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Production of hyperpolarized $^{13}\text{CO}_2$ from $[1-^{13}\text{C}]$ pyruvate in perfused liver does reflect total anaplerosis but is not a reliable biomarker of glucose production

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Abstract

In liver, $^{13}\text{CO}_2$ can be generated from $[1-^{13}\text{C}]$ pyruvate via pyruvate dehydrogenase or anaplerotic entry of pyruvate into the TCA cycle followed by decarboxylation at phosphoenolpyruvate carboxykinase (PEPCK), the malic enzyme, isocitrate dehydrogenase, or α -ketoglutarate dehydrogenase. The purpose of this study was to determine the relative importance of these pathways in production of hyperpolarized (HP) $^{13}\text{CO}_2$ after administration of hyper-polarized pyruvate in livers supplied with a fatty acid plus substrates for gluconeogenesis. Isolated mouse livers were perfused with a mixture of thermally-polarized ^{13}C -enriched pyruvate, lactate and octanoate in various combinations prior to exposure to HP pyruvate. Under all perfusion conditions, HP malate, aspartate and fumarate were detected within ~ 3 s showing that HP $[1-^{13}\text{C}]$ pyruvate is rapidly converted to $[1-^{13}\text{C}]$ oxaloacetate which can subsequently produce HP $^{13}\text{CO}_2$ via decarboxylation at PEPCK. Measurements using HP $[2-^{13}\text{C}]$ pyruvate allowed the exclusion of reactions related to TCA cycle turnover as sources of HP $^{13}\text{CO}_2$. Direct measures of O_2 consumption, ketone production, and glucose production by the intact liver combined with ^{13}C isotopomer analyses of tissue extracts yielded a comprehensive profile of metabolic flux in perfused liver. Together, these data show that, even though the majority of HP $^{13}\text{CO}_2$ derived from HP $[1-^{13}\text{C}]$ pyruvate in livers exposed to fatty acids reflects decarboxylation of $[4-^{13}\text{C}]$ oxaloacetate (PEPCK) or $[4-^{13}\text{C}]$ malate (malic enzyme), the intensity of the HP $^{13}\text{CO}_2$ signal is not proportional to glucose production because the amount of pyruvate returned to the TCA cycle via PEPCK and pyruvate kinase is variable, depending upon available substrates.

Keywords

Hyperpolarization; Liver metabolism; Hyperpolarized $^{13}\text{CO}_2$ production; Gluconeogenesis; Pyruvate cycling

1 Introduction

Carbon isotopes have been used extensively to quantify pathways involved in gluconeogenesis (GNG) (Dennis et al. 1978; Katz 1985; Magnusson et al. 1991). Since lactate and some amino acids provide the majority of substrate for net glucose synthesis from the citric acid cycle, a popular approach has been to study metabolism of $[1-^{14}\text{C}]$ enriched pyruvate to measure pyruvate carboxylation and subsequent decarboxylation of oxaloacetate in the first step of GNG (Agius and Alberti 1985; Patel et al. 1982; Sies et al. 1983). Flux through pyruvate carboxylase (PC) is necessary for glucose production, but flux through pyruvate dehydrogenase (PDH) may also be active (Agius and Alberti 1985; Katz et al. 1993). Fluxes through these competing pathways have been

estimated from appearance of $^{14}\text{CO}_2$ from ^{14}C -enriched pyruvate. Data analysis is challenging, however, because $^{14}\text{CO}_2$ derived from $[1-^{14}\text{C}]$ pyruvate can arise via one or a combination of five pathways: (1) Decarboxylation of $[1-^{14}\text{C}]$ pyruvate by PDH; (2) Flux through PC to generate $[1-^{14}\text{C}]$ oxaloacetate followed by flux through citrate synthase (CS) and decarboxylation of $[6-^{14}\text{C}]$ citrate at isocitrate dehydrogenase (IDH); (3) Flux through PC followed by randomization of label in succinate/fumarate to generate $[4-^{14}\text{C}]$ oxaloacetate followed by decarboxylation by PEPCK; (4) Decarboxylation of $[4-^{14}\text{C}]$ malate by the malic enzyme; or (5) Decarboxylation of $[1-^{13}\text{C}]\alpha$ -ketoglutarate generated from $[4-^{13}\text{C}]$ oxaloacetate and a forward turn in the TCA cycle. In spite of an extensive literature focused on metabolism of carbon-labeled pyruvate, there is surprisingly no consensus on the biochemical pathways that must be considered when interpreting data on the appearance of labeled CO_2 and bicarbonate. For example, some models of hepatic metabolism neglect flux through PDH entirely (Fernandez and Des Rosiers 1995) or conclude that flux through PDH is zero under some experimental conditions (Katz 1985), but others (Lee et al. 2013; Williamson et al. 1979) assign the appearance of labeled bicarbonate to flux through PDH. This question is especially important for hyperpolarized (HP) ^{13}C metabolic imaging because, if the appearance of $^{13}\text{CO}_2$ in livers exposed to HP $[1-^{13}\text{C}]$ pyruvate reflects flux through PEPCK exclusively, then one could potentially image glucose production from GNG in the liver in vivo.

In a previous study of perfused mouse liver, production of HP $^{13}\text{CO}_2$ from HP- $[1-^{13}\text{C}]$ pyruvate was shown to largely reflect anaplerotic entry of pyruvate into the TCA cycle followed by subsequent decarboxylation (Merritt et al. 2011). However, others have used the HP $^{13}\text{CO}_2$ signal as an index of PDH flux in the liver and suggested that a sudden increase in pyruvate after bolus injection in vivo may drive PDH flux (Lee et al. 2013). In both studies, the NMR resonances of $[1-^{13}\text{C}]$ and $[4-^{13}\text{C}]$ malate and $[1-^{13}\text{C}]$ and $[4-^{13}\text{C}]$ aspartate (Lee et al. 2013; Merritt et al. 2011) were detected so these intermediates are indeed available to produce HP $^{13}\text{CO}_2$ via a variety of decarboxylation reactions. Neither study however addressed the question, can the signal of HP $^{13}\text{CO}_2$ derived from HP $[1-^{13}\text{C}]$ pyruvate in liver be used as an index of GNG? Also, both the previous papers failed to exclude the appearance of HP $^{13}\text{CO}_2$ from a forward turn of the TCA cycle with any experimental data. In this study, we addressed these questions by quantitative measures of flux through a combination of O_2 consumption, ketone production, glucose production measurements plus evaluations of flux through PC and PDH using conventional ^{13}C NMR isotopomer methods in livers perfused with a variety of ^{13}C enriched substrates. The medium chain fatty acid, octanoate, was included in most preparations to act as a surrogate of long chain fatty acids normally present in blood. Octanoate is known to avidly produce acetyl-CoA in the liver (Williamson et al. 1979) and high levels of acetyl-CoA suppresses oxidation of pyruvate via PDH and stimulates entry of pyruvate into the TCA cycle via PC (Scrutton and Utter 1965; Scrutton and Utter 1967). The quantitative results presented in this study demonstrate that, even though the majority of HP $^{13}\text{CO}_2$ derived from HP $[1-^{13}\text{C}]$ pyruvate in livers exposed to fatty acids reflects decarboxylation of $[4-^{13}\text{C}]$ oxaloacetate (PEPCK) or $[4-^{13}\text{C}]$ malate (malic enzyme), the HP- $^{13}\text{CO}_2$ signal alone does not reflect glucose production from GNG because the amount of pyruvate recycled back into the TCA cycle is highly dependent on perfusion conditions. HP $[2-^{13}\text{C}]$ pyruvate was co-infused with

