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A switch in the source of ATP production and a loss in capacity to perform glycolysis are hallmarks of hepatocyte failure in advance liver disease

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Background & Aims: The cause of hepatic failure in the terminal stages of chronic injury is unknown. Cellular metabolic adaptations in response to the microenvironment have been implicated in cellular breakdown.

Methods: To address the role of energy metabolism in this process we studied mitochondrial number, respiration, and functional reserve, as well as cellular adenosine-5′-triphosphate (ATP) production, glycolytic flux, and expression of glycolysis related genes in isolated hepatocytes from early and terminal stages of cirrhosis using a model that produces hepatic failure from irreversible cirrhosis in rats. To study the clinical relevance of energy metabolism in terminal stages of chronic liver failure, we analyzed glycolysis and energy metabolism related gene expression in liver tissue from patients at different stages of chronic liver failure according to Child-Pugh classification. Additionally, to determine whether the expression of these genes in early-stage cirrhosis (Child-Pugh Class A) is related to patient outcome, we performed network analysis of publicly available microarray data obtained from biopsies of 216 patients with hepatitis C-related Child-Pugh A cirrhosis who were prospectively followed up for a median of 10 years.

Results: In the early phase of cirrhosis, mitochondrial function and ATP generation are maintained by increasing energy production from glycolytic flux as production from oxidative phosphorylation falls. At the terminal stage of hepatic injury, mitochondria respiration and ATP production are significantly compromised, as the hepatocytes are unable to sustain the increased demand for high levels of ATP generation from glycolysis. This impairment corresponds to a decrease in glucose-6-phosphatase catalytic subunit and phosphoglucomutase 1. Similar decreased gene expression was observed in liver tissue from patients at different stages of chronic liver injury. Further, unbiased network analysis of microarray data revealed that expression of these genes was down regulated in the group of patients with poor outcome.

Conclusions: An adaptive metabolic shift, from generating energy predominantly from oxidative phosphorylation to glycolysis, allows maintenance of energy homeostasis during early stages of liver injury, but leads to hepatocyte dysfunction during terminal stages of chronic liver disease because hepatocytes are unable to sustain high levels of energy production from glycolysis.

Keywords: Decompensated liver cirrhosis; Hepatocytes; Acute-in-chronic liver failure; Glycolysis; Mitochondria; Adenosine-5′-triphosphate.

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Abbreviations: ACLF, Acute-in-chronic liver failure; CCl_4, carbon tetrachloride; OCR, oxygen consumption rate; RCR, respiratory control ratio; ECAR, extracellular acidification rate; ATP, Adenosine-5′-triphosphate; UPLC, Ultra Performance Liquid Chromatography; FCCP, Carbonyl cyanide p-trifluoromethoxyphenylhydrazone; AABA, alpha-Aminobutyric acid; BCAA, branched chain amino acids; AAA, aromatic amino acids; TCA cycle, tricarboxylic acid cycle; G6PC, Glucose-6-phosphatase catalytic subunit; PGM1, Phosphoglucomutase 1; G6PD, Glucose-6-phosphate dehydrogenase; FBP2, Fructose-1,6-bisphosphatase 2; PCK1, Phosphoenolpyruvate carboxykinase 1; DLD, Dihydrolipoamide dehydrogenase; COY, cytochromec oxidase; ND6, NADH dehydrogenase subunit 6; PDHB, Pyruvate dehydrogenase beta.

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Introduction

Chronic injury, mediated by a number of different etiologies, produces cirrhosis of the liver [1]. End-stage cirrhosis results in more than 30,000 deaths per year in the US, which is the 6th most frequent cause of death in individuals 25–44 years of age [2]. As liver function in cirrhosis deteriorates, patients develop jaundice, encephalopathy, an increased risk of bleeding, and muscle wasting [3]. In addition, they are susceptible to episodes of acute deterioration of hepatic function with minor precipitating events [3–5]. The mechanisms responsible for deterioration of hepatic function in cirrhosis are incompletely understood.

Metabolic adaption during environmental stress is currently an area of intense investigation because of its potential relationship to human disease [6]. Alterations in lipid and amino acid metabolism are found in patients with cholestatic liver disease and such abnormalities are associated with disease progression and hepatic failure [7–10]. Thus far, however, the mechanisms responsible for these metabolomic changes have not been identified [10–16].

Oxidative phosphorylation is the major source of ATP in normal cells; however, this source of energy can change depending on microenvironment stressors [17–19,25]. In mammalian cells, a decrease in the availability of oxygen reprograms the mitochondria to generate ATP more from glycolysis instead of oxidative phosphorylation. Recent work in cancer and other disease processes has also shown that mammalian cells can switch their source of energy production from mostly oxidative phosphorylation to mostly glycolysis and back depending on the microenvironment, genetics, epigenetic changes, and exposure to toxins [6,17,19,20,25].

Since integrity of mitochondrial function is critical for both cell survival and for the generation of new cells [21], mitochondrial dysfunction could limit the survival, function, or regeneration capacity of hepatocytes in cirrhosis. Therefore, we examined the energetics and the extent of metabolic adaptation in hepatocytes from livers at various stages of liver injury.

In the present study, we demonstrate that mitochondrial energy production remains intact during the early stages of chronic liver injury despite the fact that the number of mitochondria per hepatocyte is reduced. To maintain energy homeostasis, ATP production switches from being predominantly from oxidative phosphorylation to predominantly from glycolysis. However, maintenance of energy production by this compensatory mechanism fails in hepatocytes in later stages of chronic liver injury and is associated with hepatic failure and death.

Materials and methods

Animals and chemical induced cirrhosis model

Liver cirrhosis was induced by continuous chemical treatment using phenobarbital (Sigma) and carbon tetrachloride (CCl_4, Sigma) in Lewis rats as described in our previous study [22,23]. (For detailed description please see Supplementary materials and methods.)

