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Hyperpolarized δ -[1- ^{13}C]gluconolactone as a probe of the pentose phosphate pathway

Karlos X. Moreno¹, Crystal E. Harrison¹, Matthew E. Merritt^{1,2}, Zoltan Kovacs¹, Craig R. Malloy^{1,2,3,4}, and A. Dean Sherry^{1,2,5,*}

¹Advanced Imaging Research Center, UT Southwestern Medical Center, Dallas, TX 75390

²Dept of Radiology, UT Southwestern Medical Center, Dallas, TX 75390

³Dept of Internal Medicine, UT Southwestern Medical Center, Dallas, TX 75390

⁴VA North Texas Health Care System, Dallas, TX 75216

⁵Dept of Chemistry, University of Texas at Dallas, Richardson, TX 75083

Abstract

The pentose phosphate pathway (PPP) is thought to be upregulated in trauma (to produce excess NADPH) and in cancer (to provide ribose for nucleotide biosynthesis) but simple methods for detecting changes in flux through this pathway are not available. Magnetic resonance imaging of hyperpolarized ^{13}C -enriched metabolites offers considerable potential as a rapid, non-invasive tool for detecting changes in metabolic fluxes. In this study, hyperpolarized δ -[1- ^{13}C]gluconolactone was used as a probe to detect flux through the oxidative portion of the pentose phosphate pathway (PPP_{ox}) in isolated perfused mouse livers. The appearance of hyperpolarized $\text{H}^{13}\text{CO}_3^-$ within seconds after exposure of livers to HP- δ -[1- ^{13}C]gluconolactone demonstrates that this probe rapidly enters hepatocytes, becomes phosphorylated, and enters the PPP_{ox} pathway to produce HP- $\text{H}^{13}\text{CO}_3^-$ after three enzyme catalyzed steps (6P-gluconolactonase, 6PGDH, and carbonic anhydrase). Livers perfused with octanoate as their sole energy source show no change in production of $\text{H}^{13}\text{CO}_3^-$ after exposure to low levels of H_2O_2 , while livers perfused with glucose and insulin showed a 2-fold increase in $\text{H}^{13}\text{CO}_3^-$ after exposure to peroxide. This indicates that flux through the PPP_{ox} is stimulated by H_2O_2 in glucose perfused livers but not in livers perfused with octanoate alone. Subsequent perfusion of livers with non-polarized [1,2- ^{13}C]glucose followed by ^1H NMR analysis of lactate in the perfusate verified that flux through the PPP_{ox} is indeed low in healthy livers and modestly higher in peroxide damaged livers. We conclude that hyperpolarized δ -[1- ^{13}C]gluconolactone has the potential to serve as a metabolic imaging probe of this important biological pathway.

Graphical abstract

δ -[1- ^{13}C]gluconolactone was polarized using standard DNP methods as used as a probe of the oxidative portion of pentose phosphate pathway (PPP_{ox}). The appearance of hyperpolarized

Corresponding Author: A. Dean Sherry; Advanced Imaging Research Center; 5323 Harry Hines Blvd, Dallas, TX 75390; telephone: +1 (214) 645-2730, fax: +1 (214) 645-2744; dean.sherry@utsouthwestern.edu; URL: <http://www8.utsouthwestern.edu/utsw/home/research/AIRC/index.html>.

$\text{H}^{13}\text{CO}_3^-$ within seconds after exposure of livers to the probe demonstrates that it is rapidly transported into hepatocytes and phosphorylated, enters the PPP_{ox} and produces $\text{H}^{13}\text{CO}_3^-$ after three enzyme catalyzed steps. This new probe has the potential to serve as a metabolic imaging probe of this important biological pathway.

