Acute Oxygen Supplementation Restores Markers of Hepatocyte Energy Status and Hypoxia in Cirrhotic Rats

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Accepted for publication January 23, 2000 This paper is available online at http://www.jpet.org

ABSTRACT

The oxygen limitation hypothesis states that hepatocyte hypoxia is the mechanism determining metabolic restriction in the cirrhotic liver. Therefore we studied markers of hepatocyte energy state and cellular hypoxia in livers of normal and cirrhotic rats before and after oxygen supplementation. Rats with carbon tetrachloride-induced cirrhosis and procedural control rats were exposed to either room air or a hyperoxic gas mixture for 1 h immediately before freeze clamping and perchloric acid extraction of liver tissue. Extracts were assessed by 31P NMR and enzymatic assays. Livers from cirrhotic rats breathing room air showed a reduced ratio of ATP/ADP, an increased ratio of inorganic phosphate/ATP, and a trend toward an increased ratio of lactate/pyruvate compared with procedural control livers (ATP/ADP 1.73 ± 0.35 versus 2.68 ± 0.61, P < .05; P/ATP 2.74 ± 0.48 versus 1.56 ± 0.26, P < .05; lactate/pyruvate 29.3 ± 6.4 versus 22.5 ± 7.4, P = .18). After supplementation with oxygen for 1 h, these ratios in cirrhotic livers approached control values. A variety of other metabolic markers affected by cirrhosis showed variable trends toward normal in response to oxygen supplementation, whereas minor trends toward an increase in ATP levels in control animals suggest the possibility of marginal oxygen limitation in normal livers. The data are consistent with the hypothesis that hepatocytes in cirrhotic livers have normal metabolic capacity but are constrained by a deficit in oxygen supply. Interventions aimed at increasing oxygen supply to the liver may have both short- and long-term therapeutic value in the management of cirrhosis.

The mechanisms underlying the deficits in liver function in cirrhosis are important because of the emerging potential for therapeutic intervention. The major theories of cirrhosis are based on observations of altered handling of xenobiotics and, in particular, pharmaceutical agents. However, these theories reflect differing views of the cirrhotic liver dysfunction and the potential for treatment. Traditional theories propose either a) a primary change in the hepatocytes themselves, the sick cell hypothesis (Branch and Shand, 1976; McLean and Morgan, 1991), b) a reduced mass of cells which function relatively normally, the intact hepatocyte hypothesis (Branch and Shand, 1976; McLean and Morgan, 1991) or c) a deficit in substrate supply, the impaired drug uptake hypothesis (Varin and Huet, 1985; McLean and Morgan, 1991; Morgan and McLean, 1995).

A recent theory, the oxygen limitation hypothesis, proposes that the hepatocytes are denied normal oxygen supplies because of impaired oxygen diffusion following sinusoidal capillarization (McLean and Morgan, 1991). Metabolic processes that have requirements for direct use of oxygen as a substrate and high K_m(O2) values (Jones, 1981) are predicted to be affected first, whereas processes that do not directly involve oxygen, or have low K_m(O2) values, are proposed to be relatively spared. Selective impairment of oxidative drug metabolism relative to conjugative metabolism has been advanced as an example of this principle (McLean and Morgan, 1991; Morgan and McLean, 1995).

Inhaled oxygen supplementation has been shown to increase theophylline clearance in the cirrhotic rat and human (Hickey et al., 1995; Froomes et al., 1999), and the perfused cirrhotic rat liver has also shown a marked sensitivity and rapid response to reduced oxygen delivery compared with normal livers (Hickey et al., 1996). Similarly, an increase in oxygen consumption and hepatic intrinsic clearance of propranolol has been shown to result from an increase in hepatic oxygen supply to cirrhotic rat livers by an increase in perfusion via the hepatic artery (Le Couteur et al., 1999). Although these studies have not addressed the general metabolic status of the liver, the data are supportive of the oxygen limitation hypothesis.

Received for publication October 26, 1999.

1 We acknowledge the support of the Private Practice Trust Fund of The Canberra Hospital. This work has received funding from the National Health and Medical Research Council of Australia.

