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Metabolic Response of Perfused Livers to Various Oxygenation Conditions

Mehmet A. Orman¹, Marianthi G. Ierapetritou¹, Ioannis P. Androulakis^{1,2}, and Francois Berthiaume^{2,*}

¹Department of Chemical and Biochemical Engineering, Rutgers, the State University of New Jersey, Piscataway, NJ, 08854, USA

²Department of Biomedical Engineering, Rutgers, the State University of New Jersey, Piscataway, NJ, 08854, USA

Abstract

Isolated liver perfusion systems have been used to characterize intrinsic metabolic changes in liver as a result of various perturbations, including systemic injury, hepatotoxin exposure, and warm ischemia. Most of these studies were done using hyperoxic conditions (95% O₂) but without the use of oxygen carriers in the perfusate. Prior literature data do not clearly establish the impact of oxygenation, and in particular that of adding oxygen carriers to the perfusate, on the metabolic functions of the liver. Therefore, herein the effects of oxygen delivery in the perfusion system on liver metabolism were investigated by comparing three modes of oxygenation. Rat livers were perfused via the portal and hepatic veins at a constant flow rate of 3 mL/min/g liver in a recirculating perfusion system. In the first group, the perfusate was equilibrated in a membrane oxygenator with room air (21% O₂) before entering the liver. In the second group, the perfusate was equilibrated with a 95% O₂/5% CO₂ gas mixture. In the third group, the perfusate was supplemented with washed bovine red blood cells (RBCs) at 10% hematocrit and also equilibrated with the 95% O₂/5% CO₂ gas mixture. Oxygen and CO₂ gradients across the liver were measured periodically with a blood gas analyzer. The rate of change in the concentration of major metabolites in the perfusate was measured over time. Net extracellular fluxes were calculated from these measurements and applied to a stoichiometric-based optimization problem to determine the intracellular fluxes and active pathways in the perfused livers. Livers perfused with RBCs consumed oxygen at twice the rate observed using hyperoxic (95% O₂) perfusate without RBCs, and also produced more urea and ketone bodies. At the flow rate used, the oxygen supply in perfusate without RBCs was just sufficient to meet the average oxygen demand of the liver but would be insufficient if it increased above baseline, as is often the case in response to environmental perturbations. Metabolic pathway analysis suggests that significant anaerobic glycolysis occurred in the absence of RBCs even using hyperoxic perfusate. Conversely, when RBCs were used, glucose production from lactate and glutamate, as well as pathways related to energy metabolism were upregulated. RBCs also reversed an increase in PPP fluxes induced by the use of hyperoxic perfusate alone. In conclusion, the use of oxygen carriers is required to investigate the effect of various perturbations on liver metabolism.

Keywords

liver; perfusion; oxygenation; metabolism; flux balance analysis

*Corresponding Author: Francois Berthiaume, Department of Biomedical Engineering, Rutgers University, 599 Taylor Road, Piscataway, NJ08854.

Introduction

The liver has many complex physiological functions including detoxification, lipid, protein, and carbohydrate metabolism, as well as bile and urea production. It also plays a major role in the onset and maintenance of aberrant “hyper-metabolic” patterns associated with various disease states, such as burns, infections and major trauma, which are characterized by an accelerated breakdown of skeletal muscle protein, increased resting energy expenditure, and a negative nitrogen balance. Isolated organ perfusion systems have been used to characterize the related metabolic changes at the individual organ level, including liver (Arai et al. 2001; Banta et al. 2007; Banta et al. 2005; Lee et al. 2000; Lee et al. 2003; Orman et al. 2010; Yokoyama et al. 2005). Normothermic liver perfusion systems are also being developed as an alternative to cold preservation techniques in the context of liver transplantation (Schon et al. 2001; Tolboom et al. 2007; Tolboom et al. 2009). A better understanding of the metabolic pattern in such systems may be helpful to assess the status of a liver graft before transplantation.

While the isolated perfusion approach enables one to determine the intrinsic changes in the organ by removing external influences, an obvious drawback is the artificial ex vivo environment of the perfusion which may potentially induce artifactual metabolic patterns. In particular, adequate oxygen transport and delivery is critical to correctly assess the metabolic state of organs that are suspected to undergo increased energy expenditure as a result of systemic inflammation due to disease and/or injury. Inadequate oxygenation could by itself alter gene expression levels (Rosmorduc and Housset 2010) and/or promote anaerobic pathways, including glycolysis, and would obviously limit the ability to observe a putative increase. However, the majority of liver perfusion studies were done without oxygen carriers, and some have claimed that hyperoxic oxygenation without the use of oxygen carriers is adequate to support perfused rat liver function (Bessemers et al. 2006). The importance of using oxygen carriers in liver perfusion studies remains controversial, and the full ramifications of the impact of oxygenation have not been well characterized.

