Hepatic gluconeogenesis and Krebs cycle fluxes in a CCl₄ model of acute liver failure

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Received 1 August 2001; Accepted 24 September 2001

ABSTRACT: Acute liver failure was induced in rats by CCl₄ administration and its effects on the hepatic Krebs cycle and gluconeogenic fluxes were evaluated in situ by ¹³C NMR isotopomer analysis of hepatic glucose following infusion of [U-¹³C]propionate. In fed animals, CCl₄ injury caused a significant increase in relative gluconeogenic flux from 0.80 ± 0.10 to 1.34 ± 0.24 times the flux through citrate synthase (p < 0.01). In 24-h fasted animals, CCl₄ injury also significantly increased relative gluconeogenic flux from 1.36 ± 0.16 to 1.80 ± 0.22 times the flux through citrate synthase (p < 0.01). Recycling of PEP via pyruvate and oxaloacetate was extensive under all conditions and was not significantly altered by CCl₄ injury. CCl₄ injury significantly reduced hepatic glucose output by 26% (42.8 ± 7.3 vs 58.1 ± 2.4 μmol/kg/min, p = 0.005), which was attributed to a 26% decrease in absolute gluconeogenic flux from PEP (85.6 ± 14.6 vs 116 ± 4.8 μmol/kg/min, p < 0.01). These changes were accompanied by a 47% reduction in absolute citrate synthase flux (90.6 ± 8.0 to 47.6 ± 8.0 μmol/kg/min, p < 0.005), indicating that oxidative Krebs cycle flux was more susceptible to CCl₄ injury. The reduction in absolute fluxes indicate a significant loss of hepatic metabolic capacity, while the significant increases in relative gluconeogenic fluxes suggest a reorganization of metabolic activity towards preserving hepatic glucose output. Copyright © 2002 John Wiley & Sons, Ltd.

KEYWORDS: glucose homeostasis; hepatic glucose output; intermediary metabolism; Krebs cycle flux

INTRODUCTION

Hepatic glucose metabolism is disrupted in the setting of cirrhosis.¹–⁵ In the absorptive state it is characterized by an inability to extract glucose from the portal vein circulation leading to a reduction in hepatic glycogen deposition.³,⁴,⁶ Consequently, in the postabsorptive state, hepatic glucose production from glycogenolysis is reduced. Given that fasting hypoglycemia is typically rare in cirrhosis, hepatic glucose output is believed to be maintained by a compensatory increase in gluconeogenic flux.⁵,⁶ Since gluconeogenesis is largely dependent on a functional Krebs cycle for energy and gluconeogenic precursors, it is likely that such alterations reflect a reorganization of metabolic fluxes at the Krebs cycle level. These could include changes in the relative rates of acetyl-CoA oxidation and conversion of anaplerotic substrates (such as pyruvate) to glucose. Additionally, loss of functional hepatic mass and metabolic capacity may impose limits on absolute metabolic fluxes through these pathways.

We recently developed non-invasive methods for measuring hepatic gluconeogenic and Krebs cycle fluxes following administration of ¹³C tracers.⁷–⁹ The measurements can be performed in a clinical setting since they are based on non-radioactive tracers and the withdrawal and analysis of small amounts of blood.⁹ In its simplest form, the method provides a direct measurement of relative gluconeogenic and oxidative Krebs cycle fluxes.⁷,⁹ By incorporating an additional measurement of hepatic glucose output into the assay, absolute fluxes can be derived, allowing the total metabolic capacity for

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gluconeogenesis and Krebs cycle oxidation to be evaluated. To determine the utility and sensitivity of these measurements for characterizing Krebs cycle and gluconeogenic fluxes, we applied them to a standard rat model of acute liver failure induced by CCl₄ ingestion. Although this acute injury does not constitute a model of cirrhosis, usually described as a chronic liver disease, it provides a good approach towards the validation of the above ¹³C-tracer methodologies as tools for monitoring changes in glucose metabolism in the injured liver.

MATERIALS AND METHODS

Materials

Sodium [U-¹³C]propionate and [1,6-¹³C₂]glucose were obtained from Cambridge Isotope Laboratories (Cambridge, MA, USA). Other materials were analytical grade and used without further purification (Sigma, St Louis, MO, USA).

Animals

Acute liver injury was induced both in fed and fasted animals by intragastric administration of CCl₄ mixed with corn oil (1:1) 24 h prior to experimentation (1.0 ml/240 g body weight). Four groups of animals were studied: (i) fed controls; (ii) fed CCl₄ injured; (iii) fasted control; and (iv) fasted CCl₄ injured. Male Sprague–Dawley rats (190–310 g, Sasco, Houston, TX, USA) were used throughout.