[1-¹³C]pyruvate for three reasons. First, as illustrated in Fig. 1a, metabolism of [2-¹³C]pyruvate to [2-¹³C]oxaloacetate should generate [2-¹³C]malate and [2-¹³C]aspartate and the appearance of these signals would confirm previously published resonance assignments for these intermediates (Merritt et al. 2011). Second, if significant flux of [2-¹³C]pyruvate through PDH does occur, then [5-¹³C]glutamate could be detected (Schroeder et al. 2009, 2013). Finally, after carboxylation of [2-¹³C]pyruvate to form [2-¹³C]oxaloacetate, this intermediate could condense with acetyl-CoA to generate [3-¹³C]citrate. This may be an advantage because the C3 resonance of citrate is well-resolved (at ~76 ppm) from other signals, has a relatively long T₁, and does not require ¹H decoupling for detection. We hypothesized that in the presence of HP [2-¹³C]pyruvate, detection of [3-¹³C] citrate would directly reflect flux of pyruvate into the TCA cycle via PC followed by forward flux of oxaloacetate into citrate via CS. This experimental design allows for detailed monitoring of all the pathways in the liver that might produce HP ¹³CO₂.

2 Methods

2.1 Chemicals

[U-¹³C]Octanoate, [1-¹³C]pyruvic acid, [2-¹³C]pyruvic acid, sodium [3-¹³C]pyruvate, and sodium [3-¹³C]-L-lactate were purchased from Cambridge Isotope Laboratories (Andover, MA). The radical “trityl” (tris(8-carboxyl-2,2,6,6-tetra[2-(1-hydroxyethyl)]-benzo(1,2-d:4,5-d)bis(1,3) dithiole-4-yl)methyl sodium salt) was purchased from Oxford Instruments Molecular Biotools Ltd. Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

2.2 Experimental design

The animal protocol was approved by the Institutional Animal Care and Use Committee. Female C57Bl/6 mice obtained from Charles River Laboratories were housed in the University vivarium on a 12 h light–dark cycle and had free access to standard lab chow and water. Mice ranged in weight from 18 to 22 g. Each mouse was injected with heparin (0.05 mL, intraperitoneal) 15 min prior to a 0.1 mL intraperitoneal injection of an anesthetic mixture [ketamine (Fort Dodge Animal Health) plus xylazine (Boehringer Ingelheim), 85:15, w/w]. After general anesthesia was achieved the liver was exposed via a midline laparotomy. The portal vein was cannulated and the liver was perfused with a buffered medium during dissection and subsequently suspended in the effluent at 37 °C. The perfusion medium was temperature regulated with a water jacket. Efferent and afferent pO₂ were measured with a blood gas analyzer (Instrumentation Laboratory, Lexington, MA, U.S.A.); oxygen consumption was calculated as described previously (Taegtmeyer et al. 1980). Hepatic viability was evaluated by oxygen consumption and visual inspection. Livers were rejected if they did not maintain a pO₂ gradient of at least 100 mmHg or if the tissue color changed during the study.

The perfusion medium consisted of 118 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 1.25 mM CaCl₂, plus various oxidizable substrates with different ¹³C labeling patterns shown in Table 1, each group with N = 5. Livers were supplied with a mixture of ¹³C-enriched pyruvate, lactate and octanoate in various combinations as follows: group 1, pyruvate and lactate;

group 2, octanoate; group 3, lactate, pyruvate and octanoate; group 4, octanoate and pyruvate (Table 1). Livers were perfused for ~40 min in the magnet at a perfusion column height of 15 cm H₂O. Under these conditions, livers reach steady-state ¹³C enrichment after ~ 30 min (Hausler et al. 2006). Effluent medium was collected every 15 min to measure ketone production and O₂ consumption. The concentration of ketones in the effluent was determined using a standard assay described by Williamson et al. (1962). The rates of acetoacetate and β-hydroxybutyrate production were summed to represent the rate of ketogenesis. The concentration of glucose in the effluent was determined using a standard assay described by Bergmeyer (1984).

Livers were placed in a 25 mm NMR tube and positioned in an 89 mm bore 9.4 T magnet (Agilent) equipped with a Doty ¹H/¹³C probe. Shimming was performed on the ²³Na signal. Broadband ¹H decoupling using WALTZ-16 was gated on during acquisition only. To facilitate shimming, a sodium-free perfusion medium was pumped through a separate line to the bottom of the NMR tube at a rate of ~5 mL/min. This flush served two purposes: (1) to remove extracellular sodium from the active volume of the probe to facilitate shimming and (2) to remove any metabolites excreted by the perfused liver. The HP¹³C NMR signal was collected using a series of 15° ¹³C pulses with an acquisition time of 1 and a 2 s interpulse delay. The ~ 23 mL of HP material, described below, was injected by catheter into the medium directly above the cannulated liver at a rate of ~ 15 mL/min. At the typical flow rate for these experiments, about 10 mL/min, this approach results in addition of [1-¹³C]pyruvate and [2-¹³C]pyruvate to the medium for ~ 100 s. The medium was not recirculated. Livers were perfused for an additional 15 min after completion of the hyperpolarization observations. Livers were freeze-clamped and stored at -80 °C for further processing.

2.3 Acquisition, description and analysis of the ¹³C NMR glutamate spectrum from liver extracts

After each hyperpolarization experiment, livers were freeze-clamped, acid extracted, and freeze-dried (Hausler et al. 2006; Merritt et al. 2011). The resulting powder was dissolved in ²H₂O and ¹H decoupled ¹³C NMR spectra were acquired at 14.1 T using an Agilent VNMRs system (Agilent Inc.) using a 45° pulse and a 2.5-s delay time between pulses. The relative peak areas of each glutamate multiplet were obtained by line fitting using ACD software (Advanced Chemistry Development) and the data were analyzed using tcaCALC (Malloy et al. 1990).