The main objective of the current study was to compare the effects of three modes of oxygen delivery to perfused livers: normoxic (arterial) perfusate (21% O₂), hyperoxic perfusate (95% O₂), and hyperoxic perfusate with oxygen carriers (95% O₂ + 10% hematocrit using bovine RBCs). The 21% O₂ group served as negative control where it is assumed the liver is in a hypoxic metabolic mode. Net metabolic fluxes for the important metabolites entering and exiting the liver were used as the primary measurements and applied to a stoichiometric-based optimization methodology incorporating flux balance and metabolic pathway analyses (elementary mode analysis). The flux distribution vectors as well as possible active elementary modes were uniquely identified by maximizing the activity of short pathways (Orman et al. 2011). We found that the use of hyperoxic (95% O₂) perfusate in the absence of oxygen carriers is barely sufficient to meet the oxygen demand of the liver at typical perfusion flow rates, and would insufficient if hepatic oxygen uptake increased above baseline in response to environmental perturbations. When using RBCs, we found that a 10% hematocrit was adequate to meet the highest oxygen demands reported in vivo.

Materials and Methods

Animal Model and Perfusion Experiments

Male Sprague-Dawley rats (Charles River Labs, Wilmington, MA) weighing between 150 and 200 g were used. The animals were housed in a temperature-controlled environment (25°C) with a 12-hour light-dark cycle and provided water and standard chow ad libitum. All experimental procedures were carried out in accordance with National Research Council guidelines and approved by the Rutgers University Animal Care and Facilities Committee.

The isolated perfused rat liver studies for all animals were performed following a modification of Mortimore's methods as described previously (Yamaguchi et al. 1997). The rats were anesthetized with an intraperitoneal injection of ketamine (80 to 100 mg/kg) and xylazine (12 to 10 mg/kg). The abdominal cavity was opened and heparin (1000 U/kg) was injected by transphrenic cardiac puncture, and then the liver was perfused *in situ* via the portal vein at a constant flow rate by a peristaltic pump. The hepatic artery and the suprarenal vena cava were ligated, and the liver outflow from the hepatic vein collected through the catheter which was cannulated into the inferior vena cava via the right atrium. After blood was washed out from the liver by flushing with perfusate for 10 minutes, the end of the tube connecting to the outflow catheter was placed into the perfusate reservoir to initiate the recirculating perfusion. In all perfusion experiments, the perfusion pressure was kept below 15cm H₂O while the flow rate was set to 3.0 ml/min/g liver. The perfusate consisted of Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL) used as basal medium supplemented with 3% w/v bovine serum albumin (Fraction V; Sigma-Aldrich, St. Louis, MO). The concentrations of nutrients (lactate, glucose, amino acids, etc) in the perfusate (500 ml total volume) have been reported elsewhere (Yamaguchi et al. 1997) (see "Supplementary Materials" for details). The pH of the perfusate solution was initially adjusted to 7.3, but reached the target pH of 7.4 after filling the perfusion system. Throughout the perfusion pH was close to 7.4 entering the liver but slightly lower at around 7.3 exiting the liver presumably due to production of acidic metabolites such as CO₂ and lactate. Gas exchange in the oxygenator and the buffering capacity of the perfusate were sufficient to return the perfusate to the desired pH before reentering the liver, therefore no adjustments (i.e. addition of NaOH or the like) were necessary. The temperature of the heating fluid in the heat exchanger was set to between 45 and 50°C to account for incomplete heat transfer (i.e. the perfusate did not have time to get up to the heating fluid temperature) and heat losses between the heat exchanger and the liver inlet. The temperature of the liver was frequently measured during the perfusion by inserting a thermocouple between the lobes, and the range was 37.0±0.5°C. Perfusate samples were taken from the reservoir every 10 minutes for 1 hour to measure the metabolite concentrations (Arai et al. 2001; Banta et al. 2007; Banta et al. 2005; Lee et al. 2000; Lee et al. 2003; Yokoyama et al. 2005). Samples were also withdrawn every 20 minutes for 1 hour from ports adjacent to both cannulae to measure the O₂ concentrations across the liver. In order to investigate the effect of oxygen delivery mode on liver metabolism, rats were divided into three experimental groups (n=4 for each group). The groups consisted of *Group 95% O₂*, *Group 95% O₂+10% Hct* and *Group 21% O₂*. For *Group 95% O₂*, the perfusate was oxygenated by passing through 3 m of silicone tubing in contact with a 95% O₂ / 5% CO₂ gas mixture. For *Group 95% O₂+10% Hct*, the perfusate was supplemented with washed bovine RBCs (Lampire, Pipersville, PA) at 10% hematocrit and otherwise oxygenated with the same 95% O₂ / 5% CO₂ gas mixture. For *Group 21% O₂*, perfusate medium was oxygenated using room air.

The concentrations of metabolites (glucose, urea, lactate, glutamine, glutamate, β-hydroxybutyrate, triglycerides, glycerol, and O₂) were measured as described elsewhere (Banta et al. 2005; Yamaguchi et al. 1997). Detailed explanations regarding the external flux measurements can be found in the "Supplementary Materials". Multiple comparisons among the groups were performed using analysis of variance (ANOVA) followed by Tukey's studentized range test. The criterion for statistical significance was chosen as P<0.05.

