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Assessment of mouse liver [1-¹³C]pyruvate metabolism by dynamic hyperpolarized MRS

Kasper Faarkrog Høyer^{1,2}, Christoffer Laustsen¹, Steffen Ringgaard¹, Haiyun Qi¹, Christian Østergaard Mariager¹, Thomas Svava Nielsen², Ulrik Kræmer Sundekilde³, Jonas T Treebak², Niels Jessen^{4,5,6} and Hans Stødkilde-Jørgensen¹

¹Department of Clinical Medicine, The MR Research Center, Aarhus University Hospital, Aarhus, Denmark

²Novo Nordisk Foundation Center for Basic Metabolic Research, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

³Department of Food Science, Aarhus University, Aarslev, Denmark

⁴Department of Biomedicine, Research Laboratory for Biochemical Pathology, Aarhus University, Aarhus, Denmark

⁵Department of Clinical Pharmacology, Aarhus University Hospital, Aarhus, Denmark

⁶Steno Diabetes Center Aarhus, Aarhus University Hospital, Aarhus, Denmark

Correspondence should be addressed to J T Treebak or N Jessen or H Stødkilde-Jørgensen: jttreebak@sund.ku.dk or niels.jessen@clin.au.dk or hsj@clin.au.dk

Abstract

Hyperpolarized [1-¹³C]pyruvate magnetic resonance (MR) spectroscopy has the unique ability to detect real-time metabolic changes *in vivo* owing to its high sensitivity compared with thermal MR and high specificity compared with other metabolic imaging methods. The aim of this study was to explore the potential of hyperpolarized MR spectroscopy for quantification of liver pyruvate metabolism during a hyperinsulinemic-isoglycemic clamp in mice. Hyperpolarized [1-¹³C]pyruvate was used for *in vivo* MR spectroscopy of liver pyruvate metabolism in mice. Mice were divided into two groups: (i) non-stimulated 5-h fasted mice and (ii) hyperinsulinemic-isoglycemic clamped mice. During clamp conditions, insulin and donor blood were administered at a constant rate, whereas glucose was infused to maintain isoglycemia. When steady state was reached, insulin-stimulated mice were rapidly infused with hyperpolarized [1-¹³C]pyruvate for real-time tracking of the dynamic distribution of metabolic derivatives from pyruvate, such as [1-¹³C]lactate, [1-¹³C]alanine and [1-¹³C]bicarbonate. Isotopomer analysis of plasma glucose confirmed ¹³C-incorporation from [1-¹³C]pyruvate into glucose was increased in fasted mice compared with insulin-stimulated mice, demonstrating an increased gluconeogenesis in fasted mice. The AUC ratios for [1-¹³C]alanine/[1-¹³C]pyruvate (38.2%), [1-¹³C]lactate/[1-¹³C]pyruvate (41.8%) and [1-¹³C]bicarbonate/[1-¹³C]pyruvate (169%) all increased significantly during insulin stimulation. Hyperpolarized [1-¹³C]pyruvate can be used for *in vivo* MR spectroscopy of liver pyruvate metabolism during hyperinsulinemic-isoglycemic clamp conditions. Under these conditions, insulin decreased gluconeogenesis and increased [1-¹³C]alanine, [1-¹³C]lactate and [1-¹³C]bicarbonate after a [1-¹³C]pyruvate bolus. This application of *in vivo* spectroscopy has the potential to identify impairments in specific metabolic pathways in the liver associated with obesity, insulin resistance and nonalcoholic fatty liver disease.

Key Words

- ▶ hepatic pyruvate metabolism
- ▶ glucose clamp
- ▶ dynamic nuclear polarization
- ▶ hyperpolarized carbon-13 MRI

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Introduction

The liver plays a crucial role in the regulation of whole-body glucose homeostasis and ensures a sufficient supply of substrates irrespective of food intake. During fasting, it is the primary source of endogenous glucose production (Rui 2014). This supply of glucose is crucial for adenosine triphosphate generation in the central nervous system and inadequate glucose release can have detrimental effects (Mergenthaler *et al.* 2013). In the post-absorptive state, insulin released from the pancreas reaches the liver through the portal vein, where it potently inhibits hepatic glucose production (Röder *et al.* 2016).