During exposure to ¹³C-enriched substrates, complex labeling patterns evolve in all intermediates and exchanging pools of the citric acid cycle. Most ¹³C resonances in these spectra appeared as multiplets due to ¹³C-¹³C spin-spin coupling and the areas of those multiplets enabled a measure of the relative concentration of groups of ¹³C isotopomers. The abbreviations C1, C2, C3, C4, and C5 refer to the respective carbons of glutamate while the multiplet areas within each resonance are given as C2S, singlet; C2D12, doublet due to J₁₂; C2D23, doublet due to J₂₃; C2Q, doublet of doublets due to J₂₃ and J₃₄; C3S, singlet; C3D due to either J₂₃ or J₃₄ (the coupling constants are nearly equal); C3T due to both J₂₃ and J₃₄; C4S, singlet; C4D34, doublet in C4 due to J₃₄; C4D45, doublet in C4 due to J₄₅; C4Q; doublet of doublet due to J₃₄ and J₄₅; The relative areas of the individual components of

each multiplet were measured by curve fitting and normalized to 1. The equations relating the multiplet areas to relative flux values have been described previously (Malloy et al. 1996, 1990, 1988).

2.4 TCA cycle flux (V_{TCA})

TCA cycle flux was derived from a combination of ^{13}C isotopomer data, the measured rate of oxygen consumption (V_{O_2}), and the rate of β hydroxybutyrate (βHB) and acetoacetate (AA) production ($V_{AA} + V_{\beta\text{HB}}$) (Malloy et al. 1996). In non-ketogenic tissues, the relation between V_{TCA} and oxygen consumption is given by Eq. (1):

$$V_{TCA} = \frac{V_{O_2}}{(F_{CO}R_O + F_{C1}R_1 + F_{C2}R_2 + F_{C3}R_3 + yR_a)} \quad (1)$$

where F_{CX} represents the isotopic contribution to acetyl-CoA, y is relative flux through PC (anaplerosis) and R_X is a substrate-specific proportionality factor between O_2 consumption and V_{TCA} to account for generation of reducing equivalents in reactions other than TCA cycle reactions (Malloy et al. 1996). For livers perfused with octanoate, this relationship must be modified to account for possible conversion of octanoate to ketone bodies (ketogenesis). Consequently, oxygen consumed in the generation of exported ketones must be subtracted from the total measured oxygen consumption. The number of reducing equivalents produced by ketogenesis was determined by multiplying the rate of acetoacetate production, V_{AA} , by 3 and the rate of β -hydroxybutyrate production, $V_{\beta\text{HB}}$, by 2. Applying the corrections for ketogenesis, Eq. (1) becomes:

$$V_{TCA} = \frac{V_{O_2} - 3V_{AA} - 2V_{\beta\text{HB}}}{(F_{CO}R_O + F_{C1}R_1 + F_{C2}R_2 + F_{C3}R_3 + yR_a)} \quad (2)$$

The fractional contributions of each substrate to acetyl-CoA were determined by ^{13}C isotopomer analysis of tissue extracts. Since the total perfusion period was ~45 min, steady-state conditions were assumed (Hausler et al. 2006). An example V_{TCA} calculation is presented in the online resource. PDH, PC, and pyruvate kinase (PK) flux values reported in Table I reflect average values determined for each individual perfused liver based upon its ^{13}C spectrum, measured O_2 consumption, and measured ketone production. Specifically, fitted areas of all glutamate ^{13}C isotopomers for each liver were input into tcaCALC (Malloy et al. 1990) to determine relative fluxes of PDH, PC, and PK to CS (Table S1). The reliability of the tcaCALC data was proven using tcaSIM (Malloy et al. 1988) to simulate the experimental spectra. The relative flux values were multiplied by V_{TCA} to obtain absolute flux values (see online resource for calculations).

Given that some PEP leaving the cycle via PEPCK can be recycled back into pyruvate via PK, net GNG was determined from the difference between PC and PK flux. The isotopomers of carbon 2 of lactate, coupled with tca-SIM, provided evidence for pyruvate cycling. Since two molecules of PEP are required to form one molecule of glucose, GNG is defined as $(\text{PC}-\text{PK})/2$.

2.5 Pyruvate labeling and hyperpolarization methods

A mixture of HP [1-¹³C]pyruvate and HP [2-¹³C]pyruvate was used in these experiments to maximize possible observation of HP-metabolite signals arising from influx of pyruvate into the TCA cycle via PDH versus PC. Since all downstream metabolite signals arising from either HP [1-¹³C]pyruvate or HP [2-¹³C]pyruvate would appear as singlets, this should maximize peak height-to-noise detection and simplify assignments in the crowded carbonyl region. Although doubly labeled HP-[1,2-¹³C]pyruvate would offer some advantages for interpreting metabolic pathways, the T_1 of both carbons in [1,2-¹³C]pyruvate is somewhat shorter compared to either [1-¹³C]- or [2-¹³C] pyruvate. In addition, the ¹³C J-coupling makes interpretation of the spectra in the carbonyl region difficult. The samples for dynamic nuclear polarization (DNP) were prepared by dissolving the trityl radical (15 mM) into equal volumes of [1-¹³C] and [2-¹³C]pyruvic acid. To this was added ~1 μ L of 10 mM stock solution of Prohance to give a Gd³⁺ concentration of ~1 mM. An Oxford HyperSense DNP system operating at 3.35 T was used to hyperpolarize ~10- μ L aliquots to polarization levels of ~20 % (90 min irradiation). The frozen polarized samples were dissolved using 4 mL of superheated phosphate buffered saline. After dissolution, 3 mL of the resulting solution was diluted into 20 mL of perfusate (concentrations defined in Table 1) to bring the final pH to 7.4 and assure proper oxygenation. As described previously, two perfusion columns were available. For experimental groups 1–3, the HP pyruvate mixture was injected slowly into the perfusion column in the flowing medium directly above the liver. Consequently, these experiments were considered “bolus” pyruvate because the concentration of pyruvate increased to ~4 mM for ~90 s during the exposure to HP pyruvate. For experimental group 4, one column contained [U-¹³C] octanoate plus 4 mM [3-¹³C]pyruvate while the second column contained only [U-¹³C]octanoate. During the hyperpolarization experiment, column one was switched off and column two was switched on immediately before, <1 s, the HP pyruvate was injected by catheter into the volume directly above the liver. Therefore, the nutrients provided to the liver during the experiment were 0.2 mM octanoate and 4 mM [¹³C]pyruvate. This results in an approximate steady-state concentration of pyruvate.