Keywords

pentose phosphate pathway; isolated mouse liver; ^{13}C NMR; dynamic nuclear polarization; lactone metabolism

1. Introduction

The pentose phosphate pathway (PPP) is an important pathway in glycolytic tissues. It consists of an oxidative portion (PPP_{ox}) that produces NADPH in two oxidative steps during the conversion of glucose-6-phosphate to ribulose-5-phosphate followed by a non-oxidative portion (PPP_{non}) that yields ribose-5-phosphate plus several other carbohydrates needed as biosynthetic precursors. Flux through the PPP_{ox} has been shown to be stimulated in head trauma, in cancer, and in tissues requiring continuous regeneration of glutathione, but simple methods for measuring flux of glucose through this pathway are limited. Relative flux through the PPP_{ox} has been measured using radioactive tracer studies,^{1–11} mass spectral analyses^{12–21} and NMR analyses.^{22–27} Net flux through the PPP_{ox} has been most often assessed by administration of [1,2- $^{13}\text{C}_2$]glucose and detection of singly-labeled lactate.^{14,15,17,26,27} δ -Gluconolactone, which exists in equilibrium with γ -gluconolactone in aqueous media (Scheme 1), enters mammalian cells *via* glucose transporters and phosphorylated by gluconokinase (EC 2.7.1.12) to 6-phospho- δ -gluconolactone, an intermediate in the PPP_{ox} .²⁸ Based on metabolic data obtained with ^{14}C labeled glucose and ribose in rabbit liver,²⁹ flux through the PPP_{ox} appears to be fast enough to allow detection of flux through this pathway within a time frame of 1–2 minutes. This makes δ -[1- ^{13}C]gluconolactone an attractive tracer for hyperpolarized ^{13}C studies of the PPP_{ox} because the ^{13}C atom in this derivative would be released as $^{13}\text{CO}_2$ in the second oxidative step of this pathway. Each intermediate and $^{13}\text{CO}_2/\text{HCO}_3^-$ are predicted to have a reasonably long T_1 values and so potentially can be detected by ^{13}C MRS for several minutes after injection of hyperpolarized δ -[1- ^{13}C]gluconolactone. This presents a unique opportunity to measure the flux through the PPP_{ox} by monitoring production of HP HCO_3^- by ^{13}C NMR. It is worth noting that the uptake of HP δ -[1- ^{13}C]gluconolactone will be in competition with the total glucose uptake but the rate of HP $^{13}\text{CO}_2$ production, as detected

by HP $\text{H}^{13}\text{CO}_3^-$, should in principle reflect relative flux through the PPP_{ox} (Scheme 2). It has been shown that hydrolysis of CO_2 to HCO_3^- catalyzed by carbonic anhydrase is extremely rapid under physiological conditions^{30,31} so cannot be rate limiting in the production of HCO_3^- from $[1-^{13}\text{C}]\text{gluconolactone}$. Here, we examined the use of HP- δ - $[1-^{13}\text{C}]\text{gluconolactone}$ to detect flux through PPP_{ox} by detection of HP $\text{H}^{13}\text{CO}_3^-$ in control livers and in livers pre-exposed to hydrogen peroxide to stimulate flux through this pathway. Since NMR is a non-invasive technique, translation of this method into a clinical setting may allow detection of tissues with an active PPP_{ox} pathway after a single injection of HP δ - $[1-^{13}\text{C}]\text{gluconolactone}$.

2. Materials and Methods

$[1-^{13}\text{C}]\text{Glucose}$ (99 % enriched) was purchased from Cambridge Isotope Laboratories (Andover, MA) and used without further purification. The trityl radical, 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 Molecular Biotools Ltd. (Abingdon, Oxfordshire, UK) and used without further purification. All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) at the highest quality available. Female C57BL/6 mice (20–25 g) were obtained from Charles River Laboratories (Wilmington, MA) and fed *ad libitum* prior to experimentation. The studies were performed under a protocol approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center. Liver enzyme assays, alanine aminotransferase and aspartate aminotransferase, were performed by the Mouse Metabolic Phenotyping Core at the University of Texas Southwestern Medical Center.