Metabolic Network Analysis

The liver metabolic network used in this work was originally developed for perfused livers and hepatocyte cultures (Banta et al. 2007; Chan et al. 2003; Lee et al. 2000; Lee et al. 2003) and was modified to simultaneously include both glycolytic and gluconeogenic pathways,

fatty acid synthesis and oxidation, as well as glycogenesis and glycogenolysis (see “Supplementary Materials” for details). Given the physiological properties of the liver and the perfusate composition, the network involves all major liver-specific pathways involved in central carbon and nitrogen metabolism such as gluconeogenesis, glycolysis, urea cycle, fatty acid metabolism, pentose phosphate pathway, TCA cycle, glycogen metabolism and amino acid metabolism. Given that protein metabolism only accounts for a small portion of the nitrogen metabolism in the liver during perfusion (Orman et al. 2010), a detailed description of protein metabolism was not considered herein.

The flux distribution was calculated by using the mass balances of internal metabolites following the basic assumption that these metabolites are at pseudo steady state, which is justified by the fact that metabolic transients are fast compared to environmental changes (Varma and Palsson 1994). Therefore, the mass balance is written as follows:

$$\begin{aligned} \sum_j^{n_r} S_{kj} v_j &= 0, \forall k \in n_m, \\ v_j^{\min} &\leq v_j \leq v_j^{\max}, \forall j \in n_r, \\ v_m &\geq 0, \forall m \in \{\text{irreversible fluxes}\}. \end{aligned}$$

where S is stoichiometric matrix, v is the flux vector, and n_m and n_r are total number of metabolites and total number of reactions, respectively. Any steady state flux vector v can be expressed as a linear combination of elementary modes or extreme pathways (Figure 1) (Llaneras and Picó 2007; Wiback et al. 2003):

$$v_j = \sum_i^{n_p} P_{ji} w_i, \forall j \in n_r. \quad (2)$$

where w denotes a vector representing the weight for each elementary mode; P is the matrix of elementary modes. Note that each pathway satisfies the reaction reversibility and mass constraints given in Equation 1. Reversible pathways can be split into two irreversible pathways so that the system can be defined as a non-negative linear combination of elementary modes. Substituting Equation 2 into Equation 1 results in:

$$\begin{aligned} \sum_j^{n_r} S_{kj} \left(\sum_i^{n_p} P_{ji} w_i \right) &= 0, \forall k \in n_m, \\ v_j^{\min} &\leq \sum_i^{n_p} P_{ji} w_i \leq v_j^{\max}, \forall j \in n_r, \\ w_i &\geq 0, \forall i \in n_p. \end{aligned} \quad (3)$$

where n_p is the total number of pathways. The decomposition of a steady state flux vector into pathways is not always unique, because the number of pathways is not usually equal to the dimension of the null space of the stoichiometric matrix of larger networks (Schilling et al. 2000). We previously published a comprehensive analysis including different approaches, considering the structural and physiological properties of metabolic network, aiming at a unique decomposition of the flux vector into pathways (Orman et al. 2011). Some of them had been already presented in literature such as minimizing the length of the weight vector (Schwartz and Kanehisa 2005; Schwartz and Kanehisa 2006), and maximizing the number of active pathways (Nookaew et al. 2007). It was observed that short pathways always had higher weight values given the biochemistry of the hepatic metabolic network and fluxes in perfused livers (Orman et al. 2011). In this study, we maximized the activity of short pathways in order to identify weight values and fluxes simultaneously. The quadratic optimization problem was formulated as follows:

$$\begin{aligned}
& \text{minimize } \sum_i^{n_p} (l_i w_i)^2 \\
& \text{subject to } \sum_j^{n_r} S_{kj} \left(\sum_i^{n_p} P_{ji} w_i \right) = 0, \quad \forall k \in n_m, \\
& v_j^{\min} \leq \sum_i^{n_p} P_{ji} w_i \leq v_j^{\max}, \quad \forall j \in n_r, \\
& w_i \geq 0, \quad \forall i \in n_p, \\
& l_i = \sum_{j=1}^{n_r} A_{ji}, \quad \forall i \in n_p, \\
& A_{ji} = \begin{cases} 1 & \text{if } P_{ji} \neq 0, \\ 0 & \text{if } P_{ji} = 0. \end{cases}
\end{aligned} \tag{4}$$

The length (l) of any pathway is equal to the number of reactions involved in that pathway and is calculated using the binary matrix of elementary modes, A , where A_{ji} is equal to 1 if P_{ji} is different than zero, otherwise A_{ji} is zero.

In this work, elementary modes were only used to characterize the structure of the metabolic network. Elementary modes were calculated using a MATLAB package, *CellNetAnalyzer* (Klamt et al. 2007) which is the successor and further development of *FluxAnalyzer* (Klamt et al. 2003). The quadratic programming problems were solved using GAMS/CONOPT. The constraints obtained from extracellular flux measurements of important metabolites were used in the optimization problem described above. A more detailed explanation regarding the metabolic network analysis method is provided as “Supplementary Materials.”