2.6 Symmetric redistribution of ¹³C label in the 4-carbon pools

The observed unequal ¹³C signal intensities of HP malate C1 vs. C4 enables a simple calculation of the fraction of HP [1-¹³C]oxaloacetate that undergoes reorientation (REO) or randomization in the fumarate pool. This fraction is defined here as $F_{REO} = 2 * \text{MALC4} / (\text{MALC1} + \text{MALC4})$, where MALC1 and MALC4 refer to the areas of the malate C1 and C4 resonances. If all HP-[1-¹³C]oxaloacetate equilibrates with fumarate and ¹³C the label is equally redistributed, then MALC4 = MALC1 and $F_{REO} = 1.0$. If MALC4/MALC1 = 0.5, then $F_{REO} = 0.67$, indicating that 2/3 of all HP [1-¹³C]oxaloacetate produced from [1-¹³C] pyruvate equilibrates with fumarate. Any small differences in T_1 between the MALC1 and MALC4 were not considered in this calculation.

2.7 Processing and analysis of ¹H decoupled ¹³C NMR spectra of functioning livers

After delivery of HP pyruvate to the perfusate, a series of ¹³C FIDs were collected using 15° pulses over a period of ~ 90 s. Each spectrum was zero filled to 32,768 points and the Fourier-transformed signal areas were measured by curve fitting. All metabolites were

identified to MSI level 2. The total area (total carbon signal) of all HP carbon species throughout a single experiment was obtained by summing the integrated areas of the observed peaks for the time period during which the $[1-^{13}\text{C}]$ pyruvate was observed, neglecting the signal associated with the pyruvate hydrate (~179 ppm). Signal intensities are reported as a fraction of total carbon signal as measured using the areas under the curve.

2.8 Statistics

All results are expressed as mean $\pm$ SD. Comparisons were made using a student's two-tailed t test, where $P < 0.05$ was considered significant. It was assumed that the data had equal variances (homoschedastic). As the number of variables was small, no Bonferroni correction was used.

3 Results

3.1 Contribution of exogenous substrates to oxidation and anaplerosis

Proton decoupled ^{13}C NMR spectra of carbons 2–5 of glutamate from liver extracts are shown in Fig. 2. Analysis of the glutamate multiplet areas enables a straightforward estimate of the sources of acetyl-CoA and relative anaplerosis. In the presence of $[\text{U-}^{13}\text{C}]$ lactate and $[\text{U-}^{13}\text{C}]$ pyruvate (Fig. 2a) the doublet due to J_{45} and the quartet due to J_{34} and J_{45} can only arise from oxidation of these substrates via PDH (Fig. 1b). In the next series of experiments (Figs. 2b – d), $[\text{U-}^{13}\text{C}]$ octanoate was present and the site of labeling in lactate and pyruvate was switched to the methyl carbon to simplify interpretation of the spectra. With these labeling conditions, the doublet due to J_{45} and the quartet due to J_{34} and J_{45} in carbon 4 can only arise from octanoate (Fig. 1b). Octanoate, when available, is overwhelmingly favored for oxidation as evidenced by the large doublet due to J_{45} and the quartet (a doublet of doublets) in carbon 4 (Fig. 2b – d). The contribution of octanoate to acetyl-CoA ranged from 70 to 90 % (Table 1). Oxidation of pyruvate or equivalent sources via PDH was largely but not completely suppressed (Malloy et al. 1990; Sherry et al. 1992). The glutamate C2 resonance in Fig. 2c, d is interesting because it is dominated by a large doublet with $J_{25} = \sim 3$ Hz that arises as a consequence of long-range ^{13}C - ^{13}C coupling (Carvalho et al. 1999). Inspection of this resonance alone indicates that the fractional enrichment in position 5 must be very high while the fraction of glutamate isotopomers labeled in position 2 plus an adjacent carbon ($[1,2-^{13}\text{C}]$, $[2,3-^{13}\text{C}]$ or $[1,2,3-^{13}\text{C}]$ glutamate) must also be low. This feature is consistent with entry of highly enriched acetyl-CoA into the TCA cycle plus substantial entry of an anaplerotic substrate such as $[2-^{13}\text{C}]$ pyruvate that generates only $[2-^{13}\text{C}]$ oxaloacetate or $[3-^{13}\text{C}]$ oxaloacetate (but not $[2,3-^{13}\text{C}]$ oxaloacetate). A complete quantitative analysis of these spectra is given in Table 1. The lactate carbon 2 resonances from the first three groups of livers are shown in Fig. 3 along with tca-SIM simulations of the appearance of lactate C2 with or without pyruvate kinase (PK) activity. The quartet in lactate C2 (Fig. 3a) arises directly from the $[\text{U-}^{13}\text{C}]$ lactate and $[\text{U-}^{13}\text{C}]$ pyruvate supplied to these livers but doublets labeled D23 and D12 (reflecting J_{23} and J_{12} spin-spin couplings) and the singlet (S) can only arise if PK is active. Using the relative flux values obtained from analysis of the glutamate multiplet areas (Table S1), simulated lactate spectra were obtained with and without PK active (Fig. 3). Inclusion of PK in the simulation results in both doublets, due to J_{23} and J_{12} , to appear in approximately the same ratio as in the

experimental data. Without PK, these doublets are not present. In livers exposed to [U-¹³C]octanoate only, labeling of lactate-C2 cannot occur without some PK activity. Here, enrichment of lactate-C2 is only possible after octanoate is oxidized through the TCA cycle to OAA, OAA is converted to PEP via PEPCK, and then PEP is de-phosphorylated by PK to form pyruvate and lactate. Figure 3b, c clearly demonstrate that PK is active under these perfusion conditions.

3.2 Oxygen consumption, ketone and glucose production

Ketone production, glucose production and oxygen consumption values for the four groups of perfused livers are listed in Table 1. As expected, all livers supplied with octanoate released excess acetyl-CoA as ketones and consumed the most oxygen, as reported previously (Scholz et al. 1984; Soboll et al. 1984). However, after correction for ketogenesis, all livers had similar V_{O_2} values within error limits of the measurement (Table S1). Glucose was continuously produced by all livers as expected for livers taken from fed animals with ample glycogen stores. Glucose production was relatively constant among groups 1–3 and only tended toward a slightly higher value in group 4. However, GNG from TCA cycle intermediates was relatively constant in groups 1, 3 and 4 but was significantly lower in group 2 (Table 1). Group 3 livers, perfused with the most physiological mixture of substrates showed the highest GNG flux, 0.55 $\mu\text{mol}/\text{min}/\text{gww}$. This represents about 39 % of total glucose production. In the absence of gluconeogenic substrates (group 2), GNG was only 0.12 $\mu\text{mol}/\text{min}/\text{gww}$, a result similar to that reported previously by Hausler et al. (2006). This represents only 9 % of the total glucose produced by this group of livers. In livers presented with gluconeogenic substrates but no octanoate (group 1), GNG was 0.46 $\mu\text{mol}/\text{min}/\text{gww}$ or 35 % of total glucose production. This illustrates the expected importance of lactate and pyruvate availability as precursors for GNG.