Exogenous glucose, absorbed from the gut in the post-prandial state, is transported into the liver through GLUT2. In the liver, glucose is stored as glycogen or metabolized through glycolysis to form pyruvate (Samuel & Shulman 2016). Pyruvate is a key intermediate in several metabolic pathways and can act as a substrate in hepatic glucose production (Rui 2014). Pyruvate can be converted to acetyl coenzyme A by the pyruvate dehydrogenase enzyme complex situated in the inner mitochondrial membrane (Kerr *et al.* 2012). In this process, bicarbonate is produced via CO₂ and the enzyme carbonic anhydrase. Acetyl coenzyme A is one of the main fuels for production of energy in the tricarboxylic acid (TCA) cycle. Instead of entering the TCA cycle, pyruvate can be transformed into oxaloacetate via the enzyme pyruvate carboxylase. Oxaloacetate may exit the TCA cycle through phosphoenolpyruvate carboxykinase to generate phosphoenolpyruvate and subsequently glucose. Pyruvate can be taken up by hepatocytes and converted into lactate via enzyme lactate dehydrogenase and into alanine via alanine aminotransferase mainly represented in the cytosol.

Impairment of the hepatic substrate metabolism is associated with type 2 diabetes and nonalcoholic fatty liver disease (Cerf 2013). Several existing and putative antidiabetic drugs target substrate metabolism in the liver. However, quantification of their effects is complicated due to the inaccessibility of the organ and its double afferent blood supply. Hepatic substrate turnover has successfully been measured *ex vivo* using stable isotopes (Petersen *et al.* 1996, Jones *et al.* 1997). Still, this approach does not include the integrated response at the whole-body level. Recently, dynamic nuclear polarization technology (Ardenkjaer-Larsen *et al.* 2003) has been employed to quantify hepatic substrate metabolism *in vivo*, using a hyperpolarized (HP) bioprobe as [1-¹³C]pyruvate

(Hu *et al.* 2009, Spielman *et al.* 2009). Dynamic nuclear polarization allows rapid and high-sensitivity detection of pre-polarized ¹³C compounds with more than 10,000-fold signal enhancement (Ardenkjaer-Larsen *et al.* 2003). The use of HP [1-¹³C]pyruvate opens possibilities for *in vivo* MR spectroscopy of hepatic substrate metabolism in relation to liver diseases (Hu *et al.* 2009, Wu *et al.* 2010, Wilson *et al.* 2017). Initial experiments using dynamic nuclear polarization to assess pyruvate metabolism in rodents have shown promising results. In perfused rat livers, where substrate supplies are controlled by infusion (Merritt *et al.* 2011), it was possible to detect HP TCA cycle intermediates and bicarbonate by MR. In mice, the integrated regulation of hepatic substrate metabolism has been investigated by HP [1-¹³C]pyruvate in an insulin resistance model. However, interpretation of the results obtained from these experiments are complicated due to uncontrolled glucose and insulin levels (Lee *et al.* 2013). Additionally, HP ¹³C magnetic resonance spectroscopy (MRS) was applied in a porcine liver model for studying the relation between HP metabolic data and blood insulin, glucagon and free fatty acids following glucose challenge (Kjærgaard *et al.* 2018). A generalized method for imaging pyruvate metabolism in the liver has tremendous promise for understanding human pathophysiology, hyperpolarized MRS is one such method which has recently been translated to human examinations (Nelson *et al.* 2013, Kurhanewicz *et al.* 2019). The results demonstrated the potential of HP as a future clinical method for quantification of hepatic substrate metabolism.

Glucose clamps, initially introduced in humans to study energy homeostasis, were adapted for rodents in the early 1980s. The technique is considered the gold standard for assessing whole-body insulin sensitivity (DeFronzo *et al.* 1979, Ayala *et al.* 2011). During the glucose clamp, insulin is constantly infused while the blood glucose concentration is kept stable by a variable glucose infusion. The glucose infusion rate (GIR) is determined by frequent monitoring of blood glucose concentrations. When steady state is achieved, the GIR equals glucose disposal and can be used as a measure of whole-body insulin sensitivity (Ayala *et al.* 2011). This technique allows for full suppression of endogenous glucose production (Ayala *et al.* 2006), and therefore, provides optimal conditions for assessment of hepatic substrate metabolism.