ABBREVIATIONS: CCl4, carbon tetrachloride; PCA, perchloric acid; GPE, glycerophosphoethanolamine; PE, phosphoethanolamine; PME, phosphomonoester; PDE, phosphodiester.
The metabolic status of the cirrhotic liver has been investigated using a variety of techniques that allow assessment of high-energy metabolites and disturbance of metabolic pathways (Wakashiro et al., 1989; Weiner et al., 1989; Hernandez-Munoz et al., 1990; Matsui et al., 1994; Menon et al., 1995; Taylor-Robinson et al., 1995; Jalan et al., 1996). In previous work from our laboratories, we used a carbon tetrachloride (CCl₄)-induced cirrhotic rat model in combination with rapid quenching of metabolism to allow NMR quantitation of changes in the intermediary metabolism of cirrhotic rat livers (Harvey et al., 1999a). The studies reported here used these NMR methods together with enzymatic assays to address the question of functional cellular hypoxia of cirrhotic rat livers and their responsiveness to acute oxygen supplementation.

**Experimental Procedures**

**Materials.** Phenobarbitone sodium was purchased from David Craig & Co. (Queensland, Australia), pentobarbitone sodium from Boehringer Ingelheim (Ingelheim, Germany), D₂O from Australian Nuclear Science and Technology Organization (New South Wales, Australia), and Chelex 100 from Bio-Rad Laboratories (New South Wales, Australia). Lactate and pyruvate diagnostic kits were obtained from Sigma (New South Wales, Australia). All other reagents used were of analytical grade.

**Animals and Ethical Approval.** Male Wistar rats were obtained from and housed in the John Curtin School of Medical Research. All protocols and procedures were approved by the Australian National University Animal Experimentation Ethics Committee.

**Induction of Cirrhosis.** Cirrhosis was induced according to the method described (Proctor and Chatamra, 1982) with extension of the phenobarbitonal induction and modification of CCl₄ exposure (Harvey et al., 1999a). Rats (80–100 g, 4-weeks old) were given phenobarbitone sodium (440 mg/kg) in their drinking water for approximately 10 weeks. After 2 weeks, the rats were given weekly doses of CCl₄ in corn oil (1 ml; 5–50%, v/v) administered by gavage for an average of 10 weeks. An average of 3 weeks separated the last CCl₄ dose from the experiment to avoid the effects of acute toxicity by hepatotoxin. Cirrhosis was confirmed by the presence of bridging fibrosis and nodular regeneration as determined by blinded histological examination by an independent pathologist. Littermate animals were treated as procedural controls in parallel, with phenobarbitone sodium and corn oil given in an identical manner.

**Perchloric Acid (PCA) Extraction.** PCA extraction and NMR analysis were performed as described previously (Harvey et al., 1999a). Rats were anesthetized with pentobarbitone sodium (60 mg/kg b.wt., i.p.). The liver area was exposed by laparotomy incision. The top lobe of the liver was clamped with aluminum tongs precooled in liquid nitrogen and immediately cut from the rest of the liver. The frozen portion was split into two and stored under liquid nitrogen until further treatment. Immediately after the liver clamping, a blood sample was withdrawn from the inferior vena cava, centrifuged, and the plasma stored at −20°C for liver function tests. Animals were sacrificed by exsanguination immediately after blood samples were obtained. Each portion of frozen liver was finely ground under liquid nitrogen and added to a preweighed vial of 7% PCA (5 ml) at 0°C. The mass of tissue in each portion was determined by weighing the vial and contents, and the mixture was allowed to warm to 0°C over approximately 10 min. The supernatant was removed after centrifugation (2500 rpm, 4°C, 5 min), and a second volume of PCA (3 ml) was added to the tissue. Following centrifugation as before, the supernatants were combined and neutralized with 10% KOH to pH 6.5 to 7. The potassium perchlorate precipitate was removed by centrifugation as before, and the sample was lyophilized and stored at −20°C until NMR analysis. Additional liver tissue was also sampled for histological examination.