δ - $[1-^{13}\text{C}]\text{Gluconolactone}$ was synthesized following the procedure reported for the unlabeled compound.³² A mixture of $[1-^{13}\text{C}]\text{glucose}$ (1.0 g) and Shvo's catalyst (200 mg) in cyclohexanone (25 mL) was stirred at 50 °C (bath temperature) for 24 hours under argon. The orange reaction mixture was cooled to room temperature and the precipitate was filtered on a Schlenk filter under nitrogen. The precipitate was washed with cyclohexanone (5 mL) and acetone (3×10 mL) and dried in a stream of nitrogen to give the desired product, δ - $[1-^{13}\text{C}]\text{gluconolactone}$, as a white powder (0.70 g, 71 % yield). $^{13}\text{C}\{^1\text{H}\}$ NMR (150 MHz, D_2O , δ) 173.80 ppm (C1, s), 81.58 ppm (C3, s), 72.74 ppm (C5, s), 70.95 ppm (C4, s), 67.08 ppm (C2, s), 60.06 ppm (C6, s); MS (ESI) mass calculated for $^{12}\text{C}_5^{13}\text{C}_1\text{H}_{10}\text{O}_6 = 179.1$; Found = 178.0 $[\text{M}-\text{H}]^-$.

The spin-lattice relaxation times (T_1) of carbon-1 of γ - and δ - $[1-^{13}\text{C}]\text{gluconolactone}$ and $[1-^{13}\text{C}]\text{gluconate}$ were measured at 9.4 T. The T_1 of gluconate was determined on a non-polarized sample using a conventional inversion recovery pulse sequence [d-180°- τ -90°-acquisition] with various τ times. The T_1 values of γ - and δ - $[1-^{13}\text{C}]\text{gluconolactone}$ were determined by fitting the decay of each hyperpolarized signal to:³³

$$S(\tau) = M_p (\cos\theta)^{\tau/\text{TR}} (\sin\theta) e^{-\tau/T_1}$$

where $S(\tau)$ is the signal intensity, M_p is the Z magnetization at time = 0, θ is the pulse width, τ is time, and TR is the total repetition time. The measured T_1 's of hyperpolarized γ - and δ - $[1-^{13}\text{C}]$ gluconolactone were 19.9 and 17.8 sec, respectively, while the T_1 of non-polarized $[1-^{13}\text{C}]$ gluconate was 17.3 sec. All measurements were done at 9.4T. Given that δ - $[1-^{13}\text{C}]$ gluconolactone and γ - $[1-^{13}\text{C}]$ gluconolactone were exchanging during the decay process, these values can only be considered "apparent" T_1 values. An early report on the kinetics of this interconversion demonstrated that this exchange reaches equilibrium in water only after ~25–30 s.⁴⁹ It is also interesting to note that $[1-^{13}\text{C}]$ gluconate does not appear to be an intermediate in this exchange reaction so it is safe to assume that once $[1-^{13}\text{C}]$ gluconate is formed in tissue, it does not spontaneously convert to either γ - or δ - $[1-^{13}\text{C}]$ gluconolactone.⁴⁹

δ - $[1-^{13}\text{C}]$ Gluconolactone was polarized using an Oxford HyperSense® DNP polarizer. This instrument is a "second generation" device designed for routine operation in a research lab setting.³⁴ It is equipped with a 3.35 Tesla superconducting magnet, a microwave source, a solid state nuclear magnetic resonance (NMR) spectrometer for monitoring polarization build up, and a rapid dissolution system for transporting the sample from the HyperSense to a 400 MHz NMR spectrometer for spectroscopy and imaging. ~9 mg of δ - $[1-^{13}\text{C}]$ gluconolactone was dissolved in a (3:1) water:glycerol mixture doped with the trityl radical at 15 mM. The frozen sample was lowered into the magnetic field, cooled to 1.4 K in a pumped helium bath and the microwave irradiation was turned on. After maximal polarization was achieved (1–2 hours), the irradiation was turned off and the sample was rapidly dissolved using 4 mL of hot (> 190 °C) PBS (10 mM, pH ~ 7.4) and transferred into the bore of an 89 mm vertical 9.4 T NMR spectrometer for spectral acquisition. For tissue experiments, the hyperpolarized solution was introduced into the perfusate chamber directly above a perfused liver via a catheter running to the top of the perfusion rig. The average ^{13}C signal enhancement of HP- δ - $[1-^{13}\text{C}]$ gluconolactone observed a few seconds after dissolution and placement into a 9.4T magnet was 7200; this corresponds to a polarization level of 5.6%.