Rats were anesthetized by intramuscular injection of a mixture of ketamine (85 mg/ml) and xylazine (15 mg/ml). For measurement of relative Krebs cycle and gluconeogenic fluxes an aqueous solution of sodium [U-¹³C]propionate was infused by a catheter inserted into the jugular vein at a rate of 25.0 μmol/kg/min over 1 h. For measurement of hepatic glucose output and absolute metabolic fluxes in 24 h-fasted rats, sodium [U-¹³C]propionate infusion (25.0 μmol/kg/min) was combined with a primed infusion of [1,6-¹³C₂]glucose (25.00 μmol prime, 2.75 μmol/kg/min) over 2 h.

For all measurements, blood was collected from the carotid artery and the liver was removed and freeze-clamped. Blood and tissue samples were extracted with cold perchloric acid, neutralized with KOH and freeze-dried. Glucose and carboxylic acids in the liver extract were separated from amino acids by cation exchange chromatography. The glucose/carboxylic acid fraction was treated with 100 units of glucose oxidase (6–12 h at 25 °C, Type VII-S, Sigma) and bubbled with air (about 10–20 ml/min) to quantitatively oxidize all glucose to gluconate. The reaction was terminated by lowering the pH to ~1 with concentrated perchloric acid. Plasma samples were treated with perchloric acid and prepared for NMR analysis as previously described. In separate experiments, levels of serum transaminases (GPT and GOT) were measured in a separate blood sample (16 rats, four in each group) to monitor the extent of hepatic injury induced by CCl₄.

NMR methods

Proton-decoupled ¹³C NMR spectra of liver extracts were obtained by using a 5 mm broadband probe on a 9.4 T GE-Bruker Omega spectrometer. Proton decoupled ¹³C NMR spectra of serum extracts were acquired by using a 5 mm broadband probe on a 14.1 T Unity INOVA (Varian Instruments, Palo Alto, CA). ¹H NMR spectra...
were acquired using a 5 mm indirect detection probe and the 14.1 T system. $^{13}$C broadband decoupling covering the C2–C5 region of glucose (70–76 ppm) was applied during $^1$H acquisition to eliminate all $^1$H-$^{13}$C long-range couplings in the glucose H1 resonance. Free induction decays were zero-filled and multiplied by a 0.25 Hz exponential function prior to Fourier transformation. Line fitting and spectral simulations were performed using the PC-based NMR software, NUTS (Accorn NMR, Freemont) or the standard Varian NMR software.

Measurement of hepatic fluxes using [U-$^{13}$C]propionate and $^{13}$C NMR

We have shown that [U-$^{13}$C]propionate is an effective tracer of gluconeogenesis and Krebs cycle activity in both rats and humans. $^{8,9}$ [U-$^{13}$C]propionate is metabolized via activation to propionyl-CoA, carboxylation to methylmalonyl-CoA, and entry into the Krebs cycle as succinyl-CoA. Figure 1 shows a standard model of the Krebs cycle featuring anaplerotic and gluconeogenic pathways. $^{8,9,11,12}$ Plasma glucose becomes enriched with $^{13}$C following incorporation of [U-$^{13}$C]propionate into the Krebs cycle as a consequence of gluconeogenic outflow via phosphoenolpyruvate (PEP). PEP can also be recycled via pyruvate and oxaloacetate (OAA) by a so-called futile cycle. $^{13,15}$ The major inflow of gluconeogenic carbons for PEP synthesis occurs via carboxylation of pyruvate. While these carbons can also be oxidized to acetyl-CoA via pyruvate dehydrogenase, this flux is typically very low compared to pyruvate carboxylase flux. $^{7,9,16–19}$

The labeling distribution in plasma glucose reflects the integrated activities of these pathways and given certain assumptions. Key assumptions are: (1) isotopic steady-state conditions; (2) negligible labeling of acetyl-CoA; and (3) complete randomization of OAA carbons via fumarate. Simple mathematical equations relate the glucose $^{13}$C isotopomers (as reported by the glucose C2 $^{13}$C-NMR signal) to relative fluxes for OAA $\rightarrow$ PEP, PEP $\rightarrow$ pyruvate, PEP $\rightarrow$ glucose and OAA $\rightarrow$ citrate. $^{7,9}$ These can be converted to absolute values when integrated with an independent measurement of absolute flux through any one of the pathways. $^{8}$

RESULTS

Serum transaminase levels

Conventional blood chemistry was performed on samples collected from 16 animals (four in each group). Plasma levels of glutamate-pyruvate (GPT) and glutamate-oxaloacetate (GOT) transaminases are reported in Fig. 2 for the four groups: fed control, fed CCl$_4$ injured, fasted control and fasted CCl$_4$ injured. As anticipated for animals with damaged livers, serum GPT and GOT were significantly elevated in both CCl$_4$ injured groups compared to controls.