In the present study, we investigated the metabolic fate of pyruvate in the liver during a hyperinsulinemic-isoglycemic clamp. We tested whether HP MRS combined with a glucose clamp enables quantification of

[^{13}C]pyruvate turnover while controlling hepatic glucose production. The primary aim of this study was to determine how hepatic mitochondrial pyruvate metabolism was affected in mice during insulin stimulation. To this end, we quantified [^{13}C]pyruvate turnover by HP MRS during a hyperinsulinemic-isoglycemic glucose clamp.

Methods

Animals

The experiment was conducted following the guidelines of the Animal Experiments Inspectorate in Denmark. The Ministry of Environment and Food Denmark, Danish Veterinary and Food Administration approved the study with license number: 2012-15-2934-152. WT male C57BL/6J BomTac mice (Taconic Biosciences, Ejby, Denmark), aged 10–12 weeks, were housed in a controlled environment. The room temperature was maintained at 22°C, at a 12-h light–darkness cycle (06:00 h and 18:00 h), and the mice were allowed free access to a standard chow diet (1324; Altromin, Lage, Germany) and water. Littermates housed under the same conditions were used as blood donors to ensure sufficient red blood cells during the clamp procedure.

Surgical procedure for hyperinsulinemic-isoglycemic clamp experiments

Details of the surgical procedures, the hyperinsulinemic clamp, and the determination of plasma are described elsewhere (Ayala *et al.* 2011, Hoyer *et al.* 2018). One catheter (C20PU-MJV1458; Instech Laboratories, Plymouth Meeting, PA, USA) was placed in the jugular vein and one (C10PU-MCA1459; Instech Laboratories) in carotid artery. The catheters were placed on the back of the mice, underneath the skin, during the recovery period. The hyperinsulinemic-isoglycemic clamp studies were performed 6–7 days after catheterization. For the surgical catheterization of the carotid artery and the jugular vein, the mice were anesthetized with sevoflurane (2.5% for induction, 2.0% for maintenance with Sigma Delta Vaporizer); carprofen (5 mg/kg, Rimadyl; Pfizer) was administered preoperatively for analgesia; and lidocaine (7 mg/kg Xylocain; AstraZeneca) was administered for local anesthesia during surgery. The catheters were locked with heparinized saline (50 U/mL) and the animals recovered for 6–7 days. Prior to the experiments, the following exclusion criteria were set:

weight loss of more than 10%, death during clamp procedure and blocked catheters.

Hyperpolarization, *in vivo* MR imaging and spectroscopy

[^{13}C]pyruvic acid (Sigma-Aldrich) (127 mg) mixed with 15 mM AH111501 (GE Healthcare) was polarized in a 5 T SPINLab (GE Healthcare). Pyruvic acid was polarized to a reproducible polarization of more than 35% at a final concentration of 125 mM. MR imaging and spectroscopy were performed with a field strength of 9.4 T pre-clinical MR scanner (Agilent) equipped with a $^1\text{H}/^{13}\text{C}$ dual-tuned 4 cm diameter volume coil (Rapid Biomedical, Rimpar, Germany). For MRS acquisition, TR=1 s, flip angle 10°, RF pulse bandwidth of 20 kHz were used. MR images of the liver were acquired with a 2D spoiled gradient echo sequence with a FOV of 4 × 4 cm and a matrix of 192 × 192, leading to a spatial resolution of 0.21 × 0.21 mm. Slice thickness was 1 mm. TR was 139 ms, TE was 3.5 ms, the flip angle was 20° and two averages were acquired. The carbon signal from [^{13}C]pyruvate and from the converted derivatives was acquired dynamically every second over a period of 120 s. The sequence was a slice-selective spectroscopic free-induction-decay sequence with a slice thickness of 8 mm. A spectral bandwidth of 10 kHz and 2048 points was acquired. The spectral analysis was performed in iNMR (Nucleomatica, Molfetta, Italy). Prior to Fourier transformation, the FID was zero-filled to 8 K points and apodized with an exponential line broadening of 10 Hz. Ratiometric analysis was performed by peak integration, and area under the curve (AUC) ratios were calculated as lactate (area) divided by pyruvate (area) for lactate/pyruvate. The individual signals were normalized to the pyruvate signal. The AUC ratios were measured over 60 s in the basal-fasted group and the insulin-stimulated group of mice. The representative time course graph shows the simultaneous production of downstream metabolites upon infusion of HP [^{13}C]pyruvate. The temperature inside the scanner was kept stable at ~37°C with a hot air heater (SA Instruments, Stony Brook, NY, USA). The HP sample was transferred manually to the mouse in the MR system in less than 15 s and injected by hand intravenously over 3–4 s using an injection line with an injected volume of ~100 μL [^{13}C]pyruvate (0.5 mmol/kg body weight) + 70 μL dead-space saline. All mice included in the 'Results' section (Figs 1, 2, 3 and 4) was subjected to the conventional slice-selective spectroscopy.