**NMR Analysis.** Each extract sample was eluted through a short column of Chelex 100 to remove divalent metal ions, lyophylized, and redissolved in D₂O (1.0 ml) containing 2 mM methylene diphosphonic acid. The pH (uncorrected for deuterium isotope effect) was adjusted to 7.5. 31P spectra were acquired on a Varian VXR-300 spectrometer at 121.4 MHz at 25°C using a 45° pulse, a 6-s relaxation delay, gated broad-band proton-decoupling, and approximately 2000 scans. Corrections for T₁ were made after running representative samples with a 30-s relaxation delay. Chemical shifts of phosphorus resonances are reported relative to 85% orthophosphoric acid and based on the primary internal standard of methylene diphosphonic acid (δ 17.00). Assignments were based on literature values and confirmed by addition of authentic compound to representative extract samples. Absolute metabolite levels (as micromoles per gram of wet liver) were based on three integrations of the same spectrum. Metabolite levels (as percentages) were calculated as the percentage contribution of metabolite relative to the total phosphorus in the spectrum.

**Lactate/Pyruvate Analysis.** PCA extracts, as described above, were treated with 10% KOH to raise the pH to 2 to 5, and lactate and pyruvate were enzymatically quantified according to the literature methods (Czok and Lamprecht, 1974; Gutmann and Wahlefeld, 1974).

**Oxygen Supplementation.** Rats were randomized to breathe either room air or a hyperoxic gas mixture of 95% O₂, 5% CO₂ for 1 h under a continuous flow of 2 l/min into a closed metabolic cage. For rats exposed to the hyperoxic gas mixture, liver extracts were obtained as described above while the rat continued to be exposed to direct 95% O₂, 5% CO₂ supply via a funnel over the nose and mouth. Using a separate group of control and cirrhotic rats, pO₂ levels were assessed by sampling blood from the aorta below the mesenteric artery and from the portal vein at the porta hepatis (Harvey et al., 1999b).

**Statistical Evaluation.** Statistical analysis of results was performed by Student’s unpaired t test and ANOVA with Bonferroni correction (for multiple comparisons) as appropriate. Results are expressed as mean ± 1 S.D. and considered significant at a P level of <.05.

**Results**

**Induction of Cirrhosis.** Cirrhotic rats showed smaller body weights and larger spleens when compared with procedural controls, and significant changes in plasma levels of albumin, alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase (Table 1). Hepatic cirrhosis was confirmed by histological examination.

**Physiological Responses to Oxygen Supplementation.** In both control and cirrhotic animals, hyperoxia resulted in a greater than 5-fold increase in arterial pO₂, whereas the portal vein pO₂ increased almost 2-fold (Table 2). No significant differences were detected between the pO₂ values of control and cirrhotic rats under either normoxic or hyperoxic conditions.

**TABLE 1**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 24)</th>
<th>Cirrhotic (n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>483 ± 3</td>
<td>402 ± 63*</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>19.0 ± 2.2</td>
<td>18.3 ± 4.1</td>
</tr>
<tr>
<td>Spleen weight (g)</td>
<td>1.3 ± 0.2</td>
<td>3.4 ± 0.9a</td>
</tr>
<tr>
<td>Liver function tests</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>26 ± 3</td>
<td>19 ± 4*</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/l)</td>
<td>76 ± 30</td>
<td>122 ± 42*</td>
</tr>
<tr>
<td>Aspartate aminotransferase (U/l)</td>
<td>117 ± 44</td>
<td>264 ± 131*</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/l)</td>
<td>120 ± 37</td>
<td>226 ± 74*</td>
</tr>
</tbody>
</table>

*Significantly different from controls (P < .001).
Cirrhotic rat livers showed a trend toward higher lactate/pyruvate ratios under normoxic conditions, with the ratio significantly reduced by hyperoxia (Table 2). In contrast to findings in cirrhosis, hyperoxia induced no change in the ratio in livers of control animals.

**Metabolic Data.** 31P NMR spectra of PCA liver extracts from a representative normoxic control, normoxic cirrhotic, and hyperoxic cirrhotic rat are shown in Fig. 1, A, B, and C, respectively. Of particular interest are those resonances caused by high energy metabolites such as ATP, ADP, and P. The ATP and ADP resonances reflect predominantly adenine metabolites but also contain guanine, cytosine, thymidine and uracil. The phosphomonoester (PME) region from 5.5 to 3.6 ppm contains glycolytic intermediates and phospholipid precursors, whereas the phosphodiester (PDE) region consists predominantly of glycerophosphoethanolamine (GPE) and glycerophosphocholine. All forms of nicotinamide adenine dinucleotides (NAD, NADH, NADP, and NADPH) contribute to an unresolved diphosphodiester region centered at −10.4 ppm, whereas a cluster of peaks centered at −12.0 ppm is caused by diphosphodiesters such as UDP-glucose.

