisone has been observed to upgrade transport system N for glutamine, histidine and asparagine (Gebhardt and Kleemann, 1987).

The decline in liver-specific functions of hepatocytes during plasma exposure without amino acid supplementation may be due partially to cell death (Washizu et al., 2001). However, calcein and ethidium homodimer staining of the cells indicates similar proportions of viable cells in all of the exposure conditions (Fig. 4). The loss of function during human plasma exposure is associated with intracellular triglycerol accumulation. We observed microscopically high lipid accumulation in plasma without amino acid supplementation for cultured cryopreserved hepatocytes and a reduced rate of TG accumulation with amino acid supplementation. The accumulation of TG in non-supplemented cultures can be attributed to the increased uptake of lipids in plasma (Matthew et al., 1996) and/or to a reduction in free fatty acids oxidation by cultured hepatocytes (Stefanovich et al., 1996). Therefore, we evaluated the correlation between urea synthesis and lipid accumulation through free fatty acids pathway. Total lipid (TG and FBA) uptake by cryopreserved hepatocytes was invariant with any of the experimental variables (Fig. 6). It is thus more likely that the lowered lipid accumulation under supplemented conditions was due to an increase in fatty acids conversion to acetyl-CoA. One of the end products of acetyl-CoA is ketone bodies (Fig. 6). The decrease in ketone body production suggests that more acetyl-CoA enters into the TCA cycle and produces urea, which is consistent with FBA predictions and experimental observations.

It is significant that, despite the approximations made in the FBA model, the DAA supplementation did indeed lead to a higher urea production. The fact that albumin production was increased and β-hydroxybutyrate decreased in the DAA conditions compared with exposure condition without amino acid supplementation suggests that the resulting physiological state is a metabolically healthy one for the cultured, previously cryopreserved, hepatocytes used in this study. Nonetheless, the observed synthesis rate of urea was still lower than the value predicted by the linear programming model. It is likely that one factor contributing to a lower than predicted urea output is that some designed amino acid fluxes might not be achieved due to deviations from the linear extrapolation used to design the supplementation or due to competitive transport limitations, since some amino acids make use of a common transporter. For example, lysine, arginine, and ornithine use the same cationic amino acid transporter (CATs) in cell culture of rat hepatocytes (Lerzynski et al., 2006). Regulation by amino acid transporters is an important area for future investigation, as the resulting flux-supplementation functions, which may be nonlinear in the presence of transport limitations, can be added to FBA as capacity constraints to recalculate the amino acid supplementation for maximal urea production. Furthermore, new pretreatments can be designed to enhance the capacity of needed amino acid transporters, such as adjusting sodium concentration or the hormone level (Gu et al., 2005; Kitiyakara et al., 2001).

In summary, this study presents a novel approach to design rationally an amino acid profile for enhanced hepatocyte functions during plasma exposure. The fact that the DAA promoted a metabolically healthy phenotypic with higher levels of liver-specific functions (urea and albumin synthesis), represents a proof of principle for the approach. Near-term improvements may be made in the hepatocyte cultures employed here by incorporating the amino acid transport mechanism and hormone effects regarding the metabolic capacity into the model so that a more accurate amino acid and hormone supplementation can be derived. As we characterize and incorporate such additional system

constraints into the model, the approach will move closer to the ultimate goal of achieving a supplementation that is optimal for hepatocyte cultures the general case yet also can be tuned to meet variations in operating conditions.