Isolation of rat hepatocytes and cell culture

Hepatocytes were isolated from cirrhotic and age-matched non-treated animals using a modified collagenase perfusion technique as described previously [22]. Briefly, perfusion of the portal vein using a 20G catheter (Becton, Dickinson Infusion Therapy Systems Inc., Sandy, Utah) using 0.5 mM EGTA in Leffert's buffer was performed for 10 min and then collagenase (Blendzyme, Roche, Germany) was infused at 14 units/100 ml in Leffert's solution for 10–20 min adjusted based on the consistency of liver tissue in response to collagenase digestion. After centrifugation at 50g for 3 min, the cells were re-suspended and washed twice in cold Dulbecco's Modified Eagle's Medium (DMEM). The cell quality was evaluated by viability, which was determined by trypan blue exclusion (at least 80% was required to proceed with *in vitro* experiments) and by plating efficiency, onto collagen coating plates after overnight incubation at 37 °C, 5% CO_2 in DMEM/F12 culture medium (supplemented with 5% FBS, 2 mM Glutamine, 100 U/ml Penicillin, 100 ug/ml Streptomycin, 100 nM dexamethasone, 0.872 μM insulin and 5 ng/ml epithelial growth factor).

Metabolic assays

The glucose consumption by hepatocyte groups was quantified according to the instructions of Autokit Glucose C2 (Wako) every 24 h for 7 d of culture. The absorbance was measured at 505 nm with a spectrophotometer SpectraMax M5 (Molecular Devices). Lactate secretion was quantified using a lactate kit (Trinity biotech). The absorbance measured at 540 nm was proportional to the lactate content in the sample. The results were expressed in μmol per million cells.

Mitochondrial respiration

Oxygen consumption rate (OCR) was measured with an XF24 Extracellular flux analyzer (Seahorse Bioscience) [24]. Hepatocytes were seeded in Seahorse 24 well collagen-coated microplates at a cell density of 4.10^4 cells per well. The respiration was assayed 3 days after hepatocyte isolation. The culture media was replaced by 850 μl of assay medium (DMEM 10 mM glucose, 1 mM pyruvate, 2 mM glutamine and 1% Penicillin-streptomycin without serum and bicarbonate) and incubated at 37 °C without CO_2 during 1 h. The OCR was then measured with the extracellular acidification rate (ECAR) [25], under endogenous conditions. The respiratory control ratio (RCR) was determined by using oligomycin (2 μg/ml) for F_1F_O-ATPsynthase inhibition. The maximal respiration illustrated by the uncoupled OCR was measured with 1 μM of FCCP. The cells were then treated with antimycin A, an inhibitor of complex III, in order to reveal the non-mitochondrial respiration.

Adenosine-5′-triphosphate measurements

The intracellular ATP content was measured on hepatocytes by using the CellTiter-Glo Luminescent cell viability assay (Promega) [25]. The cells were seeded in 96-well collagen coated plates (0.25 mg/ml) at 6.10^4 cells per well. After 3 d of culture, the medium was replaced by 100 μl of DMEM high glucose without phenol red. The cells were then incubated at 37 °C during 1 h in the absence or presence of oligomycin (2 μg/ml), and 2-deoxyglucose (100 mM). The ATP content was measured by luminescence with an integration time of 1 s per well, after adding 100 μL of CellTiter-Glo reagent, mixing during 2 min and incubation at room temperature during 10 min.

q-PCR array of glucose metabolism – related genes

Total RNA was extracted from isolated hepatocyte as described above. To remove genomic DNA contaminants, 5 μg total RNA was treated with 2.5 units DNAse (Roche Applied Science, Mannheim, German) for 15 min at 20 °C followed by inactivation of DNAse enzyme at 70 °C for 8 min. Then, cDNA was synthesized from total RNA using SuperScript® III First-Strand Synthesis System (Invitrogen) according to the manufacturer's protocol. 20 ng cDNA was loaded into each well in RT2 Profiler 96-well PCR array plates (PARN-006, QIAGEN, Valencia CA) and amplified using the ABI 7500 real-time PCR System. The PCR reaction was programmed as follows: initial denaturing at 95 °C for 10 min, followed by 95 °C for 15 s, 60 °C for 1 min, cycled 40 times. The median cycle threshold value (Ct) was uploaded onto the SABioscience website (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) and the fold change of each gene expression was calculated using the provided software according to manufacturer's instruction. Additionally, subsets of genes with the values of fold change larger than 1.5 across the 3 comparisons (normal *vs.* compensated cirrhotic hepatocytes, normal *vs.* decompensated cirrhotic hepatocytes, compensated *vs.* decompensated cirrhotic hepatocytes) were used to find possible signal pathways using Ingenuity Pathway Analysis software.

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Isolation of mitochondrial fraction from hepatocytes

Fifty million freshly isolated rat hepatocytes were homogenized in a tissue blender at high speed for 20 s at 4 °C in homogenization buffer (containing 25 mm Tris-HCl, pH 7.5, 100 mm KCl, 0.4 m sucrose, and protease inhibitor cocktail). The homogenate was centrifuged at 900*g* for 10 min at 4 °C, and then the supernatant containing mitochondria was subjected to centrifugation at 14,000*g* for 15 min. Following one washing with homogenization buffer, the pellet was preserved at −80 °C as a mitochondrial fraction. The samples were diluted in 1 ml buffer and the protein concentration was measured before mitochondrial assays were done.

Measurement of mitochondrial enzyme activities

Enzyme activities were measured on mitochondrial fractions isolated from hepatocytes by following protocols adapted from the methods of Brich-Machin and Turnbull [26]. (For detailed description please see Supplementary materials and methods).

Statistical analysis

The results presented are expressed in mean value of N experiments ± SD, with N >3. Comparison of the values obtained with the different groups, was performed in ANOVA and Scheffe's F test. Differences were considered to be statistically significant when p values were $\leqslant 0.05$.