The absolute concentrations of the phosphorylated energy metabolites, expressed as micromoles per gram of wet liver, are presented for control and cirrhotic rats under normoxic and hyperoxic conditions in Table 3. In normoxic cirrhotic rats, ATP was significantly reduced by the order of 50% and ATP/ADP and P/ATP ratios were changed significantly compared with normoxic control rats. The concentration of ATP rose significantly with oxygen supplementation such that differences with normoxic controls were no longer detectable (Fig. 1, C versus A, and Table 3). ATP/ADP and P/ATP ratios also changed significantly to approach the values of normoxic controls. Hyperoxia also caused an increase in the ATP/ADP ratio of control rat livers. Total phosphorus content was significantly reduced in cirrhosis, and hyperoxia caused no significant change in this trend.

In addition to high-energy metabolites, compounds such as phosphoethanolamine (PE), GPE, PME, PDE, and NADs also showed significant changes in cirrhosis with variable responses to oxygen supplementation (Table 3).

**Discussion**

The results of this study confirm the diversity of metabolic changes in the cirrhotic liver as reported by previous researchers (Wakashiro et al., 1989; Weiner et al., 1989; Hernandez-Munoz et al., 1990; Matsui et al., 1994; Menon et al., 1995; Taylor-Robinson et al., 1995; Jalan et al., 1996; Harvey et al., 1999a). Of particular interest in this study are the major changes in markers of cellular energy state and cellular hypoxia, and their reversibility with acute oxygen supplementation. These findings allow mechanistic insights and hold potential therapeutic significance.

Concentrations of high-energy metabolites in control rat livers are in good agreement with reported values (Illes et al., 1985; Desmoulin et al., 1987; Brauer and Ling, 1991; Harvey et al., 1999a). Specifically, control rat livers had mean ATP, ADP, and P concentrations of 2.48, 0.97, and 3.80 μmol/g wet liver, respectively, whereas the respective ranges of reported values are 2.16 to 2.7, 0.78 to 1.1, and 2.87 to 3.9 μmol/g wet liver (Desmoulin et al., 1987; Brauer and Ling, 1991). Similarly, the mean values of 2.68 and 1.56 for the ATP/ADP and

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**TABLE 2**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normoxia</th>
<th>Hyperoxia</th>
<th>Normoxia</th>
<th>Hyperoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterial pO2</td>
<td>77 ± 12</td>
<td>412 ± 39</td>
<td>69 ± 9</td>
<td>423 ± 51</td>
</tr>
<tr>
<td>Portal pO2</td>
<td>43 ± 6</td>
<td>82 ± 17</td>
<td>46 ± 1</td>
<td>80 ± 15</td>
</tr>
<tr>
<td>Lactate</td>
<td>2.17 ± 0.57</td>
<td>2.06 ± 0.50</td>
<td>1.84 ± 0.58</td>
<td>2.24 ± 0.65</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.11 ± 0.04</td>
<td>0.12 ± 0.05</td>
<td>0.06 ± 0.02</td>
<td>0.15 ± 0.03</td>
</tr>
<tr>
<td>Lactate/pyruvate</td>
<td>22.5 ± 7.4</td>
<td>18.6 ± 4.8</td>
<td>29.3 ± 6.4</td>
<td>15.2 ± 2.9</td>
</tr>
</tbody>
</table>

* Significantly different from normoxic group (P < .05).