Results

Extracellular Fluxes

In this study, we investigated the impact of the oxygen delivery mode, namely 21% O₂, 95% O₂, and 95% O₂ + 10% Hct, on the metabolic activity of perfused livers, for a constant flow rate of 3 mL/min/g liver. Increasing the oxygen concentration in the gas phase from 21% to 95%, and then adding 10% Hct RBCs to the perfusate, significantly increased the oxygen content of the fluid entering the liver (Table 1). Interestingly, the oxygen partial pressure of the fluid exiting the liver was similar in all three groups. Computing the oxygen consumption rate of the livers showed a parallel increase in oxygen utilization rate (Figure 2).

The concentrations of other metabolites (glucose, lactate, urea, β -hydroxybutyrate, glycerol, triglyceride, glutamate and glutamine) in the perfusate reservoir were found to change linearly as a function of time; for example, urea and β -hydroxybutyrate are shown in Figure 3, panels A and C. The data were fitted with lines to estimate the average uptake/release rates of these metabolites (Figure 3, panels B and D). Both urea and β -hydroxybutyrate production were significantly elevated by increasing oxygen partial pressure, and in turn, adding 10% Hct. Conversely, triglyceride and glycerol production rates, which were measurable in the 21% O₂ and 95% O₂ groups, decreased to near-zero values upon the addition of 10% Hct (Figure 4). A similar observation was made for lactate release from the perfused livers (Figure 4). In the 95% O₂ + 10% Hct group, some animals showed a net consumption of lactate, although on average the rate was close to zero. Glucose output was also affected by the oxygen delivery mode, increasing when going from 21% O₂ to 95% O₂, but then decreasing to a value between these two groups after adding 10% Hct. Glutamate and glutamine release/uptake rates were also measured but no significant difference among the groups was observed (see “Supplementary Materials”).

Steady State Flux Distribution

Flux vectors were uniquely determined by implementing the optimization methodology described by Equation 4 in the Materials and Methods section. Since the perfused liver was metabolically stable for the duration of the perfusion, the pseudo steady-state assumption used to formulate the Equation 4 is reasonably valid. The calculated fluxes are shown in Figure 5. Reactions 1-7 are those in the gluconeogenic pathway, although some of them are also used (in a reverse direction) by the glycolysis pathway. For example, reaction 2 (generation of glucose-6-P from fructose-6-P), and reactions 4-5 (generation of phosphoenolpyruvate [PEP] from glyceraldehyde-3-P), had negative fluxes in the 21% O₂ and 95% O₂ groups, implying that they were operating in a glycolytic mode. Conversely, these reactions had positive fluxes, indicating a gluconeogenic pattern, in the 95% O₂ + 10% Hct group. Interestingly, flux values for reaction 1 (converting glucose-6-P to glucose) were positive, consistent with a gluconeogenic pattern, while flux values for reaction 8 (glucose-6-P generation from glucose), which is strictly glycolytic, were near zero in all groups. However, glycolytic reactions 9 and 10 (generation of pyruvate from PEP and fructose-1,6-P2 from fructose-6-P, respectively) were found to be active in all groups, which results in the futile cycles with the gluconeogenic reactions 3, 6-7 which are the opposite of reactions 9-10. Reaction 11 (conversion of pyruvate into acetyl-coA) had a significantly higher flux in the 95% O₂ + 10% Hct group compared to other groups.

Reaction 12 (a lumped reaction term representing the PPP) had a higher flux value in the 95% O₂ + 10% Hct group compared to other groups. Reaction 13 is catalyzed by lactate dehydrogenase, and represents the inter-conversion of pyruvate and lactate. A negative value, as was the case for the 21% O₂ and 95% O₂ groups, indicates that the reaction was producing lactate from pyruvate, while the opposite (net conversion of lactate to pyruvate) was true in the 95% O₂ + 10% Hct group.

Reactions 14-19, which account for the TCA cycle, had their lowest fluxes in the 21% O₂ group, and increased in unison in the 95% O₂ group, and subsequently even more in the 95% O₂+10% Hct group. These changes largely reflect the observed changes in oxygen uptake rates (Figure 2), and not surprisingly parallel observed increases in the electron transport reaction fluxes (reactions 51-52). These changes are also consistent with the increased production of pyruvate and acetyl-coA by reactions 11 and 13 in the 95% O₂+10%Hct group, which are the direct precursors entering the TCA cycle.

Fluxes in the urea cycle (reactions 20-22 in Figure 5) were higher when perfusate oxygen partial pressure was increased from 21% to 95%, and then by addition of 10% Hct. Fluxes in amino acid metabolism, represented by reactions 23-42, were not significantly different and relatively small when compared to other fluxes, as previously observed (Arai et al. 2001; Banta et al. 2007; Banta et al. 2005; Lee et al. 2000; Lee et al. 2003).

Fatty acid oxidation and ketone body production (reactions 43-48) were generally lower in the 21%O₂ group compared to the other groups, but there was little difference between the 95%O₂ and 95%O₂+10%Hct groups. In all three groups, glycogen production (reaction 49) was essentially zero, while glycogen degradation (reaction 50) was very active; interestingly, this flux was significantly higher in the 95%O₂ group, but there was no difference between the 21%O₂ and 95%+10% Hct groups.