Livers isolated from C57BL/6 lab mice were perfused in an all glass perfusion system that fits entirely into the bore of an 89 mm vertical bore 9.4 T NMR spectrometer. Mice were anesthetized by an intramuscular injection (0.1 mL) of ketamine (Fort Dodge Animal Health)/xylazine (Boehringer Ingelheim) in a ratio of 85:15. Once anesthetized, a midline laparotomy was performed to expose the hepatic portal vein which was cannulated and injected with heparin (50 IU) to prevent blood clots. The hepatic vein and inferior vena cava were dissected, and flow of perfusate through portal vein was established using a peristaltic pump at ~ 8 mL/min. The excised liver was suspended in a 20 mm NMR tube attached to the perfusion apparatus maintained at 37 °C and perfused at a rate of 8 mL/min with a standard non-recirculating buffered medium (described below) and bubbled continuously with (19:1) O_2/CO_2 . Efferent and afferent $p\text{O}_2$ was measured using a blood gas analyzer (GEM Premier 3000, Instrumentation Laboratory, Lexington, MA, USA) to determine oxygen consumption.³⁵ Hepatic viability was evaluated by oxygen consumption measurements and visual inspection. The perfusion apparatus equipped with twin chambers, water jacketed for temperature control and protected by a polycarbonate outer shell was lowered into the bore of the 9.4T wide-bore magnet. The perfusion pressure was adjusted to maintain an adequate

perfusion flow through the portal vein. Hyperpolarized substrates were introduced through polyethylene tubing that terminated near the portal vein catheter. A schematic diagram of the apparatus is shown in supplementary materials.

Livers from either 15 h fasted mice or mice given full access to chow *ad libitum* were perfused with a modified Krebs-Henseleit (KH) medium containing (in mM): NaHCO_3 (25), NaCl (118), KCl (4.7), MgSO_4 (1.2), KH_2PO_4 (1.2), CaCl_2 (1.25). The perfusate presented to fasted livers also contained sodium octanoate (0.4 mM) as the only carbon energy source. Sodium octanoate was chosen because this intermediate chain-length fatty acid is avidly utilized by tissues, does not require carnitine for transport into mitochondria, and thereby insures that the livers remain in a highly reduced oxidation state. Our hypothesis was that octanoate would supply excess NADH through β -oxidation and any excess reducing equivalents would be cycled into the pool of NADPH via transhydrogenase enzymes (NADPH:NAD⁺ oxidoreductase) to replenish NADP⁺ during exposure of these livers to H_2O_2 . Thus, flux through the PPP_{ox} is predicted to be low in livers perfused with octanoate before and after exposure to H_2O_2 . Conversely, in livers from fed mice perfused with 10 mM glucose $\pm$ insulin (240 $\mu\text{U/mL}$) as their only carbon energy source, we hypothesized that flux through the PPP_{ox} would, by necessity, be stimulated to replenish NADPH in livers exposed to H_2O_2 .

After positioning each liver in a 20-mm NMR tube, the viability of the organ preparation was first confirmed by flow rate and O_2 consumption measurements before the liver was placed in the bore of a Varian VNMRs 9.4 T magnet. During a preparation and stabilization time of approximately 20 min, the NMR probe was tuned and the field homogeneity optimized using the ^{23}Na free induction decay. A ^{23}Na line width of 15 Hz or less was typically obtained. ^{13}C NMR spectra were acquired at ~ 100 MHz in a 25 mm Doty broadband probe using 66° pulses. Livers exposed to H_2O_2 were initially exposed to a perfusate lacking H_2O_2 during a preparation and stabilization period required for O_2 consumption measurements, probe tuning, and sample shimming. Following shimming, the perfusate was switched to one containing H_2O_2 for the remainder of the experiment. After 10–13 min of peroxide exposure, 8 mL of 4 mM HP δ -[1- ^{13}C]gluconolactone was injected into the perfusion chamber directly above the cannulation site over a period of ~ 30 s. Serial FIDs were acquired immediately after injection of the HP substrate using 16 K complex data points over a $\pm 32,000$ Hz bandwidth without proton decoupling to yield an acquisition time of ~ 1 s and a delay of 4.0 s, giving a repetition time of 5 s for each scan. Typically 20 separate FIDs were collected. These data were zero-filled prior to Fourier transformation and the relative peak areas were measured by integration using the ACDLabs SpecManager version 12.00 (Advanced Chemistry Development, Inc.; Toronto, Canada).

