Minimal Reaction Sets and Metabolic Pathways for Cultured Hepatocytes

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Abstract
Extracorporeal bioartificial liver (BAL) devices are the most promising technology for treatment of the liver failure. However, when exposed to plasma from the patient, hepatocytes are prone to accumulate intracellular lipids and exhibit poor liver-specific functions. Our work focuses on understanding the metabolism of cultured hepatocytes used for BAL. In this paper, a logic based programming is used to determine the important reactions in cultured hepatocytes by systemically analyzing the hepatic metabolic network, and investigating whether insulin, amino acid and hormone supplementations upregulate or downregulate certain pathways that control important liver specific functions, such as urea and albumin production. Using elementary mode analysis we were able to obtain 32 independent pathways, which are then used to analyze the results of the logic-based programming approach.

Keywords: Hepatocyte metabolism, integer programming, elementary mode analysis.

1. Introduction
Liver transplantation remains the best long-term option for the approximately 30,000 patients per year in the United States alone, while roughly 20% die of acute liver failure due to the shortage of organ donors. Extracorporeal bioartificial liver (BAL) devices employ primary hepatocytes or hepatoma cell lines to provide a whole complement of liver specific functions for the treatment of liver failure, but significant technical challenges remain to develop systems with sufficient function capacities. One key limitation is that, during BAL operation, hepatocytes are exposed to plasma from the patient and are prone to accumulate intracellular lipids and to depress liver-specific functions. It has been shown that pre-conditioning hepatocytes in low insulin plasma with supplemented amino acid dramatically improves the hepatic metabolism and increases liver-specific functions (Chan et al., 2003). Further manipulation of the culture environment is likely needed to allow hepatocytes to function at the levels necessary for productive BAL operation.

Metabolic pathway analysis has become an important tool of bioinformatics and biotechnology. Without the knowledge of kinetic parameters, it is used to determine the maximal molar yield of biotransformation and as a guideline for reconstruction of metabolic networks based on special cell functions. One of the methods that have been proposed to analyze metabolic networks is elementary mode analysis (Schuster et al., 2000). The elementary modes correspond to the pathways connecting inputs to outputs and comprise a minimal set of enzymes allowing the mass balance for each intermediate metabolite at steady state. Any steady state flux pattern can then be expressed as a non-negative linear combination of these modes to form a particular pathway.

The first aim of this work is to identify the minimal reaction set by logic-based programming for six different cultured conditions after exposure to plasma and to investigate the effects of insulin, amino acid supplementation and hormone in metabolic
network. We then use the elementary mode analysis framework to analyze the hepatic metabolic network and to investigate the relations between elementary modes and the results of logic-based programming.

2. Modeling and Computational Protocol

2.1. Logic-Based Programming

For a metabolic network comprising $M$ metabolites and $N$ reactions, the material balances result in the following set of equations:

$$\frac{dX_i}{dt} = \sum_{j=1}^{N} S_{ij} v_j, \quad i = 1, \ldots, M$$

(1)

where $X_i$ is the concentration of metabolite $i$; $S_{ij}$ is the stoichiometric coefficient for metabolite $i$ in reaction $j$, and $v_j$ is the flux of reaction $j$. The sum of the fluxes entering and exiting the metabolic network can be assumed to be zero based on pseudo steady-state assumption (Schilling, 2000):

$$\sum_{j=1}^{N} S_{ij} v_j = 0, \quad i = 1, \ldots, M$$

(2)

The metabolic network considered in this work is based on the model developed for cultured hepatocytes (Chan et al., 2003). It consists of 43 unknown fluxes and 34 measured fluxes (tryptophan uptake is also added), and 45 linearly independent mass balance equations. The unknown fluxes are calculated by using the least-square method of minimizing the square of errors of the measured fluxes.

Based on this model we used mathematical programming ideas to develop the following optimization model in order to determine the minimal number of reactions required to maintain the mass balances of cultured hepatocytes. Expressing the presence/absence of reactions by logic 0-1 variables, problem (3) is obtained:

$$\min \phi = \sum_{j=1}^{N} \lambda_j$$

Subject to:

$$\sum_{j=1}^{N} S_{ij} v_j = 0, \quad i = 1, \ldots, M, \quad v_j^{\min} \lambda_j \leq v_j \leq v_j^{\max} \lambda_j, \quad j = 1, \ldots, N$$

(3)

where $\lambda_j$ is the logic variable that correspond to the value of 1 if the reaction is active and 0 otherwise; $v_j$ is considered as variable between a lower and an upper bound $v_j^{\min}, v_j^{\max}$, respectively; which are determined based on the available experimental conditions (Chan et al., 2003). The problem is modeled in GAMS and solved using GAMS/CPLEX as the optimization solver since the problem corresponds to Mixed Integer Linear Program (MILP).