Results

Animal models for liver cirrhosis

Hepatocytes were isolated from the livers of animals (rats) with cirrhosis and normal liver function, hereafter denoted as "hepatocytes from early cirrhotic livers" and from the livers of animals (rats) with cirrhosis and sustained loss of liver function 4 weeks after they received their last dose of carbon tetrachloride, hereafter denoted "hepatocytes from failing cirrhotic livers" (Supplementary Fig. 1) [22,23]. All experiments were performed on isolated hepatocytes rather than liver tissue to accurately evaluate the hepatocyte population.

Mitochondrial function is intact in rat cirrhotic hepatocytes with normal function but is impaired in rat hepatocytes with decompensated function

To determine if alterations in mitochondrial function and activity play a role in inducing hepatocyte failure in chronic liver injury, we measured the oxygen consumption rate (OCR) as a measure of basal mitochondrial activity. The OCR in hepatocytes from early cirrhotic livers was similar to that in normal age-matched control hepatocytes, but was significantly reduced in hepatocytes from failing cirrhotic livers (Fig. 1A). We then assessed proton leak, a measure of the ability of mitochondria to transport protons, to confirm these findings. The level of proton leak in hepatocytes from early cirrhotic livers was similar to that of normal age-matched control hepatocytes, but was significantly reduced in hepatocytes from failing cirrhotic livers, a sign of mitochondrial dysfunction (Fig. 1B). The proton leak plays the important functional role of reducing reactive oxygen species (ROS) in cells. It is possible therefore that the decreased proton leak points to the inability of the hepatocytes from failing cirrhotic livers to control ROS production [27,28], which in return may expose the hepatocytes to the harmful action of increased ROS. We then assessed uncoupled OCR as a measure of maximum mitochondrial capacity. A decrease in the uncoupled OCR is considered an indicator of mitochondria dysfunction [24]. We found that maximum mitochondrial capacity was reduced from normal even in hepatocytes from early cirrhotic livers and was further depressed in hepatocytes from failing cirrhotic livers (Fig. 1C). These results show that although basal mitochondrial respiration is maintained in cirrhotic hepatocytes with compensated func-

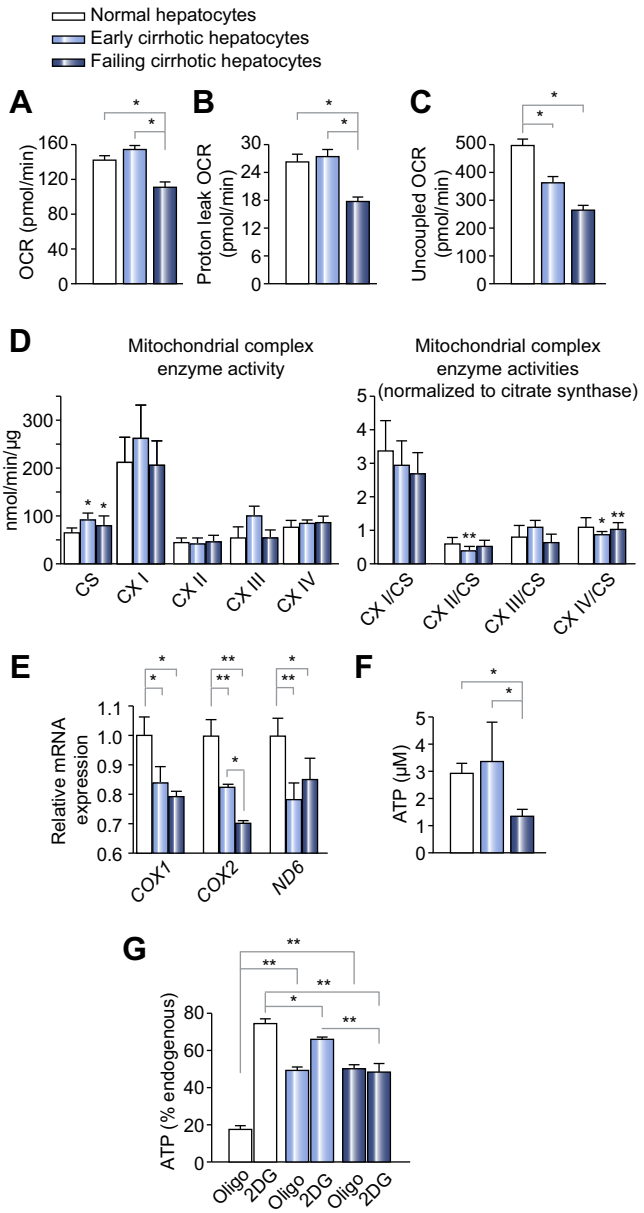

Fig. 1. Consequences of chronic liver injury on mitochondria respiration and energy production source in isolated hepatocytes. (A) Oxygen consumption rates (OCR), (B) Proton leak and (C) uncoupled OCR were measured as surrogates of mitochondria function. (D) Enzyme activities of the oxidative phosphorylation complexes were measured in nmol/min per µg of protein and normalized to the citrate synthase activity. (E) qPCR of mitochondria specific genes (*COX1*, *COX2*, and *ND6*). (F) Total ATP content and (G) the relative ATP contribution of the glycolytic and oxidative phosphorylation systems in energy production was also determined. All experiments were performed on hepatocytes isolated from normal, early and failing cirrhotic rat livers (*p <0.05; **p <0.001).

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tion their maximal mitochondrial respiration is reduced and that as cirrhosis progresses to become decompensated, mitochondrial function becomes reduced both at the basal and maximal levels. The identified mitochondrial dysfunction is probably a major contributor to the decompensated function of the hepatocytes in decompensated cirrhosis.