Interesting observations can be made by comparing the predicted values for CO₂ generation from the various relevant pathways (Table 2), namely the TCA cycle, amino acid metabolism, and PPP, and the consumption by gluconeogenesis and the urea cycle. CO₂ production from the TCA cycle increased in parallel with increasing the oxygen content in the perfusate, also consistent with similar increases in TCA cycle fluxes. CO₂ production

from the PPP increased from the 21% O₂ group to the 95% O₂ group, but then decreased in the 95% O₂+10% Hct group to less than 25% of the value in the 21% O₂ group. Values associated with amino acid metabolism were relatively small compared to contributions from the other metabolic pathways. CO₂ utilization for gluconeogenesis increased by a factor of 4 to 5 fold from the 21% O₂ group to the 95% O₂ group, and then changed little in the 95% O₂+10% Hct group. CO₂ utilization for urea synthesis increased in parallel with the increases in CO₂ production by the TCA cycle. As a result of these various contributions, overall CO₂ production increased by about 33% from the 21% O₂ group to the 95% O₂ group, but then decreased in the 95% O₂+10% Hct group to a level about 15% lower than that observed in the 21% O₂ group.

Pathway Analysis

Elementary modes were used to analyse the topology of the metabolic network. The number of all possible elementary modes of the hepatic metabolic network was found to be 134175. Among them, only 6 pathways are reversible (note that in this article we use the term “pathways” to denote the “elementary modes”). The optimization problem (Equation 4) identified possible important pathways with their weight values that characterize their importance in hepatic metabolism (Figure 6). It was observed that increasing the oxygen partial pressure from 21% to 95%, the number of active pathways whose weight values are greater than zero increased from 176 to 199. Adding 10% Hct then increased this number to 410. Furthermore, relatively longer pathways were found to be active in the 95%O₂+10% Hct group when compared to other groups, although these pathways were generally with low weight values.

In order to gain a better comprehensive understanding of the metabolic response of the liver to the oxygen delivery mode, dominant pathways (having larger weight values) were further examined and listed in Table 3. P7, a very short pathway describing glucose production from glycogen stores, was found to be active with a very high weight value in all groups. The highest weight for P7 was in the 95%O₂ group, consistent with the experimental observation that glucose output (reaction 1) was highest in that group as well. P10 is also a short pathway, linking glycogen breakdown, glycolysis, and lactate production. This pathway was nonexistent in the 95%O₂+10%Hct group, but was significant in the other two groups. P12 and P13 represent glucose production through gluconeogenesis from lactate and cysteine, respectively. These pathways were found to be more active in the 95%O₂ and 95%O₂+10%Hct groups. P237, which was found to be active in all conditions, involves the production of aspartate from glucose. P2026 represents serine and glycine inter-conversion by glycine dehydrogenase and aminomethyltransferase, and had a higher weight value in the 95%O₂+10%Hct group when compared to the other groups.

P2094 involves glutamate uptake (including glutamine uptake followed by deamination to glutamate), formation of oxaloacetate by an anaplerotic route via the TCA cycle and gluconeogenesis. The activity of this pathway was increased when perfusate oxygen content was increased. P2830 and P2842 were only active in the 95%O₂+10%Hct group and are related to energy metabolism (Table 3). They represent ATP production through electron transport chain reactions from high energy metabolites (e.g. NADH) produced by glycolysis, the TCA cycle, or fatty acid oxidation. P16876 and P16897 had higher weights in the 21%O₂ and 95% O₂ groups. These pathways show the possible paths for metabolizing intracellular glucose (generated from glycogen), namely either via the PPP or via glycolysis towards lactate. P10852 has two different pathways. One is related to glucose production from aspartate through the gluconeogenic pathway, and the other shows the inter-conversion of serine and glycine. P134172 involves lactate production from cysteine by lactate dehydrogenase, and cysteine transaminase. This pathway was upregulated in the 21% O₂ and 95% O₂ groups (Table 3). Similarly, there were other pathways (such as P47434 and

P48418, not shown) involving lactate production through glycolysis, which were also significantly upregulated in the 21% O₂ and 95% O₂ groups.

Discussion and Conclusions

Animal-based perfusion systems using hyperoxic oxygen but without oxygen carriers have been extensively used to characterize the hepatic response to various injury models (Arai et al. 2001; Banta et al. 2007; Banta et al. 2005; Lee et al. 2000; Lee et al. 2003). Herein we investigated the effect of increasing oxygen delivery by increasing oxygen partial pressure and adding bovine RBCs to the perfusate on rat liver metabolism in an ex vivo perfusion system. Increasing oxygen delivery enhanced oxygen uptake rate and fluxes through the TCA cycle and electron transport chain, suggesting an upregulation of oxidative phosphorylation and intrahepatic energy production. Concomitantly, we observed increased β -hydroxybutyrate production, and decreased lipogenesis and lactate production, consistent with an upregulation of lipid oxidation pathways and a downregulation of anaerobic glycolysis. We also found that increasing oxygen delivery increased urea cycle fluxes and gluconeogenesis. Increasing oxygen tension alone (from 21% to 95% O₂) stimulated glycogen breakdown and the PPP, but addition of RBCs reversed these changes.