Figure 1

Hyperinsulinemic-isoglycemic clamp. (A) Blood glucose levels during the hyperinsulinemic-isoglycemic clamp conditions. Control group (5-h fasted) and Clamp group (5-h fasted + hyperinsulinemic clamp). No difference between the two groups in basal fasting blood glucose (–15 min, –5 min). (B) Glucose infusion rate during the hyperinsulinemic clamp. In all panels, data are mean $\pm$ standard error of mean. $n = 7$ in the control group and $n = 5$ in the clamp group. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0159>.

Interleaved hyperpolarized ^{13}C imaging and spectroscopy

A single mouse was imaged under similar conditions (Fig. 5) to visualize the intrahepatic distribution. Imaging and spectroscopy were performed using a slice-selective multi-echo balanced steady-state free precession (BSSFP) approach, combined with a standard slice-selective FID experiment. Parameters for the BSSFP acquisitions were one axial oblique slice, nine echoes, $\text{TR} = 7.9$ ms, $\text{TE} = 1.1$ ms, $\Delta\text{TE} = 0.7$ ms, $\text{FOV} = 50 \times 50 \text{ mm}^2$, matrix = 24×24 , and flip angle = 30° for the slice-selective excitation pulses and 8 mm slice thickness. The parameters for the FID acquisitions were $\text{TR} = 206$ ms, $\text{TE} = 0.5$ ms, spectral width = 10 kHz, 2,048 complex points and a flip angle = 15° , using the same slice selection parameters as the imaging acquisition. Acquisitions were timed to one image or spectroscopic acquisition every two seconds (i.e., nine echoes acquired every four seconds and one spectrum

Figure 2

Hepatic pyruvate metabolism detected by hyperpolarized ^{13}C MRS. Integration ratios from ^{13}C spectra of 60 scans following the injection of HP [$1\text{-}^{13}\text{C}$]pyruvate into fasted and insulin-stimulated mice. Control group (5-h fasted) and Clamp group (5-h fasted + hyperinsulinemic clamp). The unpaired t -test showed a significance increase in integration ratios in insulin-stimulated mice for (A) lactate, (B) bicarbonate and (C) alanine (* $P < 0.05$, *** $P < 0.001$). (D) Significance decrease in Lactate/bicarbonate integration ratio in insulin-stimulated mice. $n = 7$ in the control group and $n = 5$ in the clamp group.

acquired every four seconds). The images and spectra were offset by two seconds. The subsequent data processing was performed in MATLAB R2016a (Mathworks, Natick, MA, USA). Using the spectral information obtained in the FID acquisition, the BSSFP data were reconstructed using an IDEAL-type method (Reeder *et al.* 2005, 2007, Wiesinger *et al.* 2012) to yield ^{13}C pyruvate, pyruvate hydrate, lactate and alanine metabolic maps for each acquired time point.

Hyperinsulinemic-isoglycemic clamp in anesthetized mice

Five-hour fasted mice underwent a hyperinsulinemic-isoglycemic clamp for 120 min (Clamp group); the steady-state assumptions were reached at time point 80–120 min. Glucose was infused at variable rates to maintain basal blood glucose levels throughout the clamp period, as described previously (DeFronzo *et al.* 1979, Ayala *et al.* 2011). On the day of the clamp experiment, all mice were fasted for 5 h starting at 07:00 h ($t = -300$ min) by placing them in a clean cage with bedding and nesting material and free access to water. All mice were anesthetized with sevoflurane (3% for induction, 2.0% for maintenance) at 200 mL/min airflow during the clamp experiment and MR scans (Sigma Delta Vaporizer, Penlon) and kept on a heat pad before the MRS scan. The catheters were flushed

Figure 3

Representative time course depicting the simultaneous production of downstream metabolites upon infusion of hyperpolarized [1-¹³C]pyruvate. Temporal patterns of the individual metabolite peak intensities observed upon infusion of hyperpolarized [1-¹³C]pyruvate in hyperinsulinemic isoglycemic clamped mice (Insulin, top) and control mice (Control, bottom). Both graphs were normalized to the maximum ¹³C pyruvate signal for clarity. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0159>.