Our data also demonstrate that, compared to control hepatocytes, the mitochondria numbers (based on citrate synthase activity, a standard marker of mitochondria numbers in cells) were increased both in hepatocytes from early and failing cirrhotic livers (Fig. 1D). The activities of the electron transport chain complexes I through IV normalized per protein content demonstrated no changes [29] in the cirrhotic hepatocytes compared to the controls (Fig. 1D). The activities of electron transport chain complexes normalized per mitochondria content (citrate synthase activity) demonstrated decreased activity of complexes II and IV in compensated cirrhosis and complex IV in decompensated cirrhosis (Fig. 1D). Further, expression level of several mitochondria-specific genes; cytochrome c oxidase 1 and 2 (*COX1*, *COX2*) and NADH dehydrogenase 6 (*ND6*) was examined via quantitative polymerase chain reaction (qPCR). We found that their expression was decreased according to the progression of liver injury (Fig. 1E). Taken together with our finding of compromised maximal mitochondrial respiration in compensated cirrhosis and compromised basal and maximal respiration in decompensated cirrhosis, these data likely point to an attempt of the diseased organs to increase the numbers of their dysfunctional mitochondria to compensate for the mitochondrial dysfunction. The dysfunction of the mitochondria is probably produced by compromised complexes II and IV. Dysfunction at the level of substrate carriers (pyruvate, malate, glutamate, succinate), electron transport molecules like coenzyme Q and cytochrome C and complex V were not assessed and may also contribute to a failure of ATP production by the electron transport chain in cirrhotic hepatocytes.

With chronic injury, the source of hepatocyte energy production undergoes a metabolic shift and hepatocyte ATP levels fall with decompensated function

Given that basal mitochondrial respiration is decreased in rat hepatocytes from failing cirrhotic livers but not in rat hepatocytes from early cirrhotic livers, we investigated whether this was also reflected in decreased mitochondrial energy production, as assessed by the levels of adenosine triphosphate (ATP). As shown in Fig. 1F, hepatocytes from early cirrhotic livers had ATP levels similar to those of normal hepatocytes, however, hepatocytes from failing cirrhotic livers had ATP levels that were significantly reduced.

To determine how chronically injured hepatocytes with normal function maintain ATP production, we examined whether energy was derived from oxidative phosphorylation or from glycolysis. We measured ATP levels in the presence of an inhibitor of mitochondrial oxidative phosphorylation (oligomycin, a F_1F_O-ATPsynthase inhibitor) or an inhibitor of glycolysis (2-deoxyglucose; 2DG) [25,30]. In normal hepatocytes, blocking mitochondrial oxidative phosphorylation causes a significant inhibition of ATP content, while blocking glycolysis has essentially no effect (Fig. 1G), demonstrating that mitochondrial oxidative phosphorylation is the major source of energy production in hepatocytes from normal livers. In cirrhotic hepatocytes, whether their function was intact or decompensated, blocking mitochondrial oxidative phosphorylation resulted in no reduction in ATP levels, and blocking glycolysis resulted in a significant reduction in ATP levels especially in hepatocytes with decompensated function (Fig. 1G). These results show that there is a metabolic shift in the source of energy production in cirrhotic hepatocytes from oxidative phosphorylation to glycolysis. Glycolysis increases in cirrhotic hepatocytes in an attempt to compensate for decreased oxidative phosphorylation in order to maintain ATP production.

Compensatory ATP production from glycolysis is severely impaired in hepatocytes from cirrhotic livers with decompensated function

Because we found that hepatocytes from failing cirrhotic livers lose their ability to generate energy through glycolysis, we examined the maximum glycolytic flux capacity. The extracellular acidification rate (ECAR) [24] was used to detect and quantitate glycolytic flux. In this assay, cells with a glycolytic phenotype exhibit significantly higher rates of proton production. Hepatocytes from early cirrhotic livers showed an increased capacity to generate ATP from glycolysis when compared to normal hepatocytes (Fig. 2A). This capacity however was severely decreased in hepatocytes from failing cirrhotic livers compared to both normal and hepatocytes from early cirrhotic livers (Fig. 2A). Consistent with these findings, glucose consumption and lactate production from anaerobic glycolysis were both elevated only in hepatocytes from early cirrhotic livers; these measures were significantly diminished in hepatocytes from failing cirrhotic livers (Fig. 2B and C).

Expression of genes in the glycolytic pathway are severely decreased in cirrhotic hepatocytes with decompensated function

Hepatocytes from failing cirrhotic livers eventually fail to generate adequate amounts of ATP because of an inability to sustain the required increase in glycolytic flux capacity. To identify possible sources for this loss in capacity, we determined the expression of genes related to glucose metabolism using qPCR array (Fig. 2D, Table 1A–C). With cirrhosis, whether associated with normal or decompensated function, there was down regulation in tricarboxylic acid (TCA) cycle genes. Compensated function early in cirrhosis was also associated with up-regulation of genes related to gluconeogenesis and glycogenolysis. However, with decompensation in function, there was down-regulation of genes related to gluconeogenesis and glycogenolysis, specifically pyruvate dehydrogenase phosphatase catalytic subunit 2 (*PDP2*), glucose-6-phosphate dehydrogenase (*G6PD*), phosphoglucomutase 1 (*PGM1*), and glucose-6-phosphatase catalytic subunit (*G6PC*) (Table 1A). We confirmed similar results in the thioacetamide-induced liver cirrhosis rat model (Supplementary Fig. 2).