Perfusion parameters critically impact on liver viability and function during ex vivo perfusion. Oxygen delivery is a particularly challenging problem due to the low solubility of oxygen in perfusion media. Supraphysiological flow rates are often used to compensate for that low solubility. For example, Bessems et al. (Bessems et al. 2006) claimed that a flow rate of 3 mL/min/g liver will adequately oxygenate a 10-12 g rat liver as long as the inlet pO₂ is greater than 500 mmHg without using any oxygen carrier. Numerous other studies have used similar flow rates (Arai et al. 2001; Banta et al. 2007; Banta et al. 2005; Lee et al. 2000; Lee et al. 2003). The data presented herein suggest that with a similar flow rate, an inlet pO₂ of ~600 mmHg results in an oxygen uptake rate of ~200 μ mol/h/g liver (Table 1 and Figure 1). These values indeed match the averaged in vivo measurements in fasted rats (Izamis et al. 2011), but are significantly lower than values reported under conditions of stress; for example, the same authors showed that the rat liver oxygen demand could be up to 400 μ mol/h/g liver after experimental burn injury. Since more than 90% of the oxygen available in the perfusate of the 95% O₂ group was used (Table 1), it would not be possible to ever match this rate without roughly doubling the flow rate, which would likely cause shear stress damage to the liver. This is consistent with other literature data suggesting that hepatic functions (e.g. bile production) and histological appearance are improved when perfusate is supplemented with RBCs or diluted blood (Alexander et al. 1995; Cheung et al. 1996; Rupenko et al. 2008). It is interesting to note, however, that the outlet oxygen partial pressure did not go below ~20 mmHg in any of the groups (Table 1), and a similar observation was made in vivo (Izamis et al. 2011). Further studies to establish whether or not this represents the lower limit for oxygen extraction by the liver are warranted.

The addition of RBCs to the perfusate at a hematocrit level of 10% is expected to increase perfusate viscosity by as much as a factor of two, although in vivo measurements such as those carried out in the classical work of Whittaker & Winton (Whittaker and Winton 1933) show little effect of hematocrit on apparent viscosity at hematocrits below 20%. Since perfusion pressure did not increase significantly (remaining around 15 cm H₂O) with the addition of RBCs, it is possible that some vasodilation occurred, and one could speculate that it may be due to shear stress induced nitric oxide production (Martini et al. 2005). It is possible that vasodilation could in turn enhance perfusion distribution and as a result increase oxygen uptake.

In this study, we focused on the impact of the mode of oxygenation on the fluxes comprising the central carbon and nitrogen metabolic pathways of the liver. Among these pathways, the ones that are most liver-specific, and therefore often used to assess specific hepatic function of the perfused liver include albumin secretion, urea synthesis, and ketone body production (Uygun et al. 2010). Albumin secretion during the 1-hour perfusion period was low, and since one cannot reliably assess the amount produced *de novo* vs. that which was already present in the cells, this parameter was not included in the mass balance analysis (Orman et al. 2010). Urea production, an important detoxification-related parameter, was very significantly increased by increasing oxygen delivery to the perfused liver, which is consistent with prior reports on cultured hepatocytes that correlate increased urea synthesis with increased oxygen uptake rate and oxygen partial pressure (Cho et al. 2007; Ledezma et al. 1999). Ketone body production (measured as β -hydroxybutyrate) was also significantly increased by increasing oxygen delivery, and are often used as an indicator of lipid oxidation. Ketone bodies are primarily produced by the liver under fasting conditions and serve as an alternative fuel during periods of glucose deficiency, and as a result may spare glucose utilization (Laffel 1999). Conversely, lower oxygen delivery increased lipogenesis, whose main source of carbon was likely TCA cycle intermediates such as citrate and acetyl-CoA. Lactate release was also increased, which is often the result of incomplete glucose oxidation. Taken together, these results support the notion that the TCA cycle was not operating properly at the lower oxygen delivery rates, most likely due to insufficient oxygen availability.

Although livers were harvested from animals in a fed state, we measured a net glucose output (as opposed to consumption) and the model predicted glycogen breakdown (as opposed to glycogen synthesis), which is more typical of a fasted pattern. These findings are in fact similar to that reported in a prior study using perfused livers isolated from fed rats (Orman et al. 2010). A plausible explanation is that in the perfusion system, the liver is not exposed to circulating factors such as insulin and glucagon that control glucose production and utilization *in vivo*. Interestingly, in this study glucose production was higher in the 95% O₂ group compared to the other groups, and the model predicted a concomitant increase in glycogen breakdown. Moreover, our analysis showed that the PPP was up-regulated in the 95% O₂ group (see Table 2 and Figure 5). The oxidative branch of the PPP produces NADPH, which is used in cells to recycle anti-oxidants and thus may indicate the presence of the oxidative stress caused by hyperoxic perfusate (Lee et al. 2003). The increased PPP flux in the 95% O₂ group is a significant draw from the glucose 6-phosphate pool and could also explain the increased glycogen breakdown in the same group.