3.3 Metabolism and symmetric redistribution of hyperpolarized [1-¹³C]pyruvate and [2-¹³C]pyruvate

After injection of the mixture of HP [1-¹³C] and [2-¹³C] pyruvate, the ¹³C resonances of [1-¹³C] and [2-¹³C] enriched pyruvate, lactate, and alanine appeared rapidly, as expected. As described in Fig. 1, [1-¹³C]pyruvate yields [1-¹³C]aspartate and [1-¹³C]malate after carboxylation of pyruvate to OAA. Simultaneous administration of [2-¹³C] pyruvate yielded equivalent amounts of [2-¹³C]malate and [2-¹³C]aspartate. All four isotopomers of malate and aspartate appeared simultaneously although the signal-to-noise in the resonances of [2-¹³C]malate and [2-¹³C] aspartate was significantly lower because of shorter T_1 values. The appearance of these resonances confirmed the assignments by Merritt et al. (Merritt et al. 2011). Representative summations, each totaling 30 scans, for each group are shown in Fig. 4. [1 or 4-¹³C]Fumarate appeared essentially simultaneously with [1-¹³C]malate and [1-¹³C] aspartate, while [4-¹³C]malate and [4-¹³C]aspartate were detected a few seconds later. The expanded carbonyl region of these spectra (Fig. 5) shows that the ratio of malate-C4 to malate-C1 was unchanged among the four groups of livers (Table 1). From the calculated areas under the curve for each separate resonance, only the resonances of HP [1- or 2-¹³C]lactate and HP $\text{H}^{13}\text{CO}_3^-$ were significantly different among the four groups. Lactate was lower in group 4 livers compared to the three other groups. Bicarbonate was significantly higher in group 1 livers compared to the other three groups ($P < .05$, Fig. 6), but

did not vary significantly among groups 2, 3 and 4. Finally, a plot of GNG versus integrated area of the HP bicarbonate signal for groups 2, 3 and 4 is presented in Fig. 7. This illustrates that there is no correlation between the amount of bicarbonate produced from HP [1-¹³C]pyruvate versus the amount of glucose produced from the TCA cycle (GNG) under these three experimental conditions. Group 1 was not included in this comparison because, in the absence of octanoate, much of the HP ¹³CO₂ produced by livers when exposed to HP [1-¹³C]pyruvate arises due to decarboxylation at PDH.

4 Discussion

This study illustrates that metabolic flux in a healthy liver can readily adapt to substrates available to the liver for oxidation and GNG. HP ¹³C NMR has recently been introduced as a novel tool for investigating conversion of pyruvate to lactate in cancers and assessing flux through PDH in the heart. The ability to monitor key reactions in real time potentially offers a new approach to detect and assess altered metabolism in liver as well but the complexity of carbon metabolism in the liver through multiple metabolic pathways offers new challenges in data interpretation. This study was designed to answer the relatively simple question, does the HP ¹³CO₂ signal derived from HP [1-¹³C]pyruvate in liver reflect GNG from the TCA cycle? This question was examined in livers perfused with a variety of ¹³C-enriched substrates for periods long enough to achieve isotopic steady-state prior delivery of HP [1-¹³C]pyruvate to measure production of HP ¹³CO₂. About 100 s after the HP experiment, each liver was freeze-clamped, acid extracted, and prepared for ¹³C NMR studies. A steady-state ¹³C isotopomer analysis of each spectrum provided a measure of PDH flux, PC flux, pyruvate cycling flux (PK), plus GNG ((PC-PK)/2). A mixture of HP [1-¹³C]pyruvate and [2-¹³C]pyruvate was simultaneously presented to livers in these experiments because the metabolic products of each labeled pyruvate yield distinctive ¹³C NMR spectra and this adds additional information for confirming flux through various pathways in the same liver (Fig. 1). The relatively low detection sensitivity of thermally-polarized ¹³C-enriched nuclei in the intact liver is a major advantage in these experiments because conventional ¹³C NMR isotopomer analysis can be performed using the same livers as used in the HP experiments. Although metabolites of the citric acid cycle were extensively labeled by the thermally-polarized ¹³C substrates, this was not detected on the three-second time scale used for acquisition of the HP data. This experimental design provided a complete metabolic profile of pyruvate metabolism in liver.

Oxygen consumption, ketone production, and glucose production measurements for the 4 groups of livers are summarized in Table 1. Hepatic function as assessed by tissue oxygen consumption was identical to prior reports (Scholz et al. 1978; Sies et al. 1983; van Dyke et al. 1983). Also, as expected, ketone production was dramatically stimulated by octanoate (Krebs et al. 1969) while glucose production was unchanged by octanoate in livers from fed animals as reported previously (Exton and Park 1967). Finally, 0.2 mM octanoate was very effective shutting down oxidation of pyruvate via PDH, again consistent with a previous report (Scholz et al. 1978).

Rapid conversion of pyruvate to OAA is essential in liver because of its key role in systemic glucose homeostasis. This study confirmed rapid carboxylation of pyruvate followed by

exchange of [1-¹³C]oxaloacetate into malate, aspartate and fumarate, all within a few seconds. Although [4-¹³C]malate] and [4-¹³C]aspartate were also observed, the ratio of C4 to C1 was consistently about 0.52, consistent with ~67 % randomization of [1-¹³C]oxaloacetate through the symmetric intermediate, fumarate (Table 1).

4.1 Influence of octanoate on PDH flux and ¹³CO₂ production from [1-¹³C]pyruvate

In the absence of octanoate, a significant fraction of acetyl-CoA was derived from [U-¹³C]lactate and [U-¹³C]pyruvate via PDH (46 %, Table 1). In the same livers, transient exposure to HP [1-¹³C]pyruvate resulted in the appearance of a large HP [¹³C]bicarbonate signal, consistent with flux through PDH. However, in these same livers, HP [1-¹³C] and [4-¹³C]malate, aspartate and fumarate were also detected, proving that PC was also active. In the presence of 0.2 mM octanoate, oxidation of lactate and pyruvate was significantly reduced. In this group, the contribution of lactate and pyruvate to acetyl-CoA dropped to 6 % while octanoate contributed 89 % of the total acetyl-CoA entering the TCA cycle. Production of HP ¹³CO₂ from HP [1-¹³C]pyruvate was strongly suppressed by octanoate (Fig. 6). For these reasons we conclude that, in the absence of octanoate, the majority of HP ¹³CO₂ appears as a result of flux of HP [1-¹³C]pyruvate through PDH while, in the presence of only 0.2 mM octanoate, this contribution drops considerably but is not zero.