To confirm the clinical relevance of these findings we examined whether the expression pattern of genes related to glucose metabolism identified in the animal model correlated with the different stages of chronic end-stage liver disease in humans according to Child-Pugh classification. A cohort of mitochondrial (*COX1* and *COX2*) and glycolytic (*PGM1*, *PDHB*, and *G6PC*) genes were examined via qPCR in liver tissue from patients with Child-Pugh classification A, B, and C (Table 1A). The gene expression results obtained from patients were consistent with our findings from the carbon tetrachloride induced liver

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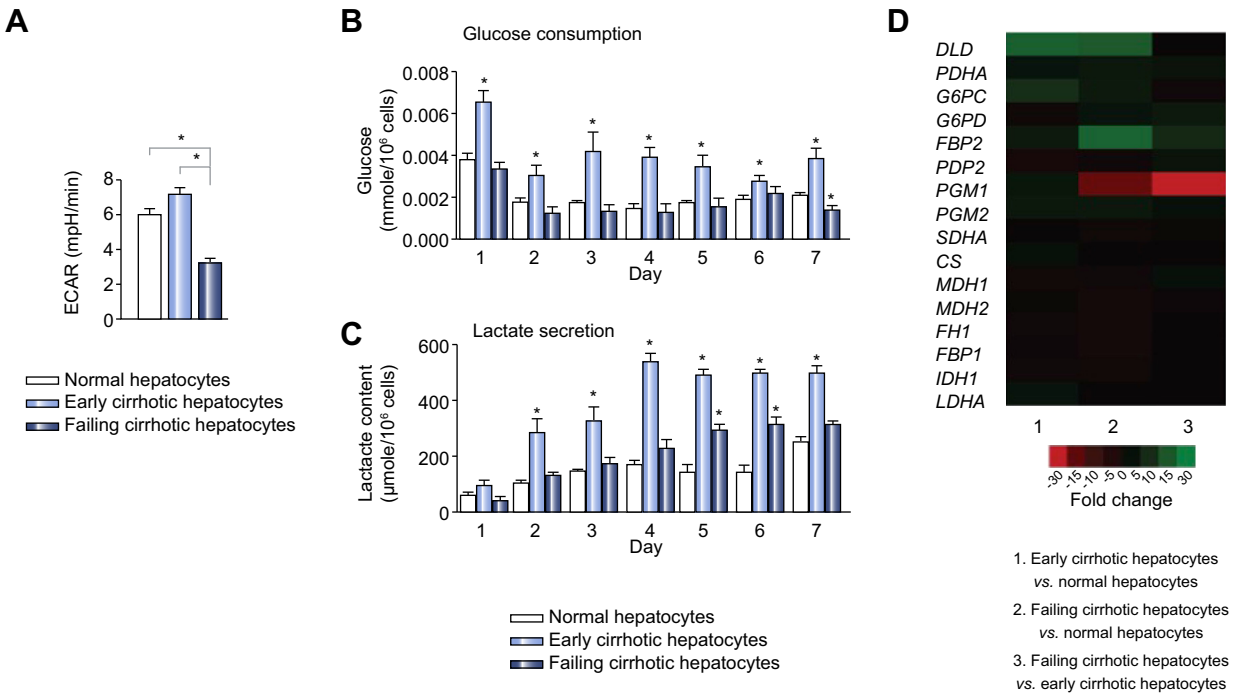

Fig. 2. Collapse of glycolytic compensation in hepatocytes from livers with terminal chronic liver injury. (A) Extracellular acidification rates (ECAR) were measured on normal, compensated, and decompensated cirrhotic hepatocytes. (B) Glucose consumption and (C) lactate secretion were also quantified every 24 h. (D) Gene expression analysis of glycolytic and mitochondrial enzymes of hepatocytes from normal, early and failing cirrhotic rat livers. The results represent fold changes and are illustrated in a heat map (*p <0.05; **p <0.001).

cirrhosis animal model. The *COX1* and *COX2* expression was decreased with the progression of liver injury. In contrast, early in cirrhosis (Child-Pugh Class A and B) human liver tissue was associated with up-regulation of genes related to gluconeogenesis and glycogenolysis (*PGM1*, *PDHB*, and *G6PC*). However, Child-Pugh Class C cirrhotic patients or decompensated liver function showed a significant down-regulation of these genes (Fig. 3).

To determine whether the expression of genes related to gluconeogenesis and glycogenolysis in early-stage cirrhosis (Child-Pugh Class A) is related to patient outcome, we examined the expression of *PGM1*, *PDHB*, and *G6PC* using microarray data available at the National Center for Biotechnology Information Gene Expression Omnibus (GSE15654) [31]. This microarray data was obtained from biopsies of 216 patients with hepatitis C-related Child-Pugh A cirrhosis who were prospectively followed for a median of 10 years and classified as good and poor prognosis (See Supplementary Materials and methods) [31]. As seen in Supplementary Fig. 3A, expression of these genes was downregulated in the group of patients with poor outcome. Performing an unbiased network analysis in the entire gene expression dataset, we found that the topmost significant down-regulated genes and functions were related to cellular amine metabolic processes and xenobiotic metabolic processes when median gene expression of Poor/Good outcome groups were compared. Genes included in this topmost down-regulated networks were alcohol dehydrogenase (*ALDH*), glucose-6-phosphatase catalytic subunit (*G6PC*), cytochrome P450 (*CYP3a4*, *CYP2a7*), growth hormone receptor (*GHR*), phosphoglycerate mutase (*PGAM4*), and transthyretin (*TTR*) (Supplementary Fig. 4 and 4A). These analyses indicated progressive loss of gene expression representing worsening of metabolic function. Specific gene networks affected in patients with poor outcome are shown in Supplementary Figs. 5–10.

