Research Article

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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.

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.