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Appendix A

The stoichiometric model for the hepatic metabolic network

No. and enzyme	Stoichiometry
1 Glucose-6-Pase	Glucose-6-P + H_2O → glucose + P_i
2 Phosphoglucose isomerase	Fructose-6-P ↔ glucose-6-P
3 Fructose-1,6-P_2ase-1	Fructose-1,6-P_2 + H_2O → fructose-6-P + P_i
4 Two steps	2-Glyceraldehyde-3-P ↔ fructose-1,6-P_2
5 Four steps	Phosphoenolpyruvate + NADH + H^+ + ATP + H_2O ↔ Glyceraldehyde-3-P + P_i + NAD^+ + ADP
6 PEPCK	Oxaloacetate + GTP → phosphoenolpyruvate + GDP + CO_2
7 Pyruvate carboxylase	Pyruvate + CO_2 + ATP + H_2O → oxaloacetate + ADP + P_i + $2H^+$
8 Lactate dehydrogenase	Lactate + NAD^+ ↔ pyruvate + NADH + H^+
9 Citrate synthase	Oxaloacetate + acetyl-CoA + H_2O → citrate + CoA + H^+
10 Isocitrate dehydrogenase	Citrate + NAD^+ ↔ α-ketoglutarate + CO_2 + NADH
11 α-ketoglutarate dehydrogenase	NAD^+ + α-ketoglutarate + CoA → succinyl-CoA + CO_2 + NADH + H^+
12 Succinyl-CoA synthetase, succinate dehydrogenase	Succinyl-CoA + P_i + GDH + FAD ↔ fumarate + GTP + $FADH_2$ + CoA
13 Fumarase	Fumarate + H_2O ↔ malate
14 Malate dehydrogenase	Malate + NAD^+ ↔ oxaloacetate + NADH + H^+
15 Arginase	Arginine + H_2O → urea + ornithine
16 Carbamoyl-P-synthetase I, ornithine transcabamylase	Ornithine + CO_2 + NH_4^+ + 2ATP + H_2O ↔ citrulline + 2ADP + $2P_i$ + $3H^+$
17 Argininosuccinase, Argininosuccinate synthetase	Citrulline + aspartate + ATP → arginine + fumarate + AMP + PP_i
18	Arginine uptake
19	NH_4^+ uptake
20	Ornithine secretion
21 Alanine aminotransferase	Alanine + $0.5NAD^+$ + $0.05NADP^+$ + H_2O → pyruvate + NH_4^+ + 0.5NADH + 0.5NADH + H^+
22	Alanine uptake
23	Serine → NH_4^+ + pyruvate
24	Serine uptake
25 3-mercaptopyruvate sulfurtransferase, transminase	Cysteine + $0.5NAD^+$ + $0.5NADP^+$ + H_2O + SO_3^{2-} ↔ pyruvate + thiosulfate + NH_4^+ + 0.5NADH + 0.5NADPH + H^+
26	Cysteine uptake
27 Serine hydroxymethyl transferase	Threonine + NAD^+ → glycine + acetyl-CoA + NADH
28 Glycine DH, Aminomethytransferase, dihydrolipoyl DH	Glycine + NAD^+ + H_4folate ↔ serine + N^5,N^{10}-CH_2-H_4folate + NADH + NH_4^+
29	Glycine uptake
30 Nine steps	Tryptophan + $3H_2O$ + $3O_2$ + CoA + $3NAD^+$ + FAD → $3CO_2$ + $FADH_2$ + 3NADH + $4H^+$ + NH_4^+ + acetoacetyl-CoA
31 Three steps	Propionyl-CoA + ATP + CO_2 → succinyl-CoA + AMP + PP_i
32 Eight steps	Lysine + $3H_2O$ + $5NAD^+$ + FAD + CoA → $2NH_4^+$ + $5H^+$ + 5NADH + $2CO_2$ + $FADH_2$ + acetoacetyl-CoA
33 Phenylalanine hydroxylase	Phenylalanine + H_4biopterin + O_2 → H_2biopterin + Tyrosine + H_2O
34 Five steps	Tyrosine + $0.5NAD^+$ + 0.5NADP + H_2O + $2O_2$ → NH_4^+ + $0.5NADH^+$ + 0.5NADPH + H^+ + CO_2 + fumarate + Acetoacetate
35	Tyrosine uptake
36 Glutamate dehydrogenase I	Glutamate + $0.5NAD^+$ + $0.5NADP^+$ + H_2O ↔ NH_4^+ + α-ketoglutarate + 0.5NADP + 0.5NADPH + H^+
37	Glutamate uptake
38 Glutaminase	Glutamine + H_2O → glutamate + NH_4^+
39 Two steps	Ornithine + NAD^+ + $NADP^+$ + H_2O → glutamate + NH_4^+ + NADH + NADPH + H^+
40 Three steps	Proline + $0.5O_2$ + $0.5NAD^+$ + $0.5NADP^+$ → glutamate + 0.5NADH + 0.5NADPH + H^+
41 Four steps	Histidine + H_4folate + $2H_2O$ → NH_4^+ + N5-formiminoH_4folate + glutamate
42 Five steps	Methionine + ATP + serine + NAD^+ + CoA → PP_i + P_i + adenosine + cysteine + NADH + CO_2 + NH_4^+ + propionyl-CoA
43 Aspartate aminotransferase	Aspartate + $0.5NAD^+$ + $0.5NADP^+$ + H_2O ↔ oxaloacetate + NH_4^+ + 0.5NADH + 0.5NADPH + H^+

(*Continued*)