Although larger scale hepatic networks have been constructed (Gille et al. 2010; Jerby et al. 2010), we used a medium scale metabolic network that has been extensively used to characterize the flux distribution in perfused rat livers, and for which there is a good consistency between the measurements and the assumed biochemistry of the network (Lee et al. 2000; Lee et al. 2003). Utilizing this network and the external flux measurements, we combined metabolic flux and pathway analysis to calculate the flux distribution vectors and weights of pathways simultaneously. In our previous study where pathway analysis was used to analyze the hepatic network, we found that higher weights were always attributed to short pathways (Orman et al. 2011). Schwarz and his co-workers (Schwarz et al. 2005) stressed the importance of short pathways since they contribute most to gene expression. Furthermore, Rutter and Zufall (Rutter and Zufall 2004) postulated that short pathways have been developed as a result of adaptive responses of organisms to environmental changes.

When we solved the optimization problem (Equation 4) by maximizing the activity of short pathways to determine the flux and weight vectors, it was observed that the fluxes in the TCA cycle, gluconeogenesis, fatty acid oxidation, and the urea cycle were increased when

oxygen delivery was increased. Our model, which allows for both glycolysis and gluconeogenesis to be simultaneously active, predicted that the glycolytic reaction 9 (generation of fructose-1,6-P2 from fructose-6-P) and reaction 10 (pyruvate production from PEP) as well as the gluconeogenic reactions 3, 6, and 7, which are the opposite of glycolytic reactions 9-10, were active in all groups, which results in futile cycles. Utilizing a single carbon tracer and mass isotopomer analysis, Marin and his co-workers (Marin et al. 2004) were able to prove the existence of futile cycles between glycolysis and gluconeogenesis. Without additional constraints or knowledge such as isotope labeling measurements, it is difficult to further resolve such futile cycles, and for those reactions that are reversible (i.e. the same reaction is used for both gluconeogenesis and glycolysis but in reverse direction), the reported values herein represent the net difference between forward and reverse rates. Increasing oxygen delivery resulted in higher fluxes in gluconeogenic reactions. Moreover, significant amounts of lactate were produced from pyruvate (reaction 13) in the 21% O₂ and 95% O₂ groups.

Elementary mode analysis showed that the number of active pathways was increased when RBCs were utilized. The added pathways (such as P2830 and P2842) are relatively longer and generally related to energy production, which is expected since RBCs also increased oxygen uptake. The pathways include glycolysis, the TCA cycle, fatty acid oxidation, and electron transport reactions. This analysis further showed that lactate production from glucose (generated from glycogen) and/or amino acids including cysteine and serine were important when the perfusate was inadequately oxygenated. On the other hand, glucose production from lactate and glutamate was increased when RBCs were used. We also found that the pathway representing lactate production from cysteine through pyruvate had higher weights in the 21% O₂ and 95% O₂ groups. The pathway related to the inter-conversion of glycine and serine, an important precursor for less common amino acids such as D-serine and taurine, was also found to be active in all three groups.

In summary, this study shows that when perfusing rat livers ex vivo, the use of hyperoxic (95% O₂) perfusate in the absence of oxygen carriers is barely sufficient to meet the oxygen demand of the liver at typical flow rates of 3 mL/min/g liver, and would be insufficient if hepatic oxygen uptake increased above baseline in response to environmental perturbations. When using RBCs, we found that a 10% hematocrit was adequate to meet the highest oxygen demands reported in vivo. Furthermore, when using 95% O₂ in the absence of oxygen carriers, urea and ketone body production were significantly decreased, and pathway analysis suggests that significant anaerobic glycolysis occurred. Conversely, when RBCs were used, glucose production from lactate and glutamate, as well as pathways related to energy metabolism were upregulated. Finally, the use of RBCs appear to temper an upregulation of the PPP triggered by the utilization of 95% O₂ in the absence of oxygen carriers, which we speculate is an indication that hyperoxic perfusate alone may cause oxidative stress that increases the demand for reducing equivalents from the PPP. The improved physiological relevance of a perfusion system using oxygen carriers makes it a more attractive tool to investigate the effect of various perturbations on liver metabolism, including the response to toxicants and drugs, as well as disease conditions known to alter liver metabolism, such as burns and trauma leading to systemic hypermetabolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Sample simplified metabolic network with its pathways (P1 and P2). Each pathway is represented by a normalized flux distribution vector. A weighted pathway (for example: $P_1 w_1$) shows the actual fluxes carried out by that pathway. A linear combination of the weighted pathways gives the net flux distribution (v) in the network.

Figure 2.

Perfused liver oxygen uptake rates (–) as a function of oxygen delivery mode at 20 min (black bar), 40 min (dark grey), and 60 min (light grey) after the beginning of perfusion. All rats were in a fed state (n=4 per group). Note: Letters indicate statistically significant ($P<0.05$) differences among groups: **a** is significantly different than either **b** or **c**; and **b** is significantly different than **c**. No significant difference is observed within the groups.

Figure 3.

Urea and β -hydroxybutyrate production during perfusion as a function of oxygen delivery mode. **A.** Urea accumulation as a function of time. **B.** Urea production rate, calculated from slope of panel **A**. **C.** β -hydroxybutyrate accumulation as a function of time. **D.** β -hydroxybutyrate production rate, calculated from slope of panel **C**. All rats were in a fed state ($n=4$ per group). Note: Letters indicate statistically significant ($P<0.05$) differences among groups: **a** is significantly different than either **b** or **c**; and **b** is significantly different than **c**.