The relative activity of the PPP_{ox} pathway was also assessed in fed livers perfused with non-polarized 10 mM [1,2- ^{13}C]glucose and insulin (240 $\mu\text{U/mL}$), with or without exposure to H_2O_2 . These livers were isolated and perfused on the bench using an identical protocol as that described for hyperpolarization experiments. After 30 minutes of perfusion, the livers were freeze-clamped and all water soluble metabolites were extracted into cold perchloric acid. Samples were reconstituted in D_2O and high resolution ^1H spectra were collected at 14.1 T.

All data are reported as mean $\pm$ 1 s.d. Statistical significance was determined by a two-tailed t-test using GraphPad Prism version 5.03 (GraphPad Prism Software, Inc, La Jolla, CA). All results are reported at the 95 % confidence level.

3. Results

Figure 1 shows stacked plots of ^{13}C NMR spectra of a mouse liver immediately after addition of 4 mM HP δ -[1- ^{13}C]gluconolactone to an aqueous perfusate feeding the liver. Four resonances were observed and assigned to 6-phospho-[1- ^{13}C]gluconate (178.6 ppm), 6-phospho- γ -[1- ^{13}C]gluconolactone (177.0 ppm), 6-phospho- δ -[1- ^{13}C]gluconolactone (173.8 ppm), and $\text{H}^{13}\text{CO}_3^-$ (160.3 ppm) by comparisons with known standards. To evaluate which of these species resulted from metabolism of HP δ -[1- ^{13}C]gluconolactone by liver, a ^{13}C spectrum of HP δ -[1- ^{13}C]gluconolactone injected into aqueous perfusate (no liver) is compared in Figure 2. In this case, the resonances of δ -[1- ^{13}C]gluconolactone and γ -[1- ^{13}C]gluconolactone were both observed but those of [1- ^{13}C]gluconate and $\text{H}^{13}\text{CO}_3^-$ were not observed. This indicates that these two metabolites were generated by metabolism of HP- δ -[1- ^{13}C]gluconolactone in liver. Both metabolites are products of the PPP_{ox} pathway. Given that the chemical shifts of these intermediates are likely insensitive to the presence of a phosphate group at position C6 and the fact that the resonance at 178.6 ppm is much larger in the liver spectrum, this resonance must reflect the phosphorylated product, 6-phospho-[1- ^{13}C]gluconate. Similarly, since 6-phospho-[1- ^{13}C]gluconate is a metabolic product of 6-phospho- δ -[1- ^{13}C]gluconolactone and $\text{H}^{13}\text{CO}_3^-$ is a metabolic product of 6-phospho-[1- ^{13}C]gluconate, we conclude that all three carbohydrate intermediates detected by ^{13}C NMR in livers must be 6-phospho derivatives of these sugars.

A comparison of the summed total intensity of the HP- $\text{H}^{13}\text{CO}_3^-$ resonances in 20 spectra collected over 50 s for four different liver groups is shown in Figure 3a. These data show that production of HP- $\text{H}^{13}\text{CO}_3^-$ in livers perfused with octanoate alone was unaltered by addition of 1% H_2O_2 while in livers perfused with glucose plus insulin alone, HP- $\text{H}^{13}\text{CO}_3^-$ production was about 50% lower in the absence of H_2O_2 but returned to basal levels after addition of H_2O_2 . In comparison, Figure 3b shows that production of 6-phospho-[1- ^{13}C]gluconate was constant in all four groups of livers. This indicates that the oxidative reaction that produces NADPH (6PGDH) is rate limiting in this portion of the PPP_{ox} pathway, while rate of production of 6PG from δ -[1- ^{13}C]gluconolactone is independent of both physiology (octanoate versus glucose perfusions) and any oxidative tissue damage caused by H_2O_2 .