Differential metabolic profiling in hepatocytes showed an increased uptake of glucogenic amino acids at early stages of chronic liver injury

Since amino acids can be converted into glucose through gluconeogenesis in states of catabolism, we asked whether the above results could be identified using differential metabolomics. We performed a systematic profile of amino acid up-take and secretion by normal and cirrhotic hepatocytes and observed a significant increase in the uptake of glucogenic amino acids in hepatocytes with compensated function compared with normal hepatocytes (Fig. 4A). Uptake of glucogenic amino acids was diminished in hepatocytes from failing cirrhotic livers when compared to hepatocytes isolated from livers with compensated function. These findings are consistent with the diminished maximum glycolytic flux capacity observed by ECAR and the loss of *G6PC* and *PGM1* expression. We also found a significant and dramatic increase in citrulline secretion in hepatocytes from failing cirrhotic livers, suggesting impairment of the urea cycle at the terminal stages of disease (Fig. 4B). There was an increase in the uptake of branched chain amino acids over aromatic amino acids (BCAA/AAA ratio) in hepatocytes from early cirrhotic livers (Fig. 4C), compared with a decrease in the BCAA/AAA ratio in hepatocytes from failing cirrhotic livers. These studies indicate that processing of BCAA, such as valine, isoleucine, and leucine, may be useful as markers for hepatic function in chronic liver disease [8].

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Table 1. List of the genes up- or down-regulated in compensated- and decompensated-cirrhotic hepatocytes. Each sample was isolated from age-matched normal, compensated and decompensated-cirrhotic hepatocytes, respectively. Changes in gene expression between groups were evaluated using RT2 Profiler 96-well PCR array plates. Data analysis was done by the $2^{-\Delta\Delta Ct}$ method on the manufacturer's Web portal (http://www.SABiosciences.com/pcrarraydataanalysis.php), (QIAGEN, CA, USA). (A) Genes differentially expressed (>1.3 fold). Normal hepatocytes *vs.* early-cirrhotic hepatocytes *vs.* failing-cirrhotic hepatocytes. (B) Up- and down-regulated genes (>2 fold). Normal *vs.* early-cirrhotic hepatocytes. (C) Up- and down-regulated genes (>2 fold). Early-cirrhotic hepatocytes *vs.* failing-cirrhotic hepatocytes.

A

Unigene	RefSeq	Symbol	Description	Fold change		
				Early *vs.* normal	Failing *vs.* early	Failing *vs.* normal
Rn.220381	NM_145091	*Pdp2*	Pyruvate dehydrogenase phosphatase catalytic subunit 2	-3.02893	1.4	-2.1
Rn.11040	NM_017006	*G6pd*	Glucose-6-phosphate dehydrogenase	-1.93483	2.5	1.3
Rn.9970	NM_017033	*Pgm1*	Phosphoglucomutase 1	1.855406	-30.5	-16.4
Rn.10992	NM_013098	*G6pc*	Glucose-6-phosphatase, catalytic subunit	5.386761	-2.1	2.5

B

Unigene	RefSeq	Symbol	Description	Fold change
Rn.86962	NM_199385	*Dld*	Dihydrolipoamide dehydrogenase	11.016
Rn.10992	NM_013098	*G6pc*	Glucose-6-phosphatase, catalytic subunit	5.3868
Rn.102424	NM_001007620	*Pdhb*	Pyruvate dehydrogenase (lipoamide) beta	3.8521
Rn.15319	NM_053716	*Fbp2*	Fructose-1,6-bisphosphatase 2	2.578
Rn.104376	NM_198780	*Pck1*	Phosphoenolpyruvate carboxykinase 1 (soluble)	2.5013
Rn.9738	NM_017328	*Pgam2*	Phosphoglycerate mutase 2 (muscle)	2.2215
Rn.3415	NM_001024743	*Ugp2*	UDP-glucose pyrophosphorylase 2	-2.0753
Rn.3561	NM_031510	*Idh1*	Isocitrate dehydrogenase 1 (NADP+), soluble	-2.091
Rn.29782	NM_017005	*Fh1*	Fumarate hydratase 1	-2.1251
Rn.13492	NM_033235	*Mdh1*	Malate dehydrogenase 1, NAD (soluble)	-2.2096
Rn.220381	NM_145091	*Pdp2*	Pyruvate dehyrogenase phosphatase catalytic subunit 2	-3.0289

C

Unigene	RefSeq	Symbol	Description	Fold change
Rn.11238	NM_012638	*Pygm*	Phosphorylase, glycogen, muscle	5.4647
Rn.15319	NM_053716	*Fbp2*	Fructose-1,6-bisphosphatase 2	4.4891
Rn.18101	XM_216091	*Pdk3*	Pyruvate dehydrogenase kinase, isozyme 3	3.406
Rn.91375	NM_012735	*Hk2*	Hexokinase 2	3.1739
Rn.11211	NM_012497	*Aldoc*	Aldolase C, fructose-bisphosphate	2.9286
Rn.11040	NM_017006	*G6pd*	Glucose-6-phosphate dehydrogenase	2.5497
Rn.50944	NM_001012130	*Pgk2*	Phosphoglycerate kinase 2	2.3271
Rn.10992	NM_013098	*G6pc*	Glucose-6-phosphatase, catalytic subunit	-2.1197
Rn.30070	NM_053551	*Pdk4*	Pyruvate dehydrogenase kinase, isozyme 4	-2.4911
Rn.104376	NM_198780	*Pck1*	Phosphoenolpyruvate carboxykinase 1 (soluble)	-2.4989
Rn.3415	NM_001024743	*Ugp2*	UDP-glucose pyrophosphorylase 2	-3.7837
Rn.145214	NM_001108703	*Rbks*	Ribokinase	-3.8254
Rn.102424	NM_001007620	*Pdhb*	Pyruvate dehydrogenase (lipoamide) beta	-4.261
Rn.9970	NM_017033	*Pgm1*	Phosphoglucomutase 1	-30.5808

Discussion

There is an abundant literature on the identification of mechanisms responsible for development of cirrhosis, but little concerning the mechanisms responsible for organ failure in terminal chronic liver disease. The present study was conducted to determine whether alterations in energy production and utilization could be linked to hepatocyte dysfunction in cirrhosis. Using a unique rat model of cirrhosis and end-stage liver failure that resembles human disease, we demonstrate that (a) mitochondrial respiration is decreased in cirrhotic hepatocytes especially at the late stages of cirrhosis, (b) cirrhotic hepatocytes undergo metabolic adaptation by shifting their ATP generation predominantly from oxidative phosphorylation to glycolysis, (c) despite a reduction in maximal respiratory capacity, mitochondrial ATP production is preserved in hepatocytes from early cirrhotic livers by increasing glycolysis to compensate for the decrease in ATP generation from oxidative phosphorylation, and (d) ATP production is severely depressed in hepatocytes from failing cirrhotic livers because both basal and maximal mitochondrial respiration are compromised and metabolic adaptation through an increase in glycolysis cannot be maintained.