with heparinized saline (10U/mL) and the mouse was connected to the infusion lines. The clamp was initiated at $t=0$ min with continuous infusions of human insulin (4mU/kg/min, Actrapid; Novo Nordisk) and saline-washed red blood cells obtained from a donor mouse to compensate for the blood loss due to repeated sampling (5μL/min of 50% RBC in 10U/mL heparinized saline). Insulin was diluted in succinylated gelatin (Gelofusine®; B. Braun Melsungen, Melsungen, Germany). Blood glucose was measured every 10 min by a handheld glucometer (Bayer Contour), and isoglycemic blood glucose levels (7–10 mM) were maintained in anesthetized mice by adjusting a variable infusion of 50% glucose. Steady state was achieved at $t=80$ min to $t=120$ min, when the GIR was equal to the glucose uptake by all tissues in the mouse.

Figure 4

Plasma ¹³C glucose and total glucose. LC/MS results from plasma. (A) ¹³C-enriched glucose in plasma, corrected for natural abundance of ¹³C glucose. Plasma samples were taken 5 min after injection of HP [1-¹³C]pyruvate. (B) Glucose concentration in plasma for control mice and insulin-stimulated mice, respectively. $n=5$ in the control group, $n=19$ in the insulin group, all measurements were done in duplicates.

At the given time, the GIR was a measure of whole-body insulin sensitivity. After the MRS scan and a final blood sample, the mice were killed by cervical dislocation. Liver tissue was collected and snap frozen in liquid nitrogen. Liver and plasma samples were processed for isotopic determination of ¹³C-labeled glucose.

Quantification of glucose and ¹³C-glucose

Twenty micrograms of each liver sample was extracted using an ultra-turrax for 15 s in ice-cold methanol and left at 4°C for 60 min before 10 min centrifugation at 10,000g. Supernatant was transferred to a new Eppendorf tube and stored at –80°C until analysis. Plasma samples were diluted 1:5 in milliQ H₂O before ultrafiltration using 10 kDa cutoff centrifugal filters (Amicon Ultra-0.5 10 kDa; Sigma-Aldrich). Filters were washed in 0.5 mL milliQ H₂O three times before use. Filtration was performed by 60 min centrifugation at 10,000g and 4°C. Filtrate was stored at –80°C until analysis. Glucose and

Figure 5

In vivo dynamic metabolite imaging of the [$1\text{-}^{13}\text{C}$]pyruvate signal from the liver in control mice. Metabolite separation combined with the high resolution and the fast acquisition provided by multi-echo BSSFP imaging. The metabolic maps are well separated with the artery (location indicated by arrows) only having a high signal in the pyruvate and pyruvate hydrate maps. The white dashed lines show the position of the liver. The pyruvate hydrate signal is always present as an impurity in the spectrum of pyruvate. A small area of signal above the artery is visible in the lactate and alanine maps, which could be due ghosting artifact from pulsatility of the aorta or due to a chemical shift artifact originating from pyruvate in the aorta. Signal from circulating blood located in the high intensity signal area in the pyruvate and pyruvate hydrate image. The scale refers to the relative ^{13}C -signal intensity of the four metabolite images shown. All the images are normalized to the maximum pyruvate signal. The pyruvate image intensity was divided by 3 in order to use the same color bar/scale for all images. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0159>.

^{13}C -enriched glucose were measured by triple quadrupole liquid chromatography/mass spectrometry (LC/MS, Agilent LC 1290 and Agilent MS 6495). Prior to analysis, all samples were diluted 1:10 in milliQ water. Chromatographic separation was achieved on a $1.7\mu\text{m}$ HILIC column (Acquity BEH HILIC $2.1 \times 100\text{mm}$; Waters, Taastrup, Denmark) at 30°C using mobile phases consisting of solution A (high-performance liquid chromatography grade acetonitrile (ACN)) and solution B (10mM ammonium formate in high-performance liquid chromatography grade water). A flow rate of $400\mu\text{L}/\text{min}$ and the following gradients were used: 0% B for 1 min, linear gradient of 0–90% B in 5 min and 90% B for 1 min. For all analyses, $1\mu\text{L}$ of sample was injected on the LC-MS. Mass spectrometry analysis ionization was achieved in the negative ion mode by atmospheric pressure chemical ionization, and data acquisition was performed as multiple reaction monitoring. The product ions monitored were m/z $179 \rightarrow 89$ and $179 \rightarrow 59$ for glucose and m/z $180 \rightarrow 89$ and m/z $180 \rightarrow 59$ for ^{13}C -enriched glucose. The contribution of natural abundant ^{13}C -glucose to total ^{13}C -glucose was corrected by subtraction of 1.1% of glucose concentration (product ion m/z 179). Standard solutions and calibration curves were made using glucose and [$1\text{-}^{13}\text{C}$]glucose obtained from Sigma-Aldrich (Sigma-Aldrich).