The kinetic rates of production of 6PG and $\text{H}^{13}\text{CO}_3^-$ from δ -[1- ^{13}C]gluconolactone in liver are compared in Figure 4. While the temporal appearance of HP-6PG did not differ significantly among the four groups (Figure 4b), the signal of HP- HCO_3^- appeared more rapidly and reached a higher amplitude in the livers perfused with octanoate compared to livers perfused with glucose plus insulin (Figure 4a). This provides further evidence that flux through 6PGDH is rate limited in livers perfused with glucose plus insulin but not rate limited in livers perfused with octanoate.

To validate that flux through the PPP_{ox} was indeed active in livers perfused with glucose, a separate group of livers from fed mice were perfused on the bench with [1,2-¹³C₂]glucose plus insulin for 30 min followed by freeze-clamping, tissue extraction and ¹H NMR. In this series, direct evidence for flux through the PPP_{ox} was shown by the ratio of [3-¹³C]lactate to [2,3-¹³C₂]lactate in the ¹H NMR spectrum of tissue extracts. [2,3-¹³C₂]lactate reflects direct conversion of [1,2-¹³C]glucose to lactate without passage through PPP_{ox} while [3-¹³C]lactate reflects metabolism of [1,2-¹³C₂]glucose through PPP_{ox} to produce [1-¹³C]ribulose-5-phosphate followed by carbon-carbon rearrangements in the PPP_{non} to produce either [1-¹³C]- or [3-¹³C]fructose-6-phosphate and ultimately [1-¹³C] or [3-¹³C]lactate. Representative ¹H spectra of tissue extracts of control and H₂O₂ treated livers perfused with [1,2-¹³C₂]glucose indicate that [2,3-¹³C₂]lactate and [3-¹³C]lactate are present in both groups of livers. The ratio of [3-¹³C]lactate/[2,3-¹³C₂]lactate is relatively small in both spectra consistent with about ~5% of glucose passing through the PPP_{ox}. Although the amount of [2,3-¹³C₂]lactate appears to be slightly lower in spectra of livers perfused with H₂O₂ (consistent lower flux through the PPP_{ox} as reported by the HP studies), this difference did not reach statistical significance.

4. Discussion

Flux through the oxidative portion of the pentose phosphate pathway (PPP_{ox}) has been reported to be as low as 1% of glycolytic flux in glial cells²⁰ to as high as 23% in rat livers.^{4,5} Absolute PPP_{ox} flux has been reported to be between 0.20 – 0.40 μmol/min/g wet weight in the rat liver^{4,5,36,37} under basal conditions, about 20 % of glycolytic flux.^{4,5,38} After an oxidative insult, flux through the PPP_{ox} typically increases to produce NADPH to replenish the reduced form of glutathione (GSH). For example, Dusick et al infused [1,2-¹³C]glucose into patients suffering from a traumatic brain injury to determine PPP_{ox} flux by collecting blood samples and analyzing the lactate by gas-chromatography/mass spectrometry.¹⁷ An increase of 7–20% of total glycolytic flux through the PPP_{ox} was observed in patients with brain injury compared to non-injured patients. Traditionally, measurement of the PPP_{ox} flux has been determined by measuring ¹⁴CO₂ production or from the labeling patterns in lactate caused by the co-infusion of [1-^{14/13}C]glucose and [6-^{14/13}C]glucose or infusion of [1,2-¹³C₂]glucose.^{3–5,7–11,13–15,17–21,26,27,39,40} These studies typically need hours to produce sufficient label through the PPP_{ox} to properly assay its flux. Hence, a HP probe that could potentially provide a quick readout of increase flux through the PPP_{ox} would be of interest.