Figure 4.

Glucose, lactate, TG and glycerol uptake (–) or production rates (+) during perfusion. All rats were fed (n=4 per group). Note: Letters indicate statistically significant (P<0.05) differences among groups: **a** is significantly different than either **b** or **c**; and **b** is significantly different than **c**. For example, lactate secretion rates in *Group 21%O₂* (**a**) and *Group 95%O₂* (**a**) are not significantly different, whereas *Group 95%O₂+10%Hct* (**b**) is significantly different than either *Group 21%O₂* (**a**) or *Group 95%O₂* (**a**).

Figure 5.
Calculated internal fluxes using Equation (4).

Figure 6. Weight values of pathways. A: *Group 21%O₂*; B: *Group 95%O₂*; C: *Group 95%O₂+10%Hct*. Note that in each method all weights values are normalized to the largest one observed among the groups. Only P7, which is not shown in this figure, has a weight value greater than 0.2 in the three experimental groups (see Table 3 for details).

Table 1

Pre- and post-liver oxygen content of perfusate solutions.

Animal Groups	Group 21%O ₂	Group 95% O ₂	Group 95% O ₂ +10% Hct
Liver inlet			
pO ₂ (mmHg)	184.3±36.1	612.3±6.1	610.3±9.2
O ₂ -Hb (mmol/L)	-	-	2.0±0.1
Total O ₂ (mmol/L)	0.315±0.062	1.047±0.01	3.05±0.1
Liver outlet			
pO ₂ (mmHg)	26.1±6.4	42.84.8	24.2±2.4
O ₂ -Hb (mmol/L)	-	-	0.78±0.005
Total O ₂ (mmol/L)	0.045±0.01	0.073±0.008	0.83±0.005

pO₂: Partial pressure of O₂.O₂-Hb: O₂ concentration carried by hemoglobin, which is calculated from the first part of the equation for total oxygen shown below.Total O₂: Total oxygen concentration calculated using the equation: $ctO_2 = 1.39 \times FO_2Hb \times ctHb + 0.00314 \times pO_2$ and then $Total\ O_2 = ctO_2 \times (0.00314)^{-1} \times 0.00171$

where ctO₂ (ml/dL) and ctHb (g/dL) are the concentrations of oxygen and hemoglobin, respectively. FO₂Hb is the fraction of hemoglobin occupied by oxygen, and pO₂ (mmHg) is the partial pressure. The value 1.39 represents the oxygen binding factor of hemoglobin and 0.00314 is the oxygen solubility coefficient which is a conversion factor from mmHg O₂ to ml O₂/dL perfusate. The value of 0.00171 is Henry's constant (mmol /mmHg-L).

Table 2

Predicted CO₂ utilization and production rates (μmol/g liver/h) by key pathways in the perfused liver.

	21 % O ₂	95 % O ₂	95 % O ₂ +10 % Hct
TCA cycle	41.1	102.4	155.8
PPP	58.9	84.7	13.6
Amino acid metabolism	10.0	5.6	6.3
Gluconeogenesis *	-28.5	-80.9	-90.1
Urea *	-15.1	-23.6	-29.1
Overall CO ₂ production	66.4	88.2	56.5

* negative values represent utilization

Table 3

Important elementary modes having larger weight values ($w > 0.01$ for each group).

Pathways	Explanation	Weights Group 21%O ₂	Weights Group 95%O ₂	Weights Group 95%O ₂ +10%Hct
P7	This is a very short pathway including glycogen breakdown, and glucose production and secretion.	0.45237	1	0.208647
P10	This pathway includes glycogen breakdown, glycolysis and lactate production.	0.079201	0.118951	0
P12	This includes glucose production from lactate through gluconeogenesis.	0	0.06365	0.026165
P23	This pathway includes glucose production from cysteine through gluconeogenesis.	0.031115	0.07476	0.07476
P237	This is related to aspartate production from gluconeogenic glucose through glycolysis.	0.02224	0.057114	0.012991
P2026	This is a very short pathway related to glycine and serine interconversion.	0.037758	0.096202	0.023511
P2094	This includes glucose production from glutamate through gluconeogenesis.	0	0.039033	0.011868
P2830	This represents energy metabolism including glycolysis, TCA cycle and electron transport reactions.	0	0	0.017946
P2842	This represents energy metabolism including fatty acid oxidation and electron transport reactions.	0	0	0.016352
P16876	This pathway includes glycogenolysis, glycolysis and PPP.	0.032322	0.084866	0.015341
P16897	This pathway includes lactate production from glycogenic glucose in addition to PPP.	0.064988	0.093428	0
P108052	This elementary mode includes two distinct extreme pathways. One represents serine and glycine inter-conversion. The other includes glucose production from aspartate through gluconeogenesis.	0	0.049932	0.013575
P134172	This pathway is related to cysteine and lactate inter-conversion.	0.058845	0.05362	0