**Fig. 1.** 121.4 MHz 31P NMR spectra of PCA liver extract of control rat (A), cirrhotic rat (B), and hyperoxic cirrhotic rat (C). 1, PE; 2, phosphocholine; 3, P; 4, GPE; 5, glycerophosphocholine; 6, ATP (αP); 7, ADP (βP); 8, ADP (αP); 9, ATP (αP); 10, diphosphodiester, mainly NAD(H) and NADP(H); 11, diphosphodiester, mainly UDP-glucose; 12, ATP (βP); PME, 5.5 to 3.6 ppm; PDE region, 2.0 to −1.0 ppm. These spectra are representative of all rats examined.
P/ATP ratios of control rat livers are supported by the literature ranges of 2 to 2.45 and 1.37 to 1.47, respectively (Iles et al., 1985; Desmoulin et al., 1987; Brauer and Ling, 1991). Hence, the significant changes observed in cirrhotic livers indicate a lower bioenergetic status in these livers, which is also consistent with previous studies (Wakashiro et al., 1989; Brauer and Ling, 1991; Desmoulin et al., 1987; Brauer and Ling, 1991). More importantly, we have observed that oxygen supplementation reversed the pattern of reduced ATP, reduced ATP/ADP ratio, and increased P/ATP ratio seen in cirrhotic livers of animals breathing room air. These observations, when combined with the arterial and portal vein pO₂ data (Table 2), allow a direct correlation between the changes in metabolic markers and acute increase in oxygen supply to the liver. This pattern of metabolic response in cirrhotic animals represents a precise mirror image of the responses of control livers to oxygen restriction reported by Brauer and colleagues (1997). We also noted an increase in the ATP content of the normal livers with oxygen supplementation, but this was only apparent when analyzed as a percentage of total phosphorus content and was not associated with any changes in other high-energy metabolites or ratios. This may suggest the presence of oxygen limitation even in normal livers although the evidence is limited.

Lactate and pyruvate concentrations of hepatic tissue extracts were determined to examine cell redox states. Our data, showing a trend toward higher hepatic lactate/pyruvate ratios in normoxic cirrhotic rats, are consistent with the findings of Hernandez-Munoz et al. (1994). The relationship between these markers and oxygenation status was also confirmed by the data showing that oxygen supplementation induced a major (2-fold) decrease in the hepatic lactate/pyruvate ratio of cirrhotic rats.

The changes detected in hepatic phosphorus content, phosphate metabolism, NAD, and phospholipid metabolism of normoxic rats confirm the literature showing major and general changes in cellular biochemistry in cirrhosis. The significant decrease in total phosphorus content observed is consistent with previous NMR findings of reduced absolute metabolite concentrations in animals and humans with cirrhosis and acute liver failure (Bates et al., 1988; Meyerhoff et al., 1989; Harvey et al., 1999a). This reduction in total phosphorus content may be accounted for by a reduced hepatocyte mass or volume in cirrhotic livers as demonstrated by morphometric analysis (Reichen et al., 1987; Matsui et al., 1994; Harvey et al., 1999a). Increased excretion of nicotinamide in the form of N-methyl nicotinamide and decreased hepatic NAD levels have been reported in previous studies (Cuomo et al., 1994, 1995) of human cirrhosis and of experimental hypoxia in perfused rat livers, with apparent linkage to the ATP content of the liver. Changes in phospholipid metabolism were detected by an increased PME/PDE ratio, reflecting a rise in PE and a decrease in GPE contributions to PME and PDE levels, respectively. Because the PME and PDE regions contain biosynthetic precursors for membrane phospholipids and hydrolytic products, respectively, it has been proposed that the change in PME/PDE ratio may be attributed to an increased rate of cell turnover as the damaged cirrhotic liver attempts to regenerate (Menon et al., 1995; Taylor-Robinson et al., 1995).

Levels of NAD and markers of phospholipid metabolism in cirrhotic livers also showed responses to acute oxygen supplementation. NAD levels showed a trend toward control values, whereas the PME/PDE ratio decreased significantly in cirrhotic livers as the total levels of PME and PDE returned to control values. The linkage of abnormal phospholipid metabolism to hypoxia has been proposed previously through activation of plasma membrane phospholipase and accelerated degradation of phospholipids (Kehrer et al., 1990). Further definition of the dependence of changes in liver metabolism on oxygen availability in cirrhosis will require studies of oxygen supplementation over longer time periods.

Oxygen supplementation and oxygen restriction have been linked to changes in hepatic metabolism of theophylline in human and animal cirrhosis (Hickey et al., 1995; Froomes et


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