Method setup in anesthetized mice

The mice were investigated during modified hyperinsulinemic-isoglycemic clamp conditions with a catheter in the jugular vein and the tip of the catheter placed in the right atrium (Supplementary Fig. 3, left,

see section on [supplementary data](#) given at the end of this article). Saline, glucose, red blood cells and insulin were infused with infusions pumps (Pump 11 Elite, Harvard Apparatus). Alterations in the classic clamp design were needed as the MRS method required the mouse to be fixed and anesthetized during the experiment (Supplementary Fig. 3, right). Injection of [$1\text{-}^{13}\text{C}$]pyruvate was initiated after the steady-state blood glucose level was reached and the mouse was relocated in the MR scanner.

Statistical analysis

All data are presented as mean $\pm$ standard error of mean. Unpaired *t*-tests were performed in GraphPad Prism 7 used for comparing integration ratios. All time-dependent analyses were evaluated by two-way repeated-measures ANOVA. Statistical significance was set at $P < 0.05$. G*Power (version 3.0.9) was used to calculate the sample size for two groups with repeated measurements. The following alpha, power and effect size were used: $\alpha = 0.05$, effect size = 0.5, power = 0.80 and a total sample size of eight, $n = 4$ in each group was necessary. Additional mice in each group were added to allow for exclusion of mice for technical reasons such as blocked catheters, failed surgeries and so forth.

Results

Insulin increases turnover of pyruvate in mouse liver *in vivo*

Fifteen mice underwent a surgical procedure, and all the mice survived the post-surgery recovery.

Mice that did not survive clamp conditions ($n=1$) or had blocked catheters ($n=2$) were omitted from analysis according to the predefined exclusion criteria. Seven mice, 24.8 ± 0.71 g, were fasted for 5 h and infused with saline as fasted controls (control group). Five mice, 24.5 ± 0.66 g, went through the hyperinsulinemic-isoglycemic clamp (Clamp group). The control group was used as a 'no insulin stimulation reference' to investigate how pyruvate metabolism changed in fasted mice compared with insulin-stimulated mice. Fasting blood glucose levels were similar between groups prior to insulin infusion (Fig. 1A). Hyperinsulinemic-isoglycemic animals remained at the same blood glucose level throughout the clamp (Fig. 1A). After 80 min of constant insulin infusion, blood glucose levels and GIR in insulin-stimulated mice remained stable throughout steady state (80–120 min, Fig. 1A and B), demonstrating the suitability of the clamp technique to induce a stable glucose homeostasis prior to HP [^{13}C]pyruvate injection. Following the clamp, integrals of 60 s MRS scans showed increased AUC ratios for [^{13}C]alanine/[^{13}C]pyruvate (38%), [^{13}C]lactate/[^{13}C]pyruvate (42%) and ^{13}C -bicarbonate/[^{13}C]pyruvate (169%) during insulin stimulation (Fig. 2A, B and C). In addition, a ~50% lower anaerobic-to-aerobic balance (lactate-to-bicarbonate ratio) was observed (Fig. 2D). Supplementary Figure 1 provides MRS spectra from clamped and control conditions with the different signals identified. Supplementary Figure 2 shows a metabolic model depicting how the [^{13}C]pyruvate signal is transferred to [^{13}C]lactate, [^{13}C]alanine and [^{13}C]bicarbonate.