In some reports, flux through PDH in the liver is assumed to be negligible (Fernandez and Des Rosiers 1995; Katz 1985). It is not surprising that PDH flux is active in the presence of lactate and pyruvate and absence of other sources of acetyl-CoA. In the current study, groups 1, 3 and 4 were provided with ¹³C-enriched lactate and pyruvate (groups 1 and 3) or pyruvate alone (group 4). The glutamate C4 signal at 34.2 ppm demonstrates that in all three groups, flux through PDH was active even in the presence of high concentrations of alternative sources of acetyl-CoA. These results indicate that flux through PDH should be assumed active in the liver, unless proven otherwise in any tracer analysis of liver metabolism.

4.2 Octanoate alters the path for generation of ¹³CO₂ from [1-¹³C]pyruvate

Previous radiotracer experiments in liver showed that production of ¹⁴CO₂ from [1-¹⁴C]pyruvate is strongly inhibited by octanoate at pyruvate concentrations of 0.5 mM (Scholz et al. 1978) or ~4 mM (Williamson et al. 1979). The current observations using HP [1-¹³C]pyruvate support this earlier conclusion but also provide additional metabolic information. Here, we show that production of HP ¹³CO₂ is significantly reduced when octanoate is present in the medium and that the bicarbonate that does appear arises largely from flux of [1-¹³C]pyruvate into the TCA cycle via PC followed by subsequent decarboxylation of 4-carbon intermediates to form HP ¹³CO₂. Given that these results were obtained after bolus administration of HP [1-¹³C]pyruvate rather than constant infusion of the label, we would expect these results to parallel in vivo conditions.

After carboxylation of [1-¹³C]pyruvate to form [1-¹³C] oxaloacetate, several possible pathways could contribute to HP ¹³CO₂. Given that liver is actively producing glucose under these perfusion conditions, the most likely pathway is backwards redistribution of the label of [1-¹³C]oxaloacetate through malate and fumarate to yield [4-¹³C]oxaloacetate followed

by decarboxylation of [4- ^{13}C]oxaloacetate to produce HP $^{13}\text{CO}_2$ via PEPCK. A number of 4-carbon intermediates were observed in the HP experiments but that alone does not verify that this is the most active pathway for production of HP $^{13}\text{CO}_2$. An alternative pathway is condensation of [1- ^{13}C]oxaloacetate with acetyl-CoA to generate HP [6- ^{13}C]citrate which after decarboxylation at IDH would also produce HP $^{13}\text{CO}_2$. Given that the C6 resonance of citrate would be difficult to detect in this experiment due to spectral overlap, the appearance of a [3- ^{13}C] citrate resonance (produced from [2- ^{13}C]pyruvate and [2- ^{13}C]oxalo-acetate) might also provide evidence that this pathway is active (Fig. 1b). Although other metabolic products of [2- ^{13}C]oxaloacetate including malate, aspartate and fumarate could be detected easily, [3- ^{13}C]citrate was not detected in most experiments. Detection of any HP ^{13}C signal from tissue is proportional to the product of (% polarization)·(^{13}C enrichment)·(concentration of metabolite). Given that citrate and malate concentrations are roughly equal in the liver (Hagopian et al. 2004), the appearance of HP malate must reflect greater flux into the 4-carbon pools via oxaloacetate compared to flux through CS. This is also consistent with the two to threefold greater flux through PC compared to CS as reported by isotopomer analyses under all four perfusion conditions (Table 1). Proton NMR spectra of tissue extracts of each liver also indicated that the concentrations of the malate and citrate were roughly equal, though both compounds were at the detection limit of the NMR system. A comparison of intensities of the [1- ^{13}C]malate and [3- ^{13}C] citrate signals (Fig. 5) indicates a ratio of at least 30:1. Assuming first order reaction rates for each step, this is consistent with exchange of labeled oxaloacetate with malate that is ~30 times faster than flux through CS. Differences in T_1 s would affect the measured ratio, but these data indicate that TCA cycle turnover is slow relative to equilibration of the 4-carbon intermediates on a 1 min time scale.

4.3 Flux through pyruvate dehydrogenase (PDH) versus pyruvate carboxylase (PC)

Partitioning of pyruvate through these alternative pathways has been studied intensively and it is generally accepted that there is reciprocal regulation of flux through PDH and PC by mitochondrial acetyl-CoA/CoA, ATP/ADP and NADH/NAD⁺ ratios. Despite exhaustive studies of these two critical pathways over decades, uncertainties remain. Although it is well-accepted that fatty acids stimulate PC activity in liver, the effects of fatty acids on PDH flux are less clear. In some studies, PDH is inactivated by fatty acids while others show either stimulation or inhibition of flux depending upon the concentration of pyruvate as described by Scholz and colleagues (1978). Although HP [1- ^{13}C]pyruvate could potentially generate HP $^{13}\text{CO}_2$ from some combination of the pathways illustrated in Fig. 1a, it is much less likely that HP [2- ^{13}C]pyruvate could contribute any HP $^{13}\text{CO}_2$ because of the more tortuous routes needed for this substrate to release $^{13}\text{CO}_2$ (Fig. 1a). The complete isotopomer analysis indicates that some pyruvate was oxidized via PDH under all conditions, but the fractional contribution was small whenever octanoate was present in the medium. The fact that [3- ^{13}C]citrate could not be detected indicates that flux through CS, relative to pyruvate carboxylation and decarboxylation of oxaloacetate, is also small.

A major and arguably the most important application of HP studies of [1- ^{13}C]pyruvate in the liver is to assess fluxes through the various pathways leading to production of $^{13}\text{CO}_2$, since it reflects a combination of energy production and GNG. One can add competitive substrates to alter flux through one pathway versus another as done here, but a further complication

arises due to likely differences in T_1 losses as HP substrates pass through the two alternative pathways. Release of HP $^{13}\text{CO}_2$ via PDH reflects a single enzyme-catalyzed step, whereas release of HP $^{13}\text{CO}_2$ after entry of HP $[1-^{13}\text{C}]$ pyruvate into the TCA cycle via PC requires at least six enzyme-catalyzed steps, plus randomization in the fumarate pool. The ^{13}C isotopomer analysis measurements provided an independent estimate of both PDH and PC flux, so if T_1 losses through the two paths to bicarbonate were equal, then the intensity of the HP $\text{H}^{13}\text{CO}_3^-$ signal should reflect the combined flux through PDH and PC under each experimental condition. For example, if one compares groups 1 and 3, one finds that PC flux was equivalent in the two groups while PDH flux was about eightfold higher in group 1 versus group 3 livers (Table 1). Thus, the simplest analysis would conclude that the much larger bicarbonate signal in group 1 livers is entirely due to differences in PDH flux. However, to use PC flux as a metric of $[^{13}\text{C}]$ bicarbonate production, the F_{REO} term (Table 1) must be included in the calculation since only $[4-^{13}\text{C}]$ oxaloacetate can produce $[^{13}\text{C}]$ bicarbonate via PEPCK. The amount of $[^{13}\text{C}]$ bicarbonate produced via PC is therefore $\text{PC} \cdot F_{\text{REO}}/2$. This leads to a rather simple relationship between the contributions of PC and PDH to HP $[^{13}\text{C}]$ bicarbonate production for each individual perfusion condition.