In the current study, exposure of HP δ-[1-¹³C]gluconolactone to livers resulted in generation of HP H¹³CO₃[–] as detected by ¹³C NMR. In this case, HP H¹³CO₃[–] could only have been generated via decarboxylation of HP 6-phospho-[1-¹³C]gluconate, a reaction catalyzed by 6PGDH in the PPP_{ox} pathway (Scheme 2). The bicarbonate fraction of total ¹³C HP signal in livers perfused with octanoate was identical to that seen in livers perfused with glucose and insulin after exposure to H₂O₂ (Figure 3) but the temporal dependence of the HP signals differed (Figure 4). The initial rate of appearance of HP HCO₃[–] in livers from fasted animals perfused with octanoate with or without H₂O₂ was more rapid, reached higher maximum intensity, then dropped off more rapidly compared to all other groups. This can be ascribed to less competition between HP δ-[1-¹³C]gluconolactone and any endogenous glucose for

metabolism in the PPP_{ox}. The fact that flux through PPP_{ox} in livers perfused with octanoate did not increase with addition of H₂O₂ suggests that this medium-chain length fatty acid supplies sufficient reducing equivalents to maintain cellular redox levels without stimulating the PPP_{ox}. This is reflected by the identical ratio of HP-bicarbonate/HP-6PG measured in livers perfused with octanoate $\pm$ H₂O₂ (Figure 3c). The data show that the kinetics of appearance of 6PG was identical in all four groups indicating that the activity of 6-phosphogluconolactonase is insensitive to perfusion conditions. 6PG is known to be an allosteric activator of PFK^{41–44} and PK^{45,46} so in livers perfused with octanoate but no glucose, the amount of available 6PG would be limited to that formed from HP-6-phospho- δ -gluconolactone plus small amounts generated from endogenous stores of glucose. In livers perfused with glucose plus insulin, one can assume that additional 6PG would be produced from non-polarized glucose in addition to that produced from HP-6-phospho- δ -[1-¹³C]gluconolactone, so higher levels of 6PG would stimulate glycolysis without necessarily stimulating flux through the PPP_{ox} pathway. In fact, in those livers, net flux through the PPP_{ox} as measured by the ratio of HP-bicarbonate/HP-6PG was lower by ~50% compared to the other groups. This was the only metabolic condition that resulted in an increase in flux through the PPP_{ox} after addition of H₂O₂ as detected by generation of HP-HCO₃⁻. In experiments designed to validate the HP results using non-polarized [1,2-¹³C]glucose plus insulin (Figure 5), PPP_{ox} flux was also found to be low (< 5%) under basal perfusion conditions. Although addition of H₂O₂ to those livers tended to decrease the amount of [2,3-¹³C]lactate compared to [3-¹³C]lactate, this decrease did not reach statistical significance. This suggests that the HP measurement is more sensitive to small changes in PPP_{ox} than is the steady-state measurement of flux from [1,2-¹³C]glucose to lactate. One should note that other mono-labeled glucose derivatives could in principle be formed in these experiments as a result of ¹³C-enriched intermediates leaving the TCA cycle via pyruvate cycling. This contribution would reduce the estimate of PPP_{ox} flux activity even lower than suggested by the ¹H NMR measurements.