Hyperpolarized ^{13}C MR provides dynamic assessment of enzymatic reactions

Representative time curves depicting the production of downstream metabolites in liver upon infusion of HP [^{13}C]pyruvate are shown in Fig. 3. The drops in metabolite signals were due to displacement of the liver caused by respiration. In order to estimate the hemodynamic differences between the groups, the time-to-peak intensity was estimated from the [^{13}C]pyruvate curves (Fig. 3). Metabolites derived from pyruvate in a single cytosolic enzyme catalyzed step, such as [^{13}C]lactate and [^{13}C]alanine, had the largest peak intensities in all spectra, while [^{13}C]bicarbonate had a lower intensity, but no differences were observed between the control and the insulin-stimulated condition.

Fasting increases the ^{13}C -labeled glucose fraction in plasma

^{13}C -labeled oxaloacetate formed via pyruvate carboxylation may exit the cycle through phosphoenolpyruvate carboxykinase generating phosphoenolpyruvate and subsequently ^{13}C -labeled glucose (Jin *et al.* 2016). However, instead of quantifying enzyme levels, gluconeogenesis was examined by substrate analysis of ^{13}C -labeled glucose from plasma (Fig. 4A). Triple quadrupole LC/MS measurements of ^{13}C -enriched glucose showed a ~50% lower fraction of ^{13}C -enriched glucose in insulin-stimulated compared to control mice (Fig. 4B). Moreover, a ~50% lower fraction of non-labeled glucose was found in the insulin-stimulated mice (Fig. 4B). We found the same tendency toward a lower ^{13}C -labeled glucose in livers from insulin-stimulated mice than in liver from control mice, but this difference did not reach statistical significance ($P=0.10$).

The metabolism of hyperpolarized ^{13}C is located to the liver

A slice-selective multi-echo BSSFP interleaved with a slice-selective FID acquisition allows for accurate generation of metabolic images, reflecting the voxel-wise metabolic signals from the individual metabolites. Figure 5 shows metabolic signal intensities overlaying anatomical ^1H MR images, representing metabolite accumulation in the different parts of the axial slice. As depicted in Fig. 5, the signal intensity for alanine, lactate, pyruvate hydrate and pyruvate after injection of [^{13}C]pyruvate was located to the liver tissue, while only little signal was detected from blood vessels. The shown metabolic maps are the sum of all time points for each metabolite.

Discussion

Here, we demonstrate for the first time that HP ^{13}C MRS and the glucose clamp technique can be combined to concomitantly quantify intrahepatic substrate fluxes and insulin sensitivity. This approach extends investigations of hepatic pyruvate metabolism to an anabolic condition with insulin and glucose levels in steady state. We demonstrate that insulin redirects hepatic pyruvate metabolism *in vivo*. Insulin induced [^{13}C]pyruvate metabolism in liver in the form of increased AUC ratios for [^{13}C]alanine, [^{13}C]lactate, and ^{13}C -bicarbonate during clamp conditions. The increase in bicarbonate signal indicates increased pyruvate dehydrogenase activity by

insulin (Jin *et al.* 2016). Like previous studies, we found that the ¹³C bicarbonate signal from [1-¹³C]pyruvate reflects PDH flux and that it is affected by fasting and by the nutritional state of the mouse (Hu *et al.* 2009, Merritt *et al.* 2011).

We detected a ~50% lower ¹³C enrichment of glucose in plasma during insulin stimulation. Concomitantly, we observed a 42% increase in the [1-¹³C]lactate/[1-¹³C]pyruvate ratio in liver stimulated with insulin. These findings could be explained by phosphoenolpyruvate carboxykinase inhibition and reduced pyruvate carboxylase activity by insulin (Samuel & Shulman 2016). In the present study, flux through phosphoenolpyruvate carboxykinase is a potential source of HP ¹³C-bicarbonate. However, it has previously been shown that a ¹³C-bicarbonate signal comes from [1-¹³C]pyruvate (Jin *et al.* 2016). In addition, given that conversion of [1-¹³C]pyruvate to ¹³C-bicarbonate via phosphoenolpyruvate carboxykinase involves at least one metabolic intermediate (Supplementary Fig. 2), it is likely that polarization is lost by the time the ¹³C-bicarbonate is generated. Previously published data in anesthetized rats showed increased hepatic pyruvate carboxylation and phosphoenolpyruvate carboxykinase activity due to prolonged fasting (21 h) and a lower [1-¹³C]alanine signal from HP [1-¹³C]pyruvate (Jin *et al.* 2016). These results support the previous reports of increased lactate production/accumulation associated with reduced gluconeogenesis by metformin treatment (Madiraju *et al.* 2014, Lewis *et al.* 2016, Qi *et al.* 2018). Our study, which was performed in 5-h fasted mice, showed a ¹³C-bicarbonate signal in contrast to signal absence in 21-h fasted rats (Nowland *et al.* 2011). This could be due to differences in fasting length and injection time of HP pyruvate. Thus, in the previous study, the rats were in an advanced state of fasting and HP pyruvate was administered over an interval that was 10-fold greater than that of our study. This may explain why they did not detect the ¹³C-bicarbonate signal (Jin *et al.* 2016). A potential source of variation and bias is in the hepatic perfusion and uptake of pyruvate, which can impact the ratiometric analysis, an issue which is particularly important in human subjects (Larson *et al.* 2018). This is however less pronounced in rodent studies (Hill *et al.* 2013, Daniels *et al.* 2016), as can be seen by the significant change in the metabolic derivative ratio's bicarbonate/lactate, which is less affected by the perfusion. As known the pyruvate is transported into the liver through monocarboxylate transporter (MCT) proteins. Several isoforms exist and both MCT1 and MCT2