No. and enzyme	Stoichiometry
44	Aspartate uptake
45 Asparaginase	Asparagine + H_2O → aspartate + NH_4^+
46 Fatty acid oxidation	Palmitate + ATP + 7FAD + 7NAD^+ → 8acetyl-CoA + 7$FADH_2$ + 7NADH + AMP + PP_i
47 Thiolase	2Acetyl-CoA ↔ acetoacetyl-CoA + CoA
48 Two steps	Acetoacetyl-CoA + H_2O → acetoacetate + CoA
49	Acetoacetate production
50 β-Hydroxyburate dehydrogenase	Acetoacetate + NADH + H^+ ↔ β-OH-butyrate + NAD^+
51 ECT	NADH + H^+ + 0.5O_2 + 3ADP → NAD + H_2O + 3ATP
52 ECT	$FADH_2$ + 0.5O_2 + 2ADP → FAD + H_2O + 2ATP
53	O_2 uptake
54 Glucose-6-P dehydrogenase	Glucose-6-P + 12$NADP^+$ + 7H_2O → 6CO_2 + 12NADPH + 12H^+ + P_i
55 Seven steps	Valine + 0.5$NADP^+$ + CoA + 2H_2O + 3.5NAD^+ + FAD → NH_4^+ + 0.5NADPH + 3H^+ + 3.5NADH + $FADH_2$ + 2CO_2 + propionyl-CoA
56 Six steps	Isoleucine + 0.5$NADP^+$ + 2H_2O + 2.5NAD^+ + FAD + 2CoA → NH_4^+ + 0.5NADPH + 3H^+ + 2.5NADH + $FADH_2$ + CO_2 + propionyl-CoA + acetyl-CoA
57 Six steps	Leucine + 0.5$NADP^+$ + H_2O + 1.5NAD^+ + FAD + ATP + CoA → NH_4^+ + 1.5NADH + 0.5NADPH + 2H^+ + $FADH_2$ + ADP + P_i + acetoacetate + acetyl-CoA
58	Threonine uptake
59	Lysine uptake
60	Phenylalanine uptake
61	Glutamine uptake
62	Proline uptake
63	Histidine uptake
64	Methionine uptake
65	Asparagine uptake
66	Valine uptake
67	Isoleucine uptake
68	Leucine uptake
69	24ARG + 32ASP + 61ALA + 24SER + 35CYS + 57GLU + 17GLY + 21TRY + 33THR + 53LYS + 26PHE + 25GLN + 30PRO + 15HIS + 6MET + 20ASN + TRP + 35VAL + 13ISO + 56LEU + 2332ATP → albumin + 2332ADP + 2332P_i
70 lipoprotein and hepatic lipase	TG + 3H_2O → glycerol + 3palmitate + 3H^+
71	Glycerol uptake
72	Palmitate uptake
73 Three steps	Glucose-6-P + UTP + H_2O → glycogen + 2P_i + UDP
74 Glycerol-3-P-dehydrogenase	Glycerol + NAD^+ ↔ glyceraldehyde-3-P + NADH + H^+
75 Cholesterol esterase	Cholesterol ester + H_2O → cholesterol + palmitate
76	Tryptophan uptake

For reversible reactions, the symbol (↔) is used between the reactants and products. For the irreversible reactions, single headed arrow (→) is used.

Appendix B

Minimum and maximum values of fluxes for measured fluxes which cover all four experiment settings and unknown fluxes based on reaction reversibility

	Bounds	
#	V_{min}	V_{max}
1	0.00	1.08
2	−100	100
3	0	100
4	−100	100
5	−100	100
6	0	100
7	0	100
8	−0.19	2.17
9	0	100
10	−100	100

(*Continued*)

	Bounds	
#	V_{min}	V_{max}
11	0	100
12	−100	100
13	−100	100
14	−100	100
15	0	100
16	−100	100
17	0	100
18	0.01	0.33
19	−0.03	0.42

(*Continued*)

#	Bounds V_{min}	V_{max}
20	0.03	0.23
21	0	100
22	−0.03	0.26
23	0	100
24	−0.29	−0.07
25	−100	100
26	−0.03	0.03
27	0	100
28	−100	100
29	0.06	0.45
30	0	100
31	0	100
32	0	100
33	0	100
34	0	100
35	−0.15	0.14
36	−100	100
37	−0.53	−0.12
38	0	100
39	0	100
40	0	100
41	0	100
42	0	100
43	−100	100
44	0.00	0.00
45	0	100
46	0	100
47	−100	100
48	0	100
49	0.05	0.54
50	0.04	0.31
51	0	100
52	0	100
53	21.89	41.37
54	0.00	0.13
55	0	100
56	0	100
57	0	100
58	−0.38	0.19
59	−0.34	0.25
60	−0.02	0.20
61	−0.09	2.54
62	0	0.14
63	−0.32	0.16
64	0.01	0.14
65	−0.04	0.02
66	−0.46	0.14
67	−0.28	0.10
68	−0.27	0.16
69	0.000048	0.0004
70	0	0.46
71	−0.07	1.28
72	−0.31	0.41
73	0	100
74	−100	100
75	0	0.94
76	−100	100

All flux values are in μmol/million cells/day. A design constraint (−100, 100) indicates lower or upper bound for reversible unknown fluxes.