From a biological perspective, an imaging method for detecting flux through 6-phosphogluconate dehydrogenase (6PGDH) should reflect flux through the entire oxidative portion of the pentose phosphate pathway (PPP_{ox}). Although the HP method presented here has been shown to be sensitive to changes in relative flux rates through the PPP_{ox}, one cannot obtain absolute flux values in units of $\mu\text{mol}/\text{min}/\text{gww}$ of tissue nor can it report the percent of total glucose contributing to glycolysis that bifurcates through the PPP_{ox} pathway first. We made no attempt to fit the kinetic curves of Figure 4 to a kinetic model largely because the conversion of HP- δ -[1-¹³C]gluconolactone to HP-bicarbonate involves four enzyme-catalyzed steps, gluconokinase, 6P-gluconolactonase, 6PGDH, and carbonic anhydrase so obtaining quantitative kinetic constants for each of these steps would require many assumptions that we cannot currently justify. Given that other investigators have demonstrated model-free area-under-the-curve (AUC) measurements may work equally well for analysis of kinetic HP data,⁴⁷ our initial observations here suggest that the ratio of AUC's for bicarbonate/6PG appears to be sensitive to changes in flux through the PPP_{ox} at least in this limited data set. Further studies will be necessary to test whether HP- δ -[1-¹³C]gluconolactone is a generally useful probe of the PPP_{ox} pathway in other tissues and *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PPP	pentose phosphate pathway
PPP_{ox}	oxidative branch of the PPP
MRS	magnetic resonance spectroscopy
MRI	magnetic resonance imaging
DNP	dynamic nuclear polarization
HP	hyperpolarized/hyperpolarization
ppm	parts per million
KH	Krebs-Henseleit
6PG	6-phosphogluconate
6PGDH	6-phosphogluconate dehydrogenase
PDH	pyruvate dehydrogenase
PEPCK	phosphoenolpyruvate carboxykinase
PFK	phosphofructokinase
PK	pyruvate kinase

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Figure 1.

A stack plot of ^{13}C NMR spectra collected on an isolated mouse liver after continuous addition of hyperpolarized δ -[1- ^{13}C]gluconolactone to the perfusion media flowing into the portal vein of the liver at 8 mL/min. The ^{13}C spectra collected every 5 sec using a 66° pulse. The resonance assignments are described in the text.

Figure 2.

^{13}C NMR spectra of hyperpolarized δ -[^{13}C]gluconolactone injected into a) a NMR tube containing perfusion medium or b) an isolated perfused mouse liver as shown in Figure 1. Both spectra reflect summed 10 summed spectra collected over 50 s. The insets show expansion of the bicarbonate region (155 – 175 ppm).

Figure 3.

a) Fraction of the summed areas of the HP-HCO_3^- signal over 50 s relative to the total area of all HP ^{13}C signals. b) Fraction of the summed area of the HP-6-phosphogluconate signal over 50 s relative to the total area of all HP ^{13}C signals. c) The ratio of HP-HCO_3^- / HP-6PG signals (AUC) as an index of PPP_{ox} . The black column represents livers perfused with octanoate without H_2O_2 ; the dark grey column reflects livers perfused with octanoate plus 1% H_2O_2 ; the light grey column reflects livers perfused with glucose plus insulin without H_2O_2 ; the striped column reflects livers perfused with glucose plus insulin plus 1% H_2O_2 . All data are reported as the mean $\pm$ 1 s.d. for four livers in each group. $P = 0.02$ for * comparisons in Fig. 3a; $P = 0.03$ for * comparisons in Fig. 3b.

Figure 4.

Time-dependent changes in a) the HP-HCO₃⁻ and b) HP-6PG after presenting HP ̢-[1-¹³C]gluconolactone to isolated, perfused livers. (—) perfused with octanoate; (- - -) perfused with octanoate plus H₂O₂; (. . .) perfused with glucose plus insulin; (— . —) perfused with glucose plus insulin plus H₂O₂. Each data point reflects the mean ± 1 s.d. at that time point for n=4 livers in each group.

Figure 5.

^1H spectra of isolated mouse livers perfused with $[1,2-^{13}\text{C}_2]\text{glucose}$ plus insulin. Top spectrum: Liver exposed to 1 mM H_2O_2 . Bottom spectrum: Control liver. The majority of lactate produced during the perfusion period was not enriched in ^{13}C . The small wings around the large unlabeled lactate doublet at 1.33 ppm show that small amounts of $[2,3-^{13}\text{C}_2]\text{lactate}$ and $[3-^{13}\text{C}]\text{lactate}$ are both present.

Scheme 1.

Structures of δ -gluconolactone and γ -gluconolactone which interconvert in aqueous solution.

Scheme 2.

The oxidative branch of the pentose phosphate pathway (PPP_{ox}).