Relative Krebs cycle and gluconeogenic flux measurements

The $^{13}$C-labeling distribution of hepatic glucose follow-
ing infusion of \([\text{U-}^{13}\text{C}]\)propionate was obtained from the \(^{13}\text{C}\) NMR spectrum of hepatic glucose following derivatization to gluconate\(^7,9,10\) and an example is shown in Fig. 3. In all cases, the gluconate C2 multiplet components were well resolved and had sufficient signal-to-noise for accurate quantitation of glucose isotopomer ratios and relative metabolic fluxes by \(^{13}\text{C}\) NMR.

Relative flux estimates from the NMR analysis of glucose are summarized in Table 1. Analysis of 24 h-fasted rat livers showed that fluxes through PEP-ck were about four times the flux through citrate synthase. In addition, a significant portion of PEP was recycled via pyruvate and OAA, the recycling flux being about 2.6 times flux through citrate synthase. Net outflow of PEP to glucose was about 1.4 times citrate synthase flux. These observations agree with our previous baseline measurements from both intact rats and perfused livers obtained from fasted rats.\(^7,8\) Compared to fasted rats, fed control rats showed a tendency toward lower PEP-ck flux and higher pyruvate kinase fluxes, resulting in significantly lower relative rates of gluconeogenesis. In fed animals, CCl\(_4\) injury caused modest but significant increases in relative gluconeogenic flux from baseline values. This resulted from non-significant increases in PEP-ck flux coupled to reductions in PEP recycling via pyruvate kinase and resembles the changes observed between fed and fasted baseline measurements. In fasted animals, while CCl\(_4\) injury also increased relative gluconeogenic flux from baseline values, this was accompanied by a tendency for increased recycling of PEP via pyruvate kinase.

**Figure 3.** 9.4 T \(^{13}\text{C}\) NMR spectra of the carboxylic acid fraction of an extract of a liver from a CCl\(_4\) injured, fasted rat. The expansion of the gluconate C2 resonance shows the resolution of multiplet components (D12, D23 and Q) whose relative areas are used to estimate relative TCA cycle and gluconeogenic fluxes

<table>
<thead>
<tr>
<th>Group</th>
<th>PEP-ck flux (OAA → PEP)</th>
<th>PK flux (PEP → pyruvate)</th>
<th>Gluconeogenic flux (PEP → glucose)</th>
<th>CS flux (OAA → citrate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed control (n = 6)</td>
<td>3.68 ± 0.42</td>
<td>2.87 ± 0.42</td>
<td>0.80 ± 0.10</td>
<td>1.00</td>
</tr>
<tr>
<td>Fed CCl(_4) injured (n = 7)</td>
<td>4.00 ± 0.59</td>
<td>2.65 ± 0.42</td>
<td>1.34 ± 0.24*</td>
<td>1.00</td>
</tr>
<tr>
<td>Fasted control (n = 6)</td>
<td>3.92 ± 0.37</td>
<td>2.55 ± 0.52</td>
<td>1.28 ± 0.16</td>
<td>1.00</td>
</tr>
<tr>
<td>Fasted CCl(_4) injured (n = 8)</td>
<td>5.03 ± 0.48</td>
<td>3.22 ± 0.49</td>
<td>1.80 ± 0.22*</td>
<td>1.00</td>
</tr>
</tbody>
</table>

* Significantly different (p < 0.005).
integrated to provide absolute flux estimates for gluconeogenic and Krebs cycle fluxes in 24 h fasted rats. A representative $^{13}$C spectrum of plasma glucose from a rat infused with [1,6-$^{13}$C$_2$]glucose and [U-$^{13}$C]propionate is shown in Fig. 4. The $\beta$-C1 resonance of glucose is shown in the expansion. The $\alpha$-H1 resonance from the $^1$H NMR spectrum of the same sample is also shown.

**Table 2. Absolute hepatic fluxes (μmol/kg/min) for 24 h fasted control and CCl$_4$ injured rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>PEP-ck flux (OAA → PEP)</th>
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<th>CS flux (OAA → citrate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control ($n = 6$)</td>
<td>356.2 ± 33.6</td>
<td>231.5 ± 47.2</td>
<td>116.2 ± 4.8</td>
<td>90.6 ± 8.0</td>
</tr>
<tr>
<td>CCl$_4$ injured ($n = 8$)</td>
<td>239.2 ± 22.8</td>
<td>153.1 ± 26.0</td>
<td>85.6 ± 14.6*</td>
<td>47.6 ± 8.0**</td>
</tr>
</tbody>
</table>

* Significantly different from control ($p < 0.01$).
** Significantly different from control ($p < 0.005$).