$$x \cdot \text{PC} \cdot F_{\text{REO}} + y \cdot \text{PDH} = S_{\text{HPbicarb}} \quad (3)$$

Here, x and y denote terms that describe the T_1 weighting introduced by each pathway to the $[^{13}\text{C}]$ bicarbonate signal intensity. Solving two simultaneous equations for groups 1 and 3 yields of $x = 0.85$ and $y = 3.11$. The nearly fourfold lower value of x compared to y suggests much greater loss of spin polarization after passage through PC and associated reactions prior to formation of bicarbonate. Using these values, one may conclude that PDH is responsible for ~ 82 % of the $[^{13}\text{C}]$ bicarbonate signal in group 1 livers (no octanoate present), while 65 % of the $[^{13}\text{C}]$ bicarbonate signal in group 3 livers originates from PC flux. In the latter case, only a small amount of octanoate (0.2 mM) was capable of largely preventing flux of pyruvate through PDH. Given the amount of free fatty acids normally present in blood in vivo, this will likely also shunt the majority of HP pyruvate away from PDH and toward the gluconeogenic pathway involving PC and PEPCK in the in vivo liver. Figure 7 makes it only too clear that HP $[^{13}\text{C}]$ bicarbonate production in the liver is a poor predictor of gluconeogenic flux.

4.4 Carbon tracers for analysis of pyruvate metabolism in the liver

Analysis of fluxes through PDH and PC using carbon tracers has attracted interest for more than 60 years. Ideally the methods should be applicable in vivo and provide sufficient information to enable analysis of the system without unrealistic mathematical assumptions. An extensive literature now exists describing various approaches to analyze hepatic metabolism using ^{14}C -enriched pyruvate (Agius and Alberti 1985; Dennis et al. 1978; Katz 1985; Magnusson et al. 1991; Patel et al. 1982; Sies et al. 1983). Multiple experiments are required in the same preparation because the information yield from a single ^{14}C tracer experiment is insufficient to solve the metabolic network. This cumbersome approach requires additional exposure to radiation, a major consideration when performing studies in human volunteers. As this study demonstrates, HP ^{13}C tracers allow direct detection of multiple reactions in real time in the intact liver. As described elsewhere (Merritt et al. 2011)

and shown here, the majority of HP $^{13}\text{CO}_2$ derived from HP $[1-^{13}\text{C}]$ pyruvate in livers exposed to octanoate reflects decarboxylation of $[4-^{13}\text{C}]$ oxaloacetate (PEPCK) or $[4-^{13}\text{C}]$ malate (malic enzyme). The HP $^{13}\text{CO}_2$ signal alone does not provide a direct measure of GNG because the amount of pyruvate generated from either PEPCK or the malic enzyme and recycled back into the TCA cycle is quite variable and depends upon the substrate mixture available to the liver.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Pyruvate and octanoate metabolism in the liver. The metabolic pathways probed in this study. Carbons are illustrated by filled (¹³C) or unfilled (¹²C) circles as follows : 1 carbon, CO₂; 2 carbons, acetyl-CoA; 3 carbons, pyruvate (Pyr) or phosphoenolpyruvate (PEP); 4 carbons, oxaloacetate (OAA); 5 carbons, α-ketoglutarate (αKG); 6 carbons, citrate (CIT); 8 carbons, octanoate (Oct). The vertical molecules are numbered from position 1 on the top. Position 1 of citrate and α-ketoglutarate is at the bottom. **a** shows metabolism of [1-¹³C]pyruvate and [2-¹³C]pyruvate through pyruvate carboxylase (PC) with complete randomization in the fumarate pool, and flux through pyruvate dehydrogenase (PDH). Flux through the malic enzyme is equivalent to flux through PEPCK. Flux through α-ketoglutarate dehydrogenase is neglected. Given the lengthy pathways involved and the relatively short half-life of the hyperpolarized intermediates, it is highly unlikely that HP ¹³CO₂ could be produced from [2-¹³C]pyruvate, but [2-¹³C]pyruvate does however provide a relatively direct pathway to [3-¹³C]citrate. **b** shows metabolism of a mixture of [3-¹³C]pyruvate and [U-¹³C]octanoate. A doublet of doublets (quartet) due to J₃₄ and J₄₅ arising from metabolism of [U-¹³C]octanoate will be generated in glutamate via α-ketoglutarate. The enrichment of carbons 1 and 2 of pyruvate are realized through pyruvate cycling, which encompasses pyruvate metabolism through PC to OAA, followed by passage through PEPCK to PEP and then through pyruvate kinase (PK) to pyruvate. In these examples small amounts of unlabeled acetyl-CoA could arise from stored glycogen or triglycerides. *Broad arrows* refer to multi-enzymatic steps, while thin *arrows* refer to single-enzymatic steps

Fig. 2.

^1H decoupled ^{13}C NMR spectra of glutamate from extracts of the liver during metabolism of octanoate, lactate and pyruvate. Results are from the four groups of perfused livers: **a** (1.5 mM) $[\text{U-}^{13}\text{C}]\text{lactate}$ and (0.15 mM) $[\text{U-}^{13}\text{C}]\text{pyruvate}$; **b** (0.2 mM) $[\text{U-}^{13}\text{C}]\text{octanoate}$; **c** (1.5 mM) $[\text{3-}^{13}\text{C}]\text{lactate}$, (0.15 mM) $[\text{3-}^{13}\text{C}]\text{pyruvate}$ and (0.2 mM) $[\text{U-}^{13}\text{C}]\text{octanoate}$. In the presence of $[\text{U-}^{13}\text{C}]\text{lactate}$ and $[\text{U-}^{13}\text{C}]\text{pyruvate}$ (**a**), the prominent signals in C4 demonstrate oxidation of the lactate plus pyruvate mixture to $[\text{1,2-}^{13}\text{C}]\text{acetyl-CoA}$. In **b-d**, the source of $[\text{1,2-}^{13}\text{C}]\text{acetyl-CoA}$ is exclusively $[\text{U-}^{13}\text{C}]\text{octanoate}$

Fig. 3. Identification of PK activity through analysis of lactate-C2 isotopomers via experimental and simulated data. *Left to right:* Experimental lactate-C2 (69.4 ppm) data acquired from liver tissue extracts; Simulated lactate-C2 data using tcaSIM with PK active; Simulated lactate-C2 data using tcaSIM without PK active. Each panel from top to bottom represents a different perfusion condition: **a** (1.5 mM) [U-¹³C]lactate and (0.15 mM) [U-¹³C]pyruvate; **b** (0.2 mM) [U-¹³C] octanoate; **c** (1.5 mM) [3-¹³C]lactate, (0.15 mM) [3-¹³C]pyruvate and

(0.2 mM) [U- ^{13}C]octanoate. The simulations were obtained by inputting fluxes relative to citrate synthase found in Table S1 into tcaSIM

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Fig. 4.