2.2. Elementary Mode Analysis

One approach for finding qualitatively distinct pathways is to calculate the elementary modes (Schuster et al., 1994). The determination of the elementary modes in a given network depends on the classification of metabolites as internal or external and the reactions as irreversible and reversible.
In this paper, the internal and external metabolites are chosen in a similar fashion as for the logic-based programming approach in the previous section. We thus consider 45 independent internal metabolites, which have to be balanced in any pathway, and 29 external metabolites, which are assumed to be unaffected by the reactions in the network, such as energy cofactors ATP, ADP, AMP and CO\textsubscript{2}, lactate, glucose, urea etc. We treat all of the reactions reversible except reactions 15 producing urea and reaction 69 producing albumin are irreversible. The dimension of the null space depends on the number of free variables in the original stoichiometric matrix (Schilling et al., 2000):

\[ \dim(\text{Null}(S)) = N - \text{rank}(S) \] \hspace{1cm} (4)

For a full rank matrix, the dimension of the null space is equal to the difference between the number of reactions and internal metabolites. In the hepatocyte network we consider, the dimension is equal to 32 \((d = N - M)\). Through the application of convex analysis, the solution of the steady-state eqn. (2) must lie in the nonnegative orthant of the space spanned by all the reactions. Due to the nonnegative constraints on the fluxes, the unique set of elementary modes is found. If the number of elementary modes is equal to the dimension of the null space, those modes are systematically independent. Because the elementary mode vectors, \(e^{(k)}\), are uniquely determined, any real flux distribution can be expressed as a linear combination of these vectors with coefficients as follows:

\[ V = \lambda_1 e^{(1)} + \lambda_2 e^{(2)} + \ldots \] \hspace{1cm} (5)

where \(V = (v_1, v_2, \ldots)\) is the vector of fluxes of reactions. If all of the reactions participating in one mode are reversible, this mode is reversible and the corresponding weight parameter \(\lambda\) can be of any sign whereas if one or more irreversible reactions are included in one mode, this mode is irreversible and in this case the weight \(\lambda\) should be positive. As will be shown in the next section, elementary modes can be used to interpret metabolic functioning and identify the importance of pathways and reactions in the production of specific metabolites.

### 3. Results and Discussion

The logic-based programming approach described in the previous section is used to estimate the minimum reactions required for maintaining the hepatic functions in different cultured conditions. In particular we investigate the following conditions for which experimental data are available (Chan et al., 2003): (a) high/low insulin preconditioned unsupplemented plasma cultures (HIP/LIP), (b) high/low insulin preconditioned with amino acid supplemented plasma (HIP\_AA/LIP\_AA), and (c) high insulin preconditioned with hormone supplemented plasma / low insulin with amino acid and hormone supplemented plasma (HIP\_H/LIP\_AA\_H). The results shown in Table 1 illustrate the dependence of the minimal reaction set on the different cultured conditions.

<table>
<thead>
<tr>
<th>HIP</th>
<th>LIP</th>
<th>HIP_AA</th>
<th>LIP_AA</th>
<th>HIP_H</th>
<th>LIP_AA_H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of MRS</td>
<td>60</td>
<td>51</td>
<td>64</td>
<td>64</td>
<td>56</td>
</tr>
</tbody>
</table>

Combining logic-based programming with stoichiometric balancing can clarify the effects of insulin on hepatic metabolism. Figure 1 (a) shows that insulin inhibits gluconeogenesis (Flux no.2 to 6) predominantly by suppressing the expression of
PEPCK (reaction 6) and G-6-Pase (reaction 1) enzymes. Specifically in HIP and HIP_AA, the flux of reaction 6 is decreased to 0.372 and 2.05 compared with 0.865 and 2.17 in the LIP and LIP_AA, respectively, and also no glucose is released out of the system in high insulin preconditioned plasma. Figure 1(a) also shows that amino acid supplementation significantly increases gluconeogenesis in HIP_AA and LIP_AA. The difference is that almost all of G-6-P is consumed to produce glycogen and no glucose is released under high insulin preconditioning, compared to about 28 percent used to produce glucose in low insulin plasma. We also found that hormone addition plays an important role in different culture conditions. In high insulin preconditioned plasma, hormones act similarly to insulin, inhibiting gluconeogenesis and changing the direction of reaction 1 to glycolysis. However, in the low insulin preconditioned plasma supplemented with amino acid, hormones increase the gluconeogenic pathway and almost all of G-6-P is used to produce Glycogen. In summary it is found that low insulin preconditioning with amino acid supplementation is the best culture condition for glucose production. Furthermore we determined that insulin and hormones have no obvious effect on the TCA cycle (flux no. 9 to 14) of the hepatic network, whereas amino acid supplementation increases significantly the TCA cycle (Figure 1b). These results from logic-based programming are in agreement with the experimental data (Chan et al., 2003) in different cultured conditions.