are believed to be involved in hepatic uptake of pyruvate (Fisel *et al.* 2018).

In order to secure a fixed location in the MR scanner, the mice were anesthetized during scanning and clamp conditions. Anesthesia can have profound effects on the whole-body glucose metabolism (Eger 2004, Kitamura *et al.* 2009). In mice, this is primarily due to impaired glucose uptake in muscle and the central nervous system, whereas hepatic insulin sensitivity remains unaffected (Hoyer *et al.* 2018). Moreover, insulin levels in sevoflurane-anesthetized mice is markedly lower, despite high blood glucose levels (Hoyer *et al.* 2018), and this leads to a lower basal hepatic gluconeogenesis. This aligns with results from human insulin levels in which pancreatic β -cell secretion is impaired during anesthesia (Tanaka *et al.* 2005). The presented results do not indicate that the clamp method should always be applied to control glucose homeostasis. Still, this approach does provide a unique possibility to quantify insulin sensitivity and control glucose metabolism *in vivo* prior to analyzing hepatic pyruvate metabolism.

Insulin stimulation had no significant influence on the time-to-peak intensity of pyruvate, lactate, alanine and bicarbonate. A tendency toward a uniform and longer mean pyruvate transit time was observed in the insulin group, but this tendency was not statistically significant. However, in future studies with larger samples sizes, this time course can potentially provide important information on the metabolic profile for overall metabolite conversion as well as the rate and timing of this conversion. MRI results demonstrate that the metabolite signal originates primarily from liver tissue, and only a low signal is from circulating blood. This indicates that the increase in hepatic [1-¹³C]lactate, [1-¹³C]alanine and [1-¹³C]bicarbonate signal did not mirror metabolic shifts in the blood pool or a direct signal from metabolism in skeletal muscle. The application of HP MRSI could potentially be used to image hepatic metabolic heterogeneity.

The increased ¹³C signal in alanine along with lactate and bicarbonate intensities in insulin-stimulated mice demonstrates that it is possible to use the combined method to investigate insulin-stimulated liver metabolism in a mouse model. This method shows potential for *in vivo* assessment of the hepatic metabolism and for investigating metabolic complications associated with liver disease, including nonalcoholic fatty liver disease, hepatitis, cirrhosis and diabetes. With the development of mouse models as a means to investigate models for human disease, this technique of assessing the murine liver

metabolism could be important for further investigation of disease mechanisms in humans.

In conclusion, we have shown that metabolic measurements in the liver based on injection of HP [^{1-13}C]pyruvate in combination with assessment of whole-body insulin sensitivity may be used to evaluate and monitor treatment of liver disease. We have quantified and visualized that insulin severely affects liver metabolism by increasing hepatic pyruvate turnover through increased PDC flux and by lowering gluconeogenesis. The findings reported here broaden the scope of the dynamic nuclear polarization technique for *in vivo* imaging of metabolism.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-19-0159>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

K F H designed experiments, researched data and wrote manuscript. C L designed experiments, researched data, contributed to discussion and reviewed/edited manuscript. S R, H Q and C Ø M researched data. U K S researched data and reviewed/edited manuscript. T S N contributed to discussion and reviewed/edited manuscript. J T T, N J and H S J designed experiments, researched data, reviewed/edited manuscript, contributed to discussion and wrote the manuscript.

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