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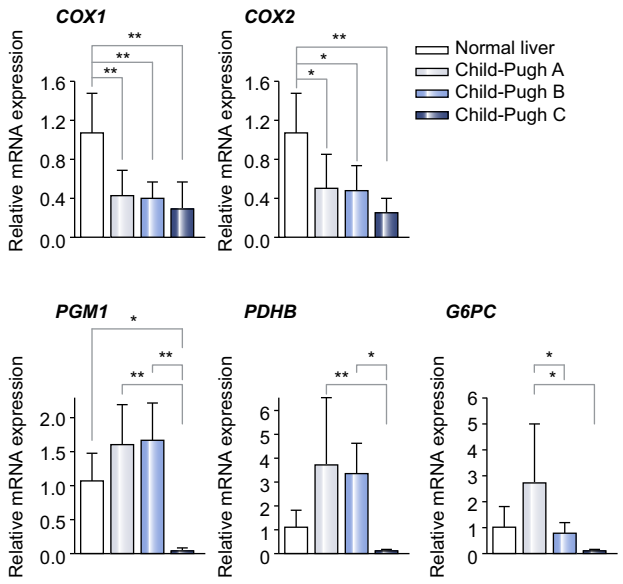

Fig. 3. Expression of mitochondrial and glycolytic genes in liver human tissue from different stages of chronic liver injury according to Child-Pugh classification. qRT-PCR analysis of mitochondrial (*COX1* and *COX2*) and glycolytic (*G6PC*, *PGM1*, *PDHB*) genes in human liver tissue from patients at different stages of chronic liver injury according to Child-Pugh classification (A–C), Child-Pugh scores are shown in Table 2 (*p <0.05; **p <0.001).

Cancer cells rely mostly on glycolysis for energy even in the presence of an adequate oxygen supply and this condition is known as the Warburg effect [32]. The shift in mitochondrial respiration to glycolysis in cancer cells has been proposed as a mechanism to avoid apoptosis [32]. The ability to maintain ATP production as a result of an increase in glycolysis may allow hepatocytes to survive additional injury in a cirrhotic liver only to make them more susceptible to becoming cancerous. In our studies, up regulation of *G6PC*, *DLD*, *PDHB*, *PCK1*, and *FBP2* was seen in compensated cirrhotic hepatocytes. If these genes sustain survival of hepatocytes that should otherwise fail and undergo apoptosis only to become tumorigenic, then up-regulation of these genes could potentially serve as a biomarker for an increased risk for the development of hepatocellular carcinoma. There is already evidence that G6PC is activated in hepatic tumors [15]. In our studies, down regulation of *PDP2*, *G6PD*, *PGM1*, and *G6PC* was associated with failure to maintain glycolysis and hepatocyte failure in cirrhosis. In this regard, polymorphisms in PGM1 have already been reported to be useful as a genetic biomarker for the development of alcoholic cirrhosis [33]. Polymorphism in the other identified genes may be useful in identifying patients with other forms of liver disease at risk for hepatic decompensation.

Table 2. Child-Pugh classification and identified etiology of cirrhotic liver tissues investigated.

Child-Pugh classification	Child-Pugh score	Etiology
A	6	HCV
A	6	HBV + HCV
A	5	HCV
A	5	HCV
A	5	HCV
B	8	HBV + HCV
B	7	HBV
B	7	HCV
B	7	HCV
B	7	HCV
C	13	Intrahepatic cholestasis
C	11	Intrahepatic cholestasis
C	11	Intrahepatic cholestasis
C	12	Biliary atresia
C	13	Intrahepatic cholestasis
C	13	Biliary atresia

It has been reported that mitochondrial metabolism is impaired in livers from rats with secondary biliary cirrhosis and activity of the electron transport chain is decreased in mitochondria from these livers [34,35]. The increase in mitochondrial content per hepatocyte was suggested as a compensatory mechanism to maintain hepatic mitochondrial function [35]. However, most of these studies were restricted to animals with early cirrhosis [34–36]. Thus far, metabolic adaptation and alterations in liver metabolism with increasing degree of cirrhosis has not been extensively studied. Our results demonstrate that mitochondrial respiration and ATP production are severely compromised in hepatocytes from failing cirrhotic livers. We confirmed our findings through gene expression of hepatocytes obtained from patients with early-stage cirrhosis (Child-Pugh Class A and B) and Child-Pugh C cirrhotic human livers. Previous studies using early-cirrhosis animal models had suggested increase in mitochondrial volume per hepatocyte and ATP production by increasing glycolysis as a compensatory mechanism in these livers [36]. In our in-depth analysis of coupled glycolysis and mitochondrial metabolism in livers with increasing degree of cirrhosis, we found that in early cirrhosis, up-regulation of glycolysis indeed supports the energetic needs of cells. However, this extra-mitochondrial ATP production itself is compromised in advanced cirrhosis and thus is unable to support energetic needs of cells. Our findings using a rat model of cirrhosis and end-stage liver failure were consistent with gene expression and metabolic network analysis in human livers. Both in our cohort analysis of liver tissue from patients with Child-Pugh classifications A, B, and C and also through microarray data obtained from 216 patients we found that glycolytic metabolism in end-stage liver was compromised. Thus, our data suggests that the failure of this adaptive pathway, leading to unmet energetic needs, is a possible cause of end-stage liver failure. Further, bioenergetic studies are needed to confirm these findings using *in vivo* models with varying degree of cirrhosis and through tissues obtained from patients.