Appendix C

Flux distribution calculated by mixed integer linear programming model III

#	Flux distribution from model III D1	D2	D3	D4	D5	D6	D7	D8
1	0.00	1.08	1.08	0.00	0.00	1.08	1.08	0.00
2	2.17	2.17	2.17	2.17	3.35	3.35	3.35	3.35
3	2.17	2.17	2.17	2.17	3.35	3.35	3.35	3.35
4	2.17	2.17	2.17	2.17	3.35	3.35	3.35	3.35
5	4.41	4.41	4.41	4.41	6.77	6.77	6.77	6.77
6	4.41	4.41	4.41	4.41	6.77	6.77	6.77	6.77
7	0.66	0.66	0.66	0.66	3.02	3.02	3.02	3.02
8	−0.19	−0.19	−0.19	−0.19	2.17	2.17	2.17	2.17
9	7.50	7.50	7.50	7.50	7.50	7.50	7.50	7.50
10	7.50	7.50	7.50	7.50	7.50	7.50	7.50	7.50
11	10.52	10.52	10.52	10.52	10.52	10.52	10.52	10.52
12	10.89	10.89	10.89	10.89	10.89	10.89	10.89	10.89
13	17.71	17.71	17.71	17.71	17.71	17.71	17.71	17.71
14	17.71	17.71	17.71	17.71	17.71	17.71	17.71	17.71
15	6.81	6.81	6.81	6.81	6.81	6.81	6.81	6.81
16	6.48	6.48	6.48	6.48	6.48	6.48	6.48	6.48
17	6.48	6.48	6.48	6.48	6.48	6.48	6.48	6.48
18	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33
19	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42
20	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
21	0.26	0.26	0.26	0.26	0.26	0.26	0.26	0.26
22	0.26	0.26	0.26	0.26	0.26	0.26	0.26	0.26
23	0.43	0.43	0.43	0.43	0.43	0.43	0.43	0.43
24	−0.07	−0.07	−0.07	−0.07	−0.07	−0.07	−0.07	−0.07
25	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17
26	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
27	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
28	0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.64
29	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45
30	3.64	3.64	3.64	3.64	3.64	3.64	3.64	3.64
31	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38
32	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
33	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34
35	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
36	3.01	3.01	3.01	3.01	3.01	3.01	3.01	3.01
37	−0.12	−0.12	−0.12	−0.12	−0.12	−0.12	−0.12	−0.12
38	2.54	2.54	2.54	2.54	2.54	2.54	2.54	2.54
39	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
40	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
41	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
42	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
43	−6.46	−6.46	−6.46	−6.46	−6.46	−6.46	−6.46	−6.46
44	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
45	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
46	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
47	−3.53	−3.53	−3.53	−3.53	−3.53	−3.53	−3.53	−3.53
48	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.36
49	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54
50	0.31	0.31	0.31	0.31	0.31	0.31	0.31	0.31
51	43.86	43.86	43.86	43.86	43.86	43.86	43.86	43.86
52	15.17	15.17	15.17	15.17	15.17	15.17	15.17	15.17
53	41.37	41.37	41.37	41.37	41.37	41.37	41.37	41.37
54	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
55	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
56	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10

(*Continued*)

#	Flux distribution from model III							
	D1	D2	D3	D4	D5	D6	D7	D8
57	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
58	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
59	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
60	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
61	2.54	2.54	2.54	2.54	2.54	2.54	2.54	2.54
62	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
63	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
64	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
65	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
66	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
67	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
68	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
69	<0.01[a]	<0.01[a]	<0.01[a]	<0.01[a]	<0.01[a]	<0.01[a]	<0.01[a]	<0.01[a]
70	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
71	−0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
72	0.00	−0.31	0.00	−0.31	0.00	0.00	−0.31	−0.31
73	2.10	1.02	1.02	2.10	3.28	2.20	2.20	3.28
74	−0.07	−0.07	−0.07	−0.07	−0.07	−0.07	−0.07	−0.07
75	0.00	0.31	0.00	0.31	0.00	0.00	0.31	0.31
76	3.64	3.64	3.64	3.64	3.64	3.64	3.64	3.64

All flux values are in μmol/million cells/day.
[a]Exact value is 0.000048.

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