At the Krebs cycle level, absolute fluxes through citrate synthase, PEP-ck and pyruvate kinase were consistent with those measured in our earlier study. As previously observed, there was extensive recycling of PEP carbons via pyruvate and oxaloacetate. Calculated to be 58.1 ± 2.4 μmol/kg/min for the control group, corresponding to a PEP → glucose flux of 116 ± 4.8 μmol/kg/min. These rates are consistent with our previous NMR measurements and the GC/MS measurements of Katz et al. with 24 h-fasted rats. At the Krebs cycle level, absolute fluxes through citrate synthase, PEP-ck and pyruvate kinase were consistent with those measured in our earlier study. As previously observed, there was extensive recycling of PEP carbons via pyruvate and oxaloacetate. Compared to controls, hepatic glucose output for the CCl$_4$-injured group was significantly lower (42.8 ± 7.3 μmol/kg/min, $p < 0.01$) reflecting a 26% reduction in PEP outflow to glucose (Table 2). The reduction
occurred despite the increase in the ratio of gluconeogenic to oxidative flux, suggesting a loss of hepatic metabolic capacity. Oxidative Krebs cycle activity suffered the greatest impact from CCl₄ injury with a 47% reduction in citrate synthase flux. Despite this significant loss of ATP synthesis capacity, the extent of PEP recycling through the so-called ‘futile cycle’ remained high.

**DISCUSSION**

Our measurements indicate that gluconeogenesis and Krebs cycle fluxes are altered in rat livers following CCl₄ intoxication. These alterations correlated with significant elevations in serum transaminase levels, standard clinical markers of liver failure. Both relative and absolute flux measurements were applied to determine their efficacy and sensitivity as metabolic indices of liver failure. Relative flux measurements have the advantage of being the simplest to implement in the clinical setting since they require a single tracer, [U-13C]propionate. In humans this can be given orally, and when combined with phenylacetate and acacetiminophen ingestion, the NMR analysis can be non-invasively performed on urinary glucuronide and phenylacetylglutamine. However, while relative flux measurements provide a detailed picture of carbon distribution between oxidative and gluconeogenic pathways, they provide no information on the metabolic capacity of the system. These limitations are significant given that cirrhosis is characterized by a loss of functional hepatic mass in addition to possible alterations in metabolic fluxes. Absolute metabolic flux measurement reflects both carbon flux distribution and the metabolic capacity of the Krebs cycle and gluconeogenic network. This measurement requires an additional primed infusion of [1,6-13C₂]glucose plus sampling of plasma glucose. Since the measurement does not account for possible contributions of glycogenolytic flux to hepatic glucose output, it can only be applied under conditions where hepatic glycogenolysis is known to be negligible. To this end, we are developing strategies that combine current 13C NMR measurements with independent 2H NMR tracer assays of hepatic glycogenolysis. Absolute metabolic flux analysis of 24 h-fasted rats revealed that hepatic citrate synthase flux was impaired to a much greater extent than gluconeogenic and PEP recycling fluxes following CCl₄ injury. CCl₄ is specifically activated in mitochondria and is known to inhibit components of mitochondrial electron transport. Studies on isolated mitochondria from livers exposed to acute CCl₄ intoxication show reduced oxygen consumption, ATP synthesis and reduction in respiratory control ratio. These are all consistent with our observation of reduced acetyl-CoA oxidation in situ. There is scant information on the effects of CCl₄ injury on gluconeogenic outflow from the Krebs cycle. While one study reported an increase in pyruvate carboxylase activity following CCl₄ administration, little is known about changes in PEP-ck and pyruvate kinase activities. Although the cytosolic location of PEP-ck and pyruvate kinase might afford some protection from the direct effects of CCl₄ toxicity, gluconeogenesis is ultimately dependent on a functional Krebs cycle and malate–aspartate shuttle to supply the bulk of substrate and reducing equivalents. To the extent that these processes are damaged by mitochondrial CCl₄ injury, gluconeogenesis will also be inhibited. Finally, CCl₄ injury causes significant reductions in hepatic blood flow and perfusion, resulting in reduced substrate and oxygen delivery. This is also likely to have a significant role in constraining absolute Krebs cycle and gluconeogenic fluxes.

In summary, NMR analysis of glucose 13C-enrichment following the administration of 13C-tracers was shown to be an effective tool for documenting alterations in Krebs cycle and gluconeogenic fluxes following hepatic injury by CCl₄. These measurements provide a direct evaluation of the total hepatic capacity for glucose synthesis and acetyl-CoA oxidation.

**Acknowledgements**

This work was supported by grants NSF/AR1 DB1-9612144 from the National Science Foundation and NIH/SIG 1-S10-RR11488 from the National Institute of Health. R.A.C. was a recipient of a PhD Grant from JNICT-Portugal (PRAXIS XXI; BD-3604/94).

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