Early identification of patients with cirrhosis at risk for developing acute decompensation could potentially improve their survival and enhance the development of therapies.

The results of the microarray analysis from early stage cirrhotic patients suggest that monitoring glycolytic gene networks may be used to predict the propensity of cirrhotic patients to worsen. Emerging metabolomics could represent an easy-to-use approach to detect metabolic abnormalities in patients with liver disease [37]. The metabolomic analysis performed in the current study revealed alterations in glycolysis and urea processing in hepatocytes from cirrhotic animals with decompensated function. In clinical practice, analysis of metabolites in the blood or other body fluids could be used.

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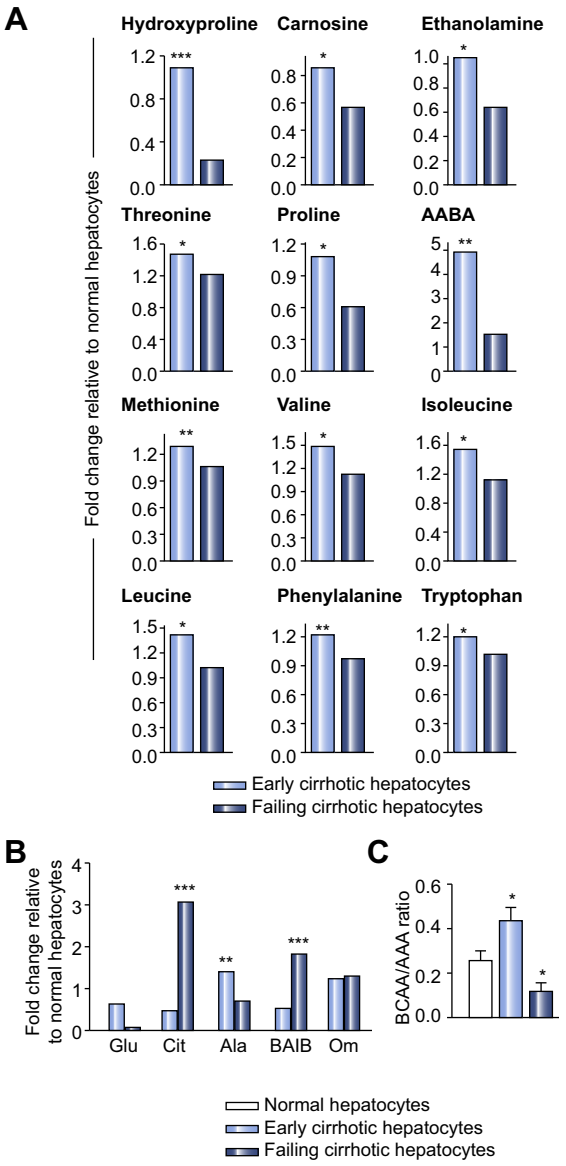

Fig. 4. Secretome flux of amino acids reflects metabolic compensation and hepatic dysfunction in cirrhotic hepatocytes. The (A) uptake and (B) secretion of amino acids were quantified by UPLC on the growth media the first 24 h after hepatocyte isolation. Each amino acid flux measured on compensated and decompensated cirrhotic hepatocytes is expressed in fold change relative to the normal hepatocytes (*p <0.05; **p <0.01 and ***p <0.001). (C) Branched chain to aromatic amino acids ratio was also determined. Each value represents mean ± SD (*p <0.05).

The alterations in energy metabolism associated with hepatocyte failure that we have identified could result not only from accumulated cellular damage but also from alteration in cellular signaling pathways that control the function of the hepatocyte. Previously, we showed that down regulation of hepatocyte nuclear factor 4α (HNF-4α) was associated with decompensated function in hepatic cirrhosis (24). HNF4α is a positive transcriptional regulator for controlling energy metabolism, xenobiotic detoxification, bile acid synthesis, and serum protein production [38,39]. More importantly, it regulates the expression of glucokinase, which plays a key role in glycolysis in the liver [40]. Thus, down regulation of HNF4α could be responsible for failure to maintain glycolysis in end-stage liver disease. Alternatively, late stages of liver cirrhosis are characterized by vascular remodeling of the liver that results in alterations in portal vein flow and ischemic injury to hepatocytes and intrahepatic endothelial cells [1,41]. Since hypoxia inducible factor 1α has been shown to control the expression of glucose transporters and glycolytic enzymes [42], it is possible that alterations in this gene might also be responsible for hepatic dysfunction in cirrhosis.

In summary, we identified two important metabolic processes in chronic liver injury: progressive impairment of mitochondrial respiration during initial stages of cirrhosis and subsequent deterioration in energy production through glycolysis in hepatocytes from failing cirrhotic livers. Further experimental dissection of the role of metabolic mediators in end-stage liver disease might be useful in the development of therapies for advanced liver disease.

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Conflict of interest

The authors who have taken part in this study declare that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' contribution

Study concept and design: A.S.-G., I.J.F., D.N.; acquisition of data: T.N., N.B., A.D., H.B., Z.Z., K.H., M.I.Y., M.O., I.P., A.S.-G., D.N., I.J.F.; analysis and interpretation of data: T.N., N.B., A.D., A.S.-G., N.B., D.N.,; microarray analysis: V.S., T.J.M., P.R.; drafting of the manuscript: T.N., N.B., A.S.-G., D.N.; critical revision of the manuscript for intellectual content: T.N., N.B., I.P., A.S.-G., I.J.F., D.N.; obtained funding: A.S.-G., I.J.F., I.P., D.N.; administrative, technical or material support: T.N., N.B., A.D., H.B., Z.Z., I.P., A.S.-G., I.J.F., D.N.; All authors contributed to the preparation of the report.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2014.02.014.

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