^{13}C NMR spectra during metabolism of hyperpolarized $[2-^{13}\text{C}]$ - and $[1-^{13}\text{C}]$ pyruvate. The spectra presented the sum of 30 broadband proton decoupled ^{13}C NMR spectra acquired under conditions 1–4, defined in Table 1. The amount of bicarbonate production differed in the four groups. $[2-^{13}\text{C}]$ fumarate and $[2-^{13}\text{C}]$ malate were observed in all conditions, and $[2-^{13}\text{C}]$ aspartate was detected in a-c, consistent with the assignments of the carbonyls of malate, aspartate and fumarate

Fig. 5. ^{13}C NMR spectra during metabolism of hyperpolarized $[2\text{-}^{13}\text{C}]$ - and $[1\text{-}^{13}\text{C}]$ pyruvate. Expanded regions of the HP spectra showing the citrate C3 region (75–78 ppm) and the carbonyl region (174–185 ppm). In addition to $[1\text{-}^{13}\text{C}]$ lactate, $[1\text{-}^{13}\text{C}]$ alanine and $[1\text{-}^{13}\text{C}]$ pyruvate hydrate, signals from $[5\text{-}^{13}\text{C}]$ glutamate, $[1\text{-}^{13}\text{C}]$ malate, $[4\text{-}^{13}\text{C}]$ malate, $[1\text{-}^{13}\text{C}]$ aspartate, $[4\text{-}^{13}\text{C}]$ aspartate, and $[1\text{ or }4\text{-}^{13}\text{C}]$ fumarate are assigned. Each *panel* represents a different perfusion condition: **a** (1.5 mM) lactate and (0.15 mM) pyruvate; **b**

(0.2 mM) octanoate; **c** (1.5 mM) lactate, (0.15 mM) pyruvate and (0.2 mM) octanoate; **d** (4 mM) pyruvate and (0.2 mM) octanoate

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Fig. 6.

Effect of octanoate on appearance of HP [^{13}C]bicarbonate. The *upper panel* shows ^{13}C NMR spectra of the bicarbonate region for all 4 groups, scaled to carbon-1 of pyruvate. *Left to right*: Group 1 (1.5 mM lactate, 0.15 mM pyruvate); Group 2 (0.2 mM octanoate); Group 3 (1.5 mM lactate, 0.15 mM pyruvate, 0.2 mM octanoate); Group 4 (0.2 mM octanoate, 4 mM pyruvate). The *lower panel* shows the total signal from HP bicarbonate and CO_2 for each group, relative to the total carbon signal. * $P < 0.05$ versus group 1

Fig. 7.

Gluconeogenic flux versus HP [^{13}C]bicarbonate signal. The *plot* shows no correlation between gluconeogenesis and the appearance of HP [^{13}C]bicarbonate in each group. (*Black square*) Group 2 (0.2 mM octanoate); (*Black up-pointing triangle*) Group 3 (1.5 mM lactate, 0.15 mM pyruvate, 0.2 mM octanoate); (*Black circle*) Group 4 (0.2 mM octanoate, 4 mM pyruvate). For each data point N = 5

Table 1

Oxygen consumption, ketone and glucose production, and ^{13}C isotopomer data for livers perfused with a variety of ^{13}C enriched substrates

Perfusion condition [Octanoate] [Lactate] [Pyruvate]	1 – 1.5 [U-^{13}C] 0.15 [U-^{13}C]	2 0.2 [U-^{13}C] –	3 0.2 [U-^{13}C] 1.5 [3-^{13}C] 0.15 [3-^{13}C]	4 0.2 [U-^{13}C] – 4 [3-^{13}C]
Total O ₂ consumption	2.56 ± 0.32	3.21 ± 1.30	4.25 ± 0.69	3.39 ± 1.45
Total ketone production	0.02 ± 0.01	0.49 ± 0.33	0.87 ± 0.18	0.35 ± 0.24
Total glucose production	1.32 ± 0.51	1.28 ± 0.56	1.41 ± 0.68	2.40 ± 1.61
Data from ^{13}C NMR extracts				
Steady state acetyl-CoA isotopic distribution				
Natural abund (F_{C0})	0.43 ± 0.03	0.17 ± 0.09	0.05 ± 0.03	0.11 ± 0.04
[1- ^{13}C] (F_{C1})	0.06 ± 0.01	0.03 ± 0.02	0.01 ± 0.01	0.03 ± 0.02
[2- ^{13}C] (F_{C2})	0.05 ± 0.02	0.03 ± 0.03	0.06 ± 0.04	0.16 ± 0.05
[1,2- ^{13}C] (F_{C3})	0.46 ± 0.03	0.77 ± 0.13	0.89 ± 0.07	0.70 ± 0.08
Absolute fluxes				
CS flux	0.61 ± 0.14	0.38 ± 0.15	0.66 ± 0.06	0.37 ± 0.20
PDH flux	0.58 ± 0.15	0.09 ± 0.04	0.07 ± 0.04	0.10 ± 0.04
PC = PEPCK flux	1.33 ± 0.45	1.14 ± 0.26	1.39 ± 0.50	1.56 ± 1.25
PK flux	0.42 ± 0.27	0.90 ± 0.11	0.30 ± 0.47	0.68 ± 0.91
GNG flux	0.46 ± 0.18	0.12 ± 0.08	0.55 ± 0.02	0.44 ± 0.21
Pyruvate cycling	32 %	79 %	22 %	44 %
Data from HP ^{13}C NMR				
Mal C4/Mal C1	0.53 ± 0.02	0.52 ± 0.07	0.51 ± 0.07	0.51 ± 0.11
F_{REO}	0.69 ± 0.02	0.68 ± 0.06	0.67 ± 0.06	0.67 ± 0.09

The " F_{REO} " value refers to the fraction of [1- ^{13}C]oxaloacetate that underwent reorientation in the fumarate and succinate pool, based on the ^{13}C signal from malate C1 and C4. All flux data are reported as mean ± SD in units of $\mu\text{mol}/\text{min}/\text{gww}$ while the isotopomer contributions to acetyl-CoA are reported as fractions (the sum of all isotopomers is unity)