As Table 2 shows, fluxes throughout the urea cycle were significantly upregulated by amino acid supplementation, but the albumin synthesis rate decreased to the minimum value. These conditions can be further improved using multiobjective optimization that results in Pareto optimal solutions for urea and albumin optimization (Sharma et al., 2005).

![Fig.1 Effect of insulin, amino acid and hormone supplementation on (a) Gluconeogenic/glycolysis pathway, (b) TCA cycle.](image_url)

<table>
<thead>
<tr>
<th>Reaction</th>
<th>LIP</th>
<th>LIP_AA</th>
<th>LIP_AA_H</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 Arginase</td>
<td>0.007</td>
<td>1.95</td>
<td>2.201</td>
</tr>
<tr>
<td>16 Urea1</td>
<td>0</td>
<td>1.822</td>
<td>2.021</td>
</tr>
<tr>
<td>17 Urea2</td>
<td>0</td>
<td>1.824</td>
<td>2.033</td>
</tr>
<tr>
<td>69 Albumin Syn</td>
<td>MAX</td>
<td>MIN</td>
<td>MIN</td>
</tr>
</tbody>
</table>
To provide a comprehensive pathway-oriented view of hepatic metabolism, the elementary modes were obtained using FluxAnalyzer (Klamt et al., 2003), which implements the iterative algorithm described by Schuster et al. (2000). Analysis of the hepatic metabolism results in 32 elementary modes. The shortest mode is mode 1, triglyceride storage, the central reaction of which does not include any internal metabolites. Mode 2 consumes glucose to produce glycogen. Modes 4, 14, 15, 16, 17, 18, 24, 25, 26, and 29 include the TCA cycle, serine-pyruvate-cysteine cycle and valine-propionyl CoA-methionine cycle. One difference is that these modes include ketone bodies except mode 4. Another difference is that modes 4, 14, 15, 16, 17, 24, 25, 26, 29 include isoleucine uptake, palmitate uptake, cholesterol esterase, glycerol update, lipid, threonine uptake, lysine uptake, tryptophan uptake, and leucine uptake, respectively. Modes 6, 7, 8, 11, 22, and 31 include parts of the TCA cycle and valine-propionyl CoA-methionine cycle. Modes 19 and 30 involve the complete electron transport system and gluconeogenesis, respectively. By considering the reactions to produce urea and albumin as irreversible, modes 3, 27, 28 involving the urea production (Figure 2) and mode 32 involving albumin productions become irreversible modes.

Since any self-consistent flux distribution can be expressed as a non-negative linear combination of elementary modes, the minimal reaction network obtained by logic-based programming can be reconstructed using the elementary modes. The coefficients of the linear combinations for the different conditions are calculated minimizing the least square error of Eqn. (5). The weights of elementary modes 7, 21, and 22 are negative in all different conditions, which mean that these three pathways will be reversed in these specific conditions investigated in cultured hepatocytes. From the values of coefficients in different culture conditions (data are not shown), we also determined the relative importance of each pathway across the various experimental conditions. For example, modes 3, 27, and 28 involve urea production, so we can determine the importance of urea production by calculating the corresponding coefficients. The sum of these coefficients under the LIP condition is 0.0097; compared to a sum of 0.1853 for LIP_AA which means that cultured hepatocytes with amino acid supplementation exhibit significantly greater urea production compared to those cultured in unsupplemented plasma. By comparing the different weight values of modes involving urea production in LIP_AA conditions, we found modes 3 and 27 are more important to produce urea than mode 28. In addition, the most important reactions in urea production can be determined to be the inhibition of enzymes carbamoyl-P synthetase I, ornithine transcarbamylase, (reaction 16) argininosuccinate synthetase, and argininosuccinase (reaction 17). Using the results of this analysis, we can investigate the effects of enzyme deletion. For example, blocking fumarase or malate dehydrogenase enzymes will lead to disruption of the TCA cycle and elimination of 19 elementary modes, including all of the modes of 9 amino acid exchange fluxes and albumin production. On the other hand, inhibiting lactate dehydrogenase is likely less important, since it results in deletion of a single elementary mode that may not be essential since its metabolites (e.g. pyruvate) can be produced from alternative pathways.

4. Summary and Future Work

In this paper we present an analysis of hepatocyte metabolism based on logic programming and elementary mode analysis. The proposed approach determines the minimal set of reactions required in various conditions, and the importance of individual enzymes. Future work involves the experimental verification of the results obtained as well as the implementation of metabolic control analysis in the hepatocytes network to
determine the main branch points and compare with the results of the logic programming model.

(a) mode 3
(b) mode27
(c) mode 28

Figure 2, Elementary modes producing urea; the red italic numbers are expressed as fluxes of reactions (value 1 is abbreviated)

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